JOÃO RENATO REBELLO PINHO

**ESTUDOS MOLECULARES SOBRE O
VÍRUS DA HEPATITE B**

Texto sistematizado apresentado à
Faculdade de Medicina da Universidade
de São Paulo para a obtenção do título de
Livre Docente junto ao Departamento de
Gastroenterologia

SÃO PAULO

2015

Dedicatória

Para

Cida

Ana

Pedro

Uma família de “Médicos de Verdade”

Agradecimentos

Todos os agradecimentos que deviam constar nesta parte trabalho estão colocados no “Memorial”. Todas aquelas pessoas que lá foram citadas foram meus colaboradores e todos tiveram sem dúvida um papel importante na minha trajetória.

*Talvez só tenham ficado de fora agradecimentos aos meus pais e meus irmãos. Minha mãe **Maria Eunice** era formada em “História e Geografia” em uma das primeiras turmas da “Universidade de São Paulo” e meu pai **Ruy** era formado em “Direito”, pela então “Universidade do Brasil”, atual “Universidade Federal do Rio de Janeiro”, onde também fez o seu exame de Livre Docência. Eles despertaram e incentivaram todo o interesse que tenho pela medicina e pelas ciências de maneira geral, que passei também para meus filhos.*

Volto a ressaltar que estou agradecendo a todas aquelas pessoas que estão citadas no memorial e seria uma lista imensa se fosse citá-las nominalmente.

*Mas não posso deixar de agradecer ao Prof. Dr. **Flair José Carrilho**, com quem sempre tive uma relação de amizade, respeito e confiança muito grande e que muito me incentivou para realizar este exame.*

*Também não posso deixar de agradecer a **Fernanda de Mello Malta** e **Michele Soares Gomes Gouvêa**, com quem trabalho desde 2000 quando ainda estava no “Instituto Adolfo Lutz”, vieram comigo para o “Laboratório de Gastroenterologia e Hepatologia Tropical”, concluíram sob minha orientação tanto o mestrado como o doutorado e foram recentemente contratadas pelo “Hospital das Clínicas” como “Monitoras de Pesquisa”. Ainda temos muito para fazer juntos..*

*Tenho também que agradecer à **Família Alves de Queiroz**, que tem apoiado as pesquisas que venho realizando há muitos anos, não só com o especial patrocínio para transformar em realidade o “Laboratório de Gastroenterologia e Hepatologia Tropical” “**João Alves de Queiroz e***

Castorina Bittencourt Alves”, como também com todo o apoio que dá a esta área de pesquisa, com auxílios que incluem também pagamentos a diferentes profissionais, reagentes e equipamentos.

*Em último lugar, tenho que agradecer a **FAPESP** , **CNPq**, e **CAPES**, instituições de pesquisa nacionais que foram fundamentais para o financiamento de meus projetos de pesquisa, na construção de infraestruturas de pesquisa e no suporte de colaboradores com bolsas de treinamento técnico, mestrado e doutorado.*

Este exame é um momento para você se voltar sobre tudo o que você fez e pensar em tudo o que você pode fazer daqui para frente.

Espero continuar contando com vocês, com todos aqueles com quem já trabalhei, com novos conhecidos com quem já temos planos para o futuro próximo e ainda com aqueles que irei conhecer, para que possamos continuar trabalhando para melhorar ainda mais os conhecimentos, as estruturas e as formas de atuação na área de medicina.

“Your whole life you are really writing one book, which is an attempt to grasp the consciousness of your time and place— a single book written from different stages of your ability.”

Nadine Gordimer

SUMÁRIO

1-INTRODUÇÃO	13
CAPÍTULO 1 – ESTUDOS DE AGENTES INFECCIOSOS POR MÉTODOS MOLECULARES ...	15
CAPÍTULO 2 - HEPATITE B	19
2-OBJETIVOS	78
3-RESULTADOS	86
1- Y. Miyamoto & J.R.R. PINHO - Análise de alguns parâmetros envolvidos com o ensaio da DNA polimerase do vírus da Hepatite B. Revista do Instituto Adolfo Lutz 50 (1): 297 – 299, 1990.....	86
2- J.R.R.PINHO, L.E.P. Fonseca, Y. Song, Y. Miyamoto, F.J. Carrilho, C.F.H. Granato, L.C. da Silva - Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/anti-HBe system, viral DNA polymerase and HBV-DNA. Revista do Instituto de Medicina Tropical de São Paulo 31(5): 328 - 335, 1989.....	90
3- J.R.R. PINHO, C.A. Santos, C.L.M. Gonzalez, L. Bassit, C.C. Barreto, A. Saez-Alquézar, A.V.C. França, F.J. Carrilho, L.E.P. Fonseca, D.A.F. Chamone, & L.C. Da Silva - Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. Revista do Instituto de Medicina Tropical de São Paulo 35 (6): 515 –520, 1993.....	98
4- Sitnik R, Paes A, Mangueira CLP, PINHO JRR. A Real-Time Quantitative Assay for Hepatitis B DNA (HBV) developed to detect all HBV genotypes. Revista do Instituto de Medicina Tropical de São Paulo,52 (3): 119-124, May-June, 2010.....	106
5- L.C. da Silva, C.L. Assis-Madruga, F.J. Carrilho, J.R.R. PINHO, A. Saez-Alquézar, C. Santos, L. Bassit, C. Barreto, L.E.P. Fonseca, V.A.F. Alves, R. Leitão, R. Vianna, R.A. Cardoso, A.V.C. França & L.C.C. Gayotto - Spontaneous Hepatitis B surface antigen clearance in long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with Hepatitis C and D virus superinfection. Journal of Gastroenterology 31 (5): 696 - 701, 1996.	113
6- Luiz C. Da Silva; João R.R. Pinho; Roberta Sitnik; Luís E. P. Fonseca; Flair J. Carrilho – Predictive factors for response to lamivudine in chronic hepatitis B. Revista do Instituto de Medicina Tropical de São Paulo 42 (4) : 179 - 186 , July/August 2000.....	120

- 7- Luiz Caetano da Silva, João Renato Rebello Pinho, Roberta Sitnik, Luís Edmundo Pinto da Fonseca & Flair José Carrilho. Efficacy and tolerability of a long term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *Journal of Gastroenterology* 36 (7): 476-85, July 2001.....129
- 8- Luiz Caetano da Silva, Maria Luiza da Nova, Suzane Kioko Ono-Nita, João Renato Rebello Pinho, Roberta Sitnik, Vera Aparecida dos Santos, Flair José Carrilho. Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic Hepatitis B. *Revista do Instituto de Medicina Tropical de São Paulo*, 51(5):261-268, September-October, 2009.....140
- 9- Mendes-Correa MC, Pinho JR, Gomes-Gouvea MS, da Silva AC, Guastini CF, Martins LG, Leite AG, Silva MH, Gianini RJ, Uip DE. Predictors of HBeAg status and hepatitis B viraemia in HIV-infected patients with chronic hepatitis B in the HAART era in Brazil. *BMC Infectious Diseases* 2011 Sep 20; 11: 247.....149
- 10- Sitnik R, Pinho JRR, Bertolini DA, Bernardini AP, da Silva LC & Carrilho FJ. Hepatitis B virus genotypes and precore and core mutations in Brazilian patients. *Journal of Clinical Microbiology* 42 (6): 2455-2460, 2004.....158
- 11- Michele S Gomes-Gouvêa, Ariana C Ferreira, Rosangela Teixeira, José R Andrade, Adalgisa SP Ferreira, Lena MF Barros, Rosamar EF Rezende, Ana CS Santos Nastri, Andrea GB Leite, Leonora Z Piccoli, Josiane Galvan, Simone RSS Conde, Manoel CP Soares, Dimas A Kliemann, Dennis A Bertolini, Aline SO Kunyoshi, André C Lyra, Marcio K Oikawa, Luciano V de Araújo, Flair J Carrilho, Maria CJ Mendes-Corrêa, João R Rebello Pinho. HBV carrying drug-resistance mutations in chronically infected treatment-naïve patients. *Antiviral Therapy* 2015 Jan 27 doi: 10.3851/IMP2938165
- 12- Maria Cássia Mendes-Correa, JOÃO RENATO REBELLO PINHO, Roberta Sitnik, Rúbia Anita Ferraz Santana, Michele Mesquita Gomes-Gouvêa, Olavo M. Leite, Luiz G. Martins, Andrea G. Leite, Mariliza H. Silva, R.J.Gianini, David E. Uip. High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. *Journal of Medical Virology*, 2010. Sep; 82 (9):1481-8.....186
- 13- Adriana Cristina da Silva, Ângela Maria Miranda Spina, Marcílio Figueiredo Lemos, Isabel Takano Oba, Cristina de Fátima Guastini, Michele Soares Gomes-Gouvêa, JOÃO RENATO REBELLO PINHO, Maria Cássia Jacintho Mendes-Correa. Hepatitis B genotype G and high frequency of lamivudine resistance mutations among

HIV/Hepatitis B virus co-infected patients in Brazil. Memórias do Instituto Oswaldo Cruz, 2010 Sep. 105 (6): 770 -778.....	195
14- Mónica Viviana Alvarado-Mora, Rúbia Anita Ferraz Santana, Roberta Sitnik, Paulo Roberto Abrão Ferreira, Cristovão Luís Pitangueira Manguiera, Flair José Carrilho, João Renato Rebello Pinho. Full-length genomic sequence of hepatitis B virus genotype C2 isolated from a native Brazilian patient. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, Vol. 106(4): 495-498, June 2011.....	205
15- Alvarado-Mora MV, Botelho-Lima LS, Santana RA, Sitnik R, Ferreira PA, do Amaral Mello F, Manguiera CP, Carrilho FJ, Rebello Pinho JR. Distribution of hepatitis B virus subgenotype F2a in São Paulo, Brazil. BMC Res Notes. 2013.....	210
16- Rosamar E. F. Rezende, Benedito A. L. Fonseca, Leandra N. Z. Ramalho, Sérgio Zucoloto, JOÃO RENATO R. PINHO, Dennis A. Bertolini, Ana L. C. Martinelli. The precore mutation is associated with severity of liver damage in chronic Hepatitis B. Journal of Clinical Virology 32(1): 53 – 59 (2005).....	216
17- Bertolini DA, Ribeiro PC, Lemos MF, Saraceni CP, Pinho JR. Characterization of a Hepatitis B virus strain in southwestern Paraná, Brazil, presenting mutations previously associated with anti-HBs Resistance. Revista do Instituto de Medicina Tropical de São Paulo, 2010 Feb;52(1):25-30.....	224
18-Bertolini DA, Gomes-Gouvêa MS, Carvalho-Mello IM, Saraceni CP, Sitnik R, Grazziotin FG, Laurino JP, Fagundes NJ, Carrilho FJ, Pinho JR. Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil. Infection, Genetics and Evolution. 2012 Volume 12, Issue 6, August 2012, Pages 1295–1304 Apr 1.....	230
19-Flair J Carrilho, Cleusa R Moraes, JOÃO RR PINHO, Isabel MVGC Mello, Dennis A Bertolini, Marcílio F Lemos, Regina C Moreira, Leda C Bassit, Rita A Cardoso, Gabriela Ribeiro-dos-Santos & Luiz C Da Silva. Hepatitis B virus infection in Haemodialysis Centres from Santa Catarina State, Southern Brazil. Predictive risk factors for infection and molecular epidemiology. BMC Public Health 4 (1):13 (2004). doi: 10.1186/1471-2458-4-13	241
20-Andre C Lyra, João RR Pinho, Isabel MVGC Mello, Fernanda de M Malta, Michele MS Gomes, Adrian M Di Bisceglie, Luiz GC Lyra, Flair J Carrilho, Luiz C da Silva. Distribution of Hepatitis B Virus (HBV) genotypes among patients with acute viral hepatitis. Journal of Clinical Gastroenterology (2005), 39 (1): 81-82	253

21-Nadia Regina Caldas Ribeiro, Gubio Soares Campos, Ana Luiza Dias Ângelo, Eduardo Lorens Braga, Nelma Santana, Michele Mesquita Soares Gomes, JOÃO RENATO REBELLO PINHO, Wilson Andrade de Carvalho, Luiz Guilherme Costa Lyra, André Castro Lyra. Distribution of Hepatitis B virus genotypes among patients with chronic infection. Liver International 26: 1 – 7 (2006).	255
22-Moura IF, Lopes EP, Alvarado-Mora MV, Pinho JR, Carrilho FJ. Phylogenetic analysis and subgenotypic distribution of the hepatitis B virus in Recife, Brazil. Infection, Genetics and Evolution. 2013 Mar;14:195-9.....	263
23-Alvarado-Mora, Monica V, Botelho, Livia, Gomes-Gouvea, Michele S, de Souza, Vanda F, Nascimento, Maria C, Pannuti, Claudio S, Carrilho, Flair J, Pinho, Joao RR Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão, Brazil. Virology journal, 8: 415, 25 August 2011.....	269
24-Barros, Lena Maria Fonseca, Gomes-Gouvêa, Michele Soares, Kramvis, Anna, Mendes-Corrêa, Maria Cássia Jacintho, Santos, Alexsandro Dos, Souza, Letícia Alana Barros, Santos, Max Diego Cruz, Carrilho, Flair José, Domicini, Arnaldo De Jesus, Pinho, João Renato Rebello, Ferreira, Adalgisa De Souza Paiva. High prevalence of hepatitis B virus subgenotypes A1 and D4 in Maranhão State, Northeast Brazil. Infection, Genetics and Evolution 2014 Jun;24:68-75.....	276
25-Alcione O Santos, Monica V Alvarado Mora, Lívia Botelho, Deusilene S Vieira, João R Rebello Pinho, Flair J Carrilho, Eduardo R Honda, Juan M Salcedo. Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. Virology Journal 2010, 7:315.....	283
26-Conde SR, Moia L de J, Barbosa MS, Amaral I do S, Miranda EC, Soares M do C, Brito EM, Souza Odo S, de Araujo MT, Demachki S, REBELLO JR, Mesquita MG, Denis AB, Ishak R. Prevalence of Hepatitis B virus genotypes and the occurrence of precore mutation A-1896 and to correlate them with the clinical presentation of chronic hepatitis, in a population group of the Eastern Amazon region. Rev Soc Bras Med Trop. 2004; 37 (Suppl 2):33-9 (2004).....	291
27-Michele Soares Gomes-Gouvêa; Manoel do Carmo Pereira Soares; Isabel Maria Vicente Guedes C Mello; Elisabete Maria Figueiredo Brito; Lizomar de Jesus Pereira Moia; Gilberta Bensabath; Heloisa Marceliano Nunes; Flair José Carrilho; JOÃO	

RENATO REBELLO PINHO. Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin. <i>Acta Tropica</i> 106: 149 – 155 (2008).....	299
28-Gomes-Gouvêa MS, Soares MC, Bensabath G, de Carvalho-Mello IM, Brito EM, Souza OS, Queiroz AT, Carrilho FJ, PINHO JR. Hepatitis B and Delta virus genotypes in Outbreaks of fulminant Hepatitis (Labrea black fever) in the Western Brazilian Amazon region. <i>Journal of General Virology</i> . 2009 Nov;90 (Pt 11):2638-2643.	307
29-Carrilho FJ, Ono-Nita SK, Cardoso RA, Cançado ELR, PINHO JRR, Alves VAF, Da Silva LC. A prospective study of Hepatitis B virus markers in patients with chronic HBV infection from Brazilian families of Western and Asian origin. <i>Brazilian Journal of Medical and Biological Research</i> 38 (9): 1399-1408 (2005).....	314
30-Cintia Mendes Clemente, Flair José Carrilho, João Renato Rebello Pinho, Suzane Kioko Ono-Nita, Luiz Caetano Da Silva, Regina Célia Moreira, Marcílio Figueiredo Lemos, Isabel Maria Vicente Guedes de Carvalho Mello. A phylogenetic study of Hepatitis B virus in chronically infected Brazilian patients of Western and Asian descent. <i>Journal of Gastroenterology</i> , 2009. 44 (6): 568-576	325
31-Sitnik R, Sette Jr H, Santana RAF, Menezes LC, Graça CHN, Dastoli GTF, Silbert S, PINHO JRR. Hepatitis B virus genotype E detected in Brazil in an African patient who is frequent traveler. <i>Brazilian Journal of Medical and Biological Research</i> (2007), 40 (12): 1689-92.....	335
32-Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvêa MS, de Azevedo Neto RS, Carrilho FJ, Pinho JR. Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population-how is the epidemiological situation? <i>PLoS One</i> . 2011 Apr 29;6(4):e18888	340
33-Alvarado-Mora MV, Romano CM, Gomes-Gouvêa MS, Gutierrez MF, Carrilho FJ, R Pinho JR. Phylogenetic analysis of complete genome sequences of hepatitis B virus from an Afro-Colombian community: presence of HBV F3/A1 recombinant strain. <i>Virology Journal</i> . 2012 Oct 24;9: 244	347
34-Mauricio Venegas, Mónica V. Alvarado-Mora, Rodrigo A. Villanueva, João R. Rebello Pinho, Flair J. Carrilho, Stephen Locarnini, Lilly Yuen, and Javier Brahm. Phylogenetic Analysis of Hepatitis B Virus Genotype F Complete Genome Sequences from Chilean Patients with Chronic Infection. <i>Journal of Medical Virology</i> 83:1530–1536 (2011). DOI: 10.1002/jmv.22129	355

35-Martinez AA, Zaldivar Y, Hong CCh, Alvarado-Mora MV, Smith R, Ortiz AY, Pinho JR, Cristina J, Pascale JM. Molecular characterisation of hepatitis B virus in the resident Chinese population in Panama City. Mem Inst Oswaldo Cruz. 2013 Aug;108(5):541-7	363
36-Mónica Viviana Alvarado Mora, Camila Malta Romano, Michele Soares Gomes-Gouvêa, Maria Fernanda Gutierrez, Flair José Carrilho, João Renato Rebello Pinho. Molecular epidemiology and genetic diversity of Hepatitis B virus (HBV) genotype E in an Afro-Colombian isolated community. Journal of General Virology 2010 Feb; 91(Pt 2):501-8	371
37-Alvarado, M.V., Romano, C. M., Gomes-Gouvea, M. S., Gutierrez, M. F., Carrilho, F. J., PINHO, J. R. R. 2010. Molecular Characterization of the Hepatitis B virus genotype in Colombia: A Bayesian Inference on the Genotype F. Infection, Genetics and Evolution 2011, 11 (1): 103-108	380
38-Godoy BA, Alvarado-Mora MV, Gomes-Gouvêa MS, Pinho JR, Fagundes NJ. Origin of HBV and its arrival in the Americas - the importance of natural selection on time estimates. Antivir Ther. 2013;18(3 Pt B):505-512.....	387
39-Bouckaert R, Alvarado-Mora MV, Pinho JR. Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods. Antivir Ther. 2013;18(3 Pt B):497-503.....	396
4-CONCLUSÕES.....	405
5-REFERÊNCIAS.....	414

INTRODUÇÃO

1-INTRODUÇÃO

Este trabalho será apresentado na forma de “**TEXTO SISTEMATIZADO SOBRE A OBRA DO CANDIDATO**”.

A parte introdutória consta de dois capítulos, o primeiro capítulo é uma introdução sobre o aparecimento da biologia molecular, desde os experimentos iniciais que levaram à descoberta dos ácidos nucleicos, a sua associação com a transmissão de informações genéticas entre diferentes gerações de organismos e os passos que foram necessários para o esclarecimento de suas estruturas e como esta estrutura se organizava para poder carregar informações genéticas.

Brevemente são descritos os progressos tecnológicos que levaram inicialmente à criação da engenharia genética, como mecanismo de estudo em geral de organismos vivos. Estes estudos levaram à clonagem de genes e expressão de proteínas recombinantes. Em paralelo, houve o desenvolvimento de uma série de metodologias que permitiram aprofundar o conhecimento da biologia humana e de seus parasitas, dentre as quais podemos destacar as técnicas de hibridação de ácidos nucleicos e principalmente a reação em cadeia da polimerase (PCR). Com esta metodologia, foi então possível se avançar de forma importante no conhecimento das patologias humanas, com suas aplicações na área de diagnóstico e de sequenciamento de ácidos nucleicos. Estas técnicas foram passando por sucessivas melhorias com o passar dos anos e agora já dispomos de metodologias de PCR em tempo real com vasta aplicação em pesquisa, assim como é o caso de metodologias de sequenciamento de nova geração que tornam possível sequenciar genomas completos.

O segundo capítulo é dedicado à Hepatite B. As hepatites virais são o principal tema de minhas pesquisas e a princípio cheguei a planejar um texto que abordassem todas elas, com as quais tenho atuado desde o início de minha carreira, mas para escrever um texto de qualidade suficiente para apresentar em um exame de livre docência, resolvi me dedicar ao vírus de hepatite B (HBV), que é o agente sobre o qual tenho mais publicações.

Neste capítulo, iniciamos com a descoberta do vírus; falamos sobre a epidemiologia da doença; descrevemos detalhes sobre a estrutura viral (tanto sobre suas proteínas como também sobre seus ácidos nucleicos) bem como sobre a replicação viral; mostramos como o HBV é classificado em função das diferenças genéticas encontradas entre os diferentes isolados virais; descrevemos sucintamente os principais aspectos clínicos e de diagnóstico e monitoramento laboratorial; mostramos as principais opções terapêuticas; e, finalmente, descrevemos as implicações da diversidade genética viral para explicar aspectos clínicos e laboratoriais da doença. Já estão inseridas como referências deste capítulo, algumas das contribuições realizadas pelo meu grupo de pesquisas nestes últimos anos.

Os trabalhos citados são então colocados na próxima seção deste texto sistematizado, mostrando as colaborações realizadas pelo meu grupo de pesquisas.

CAPÍTULO 1 – ESTUDOS DE AGENTES INFECCIOSOS POR MÉTODOS MOLECULARES

Em 1938, Warren Weaver, da Rockefeller Foundation, um cientista americano que estudava como as ferramentas da física e da química podiam auxiliar na compreensão dos processos biológicos, criou o termo **biologia molecular**.

Em 1940, George Beadle e Edward Tatum demonstraram a existência de uma relação precisa entre os genes e proteínas (1). Em 1944, Oswald Avery, Colin MacLeod, e Maclyn McCarty provaram que o ácido desoxirribonucleico (DNA) era a substância que provocava transformação bacteriana (2), explicando o fenômeno da transformação bacteriana realizado por Frederick Griffith em 1928 (3). Em 1952, Alfred Hershey e Martha Chase confirmaram que o material genético do bacteriófago, o vírus que infecta bactérias, é composto de DNA (4). Erwin Chargaff publicou em 1947 que as proporções dos quatro nucleotídeos variam entre duas amostras diferentes de DNA, mas que para pares particulares de nucleotídeos - adenina e timina, guanina e citosina - os dois nucleotídeos estão sempre presentes em proporções iguais (5).

Usando dados de difração de raios -X, assim como outros dados de Rosalind Franklin e a sua informação de que as bases eram emparelhadas, James Watson e Francis Crick chegaram ao primeiro modelo preciso da estrutura molecular do DNA em 1953 (6). A confirmação do mecanismo de replicação semiconservativa implicado pela estrutura em dupla hélice foi realizada em 1958 por Matthew Meselson e Franklin Stahl (7). Em 1961, François Jacob e Jacques Monod demonstraram que os produtos de determinados genes sobre a expressão de outros genes por ação sobre sítios específicos na borda desses genes, denominados posteriormente como promotores.

Eles também lançaram a hipótese da existência de um intermediário entre o DNA e os seus produtos proteicos, o qual denominaram RNA mensageiro (8). Neste mesmo ano, Francis Crick, Sydney Brenner, Leslie Barnett e RJ Watts-Tobin demonstraram que as três bases do código de DNA codificavam para um aminoácido no código genético. O experimento elucidou também a natureza da expressão gênica e das mutações frame-shift. Na experiência, as mutações induzidas por proflavina no gene *rIIIB* do bacteriófago T4, foram isolados. Proflavina provoca mutações inserindo-se entre as bases do DNA, resultando na inserção ou deleção de um único par de bases (9).

Em 1970, Hamilton Smith e Kent W. Wilcox descobriram a primeira enzima de restrição do tipo II, que permitiu o corte de moléculas de DNA em sítios específicos (10), que eram depois separados por eletroforese em gel. Isso permitiu aos cientistas isolar genes de genoma de um organismo (11). DNA ligases que juntam os pedaços cortados de DNA tinham sido descobertas anteriormente em 1967 (12) e pela combinação das duas enzimas foi possível cortar e colar sequências de DNA para criar DNA recombinantes. Os plasmídeos, descobertos em 1952, (13) e tornaram-se ferramentas importantes para a transferência de informações entre as células e para a replicação de sequências de DNA. Allan Maxam, Walter Gilbert e Frederick Sanger desenvolveram métodos para sequenciamento de DNA em 1977, aumentando a informação genética disponível para pesquisadores (14, 15).

Em 1972, Paul Berg utilizando enzimas de restrição e DNA ligases criou as primeiras moléculas de DNA recombinante entre o vírus SV40 de macacos com o bacteriófago λ (lambda) (16). Herbert Boyer e Stanley N. Cohen introduziram o DNA recombinante em uma célula bacteriana, através de uma enzima de restrição que

cortava o plasmídeo pSC101 em um único ponto e foram capazes de inserir e ligar um gene que confere resistência ao antibiótico tetraciclina. Cohen tinha previamente desenvolvido um método em que os plasmídeos podiam ser introduzidos nas bactérias e foram capazes de criar uma bactéria que sobrevivia na presença de tetraciclina, o que representou o primeiro organismo geneticamente modificado. Repetiram experiências demonstrando que outros genes podem ser expressos em bactérias, incluindo um gene da rã *Xenopus laevis*, que foi a primeira clonagem de um gene de organismo eucarioto (17).

A produção de proteínas recombinantes em larga escala preservando as suas funções biológicas decorrentes de processamentos pós-tradução como, por exemplo, a glicosilação, ocorre em sistemas de expressão, em organismos eucariotos como as leveduras ou em células de mamíferos, utilizando vetores virais para a expressão gênica (18) e a produção de antígenos por estas metodologias foi muito importante para a obtenção das primeiras vacinas recombinantes humanas, que foram desenvolvidas contra a hepatite B em células de mamíferos e na levedura *Saccharomyces cerevisiae* (19). Foi por muito tempo a única vacina aprovada para uso humano em larga escala baseada em engenharia genética, até recentemente, quando foi aprovada a vacinação para o Papilomavírus (20). Curiosamente, estas duas vacinas são protetoras contra os cânceres relacionados a estes vírus.

Em paralelo a isso, foram desenvolvidas metodologias para avaliar a presença de moléculas de ácidos nucleicos em amostras biológicas, baseadas na complementaridade das duas fitas de ácidos nucleicos, i.e., a partir de uma sonda de DNA de um determinado gene ou organismo, poderia se procurar a presença deste em

diferentes materiais biológicos. A primeira técnica desenvolvida para este fim recebeu o nome de *Southern blot* a partir do nome do cientista que a havia desenvolvido: amostras de DNA de alto peso molecular eram digeridas com enzimas de restrição, separadas por eletroforese em gel de agarose e transferidas para uma membrana de nitrocelulose que era então submetida a reação de hibridização com uma sonda específica de DNA (21). Outras variantes desta técnica analisando moléculas de RNA ou de proteínas foram chamadas de *Northern blot* e *Western blot*. Técnicas de *dot blot* permitiam a detecção destas diferentes biomoléculas simplesmente submetendo à hibridização amostras biológicas depositadas sobre uma membrana de nitrocelulose.

Em 1983, Kary Mullis desenvolveu a reação em cadeia da polimerase (PCR), uma tecnologia capaz de amplificar a partir de uma única cópia de DNA em várias ordens de grandeza, gerando milhares a milhões de cópias de uma sequência de DNA particular (22). Esta técnica em muito facilitou a clonagem de genes; o diagnóstico de doenças infecciosas e genéticas e a identificação de amostras biológicas, como nas ciências forenses e testes de paternidade. Diversas variantes desta técnica foram criadas com diferentes aplicações, sendo particularmente utilizadas as técnicas de PCR em tempo real para a quantificação da expressão gênica, quantificação de agentes patogênicos e detecção de polimorfismos de nucleotídeos únicos.

Na área de sequenciamento de ácidos nucleicos, a primeira mudança que ocorreu foi o desenvolvimento de sequenciadores automatizados de DNA que determinavam a sequência de ácidos nucleicos a partir da leitura de fluorescências emitidas pelas bases nitrogenadas modificadas incorporadas (23). Aliadas aos progressos na marcação fluorescente, eletroforese capilar e outras automatizações

(também na área de clonagem de ácidos nucléicos), as aplicações desta técnica foram diversas. Foi utilizada em grandes projetos de sequenciamento de genomas, como os famosos projetos do genoma humano; bem como foi incorporada em laboratórios clínicos para o sequenciamento de genes específicos para o diagnóstico de doenças genéticas e para a caracterização de genótipos e de variantes resistentes a drogas em agentes infecciosos (24).

O sequenciamento de nova geração começa a substituir o sequenciamento de Sanger especialmente porque esta metodologia possibilita a realização de reações de sequenciamento de amplas regiões do genoma em períodos de tempo muito mais curtos do que seria possível de se imaginar previamente, bem como os custos por sequência realizada caem de forma importante. Por outro lado, os equipamentos ainda são extremamente custosos e algumas das metodologias ainda apresentam taxas de erros importantes, sendo que alguns resultados ainda necessitam ser confirmados pelos sequenciadores que utilizam a metodologia de Sanger (25). Aplicações em virologia já começam a ser publicadas e se espera que estes trabalhos venham facilitar os estudos de genomas virais completos, incluindo a análise da diversidade genética viral (quasispécies) em uma só amostra (26).

CAPÍTULO 2 - HEPATITE B

1. DESCOBERTA DO VÍRUS DA HEPATITE B

A existência de uma forma de hepatite de transmissão parenteral foi documentada em 1885, transmitida por vacina antivariólica. Vários casos foram referidos na literatura, até que, em 1947, McCallum (27) definiu o termo Hepatite B para esta entidade. O mesmo experimento realizado numa instituição de deficientes mentais que permitiu a identificação da linhagem original do VHA (MS - 1) levou também à identificação de outra linhagem (MS-2) não relacionada à anterior, que causava hepatite de transmissão primordialmente parenteral e com maior período de incubação (28).

O antígeno Austrália foi descoberto por Blumberg (29), em 1963, no soro de um aborígene australiano (detectado com outro soro de um paciente norte-americano), sendo inicialmente associado com uma série de diferentes patologias. Sua associação com a hepatite B foi feita em 1968, por Prince (30) e Okochi & Murakami (31), recebendo posteriormente o nome de antígeno de superfície da hepatite B (HBsAg). Em 1970, Dane e cols. (32) visualizaram pela primeira vez a partícula viral íntegra do HBV. Posteriormente, foram descritos outros antígenos associados ao HBV, como veremos depois.

2. EPIDEMIOLOGIA DA HEPATITE B NO BRASIL E NO MUNDO

A hepatite B é uma doença de distribuição universal, presente em todos os continentes, porém com variações nos níveis de endemicidade. A Organização Mundial de Saúde estima que cerca de 2 bilhões de pessoas tenham sido infectadas pelo HBV

em algum momento de suas vidas e que cerca de 350 a 400 milhões desses indivíduos tenham se tornado portadores crônicos do vírus. Estima-se que essa infecção seja a principal causa de carcinoma hepatocelular em todo o mundo e que cerca de 600.000 mortes por ano sejam devidas à infecção aguda ou crônica pelo HBV (33).

A prevalência do HBV e os padrões de transmissão variam muito nas diferentes partes do mundo, dependendo da taxa de infecção crônica, da proporção de pessoas com o vírus replicando ativamente e da via predominante de transmissão em cada região (34). As regiões do mundo foram divididas de acordo com o padrão de endemicidade da hepatite B, que é classificado com base na prevalência do HBsAg na população geral: baixa (0,1 a 2%), intermediária (2 a 7%), alta (>8%) (35).

O HBV é transmitido principalmente através do sangue, mas outros fluidos como sêmen, secreções vaginais e saliva podem estar envolvidos na transmissão desse agente. Há três principais formas de transmissão do HBV: vertical, sexual e parenteral (7).

Nas áreas com prevalência intermediária ou alta a maioria dos indivíduos se infecta no momento do nascimento (transmissão vertical) ou durante os primeiros anos da infância pelo contato com portadores do vírus, o que ocorre com mais frequência entre membros da mesma família. Já nas áreas de baixa prevalência a infecção é adquirida primariamente durante a vida adulta, principalmente pela via sexual (33, 34).

Em geral, quando a infecção pelo HBV ocorre em indivíduos adultos, a mesma tende a evoluir para a cronicidade em cerca de 5% dos indivíduos infectados.

Entretanto, o risco de progressão para a cronicidade varia dependendo da idade de infecção, sendo inversamente proporcional à idade: acima de 90% daqueles infectados no momento do nascimento tornam-se cronicamente infectados, o que também ocorre em cerca de 30 a 50% dos indivíduos infectados durante os primeiros 5 anos (33, 34).

A introdução da vacinação contra a hepatite B tem modificado a distribuição dessa doença em diferentes regiões do mundo e no Brasil. No Brasil a prevalência de portadores de hepatite B é variável devido à larga extensão geográfica do país e à sua diversidade econômica e cultural. Em garimpeiros da Amazônia brasileira, foi encontrada uma taxa de exposição ao HBV de 82,9%, sendo 7,1% portadores crônicos. (36) Ainda na região amazônica brasileira, em certos grupos indígenas estima-se prevalência de 3,3% a 9% (37).

Recentemente um inquérito nacional de base populacional foi realizado com o objetivo, entre outros, de estimar a prevalência das hepatites A, B e C na população de 13 a 69 anos das capitais das cinco macrorregiões brasileiras e no distrito Federal. A prevalência global do HBsAg referente ao conjunto das capitais do Brasil foi de 0,37% classificando o país como uma região de baixa endemicidade (38).

As vacinas mais comumente usadas na atualidade para a prevenção dessa infecção são as que utilizam a tecnologia do DNA recombinante. As mais utilizadas são a Engerix-B (39) e a Recombivax-HB (40).

O esquema vacinal recomendado para adultos é de três doses (com intervalos de 0, 1, 6 meses), por via intramuscular. Doses maiores de vacinas ou número maior de

doses podem ser recomendadas em pacientes imunodeprimidos e em pacientes em regime de hemodiálise. A recomendação para utilização da vacina para hepatite B é universal, em diferentes regiões do mundo. Em alguns países, a recomendação se restringe a grupos de maior risco para aquisição dessa infecção.

A profilaxia pós-exposição ao HBV (acidentes percutâneos e contato sexual) deve ser realizada com a utilização de imunoglobulina específica para hepatite B (HBIG) e vacinação. Nessa situação, recomenda-se 0,06mL/kg (ou 5 mL para adultos) de HBIG, via intramuscular, logo após o contato com o caso índice de hepatite. De preferência a utilização de HBIG deverá acontecer nas primeiras 24h após o contato. Na profilaxia pós-natal, recomenda-se vacinação e HBIG, dentro das primeiras 24h de vida do neonato de mãe infectada pelo HBV (41).

3. CLASSIFICAÇÃO DO HBV

O vírus da Hepatite B está atualmente classificado dentro da família *Hepadnaviridae*, que compreende uma série de vírus hepatotrópicos que infectam outras espécies e que compartilham características estruturais e funcionais, dentro do gênero *Orthohepadnavirus* (42). Além do HBV, este gênero inclui vírus encontrados na marmota *Marmota monax* (WHV - Woodchuck Hepatitis Virus) (43), no esquilo *Spermophilus beecheyi* (GSHV - Ground Squirrel Hepatitis Virus) (44), no esquilo do ártico *Callosciurus erythracus* (ASGHV - Arctic Ground squirrel hepatitis virus) (45), no macaco barrigudo *Lagothrix lagotricia* –WMHBV Woolly Monkey Hepatitis B Virus) (46) e em diversas espécies de morcegos como *Hipposideros pomona*, *Miniopterus fuliginosus*, *Hipposideros cf. ruber* e *Uroderma bilobatum* (BHBV – Bat Hepatitis B Virus) (47). Este gênero compreende também vírus muito semelhantes ao humano, encontrados em macacos antropóides como gibões *Hylobates lar* (48), chimpanzés *Pan troglodytes verus* (49), orangotangos *Pongo pygmaeus* (50) e gorilas *Gorilla gorilla* (51). O fato de vírus análogos serem encontrados em diversas espécies de macacos leva a se levantar a hipótese de que o HBV tenha infectado a espécie humana a partir do contato com estes macacos, de forma análoga ao que foi descrito ao HIV.

Esta família compreende também o gênero *Avihepadnaviridae*, com vírus encontrados em aves: no pato *Anas domesticus* (52) (DHBV - Duck Hepatitis B Virus), na hepatite B da garça *Ardea cinerea* (53) (HHBV – Heron Hepatitis B Virus), no ganso *Anser caerulescens* e no ganso de Ross *Anser rossii* (17) (SGHV – Snow Goose Hepatitis B Virus), no grou coroadado *Balearica regulorum* (CCHBV – Grey crowned crane Hepatitis B Virus), (54) na cegonha *Ciconia ciconia* (STHBV – Stork Hepatitis B Virus) (55) e nos

papagaios *Psittacula krameri*, *Psittacus erithacus*, *Psittacula eupatria* e *Platycercus elegans* (PHBV – Parrot Hepatitis B Virus) (56, 57).

As características comuns aos hepadnavírus são: seu tropismo por células hepáticas, partículas virais envelopadas com nucleocapsídeo de simetria icosaédrica, genoma formado por DNA fita dupla parcial e replicação através de intermediários RNA, via transcrição reversa (58).

4. ESTRUTURA DO HBV

As células infectadas pelo HBV produzem diferentes tipos de partículas relacionadas ao vírus, as quais podem ser identificadas por microscopia eletrônica no soro de indivíduos infectados: 1- Partículas completas infecciosas (Partículas de Dane) com 42 a 47 nanômetros (nm) de diâmetro, formadas pelo envelope e um nucleocapsídeo icosaédrico de 27 nm contendo a molécula de DNA e a polimerase viral; 2- Partículas incompletas esféricas de 17 a 25 nm; 3- Partículas incompletas cilíndricas (tubulares) com cerca de 20 a 22 nm e tamanho variável. As partículas incompletas são constituídas apenas pelo envelope viral, e não contém DNA, portanto não são infecciosas. Essas partículas são secretadas em grande quantidade, excedendo a produção das partículas completas em 1.000 a 10.000 vezes (59).

4.1. PROTEÍNAS VIRAIS

4.1.1. ANTÍGENOS DE SUPERFÍCIE (ENVELOPE)

O principal antígeno do envelope do HBV é o HBsAg, mas também encontramos as proteínas L (“large”) e M (“medium”), que contém os antígenos correspondentes às regiões pré S1 e pré S2 do genoma viral, além de compartilharem as regiões antigênicas do HBsAg. O HBsAg é encontrado no envelope viral em sua forma não - glicosilada (p25) e glicosilada (gp29), a proteína M em duas formas glicosiladas (gp33 e gp36) e a proteína L em uma forma não - glicosilada (p39) e outra glicosilada (gp42) (60).

A principal proteína do envelope do HBV é denominada de HBsAg, é altamente imunogênica e é a base da vacina utilizada contra a infecção pelo HBV, que inicialmente consistia em HBsAg isolado do plasma de indivíduos infectados e atualmente são utilizadas proteínas HBs purificadas produzidas por engenharia genética. Os anticorpos anti-HBs gerados pela vacinação ou que constituem a imunoglobulina hiperimune são predominantemente dirigidos contra um epítopo antigênico presente no HBsAg denominado de determinante “a” (59, 61).

As proteínas L e M estão presentes em maior proporção nas partículas virais íntegras e partículas ocas cilíndricas e têm um papel fundamental na infecção pelo HBV, participando no processo de captação do vírus pelas células hepáticas e do processo de morfogênese da partícula viral (62).

As proteínas L, M e S compartilham um domínio na extremidade carboxila com quatro hélices putativas para ancoragem na membrana. As duas extensões terminais

(pre-S2 e preS-1/2) de L e M servem diversas funções, tais como a ligação do nucleocapsídeo durante o envelopamento (63) e o reconhecimento do receptor (64).

A penetração do HBV dentro das células hepáticas parece ser mediada por sequências de aminoácidos encontradas nas regiões pré S1 e pré S2. A adesão do HBV na superfície do hepatócito requer a ligação com proteoglicanas de sulfato de heparana (HSPG) como um pré-requisito para a ligação específica com o polipeptídeo de co-transporte taurocolato de sódio (NTCP), através da extremidade amina do domínio pré S1. NTCP é especificamente presente na membrana sinusoidal/basolateral do hepatócito, desempenhando um papel importante no hepatotropismo do HBV e na especificidade do hospedeiro e, presumivelmente, participa do processo de endocitose do HBV. Após a fusão por um mecanismo desconhecido, o nucleocapsídeo é liberado e transportado ao longo dos microtúbulos para o núcleo (65). Cotransportadores de sódio/ácido biliar são glicoproteínas integrais de membrana que participam da circulação entero-hepática dos ácidos biliares. Dois transportadores homólogos estão envolvidos na reabsorção de ácidos biliares, uma absorção a partir do lúmen intestinal, o ducto biliar, e o rim com uma localização apical (SLC10A2), e o outro nas membranas basolaterais de hepatócitos (SLC10A1) (66).

Existem pelo menos três determinantes para a capacidade de infecção nas proteínas L e S:

1) um está localizado na alça antigênica do domínio S que é dependente da formação de pontes dissulfeto formando a estrutura terciária. (67, 68) A proteção através de anticorpos por imunidade induzida adquirida de infecção resolvida ou por vacinas contra o HBV é mediada por epítopos conformacionais dentro deste domínio;

2) O segundo determinante é composto de três grupos hidrofóbicos (ϕ) no âmbito da primeira região transmembrana da proteína L que provavelmente participa na fusão da membrana (69);

3) O determinante melhor caracterizado é um motivo miristoilado na extremidade amina do subdomínio preS1, no qual os mutantes com deficiência de miristoilação montam a partícula viral, mas não são infecciosos (70, 71).

Inserções e/ou deleções de aminoácidos (a.a.) nos domínios pré S1 e pré S2 da proteína L destacam o papel essencial para os a.a. 2-75 na infectividade da partícula. (72, 73) A sequência a jusante do a.a. 75 não é importante para a entrada do vírus; no entanto, contém o domínio de ligação com o nucleocapsídeo e fornece um espaçador eficaz para permitir o envelopamento (74). Um peptídeo sintético dos a.a. 2-78 com ácido mirístico na extremidade amino inibe a infecção por HBV (75). A extremidade carboxila diminuída em relação ao a.a. 48 aumenta a atividade anti-viral (76) e é um potente inibidor do HBV *in vivo* (77). Estudos recentes demonstram a alta afinidade dos peptídeos Pre S contendo a região conservada de a.a.9 - NPLGF(F/L) P-15 com os hepatócitos (64) e com o receptor recentemente descoberto, polipeptídeo de co-transporte taurocolato de sódio (NTCP) (78). Estes peptídeos, com o nome de Myrcludex B está atualmente sendo testado em ensaios clínicos fase II para o tratamento de pacientes com hepatite crônica B e delta (65).

A maior hidrofiliabilidade e localização externa das regiões pré S1 e pré S2 lhes conferem grande imunogenicidade (63). Anticorpos protetores específicos contra epítomos destas regiões foram descritos (60), bem como a evolução favorável da doença com o aparecimento desses anticorpos (79). Estas observações sugerem que a

presença das regiões pré S1 e pré S2 pode superar a ausência de resposta à vacina em alguns indivíduos (80).

As proteínas M e L possuem também atividade transativadora de outros promotores, podendo participar do processo de carcinogênese induzida pelo HBV. (81, 82) A utilização de peptídeos sintéticos e antígenos recombinantes correspondentes às regiões pré S1 e pré S2 permitiu estabelecer uma correlação entre estes antígenos e a presença de replicação viral, estabelecendo um valor prognóstico destes antígenos na evolução da hepatite B (83).

4.1.2. ANTÍGENOS CENTRAIS

4.1.2.1. ANTÍGENO DO CAPSÍDEO (HBcAg) E HBeAg

Nas partículas virais íntegras são encontrados outros antígenos relacionados ao nucleocapsídeo viral, sendo seu principal constituinte o HBcAg, que é uma proteína de peso molecular 22kDa codificada pelo gene C, fosforilada em resíduos de serina presentes no terço carboxi-terminal. Anticorpos contra esta proteína não são protetores, e podem mesmo facilitar o desenvolvimento de hepatite em recém-nascidos de mães portadoras do HBV. Em contraste, chimpanzés imunizados com HBcAg ficaram protegidos contra a infecção viral. Exceto em casos de transmissão vertical, este antígeno estimula células T auxiliares na produção de anticorpos contra proteínas do envelope viral quando apresentadas conjuntamente na mesma partícula e induz células T citotóxicas específicas (60, 84).

Outro antígeno associado ao core viral encontrado em forma solúvel no soro de indivíduos infectados pelo HBV é o HBeAg. A proteína HBe não faz parte da estrutura da partícula viral, podendo ser encontrada apenas em forma solúvel no soro de indivíduos infectados e sua detecção é indicativa de altos níveis de replicação viral (59). Apesar do HBcAg poder ser transformado em HBeAg por ação enzimática, é pouco provável que este seja o mecanismo principal de sua formação *in vivo*. O HBeAg é um derivado da proteólise de um precursor com regiões comuns ao HBcAg (p25), produzido quando a tradução se inicia a montante da região C do genoma viral, englobando a região pré C (85). A região pré C codifica um peptídeo sinal que leva à translocação da proteína produzida através do retículo endoplasmático, conduzindo-a às vias de secreção de proteínas pela célula. O HBeAg maduro é formado pela clivagem do peptídeo sinal e da porção carboxi-terminal, possivelmente pela ação de protease celular (86, 87). Esta clivagem pode ser catalisada por proteases celulares ou pelo próprio precursor, que contém sequências análogas a encontradas em outras enzimas desta classe. Além do HBeAg, esta última clivagem origina uma pequena proteína rica em arginina, semelhante à protamina (87), que participaria do processo de encapsidação do complexo RNA pré-genômico acoplado à transcriptase reversa. (88) De qualquer forma, o HBeAg não é essencial para o vírus, pois existem mutantes não produtores deste antígeno, associados com formas graves de hepatite, como será visto detalhadamente mais adiante.

O HBeAg solúvel encontrado no soro deve modular a atividade de células T citotóxicas anti-HBc, através de epítomos comuns, contribuindo para o estabelecimento da persistência da infecção. Por outro lado, o aparecimento de

anticorpos contra este antígeno sinaliza evolução favorável da doença. Este fato, aliado aos efeitos protetores do HBcAg na prevenção da Hepatite B em chimpanzés, levou alguns autores a proporem a inclusão de peptídeos derivados do HBcAg ou HBeAg nas vacinas contra esta doença, que permitissem aumentar a sua imunogenicidade, evitando ao mesmo tempo as possíveis regiões supressoras encontradas no HBcAg (80).

O HBeAg atua como um tolerógeno de células T regulando a resposta imune do hospedeiro ao HBcAg presente nos hepatócitos. Também tem a capacidade de atravessar a barreira placentária e induzir tolerância imunológica in utero. Tal ação se dá pelo fato do HBeAg possuir epítomos em comum com o HBcAg, os quais são reconhecidos pelas células T citotóxicas envolvidas no dano celular durante a infecção (89).

A capacidade de expressão do HBeAg é importante para o estabelecimento de infecção crônica, mas não é essencial para a replicação viral. A regulação imunológica exercida pelo HBeAg pode predispor à evolução para a cronicidade nos casos de infecção perinatal e prevenir o dano hepático nos casos de infecção durante a vida adulta (89).

A soroconversão do HBeAg para o anti-HBe geralmente indica a redução da fase de replicação ativa do vírus, sendo um dos objetivos no tratamento de indivíduos HBeAg positivos. Durante a fase crônica, a soroconversão para anti-HBe é frequentemente acompanhada pelo aumento da atividade inflamatória e necrose com elevação de transaminases, podendo em alguns casos evoluir para uma hepatite

fulminante. A soroconversão durante a fase aguda da infecção geralmente ocorre sem complicações e indica que o indivíduo está progredindo para a recuperação (90).

A detecção do HBeAg pode ser usada clinicamente como um marcador de replicação viral, infectividade, severidade da doença e resposta ao tratamento. Entretanto, algumas cepas possuem mutações no genoma que promovem a interrupção ou redução da síntese desse antígeno. Pacientes portadores dessas cepas irão apresentar soroconversão para anti-HBe e manutenção da replicação viral, que geralmente apresenta níveis inferiores àqueles encontrados nos pacientes HBeAg positivos (90).

4.1.2.2. ANTÍGENO HBx (HBxAg)

Além destas proteínas, pelo menos duas outras são produzidas pelo HBV, o HBxAg e a DNA polimerase viral. A presença da proteína HBxAg foi inferida pela descoberta de uma fase de leitura aberta na sequência do genoma viral. Posteriormente, a clonagem de segmentos desta sequência em organismos eucarióticos ou procarióticos confirmou a presença desta proteína durante a infecção natural, sendo associada com o aparecimento de carcinoma hepatocelular (91). O HBxAg é essencial para a iniciação da transcrição do DNA e manutenção do DNA circular covalentemente fechado (cccDNA) que se acumula no núcleo dos hepatócitos infectados: Este antígeno estimula a acetilação de histonas associadas com cccDNA e é necessário para a transcrição no contexto de infecções por HBV (92). HBxAg promove a expressão dos genes virais recrutando a histona acetilases CBP / p300 e PCAF / GCN5 ao cccDNA (93). Adicionalmente, um estudo recente mostrou que HBx promove a

expressão de gene do cccDNA, mas não a partir de uma cópia cromossomicamente integrada de HBV (94). A replicação do HBV é regulada pelo estado de acetilação das histonas H3 e H4 ligadas ao cccDNA (95), bem como as acetiltransferases celulares p300 e CBP e a deacetilase celular HDAC1, que se ligam ao cccDNA (93). Este tema foi revisto recentemente por Ezzikouri e cols. (96).

A demonstração de atividade transativadora do HBxAg sobre promotores de genes do HBV, bem como derivados de outros vírus, veio substanciar a hipótese do papel desta proteína na carcinogênese induzida pelo HBV (97). Foi também demonstrado que esta proteína interfere com a capacidade de reparo de DNA pela célula, o que potencializa seu efeito oncogênico (98).

Como um transativador, HBx pode modular NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) e fator de transcrição AP-2. Além disso, HBx pode afetar RNAs não-codificantes reguladores (ncRNAs), incluindo microRNAs e RNAs longos não codificantes (lncRNAs), como miRNA-205 e HULC (highly upregulated in liver cancer). HBx também está envolvido em modificações epigenéticas, incluindo metilação e acetilação. HBx interage com várias vias de transdução de sinal, tais como Akt, Wnt/ β -catenina, o transdutor proteína quinase B / sinal e o ativador da transcrição, e as vias de PKC, raf ou NF- κ B. Além disso, HBx afeta o destino celular, deslocando o equilíbrio para a sobrevivência da célula: pode levar à perda de funções apoptóticas ou diretamente contribuir para a oncogênese através de funções transformadoras, que induzem hepatocarcinogênese (46, 91).

4.1.2.3. DNA POLIMERASE

A DNA polimerase viral (P) teve sua atividade constatada em partículas virais purificadas (99) e sua atividade foi confirmada após expressão em *S. cerevisiae*. (100) Surpreendentemente, se demonstrou que esta enzima tinha a atividade de transcriptase reversa (RT), i.e., era capaz de gerar moléculas de DNA complementar e a partir de moléculas RNA (43).

Comparando-se a sequência do HBV com a sequência de retrovírus, determinou-se a região do genoma responsável por sua codificação, bem como a localização de seus domínios (101). Estes domínios são:

1 – na extremidade amino-terminal, encontra-se a região que se liga à extremidade 5' do DNA viral e é essencial para o início da síntese do DNA, sendo também conhecida como primase terminal – TP, que contém o resíduo de tirosina na posição 63 que é essencial para a iniciação (102);

2 – o segundo domínio não tem nenhuma função específica, além de funcionar com um espaçador (103) – esta parte corresponde numa ORF diferente, com a região pré S, que é o segmento mais variável entre os genomas dos diferentes hepadnavírus (104), possivelmente refletindo uma adaptação à infecção de hospedeiros diferentes;

3- o terceiro domínio é a DNA polimerase em si, com atividade de transcriptase reversa;

4- o domínio carboxi-terminal é a RNase H que cliva RNA apresentado na forma de híbrido com DNA, como acontece durante o ciclo de replicação do HBV (100).

Com cerca de 90 kDa em tamanho, as proteínas P de hepadnavírus são muito maiores do as RTs típicas. Alinhamentos com a sequência primária revelaram a

presença de motivos de RT universalmente conservados (105) e uma menos pronunciada homologia embora ainda significativa a RNase H (106).

Os domínios RT das DNAs polimerase de HBV e HIV-1 parecem globalmente semelhantes, como é ainda apoiada pela frequente ocorrência de mutações de resistência, inevitavelmente emergentes após tratamento prolongado com outras drogas antivirais de ação direta, em posições equivalentes em ambas as enzimas, frequentemente próximas ao motivo YMDD(Tyr-Met-Asp-Asp). Este tema será largamente abordado em outra seção a seguir.

A região da TP, incluindo o motivo altamente conservado T3 - com vários resíduos aromáticos AGILYKR ou Ala-Gly-Ile-Leu-Tyr-Lys, é o principal responsável para a insolubilidade da proteína recombinante. Assim, nesta região resíduos de aminoácidos hidrofóbicos, incluindo Arg183, parecem ser cruciais para interações intra- α e / ou α -RNA que ocorrem durante a formação do complexo de iniciação (107).

Muitas RTs retrovirais produzem cDNA a partir de praticamente qualquer RNA se provida de iniciador e dNTP em um tampão adequado; mas não há tal atividade com as proteínas P hepadnavirais, mesmo depois que se tornaram acessíveis em quantidades maiores por expressão recombinante (26). A razão por trás é a dependência estrita da atividade da proteína P de chaperonas celulares (proteínas de choque térmico, Hsp). As chaperonas Hsc70 (forma constitutiva de Hsp70) e Hsp40, com ATP, são essenciais para a ativação da proteína P. No entanto, Hsp90 com Hop, bem como a Hsp70 fator de troca de nucleotídeos Bag-1, podem estimular ainda mais a ativação por quatro a cinco vezes (7).

4.2. O GENOMA DO HBV

O genoma do HBV consiste de uma molécula de DNA de cerca de 3200 nucleotídeos, sendo um dos menores genomas de DNA conhecidos entre os vírus animais. Sua estrutura é peculiar: a molécula de DNA é circular, em parte fita dupla, mas com uma região de fita simples de extensão variável. A fita longa ou L(-) possui um corte ("nick") constante na posição 1818 (tomando-se o início do sítio único para *EcoRI* como nucleotídeo 1), enquanto a fita curta ou S(+), de extensão que varia 1700 e 2800 nucleotídeos nas diferentes moléculas, possui extremidade 5' fixa por volta da posição 1620 e extremidade 3' variável (58, 59).

Sua organização genética já foi determinada, identificando-se as diferentes sequências de nucleotídeos dos diferentes subtipos do HBsAg. A capacidade codificadora está restrita à fita L(-), na qual foram identificadas 4 regiões codificadoras: S, C, P e X. A região S é dividida em S e pré S, sendo aquela responsável pela codificação da proteína principal do envelope, o HBsAg (58, 59). A região pré S, na mesma fase de leitura codifica para as proteínas L (pré S1 + pré S2 + S) e M (pré S2 + S) do envelope viral. A região C codifica para a principal proteína do nucleocapsídeo (core) viral. As regiões pré C e C, quando traduzidas conjuntamente, originam o HBeAg, pelos mecanismos discutidos anteriormente. A região P se estende por cerca de 80% do genoma viral e codifica a DNA polimerase viral. Finalmente, a região X codifica o HBxAg (58).

4.2.1. PROMOTORES DOS GENES DO HBV

Diferentes promotores são responsáveis pelo controle da expressão dos genes do HBV.

Existem dois diferentes promotores para as proteínas de envelope.

1 – o promotor do gene da proteína de envelope L situa-se logo antes da região pré S1 (108). Este promotor, conhecido também como SPI (S promoter I) tem um “TATA box” típico e transcreve um mRNA com extremidade 5’ bem definida. Todos os outros promotores têm sítios múltiplos para início da transcrição. O promotor pré S1 para LHB mRNA contém sítios de ligação para fatores de transcrição específicos do fígado, por exemplo, HNF1 (109) e HNF3 (110), e é relativamente fraco em comparação com o promotor do gene das proteínas de envelope S e M. É também funcional em células não hepáticas após transfecção (111).

2 - o promotor do gene das proteínas de envelope S e M (-103 - +30) (112) localiza-se dentro da região pré S1, originando mRNAs de cerca de 2,1 kb (111), tem sua atividade aumentada pelo “enhancer”-I (113) e pela dexametasona (via fator de transcrição NF-1) (114). Este promotor, também conhecido como SPII (S promoter II), produz 3 mRNAs diferentes, mas apenas o maior contém o códon de iniciação para a proteína M. Ambos os promotores das proteínas de envelope são mais ativos em hepatócitos diferenciados (17). Existe um composto chamado helioxantina (HE-145) que inibe este promotor bem como o promotor do core que pode potencialmente ser utilizado para o tratamento da hepatite B e suas complicações (115).

A síntese das proteínas do nucleocapsídeo viral e do mRNA pré-genômico é controlada pelo promotor básico do core (BCP) (116). Este promotor se situa na região

X (1573-1604) (117) e regula a transcrição de três mRNAs de cerca de 3,6 kb (118). O promotor possui 4 regiões ricas em TA que funcionam simultaneamente como “TATA box” e sítio de iniciação da transcrição para estes mRNAs (119). Os dois maiores mRNAs, cuja transcrições se iniciam cerca de 30 bp acima do menor, codificam a proteína precursora pré Core/Core. A transcrição do menor mRNA se inicia quatro bases após o códon de iniciação pré Core, e, portanto, este mRNA não codifica o precursor pré C, mas pode codificar a proteína C. A regulação da transcrição destes mRNAs é diferente, pois fatores de transcrição, como o fator nuclear hepatocítico NHF4, reprimem o promotor pré C e outros ativam o promotor pré-genômico, como o fator ativador de transcrição Sp1 (120, 121). O promotor possui também um elemento regulatório negativo NRE, e normalmente só está ativado por fatores celulares presentes nos hepatócitos e algumas outras linhagens celulares (122). O promotor também é normalmente inibido pela p53 selvagem, mas não pela p53 mutante presente em hepatocarcinomas (G→T 249) (123). Este promotor também é inibido pela helioxantina (HE-145) (115).

Finalmente, o promotor do gene X (1042-1354) origina um transcrito de 0,7 kb, que codifica apenas essa proteína (124, 125), mas já foram descritos transcritos de menores originados pelo processamento deste mRNA que poderiam originar proteínas diferentes com função ainda não determinada (126).

Para a terminação da transcrição, os genomas de todos os *Orthohepadnavirus* possuem um sinal de poliadenilação e terminação TATAAA, denominado PS2, logo no início do gene C. Este sinal só se torna ativo quando o sítio de iniciação do RNA está a mais de 400 bp a montante pois ele precisa de outro elemento presente no BCP para

ser ativo. Este sinal de terminação é portanto ignorado na primeira passagem da RNA polimerase II na síntese do RNA pré-genômico. Este sinal é também comum para todos os outros mRNAs, exceto para o mRNA do gene X. Esta estratégia de expressão utilizando apenas um terminador para vários promotores é exclusiva dos hepadnavírus (126).

4.2.2. OUTRAS SEQUÊNCIAS REGULATÓRIAS NO GENOMA DO HBV:

Algumas estruturas peculiares são encontradas no genoma do HBV que merecem ser ressaltadas. Muitas delas têm um papel importante na replicação viral e sua função será detalhada em uma seção posterior sobre a replicação viral.

1 – as pequenas regiões repetitivas DR1 e DR2, participam do processo de encapsidação do genoma viral (127), e são os sítios de integração do vírus no genoma das células hospedeiras (128). Além disso, DR1 possui uma estrutura secundária importante para a replicação e transcrição viral (129).

2 – o sinal de encapsidação, conhecido pela letra grega épsilon (ϵ), que forma uma estrutura secundária em alça no DNA viral (130), localizado na região pré - Core, fundamental para o início da replicação viral, através da interação com a polimerase viral (131). Os sinais ϵ tem duas hastes, uma estrutura em bojo intermediária e uma alça superior (132). Os efeitos encontrados nos vírus mutantes pré C são também devidos às alterações causadas na encapsidação do HBV, que teria sua estabilidade aumentada e uma replicação viral ainda mais efetiva (133), o que justificaria a

associação deste tipo de mutante com formas mais graves de hepatites, como será visto ulteriormente.

3 – “enhancers” são segmentos de ácidos nucleicos que possuem a capacidade de aumentar a transcrição de determinados genes, podendo atuar mesmo à distância dos genes ativados e em qualquer orientação. Os “enhancers” e os promotores atuam conjuntamente na ativação da transcrição gênica, mas estes só são capazes de atuar nos genes contíguos e sempre se localizam à montante destes. O HBV apresenta dois “enhancer”s que podem ativar independentemente a transcrição de todos os genes virais. A presença de dois “enhancers” regulados de forma diferente no HBV deve refletir uma estratégia para garantir replicação eficiente em hepatócitos indiferenciados, em regeneração ou mesmo durante a hepatocarcinogênese (133). O “enhancer” I aumenta a transcrição de HBV em 10 a 50 vezes nas células hepáticas e o efeito do “enhancer” II é ainda mais específico do fígado. Estes enhancers são ativados por diferentes fatores de transcrição. Os promotores e estas estruturas controlam o nível de transcrição do HBV, a expressão de seus genes bem como sua replicação e podem ser influenciados por substâncias ativas antivirais, por exemplo, a interleucina 6 (IL-6) ou análogos de helioxantina ou pelo estado de diferenciação dos hepatócitos. O vasto número de substâncias que podem desta forma influenciar a atividade do HBV foi revisto recentemente (134).

As localizações e funções dos “enhancers” virais bem como a interação de algumas destas substâncias com os “enhancers” do HBV será descrita abaixo:

a - o “enhancer” I localiza-se 450 bp (1080-1234) à montante do promotor core (135), é especialmente ativo em hepatócitos (136), mas também

atua em células hematopoiéticas (137), ativando os promotores dos genes S, C e X (113), interagindo com fator de ativação celular específico (138). O hepatotropismo do HBV parece ser definido pela interação do “enhancer” I com fatores nucleares hepáticos, como NHF3 and NHF4 e o receptor retinóide $X \alpha$ (RXR α) ativando o promotor X (139). O “enhancer” I se relaciona com o processo de carcinogênese induzida pelo HBV (140), pois a p53 normalmente o reprime, mas algumas mutações no “enhancer” I fazem com que ela passe a ter efeito ativador (141). Na ausência de atividade do “enhancer” I, a transcrição do RNA pré-genômico viral é bastante reduzida (26). Uma região dentro deste enhancer é um transativador transcricional do receptor de andrógenos e pode ser envolvido com a maior frequência de evoluções mais graves da hepatite B no sexo masculino, incluindo o desenvolvimento de câncer (142).

b – o “enhancer” II localiza-se na região X (1627 – 1741), parece ser específico de hepatócitos (143), contém 2 elementos diferentes que necessitam estar juntos para sua função: elemento “e II-A” - 1636 a 1671 e elemento “e II-B” (1704 –1741) (144). Mutações na região deste “enhancers” podem estar envolvidas com maior risco de evolução para carcinoma hepatocelular (145).

4 - elemento responsivo à glicocorticóide (GRE) (110), localizado na posição 341-370, contendo duas cópias da sequência de hexanucleotídeos específica (5'-TGTTCT-3') (145, 146). Não funciona como um “enhancer” independente, mas pode explicar o efeito marcante do tratamento com corticóide sobre a replicação do HBV (147).

4.3. REPLICAÇÃO VIRAL

A encapsidação seletiva do RNA pregenômico (pgRNA) dentro do capsídeo icosaédrico é mediada por interações específicas com a transcriptase reversa (RT) ou DNA polimerase viral, chamada proteína P, ao mesmo tempo em que se inicia a síntese de DNA (148). Apenas um único pgRNA e uma única proteína P são embalados por partícula (131).

A iniciação da síntese de DNA em hepadnavírus ocorre por uma única proteína, isto se reflete na presença na proteína P de um terminal adicional de proteína (TP) não encontrado em qualquer outra RT (149). A proteína P depende de moléculas chaperonas para conseguir competência para se ligar ao RNA molde e iniciar a síntese de DNA. Durante a replicação, são também necessárias interações de longa distância entre sítios nos ácidos nucleicos moldes para gerar o genoma viral circular relaxado (RC), o que pode explicar o achado de recombinantes entre cepas diferentes do HBV (150).

O receptor celular para a penetração do vírus nos hepatócitos é o polipeptídeo de co-transporte taurocolato de sódio (NTCP) e o processo de entrada e adesão do vírus ao receptor celular durante o processo de replicação foi descrito anteriormente (65).

Os nucleocapsídeos são transportados para o poro nuclear e o genoma de RC-DNA é liberado no nucleoplasma e convertido em cccDNA. Este cccDNA episomal é o intermediário intracelular central em hepadnavírus para a replicação, funcionalmente equivalente ao DNA proviral. Ele serve como molde de transcrição para a RNA polimerase II celular produzir vários RNAs subgenômicos que codificam as proteínas do

envelope e o HBx, além de duas moléculas de comprimento maior que o genoma. Do RNAm pré-core mais longo, uma versão maior da proteína do nucleocapsídeo é sintetizada, que após processamento é secretada como HBeAg; o RNA pré-genômico (RNAp_g) mais curto serve como mRNA para a proteína da cápside e proteína P. A proteína P liga-se preferencialmente à molécula pgRNA a partir da qual foi traduzida, especificamente na cópia 5' de uma estrutura terciária chamada ϵ (épsilon), o que inicia a encapsidação (daí o nome ϵ) e a transcrição reversa. Os sinais ϵ tem duas hastes, uma estrutura em bojo intermediária e uma alça superior. O RNAm pré-core é excluído da encapsidação provavelmente porque a interação P- ϵ é impedida pelos ribossomos que traduzem a região pré-core. Dentro dos nucleocapsídeos recém-formados, o RNA é reversamente transcrito pela proteína P co-empacotada em RC-DNA. Estes nucleocapsídeos recém formados podem tanto devolver seus genomas aos núcleos para criar um conjunto de cerca de 10-100 cópias cccDNA ou interagir com as proteínas do envelope e serem secretados como novos vírions infecciosos. Daí as três formas em que a informação genética de hepadnavírus armazenados são o RC-DNA em vírions; e cccDNA e pgRNA na célula (107).

Para HBV, a estrutura ϵ apenas media a encapsidação e pode ser a origem de replicação para a fita complementar de DNA. A síntese de DNA começa com um sistema de iniciação com o domínio TP que tem um resíduo de tirosina que se liga de forma covalente a extremidade 5' de um pequeno oligonucleotídeo de DNA, o qual posteriormente move-se para a região repetitiva DR1 para iniciar a síntese da outra fita de DNA. A síntese do DNA ocorre em grande parte dentro dos nucleocapsídeos formados e depende de alguma forma da proteína C (151).

O cccDNA nuclear produzido a partir do RC-DNA genoma serve como molde para a transcrição pela RNA polimerase II celular. Portanto, todos os transcritos virais, incluindo o pgRNA de 3,5 kb, terá um cap 5' e uma cauda 3' poliadenilada. O sinal de poliadenilação no cccDNA é ignorado durante o primeiro encontro pela RNA polimerase de RNA e utilizado apenas durante o segundo encontro. Consequentemente, o pgRNA contém uma redundância terminal no qual o elemento de DR1 e o início da fase de leitura aberta (ORF) para o nucleocapsídeo estão presentes uma segunda vez (126). A enzima de reparo de DNA humano tirosil-DNA-fosfodiesterase 2 (TDP2) está envolvida na biogênese do reservatório do cccDNA devido as similaridades estruturais entre o RC-DNA do HBV e os adutos entre topoisomerase e DNA humano (152).

5. DIVERSIDADE GENÉTICA VIRAL

5.1. TIPOS

Diferenças antigênicas no HBsAg definem quatro diferentes subtipos, que foram posteriormente subdivididos na seguinte classificação: *adw* (*adw2*, *adw4*); *ayw* (*ayw1*, *ayw2*, *ayw3*, *ayw4*); *adr* (*adrq+*, *adrq-*) e *ayr* (153). O subtipo *a*, comum à maioria dos vírus, é atribuído a um epítipo descontínuo, ao qual são dirigidos anticorpos neutralizantes. Desta forma, vacinas feitas contra um subtipo são também eficazes para os outros subtipos, a não ser em variantes recentemente descritas nas quais este epítipo está modificado, como veremos no próximo item.

A divergência das sequências do genoma completo dentro de um mesmo subtipo é de aproximadamente 8%, semelhante à encontrada entre diferentes

subtipos. Assim, a subtipagem não reflete uma verdadeira variação genotípica nas sequências codificadoras da proteína S. Por esta razão outro método de classificação de genótipos do HBV baseados na sequência de nucleotídeos do HBsAg passou a ser adotado (154). O genótipo A inclui vírus dos subtipos *adw2* e *ayw1*; genótipo B, subtipos *adw* e *ayw1*; genótipo C, subtipos *adw*, *ayr* e *adr*; genótipo D, subtipos *adw*, *ayw2* e *ayw3* (155). Genótipos E e F, correspondente aos subtipos *ayw4* e *adw4*, respectivamente, pela análise da sequência do gene S (153, 155).

5.2. GENÓTIPOS E SUBGENÓTIPOS

A classificação dos genótipos do HBV é baseada no percentual de divergência da sequência do genoma completo, que deve ser maior que 7,5% entre os diferentes genótipos. Os subgenótipos representam variantes de um mesmo genótipo cujo percentual de divergência baseado na sequência de nucleotídeos do genoma completo é superior a 4% (156).

Atualmente o HBV está classificado em 10 genótipos (A, B, C, D, E, F, G, H, I e J). Os genótipos A-H estão bem estabelecidos, já o I e o J foram recentemente descritos e sua denominação como novos genótipos ainda é fruto de continuas discussões entre os pesquisadores da área. Tal indefinição se dá pelo fato desses supostos novos genótipos serem resultantes de recombinações entre genomas de diferentes genótipos do HBV isolados em humanos (genótipo I) ou entre genótipos isolados em humanos e em primatas não humanos (genótipo J) (157-159).

Em relação aos subgenótipos, estes só foram identificados entre os genótipos A, B, C, D e F (156).

A maioria dos genótipos e alguns subgenótipos apresentam um padrão de distribuição geográfica característico, e são associados com diferentes etnias (156). O genótipo A predomina em algumas regiões da África, regiões norte e central da Europa e na América do Norte. Para o genótipo A, inicialmente dois subgenótipos foram descritos: o A1, que é encontrado principalmente em algumas regiões dos continentes Africano (África Oriental e do Sul), Asiático (Índia, Bangladesh) e Americano (Brasil, Haiti, Martinica) e o A2, que predomina na Europa (156). Posteriormente, mais cinco subgenótipos foram propostos para a classificação de cepas isoladas em diferentes regiões da África (A3, A4, A5 e A7) e na Bélgica entre pacientes de origem Africana (A6) (159). Esses subgenótipos não parecem estar disseminados em outras regiões do mundo, com exceção do A5, que foi recentemente identificado com elevada frequência no Haiti (160).

Os genótipos B e C são comuns no Sudeste Asiático, China e Japão. Quanto aos subgenótipos, diversos já foram descritos para o genótipo B: B1 (Japão), B2 (Japão, China), B3 (Indonésia), B4 (Vietnã), B5 (Filipinas) e B6 (populações nativas do Ártico incluindo Alasca, norte do Canadá e Groenlândia), B7-B9 (Indonésia); e da mesma forma para o genótipo C: C1 (Sudeste da Ásia, Sul da China), C2 (Sudeste Asiático, China e Japão), C3 (Oceania, Indonésia), C4 (Austrália), C5 (Filipinas, Vietnã, Indonésia), C6 (Indonésia, Filipinas), C7 (Filipinas), C8-C16 (Indonésia) (159).

O genótipo D tem uma ampla distribuição geográfica, mas é mais frequente em países localizados na bacia do Mediterrâneo e Oriente Médio. Este genótipo também

apresenta uma grande diversidade genética caracterizada pelos diferentes subgenótipos já identificados: D1 (Bacia do Mediterrâneo, Oriente Médio, Índia, China), D2 (Japão, Norte da Europa), D3 (Índia, África do Sul, Sul da Europa), D4 (Oceania, Haiti), D5 (Índia, Japão), D6 (Indonésia) e D7 (Tunísia), D8 (Nigéria) (159, 161).

O genótipo E é considerado nativo da África e predomina na região Ocidental desse continente (162, 163). Estudos sugerem que a ampla disseminação do genótipo E na África parece ser um evento relativamente recente resultante, principalmente, da aplicação de injeções em massa (campanhas de vacinação, aplicação de medicamentos) usando as mesmas agulhas e seringas, realização de rituais tribais envolvendo o uso de objetos perfuro cortantes, além de outros mecanismos de transmissão horizontal pelo contato próximo com indivíduos infectados e da transmissão sexual (162). Apesar de uma grande população Africana ter sido introduzida em outros continentes, particularmente nas Américas, casos de infecção com o genótipo E fora do continente africano têm sido encontrados apenas em indivíduos com contato recente com essa região ou com indivíduos de lá provenientes (162). Exceção a esse perfil foi observada em Quibdó, Colômbia, onde o genótipo E foi encontrado entre indivíduos afrodescendentes sem histórico de viagens para a África ou contato recente com pessoas procedentes de qualquer país Africano (164). As cepas do HBV genótipo E caracterizadas até o presente momento apresentam pouca variabilidade genética e, portanto não foram identificados subgenótipos (162, 163).

O genótipo F é característico da América do Sul, sendo o único genótipo encontrado entre populações indígenas isoladas (165, 166) e amplamente distribuído

nos países com forte contribuição desse grupo racial em sua formação, tais como: Venezuela (165), Colômbia (167) e Chile (168). As cepas do genótipo F isoladas em diferentes regiões geográficas são classificadas em 4 subgenótipos: F1, cujo clado é subdividido em F1a (América Central) e F1b (Argentina), F2, que também tem uma subdivisão em F2a (Venezuela, Argentina) e F2b (Venezuela), F3 (Venezuela, Colômbia) e F4 (Argentina) (169).

A distribuição dos genótipos G e H ainda não é bem conhecida: o genótipo G foi identificado inicialmente na Europa (Alemanha e França) e posteriormente nas Américas (Canadá, EUA, México, Colômbia), além de alguns poucos casos na Ásia (170). Já o genótipo H foi encontrado na América Central, sobretudo no México, onde é encontrado com elevada frequência (26, 171).

A infecção pelo genótipo G apresenta algumas particularidades: 1- tem sido encontrada mais frequentemente entre homens que fazem sexo com homens e entre os casos de coinfecção com o Vírus da Imunodeficiência humana (HIV); 2- ocorre quase sempre em coinfecção com outro genótipo do HBV, sendo a coinfecção com os genótipos A e H a mais descrita até o momento (170, 172, 173).

O genótipo I foi encontrado no Vietnã, Laos e China e o J em um único caso no Japão (170).

No Brasil os genótipos A, D e F circulam em todas as regiões, sendo que o genótipo A é o mais prevalente em todas elas com exceção da região Sul, onde o genótipo D predomina (174, 175). Apenas os subgenótipos A1 e A2 já foram identificados, sendo que o subgenótipo A1 é o mais frequente em todas as regiões.

Entre os subgenótipos de D, os subgenótipos D2 e D3 são os mais frequentes no Sul e Sudeste do país, por outro lado o subgenótipo D4 foi o principal subgenótipo de D identificado no Maranhão (166, 174-176).

O genótipo F é encontrado com maior frequência no norte do país, especialmente entre populações indígenas, sendo o subgenótipo F2a o mais frequente (166).

Os genótipos B e C também circulam no Brasil, porém praticamente restritos a indivíduos com origem oriental ou seus descendentes (175, 177), apesar de que já foi identificado em brasileiros que moraram no leste da Ásia (26).

O genótipo E é muito raro em nosso país, sendo só encontrado entre imigrantes Africanos (178) ou naqueles que passaram algum período neste continente (179).

O genótipo G também já foi descrito em alguns casos no Rio de Janeiro (180), São Paulo (179, 181, 182) e Acre (183).

O genótipo H até o momento só foi encontrado em um caso no Sul do Brasil (174).

6. ASPECTOS CLÍNICOS

O HBV causar uma ampla gama de manifestações clínicas, desde o estado de portador assintomático até outras formas de doença, como hepatite aguda, fulminante ou crônica, cirrose hepática e carcinoma hepatocelular.

O período de incubação varia de 1 a 4 meses e o a infecção depende de vários fatores que influenciam a resposta imune (idade no momento da aquisição da infecção, fatores genéticos do hospedeiro e a variabilidade genética do vírus) (184).

Após a infecção, esta pode evoluir para: 1- hepatite fulminante que é rara, mas de elevada gravidade e letalidade; 2- resolução da infecção; 3- infecção crônica (184).

A resolução da infecção aguda pelo HBV é caracterizada pelo desaparecimento do HBsAg e detecção dos anticorpos anti-HBs dentro de um período de 6 meses. Contudo, é importante ressaltar que essa resolução não significa eliminação completa do vírus e sim um eficiente controle da replicação pelo sistema imune do hospedeiro, o que pode resultar em reativação da infecção na ocorrência de imunossupressão (184).

O diagnóstico de infecção crônica se caracteriza pela detecção do HBsAg por período superior a 6 meses, o que é observado na maioria dos indivíduos infectados antes dos 5 anos de idade e na minoria daqueles infectados durante a fase adulta. Alguns fatores clínicos, demográficos e epidemiológicos têm sido associados à evolução da doença: idade avançada, idade no momento da infecção, sexo masculino, coinfecção com os vírus das hepatites C, D ou com o HIV, níveis de carga viral do HBV, consumo de álcool, genótipo do HBV e presença de determinadas mutações no genoma viral (185).

No curso natural da infecção crônica pelo HBV são definidas quatro fases, nem sempre identificadas em todos os pacientes e com durações temporais extremamente diferentes (186).

A primeira fase denominada fase de tolerância imune é caracterizada pela presença do HBeAg, elevados níveis de carga viral (> 20.000 UI/ml e comumente acima

de 107-8 UI/ml), níveis de aminotransferases persistentemente normais e sem lesões hepáticas significantes. Esta fase é vista com mais frequência e mais longa entre indivíduos infectados pela via perinatal, nos quais pode perdurar por 30 anos ou mais.

Posteriormente, segue-se a fase de clareamento imune, também chamada de fase hepatite B crônica. Nesta fase o sistema imune passa a reconhecer o HBV como um agente estranho e inicia intensa resposta imunológica, o que resulta em lesão hepática. Nesta fase ainda se detecta o HBeAg no soro, altos níveis de HBV DNA (> 2.000 UI/ml), assim como aumento persistente nos níveis das aminotransferases, especialmente a alanina aminotransferase (ALT), e inflamação hepática ativa. Durante a fase de clareamento imune pode ocorrer a soroconversão do HBeAg para anti-HBe espontaneamente (taxa de 10 a 20% ao ano) ou resultante da terapia antiviral, o que reduz o risco de descompensação hepática. A soroconversão para anti-HBe pode ser precedida por exacerbação (“flare”) aguda da hepatite B crônica. Esta fase da infecção pode durar vários anos, e pode resultar no desenvolvimento de cirrose hepática e complicações (187).

A terceira fase, denominada fase de portador inativo, é caracterizada pela negatificação do HBeAg e presença do anti-HBe, níveis de HBV DNA baixos (< 2.000 UI/ml) ou indetectáveis e níveis de ALT persistentemente normais resultante da resolução do processo necroinflamatório. O paciente pode permanecer neste estado por longos anos. A resolução completa da infecção com soroconversão HBsAg/anti-HBs (taxa de 0,5% ao ano) pode ocorrer, assim como o retorno à fase replicativa com reativação da doença caracterizando a fase de reativação.

Esta última fase é também conhecida como hepatite B crônica HBeAg negativo e tem como característica a ausência do HBeAg, presença do anti-HBe, níveis de ALT e HBV DNA persistentemente elevados e inflamação hepática ativa. Pacientes nesta fase geralmente são mais velhos e apresentam doença hepática mais avançada que aqueles em outras fases da doença. A presença de variantes do HBV com mutações no genoma que abortam a síntese do HBeAg é comum em pacientes nesta fase (186).

7. DIAGNÓSTICO E MONITORAMENTO

O HBsAg é o primeiro marcador sorológico viral detectado após a infecção aguda pelo HBV, o qual se torna detectável cerca de 6 a 12 semanas após a exposição, podendo surgir antes do aparecimento dos sintomas. Os anticorpos contra os antígenos virais começam a aparecer durante a fase sintomática e os primeiros anticorpos a serem detectados são os da classe IgM direcionados contra o antígeno do nucleocapsídeo viral (anti-HBc IgM). Os anticorpos anti-HBc IgM tendem a desaparecer após 3 a 12 meses, independente da evolução da doença, enquanto que os títulos de anticorpos anti-HBc IgG aumentam e permanecem detectáveis pelo resto da vida do indivíduo (188).

Anticorpos contra o HBeAg (anti-HBe) podem ser detectados juntamente com o anti-HBc IgM ou logo após o seu aparecimento. A soroconversão do HBeAg para o anti-HBe coincide com um intenso aumento da ALT, o que é devido à lise das células infectadas pela resposta imunológica. Geralmente o anti-HBe persiste por anos, mas na ausência de replicação viral ativa, os títulos tendem a reduzir. Por fim surgem os anticorpos contra o HBsAg (anti-HBs), que são anticorpos neutralizantes e são

reconhecidos como um marcador de imunidade. O aparecimento dos anticorpos anti-HBs não deve ser interpretado como eliminação completa da infecção, uma vez que o cccDNA viral permanece nos hepatócitos infectados, mas sim como o resultado de um controle eficiente da infecção viral pelo sistema imune do hospedeiro. Alguns pacientes podem não produzir níveis detectáveis de anti-HBs, apesar do controle eficiente da replicação viral. O anti-HBs também é um importante marcador que confirma a eficiência da vacinação contra o HBV (188).

O HBV DNA pode ser encontrado no soro poucas semanas após a infecção usando técnicas de amplificação como a reação em cadeia da polimerase (PCR), contudo a detecção desse marcador não tem muita importância no diagnóstico da infecção aguda (188).

A persistência do HBsAg por 6 meses ou mais após a infecção aguda indica que a infecção evoluiu para a cronicidade. A replicação viral nos estágios iniciais da infecção crônica pode ser identificada pela presença do HBeAg e do HBV DNA. Nesse estágio a resposta imune humoral se caracteriza pela presença de anticorpos anti-HBc IgG, que são detectados como anti-HBc total (188).

A soroconversão do HBeAg para anti-HBe caracteriza uma fase de pouca replicação viral com redução dos níveis de HBV DNA circulantes. Contudo, alguns casos podem permanecer com altos níveis de carga viral apesar da soroconversão para anti-HBe, o que é resultado da presença das variantes virais com mutações que reduzem ou eliminam a síntese do HBeAg, conforme descrito anteriormente (188).

Nos casos de hepatite crônica, os níveis de HBV DNA necessitam ser constantemente analisados (a cada 6 meses ou anualmente nos casos de portadores

inativos) para auxiliar no monitoramento da evolução da doença hepática, na avaliação da necessidade de introdução de tratamento antiviral e no monitoramento da resposta ao tratamento (188).

O clareamento do HBsAg e a detecção dos anticorpos anti-HBs pode ocorrer espontaneamente durante a hepatite crônica ou como resultado da terapia antiviral (187). A quantificação do HBsAg tem sido utilizada como um preditor de evolução da doença hepática e resposta ao tratamento, particularmente com Interferon (189).

Atualmente são empregados ensaios comerciais para a quantificação dos níveis de HBsAg no soro, dentre os quais os mais comumente utilizados são o ARCHITECT HBsAg QT (Abbott Laboratories, Rungis, França) e o Elecsys HBsAg II Quant (Roche Diagnostics, Penzberg, Alemanha). Esses ensaios são completamente automatizados e os títulos de HBsAg são expressos em unidades internacionais (UI)/ml.

A quantificação do HBeAg chegou a ser proposta para esta finalidade (190) mas seu uso era restrito aos pacientes positivos para este marcador, o que reduzia a utilização, pois muitos pacientes era, anti-HBe positivos. Em um trabalho, demonstramos que a quantificação do HBeAg simultaneamente a quantificação do HBV DNA poderia prever o padrão de resposta dos pacientes ao tratamento com interferon e ribavirina (191).

O primeiro marcador proposto para a detecção da replicação do HBV foi a técnica de detectar a atividade da DNA polimerase (99), criada em 1973, que foi depois simplificada evitando-se o passo da ultracentrifugação pela inibição específica da polimerase viral pelo ácido fosfonofórmico (192), com a qual chegamos a trabalhar na época (193).

Posteriormente, surgiu a técnica da hibridação molecular para a detecção do DNA viral no soro anos (194), que exigia que se trabalhasse com compostos radioativos, mas mesmo assim não alcançava uma boa sensibilidade, pois muitas amostras positivas para o HBeAg continuavam sem o DNA viral detectável (26). Um importante incremento na sensibilidade de tal reação só foi possível com o desenvolvimento da técnica de reação em cadeia da polimerase (PCR) para detectar o DNA viral, que apresentava o problema inverso, ou seja, quase todas as amostras HBsAg eram positivas e ela perdia o poder de discriminar as diferentes fases evolutivas da hepatite B descritas acima (26). Desta forma, o uso de técnicas quantitativas para serem utilizadas em rotina se tornava obrigatória para o acompanhamento da hepatite B.

Com o desenvolvimento da técnica de PCR em tempo real, vários grupos de pesquisa desenvolveram técnicas de quantificação desenvolvidas no próprio laboratório pois as empresas que geralmente desenvolvem estas metodologias por uma série de motivos acabaram demorando muito para liberar estas reações em rotina para o HBV. Estas metodologias incorporavam o uso de controles internos para certificar-se de que não existiam diferenças de amplificação dentro de cada amostra que poderiam influenciar os resultados. Estas metodologias por muito tempo eram as únicas opções disponíveis para a quantificação do DNA do HBV, em especial no nosso país. Este método detecta baixas cargas virais e, com a disponibilização de um padrão pela Organização Mundial de Saúde que expressa os resultados em UI/ml, permite dessa forma a comparação entre os diferentes ensaios (195).

Atualmente já estão disponíveis no mercado brasileiro reações baseadas nos testes mais comumente utilizados atualmente no mundo: o bDNA Bayer VERSANT HBV 3.0 TEST, que emprega passos de hibridizações sequenciais para produzir um sinal quimioluminescente que é proporcional à quantidade de HBV DNA inicial presente na amostra, sendo seu limite de detecção inferior de 357 UI/ml e superior de 17.000.000 UI/ml; e outros três testes baseados em amplificação por reação em cadeia da polimerase em tempo real: Abbott Real Time HBV (linearidade de 10 a 10^9 UI/mL) (196); Roche COBAS TaqMan HBV (linearidade de 30 UI/ml a $> 1,1 \times 10^8$ UI/ml) e Artus RealArt HBV LC PCR (linearidade de 2×10^2 UI/ml a 10^9 UI/ml) (197).

Alguns pacientes poderão apresentar perfis sorológicos diferentes do padrão esperado. Baixos níveis de anti-HBc IgM podem algumas vezes ser detectados em casos de reativação da infecção crônica. Além disso, uma pequena proporção de pacientes cronicamente infectados podem apresentar soropositividade simultânea para o HBsAg e anti-HBs. A presença de HBV DNA também pode ser detectada na ausência de do HBsAg caracterizando a chamada hepatite B oculta, nesses casos frequentemente anticorpos anti-HBc são encontrados isoladamente (197).

8. TRATAMENTO

O tratamento da Hepatite B crônica tem como objetivo a supressão permanente da replicação do HBV e consequentemente a remissão da doença hepática, prevenindo dessa forma a progressão para cirrose e CHC.

Atualmente, há sete medicamentos aprovados, que incluem duas apresentações de interferon - INF (interferon-alfa e interferon-alfa peguilado) e cinco

análogos nucleos(t)ídeos (AN): lamivudina, adefovir, entecavir, telbivudina e tenofovir (198).

As apresentações de interferon (convencional ou forma peguilada) apresentam ação antiviral direta e ação imunomoduladora. Cerca de 30% dos pacientes com hepatite crônica HBeAg positivo, e 40% dos pacientes com hepatite crônica HBeAg negativo apresentam resposta virológica sustentada após utilização do interferon-alfa peguilado . Além da baixa taxa de resposta, os pacientes tratados com INF experimentam efeitos adversos durante a terapia que muitas vezes dificultam a finalização da mesma (198).

Os análogos nucleos(t)ídeos (AN) são medicamentos de uso oral que atuam primariamente na inibição da transcrição do RNA pg para formação da primeira fita do DNA viral e secundariamente inibindo a formação da fita complementar. O uso desses medicamentos promove inibição rápida e potente da replicação viral, de forma segura e com poucos efeitos colaterais, resultando na redução da progressão da fibrose hepática, da evolução para a cirrose e do desenvolvimento de CHC (198).

Embora os AN sejam eficazes na inibição da replicação do HBV eles não são capazes de eliminar totalmente o vírus das células hepáticas infectadas. Isso ocorre devido à presença do cccDNA no núcleo dessas células, o qual não sofre a ação dos AN e funciona como um reservatório celular para a formação de novas moléculas de RNA pré-genômico. Portanto, a manutenção da supressão viral só é possível com o uso de AN por longos períodos.

Essa necessidade de tratamento em longo prazo leva a uma séria consequência, a seleção de cepas do HBV resistentes à ação desses medicamentos, o

que representa um dos principais obstáculos para o sucesso do tratamento com AN. A emergência de cepas resistentes geralmente resulta na perda de todos os benefícios alcançados anteriormente com o tratamento podendo resultar em episódios de exacerbação e até descompensação hepática (198).

Os AN aprovados para o tratamento da HBC apresentam diferenças na sua potência de inibição da replicação viral e na sua capacidade de manter essa potência na presença de alterações que ocorrem na polimerase viral em decorrência de mutações no gene codificante da mesma (barreira genética) (198).

O primeiro análogo nucleosídeo aprovado para o tratamento foi a lamivudina. A lamivudina promove eficaz redução da replicação do HBV, porém, longos períodos de tratamento estão relacionados com a seleção de HBV resistentes ao medicamento. A resistência a esse AN aumenta progressivamente ao longo do tratamento, após 48 meses de tratamento cerca de 80% dos pacientes apresentam vírus resistentes (198). Em um trabalho de nosso grupo com a lamivudina, observamos uma taxa de resposta de 65,7% após um ano de tratamento (199). Em outro trabalho, utilizando uma dose maior de lamivudina no primeiro ano de tratamento, envolvendo tanto pacientes cirróticos com não cirróticos, a taxa de resposta foi semelhante e pudemos verificar o aparecimento das mutações de resistência no sítio ativo YMDD (Tyr– Met- Asp-Asp) associada com o aparecimento da resistência (199). A elevada frequência de mutações de resistência a lamivudina também foi verificada em pacientes co-infectados pelo HBV e HIV, dos quais muitos usavam a lamivudina por um longo prazo para o tratamento da aids: foram encontradas em 50% dos pacientes virêmicos para o HBV

(181) Mutações de resistência a lamivudina foram encontradas em 7/702 (1%) pacientes não tratados no Brasil (179).

Em 2002 foi introduzido o adefovir, um análogo nucleotídeo que se mostrou efetivo nos casos com resistência à lamivudina. No entanto, a monoterapia com adefovir está associada a seleção de cepas do HBV resistentes ao ADV, o que ocorre em cerca de 2% dos pacientes com HBeAg negativo após dois anos de terapia, aumentando para 30% após 5 anos de tratamento em monoterapia. Nos casos de tratamento de pacientes com HBV resistentes à lamivudina, a resistência ao adefovir é observada mais precocemente e cerca de 20% apresentam resistência após 12 meses de terapia (198). Mutações de resistência a lamivudina foram encontradas em 2/702 (0,3%) pacientes não tratados no Brasil (179), muito provavelmente por que esta droga foi muito raramente utilizada no país.

Em 2005 foi aprovado o entecavir, um análogo nucleosídeo com atividade antiviral bem mais potente do que os dois anteriores e com elevada barreira genética. As taxas de resistência ao entecavir são bem mais baixas, vírus resistentes foram descritos em 0,1% dos casos após um ano de terapia, chegando a apenas 1,2% dos casos após 6 anos. Todavia, essa taxa aumenta quando pacientes com vírus resistentes à lamivudina são tratados, indo de 6% em um ano a quase 60% após 6 anos de tratamento (198). Mutações de resistência ao entecavir foram encontradas em 2/702 (0,3%) pacientes não tratados no Brasil (179).

A telbivudina foi um outro análogo nucleosídeo aprovado em 2006 para o tratamento da HCB, que apresenta capacidade de supressão da replicação comparável à lamivudina. Embora a barreira genética da telbivudina seja um pouco mais alta que a

da lamivudina, as taxas de desenvolvimento de resistência são bem elevadas: 21,6% em pacientes com HBeAg positivo e 8,6% naqueles com HBeAg negativo após 1 e 2 anos de terapia, respectivamente (198).

O tenofovir é o mais novo medicamento aprovado para o tratamento da hepatite B, é um análogo nucleotídeo estruturalmente relacionado ao adefovir, porém muito mais potente em sua ação de supressão da replicação viral. Desde sua introdução em 2008 até o momento não houveram relatos de seleção de cepas do HBV com mutações que promovam resistência viral completa à ação desse AN (198). Por sua potência e elevada barreira genética o tenofovir, assim como o entecavir, são indicados atualmente como primeira opção de tratamento (198).

O tratamento não é indicado para todos os pacientes com hepatite crônica, a indicação é baseada em três critérios principais: 1- níveis de HBV DNA; 2- níveis de transaminases hepáticas; 3- gravidade e estágio da lesão hepática (200).

Também o tratamento está indicado em pacientes com hepatite B aguda, nas formas fulminantes ou agudas graves. Na verdade, pacientes com essas apresentações de doença grave deveriam ser avaliados para transplante de fígado e deveriam receber medicação específica, sendo entecavir ou tenofovir os medicamentos de escolha nesse caso (200).

Há ainda situações particulares de tratamento da infecção pelo HBV, como em co-infectados pelo HIV ou em gestantes, em situações de falha medicamentosa devido a presença de mutantes resistentes aos esquemas utilizados onde os esquemas preconizados são distintos.

Vale ainda lembrar a necessidade de profilaxia da reativação dessa infecção em indivíduos portadores de HBsAg ou em indivíduos com marcadores sorológicos de infecção prévia, quando submetidos a imunossupressão. Também nessa situação esquemas específicos são recomendados (201).

9. IMPLICAÇÕES DA DIVERSIDADE GENÉTICA DO HBV

Têm crescido o número de estudos relatando que, além das diferenças no padrão distribuição, alguns genótipos e subgenótipos do HBV apresentam diferenças na forma de transmissão, na capacidade de desenvolver infecção crônica, na severidade da doença hepática e na resposta ao tratamento. Contudo, vale ressaltar que ainda é necessário cautela no uso dessas informações na prática clínica, uma vez que há uma série de limitações e controvérsias nesse campo.

Os diferentes genótipos e alguns subgenótipos parecem estar mais associados a algumas vias específicas de transmissão e uma das razões para explicar essas diferenças está relacionada com diferenças no tempo de conversão do HBeAg para o anti-HBe. Indivíduos com HBeAg positivo apresentam níveis de carga viral mais elevado e consequentemente maior possibilidade de transmissão, particularmente pela via vertical (20, 171, 202-205). Os genótipos B e C estão mais envolvidos em casos de transmissão vertical do que outros genótipos, que têm sido mais associados com a transmissão horizontal (sexual e parenteral). O subgenótipo A2 parece estar mais associado com transmissão sexual, assim como genótipo G, cuja transmissão é mais frequente entre homossexuais masculinos. Na Europa as infecções pelo HBV

transmitidas pelo uso de drogas endovenosas estão relacionadas com o genótipo D (202, 203).

Em relação ao papel dos genótipos na evolução da doença a maioria dos estudos publicados até agora foram realizados na Ásia e restringem-se a comparar os genótipos B e C, que predominam nessa região. Esses estudos associam as formas mais graves de doença hepática e o aparecimento de cirrose ao genótipo C. Além disso, os pacientes com genótipo C parecem apresentar mais frequentemente a presença do HBeAg e níveis mais altos de viremia, o que pode contribuir para uma pior evolução da doença (171, 202, 203, 205, 206).

Embora os subgenótipos C1 e C2 estejam associados com desenvolvimento de CHC, apenas o subgenótipo C2 foi independentemente associado a maior risco de evolução para esse quadro. Na China e em Taiwan, a infecção com o subgenótipo B2 foi associada com desenvolvimento ou recorrência de CHC em pacientes jovens, enquanto que a infecção pelo subgenótipo B1 é frequentemente associada a episódios de hepatite fulminante no Japão (171, 202, 203, 205, 206).

Estudos realizados em regiões (principalmente Europa) onde os genótipos A e D são mais prevalentes têm sugerido que a infecção pelo genótipo A tem maior evolução para a cronicidade do que a infecção pelo genótipo D (203). Por outro lado, um estudo realizado na Espanha entre pacientes com hepatite crônica, mostrou que os casos com infecção pelo genótipo A (provavelmente A2) estiveram mais associados com elevada taxa de remissão bioquímica sustentada, clareamento do HBV DNA e do HBsAg do que aqueles infectados pelo genótipo D (205).

Um estudo realizado na Índia mostrou maior relação do genótipo D com severidade da doença hepática e evolução para CHC em relação ao genótipo A (205).

Comparações feitas entre indivíduos infectados pelo subgenótipo A2 e genótipo D mostraram que a taxa de complicações da doença hepática, incluindo CHC foi menor nos casos de A2 (203, 205).

O subgenótipo A1 tem sido associado com elevadas taxas de CHC na África-Subsaariana. Um estudo caso-controle com pacientes africanos demonstrou que o risco de desenvolver CHC foi 4,5 vezes maior naqueles infectados com o HBV/A1 do que entre aqueles infectados com outros genótipos (171, 203, 205).

No Alasca, em um estudo que acompanhou pacientes com HBC por cerca de 20 anos, uma proporção significativamente alta dos pacientes infectados com os subgenótipos F1 ou C2 desenvolveram CHC em comparação com aqueles infectados com A2, B6 ou D (203, 205).

No México o genótipo H foi encontrado em um grande número de casos de hepatite B oculta, caracterizada por baixos níveis de carga viral, HBsAg negativo e com presença ou não de evidências de infecção prévia pelo HBV (Anti-HBs e Anti-HBc + ou Anti-HBc + isoladamente) (207).

Em relação ao tratamento, pacientes com genótipos A e B apresentaram maior taxa de resposta ao tratamento com interferon (soroconversão do HBeAg e negativação do HBV DNA) do que aqueles infectados com genótipos D ou C. Contudo, nenhuma diferença significativa foi observada na resposta ao tratamento com análogos nucleos(t)ídeos (203-205, 208).

9.1. MUTAÇÕES NO GENE S

A proteína S é constituída por 226 aminoácidos e apresenta três regiões: região N-Terminal (aa 1 – 99), a principal região hidrofílica (MHR – do inglês “major hydrophilic region”) (aa 100 – 169) e a região C-terminal (aa 170 – 226). Dentro da MHR está localizada uma região conhecida como determinante “a”, que é o principal sítio de reconhecimento dos anticorpos neutralizantes anti-HBs. Essa região está localizada entre os aminoácidos 124 – 147 e nela são encontrados cinco resíduos de cisteína (C) ligados por pontes dissulfeto (C 121 – 124; C 124 – 137 e C139 – 147), as quais são responsáveis pela estabilidade e estrutura conformacional que conferem a imunogenicidade ao HBsAg (209).

Mutações localizadas no gene S do HBV podem resultar na alteração da estrutura antigênica do HBsAg com consequente perda da imunogenicidade. Vírus com mutações no gene S, principalmente na região do determinante “a”, podem não ser neutralizados pelos anticorpos induzidos pela vacina e causar infecção em pessoas vacinadas. Além disso, alguns desses mutantes podem não ser detectados pelos testes imunoenzimáticos utilizados na detecção do HBsAg (209).

Cepas do HBV com mutações na região que codifica o determinante “a” são selecionadas, sobretudo, pela pressão seletiva exercida pela vacinação ou tratamento com imunoglobulina hiperimune (209, 210). Alguns trabalhos têm demonstrado que a seleção dessas cepas também pode ocorrer durante o curso natural da infecção crônica sem a interferência de agentes seletivos exógenos, em alguns desses casos tem sido observada a coexistência de HBsAg e Anti-HBs (211) e em outros a ocorrência de hepatite B oculta (212).

A mutação mais comumente descrita que induz escape vacinal é a substituição do aminoácido Glicina (G) por Arginina (R) na posição 145 (sG145R) do HBsAg, a qual está localizada na segunda alça do determinante “a”. Este mutante foi identificado pela primeira vez na Itália infectando em uma criança nascida de uma portadora do HBsAg e HBeAg. Neste caso a criança havia recebido imunoprofilaxia adequada (vacina e imunoglobulina hiperimune) e apresentava anti-HBs (213). O DNA do HBV podia ser detectado neste caso e a sequência nucleotídica identificou esta mutação no epítopo *a* do AgHBs, para o qual são dirigidos os anticorpos neutralizantes, bem como os principais anticorpos utilizados nos testes para a detecção do HBsAg (214). Outros casos semelhantes foram também descritos em outras regiões, após o uso de vacina, imunoglobulina (206) ou anticorpo monoclonal (205) e foram associados com hepatites pós- transfusionais (215-219) soronegativas para o AgHBs com os testes utilizados. Testes sorológicos aprimorados, contendo anticorpos também contra outros epítomos do HBsAg permitem a detecção deste mutante (220).

Cepas do HBV com a mutação sG145R podem também ser transmitidas horizontalmente e permanecer replicando de forma estável por vários anos, embora a capacidade replicativa seja inferior à observada nas cepas selvagens, o que está relacionado com o fato dessa mutação resultar em secreção de vírions deficiente e redução de sua estabilidade (210).

Diversas outras mutações dentro da MHR associadas com escape vacinal, escape aos anticorpos utilizados no tratamento com imunoglobulina hiperimune (HBIG) ou ao escape aos anticorpos utilizados nos testes para a detecção do HBsAg têm sido descritas, entre elas as mais comuns são: sP120E/S/T, sT123N, sC124R/Y,

sI/T126A/N/S, sQ129H/L, sG130D/R, sM133L, sF/Y134N/R, sK141E/I, sP142S, sD144A/E, sN146S, sT148I, sC149R, sA157D/R, sF158Y, sE164D (209).

A prevalência de cepas do HBV com mutações no determinante “a” tem sido relatada com maior frequência entre crianças que receberam vacinas recombinantes do que entre aquelas vacinadas com vacinas derivadas de plasma. Além disso, também foram encontradas com maior frequência em países que implementaram a vacinação universal contra o HBV, entretanto com prevalência variadas: 39% em Singapura, 22% em Taiwan, 12% no Reino Unido e 0% na África do Sul (209, 210).

No Paraná, analisando cepas de 25 crianças que tinham recebido imunoprofilaxia porque eram filhas de mães portadoras do HBV, apenas uma delas tinha sido infectada por este vírus. Esta amostra apresentava mutações previamente associadas com o surgimento da doença em transplantados de fígado que haviam realizado a imunoprofilaxia e com o aparecimento da doença em crianças imunizadas em Cingapura. Este achado demonstra a possibilidade de circulação de cepas resistentes à imunoprofilaxia no sudoeste do Paraná (221).

Em um trabalho envolvendo 702 amostras de diferentes regiões brasileiras, 57 (8,5%) das amostras analisadas apresentaram cepas do HBV com mutações na região MHR previamente relacionadas com alteração da antigenicidade do HBsAg e escape dos anticorpos anti-HBs ou com prejuízo na secreção do HBsAg, e consequentemente das partículas virais. Essas mutações foram observadas em 20 posições da região MHR do HBsAg (dentro e fora do determinante “a”): Y100S, Q101H, P105R, T118R, V118A, G119R, G119E, P120T, P120S, K122N, T126I, A128T, Q129H, Q129R, G130R, G130N, M133T, M133L, M133I, Y134N, F134L, F134Y, S136P, T140I, S140T, S143L, 144E,

D144A, D144G, G145R, G145A, E164D, R169P. A mutação G145R, a mais importante de todas e relatada em casos de transmissão de hepatites mesmo após vacinação em vários países, foi relatada em 3 casos, mas ainda não existem outros dados sobre estes casos (222).

9.2. MUTAÇÕES NO GENE P

As mutações de maior importância no gene da polimerase ocorrem na região com função de transcriptase reversa (RT) e estão relacionadas com resistência aos AN utilizados no tratamento da hepatite B crônica. Assim como as demais mutações, estas surgem naturalmente em decorrência de erros inerentes ao processo de transcrição reversa, o que pode dar-se durante o curso natural da HBC ou durante o tratamento com AN. Neste último caso o surgimento de cepas com mutações de resistência ocorre devido à supressão incompleta da replicação, que pode ser resultado da baixa potência da droga, baixa dosagem ou aderência inadequada ao tratamento (223).

O tratamento com AN promove a pressão seletiva necessária para a emergência das cepas com mutações de resistência, as quais pela sua vantagem seletiva de se replicar na presença do antiviral vão se tornando dominantes sobre a população de vírus selvagens e se estabelecendo lentamente pela substituição do arquivo genético de cccDNA da cepa selvagem (223).

A identificação das mutações na polimerase é realizada através de uma nomenclatura padronizada que numera de forma independente os aminoácidos de cada uma das quatro regiões da polimerase (amino-terminal, espaçadora, RT e carboxi-

terminal). Essa padronização foi sugerida devido a diferenças no tamanho do genoma observadas entre os genótipos do HBV (presença de inserções ou deleções principalmente na região dos genes pré-S1 e polimerase), o que resultava em divergências no tamanho das polimerases e consequentemente na localização dos aminoácidos. O domínio de RT da polimerase do HBV de todos os genótipos inicia com uma sequência de aminoácidos padrão altamente conservada entre eles (EDWGPCDEHG) e possui 344 aminoácidos (224). As mutações são identificadas usando as letras rt para identificar o gene, o aminoácido original, o número da posição do aminoácido e o novo aminoácido gerado pela mutação, por exemplo rtM204I (225).

As mutações estabelecidas como causadoras de resistência do HBV aos AN ocorrem em 8 códons localizados nos diferentes domínios da região de RT da polimerase viral: domínio B (rtI169, rtL180, rtA181, rtT184), domínio C (rtS202, rtM204), domínio D (rtN236) e domínio E (rtE250). Existem outras mutações de grande importância no âmbito da resistência aos análogos, que são as mutações chamadas de compensatórias ou secundárias. Essas mutações têm a função de restaurar completamente ou parcialmente a capacidade replicativa das cepas resistentes, que geralmente é reduzida em comparação com as cepas selvagens devido às alterações na estrutura da polimerase. As mutações compensatórias são de grande importância para as cepas resistentes porque promovem a fixação das mutações de resistência no arquivo de cccDNA através do aumento dos níveis de replicação (223, 226).

A mais importante mutação responsável pela resistência à lamivudina localiza-se no locus YMDD (domínio C) e é caracterizada pela substituição de uma Metionina (M) para uma Isoleucina (I), para Valina (V) ou para Serina (S) na posição 204:

rtM204I/V/S. Posteriormente, a mutação rtA181T/V/S também foi relacionada com redução da susceptibilidade viral a esse NA (223, 227, 228). Além dessas mutações, diversas mutações compensatórias em diferentes domínios da RT já foram identificadas em cepas com resistência à lamivudina: rtL80V/I, rtV173L, rtL180M/C (223, 228), rtA200V (229), rtV/F/L/M207I (230), rtQ215S (231, 232), rtL217P e rtL229F (233).

A resistência ao adefovir foi inicialmente caracterizada pelas mutações rtA181V/T (domínio B) e/ou rtN236T (domínio D) (223, 226, 232).

Posteriormente, outras mutações foram associadas com resistência ou susceptibilidade reduzida ao adefovir: rtL80V/I (228, 234), rtV84M, rtS85A (228, 232), rtL180M (235), rtA181S (236), rtV214A, rtQ215S (228), rtL217R (229), rtE218G (237), rtI233V (223), rtP237H (228, 232), rtN238T/D/R, rtT240Y, rtN248H (228, 232, 238). Entre essas a rtV214A e a rtQ215S também foram associadas com falhas na resposta ao tratamento com lamivudina (48, 239). Entretanto o papel dessas mutações na resistência a esses AN não está estabelecido, em alguns casos os estudos ainda são escassos e em outros os resultados apresentados por diferentes estudos são contraditórios. Algumas dessas mutações tem sido confirmadas como polimorfismos de determinados genótipos como é o caso da rtL217R, que é encontrada em praticamente todas as cepas de A2 (182) e das mutações na posição rt215, que são encontradas com frequência bem maior nas cepas genótipo D (240).

A resistência ao entecavir ocorre quando há a combinação das mutações de resistência à lamivudina (rtM204V/I) com uma das seguintes mutações: rtI169T, rtT184G/A/I/S/C/L/F/M, rtS202G/I/C/A ou rtM250V/I/L/C (223, 241). Estas mutações

isoladamente conferem redução mínima da susceptibilidade ao entecavir (223). Um estudo publicado recentemente associou a presença de duas novas mutações (rtS219A e rtY245H) à redução da susceptibilidade ao entecavir (227).

Para o tenofovir nenhuma mutação foi definitivamente associada à resistência, todas aquelas associadas com redução da susceptibilidade descritas até o momento ainda são alvo de muita controvérsia ou foram pouco estudadas. Por outro lado, já se conhece que as mutações que conferem resistência ao adefovir (rtN236T e rtA181V/T) promovem a redução da eficácia do tratamento com tenofovir, uma vez que essas drogas são estruturalmente relacionadas (223, 226).

Há uma discussão na literatura acerca do papel da mutação rtA194T na redução da susceptibilidade a esse medicamento. Essa mutação foi encontrada em cepas do HBV isoladas de dois pacientes coinfectados pelo HIV que estavam em tratamento com tenofovir (242). Dois estudos que realizaram testes in vitro para avaliar a susceptibilidade dessas cepas ao tenofovir demonstraram que a mutação rtA194T causa redução na susceptibilidade ao tenofovir e também à lamivudina (240, 242), contudo outros dois estudos não observaram esses resultados (243, 244).

Recentemente um estudo testou uma série de mutações que poderiam interferir na ligação da polimerase do HBV com o tenofovir, as quais foram selecionadas após análises com métodos de bioinformática tendo como base a homologia existente entre as polimerases do HBV e HIV. Nesses estudos os autores concluíram que cepas do HBV com as mutações rtP177G ou rtF249A apresentam redução na susceptibilidade ao tenofovir (245).

A diversificação do arsenal terapêutico para o tratamento da hepatite B trouxe grandes benefícios para os pacientes não respondedores ao tratamento com interferon e àqueles que inicialmente se beneficiaram com a introdução da lamivudina, mas que logo foram surpreendidos pelo surgimento dos vírus resistentes. Por outro lado, o manejo do tratamento da hepatite B se tornou muito mais complexo, exigindo cada vez mais a disponibilidade de exames que permitam avaliar a resposta ao tratamento e a emergência de cepas resistentes. Esse monitoramento é fundamental no acompanhamento dos pacientes em tratamento com AN pelo fato de algumas mutações conferirem resistência cruzada a vários medicamentos. Por exemplo, cepas do HBV resistentes à lamivudina com as mutações rtL180M ± rtM204V/I, que desenvolvam mutações nas posições rtT184, rtS202 ou rtM250 serão resistentes também ao entecavir. A mutação rtA181T/V, que pode ser selecionada durante o tratamento com lamivudina, confere resistência ao adefovir e redução da susceptibilidade ao tenofovir (223, 226).

9.3. EFEITOS DE MUTAÇÕES NO GENE P NO GENE S

Além da redução da susceptibilidade do vírus ao tratamento, as mutações que ocorrem no gene da polimerase têm uma importante implicação epidemiológica uma vez que podem gerar alterações no HBsAg. Algumas dessas alterações podem reduzir a antigenicidade do HBsAg a ponto de promover o escape dos anticorpos neutralizantes anti-HBs desenvolvidos em resposta ao HBsAg selvagem, de forma similar ao que ocorre com as cepas mutantes selecionados pela vacina ou tratamento com imunoglobulina.

Isso ocorre devido à organização genômica do HBV, cujas regiões codificantes são sobrepostas, ou seja, uma mesma sequência de nucleotídeos é utilizada para a codificação de duas ou mais proteínas. A parte do genoma utilizada para codificar as proteínas do envelope também é utilizada para codificar a polimerase viral, logo substituições de nucleotídeos que provocam alterações em uma proteína podem também alterar a outra.

A capacidade de ligação dos anticorpos anti-HBs a proteínas HBs com algumas dessas mutações foi avaliada em um estudo que demonstrou elevada redução da afinidade na presença das mutações sE164D+sI195M, a qual foi similar àquela produzida pela presença da mutação sG145R (209). Essas mutações são geradas pela ocorrência das mutações de resistência à lamivudina rtV173L+rtM204V, que no geral são observadas em conjunto com a mutação rtL180M constituindo o padrão rtV173L+rtL180M +rtM204V encontrado em cerca de 20% dos casos com resistência à lamivudina (223).

Proteínas HBs com as mutações sE164D, sI195M, sW196S e sM198I também apresentam redução na capacidade de ligação com o anti-HBs, embora em menor grau (209).

Outro tipo de alteração que pode ser provocada em decorrência das mutações na polimerase é a introdução de mutações que geram códons de parada (*) prematura no gene S, o que leva à síntese de proteínas de envelope de tamanho menor do que o esperado. No quadro 5 são descritos aqueles resultantes de mutações relacionadas à resistência aos AN, contudo a presença dessa alteração já foi encontrada em outras

posições do HBsAg (sL15*, sL21*, sL95*, sW156*, sW163*, sW165*, sW182*, sW191*, sL216*) (210, 223, 234, 246, 247).

Essas proteínas truncadas prejudicam a secreção de partículas virais e consequentemente a infectividade do vírus. Apesar disso, as cepas com genomas produtores dessas proteínas conseguem se propagar e se manter na população viral através dos mecanismos de transcomplementação, onde os vírus mutantes utilizam as proteínas produzidas por cepas selvagens para montagem da partícula viral e infecção de outras células. Por outro lado, a não utilização dessas proteínas defeituosas resulta em acúmulo das mesmas no interior dos hepatócitos resultando em stress celular e ativação de vias celulares oncogênicas. Alguns estudos tem demonstrado a associação dessas mutações com risco aumentado de desenvolvimento de CHC (234, 246, 247).

9.4. MUTAÇÕES NOS GENE PRÉ-CORE/CORE E X & ALTERAÇÃO NA SÍNTESE DO HBeAG, NÍVEIS DE REPLICAÇÃO E EVOLUÇÃO PARA CHC

A região pré-core tem um importante papel na replicação viral, pois o RNAPg forma uma estrutura em alça (“hairpin”) nesta região através do pareamento entre os nucleotídeos das posições 1855 a 1858 com os nucleotídeos das posições 1896 a 1899. Esta estrutura é o local de interação entre a polimerase e o RNAPg, o que constitui o primeiro passo da encapsidação do RNAPg nas partículas de core. A mutação mais comumente descrita no gene pré-core é a substituição de guanina (G) por adenina (A) na posição 1896 (G1896A), que gera um códon de parada prematura e aborta a síntese do HBeAg. Esta mutação também pode aumentar a estabilidade da estrutura em alça

do sinal de encapsidação (ϵ) do RNAPg, o que aumenta a capacidade replicativa do vírus e, conseqüentemente, agrava o dano hepático (246).

A frequência da mutação G1896A varia entre os diferentes genótipos, pois depende do nucleotídeo na posição 1858, oposta ao nucleotídeo 1896 na alça do sinal de encapsidação. Portanto, a mutação G1896A raramente é encontrada nos genótipos A, F e H, nos quais G na posição 1896 normalmente pareia com citosina (C) na posição 1858. Nestes genótipos a substituição G1896A resulta no pareamento de bases C-A, o que desestabiliza a estrutura em alça do sinal ϵ . Porém, na América Central foram descritas cepas do HBV pertencente ao genótipo F com uma T na posição 1858 juntamente com a mutação G1896A (246).

Os demais genótipos apresentam uma timina (T) na posição 1858 que forma um pareamento estável com uma A na posição 1896, estabilizando a estrutura secundária do ϵ e, conseqüentemente, aumentando a replicação viral. No entanto, foram observadas cepas do HBV pertencentes ao genótipo C com uma C na posição 1858 em pacientes Asiáticos.

O papel da mutação G1896A no curso clínico da infecção ainda é bastante controverso. Inicialmente, alguns trabalhos demonstram que este mutante está associado a maior dano hepático e com surtos de hepatite fulminante, mas esse mutante também foi detectado em portadores assintomáticos do HBV (20, 248).

Uma mutação associada à negatividade do HBeAg em que também há uma substituição de G por A foi observada na posição 1899, podendo ocorrer isoladamente, em associação com a G1896A ou com outras mutações. Essa mutação também aumenta a estabilidade do ϵ por gerar um pareamento estável adicional (248).

Outras mutações menos comuns na região pré-core também podem resultar na ausência de síntese do HBeAg, tais como mutações no códon de iniciação do gene localizado nas posições 1814 a 1816.

As mutações A1762T e G1764A, localizadas dentro do promotor basal do core (BCP), estão envolvidas na supressão da síntese do HBeAg. Essas alterações provocam interferência na transcrição do RNA mensageiro dessa proteína, que é regulada pelo BCP (249, 250).

Mutações nas posições 1809 a 1812, que se localizam um pouco antes do códon de iniciação do gene pré-core, têm sido relacionadas com prejuízos na expressão do HBeAg. Experimentos de transfecção demonstraram que a expressão do HBeAg foi severamente prejudicada pela presença de mutações concomitantes nas posições 1809, 1811 e 1812 (G1809T, A1811T/C e C1812T), e sofreu redução moderada (semelhante à observada na presença das mutações A1762T/G1764A) pela presença de dupla mutação nas posições 1809 e 1812 (G1809T/A e C1812T) (251). Mutações nessa região têm sido descritas como características das cepas HBV/A1 circulantes na África, particularmente África do Sul. Dupla ou tripla mutação na região 1809-1812 foram encontradas em cerca de 80% das cepas HBV/A1 caracterizadas na África do Sul (176).

A presença de uma Timina (T) na posição 1653 caracterizando a mutação C1653T tem sido associada com risco aumentado de desenvolvimento de carcinoma hepatocelular, assim como a alteração de um T na posição 1753 para V (C, A ou G) (252, 253).

Duas outras mutações associadas à evolução clínica da infecção são as que ocorrem nas posições 1766 e 1768. A alteração de C para T na posição 1766 (C1766T) tem sido relacionada principalmente com aumento da replicação viral quanto associada à dupla mutação A1762T/G1764A. Alguns estudos têm relacionado a presença dessa mutação com evolução da infecção pelo HBV para insuficiência hepática aguda. Na posição 1768 a mutação que comumente tem sido encontrada é a substituição de T por A (T1768A) e a presença da mesma tem sido associada com desfechos similares aqueles descritos para a mutação na posição 1766 (250). Essas mutações também têm sido associadas com o desenvolvimento de CHC (254).

Um estudo realizado na Índia mostrou que as mutações C1766T e T1768A foram significativamente associadas com CHC e encontradas predominantemente no subgenótipo A1 (254). Vale ressaltar que o subgenótipo A1 tem sido associado com elevadas taxas de CHC na África-Subsaariana, o que também foi observado em outro estudo realizado na Índia. Foi avaliada a associação de CHC com a presença de mutações no genoma do HBV e as mutações C1766T/T1768A foram significativamente associadas com o genótipo A1 e com o CHC (254).

OBJETIVOS

2-OBJETIVOS

✓ **Desenvolver metodologias moleculares aplicadas para a detecção do vírus da hepatite B**

1. Y. Miyamoto & J.R.R. PINHO - Análise de alguns parâmetros envolvidos com o ensaio da DNA polimerase do vírus da Hepatite B. Revista do Instituto Adolfo Lutz 50 (1) : 297 – 299, 1990
2. J.R.R.PINHO, L.E.P. Fonseca, Y. Song, Y. Miyamoto, F.J. Carrilho, C.F.H. Granato, L.C. da Silva - Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/anti-HBe system, viral DNA polymerase and HBV-DNA. Revista do Instituto de Medicina Tropical de São Paulo 31(5): 328 - 335, 1989.
3. J.R.R. PINHO, C.A. Santos, C.L.M. Gonzalez, L. Bassit, C.C. Barreto, A. Saez-Alquézar, A.V.C. França, F.J. Carrilho, L.E.P. Fonseca, D.A.F. Chamone, & L.C. Da Silva -Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. Revista do Instituto de Medicina Tropical de São Paulo 35 (6): 515 –520, 1993.
4. Sitnik R, Paes A, Mangueira CLP, PINHO JRR. A Real-Time Quantitative Assay for Hepatitis B DNA (HBV) developed to detect all HBV genotypes. Revista do Instituto de Medicina Tropical de São Paulo,52 (3): 119-124, May-June, 2010

✓ **Avaliar a resposta ao tratamento da hepatite B utilizando marcadores moleculares**

5. L.C. da Silva, C.L. Assis-Madruga, F.J. Carrilho, J.R.R. PINHO, A. Saez-Alquézar, C. Santos, L. Bassit, C. Barreto, L.E.P. Fonseca, V.A.F. Alves, R. Leitão, R. Vianna, R.A. Cardoso, A.V.C. França & L.C.C. Gayotto - Spontaneous Hepatitis B surface antigen clearance in long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with Hepatitis C and D virus superinfection. Journal of Gastroenterology 31 (5): 696 - 701, 1996.
6. Luiz C. Da Silva; João R.R. Pinho; Roberta Sitnik; Luís E. P. Fonseca; Flair J. Carrilho – Predictive factors for response to lamivudine in chronic hepatitis B. Revista do Instituto de Medicina Tropical de São Paulo 42 (4) : 179 - 186 , July/August 2000.

7. Luiz Caetano da Silva, João Renato Rebello Pinho, Roberta Sitnik, Luís Edmundo Pinto da Fonseca & Flair José Carrilho. Efficacy and tolerability of a long term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *Journal of Gastroenterology* 36 (7): 476-85, July 2001.
8. Luiz Caetano da Silva, Maria Luiza da Nova, Suzane Kioko Ono-Nita, João Renato Rebello Pinho, Roberta Sitnik, Vera Aparecida dos Santos, Flair José Carrilho. Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic Hepatitis B. *Revista do Instituto de Medicina Tropical de São Paulo*, 51(5):261-268, September-October, 2009
9. Mendes-Correa MC, Pinho JR, Gomes-Gouvea MS, da Silva AC, Guastini CF, Martins LG, Leite AG, Silva MH, Gianini RJ, Uip DE. Predictors of HBeAg status and hepatitis B viraemia in HIV-infected patients with chronic hepatitis B in the HAART era in Brazil. *BMC Infectious Diseases* 2011 Sep 20; 11: 247.

✓ **Avaliar os diferentes genótipos e/ou subgenótipos virais e a presença de mutações com impacto clínico em diferentes grupos populacionais do Brasil e de outros países da América Latina (Chile, Colômbia e Panamá)**

a) Brasil

10. Sitnik R, Pinho JRR, Bertolini DA, Bernardini AP, da Silva LC & Carrilho FJ. Hepatitis B virus genotypes and precore and core mutations in Brazilian patients. *Journal of Clinical Microbiology* 42 (6): 2455-2460, 2004.
11. Michele S Gomes-Gouvêa, Ariana C Ferreira, Rosângela Teixeira, José R Andrade, Adalgisa SP Ferreira, Lena MF Barros, Rosamar EF Rezende, Ana CS Santos Nastri, Andrea GB Leite, Leonora Z Piccoli, Josiane Galvan, Simone RSS Conde, Manoel CP Soares, Dimas A Kliemann, Dennis A Bertolini, Aline SO Kunyoshi, André C Lyra, Marcio K Oikawa, Luciano V de Araújo, Flair J Carrilho, Maria CJ Mendes-Corrêa, João R Rebello Pinho. HBV carrying drug-resistance mutations in chronically infected treatment-naïve patients. *Antiviral Therapy* 2015 Jan 27 doi: 10.3851/IMP2938

b) São Paulo (capital)

12. Maria Cássia Mendes-Correa, JOÃO RENATO REBELLO PINHO, Roberta Sitnik, Rúbia Anita Ferraz Santana, Michele Mesquita Gomes-Gouvêa, Olavo M. Leite,

- Luiz G. Martins, Andrea G. Leite, Mariliza H. Silva, R.J.Gianini, David E. Uip. High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. *Journal of Medical Virology*, 2010. Sep; 82 (9):1481-8
13. Adriana Cristina da Silva, Ângela Maria Miranda Spina, Marcílio Figueiredo Lemos, Isabel Takano Oba, Cristina de Fátima Guastini, Michele Soares Gomes-Gouvêa, JOÃO RENATO REBELLO PINHO, Maria Cássia Jacintho Mendes-Correa. Hepatitis B genotype G and high frequency of lamivudine resistance mutations among HIV/Hepatitis B virus co-infected patients in Brazil. *Memórias do Instituto Oswaldo Cruz*, 2010 Sep. 105 (6): 770 -778.
14. Mônica Viviana Alvarado-Mora, Rúbia Anita Ferraz Santana, Roberta Sitnik, Paulo Roberto Abrão Ferreira, Cristovão Luís Pitangueira Manguiera, Flair José Carrilho, João Renato Rebello Pinho. Full-length genomic sequence of hepatitis B virus genotype C2 isolated from a native Brazilian patient. *Memórias do Instituto Oswaldo Cruz*, Rio de Janeiro, Vol. 106(4): 495-498, June 2011
15. Alvarado-Mora MV, Botelho-Lima LS, Santana RA, Sitnik R, Ferreira PA, do Amaral Mello F, Manguiera CP, Carrilho FJ, Rebello Pinho JR. Distribution of hepatitis B virus subgenotype F2a in São Paulo, Brazil. *BMC Res Notes*. 2013

c) São Paulo – Ribeirão Preto

16. Rosamar E. F. Rezende, Benedito A. L. Fonseca, Leandra N. Z. Ramalho, Sérgio Zucoloto, JOÃO RENATO R. PINHO, Dennis A. Bertolini, Ana L. C. Martinelli. The precore mutation is associated with severity of liver damage in chronic Hepatitis B. *Journal of Clinical Virology* 32(1): 53 – 59 (2005).

d) Paraná

17. Bertolini DA, Ribeiro PC, Lemos MF, Saraceni CP, Pinho JR. Characterization of a Hepatitis B virus strain in southwestern Paraná, Brazil, presenting mutations previously associated with anti-HBs Resistance. *Revista do Instituto de Medicina Tropical de São Paulo*, 2010 Feb;52(1):25-30
18. Bertolini DA, Gomes-Gouvêa MS, Carvalho-Mello IM, Saraceni CP, Sitnik R, Graziotin FG, Laurino JP, Fagundes NJ, Carrilho FJ, Pinho JR. Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil. *Infection, Genetics and Evolution*. 2012 Volume 12, Issue 6, August 2012, Pages 1295–1304 Apr 17

e) Santa Catarina

19. Flair J Carrilho, Cleusa R Moraes, JOÃO RR PINHO, Isabel MVGC Mello, Dennis A Bertolini, Marcílio F Lemos, Regina C Moreira, Leda C Bassit, Rita A Cardoso, Gabriela Ribeiro-dos-Santos & Luiz C Da Silva. Hepatitis B virus infection in Haemodialysis Centres from Santa Catarina State, Southern Brazil. Predictive risk factors for infection and molecular epidemiology. BMC Public Health 4 (1):13 (2004). doi: 10.1186/1471-2458-4-13

f) Bahia

20. Andre C Lyra, João RR Pinho, Isabel MVGC Mello, Fernanda de M Malta, Michele MS Gomes, Adrian M Di Bisceglie, Luiz GC Lyra, Flair J Carrilho, Luiz C da Silva. Distribution of Hepatitis B Virus (HBV) genotypes among patients with acute viral hepatitis. Journal of Clinical Gastroenterology (2005), 39 (1): 81-82.
21. Nadia Regina Caldas Ribeiro, Gubio Soares Campos, Ana Luiza Dias Ângelo, Eduardo Lorens Braga, Nelma Santana, Michele Mesquita Soares Gomes, JOÃO RENATO REBELLO PINHO, Wilson Andrade de Carvalho, Luiz Guilherme Costa Lyra, André Castro Lyra. Distribution of Hepatitis B virus genotypes among patients with chronic infection. Liver International 26: 1 – 7 (2006).

g) Pernambuco

22. Moura IF, Lopes EP, Alvarado-Mora MV, Pinho JR, Carrilho FJ. Phylogenetic analysis and subgenotypic distribution of the hepatitis B virus in Recife, Brazil. Infection, Genetics and Evolution. 2013 Mar;14:195-9.

h) Maranhão

23. Alvarado-Mora, Monica V, Botelho, Livia, Gomes-Gouvea, Michele S, de Souza, Vanda F, Nascimento, Maria C, Pannuti, Claudio S, Carrilho, Flair J, Pinho, Joao RR Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão, Brazil. Virology journal, 8: 415, 25 August 2011
24. Barros, Lena Maria Fonseca, Gomes-Gouvêa, Michele Soares, Kramvis, Anna, Mendes-Corrêa, Maria Cássia Jacintho, Santos, Alexsandro Dos, Souza, Letícia Alana Barros, Santos, Max Diego Cruz, Carrilho, Flair José, Domicini, Arnaldo De Jesus, Pinho, João Renato Rebello, Ferreira, Adalgisa De Souza Paiva. High prevalence of hepatitis B virus subgenotypes A1 and D4 in Maranhão State, Northeast Brazil. Infection, Genetics and Evolution 2014 Jun;24:68-75

i) Rondônia

25. Alcione O Santos, Monica V Alvarado Mora, Livia Botelho, Deusilene S Vieira, João R Rebello Pinho, Flair J Carrilho, Eduardo R Honda, Juan M Salcedo. Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virology Journal* 2010, 7:315

j) Amazônia

26. Conde SR, Moia L de J, Barbosa MS, Amaral I do S, Miranda EC, Soares M do C, Brito EM, Souza Odo S, de Araujo MT, Demachki S, REBELLO JR, Mesquita MG, Denis AB, Ishak R. Prevalence of Hepatitis B virus genotypes and the occurrence of precore mutation A-1896 and to correlate them with the clinical presentation of chronic hepatitis, in a population group of the Eastern Amazon region. *Rev Soc Bras Med Trop.* 2004; 37 (Supl 2):33-9 (2004).
27. Michele Soares Gomes-Gouvêa; Manoel do Carmo Pereira Soares; Isabel Maria Vicente Guedes C Mello; Elisabete Maria Figueiredo Brito; Lizomar de Jesus Pereira Moia; Gilberta Bensabath; Heloisa Marceliano Nunes; Flair José Carrilho; JOÃO RENATO REBELLO PINHO. Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin. *Acta Tropica* 106: 149 – 155 (2008),
28. Gomes-Gouvêa MS, Soares MC, Bensabath G, de Carvalho-Mello IM, Brito EM, Souza OS, Queiroz AT, Carrilho FJ, PINHO JR. Hepatitis B and Delta virus genotypes in Outbreaks of fulminant Hepatitis (Labrea black fever) in the Western Brazilian Amazon region. *Journal of General Virology.* 2009 Nov;90 (Pt 11):2638-2643.

k) Imigrantes Orientais

29. Carrilho FJ, Ono-Nita SK, Cardoso RA, Cançado ELR, PINHO JRR, Alves VAF, Da Silva LC. A prospective study of Hepatitis B virus markers in patients with chronic HBV infection from Brazilian families of Western and Asian origin. *Brazilian Journal of Medical and Biological Research* 38 (9): 1399-1408 (2005).
30. Cintia Mendes Clemente, Flair José Carrilho, João Renato Rebello Pinho, Suzane Kioko Ono-Nita, Luiz Caetano Da Silva, Regina Célia Moreira, Marcílio Figueiredo Lemos, Isabel Maria Vicente Guedes de Carvalho Mello. A phylogenetic study of Hepatitis B virus in chronically infected Brazilian patients of Western and Asian descent. *Journal of Gastroenterology*, 2009. 44 (6): 568-576

l) Imigrantes Africanos

31. Sitnik R, Sette Jr H, Santana RAF, Menezes LC, Graça CHN, Dastoli GTF, Silbert S, PINHO JRR. Hepatitis B virus genotype E detected in Brazil in an African patient who is frequent traveler. *Brazilian Journal of Medical and Biological Research* (2007), 40 (12): 1689-92.

m) Colômbia

32. Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvêa MS, de Azevedo Neto RS, Carrilho FJ, Pinho JR. Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population-how is the epidemiological situation? *PLoS One*. 2011 Apr 29;6(4):e18888
33. Alvarado-Mora MV, Romano CM, Gomes-Gouvêa MS, Gutierrez MF, Carrilho FJ, R Pinho JR. Phylogenetic analysis of complete genome sequences of hepatitis B virus from an Afro-Colombian community: presence of HBV F3/A1 recombinant strain. *Virology Journal*. 2012 Oct 24;9: 244

n) Chile

34. Mauricio Venegas, Mónica V. Alvarado-Mora, Rodrigo A. Villanueva, João R. Rebello Pinho, Flair J. Carrilho, Stephen Locarnini, Lilly Yuen, and Javier Brahm. Phylogenetic Analysis of Hepatitis B Virus Genotype F Complete Genome Sequences from Chilean Patients with Chronic Infection. *Journal of Medical Virology* 83:1530–1536 (2011). DOI: 10.1002/jmv.22129

o) Panamá

35. Martinez AA, Zaldivar Y, Hong CCh, Alvarado-Mora MV, Smith R, Ortiz AY, Pinho JR, Cristina J, Pascale JM. Molecular characterisation of hepatitis B virus in the resident Chinese population in Panama City. *Mem Inst Oswaldo Cruz*. 2013 Aug;108(5):541-7

✓ **Avaliar os tempos de infecção pelo vírus da hepatite B em diferentes grupos populacionais utilizando métodos de análise filogenéticos**

36. Mónica Viviana Alvarado Mora, Camila Malta Romano, Michele Soares Gomes-Gouvêa, Maria Fernanda Gutierrez, Flair José Carrilho, João Renato Rebello Pinho. Molecular epidemiology and genetic diversity of Hepatitis B virus (HBV) genotype E in an Afro-Colombian isolated community. *Journal of General Virology* 2010 Feb; 91(Pt 2):501-8
37. Alvarado, M.V., Romano, C. M., Gomes-Gouvea, M. S., Gutierrez, M. F., Carrilho, F. J., PINHO, J. R. R. 2010. Molecular Characterization of the Hepatitis B virus genotype in Colombia: A Bayesian Inference on the Genotype F. *Infection, Genetics and Evolution* 2011, 11 (1): 103-108
38. Godoy BA, Alvarado-Mora MV, Gomes-Gouvêa MS, Pinho JR, Fagundes NJ. Origin of HBV and its arrival in the Americas - the importance of natural selection on time estimates. *Antivir Ther.* 2013;18(3 Pt B):505-512.
39. Bouckaert R, Alvarado-Mora MV, Pinho JR. Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods. *Antivir Ther.* 2013;18(3 Pt B):497-503

RESULTADOS

3-RESULTADOS

- 1- Y. Miyamoto & J.R.R. PINHO - Análise de alguns parâmetros envolvidos com o ensaio da DNA polimerase do vírus da Hepatite B. Revista do Instituto Adolfo Lutz 50 (1): 297 – 299, 1990**

Rev. Inst. Adolfo Lutz,
50(1/2): 297-299, 1989.

ANÁLISE DE ALGUNS PARÂMETROS ENVOLVIDOS COM O ENSAIO DA DNA POLIMERASE DO VÍRUS DA HEPATITE B (*)

Yuriko MIYAMOTO**
João Renato Rebello PINHO***

RIALA6/695

MIYAMOTO, Y. & PINHO, J.R.R. — Análise de alguns parâmetros envolvidos com o ensaio de DNA polimerase do vírus da hepatite B. *Rev. Inst. Adolfo Lutz*, 50(1/2): 297-299, 1990.

RESUMO: O micrométodo para detecção da atividade da DNA polimerase do vírus da hepatite B descrito por LIN et alii² foi modificado, e realizou-se um estudo comparativo entre as duas técnicas. Além disso, foram comparados dois tipos diferentes de papel de filtro, o GF/C e o AP20. Todos os testes apresentaram resultados viáveis; ademais, podemos verificar que a técnica de Lin modificada e o filtro AP20 mostraram maiores diferenciais, sendo recomendados para este tipo de trabalho.

DESCRIPTORES: DNA polimerase; vírus da hepatite B; hibridização molecular; papel de filtro com fibra de vidro.

INTRODUÇÃO

A atividade da DNA polimerase (DNAP) do vírus da hepatite B (HBV) no soro é um dos marcadores que permitem detectar a replicação viral, através da monitoração da incorporação de nucleotídeos radioativos no genoma viral. Inicialmente, o método foi descrito por KAPLAN et alii¹, que utilizavam uma etapa de ultracentrifugação no preparo das amostras. Em 1984, LIN et alii² desenvolveram um micrométodo dispensando essa etapa, e utilizando ácido fosfonofórmico (PFA) como inibidor da DNAP viral.

Em nosso laboratório, utilizamos a técnica de Lin, porém com algumas modificações, e desenvolvemos uma análise comparativa entre o nosso método e o originalmente proposto pelo autor.

Além disso, realizamos um estudo com dois tipos diferentes de papéis de filtro com fibra de vidro, o GF/C, normalmente utilizado em nossas reações, e o AP20, para teste.

Algumas das amostras haviam sido anteriormente analisadas por hibridização molecular (HBV-DNA), conforme PINHO et alii³, e também por DNAP.

MATERIAL E MÉTODOS

No estudo das duas técnicas, utilizamos oito soros anteriormente testados por HBV-DNA, dos quais seis haviam também sido analisados por DNAP, sendo um soro positivo e um negativo para essas duas técnicas, três soros com resultados discordantes (um positivo para HBV-DNA e negativo para DNAP, e dois positivos para DNAP, mas negativos para HBV-DNA), um soro duvidoso com a hibridização e negativo para DNAP, e, finalmente, um positivo e um negativo testados apenas para HBV-DNA (Tabela 2).

Os soros foram incubados a 37°C por 16 horas, em solução de reagentes, de acordo com a tabela 1.

* Realizado pelo Laboratório de Biologia Molecular do Instituto Adolfo Lutz.

** Instituto de Medicina Social e de Criminologia do Estado de São Paulo.

*** Laboratório de Biologia Molecular do Instituto Adolfo Lutz.

MIYAMOTO, Y. & PINHO, J.R.R. — Análise de alguns parâmetros envolvidos com o ensaio de DNA polimerase do vírus da hepatite B. *Rev. Inst. Adolfo Luiz*, 50(1/2): 297-299, 1990.

Cada amostra foi incubada em duplicata, um tubo contendo PFA e outro sem PFA. Em seguida, foram adicionados 25µl de Pronase E, para parar a reação, incubando-se 40°C por uma hora.

As amostras foram então transferidas para papéis de filtro GF/C, recortados em círculos de 25 mm de diâmetro e secos em estufa a 70°C. A seguir, os filtros foram lavados cinco vezes em TCA 5% + Pirofosfato de Sódio 50 mM por dez minutos e uma vez em etanol por quinze minutos, sob agitação. Foram novamente secos a 70°C, e colocados em 8 ml de líquido de cintilação, para contagem da radioatividade por um minuto, em contador beta. O soro é considerado positivo se apresentar uma diferença significativa entre os dois tubos.

O intervalo entre a dosagem prévia de DNAP e este ensaio foi de cerca de seis meses.

Para a comparação dos dois tipos de filtros, foram utilizados um soro positivo e outro negativo, e papéis de filtro com fibra de vidro GF/C (Whatman) e AP20 (Millipore), analisados pelo método de Lin modificado.

RESULTADOS

Em todas as amostras, obtivemos resultados concordantes nas duas técnicas de DNAP, e também com a DNAP anterior. Das duas amostras que haviam sido testadas anteriormente somente por hibridização, uma foi igualmente negativa para DNAP, e a outra discordante (negativa para DNAP, e positiva para HBV-DNA), como mostra a tabela 2.

Os dois tipos de papéis de filtro apresentaram os resultados esperados, de acordo com a tabela 3.

TABELA 1

Solução de Reagentes

REAGENTES	MÉTODO DE LIN	
	Modificado	Original
Tris HCl pH 7,6	-	77mM
Tris HCl pH 7,4	100mM	-
KCl	154mM	400mM
MgCl ₂	38mM	-
Mg (CH ₃ COO) ₂	-	2,5mM
NTL ₂ Cl	57mM	-
2-Mercaptoctanol	0,14%	0,1%
Nonidet P-40	0,89%	1,0%
dNTPs (A+C+G) cada	0,1mM	0,3mM
³ H-TTP	1,5µCi	2,0µCi
PFA	2,5mM	1,0mM
Soro	50ul	25ul
Solução de reagentes	25ul	50ul

TABELA 2

Resultados dos Testes de DNAP e HBV-DNA

Soro	DNAP			HBV-DNA	
	1	2	3	1	2
1	+(532)	+(286)	+(346)	-	+++
2	N.R.	-(22)	-(28)	++	++
3	-(<0)	-(21)	-(36)	+/	-
4	-(<0)	-(48)	-(19)	+	-
5	+(846)	+(862)	+(399)	-	++++
6	N.R.	-(<0)	-(<0)	-	N.R.
7	+(646)	+(564)	+(285)	+++	N.R.
8	-(7)	-(2)	-(<0)	-	-

DNAP: 1- DNAP prévia; 2- Método de Lin modificado; 3- Método de Lin original. Os números entre parênteses indicam o diferencial: até 100 = negativo; de 100 a 200 = incorporação baixa; de 200 a 300 = incorporação moderada; acima de 300 = incorporação elevada.

HBV-DNA: 1- Hibridização anterior; 2- hibridização realizada posteriormente. O número de cruzes indica semi-quantificação da positividade (+ a ++++).

N.R.: Não realizado

TABELA 3

Comparação dos Papéis de Filtro

Soro	GF/C	AP20
+	+(4210)	+(5585)
-	-(<0)	-(14)

DISCUSSÃO

Os resultados mostram que tanto a técnica de Lin modificada como a original são viáveis, embora a DNAP prévia talvez apresente maior sensibilidade, como se nota pelos diferenciais obtidos (Tabela 1).

A discordância entre a amostra positiva para HBV-DNA e negativa para DNAP deve-se talvez à maior sensibilidade da técnica. Quanto às duas amostras negativas para hibridização, e que foram positivas para DNAP, um novo teste de HBV-DNA foi realizado, cujo resultado comprovou a positividade de ambas. A amostra duvidosa e a positiva(+) para HBV-DNA, que era negativa para DNAP, numa segunda hibridização, foi também negativa (Tabela 2).

Pudemos observar também que, após seis meses de armazenamento a -20°C, os resultados obtidos por DNAP foram praticamente os mesmos.

No teste dos dois papéis de filtro, ambos apresentaram os resultados esperados, sendo que

MIYAMOTO, Y. & PINHO, J.R.R. — Análise de alguns parâmetros envolvidos com o ensaio de DNA polimerase do vírus da hepatite B. *Rev. Inst. Adolfo Lutz*, 50(1/2): 297-299, 1990.

o filtro AP20 mostrou um diferencial maior que o GF/C.

Nossos resultados demonstram também a utilidade da realização das duas técnicas, a DNAP e o

HBV-DNA, para a monitoração da replicação do HBV, pois a detecção da atividade da DNA polimerase funciona como um eficiente controle de qualidade da técnica de hibridização, ao menos durante a padronização desta.

RIALA6/669

MIYAMOTO, Y. & PINHO, J.R.R. — Some parameters for the assay of the DNA polymerase of the hepatitis B virus. *Rev. Inst. Adolfo Lutz* 50 (1/2): 297-299, 1990.

ABSTRACT: The micromethod for detection of the hepatitis B virus DNA polymerase activity described by LIN et alii was modified, and a comparative study between this two techniques was performed. Besides, two different kinds of filter paper utilized in the reaction, the GF/C and the AP20, were compared. All the tests showed viable results, although the modified Lin's technique and the filter AP20 showed larger differentials.

DESCRIPTORS: DNA polymerase; hepatitis B virus; molecular hybridization;

REFERÊNCIAS BIBLIOGRÁFICAS

1. KAPLAN, P.M.; GREENMAN, R.L.; GERIN, J.L.; PURCELL, R.H. & ROBINSON, W.S. — DNA polymerase associated with human hepatitis B antigen. *J. Virol.*, 12: 995-1005, 1973.
2. LIN, H.J.; WU, P.C.; LAI, C.L. & CHAK, W. — Micromethod for phosphonoformic inhibition assay of hepatitis B viral DNA polymerase. *Clin. Chem.*, 30: 549-552, 1984.
3. PINHO, J.R.R.; FONSECA, L.E.P.; SONG Y.; MIYAMOTO, Y.; CARRILHO, F.J.; GRANATO, C.F.H. & SILVA, L.C. — Comparison of serum hepatitis B virus replication markers in chronic patients: studies on HBeAg/Anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. São Paulo*, 31: 328-335, 1989.

Recebido para publicação em 23 de março de 1990

2- J.R.R.PINHO, L.E.P. Fonseca, Y. Song, Y. Miyamoto, F.J. Carrilho, C.F.H. Granato, L.C. da Silva - Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/anti-HBe system, viral DNA polymerase and HBV-DNA. Revista do Instituto de Medicina Tropical de São Paulo 31(5): 328 - 335, 1989.

Rev. Inst. Med. trop. São Paulo
31 (5): 328-335, setembro-outubro, 1989

COMPARISON OF SERUM HEPATITIS B VIRUS REPLICATION MARKERS IN PATIENTS WITH CHRONIC HEPATITIS B: STUDIES ON HBeAg/ANTI-HBe SYSTEM, VIRAL DNA POLYMERASE AND HBV-DNA

João Renato Rebello PINHO (1), Luis Edmundo Pinto da FONSECA (2), Yu SONG (1), Yuriko MIYAMOTO (1), Flair José CARRILHO (2), Celso Francisco Hernandez GRANATO (3) & Luiz Caetano da SILVA (2)

SUMMARY

The detection of HBV-DNA in serum by molecular hybridization is the most sensitive and specific marker of replication and infectivity of hepatitis B virus and currently is proposed as a routine diagnostic technique in the follow-up of HBV-related diseases. Comparing different techniques already described, we found that direct spotting of serum samples on nitrocellulose membranes under vacuum filtration, followed by denaturing and neutralizing washes is more practical, simple, sensible and reproducible.

DNA polymerase assay using phosphonoformic acid as specific viral inhibitor has shown 86.8% of concordance with HBV-DNA detection, and so, it is an useful alternative in the follow-up of hepatitis B chronic patients.

We found 19.2% HBeAg positive samples with no other markers of viral replication and no anti-HBe positive sample had detectable HBV-DNA. Discordance between the 2 systems have been extensively described, and we confirm this for the first time in our country. Molecular biological techniques are essential to determine the replication status of chronic hepatitis B patients.

KEY WORDS: Hepatitis B; Molecular hybridization; DNA polymerase; HBV-DNA; HBV replication markers; Chronic hepatitis B.

INTRODUCTION

Hepatitis B is a worldwide public health problem involving more than 200 million people as Hepatitis B Virus (HBV) carriers, with high risk of developing severe chronic liver disease, cirrhosis and hepatocellular carcinoma²⁷. Its aetiological agent has been extensively studied and characterized and is now classified in the new group of Hepadnaviridae, which includes other

animal partial double-strand DNA hepatotropes virus that share many other common features with the human virus¹⁴.

During the natural history of this disease, there is a initial period with active viral replication, corresponding to the acute phase. In most of the cases, the evolution is benign, and the

(1) Molecular Biology Branch, Division of Virology, Instituto Adolfo Lutz, São Paulo, SP, Brazil.

(2) Clinical Hepatology Branch (LIM 07), Department of Gastroenterology and Hepatology Laboratory (LIM 47), Instituto de Medicina Tropical, Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil.

(3) Hepatitis Branch, Division of Virology, Instituto Adolfo Lutz, São Paulo, SP, Brazil.

Address for correspondence: Dr. Luiz Caetano da Silva, Instituto de Medicina Tropical, Faculdade de Medicina da Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 470, CEP 05403 São Paulo, SP, Brazil.

PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, Yu; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.

disease is aborted at this stage. In some cases, however, the host immune system is not capable to neutralize viral replication, and the disease progresses with continued viral replication for more than 6 months, characterizing the chronic forms of hepatitis B. In other cases, the viral DNA becomes integrated in the host cellular genome, and the viral replication ceases or decreases to undetectable levels¹².

The assessment of HBV replication status in following these patients is very important for prognosis, infectivity monitoring, and, furthermore, for indication and follow-up of antiviral or corticosteroid therapy⁶.

Many serum markers have been developed to characterize viral replication. Initially, it was shown that the detection of HBeAg in sera was related in many cases with other evidences of viral replication²³. HBeAg, detected by standard immunoassays, is a soluble antigen, and its synthesis occurs in the liver generally when the virus is actively replicating¹³. However, some direct methods to detect Dane particles constituents have shown discordance between the presence of viral replication and the detection of HBeAg⁸.

KAPLAN et al., 1973¹⁶ developed a technique to detect the HBV DNA polymerase activity in serum after ultracentrifugation. This technique is very cumbersome and not suitable for routine screening. LIN et al., 1984¹⁹ avoided the ultracentrifugation step using phosphonoformic acid as a specific viral polymerase inhibitor, creating a simpler and more sensitive method suitable for routine screening.

Detection of HBV-DNA in serum by molecular hybridization was developed by some groups in the early 80's^{1, 3, 18, 30} and is currently proposed as a routine diagnostic assay in the follow-up of HBV related diseases. The superiority of this technique has led to the detection of a more sensitive and specific marker of replication and infectivity of HBV⁹. The use of this technique allowed the detection of HBV homologous sequences in HBsAg negative chronic hepatitis cases^{6, 10}. Since then discrepancies between the HBeAg/anti-HBe system and direct HBV-DNA detection have been described.

Hybridization techniques described so far vary in many crucial features: direct immobilization of samples onto nitrocellulose membranes¹⁸, pre-treatment of samples with proteinase K²², previous denaturation of samples with Sodium hydroxide²⁶, utilization of Dextran-Sulphate in washes²¹. WALTER et al., 1986²⁸ compared various techniques and proposed pre-treatment of samples with formalin-sarkosyl as the most sensitive one, with no other treatment. Other groups have also shown that proteases digestion has adverse effects in sensitivity³¹.

Comparisons of the "e" system with others parameters of viral replication has led to variable results. Some groups found 67%²² and 68%⁶ HBeAg positive cases also HBV-DNA positive. For other groups, about 90 to 100% HBeAg positive cases have detectable DNA^{2, 18, 28, 30}. Considering anti-HBe cases, less concordance is found, with very high geographic variability¹⁷: in Northern Europe and USA anti-HBe positive patients were rarely HBV-DNA positive^{15, 28}, but in Italy², Greece¹⁸ and Taiwan⁷ more than half anti-HBe positive patients have detectable serum viral genomes.

Considering the data discussed above, the aims of our study are: first, compare the hybridization technique proposed by WALTER et al. with Scotto's technique proposed by ZELDIS et al. and the technique utilized by us; second, compare the DNA polymerase assay developed by LIN et al. with the detection of HBV-DNA; and, finally, compare the detection of HBeAg, anti-HBe, viral DNA polymerase and HBV-DNA in 38 serum samples from chronic hepatitis B patients.

MATERIAL AND METHODS

REAGENTS — Restriction endonucleases for isolating HBV-DNA from the vector DNA were supplied by Pharmacia and Sigma. Radioactive probes were labeled to specific activities between 10⁷ and 10⁸ cpm/ug with DNase I and DNA polymerase I from Sigma using alpha — ³²P — dATP manufactured at Instituto de Química da USP (a gentle gift of Dr. J.C.C. Maia and Dr. M.H. Juliani) (800 mCi/mmol). Nitrocellulose membranes BA85 were supplied from Schleicher & Schuell. Samples were concentrated using a

PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, Yu; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.

manifold apparatus manufactured at Tecnica Permatron (São Paulo, SP). Hybridizations were performed in heat-sealed plastic bags. Autoradiographies were performed with Kodak QH-S film and intensifying screens. ^3H — dTTP was supplied from Amersham. Other reagents were mainly supplied by Sigma or Merck.

PREPARATION OF HBV-DNA PROBES

— Cloned HBV-DNA was prepared in *Escherichia coli* HB101 transformed by p211 (a gentle gift of Dr. Francis Galibert, Laboratoire D'Hématologie Experimentale, Centre Hayem, Hôpital St. Louis, Paris, France) according to MANIATIS et al, 1982²⁰. This plasmid contains the entire HBV genome cloned in the EcoRI site of pBR322. Probes were labelled by nick-translation as described by RIGBY et al. (1976)²⁴. Standards were prepared by serial dilutions of purified HBV-DNA in water in a way that each 25 μl of solution contains always 100 μg of sheared herring sperm DNA and HBV-DNA quantities cited in the text. Serum samples standard were made by diluting a strong positive sample with a negative sample to the concentrations cited in the figures.

PREPARATION OF SAMPLES FOR HYBRIDIZATION:

TECHNIQUE A — Routine laboratory technique is a modified Liebermann's technique. Samples (25 μl) were directly applied onto a nitrocellulose membrane (previously soaked in water) using a manifold system. The filters were then submitted to a denaturing treatment with NaOH 0.5 M, NaCl 1M for 20 minutes, neutralized with Tris HCl 0.1 M pH 7.4, NaCl 0.5 M for 5 minutes twice and then rinsed with 2 x SSC 5 minutes twice.

TECHNIQUE B — A modified Scotto's technique. Samples (25 μl) were incubated previously with 25 μl 2 M NaCl and 50 μl 1 M NaOH for 10 minutes in microtitration plaques wells and then filtered through a nitrocellulose membrane (previously soaked in 6 x SSC) using a manifold system. Filters were then neutralized as described above.

TECHNIQUE C — Walter's technique — samples (25 μl) were denatured in 10 x SSC, 2 N NaOH, 15% formaldehyde and 0.5% Sarkosyl at 70°C for 10 minutes and then filtered through a nitrocellulose membrane using the manifold system. Filters were then neutralized as described above.

Whatever was the technique used, filters were air dried and then baked for 2 hours at 80°C in vacuum oven. Procedures were performed at room temperature, unless other temperature is cited.

PRE-HYBRIDIZATION AND HYBRIDIZATION

PRE-HYBRIDIZATION was performed submerging the filter in 20 ml of 5 x SSC, 1 x Denhardt, 0.1 M Sodium Phosphate pH 7.5, 1 mM EDTA, 0.1 mg/ml freshly heat denatured sheared herring sperm DNA at 65°C for at least 2 hours.

HYBRIDIZATION was performed with 10 ml of 4 x SSC, 2.5 x Denhardt, 0.1 M Sodium phosphate pH 7.5, 1 mM EDTA, 0.2 mg/ml freshly denatured sheared herring sperm DNA, freshly heat-denatured nick-translated probe (10^6 - 10^7 cpm/ml) gentle shaking at 65°C overnight.

REMOVAL OF NON-SPECIFIC HYBRIDIZATION was made by filter treatment twice in 2 x SSC, 0.1% SDS, 65°C, 10 minutes each, 1 x SSC, 0.1% SDS, 65°C, 10 minutes, and 0.1 x SSC, 0.1% SDS, 65°C, 10 minutes.

AUTORADIOGRAPHY — Filters were air dried and exposed for 2 and 7 days to film and intensifying screens cited above.

DETERMINATION OF VIRAL POLYMERASE ACTIVITY — Lin's technique modified — in two 1.5 ml plastic tubes each serum sample (25 μl) is incubated at 37°C for 16 hours in 1 M Tris pH 7.4; KCl 0.15 M; MgCl₂ 45 mM; NH₄Cl 55 mM; 2 — mercaptoetanol 0.15%; Nonidet P-40 0.8%, 20 nM each non-radiolabelled nucleotide and 1 μCi 3 H-dTTP. In one of the 2 tubes was added 10 mM phosphonoformic acid as specific viral polymerase inhibitor. The reaction was

PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, Yu; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.

then stopped adding Pronase 1 mg/ml, that breaks viral capsid. Samples were then deposited on 2.5 cm diameter GF/C filters and non-incorporated nucleotides were removed by three washings in 5% trichloroacetic acid, 50 mM Sodium Pirophosphate. After drying, filters were counted in scintillation solution in a beta-counter. Samples were considered positive when there is a significant difference between the 2 tubes, compared to negative controls.

DETECTION OF HBeAg AND ANTI-HBe was performed with Abbott diagnostic Kits.

SAMPLES — 38 HBsAg positive serum samples from chronic hepatitis B patients were assayed by the 3 methods described above.

RESULTS

Comparative results between the three samples preparation procedures are shown in figure 1. In terms of detection of purified cloned HBV-DNA, all the techniques could detect as little as 100 pg of DNA diluted as described above. No hybridization signal was detected with the negative control sera. When comparing the detection of our standard serum sample dilutions, techniques A and B gave signals till the 1/16 dilution, but technique C shown no signal, even with the half-diluted sample.

Operationally the most simple technique is by far technique A that needs no separated treat-

Fig. 1 — Autoradiographies after 1 day of exposure of hybridized nitrocellulose filters submitted to spotting techniques A, B and C as described in text. From left to right: purified cloned DNA: 10ng, 1ng, 100pg, 50pg, 25pg, 10pg, positive serum sample: 1/2, 1/4, 1/8, 1/16; negative serum sample.

ment of individual serum samples and allows a rapid and efficient filtration of samples. Technique B, besides a longer operation time, elicits in some samples the appearance of protein clots that block total filtration of samples in commercial manifold systems. Considering these results, the other hybridizations were performed according to technique A. Longer autoradiographic exposures (7 days) allow the detection of as little as 10 pg of purified DNA, as shown in figure 2, without compromising specificity.

The results of the analysis of 38 serum samples by the four serologic methods described above, are shown in table 1. Hybridization signals with varied intensity over a clear background from some of these samples were shown in figure 3 even with some spillage and no round spot caused by the manifold apparatus available.

Fig. 2 — Autoradiography of hybridized nitrocellulose filter after 7 days of exposition performed according to technique A. Serial dilutions of HBV DNA as described in text. From left to right 10ng, 1ng, 100pg, 50pg, 25 pg, 10 pg, 1 pg. Positive serum sample dilutions 1/2, 1/4, 1/8, 1/16; negative serum sample.

TABLE 1

	DNA and Pol. +	DNA + Pol. -	DNA - Pol. +	DNA and Pol. -	Total
I. HBeAg +	17	3	1	5	26
Anti HBe -	65.5%	11.5%	3.9%	19.7%	
II. HBeAg +	0	0	0	1	1
Anti HBe +					
III. HBeAg -	0	0	1	8	9
Anti HBe +			11.1%	88.9%	
IV. HBeAg -	0	0	0	2	2
Anti HBe -					
Total	17	3	2	16	38

Comparative results of hybridization (DNA) and DNA polymerase (POL) assays in 4 different groups according to HBeAg/anti HBe status.

DISCUSSION

Development of molecular biology allowed the standardization of very specific and sensitive

PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, Yu; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.

Fig. 3 — Autoradiography of a nitrocellulose filter containing spotted serum samples and hybridized as described in the text. The filter contains negative samples and positive samples of varying intensity spotted in alternate wells. Results were expressed from + to ++++ according to the signal intensity.

methods driven to the direct detection of complete HBV particles components, such as DNA polymerase and viral DNA. Initially restrained to research laboratories, these techniques have been so simplified that, nowadays, the detection of HBV-DNA and DNA polymerase are considered essential diagnostic tools for determining the replication status of chronic hepatitis B patients, necessary for indication, follow-up and prognosis of antiviral or corticosteroids therapy⁶.

Since its first application in the beginning of this decade, many groups proposed several different procedures for serum HBV-DNA detection. Recently, WALTER et al., 1987²⁹ and ZELDIS et al., 1986³¹ compared some different techniques, and each proposed one different technique as the best one. We compared the Scotto's technique proposed by ZELDIS et al. and Walter's technique with our routine technique, which is a modification of Liebermann's technique. We got disappointing results with Walter's technique, and, comparable results using Scotto's procedures.

In our hands, previous denaturation of serum samples could elicit appearance of protein clots that will block filtration in commercial manifold apparatus. Other group using this technique utilise a self-planned equipment with larger wells, that will not be blocked by these clots²⁶. Most of the groups proposing direct spotting of

serum samples do not perform vacuum filtration to concentrate them, as the porous diameter of nitrocellulose membranes (220 or 450 nm) is larger than the diameter of Dane particle (42 nm), and some virus are not retained on nitrocellulose. WALTER et al., 1987²⁹ have even showed positive hybridization signals in four overloaded membranes submitted to vacuum filtration. However, these groups had to work with smaller quantities of serum (5 µl) than us (25 µl), because samples diffuse rapidly through nitrocellulose (5 µl sample directly spotted corresponds to about 1 cm diameter spot). So, even with the loss of some virus through nitrocellulose membrane porous, we can assay a quantity of serum 5-fold higher than simple direct spotting, allowing greater sensitivity. Unlike other groups^{26, 29}, we did not identify any serum inhibitor of DNA binding to nitrocellulose, as our sensitivity was increased when performing direct spotting. ZELDIS et al., 1986³¹ suggest that the 5' binding protein could work as a bridge to the membrane. Perhaps interactions between viral envelope and core proteins with the nitrocellulose, involving or not polymerised serum albumin, enhance the retention of viral particles.

Other method proposed to HBV replication monitoring is the detection of serum viral DNA polymerase activity. LIN et al., 1984¹⁹ developed a simplified technique, slightly modified by us. To our knowledge, Lin's method was never compared with HBV-DNA detection. This assay has shown a good correlation with hybridization (33/38 - 86.8%) and HBeAg positivity (29/40 - 72.5%). We found 3 sera HBV-DNA positive and DNA polymerase negative, and 2 other with inverse parameters. The former were all HBeAg positive and the latter were one HBeAg positive and one anti-HBe positive. This last serum was also anti-delta positive, and, it might represent a false positive for DNA polymerase. Another possibility is that the virus delta interferes with the results of this assay. Studies involving more anti-delta positive cases will be performed to enlarge our knowledge about this.

In conclusion, assaying viral DNA polymerase using the technique developed by LIN et al. is a good marker for HBV replication, although false positive samples could rarely ap-

PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, Yu; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.

pear. Comparing to hybridization, it has the advantage of involving tritium labelled nucleotides, that are less hazardous, easier to handle, and have longer half-life. Careful storage of sera is very important because the enzyme is very labile¹³. We are now trying to simplify the precipitation and washing steps, optimize positive/negative ratio and reproducibility, which is a common problem involving enzymatic reactions.

For the first time in our country, the comparison between HBeAg/anti-HBe system and molecular biological replication markers in chronic hepatitis B patients was performed. This is very important due to high geographic variability of this correlation. Our results show that among HBeAg positive/anti-HBe negative patients, 19.2% do not show other serum replication marker and 80.8% show one of them at least. On the other hand, 1/9 anti-HBe positive patient had detectable DNA polymerase but no HBV-DNA. This sample was already discussed above. None of our anti-HBe positive sample shown HBV-DNA.

These results are intermediate to those from other groups, where HBV replication markers were found in most (67 - 100%) HBeAg positive samples and in few to more than half anti-HBe positive samples. Many reasons may explain our results. First, we studied chronic hepatitis B patients, some of them evolving by spontaneous or induced HBeAg/anti-HBe seroconversion, that could explain the HBeAg positive/DNA negative sera. Second, some patients in advanced stages of disease could maintain this serological pattern^{6, 12}. Third, São Paulo is a region where the HBV prevalence is about 2%⁹, corresponding to medium endemicity region, with a paucity of studies through the world at this point. This could explain the intermediate characteristic of our population, with more HBeAg/DNA negative patients than Northern Europe and USA, and with fewer anti-HBe/DNA positive patients than found in highly endemic areas. Studies with larger populations, from different ethnic and regional origins will be performed in a larger study.

Anyway, these data clearly demonstrate some disagreement between HBeAg and HBV-DNA or DNA polymerase detection in chronic hepatitis B patients and reinforce the necessity

of monitoring them in the follow-up of these patients. Comparison between sorologic markers and detection of tissual viral antigens and in situ hybridization²⁵ are under investigation in our laboratories.

RESUMO

Comparação dos marcadores sorológicos da replicação do vírus da hepatite B: estudos sobre o sistema AgHBe/anti-HBe, DNA polimerase viral e HBV-DNA.

A detecção do genoma do HBV no soro por hibridização molecular é o mais sensível e específico marcador da replicação e infectividade do HBV, sendo sua utilização proposta como técnica rotineira no acompanhamento de doenças relacionadas a este vírus. Comparando diferentes técnicas descritas anteriormente, escolhemos a deposição direta das amostras séricas sobre a membrana de nitrocelulose sob filtração a vácuo, seguida de banhos desnaturantes e neutralizantes como mais prática e simples, com sensibilidade equivalente.

O ensaio da DNA polimerase usando ácido fosfonofórmico como inibidor viral específico mostrou 86.8% de concordância com a detecção direta do DNA viral, sendo, portanto, uma alternativa viável no acompanhamento de pacientes com hepatite crônica B.

Encontramos 19.2% das amostras AgHBe positivos sem outros marcadores de replicação viral. Por outro lado, nenhuma amostra anti-HBe positiva teve HBV-DNA detectável. Discordância entre estes dois sistemas foi extensamente descrita, e confirmamos pela primeira vez este fato em pacientes com hepatite crônica B em nosso país. Técnicas de biologia molecular são, portanto, fundamentais na determinação da replicação viral em cada paciente.

ACKNOWLEDGEMENTS

We are grateful to Doctors José Carlos da Costa Maia and Maria Helena Juliani for the alpha — 32 P - dATP, and to Doctors Francis Galibert and Christian Trépo for the plasmid containing the cloned HBV genome, to whom we are deeply indebted.

PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, YU; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.

ABREVIATIONS

HBV — Hepatitis B Virus

1 x SSC — 0.15 M NaCl, 0.015 M Sodium Citrate

1 x Denhardt — 0.02% Ficoll, 0.02% Polivinylpyrrolidone, 0.02% BSA

BSA — Bovine serum albumin.

REFERENCES

- BERNINGER, M.; HAMMER, M.; HOYER, B. & GERIN, J. L. — An assay for detection of the DNA genome of hepatitis B virus in serum. *J. med. Virol.*, 9: 57-66, 1982.
- BONINO, F.; HOYER, B. & NELSON, J. — Hepatitis B virus DNA in the sera of HBs antigen carriers: a marker of active hepatitis B virus replication in the liver. *Hepatology*, 1: 388-391, 1981.
- BRECHOT, C.; HADCHOUEL, M.; SCOTTO, J.; DEGOS, F.; CHARNAY, P.; TREPO, C. & TIOLLAIS, P. — Detection of hepatitis B virus DNA in liver and serum: a direct appraisal of the chronic carrier status. *Lancet*, 2: 756-758, 1981.
- BRECHOT, C.; DEGOS, F.; LUGASSY, C.; THIERS, V.; ZAFRANI, S.; FRANCO, D.; BENHAMOU, J. P.; WANDS, J.; ISSELBACHER, K.; TIOLLAIS, P. & BERTHELOT, P. — Hepatitis B virus DNA in patients with chronic liver disease and negative tests for hepatitis B surface antigen. *New Engl. J. Med.*, 312: 270-276, 1985.
- CARRILHO, F. J. & DA SILVA, L. C. — Epidemiologia. In: DA SILVA, L. C., ed. — *Hepatites agudas e crônicas*. São Paulo, Sarvier, 1986, p. 47-69.
- CHAUSSEADE, S.; THIERS, V.; BONOC, B.; HOUSSET, C.; BRECHOT, C. & DEGOS, F. — L'antigène HBe et l'ADN du virus B chez les malades d'hépatite chronique active. *Gastroenterol. clin. biol.*, 10: 744-747, 1986.
- CHU, C. M.; KARAYIANNIS, P.; FOWLER, M. J. F.; MONJARDINO, J.; LIAW, Y. F. & THOMAS, H. C. — Natural history of chronic hepatitis B virus infection in Taiwan: studies of hepatitis B virus DNA in serum. *Hepatology*, 5: 431-434, 1985.
- CRAXI, A.; WELLER, I. V. D. & BASSENDINE, M. F. — Relationship between HBV specific DNA polymerase and HBe antigen/antibody system in chronic HBV infection: factors determining selection of patients and outcome of antiviral therapy. *Gut*, 24: 143-147, 1983.
- FEINMAN, S. V.; BERRIS, B.; GUHA, A.; SOOKNANAN, R.; BRADDLEY, D. W.; BOND, W. W. & MAYNARD, J. E. — DNA: DNA hybridization method for the diagnosis of hepatitis B infection. *J. virol. Meth.*, 8: 199-206, 1984.
- FIGUS, A.; BLUM, H. E.; VYAS, G. N.; DE VIROHIS, S.; CAO, A.; LIPPI, M.; LAI, E. & BALESTRIERI, A. — Hepatitis B viral sequences in non A, non B or hepatitis B virus related chronic liver disease. *Hepatology*, 4: 364-368, 1984.
- GENESCA, J.; JARDI, R.; PRAT, S.; BUTI, M.; STEBAN, R.; HERNANDEZ, J. M.; LOPEZ VIVANCOS, J.; PUIG DOMENICH, P. & GUARDIA, J. — Valor de la determinación sérica del DNA del virus de la hepatitis B como marcador de replicación vírica. *Med. clín. (Barcelona)*, 87: 665-668, 1986.
- GERBER, M. A. & THUNG, S. N. — Biology of disease: molecular and cellular pathology of hepatitis. *Lab. Invest.*, 52: 572-590, 1985.
- GRANATO, C. F. H. — Avaliação do grau de replicação viral em indivíduos aguda ou cronicamente infectados pelo vírus da hepatite B: estudo sobre o antígeno HBe. São Paulo, 1988. (Dissertação de mestrado — Faculdade de Medicina da Universidade de São Paulo).
- GUST, I. D.; BURRELL, C. J.; COULEPIS, A. O.; ROBINSON, W. S. & ZUCKERMAN, A. J. — Taxonomic classification of human hepatitis B virus. *Intervirology*, 25: 14-29, 1986.
- HARRISON, T. J.; BAL, V.; WHEELER, E. G.; MEACOCK, J. J.; HARRISON, J. P. & ZUCKERMAN, A. J. — Hepatitis B virus DNA and e antigen in serum from blood donors in the United Kingdom positive for hepatitis B surface antigen. *Brit. med. J.*, 290: 663-664, 1985.
- KAPLAN, P. M.; GREENMAN, R. L.; GERIN, J. L.; PURCELL, R. H. & ROBINSON, W. S. — DNA polymerase associated with human hepatitis B antigen. *J. Virol.*, 12: 995-1005, 1973.
- KARAYIANNIS, P.; FOWLER, M. J. F.; LOK, A. S. F.; GREENFIELD, C.; MONJARDINO, J. & THOMAS, H. C. — Detection of serum HBV-DNA by molecular hybridization: correlation with HBeAg/anti-HBe status, racial origin, liver histology and hepatocellular carcinoma. *J. Hepat.*, 1: 99-106, 1985.
- LIEBERMANN, H. M.; LEBRECQUE, D. P.; KEW, M. C.; HADZIYANNIS, S. J. & SHAFRITZ, D. A. — Hepatologic detection of hepatitis B virus DNA directly in human serum by a simplified molecular hybridization test: comparison to HBeAg anti-HBe status in HBsAg carriers. *Hepatology*, 3: 285-291, 1983.
- LIN, H. J.; WU, P.; LAI, C. L. & CHAK, W. — Micromethod for phosphonoformic inhibition assay of hepatitis B viral DNA polymerase. *Clin. Chem.*, 30: 549-552, 1984.
- MANIATIS, T.; FRITSCH, E. F. & SAMBROOK, J. — *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1982.
- MEINKOTH, J. & WAHL, G. — Review: hybridization of nucleic acids immobilized on solid supports. *Analyt. Biochem.*, 138: 267-284, 1984.
- MORACE, G.; VON DER HELM, K. & DEINHARDT, P. — Detection of hepatitis B virus DNA in serum by a rapid filtration-hybridization assay. *J. virol. Meth.*, 12: 235-242, 1985.

- PINHO, J. R. R.; FONSECA, L. E. P. da; SONG, Yu; MIYAMOTO, Y.; CARRILHO, F. J.; GRANATO, C. F. H. & SILVA, L. C. da — Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/Anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31(5): 328-335, 1989.
23. NORDENFELT, E. I. & KJELLEN, L. — Dane particles, DNA polymerase, and e antigen in two distinct categories of hepatitis B antigen carriers. *Intervirology*, 5: 225-232, 1975.
24. RIGBY, P. W. J.; DIECKMAN, M.; RHODES, C. & BERG, P. — Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. molec. Biol.*, 113: 237-251, 1976.
25. RINTJES, P. J. M.; VAN DITZHUIJSEN, J.; VAN LOON, A. M.; VAN HAELEST, V. J. G. M.; BRONKHORST, F. B. & YAP, S. H. — Hepatitis B virus DNA detected in formalin fixed liver specimens and its relation to serologic markers and histopathologic features in chronic liver disease. *Amer. J. Path.*, 120: 411-418, 1985.
26. SCOTTO, J.; HADCHOUEL, M.; HERY, C.; YVART, J.; TIOLLAIS, P. & BRECHOT, C. — Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique: comparison with results for other viral markers. *Hepatology*, 3: 279-284, 1984.
27. STANDRING, D. N. & RUTTER, W. J. — The molecular analysis of hepatitis B virus. In: POPPER, H. & SCHAFFNER, F., ed. — *Progress in liver diseases*. Orlando, Grune & Stratton, 1986. v. 8, p. 311-331.
28. VAN DITZHUIJSEN, T. J. M.; SELTEN, G. C. M.; VAN LOON, A. M.; WOLTERS, G.; MATTHYSSEN, L. & YAP, S. H. — Detection of Hepatitis B virus DNA in serum and relation with IgM class anti-HBe titers in hepatitis B virus infection. *J. med. Virol.*, 15: 49-56, 1985.
29. WALTER, E.; BLUM, H. E.; OFFENSBERGER, W. B.; ZESCHNIOK, C.; OFFENSBERGER, S. & GEROK, W. — Spot blot hybridization assay for the detection of hepatitis B virus DNA in serum: factors determining its sensitivity and specificity. *Hepatology*, 7: 557-562, 1987.
30. WELLER, I. V. D.; FOWLER, M. J. F.; MONJARDINO, J. & THOMAS, H. C. — The detection of HBV DNA in serum by molecular hybridization: a more sensitive method for the detection of complete HBV particles. *J. med. Virol.*, 9: 273-280, 1982.
31. ZELDIS, J. B.; BEN-PORATH, E.; ENAT, R.; KIRSCH, K. & WANDS, J. — Correlation of HBV DNA and monoclonal reactivity to HBsAg in serum of patients with HBV infection. *J. virol. Meth.*, 14: 153-166, 1984.

Recebido para publicação em 13/6/1989.

- 3- J.R.R. PINHO, C.A. Santos, C.L.M. Gonzalez, L. Bassit, C.C. Barreto, A. Saez-Alquézar, A.V.C. França, F.J. Carrilho, L.E.P. Fonseca, D.A.F. Chamone, & L.C. Da Silva -Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. Revista do Instituto de Medicina Tropical de São Paulo 35 (6): 515 –520, 1993.**

DETECTION OF HEPATITIS B VIRUS DNA BY THE POLYMERASE CHAIN REACTION IN ANTI-HBe POSITIVE CHRONIC HEPATITIS B PATIENTS

J.R.R. PINHO (1,2,3), C.A. SANTOS (2), C.L.M. GONZALEZ (4), L. BASSIT (1), C.C. BARRETO (1), A. SAEZ-ALQUEZAR (1), A.V.C. FRANÇA (4), F.J. CARRILHO (4), L.E.P. FONSECA (4), D.A.F. CHAMONE (1) & L. C. da SILVA (3).

SUMMARY

Detection of HBV-DNA by PCR was compared with other serological markers (HBsAg, HBeAg and anti-HBe) in a series of 49 Chronic Hepatitis B patients, including 12 with a spontaneous clearance of HBsAg. None of these HBsAg negative cases were PCR positive, but 33/37 (89.2%) HBsAg positive cases were PCR positive ($p < 0.0001$). Among HBeAg positive samples, nine cases were HBeAg positive and anti-HBe negative, all of them PCR positive. Other 3 patients were HBeAg and anti-HBe positive and these cases were also found PCR positive. A third group included 21 patients anti-HBe positive and HBeAg negative: 19 of them were PCR positive and 2 were PCR negative. The last 4 cases were HBeAg and anti-HBe negative, two of them were PCR positive.

The detection of anti-HBe viremic cases in the present series suggest that preC variants could occur in our country. In conclusion, the integrated phase of chronic hepatitis B seems to be less frequent than it was assumed, when only HBeAg or dot blot hybridization techniques were used. The new term "low replication phase" might favorably replace the former "integrated phase".

KEYWORDS: HBV; PCR; replication Markers; chronic Hepatitis

INTRODUCTION

The natural history of Hepatitis B has been extensively studied in the last two decades. According to a recent proposed model, two different phases during the evolution of Chronic Hepatitis B (CHB) are observed, i.e. the replicative and the integrated phases²². The latter is characterized by the presence of serum HBsAg derived from integrated defective viral genomes expressing only this protein without active viral replication. The replicative phase is distinguished by detection of serum HBeAg, liver HBeAg and serum HBV-DNA by dot-blot hybridization²².

In spite of a good correlation among these replicative markers, some discrepancies have been observed between serum HBV-DNA and the HBe/anti-HBe system. Thus, serum viral genomes were detected in more than half of the anti-HBe positive patients in Italy², Greece^{15,18} and Taiwan⁸. According to a preliminary paper, based on dot blot hybridization performed in Brazilian patients with CHB, this pattern was not found in our country¹⁹.

Anti-HBe viremic cases were recently elucidated

(1) Fundação Pró-Sangue Hemocentro de São Paulo, São Paulo, SP, Brasil.

(2) Virology Branch - Instituto Adolfo Lutz, São Paulo, SP, Brasil.

(3) Virology and Hepatology Laboratories - Instituto de Medicina Tropical de São Paulo, São Paulo, SP, Brasil.

(4) Gastroenterology Branch - Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brasil.

Address for correspondence: João Renato Rebello Pinho, Instituto de Medicina Tropical de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 470, 05403-000 São Paulo, SP, Brasil.

PONTO, J.R.R.; SANTOS, C.A.; GONZALEZ, C.L.M.; BASSIT, L.; BARRETO, C.C.; SAEZ-ALQUEZAR, A.; FRANÇA, A.V.C.; CARILHO, F.J.; FONSECA, L.E.P.; CHAMONE, D.A.F. & SILVA, L.C. da - Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev. Inst. Med. trop. S. Paulo*, 35 (6):515-520, 1993.

by identification of HBV variants with mutations in the pre C region of the genome. These mutations can abolish HBeAg expression from infected cells and are now established as the cause of anti-HBe positive patients with circulating HBV - DNA^{3,4,5}.

The development of Polymerase Chain Reaction (PCR)¹⁰ allowed the detection of viral genomes in a larger number of samples, including some HBsAg negative^{14,22,28}. In anti-HBe positive samples, the incidence of HBV-DNA positivity determined by PCR has been reported by several groups ranging from 64 to 100%^{7,9,10,12,13}.

PCR based methodology was utilized by many different groups^{1,6,7,18}. When this technique is used in its most sensitive way (i.e. the final detection step with radioactive Southern blot hybridization), some groups did not find any correlation between the detection of viral DNA with any other immunologically detected viral antigen related to the presence of circulating viral particles (HBeAg, preS1 and preS2 antigens)^{7,10}. In a long term follow-up study, KORENMAN et al.¹⁶, showed that HBV-DNA usually disappeared at or around the time which HBsAg became undetectable. On the other hand, CARMAN et al.⁶, in a shorter follow-up study, found that all patients successfully treated with alpha-IFN have no viral DNA detectable by PCR after 18 months of the end of the therapy, independently of their HBsAg status.

KANEKO et al.^{13,18} developed a simplified non-radioactive nested PCR for the detection of HBV - DNA in serum that would be suitable for clinical application and is as sensitive as the PCR technique followed by the Southern blot hybridization.

Our aim was to compare the detection of serum HBV-DNA by PCR with HBsAg, HBeAg and anti-HBe in patients with CHB and to analyse the impact of the introduction of the PCR in the interpretation of CHB serological profile.

MATERIAL AND METHODS

Samples - Serum samples from 49 patients with CHB attended at an outpatient department of Gastroenterology with a follow-up of 1 to 19 years were collected. Twelve out of these 49 patients had previously been studied²¹ because of a spontaneous clearance of HBsAg. A control group of 11 healthy individuals, including 7 with isolated anti-HBe,

was also analyzed to ensure the specificity of the PCR results.

Serological tests - HBsAg, HBeAg and anti-HBe were detected by ELISA using commercial available reagents (Abbott Laboratories, USA).

Anti-HCV was detected using second generation ELISA kits (Ortho or Diagnostics Pasteur). Positive results were confirmed with the 2nd generation RIBA (Ortho). Anti-HIV was detected using both viral lysate (Genetic Systems) and recombinant (Wellcome) ELISA kits, and results were confirmed with the Western blot test (Dupont/Biotech). Anti-HDV was detected using Organon ELISA kit. Only patients negative for anti-HCV, anti-HDV and anti-HIV were analyzed.

Polymerase Chain Reaction - For the detection of HBV-DNA in serum by PCR, the technique developed by KANEKO et al.¹³ was used with minor modifications. To avoid carry-over between samples, the procedures described by KWOK & HIGUCHI¹⁷ were strictly followed.

Sample preparation - in a 0.5ml GeneAmp reaction tube (Perkin Elmer), serum (10µl) was mixed with 2.5µl NaOH 0.5M (Sigma), overlaid with 100µl of mineral oil and incubated for 1 hour at 37 °C. After this time, the solution was neutralized by adding 2.5µl HCl 0.5M (Merck).

OLIGONUCLEOTIDES:

Oligonucleotides covering a well conserved sequence of the C gene were synthesized by Genomics, São Paulo, Brazil, according to the sequences proposed by KANEKO et al.¹³, as shown below.

1763: 5' GCT TTG GGG CAT GGA CAT TGA CCC GTA TAA 3' (30mer)

2032R: 5' CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT 3' (30mer)

1778-E: 5' GAC GAA TTC CAT TGA CCC GTA TAA AGA ATT 3' (30mer)

2017R-B: 5' ATG GGA TCC CTG GAT GCT GGG TCT TCC AAA 3' (30mer)

AMPLIFICATION:

FIRST PCR: using the GeneAmp PCR kit (Perkin Elmer), the volumen as completed to 100µl, containing 2.5U Taq polymerase, 200 µM each deoxynucleotide, 1µM primers 1763 and 2032R, 50mM KCl, 50mM TrisHCl pH 8.3, 1.5mM MgCl₂ and 0.01% gelatin.

PINHO, J.R.R.; SANTOS, C.A.; GONZALEZ, C.L.M.; BASSIT, L.; BARRETO, C.C.; SAEZ-ALQUEZAR, A.; FRANÇA, A.V.C.; CARRILHO, F.J.; PONSECA, L.E.P.; CHAMONE, D.A.F. & SILVA, L.C. *de* - Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev. Inst. Med. trop. S. Paulo*, 35 (6):515-520, 1993.

The amplification was performed placing the tubes in a DNA Thermal Cycler 480 (Perkin Elmer), programmed to 30 cycles (denaturation 94 °C 1.5 min, annealing 42 °C 1.5 min, extension 72 °C 1.5 min - total duration: 3 hours).

Second PCR: after brief centrifugation, 10 µl from the 1st amplification are transferred to another Gene Amp tube, and amplified with primers 1778-F and 2017R-B in the same way as described above.

Analysis of the PCR: after brief centrifugation, 15 µl aliquots from the 1st and 2nd PCR were analysed in an agarosis gel 1% (Sigma) in 0.5xTBE and 0.5 µg/ml Ethidium bromide (Sigma).

Samples were considered positive when a 258bp band was visualized after the 2nd PCR and, sometimes, a 270bp band was also visualized after the 1st PCR (Figure 1).

Statistics - The Fisher exact test was utilized for statistical analysis of the results.

RESULTS

Out of 49 patients studied, 37 (75.5%) were HBsAg positive and 12 (24.5%) were HBsAg negative (spontaneous clearance during follow-up). None of the HBsAg negative cases were PCR positive, but 33/37 (89.2%) HBsAg positive cases were PCR positive ($p < 0.0001$) (Figure 2). No patient in the control group was PCR positive.

Another analysis considered only the 37 HBsAg positive patients (Figure 3). Nine of these patients were HBeAg positive and anti-HBe negative, all of them PCR

Fig. 1 - Detection of HBV-DNA by PCR. Two negative samples 1 and 2, 8 and 9 (1st and 2nd amplifications, respectively). Three positive samples in lanes 3 and 4, 5 and 6, 10 and 11 (1st and 2nd amplifications, respectively). Molecular weight standard (pBR322 Hae III digested) in lane 7.

HBsAg AND PCR

Fig. 2 - Detection of HBV-DNA by PCR according to the presence (+) or absence (-) of serum HBsAg.

HBeAg/anti-HBe and PCR

Fig. 3 - Detection of HBV-DNA by PCR in HBsAg(+) samples according to HBeAg/anti-HBe status.

positive. Other 3 patients were HBeAg and anti-HBe positive and these cases were also found PCR positive. A third group included 21 patients anti-HBe positive and HBeAg negative: 19 of them were PCR positive and 2 were PCR negative. The last 4 cases were HBeAg and anti-HBe negative, two of them were PCR positive.

DISCUSSION

In this study, we compared the detection of routine serologic HBV markers (HBsAg, HBeAg and anti-HBe) with the detection of viral DNA by PCR. The utilization of this latter technique substantially modifies the interpretation of serological results. An absolute concordance between the detection of HBeAg and HBV-DNA by PCR was observed in this study. On the other hand, the seroconversion of HBeAg to anti-HBe may not correspond to a complete interruption of viral replication, as

PINHO, J.L.R.; SANTOS, C.A.; GONZALEZ, C.L.M.; BASSIT, L.; BARRETO, C.C.; SAEZ-ALQUEZAR, A.; FRANÇA, A.V.C.; CARRILHO, F.J.; FONSECA, L.E.P.; CHAMONE, D.A.F. & SILVA, L.C. da - Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev. Inst. Med. trop. S. Paulo*, 35 (6):515-520, 1993.

viral DNA in serum was detected in most of our anti-HBe positive cases. The impact of PCR in the interpretation of serological patterns of chronic hepatitis B can be clearly seen if we compare the present data with our previous results: HBV-DNA was detected by dot-blot hybridization in 67% of HBeAg positive and none of anti-HBe positive patients with CHB¹⁸.

HBV-DNA was detected by PCR in most of our HBeAg positive cases, independently of their HBeAg / anti-HBe status, while no viral DNA was detected in the patients with CHB who cleared serum HBeAg. These data reinforce the recent hypothesis that the presence of circulating HBeAg correlates with active viral replication¹¹. The few positive HBeAg and negative PCR cases might be associated with the integration of viral DNA in the genome of host cells, with exclusive HBeAg expression, without active viral replication. In such cases, we cannot rule out the possibility of viral particles circulating at lower levels than the PCR detection threshold.

Two different hypotheses may explain the concomitance of anti-HBe and HBV-DNA in HBeAg positive patients. First, we have to consider the high sensitivity of PCR. This technique detect as less as 3 viral particles¹², even when the DNA is contained inside viral particles complexed with immunoglobulins. In this way, viral particles could still be present in very low levels, beyond the detection threshold of serological methods. Free DNA in serum derived from lysed hepatocytes or peripheral mononuclear blood cells was also suggested as the source of PCR positivity in some cases¹.

The second hypothesis refers to the presence of viral variants with preC mutations. These variants were initially found in countries where anti-HBe viremic cases were very frequent and a mutation introducing a stop codon in the preC region has been identified in patients with this serological pattern^{2,13}. The detection of anti-HBe viremic cases in the present series suggests that these variants could occur in our country; however, further molecular studies are needed to confirm this hypothesis.

The clinical approach for anti-HBe and PCR positive cases ("low replication phase") is a crucial problem arising from these results. The presence of preC variants in the presence of ongoing active disease and active replication, may indicate antiviral treatment. On the other hand, if these results are only due to the

extremely high sensitivity of PCR, a expectant approach would be desirable with careful clinical and serological follow-up of these patients to assess the disease activity. As a matter of fact, it has been observed that PCR positive patients show higher ALT levels than PCR negative ones, indicating the presence of hepatocyte damage¹.

In conclusion, the integrated phase of chronic hepatitis B seems to be less frequent than it was assumed, when only HBeAg or dot blot hybridization techniques were used, as recently suggested by HOOFNAGLE¹¹.

The new term "low replication phase", used by ZOULIM et al.²⁵ might favorably replaces the former "integrated phase", as integration without replication seems to be very rare in Hepatitis B, at least in some geographic areas. Nevertheless, on the clinical standpoint, it is still controversial how important is it to separate the disease in these two new phases. Therefore, the development of sensitive methods to quantify serum HBV-DNA should improve the follow-up of these patients, allowing a direct method to evaluate viral replication and thus providing a better definition of these two different phases.

RESUMO

Deteção do DNA do vírus da Hepatite B pela reação em cadeia da polimerase em pacientes com Hepatite Crônica B soropositivos para o anticorpo Anti-HBe

A detecção do DNA do Vírus da Hepatite B pela Reação em Cadeia da Polimerase (PCR) foi comparada com os outros marcadores sorológicos virais (AgHBs, AgHBe e anti-HBe) numa série de 49 pacientes com hepatite crônica B, incluindo 12 que apresentaram esclarecimento espontâneo do AgHBs. Nenhum caso AgHBs negativo foi PCR positivo, mas 33/37 (89,2%) dos casos AgHBs positivos foram PCR positivos ($p < 0,0001$). Entre as amostras AgHBs positivas, 9 foram AgHBe positivas e anti-HBe negativas, todas elas PCR positivas. Outros 3 pacientes foram AgHBe e anti-HBe positivos, todos também PCR positivos. Um terceiro grupo incluía 21 pacientes anti-HBe positivos e anti-HBe negativos: 19 foram PCR positivos e 2 PCR negativos. Os últimos 4 casos foram AgHBe e anti-HBe negativos, sendo 2 destes PCR positivos. A detecção de casos virêmicos anti-HBe positivos sugere que as va-

PINHO, J.R.R.; SANTOS, C.A.; GONZALEZ, C.L.M.; BASSIT, L.; BARRETO, C.C.; SAEZ-ALQUEZAR, A.; FRANÇA, A.V.C.; CARRILHO, F.J.; FONSECA, L.E.P.; CHAMONE, D.A.F. & SILVA, L.C. da - Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev. Inst. Med. trop. S. Paulo*, 35 (6):515-520, 1993.

riantes preC do VHB podem também estar presentes em nosso país. Em conclusão, a "fase de integração viral" da hepatite crônica B parece ser muito menos frequente do que se imaginava previamente, quando apenas se dispunha das técnicas de detecção de AgHBe e DNA por hibridização. O novo termo "fase de baixa replicação baixa" parece ser melhor que o anterior "fase de integração viral".

REFERENCES

- BAKER, B.L.; BISCEGLIE, A.M.; KANEKO, S.; MILLER, R.; FEINSTONE, S.M.; WAGGONER, J.G. & HOOFNAGLE, J.H. - Determination of hepatitis B virus DNA in serum using the polymerase chain reaction: clinical significance and correlation with virological and biochemical markers. *Hepatology*, 13: 632-636, 1991.
- BONINO, F.; HOYER, B. & NELSON, J. - Hepatitis B virus DNA in the sera of HBs antigen carriers: a marker of active hepatitis B virus replication in the liver. *Hepatology*, 1: 386-391, 1981.
- BRUNETTO, M.R.; STEMLER, M.; SCHODEL, F.; WILL, H.; OTTOBRELLI, A.; RIZZETTO, M.; VERME, G. & BONINO, F. - Identification of HBV variants which cannot produce precore derived HBeAg and may be responsible for severe hepatitis. *Ital. J. Gastroenterol.*, 21: 151-154, 1989.
- BRUNETTO, M.R.; STEMLER, M.; BONINO, F.; SCHODEL, F.; OLIVERI, F.; RIZZETTO, M.; VERME, G.; WILL, H. - A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B. *J. Hepatol.*, 10: 258-261, 1990.
- CARMAN, W.F.; JACZYNA, M.R.; HADZIYANNIS, S.; KARAYIANNIS, P.; MCGARVEY, M.J.; MAKRI, A. & THOMAS, H.C. - Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet*, 2: 588-591, 1989.
- CARMAN, W.F.; DOURAKIS, S.; KARAYIANNIS, P.; CROSEY, M.; DROBNER, R. & THOMAS, H.C. - Incidence of hepatitis B viraemia, detected using the polymerase chain reaction, after successful therapy of hepatitis B virus carriers with interferon - alpha. *J. Med. Virol.*, 34: 114-118, 1991.
- CHÉMIN, I.; BAGINSKY, I.; PETIT, M.A.; ZOULIM, F.; PICHOU, C.; CAPEL, F.; HANTZ, O. & TREPO, C. - Correlation between HBV DNA detection by polymerase chain reaction and pre-S1 antigenemia in symptomatic asymptomatic hepatitis B virus infections. *J. Med. Virol.*, 33: 51-57, 1991.
- CHU, C.M.; KARAYIANNIS, P.; FOWLER, M.J.F.; MONJARDINO, J.; LIAN, Y.F. & THOMAS, H.C. - Natural history of chronic hepatitis B infection in Taiwan: studies of hepatitis B virus DNA in serum. *Hepatology*, 5: 431-434, 1985.
- GARBUGLIA, A.R.; MANZIN, A.; BUDKOWSKA, A.; TALIANI, G.; CLEMENTI, M.; DELFINI, C. & CARLONI, G. - Levels of pre-S antigens and HBV DNA in sera from high and low viraemic HBV carriers. *J. med. Virol.*, 35: 273-282, 1991.
- GERKEN, G.; PATERLINI, P.; MANN, M.; HOUSSET, C.; TERRE, S.; DIENES, H.P.; HESS, G.; GERLICH, W.H.; BURTHELOT, P.; MEYER ZUM BUSCHENFELDE, K.H. & BRECHOT, C. - Assay of hepatitis B virus DNA by polymerase chain reaction and its relationship to pre-S and S- encoded viral surface antigens. *Hepatology*, 13: 158-166, 1991.
- HOOFNAGLE, J.H. - Chronic hepatitis B. *New Engl. J. Med.*, 323: 337-339, 1990.
- KANEKO, S.; MILLER, R.H.; FEINSTONE, S.M.; UNOURA, M.; KOHAYASHI, K.; HATTORI, N. & PURCELL, R. - Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. *Proc. Nat. Acad. Sci. (Wash.)*, 86: 312-315, 1989.
- KANEKO, S.; FEINSTONE, S.M. & MILLER, R.H. - Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. clin. Microbiol.*, 27: 1930-1933, 1989.
- KANEKO, S.; MILLER, R.H.; DI BISCEGLIE, A.M.; FEINSTONE, S.M.; HOOFNAGLE, J.H. & PURCELL, R.H. - Detection of hepatitis B virus DNA in serum by polymerase chain reaction: application for clinical diagnosis. *Gastroenterology*, 99: 799-804, 1990.
- KARAYIANNIS, P.; FOWLER, M.J.F.; LOK, A.S.F.; GREENFIELD, C.; MONJARDINO, J. & THOMAS, H.C. - Detection of serum HBV DNA by molecular hybridization: correlation with HBeAg/anti-HBe status, racial origin, liver histology and hepatocellular carcinoma. *J. Hepatol.*, 4: 99-106, 1985.
- KORENMAN, J.; BAKER, B.; WAGGONER, J.; EVERHART, J.E.; DI BISCEGLIE, A.M. & HOOFNAGLE, J.H. - Long term remission of chronic hepatitis B after alpha-interferon therapy. *Ann. Intern. Med.*, 114: 629-634, 1991.
- KWOK, S. & HIGUCHI, R. - Avoiding false positives with PCR. *Nature*, 339: 237-238, 1989.
- LIEBERMANN, H.M.; LEBRECQUE, D.P.; KEW, M.C.; HADZIYANNIS, S.J. & SHAFRITZ, D.A. - Hepatologic detection of hepatitis B virus DNA directly in human serum by a simplified molecular hybridization test: comparison to HBeAg/anti-HBe status in HBeAg carriers. *Hepatology*, 3: 285-291, 1983.
- PINHO, J.R.R.; FONSECA, L.E.P.; SONG, Y.; MIYAMOTO, Y.; CARRILHO, F.J.; GRANATO, C.F.H. & SILVA, L.C. - Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: studies on HBeAg/anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev. Inst. Med. trop. S. Paulo*, 31: 328-335, 1989.
- SAIKI, R.K.; GELFAND, D.H.; STOFFEL, S.; SCHARF, S.; HIGUCHI, R.; HORN, G.; MULLIS, K. & ERLICH, H. - Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239: 487-491, 1988.
- SILVA, L.C.; MADRUGA, C.L.A.; CARRILHO, F.J.; PINHO, J.R.R.; SAEZ-ALQUEZAR, A.; SANTOS, C.A.; BASSIT, L.; BARRETO, C.C.; ALVES, V.A.F.; GAYOTTO, L.C.C. & CHAMONE, D.A.F. - Spontaneous clearance (SC) of serum HBsAg in twenty patients with chronic hepatitis B (CHB): HBV-DNA status by Polymerase Chain Reaction (PCR) and role of viral superinfection. *Gastroenterology*, 104: A688, 1993.
- SHERLOCK, S. - The natural history of hepatitis B. *Postgrad. med. J.*, 63 (suppl. 2): 7-11, 1987.

PINHO, J.R.R.; SANTOS, C.A.; GONZALEZ, C.L.M.; BASSIT, L.; BARRETO, C.C.; SAEZ-ALQUEZAR, A.; FRANÇA, A.V.C.; CARRILHO, F.J.; FONSECA, L.E.P.; CHAMONE, D.A.F. & SILVA, L.C. da - Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev. Inst. Med. trop. S. Paulo*, 35 (6):515-520, 1993.

23. SHIH, L.N.; SHEU, J.C.; WANG, J.T.; HUANG, G.T.; YANG, P.M.; LEE, H.S.; SUNG, J.L.; WANG, T.H. & CHEN, D.S. - Serum hepatitis B virus DNA in healthy HBsAg negative Chinese adults evaluated by polymerase chain reaction. *J. med. Virol.*, 32: 257-260, 1990.
24. WANG, J.T.; WANG, T.H.; SHEU, J.C.; SHIH, L.N.; LIN, J.T. & CHEN, D.S. - Detection of hepatitis B virus DNA by polymerase chain reaction in plasma of volunteer blood donors negative for hepatitis B surface antigen. *J. Infect. Dis.*, 163: 397-399, 1991.
25. ZOULIN, F.; MIMMS, L.; FLOREANI, M.; PICHOU, C.; CHÉMIN, L.; KAY, A.; VITVITSKI, L. & TREPO, C. - New assays for quantitative determination of viral markers in management of chronic hepatitis B virus infection. *J. clin. Microbiol.*, 30: 1111-1119, 1992.

Recebido para publicação em 22/06/1993

Aceito para publicação em 27/08/1993.

4- Sitnik R, Paes A, Mangueira CLP, PINHO JRR. A Real-Time Quantitative Assay for Hepatitis B DNA (HBV) developed to detect all HBV genotypes. Revista do Instituto de Medicina Tropical de São Paulo, 52 (3): 119-124, May-June, 2010

A REAL-TIME QUANTITATIVE ASSAY FOR HEPATITIS B DNA VIRUS (HBV) DEVELOPED TO DETECT ALL HBV GENOTYPES

Roberta SITNIK(1), Ângela PAES(2), Cristóvão Pitangueira MANGUEIRA(1,3) & João Renato Rebello PINHO(1,4)

SUMMARY

Hepatitis B virus (HBV) is a major cause of chronic liver disease worldwide. Besides genotype, quantitative analysis of HBV infection is extensively used for monitoring disease progression and treatment. Affordable viral load monitoring is desirable in resource-limited settings and it has been already shown to be useful in developing countries for other viruses such as Hepatitis C virus (HCV) and HIV. In this paper, we describe the validation of a real-time PCR assay for HBV DNA quantification with TaqMan chemistry and MGB probes. Primers and probes were designed using an alignment of sequences from all HBV genotypes in order to equally amplify all of them. The assay is internally controlled and was standardized with an international HBV panel. Its efficacy was evaluated comparing the results with two other methods: Versant HBV DNA Assay 3.0 (bDNA, Siemens, NY, USA) and another real-time PCR from a reference laboratory. Intra-assay and inter-assay reproducibilities were determined and the mean of CV values obtained were 0.12 and 0.09, respectively. The assay was validated with a broad dynamic range and is efficient for amplifying all HBV genotypes, providing a good option to quantify HBV DNA as a routine procedure, with a cheap and reliable protocol.

KEYWORDS: HBV DNA quantification; Real Time PCR; Hepatitis B virus; Viral load.

INTRODUCTION

Hepatitis B virus (HBV) is a major cause of chronic liver disease worldwide. This virus is a small DNA virus with a partially double-stranded 3.2 kb genome and has been classified into eight genotypes (A-H) based on sequence divergence over the entire genome¹⁶⁻¹⁸. These genotypes have a distinct and well established geographic distribution¹⁹ and impact disease prognosis and progression. In Brazil, genotypes A, D and F are the most prevalent, but genotypes B, C, E and G have also been reported^{3,5,23,25,26}.

Besides genotype, the quantitative analysis of HBV DNA is extensively used for monitoring disease progression and treatment of HBV infection. Commercial assays are available for quantification of HBV DNA, such as the Versant HBV DNA Assay 3.0 (bDNA - Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) with a dynamic range from 2×10^3 to 1×10^8 copies/mL²⁰ and the Amplicor HBV Monitor Test (Roche, Alameda, CA, USA), with a dynamic range from 2×10^2 to 2×10^8 copies/mL¹¹. Recently, several real-time PCR-based HBV DNA quantification assays have also been developed with the advantages of increased sensitivity, broader linearity and less carryover contamination risks^{13,20}. Real time PCR based kits for HBV detection have also been launched by diagnostic companies and are currently replacing former HBV DNA amplification kits, such as the COBAS TaqMan 48 real-time

PCR system (Roche Molecular Systems, Branchburg, NJ), and the Abbott Real Time HBV Quantification kit (Abbott Laboratories, USA)^{23,28}.

Affordable viral load monitoring is desirable in resource-limited settings and it has already been shown to be very useful in developing countries for the Hepatitis C virus (HCV) and HIV^{7,8,22,24,27}. In this paper, we describe the validation of a real-time PCR quantitative assay (qPCR) for HBV DNA based on TaqMan technology and MGB probe, which have a large dynamic range and can amplify all known HBV genotypes.

MATERIALS AND METHODS

Patients and clinical samples: For initial standardization, two international HBV reference plasmas developed by BBI Diagnostics (West Bridgewater, MA, USA) were used: PHD801 (concentration of 4.3×10^6 IU/mL diluted from 1 to 500,000X, genotype A) and QHD701 (concentration of 1.19×10^6 IU/mL diluted from one to 15,000X, genotype A).

Clinical samples stored at -80°C were obtained from 95 patients from other studies. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Ethical Committee from Hospital Israelita Albert Einstein, São Paulo, SP, Brazil. Written informed consent was obtained from each patient in the study.

(1)Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil.

(2) Departamento de Estatística, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil.

(3) Laboratório de Patologia Clínica, Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil.

(4) Laboratório de Gastroenterologia e Hepatologia Tropical, Instituto de Medicina Tropical de São Paulo, Departamento de Gastroenterologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil.

Correspondence to: Roberta Sitnik. Av. Albert Einstein, 627, Laboratório de Técnicas Especiais, Departamento de Patologia Clínica, 1º andar, 05651-901 São Paulo, SP, Brasil. Phone: +55 11 37472105, Fax: +55 11 3747 2420, E-mail: rositnik@hotmail.com

SITNIK, R.; PAES, A.; MANGUEIRA, C.P. & PINHO, J.R.R. - A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev. Inst. Med. Trop. São Paulo*, 52(3):119-24, 2010.

HBV genotypes had already been determined as previously described²⁵ and samples representing genotypes A, B, C, D, E and F were chosen to verify the detection of them with the new assay. Genotype distribution was: A - 36.1%; B - 2.8%; C - 25.0%; D - 25.0%; E - 2.8% and F - 8.3%. For specificity analysis, samples obtained from 40 healthy blood donors provided by the Hospital Albert Einstein Blood Bank were assayed. These samples were seronegative for HBsAg, anti-HBc, anti-HCV, anti-HIV, *Treponema pallidum* and *Trypanosoma cruzi*.

Primers and probe design: The PCR primers and probe were designed in order to equally amplify all known HBV genotypes. In this way, an alignment with sequences of all HBV genotypes described in a previous study with Brazilian patients²⁵ and sequences from GenBank was carried out and a well conserved region among all genotypes in the S gene was selected. After this, PCR primers and TaqMan MGB probe sequences were checked concerning base composition, melting temperatures, GC content, internal folding using Primer Express software (Applied Biosystems, Foster City, CA, USA).

DNA extraction: For the isolation of HBV DNA from plasma, the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) was used, following manufacturer's instructions. DNA was extracted from 200 µL plasma with 1 µL of the internal control (1X IPC DNA; Applied Biosystems, Foster City, CA, USA) and eluted in 50 µL buffer.

HBV quantification by versant HBV DNA assay: The Versant HBV DNA Assay version 3.0 (bDNA - Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) was performed in accordance with the manufacturer's protocol.

Real-Time PCR assay (qPCR): Amplification was performed in a 50 µL reaction mixture containing: 1X *TaqMan Universal PCR Master Mix*, 20 µM of each primer and probe, and 5 µL of extracted DNA. All samples were performed in duplicate. As an internal control, we used the *TaqMan Exogenous Internal Positive Control Reagents kit* with 1X IPC Mix (primers and TaqMan probe labeled with VIC) and 1X IPC DNA. All reagents were obtained from Applied Biosystems (Foster City, CA, USA).

Absolute quantification of HBV DNA was performed with ABI PRISM 7500 Real Time (Applied Biosystems, Foster City, CA, USA). Amplification starts with an incubation at 50 °C for two min for uracil N³-glycosylase inactivate possible contaminating amplicons, followed by 10 min at 95 °C that activates AmpliTaq Gold Polymerase and inactivates uracil N³-glycosylase. PCR cycling program consisted of 45 two-step cycles of 15 s at 95 °C and 60 s at 60 °C (universal conditions).

For validation of the method, qPCR assay for HBV DNA was first validated using two international HBV reference plasmas developed by BBI Diagnostics (West Bridgewater, MA, USA): PHD801 (concentration of 4.3 X 10⁶ IU/mL diluted from one to 500,000X, genotype A) and QHD701 (concentration of 1.19 X 10⁶ IU/mL diluted from one to 15,000X, genotype A). Afterwards, linearity of the test was also performed with samples of genotype D and F.

qPCR results were also compared with one other available assay: Versant HBV DNA Assay version 3.0 (bDNA - Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) (n = 58) and samples were also sent to a reference laboratory (Quest diagnostics, San Juan

Capistrano, CA, USA) (n = 56). The method used in this test is real-time PCR of the precore/core region of the HBV genome. It has a linear range from 10² to 5 X 10⁸ IU/mL and was developed by Quest Diagnostics Nichols Institute (San Juan Capistrano, CA, USA).

Inter- and intra-assay reproducibilities were examined using two clinical samples (A - 7.25 log IU/mL, Genotype C; and B - 7.40 log IU/mL, Genotype A) at different dilutions. Detection limit was established by testing samples of the most common genotypes found in Brazil (A, D and F) in different concentrations (20, 50, 100 and 150 IU/mL).

Preparation of standard: One sample with a very high viral load (6.27 log IU/mL, genotype C) was selected and diluted in normal plasma to be used as a quantification standard. This diluted sample was frozen at -80 °C in 200 µL aliquots in order to preserve its viral load. It was then tested in triplicate using the international panel as a quantification control, and the obtained result was compared to the external reference laboratory and bDNA. In every routine, this sample was extracted, diluted from 10⁻¹ to 10⁻⁶ and used as a quantification control.

Statistical analysis: Validation of qPCR was analyzed by scatter, Bland-Altman plots⁴ and linear regression. Intercept was not considered in order to evaluate the slope of fitted line. For reproducibility analyses, viral loads were summarized by means of standard deviations (SD) and coefficient of variation (CV). Scatter and Bland-Altman plots were also used to compare qPCR with other methods. Pearson's correlation coefficients were computed. All analyzes were performed using Excel and SAS statistical package, version 9.01.

RESULTS

Primers and probe design: After alignment of sequences previously described in the GenBank, conserved regions in the S gene region of HBV DNA were selected and analyzed by Primer Express software (Applied Biosystems, Foster City, CA, USA), considering the best fitted combination of primers and probe.

The selected sequences are:
Primer forward: 5' CCT GGY TAT CGY TGG ATG TGT 3'
Primer Reverse: 5' GGA CAV ACG KGC AAC ATA CCT T 3'
Probe: 5' 6FAM CTA TGC CTC ATC TTC TT MGB NFQ 3'

PCR product is 116 bp and position of primers and probe on reference HBV strains of different genotypes is shown in Fig. 1.

Validation with international panels: The log₁₀ values of all international HBV reference plasmas samples were plotted against expected results and regression analysis (without intercept) showed that the slope approached 1.0 with R² = 0.996 (Fig. 2A). Bland-Altman plot shows most of points are within 95% limits of agreement (Fig. 2B).

Results of genotypes A, D and F samples diluted at different viral load levels showed that the test had a linear range from 50 to 1 X 10⁸ IU/mL, or 1.70 to 8.0 log IU/mL. Samples below 50 IU/mL could be detected, but not every time tested, and detection limit was established as 50 IU/mL, with 92% confidence interval. Samples with viral loads higher than 10⁸ IU/mL had results below the expected, and must be diluted (data not shown).

SITNIK, R.; PAES, A.; MANGUEIRA, C.P. & PINHO, J.R.R. - A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev. Inst. Med. Trop. São Paulo*, 52(3):119-24, 2010.

Fig. 1 - Alignment of different HBV genotypes and subgenotype strains from GenBank showing the localization of primers and probe selected for the HBV qPCR.

Fig. 2 - Scatter plot (A) and Bland-Altman (B) of the comparisons between qPCR and International HBV reference plasmas. In Fig. A, the dotted line refers to diagonal ($y = x$).

Influence of Internal Control primers and probe was tested by parallel reactions performed with and without them. HBV viral load was the same in both reactions showing no competition. Besides, internal control was detected in all samples with viral load below 10^6 IU/mL.

Reproducibility: Interassay variability was determined by running

the samples for 12 consecutive days and intra-assay variability was evaluated by running 10 determinations for each sample. Results of viral load means, standard deviation (SD) and coefficient of variation (CV) are shown in Table 1. One sample (B) was diluted to 1.40 log IU/mL and could not be detected in all runs, but it was below the established detection limit of 1.70 log IU/mL.

Table 1
Reproducibility of real time assay for hepatitis B virus (HBV) detection

	n times	Input HBV DNA (log IU/mL)	Observed HBV DNA (log IU/mL) Mean (SD)	CV ^{a,b}
Interassay				
High (pure) - A	12	7.25	7.13 (0.07)	0.01
High (pure) - B	12	7.40	7.29 (0.07)	0.01
Low (10^{-5}) - A	12	2.25	2.23 (0.31)	0.14
Low (10^{-6}) - B	10	1.40	1.33 (0.35)	0.26
Intra-assay				
High (pure) - A	10	7.25	7.00 (0.04)	0.01
High (pure) - B	10	7.40	6.79 (0.05)	0.01
Intermediate (10^{-5}) - A	10	4.25	4.34 (0.08)	0.02
Intermediate (10^{-5}) - B	10	4.40	4.14 (0.04)	0.01
Low (10^{-5}) - A	10	2.25	2.73 (0.16)	0.06
Low (10^{-6}) - B	8	1.40	1.63 (0.15)	0.09

^a mean CV interassay: 0.12; ^b mean CV intra-assay: 0.03

Specificity: None of the 40 samples from healthy blood donors had detectable results for HBV DNA, tested in duplicate. Negative controls (NC) were also processed in every reaction in a proportion of one NC for every five-tested sample. Negative results were obtained every time for those samples.

SITNIK, R.; PAES, A.; MANGUEIRA, C.P. & PINHO, J.R.R. - A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev. Inst. Med. Trop. São Paulo*, 52(3):119-24, 2010.

Fig. 3 - Comparisons between qPCR and reference laboratory (A and B) and bDNA (C and D). In Fig. A and C (Scatter plots), the dotted lines refer to minimum detection limit for each method while Figures B and D show Bland-Altman comparisons between the two methods.

Correlation between real time and other HBV DNA assays: The HBV DNA real-time PCR detection assay was compared with Versant HBV DNA Assay version 3.0 (bDNA - Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) ($n = 58$) and samples were also sent to a reference laboratory (Quest diagnostics, San Juan Capistrano, CA, USA) ($n = 56$). The log of viral load values (IU/mL) obtained was plotted against values obtained from reference laboratory (Fig. 3A) and bDNA (Fig. 3C). It is noteworthy that some samples were detected only by the qPCR here described. Linear correlation coefficient based on only detectable (or undetectable) viral loads by both methods were high ($r = 0.984$ for reference laboratory and $r = 0.966$ for bDNA). In Bland-Altman plots (Fig. 3B and 3D), only one sample with high viral load in Figure 3D had absolute difference between qPCR and bDNA, higher than expected. This sample was genotype A and had a viral load of $9.82 \log \text{ IU/mL}$, above our linear range, and should be diluted. Other samples that exceed 95% limits of agreement correspond to low viral load samples, which were detected only by qPCR method. Samples with different viral load levels were tested for each genotype.

DISCUSSION

HBV viral load measuring is a very important tool for monitoring HBV infected patients. There is an increasing trend for the use of combination of nucleotide analogs, which lead to a fast dynamic of viral load. It is expected that monitoring the HBV viral load together with the detection of resistance related mutations in the HBV genome will be able to provide clinicians with important guidelines for the HBV treatment.

The most direct and reliable measurement of viral replication is HBV DNA quantification that can replace other indirect methods to assess the efficacy of antiviral therapy used to treat HBV infected patients, such as serologic markers or measurement of liver enzyme function. Histology is adequate to evaluate the efficacy of a treatment, however, lacks the ability to discriminate rapidly any difference at multiple time points in clinical trials, comparing active drugs or combination therapy. Monitoring the HBV viral load can predict the evolution to cirrhosis and hepatocellular carcinoma⁶, as well as a rapid and sustained response to treatment, as a predictive factor for a favorable treatment outcome. It can also provide an early detection of treatment failure that may be related to poor adherence to therapy or selection of a resistant virus. The likelihood of resistance to nucleos(t)ide analogues is very low when HBV-DNA level is undetectable during therapy and increases proportionally to the HBV-DNA level²¹.

An assay with a lower limit of detection of 10^3 IU/mL may be sufficient to monitor and manage the patient but, in some instances a more sensitive assay with a lower limit of detection of the order of 10^1 IU/mL may be required to ensure detection of the emergence of resistance as early as possible. Our results have shown that we could detect samples with viral load below 50 IU/mL . An example can be seen in Table 1, where Sample B - Low (10^4) could not be detected in 2/12 times in the interassay test and in 2/10 times in the intra-assay test. A dynamic range of quantification of at least $5 \log_{10}$ is also recommended and samples with an HBV-DNA level above the upper limit of detection of the assay should be diluted and retested to provide an end point. For all of these reasons, real-time PCR quantification assays are strongly recommended

SITNIK, R.; PAES, A.; MANGUEIRA, C.P. & PINHO, J.R.R. - A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev. Inst. Med. Trop. São Paulo*, 52(3):119-24, 2010.

over other technologies, especially in clinical trials, because they are very sensitive and have a broad dynamic range of quantification^{1,13,14,19,21}.

Early predictors of the treatment efficacy at week 24 include (1) complete virologic response, defined as a decrease in HBV DNA to <60 IU/mL; (2) partial virologic response (HBV DNA 60 to <2000 IU/mL); or (3) inadequate virologic response (HBV DNA $\geq$ 2000 IU/mL). Treatment recommendations vary depending on which of these categories a patient falls into. For patients who have an ideal response to therapy (complete virologic response), the panel recommends maintaining treatment with no change and broadening the interval for monitoring. Treatment should be continued without change for patients who have an adequate response to therapy (partial virologic response) and monitoring should be more frequent. For patients who have a suboptimal response or no response to therapy, the panel recommends switching to or adding alternative therapy with no overlapping resistance mutations and continuing to monitor frequently¹⁰.

Although we have only tested this assay with samples harboring genotypes A to F during the validation process, the primer sequences chosen were also covered for genotypes G and H, which are more similar to genotypes A and F, respectively. Afterwards, the protocol was established in the routine laboratory and more than 1,500 clinical samples were already analyzed, including genotypes A to G. Proficiency test samples (Hepatitis Viral Load Survey from the College of American Pathologists - CAP and Molecular Biology Panel from ControlLab, SBPC/ML) were also analyzed with satisfactory results in the last two years.

In Brazil, only a few commercial HBV quantification assays are available; and several *in house* assays described before have some limitations, such as lack of internal control, a small linear range or allowing analysis of only a few HBV genotypes^{1,13,14,20}.

When conducting this work, we were able to compare our test with the only commercial available kit (bDNA) and it was possible to verify its low sensibility, linked to the principle of the method. Nowadays, other commercial kits have been released, such as the Cobas Taqman HBV test (Roche) and Abbott Real Time HBV assay, but these kits are more expensive and not commercially available in many countries. Otherwise, this is an open protocol that allows HBV quantitation in resource-limited settings, providing a cheap and effective alternative to existing HBV assays. It has already been shown in other studies that in house testing for HCV and HIV detection and quantitation^{7,8,22,24,27} can be used routinely, provided high level quality control rules are followed.

An underestimation of the HBV viral load of genotype F samples with the Cobas Monitor PCR system has been reported²². As genotype F is mainly present in Central and South America and is relatively common among our patients in this case²⁵, quantification of this genotype is very important in Brazil. In the COBAS Taqman HBV Test this problem seems to be solved and it equally amplifies genotypes A-G. The linear range of this test is from 29 to 1.1×10^8 IU/mL and the probes are directed to the pre-core/core region of HBV^{2,29}.

The Abbott Real Time HBV kit is also a real time PCR based kit, all HBV genotypes (A-H) can be adequately detected, results in IU/mL or copies/mL, dynamic range from 10^1 to 10^9 IU/mL. The probes are in the S region of HBV genome²⁸.

The standardization of quantification units is another important issue when measuring HBV viral load. Serum HBV-DNA levels are reported in many different units depending on the method used and the manufacturer of the assay (eg, copies/mL, genome equivalents - Eq/mL, mega-equivalents - MEq/mL, or international units - IU/mL). The World Health Organization has defined an international standard for HBV DNA nucleic acid amplification techniques that has been used to calibrate the IU/mL. Several HBV-DNA quantification assays that are available have been normalized to the World Health Organization international standard, such as the Abbott Real Time HBV kit and the COBAS Taqman HBV Test. Serum HBV-DNA levels should now be expressed universally in IU/mL in all available assays to ensure comparability between the assays, between different trials in which different assays have been used, and to allow the creation of guidelines that can be applied to whatever assay was used²¹.

In the test described here, many limitations of other methods were considered. Since conserved regions were chosen from the alignments of all HBV genotypes (A-H) during primer and probe design; and genotypes A-F infected patients were tested, this assay showed efficacy to amplify all genotypes circulating in Brazil. The use of an internal control, extracted together with the sample, is able to detect the presence of inhibitors in the sample. The standard curve was normalized to IU/mL, and this qPCR is sensitive, accurate and reproducible allowing the quantification of HBV DNA, particularly useful for following chronic HBV carriers. The assay was implemented in the diagnostic routine from a clinical laboratory and has proved to be highly cost efficient.

RESUMO

Ensaio quantitativo em tempo real para o DNA do vírus da hepatite B (HBV) desenvolvido para detectar todos os genótipos do HBV

O vírus da Hepatite B (HBV) é uma das principais causas de doença crônica do fígado no mundo. Além do genótipo, a análise quantitativa do HBV é amplamente utilizada para monitorar a progressão da doença e o tratamento. Em locais com recursos escassos, métodos baratos para o monitoramento da carga viral são desejáveis e, em países em desenvolvimento, sua utilidade já foi demonstrada para outros vírus, como o da Hepatite C e HIV. Neste trabalho, descrevemos a validação de um teste de PCR em Tempo Real para a quantificação do DNA do HBV utilizando sondas Taqman/MGB. Os oligos e sondas foram escolhidos usando um alinhamento contendo seqüências de todos os genótipos do HBV para garantir uma amplificação igual de todos eles. O teste possui um controle interno e foi padronizado com um painel internacional de HBV. Sua eficácia foi testada comparando-se os resultados com outros dois métodos: Versant HBV DNA Assay 3.0 (bDNA, Siemens, NY, USA) e outro PCR em tempo real realizado em um laboratório de referência. As reprodutibilidades intra e inter-ensaio foram determinadas e a média dos valores de CV obtidos foram de 0,12 e 0,09, respectivamente. O teste foi validado com uma ampla faixa dinâmica e amplificou com eficiência os diferentes genótipos de HBV, fornecendo uma boa opção para a quantificação de rotina do DNA do HBV, com um protocolo barato e confiável.

ACKNOWLEDGMENTS

We would like to thank the Molecular Pathology Department from Hospital Israelita Albert Einstein, in particular: Cinthia Bachir Moyses,

SITNIK, R.; PAES, A.; MANGUEIRA, C.P. & PINHO, J.R.R. - A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev. Inst. Med. Trop. São Paulo*, 52(3):119-24, 2010.

Cláudia Huerta de Nardi Graça, Gregorio Tadeu Fernando Dastoli, Leticia Oyakawa, Liana Carballo Menezes, and Rubia Anita Ferraz Santana. We are also thankful to Michele Gomes-Gouvêa of the Tropical Medicine Institute/FMUSP.

Funding: *Instituto de Ensino e Pesquisa and Instituto de Responsabilidade Social from Hospital Israelita Albert Einstein, São Paulo, SP, Brazil.* The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, *et al.* Quantitation of Hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol.* 1999;37:2899-903.
2. Alice T, Cerutti F, Pittaluga F, Varetto S, Gabella S, Marzano A, *et al.* COBAS AmpliPrep-COBAS TaqMan hepatitis B virus (HBV) test: a novel automated real-time PCR assay for quantification of HBV DNA in plasma. *J Clin Microbiol.* 2007;45:828-34.
3. Araujo NM, Mello FC, Yoshida CF, Niel C, Gomes SA. High proportion of subgroup A' (genotype A) among Brazilian isolates of Hepatitis B virus. *Arch Virol.* 2004;149:1383-95.
4. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet.* 1986;1:307-10.
5. Bottecchia M, Souto FJ, Ó KM, Amendola M, Brandão CE, Niel C, *et al.* Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *BMC Microbiol.* 2008;22:8-11.
6. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA.* 2006;295:65-73.
7. Drexler JF, Kupfer B, Petersen N, Grotto RM, Rodrigues SM, Grywna K, *et al.* A novel diagnostic target in the hepatitis C virus genome. *PLoS Med.* 2009;6:e31.
8. Fiscus SA, Cheng B, Crowe SM, Demeter L, Jennings C, Miller V, *et al.* HIV-1 viral load assays for resource-limited settings. *PLoS Med.* 2006;3:e417.
9. Gordillo RM, Gutiérrez J, Casal M. Evaluation of the COBAS TaqMan 48 real-time PCR system for quantitation of hepatitis B virus DNA. *J Clin Microbiol.* 2005;43:3504-7.
10. Keefe EB, Zeuzem S, Koff RS, Dieterich DT, Esteban-Mur R, Gane EJ, *et al.* Report of an International Workshop: roadmap for management of patients receiving oral therapy for chronic hepatitis B. *Clin Gastroenterol Hepatol.* 2007;5:890-7.
11. Kessler HH, Pierer K, Dragon E, Lackner H, Santner B, Stünzner D, *et al.* Evaluation of a new assay for HBV DNA quantitation in patients with chronic hepatitis B. *Clin Diagn Virol.* 1998;9:37-43.
12. Laperche S, Thibault V, Bouchardaud F, Alain S, Castelain S, Gassin M, *et al.* Expertise of laboratories in viral load quantification, genotyping, and precore mutant determination for hepatitis B virus in a multicenter study. *J Clin Microbiol.* 2006;44:3600-7.
13. Lole KS, Arankalle VA. Quantitation of Hepatitis B virus DNA by real-time PCR using internal amplification control and dual TaqMan MGB probes. *J Virol Met.* 2006;135:83-90.
14. Mukaide M, Tanaka Y, Katayose S, Tano H, Murata M, Hikata M, *et al.* Development of real-time detection direct test for hepatitis B virus and comparison with two commercial tests using the WHO international standard. *J Gastroenterol Hepatol.* 2003;18:1264-71.
15. Norder H, Hammas B, Lee SD, Bile K, Couroucé AM, Mushahwar IK, *et al.* Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol.* 1993;74:1341-8.
16. Norder H, Couroucé AM, Magnius LO. Complete genomes, phylogenetic relatedness and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology.* 1994;198:489-503.
17. Norder H, Couroucé AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, *et al.* Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology.* 2004;47:289-309.
18. Okamoto H, Tsada F, Sakugawa H, Sastroewigjo RI, Imai M, Miyakawa Y, *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol.* 1988;69:2575-83.
19. Pas SD, Fries E, de Man RA, Osterhaus ADME, Niesters HGM. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. *J Clin Microbiol.* 2000;38:2897-901.
20. Pas SD, Niesters HGM. Detection of HBV DNA using real time analysis. *J Clin Virol.* 2002;25:93-4.
21. Pawlotsky JM, Dusheiko G, Hatzakis A, Lau D, Lau G, Liang TJ, *et al.* Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. *Gastroenterology.* 2008;134:405-15.
22. Phillips AN, Pillay D, Miners AH, Bennett DE, Gilks CF, Lundgren JD. Outcomes from monitoring of patients on antiretroviral therapy in resource-limited settings with viral load, CD4 cell count, or clinical observation alone: a computer simulation model. *Lancet.* 2008; 371:1443-51.
23. Ribeiro NR, Campos GS, Angelo AL, Braga EL, Santana N, Gomes MM, *et al.* Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver Int.* 2006;26:636-42.
24. Roth WK, Weber M, Seifried E. Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. *Lancet.* 1999;353:359-63.
25. Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, da Silva LC, Carrilho FJ. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol.* 2004;42:2455-60.
26. Sitnik R, Sette H Jr, Santana RA, Menezes LC, Graça CH, Dastoli GT, *et al.* Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. *Braz J Med Biol Res.* 2007;40:1689-92.
27. Stramer SL, Glynn SA, Kleinman SH, Strong DM, Caplioti S, Wright DJ, *et al.* Detection of HIV-1 and HCV infections among antibody-negative blood donors by nucleic acid-amplification testing. *N Engl J Med.* 2004;351:760-8.
28. Thibault V, Pichoud C, Mullen C, Rhoads J, Smith JB, Bibol A, *et al.* Characterization of a new sensitive PCR assay for quantification of viral DNA isolated from patients with hepatitis B virus infections. *J Clin Microbiol.* 2007;45:3948-53.
29. Weiss J, Wu H, Farrenkopf B, Schultz T, Song G, Shah S, *et al.* Real-Time TaqMan PCR detection and quantitation of HBV genotypes A-G with the use of an internal quantitation standard. *J Clin Virol.* 2004;30:86-93.
30. Yao JD, Beld MG, Oon LL, Sherlock CH, Germer J, Menting S, *et al.* Multicenter evaluation of the VERSANT hepatitis B virus DNA 3.0 assay. *J Clin Microbiol.* 2004;42:800-6.

Received: 20 August 2009

Accepted: 22 April 2010

- 5- L.C. da Silva, C.L. Assis-Madruga, F.J. Carrilho, J.R.R. PINHO, A. Saez-Alquézar, C. Santos, L. Bassit, C. Barreto, L.E.P. Fonseca, V.A.F. Alves, R. Leitão, R. Vianna, R.A. Cardoso, A.V.C. França & L.C.C. Gayotto - Spontaneous Hepatitis B surface antigen clearance in long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with Hepatitis C and D virus superinfection. *Journal of Gastroenterology* 31 (5): 696 - 701, 1996.

Spontaneous hepatitis B surface antigen clearance in a long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with hepatitis C and D virus superinfection

LUIZ CAETANO DA SILVA,^{1,4} CARMEN LÚCIA DE ASSIS MADRUGA,^{1,4} FLAIR JOSÉ CARRILHO,^{1,4} JOÃO RENATO REBELLO PINHO,^{3,4} AMADEO SAÉZ-ALQUEZAR,³ CARLOS SANTOS,³ LEDA BASSIT,³ CLAUDIA BARRETO,³ LUIS EDMUNDO PINTO DA FONSECA,^{1,4} VENÂNCIO AVANCINI FERREIRA ALVES,² REGINA LEITÃO,² REGINA VIANNA,² RITA ANTONELLI CARDOSO,⁴ ALEX VIANEY CALLADO FRANÇA,^{1,4} and LUIZ CARLOS DA COSTA GAYOTTO²

Departments of Gastroenterology (Hepatology Branch)¹ and Pathology², Pro-Sangue Foundation³, and Institute of Tropical Medicine (LIM-47 and 52)⁴, University of São Paulo School of Medicine, São Paulo, Brazil

Abstract: We investigated the frequency of HBsAg clearance and the possible role of viral superinfection in a long-term follow-up of 184 patients with chronic hepatitis B (CHB). Our subjects were 184 patients with chronic hepatitis B and the follow-up was 12-216 months (mean 66.2 ± 53.7 months). The investigative methods used were: immunoenzymatic assays for HBV, HCV, HDV, and HIV markers; polymerase chain reaction (PCR) for HBV DNA; and liver biopsy and immunoperoxidase. During the follow-up, 20 of the 184 patients cleared serum HBsAg. A comparison of patients with persistent HBsAg (group I) and of those who cleared this marker (group II) showed a significant difference in mortality ($P = 0.002$) between the two groups and a tendency to a more severe exacerbation (flare) in group II ($P = 0.07$). Antibodies to hepatitis C and D virus as well as antibodies to HIV were equally distributed in both groups. Thirteen patients (7.9%) from group I, but none from group II, subsequently developed hepatocellular carcinoma. These results suggest that the frequency of spontaneous clearance of HBsAg during chronic HBV infection is low. No determinant factor for the clearance was found, including the presence of liver cirrhosis. Serum HBV DNA was undetectable by PCR after clearance in 16 out of 17 patients.

Key words: hepatitis B, chronic hepatitis B, spontaneous HBsAg clearance

Introduction

During the natural history of chronic hepatitis B (CHB), seroconversion from HBeAg to anti-HBe is frequently observed at the same time as or preceded by a flare of the hepatitis.¹ After this seroconversion, HBV DNA may be absent or may be found in small amounts in serum, particularly by the polymerase chain reaction (PCR) technique. Despite the frequency of this seroconversion, the spontaneous clearance of HBsAg is considered to be an unusual event.² In a study of 204 blood samples with serum HBsAg in patients followed-up for 3-44 months, Sampliner et al.³ found that the annual clearance rate of this antigen was 1.7%. A lower incidence (0.1%/year) of HBsAg clearance was described in Taiwan,⁴ a hyperendemic area where HBV infection usually occurs perinatally or during early childhood.² In a series of 100 carriers from Britain, HBsAg was undetectable in one healthy carrier and one cirrhotic carrier (2%) after a follow-up of 14 and 60 months, respectively;⁵ 77 of these carriers were male homosexuals. A higher frequency of HBsAg clearance (10.7%) was observed by Poralla et al.⁶ in 65 patients with CHB during a 3-year period.

Several questions remain unanswered regarding the clearance of HBsAg in CHB, due to the wide variety in approach of the studies, particularly the incomplete characterization of the entire group of patients among whom clearance was detected, and the incomplete characterization of their morphological patterns.

According to some authors,^{7,8} superinfection with hepatitis C virus (HCV) may suppress HBV infection and produce the clearance of serum HBsAg.

The Brazilian population includes individuals of different ethnic origins. As far as age, sex, race, and viral infections of the liver are concerned, these characteristics of our subjects permitted us to make a comparison between patients with CHB who cleared serum HBsAg and those who showed persistent serum

Offprint requests to: L. Caetano Da Silva

(Received Aug. 14, 1995; accepted Feb. 6, 1996)

Some of these findings were presented at the Biennial Scientific Meeting of the International Association for the Study of the Liver, held in Brighton, UK, in June 1992.

HBsAg. The annual rate of clearance could also be determined.

The aim of the present study was to analyze clinical, biochemical, serological, and morphologic parameters of 184 chronic hepatitis B patients with a long follow-up, comparing 20 patients who cleared serum HBsAg with the remaining 164 patients with persistent HBV surface antigenemia.

Subjects and methods

This was a retrospective study of 184 patients with chronic hepatitis B consecutively followed at our institution. Criteria for inclusion were: (1) Diagnosis of CHB; persistence of HBsAg for at least 1 year and histological diagnosis of chronic hepatitis; (2) absence of hepatotoxic drug ingestion, chronic alcoholism, and other non-viral chronic liver diseases; (3) repeated detection of serum HBV markers. Of 214 patients with chronic hepatitis B followed at our institution from 1973 to 1991, 30 did not fulfill all these criteria and were excluded. The study population consisted of 184 HBsAg-positive patients with chronic hepatitis B. All were known to have been HBsAg-positive for at least 12 months when they entered the study; there were 159 men and 25 women, with a mean age of 40.2 years (± 15.3 years).

In a follow-up of 12–216 months (mean 66.2 ± 53.7 months) 20 patients (10.8%) showed permanent clearance of HBsAg. Two of the 20 patients (10%) admitted homosexual practices. These 20 patients form the basis of the present report.

Criteria for HBsAg clearance

Absence of serum HBsAg determined by both radioimmunoassay (RIA; polyclonal) and enzyme-linked immunosorbent assay (ELISA) (monoclonal antibodies) in at least three consecutive tests.

Serological studies

Routine follow-up studies included complete clinical and liver biochemical evaluation and serological tests performed at each visit at 3- to 6-month intervals. From 1973 to 1985, HBsAg was serially tested by RIA (Ausria II; Abbott Laboratories, Chicago, IL, USA). Testing for anti-HBs and anti-HBc was done by RIA (Ausab and Corab, respectively; Abbott Laboratories). From 1986 on, HBsAg was tested by ELISA using monoclonal antibodies (Abbott Laboratories). Sera were also tested for HBeAg, anti-HBe, anti-HBs, and anti-HBc IgM, using ELISA (Abbott Laboratories). Determination of viral polymerase activity and detection of HBV DNA in

serum by molecular hybridization (dot-blot) were performed as previously described.⁹ Anti-HCV was determined using second generation ELISA kits (Ortho, Raritan, NJ, USA or Diagnostics Pasteur, Marne-la-Coquette, France). Positive results were confirmed by second generation RIA (Ortho USA). Anti-HIV was detected using both viral lysate (Genetic Systems, Redmond, WA, USA) and recombinant (Wellcome, Kent, UK) ELISA kits, and results were confirmed by Western blot test (Dupont Biotech, Boston, MA, USA). Anti-HDV was detected using an Organon (Boxtel, Netherlands) ELISA kit. These techniques were performed on sera kept at -20°C or collected during an outpatient visit.

Polymerase chain reaction (PCR)

For the detection of HBV-DNA in serum by PCR, the technique developed by Kaneko et al.¹⁰ was used, with minor modifications. To avoid carry-over between samples, the procedures described by Kwok and Higuchi¹¹ were strictly followed. Sample preparation: in a 0.5-ml Gene Amp reaction tube (Perkin Elmer, Branchburg NJ, USA), serum (10 μl) was mixed with 2.5 μl 0.5 M NaOH (Sigma, St. Louis, MO, USA), overlaid with 100 μl of mineral oil and incubated for 1 hour at 37°C . After this time, the solution was neutralized by adding 2.5 μl 0.5 M HCl (Merck, Darmstadt, Germany).

Oligonucleotides covering a well conserved sequence of the C gene were synthesized by Genomics (São Paulo, Brazil), according to the sequences proposed by Kaneko et al.,¹⁰ as shown below:

1763: 5' GCT TTG GGG CAT GGA CAT TGA CCC GTA TAA 3'
(30mer)
2032R: 5' CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT 3'
(30mer)
1778-E: 5' GAC GAA TTC TGA CCC GTA TAA AGA ATT 3'
(30mer)
2017R-B: 5' ATG GGA TCC CTG GAT GCT GGG TCT TCC
AAA
3' (30mer)

For amplification, the first PCR employed the Gene Amp PCR kit (Perkin Elmer, USA). The final volume was 100 μl and contained 2.5 U Taq polymerase, 200 μM each deoxynucleotide, 1 μM primers 1763 and 2032R, 50 mM KCl, 50 mM TrisHCl pH 8.3, 1.5 mM MgCl_2 , and 0.01% gelatin. The amplification was performed by placing the tubes in a DNA Thermal Cycler 480 (Perkin Elmer), programmed for 30 cycles (denaturation at 94°C for 1.5 min, annealing at 42°C for 1.5 min, and extension at 72°C for 1.5 min—total duration, 3 h). For the second PCR, after brief centrifugation, a 10- μl vol-

ume from the first amplification was transferred to another Gene Amp tube, and amplified with primers 1778-E and 2017R-B in the same way as described above. For PCR analysis, after brief centrifugation, 15- μ l aliquots from the first and second PCR were analyzed on 1% agarose gel (Sigma, USA) in 0.5 $\times$ TBE and 0.5 μ g/ml ethidium bromide (Sigma, USA). Samples were considered positive when a 258-bp band was visualized after the second PCR; sometimes, a 270-bp band was also visualized after the first PCR.

Criteria for exacerbation

Biochemical exacerbation of liver disease was defined as an abrupt elevation of serum alanine aminotransferase (ALT) level to 200 IU/l or more (normal 50 IU/l) or a greater than threefold increase in the previous abnormal ALT level.¹² Severe exacerbation was defined as an abrupt elevation of serum ALT of more than ten times the upper normal limit.

Histological and immunohistochemical studies

Liver biopsy and/or laparoscopy, performed with a Tru-cut needle (Baxter, Deerfield, IL, USA) were done in all but one patient. Repeat liver biopsies were obtained from three patients after HBsAg clearance. Besides the routine staining techniques, immunohistochemical methods¹³ were used for the detection of HBsAg and HBcAg, with avidin biotin-peroxidase amplification.

Clinical features

The clinical features, studied in patients with persistent HBsAg (group I) and in those showing serum HBsAg clearance (group II), were: sex, age, ethnic group (Occidental and Oriental, all Orientals being of Japanese origin), clinical and/or morphological data, total time of follow-up, time after HBeAg clearance, and severity of biochemical exacerbation (Table 1). Informed consent was obtained from all patients and the study was approved by the Ethics Committee of the Department of Gastroenterology, University of São Paulo School of Medicine.

Statistics

Results were expressed as means and SD and analyzed using the following two-tailed tests: *t*-test or Wilcoxon's rank-sum test¹⁴ and Fisher's exact test to compare proportions between groups. *P* values less than 0.05 were considered significant. Calculations were performed using Statistical Analysis System (SAS) (Cary, NC, USA) software.

Results

Twenty patients (10.8%) lost serum HBsAg. The annual clearance rate was 2.1%. In only one patient was reactivation of the disease observed, this occurring 4 years after clearance, in association with AIDS and multiple myeloma; the clinical details of this patient will

Table 1. Clinical and morphological data in patients showing persistent HBsAg (group I) and clearance of serum HBsAg (group II)

	Group I (n = 164)	Group II (n = 20)	P
Sex (% male)	85.4	95.0	0.319 ^a
Age (years, mean $\pm$ SD)	38.5 $\pm$ 15.6	41.9 $\pm$ 15.1	0.354 ^a
Race (percent Caucasians)	82.3	95.0	0.208 ^a
Chronic active hepatitis (%)	42.7	65.0	0.094 ^a
Liver cirrhosis (%)	42.1	30.0	0.344 ^a
Hepatocellular carcinoma (%)	7.9	0	0.212 ^a
Death (%)	18.2	0	0.022 ^a
Time of follow-up, in years (mean $\pm$ SD)	5.1 $\pm$ 4.4	5.7 $\pm$ 4.0	0.290 ^d
Time of follow-up after HBeAg clearance			
no. of patients ^a	29	7	
Months (mean $\pm$ SD)	41.0 $\pm$ 41.0	37.6 $\pm$ 25.1	0.731 ^e
Severe exacerbation (%) ^b	35/53 (66)	17/19 (89)	0.072 ^c

^aNumber of patients showing serum HBcAg upon admission

^bAbrupt elevation of AST and ALT to more than ten times the upper normal limit

^c*t*-test

^dWilcoxon's rank sum test

^eFisher's exact test

Fig. 1. Follow-up of patients with chronic hepatitis B and clearance of HBsAg

be reported elsewhere. Seroconversion to anti-HBs was observed in 14 of the 20 patients (70%). HBV DNA, determined by PCR was negative in 16 out of 17 patients and positive in the 1 patient with viral reactivation, as mentioned above. After clearance, liver biopsy, performed in 3 patients, showed a striking decrease in inflammatory activity. No HBV antigens were detected in their livers by the immunoperoxidase technique. The follow-up time of patients who cleared HBsAg is shown in Fig. 1. It is worth emphasizing that, despite the diagnosis of chronic active hepatitis and/or liver cirrhosis in all but two patients and the long-term follow-up of most, all but 2 are asymptomatic and show normal serum aminotransferase activity. Both exceptions are related to HCV superinfection. As shown in Table 1, only 7 patients from group II were HBeAg-positive upon admission.

When groups I and II were compared (Table 1), the only statistically significant difference was the number of deaths, which was lower in group II ($P = 0.022$). No other significant differences were found between groups in terms of the clinical, biochemical, or morphological parameters studied (including the presence of liver cirrhosis), except for a tendency to a more severe flare in group II ($P = 0.072$).

As shown in Table 2, the frequency of detection of antibodies to HCV, HDV, and HIV was similar in both groups.

Discussion

Despite the frequency of seroconversion of HBeAg to anti-HBe, spontaneous clearance of serum HBsAg during the course of untreated chronic hepatitis B is considered to be an unusual event,^{2,15-20} which seems to be more frequent in Caucasian than in Oriental patients. The annual rate of spontaneous clearance in Caucasians has been reported to vary from 1% to 2%,^{3,15} whereas in Orientals it seems to be between 0.5% and 0.8%/year.² The reported frequency of clearance varies widely, with figures being as low as 0.1% in Taiwan,⁴ a hyperendemic area for HBV infection that usually occurs perinatally or during early childhood,² and as high as 10.7%.⁶

In our study, 20 of 184 patients (10.8%) cleared serum HBsAg, at an annual rate of 2.1%. Seroconversion to anti-HBs was observed in 14 of the 20 patients (70%). According to some authors,²¹ the subsequent development of anti-HBs may be necessary to confirm that the infection has terminated; otherwise, the clearance may represent an HBsAg titer below the detection threshold. However, detection of anti-HBe alone for long periods after clearance has been observed by others²² and may represent a protracted "window" period or an inability to develop anti-HBs²² or, yet again, a low level of anti-HBs undetectable by ELISA.² Development of anti-HBs after vaccination in those individuals with isolated anti-HBe is strong evidence against a chronically infected, low-level HBsAg carrier state.²³ In our study, the importance of the clearance of HBsAg is emphasized by the absence of HBV DNA on PCR, even in those who did not develop detectable anti-HBs. Furthermore, in another series, we did not detect viral DNA by dot blot hybridization and PCR in 10 individuals with isolated serum anti-HBe determined by ELISA. It is worth mentioning that our criteria for HBsAg clearance were based on three consecutive negative results by RIA and ELISA, with polyclonal and monoclonal antibodies, respectively.

The low rate of detection of HBV DNA by PCR in our patients is in accordance with the results published by Baker et al.,²⁴ Koremman et al.,²⁵ and Hsu et al.²⁶ However, higher figures were reported by Liaw et al.² and Adachi et al.²⁷

Table 2. Antibodies to HCV, HDV, and HIV in 164 patients with persistent serum HBsAg (group I) and in 20 patients with clearance of HBsAg (group II)

Antibodies	Group I n (%)	Group II n (%)	P ^a
Anti-HCV	8/87 (9.2)	2/16 (12.5)	0.652
Anti-HDV	1/77 (1.3)	0/16 (0)	1.000
Anti-HIV	7/79 (8.8)	2/17 (11.7)	0.658

^aFisher's exact test

The pathogenesis of serum HBsAg clearance is not well understood. Its appearance at different times after HBeAg clearance, and various clinical and laboratory observations, show that it frequently follows an acute exacerbation, characterized by clinical symptoms or laboratory changes quite similar to those found in acute hepatitis or in a sudden downhill course of liver cirrhosis. Severe spontaneous exacerbations, characterized by an abrupt elevation of serum aminotransferases (exceeding ten times the upper normal limit) were observed in 17 of 19 patients (89%). As shown in Table 1, this phenomenon tended to be more frequent ($P = 0.072$) in patients who had cleared HBsAg (group II). HBeAg was detected in 7 patients upon admission, whereas the others were anti-HBe-positive. Interestingly, in patients who did not show HBsAg clearance (group I), the time elapsed between the disappearance of HBeAg and the clearance of HBsAg varied widely and did not differ from the time of follow-up of group II patients. According to some authors,¹⁵ HBsAg clearance tends to occur more often in females. This phenomenon was not observed in our subjects, where the male:female ratio was 19:1.

Although our virological findings stress the strong relationship of HBsAg clearance with the end of the infection, as assessed by the absence of viral DNA on detection by PCR, this phenomenon occurred late in the course of the illness, when severe histological changes had already occurred in 90% of the patients, the vast majority of them being asymptomatic.

These findings reduced the level of enthusiasm about the higher probability of HBsAg clearance in asymptomatic carriers described by Liaw et al.,² since those authors did not perform biopsies, and therefore past necro-inflammatory changes could not be excluded.

Chronic active hepatitis occurring while a patient is HBsAg-positive usually indicates vigorous immune reactivity in an attempt to clear HBV-infected hepatocytes. Loss of both HBeAg and HBsAg, therefore, would reasonably be more likely to occur in this situation, particularly in patients who clear HBsAg shortly after HBeAg,²⁶ as was seen in some of our patients (Table 1).

The possibility that HCV superinfection is a cause of HBsAg clearance was mentioned by some authors.^{7,8,28} In our patients, however, the detection of anti-HCV was similar in both patients who demonstrated clearance and those who did not (Table 2). Also, no difference was found with respect to anti-HDV and anti-HIV.

The clinical implications of HBsAg clearance in CHB are rather obvious. First, this phenomenon is always observed after HBeAg disappearance, which may rep-

resent a reduction or interruption of HBV replication. HBV DNA was not detected, even by PCR, in most patients who cleared HBsAg. Second, serum levels of aminotransferase were normalized, suggesting an interruption of the inflammatory process; our two exceptions had superinfection with HCV. Liver biopsies tended to show an inactive process with disappearance of HBcAg in the tissue. Third, the clearance of HBsAg is often followed by the appearance of anti-HBs, which is considered to be a protective antibody. Finally, a reduced tendency to evolve to hepatocellular carcinoma (HCC) was another important feature. Adachi et al.²⁷ reported evolution to HCC in 3 out of 7 patients with CHB, within 2–9 years after clearance of serum HBsAg. Serum HBV DNA, however, was detected by PCR in all 3. These results contrast sharply with our experience, based on 20 patients with a follow-up of 1–11 years after clearance; no evolution to HCC has been observed thus far, whereas this evolution was seen in 13 patients (7.9%) from group I. Furthermore, there was a fatal outcome in 30 of 164 patients (18.2%) from group I and in none from group II ($P = 0.022$), indicating a better prognosis for patients who clear their serum HBsAg.

The fate of HBV in CHB after sustained loss of HBsAg was recently investigated.^{29–31} Fong et al.²⁹ and Kuhns et al.³⁰ concluded that HBV DNA may be detectable by PCR in liver tissue after the disappearance of HBsAg, even in the absence of detectable HBV DNA in serum. Some investigators^{30,32–34} have detected HBV DNA in peripheral blood mononuclear cells. This finding was more often observed in immunocompromised^{35,36} than in immunocompetent patients.^{31,37} Interestingly, serum anti-HIV was detected 2 years before death in the only patient in our series who presented with HBV reactivation, this being 4 years after HBsAg clearance.

Marcellin et al.³¹ detected HBV DNA in liver tissue and serum but not in mononuclear blood cells collected after seroconversion from HBsAg to anti-HBs.

In conclusion, the annual rate of HBsAg clearance in patients with CHB is low in this series of Brazilian subjects. Despite a trend to more severe exacerbation of hepatitis activity observed in patients from this group during follow-up, no statistically significant difference was found between our two groups, except for a higher mortality in patients with persistent serum HBsAg (group I). HBV DNA was usually undetectable by PCR and seroconversion to anti-HBs was observed in most patients. The suggestion of possibly lower rates of development of HCC in these patients should be addressed in future studies.

Acknowledgments. The authors thank Dr. Celso Granato for some assays of serum HBsAg.

References

- Hoofnagle JH, Seeff LB. Natural history of chronic type B hepatitis. In: Popper H, Schaffner F (eds) *Progress in liver diseases*, vol. 7. New York: Grune and Stratton, 1982; 469-479.
- Liaw Y-F, Sheen I-S, Chen T-J, et al. Incidence, determinants and significance of delayed clearance of serum HBsAg on chronic hepatitis B virus infection: A prospective study. *Hepatology* 1991;13:627-631.
- Sampliner RE, Hamilton FA, Iseri OA, et al. The liver histology and frequency of clearance of the hepatitis B surface antigen (HBsAg) in chronic carriers. *Am J Med Sci* 1979; 277:17-22.
- Wu TT, Hsu HC, Chen DS, et al. Clearance of hepatitis B surface antigen (HBsAg) after surgical resection of hepatocellular carcinoma. *J Hepatol* 1987;4:45-51.
- Viola LA, Coleman JC, Fluker JL, et al. Natural history of liver disease in chronic hepatitis B surface antigen carriers. Survey of 100 patients from Great Britain. *Lancet* 1981; II:1156-1159.
- Poralla T, Manns M, Hutteroth TH, et al. HBsAg clearance in patients with long-standing chronic active hepatitis B and hepatitis B virus-induced liver cirrhosis. *Digestion* 1986;33:53-60.
- Liaw Y-F, Lin S-M, Sheen I-S, et al. Acute hepatitis C virus superinfection followed by spontaneous HBeAg seroconversion and HBsAg elimination. *Infection* 1991;19:250-251.
- Sheen I-S, Liaw I-F, Chu C-M, et al. Role of hepatitis C virus infection in spontaneous hepatitis B surface antigen clearance during chronic hepatitis B virus infection. *J Infect Dis* 1992; 165:831-834.
- Pinho JRR, Fonseca LEP, Miyamoto Y, et al. Comparison of serum hepatitis B virus replication markers in patients with chronic hepatitis B: Studies on HBeAg/anti-HBe system, viral DNA polymerase and HBV-DNA. *Rev Inst Med Trop S Paulo* 1989;31:328-325.
- Kaneko S, Miller RH, Feinstone SM, et al. Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. *Proc Natl Acad Sci USA* 1989;86:312-316.
- Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989;239:237-238.
- Lok ASF, Lai CL, Wu PC, et al. Spontaneous hepatitis B e antigen to antibody seroconversion and reversion in Chinese patients with chronic hepatitis B virus infection. *Gastroenterology* 1987; 92:1839-1843.
- Stemberger LA, Hardy Jr PH, Cuculis JJ, et al. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (PAP) and its use in identification of spirochetes. *J Histochem Cytochem* 1970;8:315-334.
- Rosner B. *Fundamentals of biostatistics*. 2nd ed. Boston: PWS Publishers, 1982;288-293.
- Alward WLM, McMahon BJ, Hall DB, et al. The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. *J Infect Dis* 1985;151:604-609.
- Dooley JS, Davis GL, Peters M, et al. Pilot study of recombinant human alpha-interferon for chronic type B hepatitis. *Gastroenterology* 1986;90:150-157.
- Realdi G, Alberti A, Rugge M, et al. Seroconversion from hepatitis B e antigen to anti-HBe in chronic hepatitis B virus infection. *Gastroenterology* 1980;79:195-199.
- Bortolotti F, Cadrobbi F, Crivellaro C, et al. Chronic hepatitis type B in childhood: longitudinal study of 35 cases. *Gut* 1981; 22:499-504.
- Dormeyer HH, Arnold W, Schonborn H, et al. The significance of serologic, histologic, and immunohistologic findings in the prognosis of 88 asymptomatic carriers of hepatitis B surface antigen. *J Infect Dis* 1981;144:33-37.
- Sánchez-Tapias JM, Vilar HJ, Hosta J, et al. Natural history of chronic persistent hepatitis B. Relationship between hepatitis B virus replication and the course of the disease. *J Hepatol* 1984;1:15-27.
- Liaw Y-F, Sheen I-S, Chen T-J, et al. Incidence, determinants and significance of delayed clearance of serum HBsAg in chronic hepatitis B virus infection: A prospective study. *Hepatology* 1991;13:627-631.
- Morgan TR, Redeker AG, Yamada S, et al. HBsAg clearance in chronic active hepatitis B. A possible cause of cryptogenic cirrhosis. *Dig Dis Sci* 1986;31:700-704.
- Draeos M, Morgan T, Schiffman RB, et al. Significance of isolated antibody to hepatitis B core antigen determined by immune response to hepatitis B vaccination. *JAMA* 1987;258: 1193-1195.
- Baker BL, Di Bisceglie AM, Kaneko S, et al. Determination of hepatitis B virus DNA in serum using the polymerase chain reaction: Clinical significance and correlation with serological and biochemical markers. *Hepatology* 1991;13:632-636.
- Koremman J, Backer B, Waggoner J, et al. Long-term remission of chronic hepatitis B after alpha-interferon therapy. *Ann Int Med* 1991;114:629-634.
- Hsu H-Y, Chang M-H, Lee C-Y, et al. Spontaneous loss of HBsAg in children with chronic hepatitis B virus infection. *Hepatology* 1992;15:382-386.
- Adachi H, Kaneko S, Matsushita E, et al. Clearance of HBsAg in seven patients with chronic hepatitis B. *Hepatology* 1992;16:1334-1337.
- Fong TL, Di Bisceglie AM, Waggoner JG, et al. The significance of antibody to hepatitis C virus in patients with chronic hepatitis B. *Hepatology* 1991;14:264-267.
- Fong TL, Di Bisceglie AM, Feinstone SM, et al. Persistent hepatic HBV-DNA after clearance of HBsAg from serum of patients with chronic hepatitis B. *Hepatology* 1991;14:130A.
- Kuhns M, McNamara A, Mason A, et al. Serum and liver hepatitis B virus DNA in chronic hepatitis B after sustained loss of surface antigen. *Gastroenterology* 1992;103:1649-1656.
- Marcellin P, Martinot-Peignoux M, Lioriot M-A, et al. Persistence of hepatitis B virus DNA demonstrated by polymerase chain reaction in serum and liver after loss of HBsAg induced by antiviral therapy. *Ann Int Med* 1990;112:227-228.
- Mason A, Yoffe B, Noonan C, et al. Hepatitis B virus DNA in peripheral blood mononuclear cells in chronic hepatitis B after HBsAg clearance. *Hepatology* 1992;16:36-41.
- Hadchouel M, Pasquinelli C, Fournier JG, et al. Detection of mononuclear cells expressing hepatitis B virus in peripheral blood from HBsAg positive and negative patients by in situ hybridization. *J Med Virol* 1988;24:27-32.
- Pasquinelli C, Laure F, Chatenoud L, et al. Hepatitis B virus DNA in mononuclear blood cells. *J Hepatol* 1986;3:95-103.
- Laure F, Zagury D, Saimot AG, et al. Hepatitis B virus DNA sequences in lymphoid cells from patients with AIDS and AIDS-related complex. *Science* 1985;229:561-563.
- Laure F, Chatenoud L, Pasquinelli C, et al. Frequent lymphocyte infection by hepatitis B virus in haemophiliacs. *Br J Haematol* 1987;65:181-185.
- Lic-Injo LE, Balase-Garam M, Lopez C, et al. Hepatitis B DNA in liver and white blood cells of patients with hepatoma DNA cells. *Biology* 1983;2:301-308.

6- Luiz C. Da Silva; João R.R. Pinho; Roberta Sitnik; Luís E. P. Fonseca; Flair J. Carrilho – Predictive factors for response to lamivudine in chronic hepatitis B. Revista do Instituto de Medicina Tropical de São Paulo 42 (4) : 179 - 186 , July/August 2000.

PREDICTIVE FACTORS FOR RESPONSE TO LAMIVUDINE IN CHRONIC HEPATITIS B

Luiz Caetano DA SILVA(1), Luis Edmundo Pinto da FONSECA(1), Fláir José CARRILHO(1), Venâncio Arancini Ferreira ALVES(2, 3),
Roberta SITNIK(5) & João Renato Rebello PINHO(4, 5)

SUMMARY

Background: Lamivudine has been shown to be an efficient drug for chronic hepatitis B (CHB) treatment.

Aim: To investigate predictive factors of response, using a quantitative method with high sensitivity.

Methods: We carried out a prospective trial of lamivudine in 35 patients with CHB and evidence for viral replication, regardless to their HBeAg status. Lamivudine was given for 12 months at 300 mg daily and 150 mg thereafter. Response was considered when DNA was undetectable by PCR after 6 months of treatment. Viral replication was monitored by end-point dilution PCR. Mutation associated with resistance to lamivudine was detected by DNA sequencing in non-responder patients.

Results: Response was observed in 23/35 patients (65.7%) but only in 5/15 (33.3%) HBeAg positive patients. Only three pre-treatment variables were associated to low response: HBeAg ($p = 0.006$), high viral load (DNA-VHB $> 3 \times 10^6$ copies/ml) ($p = 0.004$) and liver HBcAg ($p = 0.0028$). YMDD mutations were detected in 7/11 non-responder patients.

Conclusions: HBeAg positive patients with high viral load show a high risk for developing drug resistance. On the other hand, HBeAg negative patients show a good response to lamivudine even with high viremia.

KEYWORDS: Hepatitis B; HBV; Lamivudine; HBV-DNA; PCR; bDNA

INTRODUCTION

Chronic hepatitis caused by hepatitis B virus (HBV) may lead to cirrhosis, liver failure and hepatocellular carcinoma. The current treatment with interferon- α (IFN- α) remains unsatisfactory, as it is effective in fewer than 40 percent of patients¹⁴, and previous treatment with corticosteroid is still in use to improve results¹⁵.

Lamivudine (2', 3'-dideoxy-3'-thiacytidine) is a potent inhibitor of viral DNA polymerase and is considered one of the most promising nucleoside analog. Following entering the cells, lamivudine is phosphorylated to its active metabolite, lamivudine-5'-triphosphate, by the 2'-deoxycytidine kinase and other cellular kinase enzymes¹⁶. It, then, interferes with the reverse-transcriptase activity of the HBV polymerase, terminating the nascent viral DNA chain and inhibiting DNA synthesis¹⁷.

According to a preliminary study carried out by DIENSTAG *et al.*, daily doses of 100 and 300 mg of lamivudine reduced HBV-DNA to undetectable levels in all patients during a six month therapy; but

sustained undetectable levels of HBV-DNA and hepatitis-B e antigen (HBeAg) occurred in only 5-12% of the treated patients⁶. In an extended study for up to 18 months, with 23 patients who had remained HBeAg positive after six months of therapy, the same research group observed that HBV-DNA suppression was maintained during therapy in 20 (87%) of these patients. Whereas, HBeAg became negative in 39% of the patients⁷.

A recent report showed that HBV-DNA levels in serum may decrease below the detection threshold of the Polymerase Chain Reaction (PCR) assay in 46% of 51 patients, after a 24-week course of lamivudine¹². A trend towards a deeper suppression of viral replication with a daily dose of 300 mg was also observed¹². Such efficacy of lamivudine to impair HBV replication was also shown in HIV-infected⁸ and in transplanted patients^{23, 24, 25}.

Daily doses of lamivudine in patients with chronic hepatitis B (CHB) has varied widely, ranging from 5 to 600 mg. Doses above 5 mg reproducibly decreased HBV-DNA levels in serum⁶, but doses of 100 mg and 300 mg can reduce viremia to an undetectable level⁶. Daily doses

(1) Departamento de Gastroenterologia do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brasil.

(2) Departamento de Patologia do Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brasil.

(3) Laboratório de Histopatologia do Instituto Adolfo Lutz, São Paulo, SP, Brasil.

(4) Laboratório de Biologia Molecular do Instituto Adolfo Lutz, São Paulo, SP, Brasil.

(5) Laboratório Bioquímico Jardim Paulista, São Paulo, SP, Brasil.

Correspondence to: Luis Edmundo Pinto da Fonseca, M.D., PH.D. Rua Augusta 2709, CJ 123, 01413-100 São Paulo, SP, Brasil. Phone +55.11. 3082.6979, Fax +55.11.3083.3597. e-mail: edmundofonseca@uol.com.br

DA SILVA, L.C.; FONSECA, L.E.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

of 300 mg may lead to an earlier⁶ and more profound suppression of HBV DNA¹².

Viral resistance to lamivudine is associated with mutations which lead to amino acid substitutions in the highly conserved Tyr-Met-Asp-Asp (YMDD) motif, in the active site of the polymerase²². The appearance of such mutation (Methionine to Isoleucine or Valine) may explain the viral "breakthrough" during lamivudine therapy^{6, 7, 23, 28}.

Despite the reported efficacy of lamivudine in patients not previously treated and in those who had not responded to interferon therapy⁶, additional data are needed to evaluate whether there is a difference in response to lamivudine in these two groups of patients.

The level of viral nucleic acid in serum appears to be an early indicator for the effectiveness of therapy³¹. Few studies have evaluated the efficacy of lamivudine with more sensitive quantitative methods, including the end-point dilution method²⁷, specially in patients undergoing long-term treatment¹².

The aim of this study was to assess the efficacy and safety of lamivudine in two groups of Brazilian patients with different degrees of CHB; one who had not been previously treated with any drug (12 patients), and another after failure of IFN- α treatment (23 patients).

The present study revealed an as yet not described correlation between viral load and response to lamivudine.

PATIENTS AND METHODS

Patients

We carried out an open and prospective trial of lamivudine in 35 consecutive patients with CHB with evidence of viral replication. Eligible patients included 30 men and 5 women, ranging from 9 to 72 years old. All had hepatitis B surface antigen (HBsAg) in serum for at least six months, detectable HBV-DNA in serum (34 by PCR and one by bDNA) and alanine aminotransferase (ALT) levels ranging from normal levels to 10 times the upper normal limit (UNL).

The inclusion criteria were broad, allowing the enrollment of patients with low transaminase levels as well as patients with low degree of hepatitis, on one side, and patients with compensated (Child-Pugh A) and decompensated (Child-Pugh B) cirrhosis on the other side³⁰.

Patients were excluded if they were also infected with hepatitis C virus, hepatitis D virus or human immunodeficiency virus (HIV), had other serious medical illness, another type of liver disease or an advanced decompensated liver disease (Child-Pugh C), or were pregnant or lactating.

Patients consecutively attended from March 1996 to July 1997 were considered for treatment with lamivudine on a daily dose of 300 mg for 12 months and 150 mg thereafter. Only 4 patients with decompensated liver cirrhosis (Child-Pugh B) received 150 mg from the beginning.

Thirty five patients have been enrolled, being 30 under lamivudine treatment and most of them for longer than 6 months (long term treatment). Lamivudine was withdrawn from five patients who presented

YMDD mutation after nine months of treatment (4 with YVDD and one with YIDD mutations).

Patients were divided in two groups: Group I included 12 patients who had not been previously treated ("naive") and, Group II, 23 patients who had been previously treated with interferon- α (IFN- α). Resistance to IFN- α was considered as partial in case of HBeAg clearance and persistence of HBV-DNA detected by PCR (15 patients). Complete resistance was defined as persistence of HBeAg and HBV-DNA (8 patients). None of these patients received immunosuppressive or antiviral therapy at least 6 months before lamivudine therapy.

Biopsies

Liver biopsy was performed using a Tru-CutTM needle in 25 patients, but not in the remaining 10, due to coagulation disturbances or ascitis. In the latter cases, a clinical and ultrasonographic diagnosis of cirrhosis was performed. Liver histology was blindly evaluated by two specialized pathologists.

Immunohistochemistry

HBsAg and Hepatitis B core antigen (HBcAg) detection in liver tissue was carried out using monospecific polyclonal antibodies in the high-sensitive streptavidin-biotin-peroxidase system (LSAB, Dako, USA), after blockage of endogenous biotin and peroxidase³².

Serology

Detection of HBsAg, HBeAg and antibodies to hepatitis B e antigen (anti-HBe) in serum samples were carried out by ELISA, using commercially available kits (Abbott Laboratories, North Chicago, IL, USA).

Detection of HBV-DNA by Polymerase Chain Reaction (PCR)

HBV-DNA was detected by a nested PCR as previously described¹⁵. Serum samples (10 μ l) were denatured with 2.5 μ l of NaOH 0.5 M and incubated at 37 °C for one hour. Samples were neutralized with 2.5 μ l of HCl 0.5 M. For the first round, 1 μ M of each primer [1763 (5'-TTG GGG CAT GGA CAT TGA CCC GTA TAA 3') and 2032R (5'-CTG ACT AAT TCC CTG GAT GGG TCT 3')] were added to the mixture. Amplification was achieved in a thermocycler model 480 (Perkin Elmer, USA), in 25 cycles of 94 °C for 1 minute (min.), 42 °C for 1 min. and 72 °C for 2 min., followed by an extension step at 72 °C for 5 min. In the second round, 1:10 of the first round product was amplified by primers 1778 (5'-CAT TGA CCC GTA TAA AGA ATT 3') and 2017 (5'-CTG GAT GCT GGG TCT TCC AAA 3'), in the same conditions as above. Amplified products from the second round were electrophoresed in a 2% agarose gel, stained with Ethidium Bromide and visualized under ultra-violet light.

To avoid false-positive results, strict procedures proposed for nucleic acid amplification techniques were followed¹⁹.

Detection of mutations associated with resistance to Lamivudine

HBV-DNA positive samples were also amplified with primers

DA SILVA, L.C.; FONSECA, L.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

corresponding to the HBV polymerase gene, targeted to the region of mutations associated with lamivudine resistance. Serum samples were denatured and neutralized as described above and submitted to nested PCR. The outer primers 5'LAM 5 (5'TGC RYY TGT ATT CCC ATC CCA TC 3') and 3'LAM 2 (5'GTT TTG TTA GGG TTT AAA TG 3') were used in the first round, while the inner primers L840 (5'ACC CCA TCT TTT TGT TTT GTT AAG 3') and L372 (5'TCG CTG GAT GTG TCT GCG GCG TTT TAT 3') were used in the second round. Amplification for the first and second round was carried out at 94 °C for 1 min., followed by 35 cycles of 94 °C for 30s, 42 °C for 30s and 72 °C for 40s and a final extension step at 72 °C for 7 min in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA)^{1,2}.

Sequencing

PCR products were submitted to cycle sequencing reactions, using the second round primers described above and the ABI PRISM[®] BigDye[™] Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq[®] DNA Polymerase, FS (PE Biosystems, Foster City, CA, USA). Nucleotide sequences from both strands were determined in an Automated DNA sequencer model ABI 310 FS (PE Biosystems, Foster City, CA, USA). Sequences were compared with those previously described in the literature using the Lasergene program (DNASTar, Madison, WI, USA).

Quantification of HBV DNA

HBV viremia was followed in sequential samples from the patients using an in-house end-point dilution PCR, or simply EPD method.

Samples were initially diluted in water to a 10-fold series till 10¹⁰. Each dilution was submitted to the same PCR protocol described above for detection of HBV-DNA. As the sensitivity of the method was reported to be around 3 copies per 10 µl or 300 copies/ml^{15,16}, quantitative results were estimated by multiplying the least positive dilution by 3 x 10³ copies/ml.

Assessment of response

Patients were considered as responders if HBV-DNA was undetectable by PCR at the sixth month of treatment.

Other endpoints such as HBeAg clearance, HBeAg to anti-HBe seroconversion and disappearance of HBsAg were also investigated. HBeAg clearance was defined as the absence of HBeAg in two consecutive samples.

Statistical analysis

Univariate analysis for non-responder and responder patients were performed according to the Chi-square (χ^2) and Fisher's Exact Test, taking into consideration the following variables: sex, previous treatment with IFN- α , viremia, presence of serum HBeAg, HBcAg *in situ*, and liver cirrhosis. Age and pre-treatment ALT levels were analysed using the Student's t-test. Pre-treatment ALT levels were also analysed by the non-parametric Mann-Whitney-Wilcoxon test. This test was also used to analyse the viral load median (represented by the log of the number of genomes/ml) in the two groups. For viral load analysis, we also compared two groups with high viremia ($> 3 \times 10^6$) and low viremia ($\leq 3 \times 10^6$) by

the Chi-square test.

The logistic regression technique was performed to estimate the probability of non-response based on variables that demonstrated statistical significance in the univariate analyses. The stepwise method was used to select the variables more associated with the probability of non-response.

The level of significance was 5% ($\alpha = 0.05$); and the SAS software (Statistical Analysis System, SAS Institute, NC, USA) was used.

All patients gave informed consent for the study, which was approved by the Local Ethics Committee.

RESULTS

Therapy efficacy

Lamivudine therapy induced a rapid decrease in serum HBV-DNA concentration in 27 out of 29 patients (93.1%) whose quantification was performed after 12 weeks of treatment: 14 (51.9%) had undetectable HBV-DNA, and 13 (48.1%) showed a decrease in viremia. The other 2 patients presented no change in the HBV-DNA concentration (Table 1 and Table 2). At the sixth month of treatment, chosen as the time point to define response in this study, HBV-DNA was undetectable in the serum

Table 1
HBV-DNA levels (log of genomes/ml) in the Responder Group

Patient	Sex	Age	Group	PCR (log genomes/ml)		
				Pre	12 weeks	24 weeks
1	M	17	I	3	NEG	NEG
3	M	29	I	4	NEG	NEG
5	M	72	I	10	3	NEG
6	M	62	I	12	4	NEG
8	F	9	I	8	NEG	NEG
9	M	59	I	9	2	NEG
10	M	47	I	4	NEG	NEG
12	M	67	I	6	3	NEG
14	M	33	II	3	NEG	NEG
15	M	27	II	4	NEG	NEG
19	M	48	II	3	ND	NEG
20	M	46	II	8	NEG	NEG
24	M	29	II	3	NEG	NEG
25	M	72	II	3	NEG	NEG
26	M	37	II	10	6	NEG
27	F	18	II	3	NEG	NEG
28	F	37	II	6	6	NEG
30	M	49	II	6	ND	NEG
31	M	43	II	3	NEG	NEG
32	M	32	II	3	NEG	NEG
33	M	24	II	8	3	NEG
34	M	50	II	4	ND	NEG
35	M	45	II	6 ^(a)	4	NEG

Sex (M = male, F = female); Age (years); Group (I = no previous treatment; II = previous treatment with Interferon- α); ^(a) = bDNA; ND = not done; NEG = negative.

DA SILVA, L.C.; FONSECA, L.E.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

Table 2
HBV-DNA levels (log of genomes/ml) in the Non-Responder Group

Patient	Sex	Age	Group	PCR (log genomes/ml)		
				Pre	12 weeks	24 weeks
2	F	22	I	10	3	4
4	M	42	I	10	4	3
7	M	51	I	12	NEG	4
11	M	11	I	10	6	4
13	M	27	II	10	10	6
16	M	30	II	8	NEG	4
17	M	65	II	12	4	7
18	M	48	II	10	4	3
21	M	44	II	10	ND	3
22	F	23	II	3	ND	4
23	M	25	II	3	ND	3
29	M	60	II	10	6	4

Sex (M = male, F = female); Age (years); Group (I = no previous treatment; II = previous treatment with Interferon- α); ND = not done; NEG = negative.

of 23 out of 35 patients (65.7%) (responder group). The relevant characteristics of the 23 responder and the 12 non-responder patients are shown in Table 3.

There were no significant differences between the two groups (responders and non-responders) with respect to age, gender, response to previous interferon treatment, base-line alanine aminotransferase levels and degree of liver injury, as shown by the semi-quantification of the stage or grade of inflammation in each liver compartment.

Only three pre-treatment variables were statistically associated with low response to lamivudine: HBeAg positivity ($p = 0.006$), high viral load ($p = 0.004$) and HBcAg *in situ* immune-expression ($p = 0.028$) (Table 3). Fourteen (93%) out of the 15 HBeAg positive serum presented high viremia at baseline (greater than 3×10^6 genomes/ml); and response to lamivudine at the sixth month was observed in 5 (36%) of these 14 patients. Baseline low viremia was observed in one HBeAg-positive patient who responded to lamivudine. Of the 20 HBeAg-negative at the baseline, 3 (15%) patients presented high and 17 (85%) low viremia, of whom, 2 and 15, respectively, responded to lamivudine after the sixth month. As a whole, a lack of parallelism was seen between HBeAg positivity and degree of viremia in 4 (11%) out of 35 patients. The presence of HBcAg was significantly related to the presence of HBeAg in sera (Fisher's Exact Test: $p = 0.002$).

Levels of viral load at pre-treatment was correlated with response to lamivudine in two ways: when comparing patients with high ($> 3 \times 10^6$ genomes/ml) and low viremia ($\leq 3 \times 10^6$ genomes/ml) ($p = 0.004$), and when analyzing the log of number of such values using a non-parametric test ($p = 0.006$).

The stepwise method selected two variables associated with response to lamivudine: high or low viremia (genomes/ml) and level of viremia (log genomes/ml). With the first variable, the probability of non-response to lamivudine is 11.4 times higher in patients with high viremia ($> 3 \times 10^6$ genomes/ml), with a very wide 95% confidence interval (1.938 to

66.355). For this reason, the variable log of number of genomes (G), as determined by EPD-PCR, was selected and the model was written as follows, where p is the probability of non-response to lamivudine.

$$p = \frac{\exp(-3.507 + 0.387G)}{1 + \exp(-3.507 + 0.387G)}$$

In this model, the variable G assumes continuous values from 3 to 12. The estimated odds-ratio (1.473; 95% confidence interval, 1.109 to 1.958) showed that the chance of non-response increases 1.5 times for each increase of one log in the viral load, as shown in Figure 1.

The HBV-DNA levels (log of genomes/ml) found for responders are shown in Table 1 and for non-responders in Table 2.

All but one non-responder patients at the sixth month persisted as PCR-positive at the twelfth month, regardless of their viremia level by PCR. On the other hand, all but one responder patients were PCR negative at the twelfth month.

Serum HBV markers

At the sixth month of therapy, HBeAg became negative in only 3 (20%) out of 15 patients. Among the remaining 12 HBeAg positive, 5 (42%) became negative after one year of therapy. HBeAg negativation was followed by anti-HBe seroconversion in all responder patients; in one patient, HBeAg disappeared only after 21 months of therapy. Interestingly, despite the detection of HBV-DNA and YVDD mutation, the HBeAg antigen became negative in one non-responder. Moreover, HBsAg clearance was observed in only one responder patient, after 20 months of therapy.

Fig. 1 - Probability of non-response according to the regression model.

DA SILVA, L.C.; FONSECA, L.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

Serum alanine aminotransferase (ALT) activity:

Serum ALT activity was determined in 33 patients at the sixth month. Of 18 patients with increased ALT level at baseline, 12 became normal (including three non-responders). Whereas of 15 patients with normal ALT at baseline, only one non-responder showed an increased ALT level after 6 months of treatment. Therefore, lamivudine was equally effective at suppressing viral load among patients with normal and elevated ALT levels (Table 3).

The seven patients with lamivudine resistance and YMDD mutations (see below) showed a slight elevation of the ALT level (0.5 to 3.0 times the upper normal limit) with abnormal values in four of them. No biochemical flare was observed, except in one patient during a one-month association of famciclovir.

Previous treatment with Interferon- α

As shown in Table 3, no difference was found between Group I (naive of treatment) and Group II [resistant to Interferon- α (IFN- α)]. However, considering the pattern of previous response to IFN- α , we observed a trend (Fisher's Exact Test: $p = 0.058$) to a better response to lamivudine in patients who had shown a partial response (12 out of 15 patients) as compared to those who did not respond (3 out of 8 patients) to IFN- α , respectively.

As for HBV-DNA levels, a baseline high viremia was detected in 8 out of 12 patients naive of treatment and in 9 out of 23 interferon-resistant patients, with a response to lamivudine of 50% and 33%, respectively. On the other hand, a baseline low viremia was observed in 4 out of 12

Table 3
Characteristics of the 35 patients at baseline and comparison between responder and non-responder. Patients were considered "responders" if presented undetectable HBV-DNA by PCR at the sixth month of treatment

Variable	Responder (%) (23 patients)	Non-responder (%) (12 patients)	Total	p	Test
Male/female	20 : 3	10 : 2	35	1.00	Fisher
Age (mean; SD)	41.4 ; 17.5	37.3 ; 16.8	35	0.51	Student's
HBsAg positive	6 (40)	9 (60)	15	0.006	χ^2
HBsAg negative	17 (85)	3 (15)	20		
Non-cirrhosis	15 (65)	8 (35)	23	1.00	Fisher
Cirrhosis	8 (67)	4 (33)	12		
Previous IFN- α : Yes	15 (65)	8 (35)	23	1.00	Fisher
No	8 (76)	4 (33)	12		
Pre-treatment ALT (x UNL) (mean; SD)	2.66 ; 2.95	1.33 ; 0.86	35	0.054	Student's
Pre-treatment ALT (x UNL ^(*)) (median)	1.26	0.97	35	0.37	Mann-Whitney-Wilcoxon
Viral load (median of log genomes/ml)	4	10	34 (*)	0.006	Mann-Whitney-Wilcoxon
Viremia (genomes/ml):				0.004	χ^2
High ($>3 \times 10^6$)	7 (41)	10 (59)	17		
Low ($\leq 3 \times 10^6$)	15 (88)	2 (12)	17		
HBcAg <i>in situ</i> :				0.028	Fisher
Positive	4 (40)	6 (60)	10		
Negative	13 (87)	2 (13)	15		

(*) One patient was excluded because his pre-treatment viral load was determined only by bDNA. SD = Standard Deviation; UNL = upper normal limit.

DA SILVA, L.C.; FONSECA, L.E.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

naive patients and in 14 out of 23 Interferon - resistant patients, with a response frequency of 100% and 86%, respectively. No statistical significant difference was found in both cases.

Resistance to Lamivudine

Of the 12 non-responders to lamivudine, samples of 11 were sequenced for the YMDD motif of the C region, and 7 (64%) of them showed mutations associated with lamivudine resistance. Six (55%) patients showed a YVDD mutation and one (9%) of them a YIDD mutation. The other 4 (36%) patients presented the wild type virus, and in the 12th patient, sequencing was not possible as no DNA amplification of this region was obtained after several PCR attempts.

At the time samples were collected for sequencing the YMDD motif, 8 out of the 11 patients were under lamivudine treatment (period of 6 to 24 months, average 12.5 months). In the other 3 patients, treatment had been withdrawn at 2 (one patient) and 8 months (2 patients) before the mutation analysis, and all showed the wild type YMDD motif.

All 7 patients with resistance mutation were HBeAg positive at baseline. A detailed analysis of YMDD mutations during and after withdrawal of lamivudine will be described elsewhere.

DISCUSSION

The recent introduction of the drug lamivudine added a new expectative to the treatment of Chronic Hepatitis B (CHB). In the present study, in agreement with previous results using Abbott Genostics liquid hybridization assay²⁶, a gradual or a rapid decrease in HBV-DNA levels was observed. This pattern was detected by the three different quantitative methods: end-point dilution (EPD), AmpliCor Monitor and branched DNA (bDNA). The EPD method with 10-fold serial dilutions was also used by YOTSUYANAGI *et al.* who described a highly sensitive detection limit (10 copies per reaction)²⁶.

Other studies reported higher rates of HBV-DNA suppression after lamivudine therapy at week 24 or 52^{7,11,20}. However, their quantification methodology was based on the solution hybridization assay (Abbott), with a detection limit of 1.6 to 2.0 pg/ml, corresponding to 4.5x10⁵ to 5.6x10⁵ genomes/ml^{4,20,31}. Concentrations calculated by the Abbott assay were approximately 40 to 100 fold and 35 fold lower than those obtained when the same sample was tested by the Quantiplex and Hybrid Capture System HBV-DNA assays, respectively^{4,17,31}. On the other hand, like us, LAU *et al.* observed a lower rate of response when using a quantitative PCR method²¹.

The HBV-DNA levels were found to be low or undetectable at the sixth month of therapy in our patients. However, in patients with low HBV-DNA levels at base line, only the quantitative PCR-based methodology was able to show variation in viremia. Therefore, some patients with low levels at baseline would not have been treated if selection had been based only on bDNA determinations. As emphasized by HADZIYANNIS¹⁰, quantification of HBV-DNA in sera by techniques with a detection limit of at least 1,000 copies/ml is recommendable for both the inclusion of low-viremia patients in treatment trials as well as for evaluating therapy efficacy.

Supporting data for the hypothesis that persistent low degree of viremia (detected only by PCR quantitative methods) is clinically relevant were shown by the evolution of our non-responder patients at the sixth month. All but one of the 12 non-responder patients persisted with positive PCR and a viral load from 10³ to 10⁷ genomes/ml and out of these 11, seven patients showed a progressive increase of viremia and a resistance mutation in the YMDD motif. Conversely, all but one of the 23 responder patients (PCR negative at the sixth month) persisted as such after 12 months of therapy. These results suggest that highly sensitive PCR assays will be useful for evaluating new drugs and therapy regimens³¹ and that a negative PCR at the sixth month is an useful criterion for determining response to lamivudine.

Our results also showed that non-response to lamivudine is mainly observed in serum of patients with high viremia and, most of them, positive for HBeAg. This correlation was so significant that a proportion between probability of non-response and increase in the logarithm of genome copies per ml could be established (Figure 1). MUTIMER *et al.*²⁴ showed that high titers of hepatitis B virus in serum of pre-treated patients predicted failure of lamivudine prophylaxis and graft re-infection after liver transplantation.

Mutations in the YMDD motif were observed only in non-responder patients with high viremia and positive HBeAg. If we take into consideration only the first 12 months of treatment, 33% of our patients (5/15) presented such mutation - which is comparable to the frequency obtained by HONKOOP *et al.* (39%, in 14 patients)¹³, but higher than the one reported by LAI *et al.* (14%, in 335 patients)²⁰ - both within the same therapy period. Furthermore, if we consider the full length of our treatment (24 months), the frequency of mutation carriers was even higher: 47% (7 out of 15 patients).

In a later paper, HONKOOP *et al.* calculated an actuarial cumulative incidence of YMDD mutation carriers of 32% and 53% after 52 and 78 weeks, respectively, of lamivudine therapy in 15 patients treated for more than 12 months¹⁴.

According to LAU *et al.*, lamivudine resistance tends to appear specially in patients whose serum levels of HBV-DNA persist above 1,000 Eq/ml²¹.

The response frequency found in HBeAg-negative patients was 85%; higher than that reported by TASSOPOULOS *et al.* patients with the same serological profile (63%), probably because we included patients with low viremia, not detected by the bDNA method employed by these workers³³. Interestingly, 27% of patients in the Tassopoulos' study showed YMDD variant at week 52. This mutation was not observed by us in HBeAg negative patients.

It is worth mentioning that one patient became HBeAg negative, despite the detection of a YVDD mutation to lamivudine resistance. This fact was also observed by GARRETT *et al.*⁹.

Another important point is that the response to lamivudine did not depend on the presence or absence of liver cirrhosis. Furthermore, the tolerance to lamivudine was equally good in both situations, even in patients with ascites (Child Pugh B).

DA SILVA, L.C.; FONSECA, L.E.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

Summing up, two important conclusions arose from our observations: first, patients positive for HBeAg and with a high viral load are less prone to respond to lamivudine as monotherapy due to the high risk of drug resistance and selection of mutation in the YMDD motif; second, patients negative for HBeAg generally showed good response to lamivudine, even in case of liver cirrhosis or of high viremia. A longer follow-up for these and other patients with YMDD mutation will be described elsewhere.

RESUMO

Fatores preditivos para resposta da lamivudine na hepatite crônica B

Introdução: A Lamivudina tem-se mostrado útil no tratamento da hepatite crônica pelo vírus B (HC-VHB).

Objetivo: Investigar os fatores preditivos da resposta à Lamivudina na HC-VHB.

Material e Métodos: Estudo prospectivo com Lamivudina em 35 pacientes com HC-VHB e evidência de multiplicação viral, independentemente do resultado do AgHBe. Administrou-se a Lamivudina na dose diária de 300 mg por 12 meses, seguida de 150 mg diários. Critério de resposta: DNA-VHB negativo (por técnica de PCR) aos 6 meses de tratamento. Nos pacientes não respondedores, pesquisaram-se mutações associadas com resistência à Lamivudina, através do sequenciamento do DNA viral.

Resultados: Observou-se resposta em 23/35 pacientes (65,7%). Dos 15 pacientes com AgHBe positivo antes do tratamento, apenas 5 (33,3%) responderam. As variáveis prévias ao tratamento que puderam prever uma má resposta foram: AgHBe positivo ($p = 0,006$), carga viral elevada ($> 3 \times 10^6$ genomas/ml) ($p = 0,004$) e AgHBc no tecido positivo ($p = 0,0028$). Mutações na região YMDD foram detectadas em 7/11 pacientes não respondedores.

Conclusões: Pacientes com AgHBe positivo e com alta carga viral apresentam um alto risco de desenvolver resistência à Lamivudina. Por outro lado, pacientes com AgHBe negativo, mesmo com alta carga viral, mostraram uma boa resposta à Lamivudina.

ACKNOWLEDGMENT

This research was supported by a grant from Arisco Industrial Ltd.

REFERENCES

1. AYE, T.T.; BARTHOLOMEUSZ, A.; SHAW, T. *et al.* - Hepatitis B virus polymerase mutations during antiviral therapy in a patient following liver transplantation. *J. Hepat.*, 26: 1148-1153, 1997.
2. BARTHOLOMEW, M.M.; JANSSEN, R.W.; JEFFERS, L.J. *et al.* - Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation. *Lancet*, 349: 20-22, 1997.
3. BENHAMOU, Y.; KATLAMA, C.; LUNEL, F. *et al.* - Effects of lamivudine on replication of hepatitis B virus in HIV-infected men. *Ann. intern. Med.*, 125: 705-712, 1996.
4. BUTTERWORTH, L.-A.; PRIOR, S.L.; BUDA, P.J. *et al.* - Comparison of four methods for quantitative measurement of hepatitis B viral DNA. *J. Hepat.*, 24: 686-691, 1996.
5. CHAYAMA, K.; SUZUKI, Y.; KOBAYASHI, M. *et al.* - Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. *Hepatology*, 27: 1711-1716, 1998.
6. DIENSTAG, J.L.; PERRILLO, R.P.; SCHIFF, E.R. *et al.* - A preliminary trial of lamivudine for chronic hepatitis B infection. *New Engl. J. Med.*, 333: 1657-1661, 1995.
7. DIENSTAG, J.L.; SCHIFF, E.R.; MITCHELL, M. *et al.* - Extended lamivudine retreatment for chronic hepatitis B. *Hepatology*, 24: 188A, 1996 [Abstract].
8. DUSHEIKO, G.M. - New treatments for chronic viral hepatitis B. In: ARROYO, V.; BOSCH, J.; BRUGUERA, M. & RODES, J., ed. *Therapy in liver diseases: the pathophysiological basis of therapy*. Barcelona, Masson, 1997. p. 317-330.
9. GARRETT, L.; DIENSTAG, J.L.; GAUTHIER, J. *et al.* - Hepatitis B e-antigen (HBeAg) seroconversion in two patients with evidence of genotypic resistance following extended lamivudine treatment. *Hepatology*, 26: 431A, 1997 [Abstract].
10. HADZYANNIS, S.J. - Natural course and therapy of anti-HBe-positive chronic hepatitis B. In: ARROYO, V.; BOSCH, J.; BRUGUERA, M. & RODES, J., ed. *Therapy in liver diseases. The pathophysiological basis of therapy*. Barcelona, Masson, 1997. p. 301-308.
11. HONKOOP, P.; DE MAN R.A.; ZONDERVAN, P.E. *et al.* - Histological improvement in patients with chronic hepatitis B virus infection treated with lamivudine. *Liver*, 17: 103-106, 1997.
12. HONKOOP, P.; DE MAN R.A.; NIESTERS, H.G.M. *et al.* - Quantitative hepatitis B virus DNA assessment by the limiting-dilution polymerase chain reaction in chronic hepatitis B patients: evidence of continuing viral suppression with longer duration and higher dose of lamivudine therapy. *J. viral Hepatitis*, 5: 307-312, 1998.
13. HONKOOP, P.; NIESTERS, H.G.; DE MAN R.A. *et al.* - Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. *J. Hepat.*, 26: 1393-1395, 1997.
14. HONKOOP, P.; DE MAN, R.A.; NIESTERS, H.G.M. *et al.* - Incidence, characteristics and clinical impact of lamivudine resistance in chronic hepatitis B. *J. Hepat.*, 28(suppl.1): 48, 1998 [Abstract].
15. KANEKO, S.; FEINSTONE, S.M. & MILLER, R.H. - Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. clin. Microbiol.*, 27: 1930-1933, 1989.
16. KANEKO, S.; MILLER, R.H.; DI BISCEGLIE, A.M. *et al.* - Detection of hepatitis B virus DNA in serum by polymerase chain reaction. Application for clinical diagnosis. *Gastroenterology*, 99: 799-804, 1990.
17. KAPKE, G.E.; WATSON, G.; SHEFFLER, S. *et al.* - Comparison of the Chiron Quantiplex branched DNA (bDNA) assay and the Abbott Genostics solution hybridization assay for quantification of hepatitis B viral DNA. *J. viral Hepatitis*, 4: 67-75, 1997.
18. KROGSGAARD, K.; MARCELLIN, P.; TREPO, C. *et al.* - Prednisolone withdrawal therapy enhances the effect of human lymphoblastoid interferon in chronic hepatitis B. *J. Hepat.*, 25: 803-813, 1996.
19. KWOK, S. & HIGUCHI, R. - Avoiding false positives with PCR. *Nature (Lond.)*, 339: 237-238, 1989.
20. LAI, C.-L.; CHIEN, R.-N.; LEUNG, N.W.Y. *et al.* - A one-year trial of lamivudine for chronic hepatitis B. *New Engl. J. Med.*, 339: 61-68, 1998.
21. LAU, D.T.-Y.; DOO, E.; GHANY, M.G. *et al.* - Lamivudine for chronic hepatitis B with typical and atypical serology. *Hepatology*, 26: 429A, 1997 [Abstract].

DA SILVA, L.C.; FONSECA, L.E.P.; CARRILHO, F.J.; ALVES, V.A.F.; SITNIK, R. & PINHO, J.R.R. - Predictive factors for response to Lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42(4): 189-196, 2000.

22. LING, R.; MUTIMER, D.; AHMED, M. *et al.* - Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology*, 24: 711-713, 1996.
23. MARKOWITZ, J.; PAKRASI, A.; HOLLIS, P. *et al.* - Efficacy of lamivudine for prophylaxis and treatment of hepatitis B in liver transplant patients. *Hepatology*, 24: 182A, 1996 [Abstract].
24. MUTIMER, D.; PILLAY, D.; DRAGON, E. *et al.* - High pre-treatment serum hepatitis B virus titre predicts failure of lamivudine prophylaxis and graft re-infection after liver transplantation. *J. Hepat.*, 30: 715-721, 1999.
25. NAOUMOV, N.V.; CHOKSHI, S.; SMITH, H.M. *et al.* - Emergence and characterization of lamivudine-resistant hepatitis B virus variant. *Hepatology*, 24: 282A, 1996 [Abstract].
26. NEVENS, F.; MAIN, J.; HONKOOP, P. *et al.* - Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. *Gastroenterology*, 113: 1258-1263, 1997.
27. NITSUMA, H.; ISHII, M.; MIURA, M. *et al.* - Low level hepatitis B viremia detected by polymerase chain reaction accompanies the absence of HBe antigenemia and hepatitis in hepatitis B virus carriers. *Amer. J. Gastroent.*, 92: 119-123, 1997.
28. PERRILLO, R.; RAKELA, J.; MARTIN, P. *et al.* - Lamivudine for hepatitis B after liver transplantation. *Hepatology*, 24: 182A, 1996 [Abstract].
29. PERRILLO, R.P. - Antiviral therapy of transplant patients with recurrent hepatitis B. In: ARROYO, V.; BOSCH, J.; BRUGUERA, M. & RODES, J., ed. *Therapy in liver diseases: the pathophysiological basis of therapy*. Barcelona, Masson, 1997. p. 347-350.
30. PUGH, R.N.; MURRAY-LYON, I.M.; DAWSON, J.L. *et al.* - Transection of the oesophagus for bleeding oesophageal varices. *Brit. J. Surg.*, 60: 646-649, 1973.
31. ROSENSTRAUS, M.; GUTEKUNST, K. & DALE, B. - Utility of hepatitis virus nucleic acid assays in therapeutic drug trials. In: SCHINAZI, R.F.; SOMMADOSSI, J.-P. & THOMAS, H.C., ed. *Therapies for viral Hepatitis*. London, International Medical Press, 1998. p. 115-127.
32. SANTOS, R.T.M.; ALVES, V.A.F.; WAKAMATSU, A. *et al.* - Chronic hepatitis B immunohistochemistry - comparison of the amplification systems PAP and ABC for the detection of HBsAg and HBeAg. *Arch. argent. Enferm. Apar. dig.*, 10: 10, 1996.
33. TASSOPOULOS, N.C.; VOLPES, R.; PASTORE, G. *et al.* - Efficacy of lamivudine in patients with hepatitis B e antigen - negative / hepatitis B virus DNA - positive (precore mutant) chronic hepatitis B. *Hepatology*, 29: 889-896, 1999.
34. WONG, D.K.H.; CHEUNG, A.M.; O'ROURKE, K. *et al.* - Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B: a meta-analysis. *Ann. intern. Med.*, 119: 312-323, 1993.
35. WONG, W.W.S. & TYRRELL, D.L.J. - Treatment of hepatitis B virus with lamivudine. In: SCHINAZI, R.F.; SOMMADOSSI, J.-P. & THOMAS, H.C., ed. *Therapies for viral Hepatitis*. London, International Medical Press, 1998. p. 353-363.
36. YOTSUYANAGI, H.; YASUDA, K.; INO, S. *et al.* - Persistent viremia after recovery from self-limited acute hepatitis B. *Hepatology*, 27: 1377-1382, 1998.
37. ZEUSEM, S.; DE MAN, R.A.; HONKOOP, P. *et al.* - Dynamics of hepatitis B virus infection *in vivo*. *J. Hepat.*, 27: 431-436, 1997.

Received: 15 December 1999

Accepted: 06 June 2000

- 7- Luiz Caetano da Silva, João Renato Rebello Pinho, Roberta Sitnik, Luís Edmundo Pinto da Fonseca & Flair José Carrilho. Efficacy and tolerability of a long term therapy using high lamivudine doses for the treatment of chronic hepatitis B. Journal of Gastroenterology 36 (7): 476-85, July 2001.**

Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B

LUIZ CAETANO DA SILVA¹, JOÃO RENATO REBELLO PINHO^{2,3}, ROBERTA SITNIK², LUÍS EDMUNDO PINTO DA FONSECA¹, and FLAÍR JOSÉ CARRILHO¹

¹Departamento de Gastroenterologia da Faculdade de Medicina da Universidade de São Paulo, São Paulo-SP, Brazil

²Laboratório Bioquímico Jardim Paulista, São Paulo-SP, Brazil

³Instituto Adolfo Lutz, São Paulo-SP, Brazil

Editorial on page 515

Purpose. A long-term follow-up study was carried out to evaluate the tolerability and efficacy of long-term therapy (1 to 3 years) with high doses (150 or 300 mg daily) of lamivudine for chronic hepatitis B. **Methods.** Thirty-two patients were studied, including those who were seronegative for hepatitis B e antigen (HBeAg), as well as those with decompensated liver cirrhosis. Viral DNA clearance was monitored by using end-point dilution polymerase chain reaction (PCR), a highly sensitive method. Hepatitis B virus (HBV) polymerase gene mutations associated with resistance were determined by sequencing. **Results.** Response to lamivudine in the sixth month was observed in 19/32 (59.4%) patients. With one exception, viral DNA results observed at this time were maintained. The YMDD mutation was detected in 12 nonresponder patients (9 YVDD, 2 YIDD, and 1 mixed population Y(V/I)DD), generally associated with the L528M mutation. Re-takeover by the wild type was observed 6 to 18 months after lamivudine withdrawal. Lamivudine response rates in noncirrhotic and cirrhotic patients were 9/18 (50%) and 10/14 (71.4%), respectively. HBeAg to anti-HBe seroconversion was found after different periods in all responder patients. Hepatitis B surface antigen (HBsAg) clearance and anti-HBs seroconversion were occasionally found. **Conclusions.** In nonresponder patients, resistant mutants appeared up to the second year of lamivudine therapy. In spite of the presence of resistant mutants, maintenance of therapy was usually associated with a lower viral load. In responder patients, maintenance of

therapy was associated with continued absence of detectable HBV DNA in serum, as monitored by highly sensitive methods. No significant side effects caused by lamivudine were observed in our patients, even in those with liver cirrhosis.

Key words: lamivudine, 3TC, HBV, PCR, chronic hepatitis B, treatment

Introduction

Lamivudine or 2'-deoxy-3'-thiacytidine (3TC) monotherapy has been widely used in the treatment of chronic hepatitis B during the past few years, leading to undetectable hepatitis B virus (HBV) DNA by hybridization analysis in many patients.^{1–5} Efficacy was similar for those receiving doses of 100 mg/day and higher doses, such as 300 mg/day,^{2,5,6} but time for HBV DNA suppression was shorter with 300 mg/day.^{1,2,7} HBV therapy focuses on the prevention of antiviral resistant selection, using the maximum tolerated antiviral dose.⁸ Lamivudine was also effective in hepatitis B e antigen (HBeAg)-negative patients,⁹ in those with decompensated cirrhosis,^{10–12} in those with fibrosing cholestatic hepatitis,^{13,14} in the retreatment of patients persistently positive for HBeAg/HBV DNA,¹⁵ and in inhibiting HBV replication in patients with hepatitis flare after lamivudine withdrawal.¹⁶ Despite these promising antiviral results, there are some unsolved issues, including the optimum treatment duration required and the management of drug-resistant virus.¹ Furthermore, various alterations in serum alanine aminotransferase (ALT) have been described; some authors mention only slight changes in its levels,⁴ while others point to frequent ALT exacerbations.¹⁷

It is an open question whether patients with chronic liver disease but normal serum ALT should be treated,

Received: October 13, 2000 / Accepted: January 26, 2001

Reprint requests to: J.R. Rebello Pinho

Serviço de Virologia—Instituto Adolfo Lutz, Avenida Doutor Arnaldo, 355 01246-902 São Paulo-SP, Brazil

as some will proceed to more severe liver disease.¹⁸ Clearance of hepatitis B surface antigen (HBsAg) in chronic hepatitis B has also been related to favorable clinical and histological evolution.^{19,20} Furthermore, low viremia levels have also been considered an important goal for the treatment of chronic hepatitis in HBeAg-negative patients.²¹ Therefore, a therapeutic trial of lamivudine that includes patients with low viremia and different degrees of liver disease would be desirable.

Lamivudine resistance arising from mutations at the YMDD motif was recognized in liver transplant recipients and in patients with chronic hepatitis B.²²⁻²⁵ Following the emergence of resistance, a delayed rise in HBV DNA and serum ALT to below pretreatment levels was observed, suggesting that the resistant virus is less replication-competent.²⁶ The natural history of genetic resistance to lamivudine and the ideal strategy for its management are still unknown. Different approaches to cope with resistant strains have been proposed: to maintain lamivudine treatment, to retreat after initial lamivudine failure¹, or to introduce other antiviral drugs.²⁷ As a matter of fact, when lamivudine is withdrawn, generally, the wild-type virus rapidly becomes the predominant species over resistant ones, leading to clinical deterioration.²⁸

The aim of this study was to evaluate the tolerability and efficacy of long-term therapy (1 to 3 years) with high doses (150 or 300 mg daily) of lamivudine for chronic hepatitis B. Highly sensitive methods were used to monitor viral DNA clearance. Resistance or nonresponse was defined by HBV DNA persistence or rebound in serum ("breakthrough") during lamivudine therapy, while response was characterized by a negative polymerase chain reaction (PCR) result at the sixth month.²⁵ The inclusion criteria were broad, allowing the inclusion of patients with low transaminase levels, as well as patients with mild degrees of chronic hepatitis and patients with compensated (Child-Pugh A) and decompensated cirrhosis (Child-Pugh B). This was done in order to generate results that would apply to patients seen in daily clinical practice, and to increase the probability of HBsAg clearance.

Patients and methods

Patients

We carried out an open, prospective, trial of lamivudine in 32 consecutive patients with chronic hepatitis B and molecular evidence of viral replication. Of these eligible patients, 30 were male and 2, female. Their ages ranged from 9 to 72 years (mean, 45.8 ± 15.7 years; median, 47 years) and they were studied during the period March

1996 to May 1999. All had HBsAg in serum for at least 6 months and detectable HBV DNA in serum. Alanine aminotransferase (ALT) levels ranged from normal to nine times the upper normal limit. Patients were excluded if they were also infected with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus (HIV); also if they had other serious illness; had advanced decompensated liver disease (Child-Pugh C) or another type of liver disease; or were pregnant or lactating. Also excluded were patients who withdrew prematurely from the study or had only minimal lesions ("healthy carriers").

Patients consecutively attending from March 1996 to March 1998 were considered for treatment with lamivudine at a daily dose of 300 mg for 12 months and 150 mg thereafter. Seven patients with decompensated liver cirrhosis (Child-Pugh B) received 150 mg/day from the beginning. All patients gave informed consent for the study, which was approved by the Local Ethics Committee.

Only patients followed up for more than 52 weeks were included. In this study, follow-up ranged from 86 to 182 weeks (mean, 130 weeks). Thirty-two patients were enrolled, of whom 21 are still receiving lamivudine treatment. This drug was withdrawn from 10 of the 13 HBeAg-positive patients who presented with the YMDD mutation (see below) and from 1 HBeAg-negative patient who presented with clearance of serum HBsAg and seroconversion to anti-HBs. Two patients (nos. 21 and 25) did not want to continue therapy and temporarily stopped taking the medication. As a whole, lamivudine was administered for periods varying from 52 to 156 weeks (mean, 109 weeks).

No treatment was previously given to 13 (40.6%) of the 32 patients ("naive"), whereas 19 patients (59.4%) had been previously treated with interferon- α (IFN- α). Previous resistance to IFN- α was considered as partial in patients with disappearance of HBeAg but with persistence of HBV DNA by PCR (9 patients; 47.4%). Complete resistance was defined as persistence of HBeAg (10 patients; 52.6%). None of these patients had received immunosuppressive or antiviral therapy for at least 6 months before lamivudine therapy.

Efficacy endpoints

The primary efficacy endpoint in this study was undetectable serum HBV DNA by an in-house PCR after 24 weeks of treatment. Secondary endpoints were: disappearance of HBeAg in previously HBeAg-positive patients and normalization of serum ALT level. HBeAg and HBsAg clearance was defined when the antigens were not detected in two consecutive samples at least 1 month apart.

478

Biopsies

Liver biopsy was performed with a Tru-Cut (Baxter Health Care, Deerfield, IL, USA) needle in 27 patients, but not in the remaining 5 patients, because of coagulation disturbances or ascites. In these patients, clinical and ultrasonographic diagnosis of cirrhosis was performed instead.

Hepatitis B serology

HBsAg, and HBeAg and their corresponding antibodies (anti-HBs and anti-HBe) were tested using commercially available enzyme-linked immunoassays (Abbott Laboratories, North Chicago, IL, USA).

Alanine aminotransferase (ALT) and amylase levels

Serum ALT and amylase levels were tested using commercially available assays (Abbott Laboratories). Results were expressed as "times the upper normal limit" ($\times$ UNL) values.

Detection of HBV DNA by polymerase chain reaction (PCR)

HBV DNA was released by alkaline treatment and amplified, using primers covering the core region, by a nested PCR, as previously described.^{29,30} To avoid false-positive results, strict procedures proposed for nucleic acid amplification techniques were followed.³¹

Detection of mutations associated with resistance to lamivudine

HBV DNA-positive samples were also amplified with primers corresponding to the polymerase gene, targeted to the region of mutations associated with lamivudine resistance. Samples were denatured as described above and submitted to nested PCR. Outer primers [5'-LAM 5 (5'-TGC RYY TGT ATT CCC ATC CCA TC 3') and 3'-LAM 2 (5'-GTT TTG TTA GGG TTT AAA TG 3')] and inner primers [L840 (5'-ACC CCA TCT TTT TGT TTT GTT AAG 3') and L372 (5'-TCG CTG GAT GTG TCT GCG GCG TTT TAT 3')] were used. Amplifications were carried out at 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 42°C for 30 s and 72°C for 40 s, and a final extension at 72°C for 7 min in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA). This method is based on previously published methods,³²⁻³⁴ incorporating primer 5'-LAM5 developed by us, to ensure better amplification of different HBV genotypes and sequencing of a larger fragment of the polymerase gene.

PCR products were submitted to cycle sequencing reactions, using the second-round primers described

L.C. da Silva et al.: Long-term high-dose lamivudine therapy

above and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences from both strands were determined in an Automated DNA sequencer model ABI 310 (Applied Biosystems). Sequences were compared with those previously published, using the Lasergene package (DNASTar, Madison, WI, USA).

Quantification of HBV DNA by endpoint dilution (EPD) PCR

Samples were initially diluted in water to a tenfold series up to 10^{-10} . Each dilution was submitted to the same PCR protocol as that described above for the detection of HBV-DNA. The sensitivity of the method was reported to be around 3 copies per 10 μ l, or 300 (or $10^{2.48}$) copies/ml.^{29,30} Quantitative results were estimated by multiplying the least positive dilution by 3×10^2 (or $10^{2.48}$) copies/ml.

Laboratory follow-up and clinical evaluation

HBV DNA, ALT levels, and the HBeAg-antiHBe system were assayed at the following time points: pre-treatment study start; at 3, 6, 12, 18, 24, 30, and 36 months of therapy, and every 3 months after stopping lamivudine in ten patients with drug resistance. ALT levels were compared in different periods before, during lamivudine therapy and, in nonresponder patients, after lamivudine withdrawal, as well. Furthermore, ALT level was analyzed according to the presence or absence of HBeAg. Acute exacerbation or ALT flare was defined as an abrupt increase in ALT level by twofold, to a level greater than $5 \times$ UNL.¹⁷ Safety was monitored by clinical and routine laboratory evaluation, including serum amylase at baseline, 2 and 4 weeks after treatment start, and every 4–12 weeks thereafter. Compliance was assessed by questioning the patient.

Statistical analysis

Descriptive measures to express continuous data were: median, mean, and SD ($X \pm$ SD). Nonparametric tests were chosen because of the non-normality of data. The Mann-Whitney test was used to compare two patient groups and the Kruskal-Wallis test to compare three groups. Viral load changes over time were analyzed using Friedman's test. Fisher's exact test (two-tailed) and Pearson's χ^2 (Yates corrected) test were used to compare proportions of patients with certain characteristics. The SPSS statistical package (SPSS, Chicago, IL, USA) was used for all statistical calculations, and the significance level was established at 0.05.

Table 1. Clinical profile and baseline data of the 20 HBeAg-positive patients, and response to lamivudine

Patient	Age (years)/ Sex	Diagnosis	Previous IFN therapy	ALT (IU/l) ($\times$ UNL)	HBV DNA, log (genomes/ml)	Response to lamivudine	Mutation during treatment	Evolution of mutation
1	27/F	LC	Yes	0.8	10.48	Poor	YVDD	PM
2	9/F	CH	No	3.3	8.48	Poor	YIDD	PM
3	59/M	LC	No	8.7	4.48	Good	NA	NA
4	62/M	CH	No	1.7	12.48	Good	NA	NA
5	11/M	CH	No	0.7	10.48	Poor	YMDD	*
6	65/M	LC	No	3.6	12.48	Good	NA	NA
7	30/M	CH	Yes	2.9	8.48	Poor	YVDD	RT
8	45/M	LC	Yes	2.3	6.48	Good	NA	NA
9	60/M	LC	Yes	1.8	10.48	Poor	YVDD	RT
10	44/M	CH	Yes	1.2	10.48	Poor	YVDD	RT
11	48/M	CH	No	2.0	6.48	Poor	YVDD	*
12	65/M	CH	Yes	1.7	12.48	Poor	YVDD	RT
13	37/F	CH	Yes	8.8	6.48	Good	NA	NA
14	48/M	CH	No	1.7	8.48	Poor	YVDD	PM
15	37/M	CH	Yes	4.5	10.48	Good	NA	NA
16	42/M	CH	No	3.1	10.48	Late	NA	NA
17	49/M	CH	Yes	8.0	9.48	Poor	YVDD	^b
18	52/M	LC	No	1.5	8.48	Poor	Mixed	*
19	48/M	CH	Yes	1.3	10.48	Poor	YVDD	RT
20	51/M	LC	No	0.7	12.48	Poor	YIDD	PM
Mean				3.01	9.53			
SD				2.57	2.28			
Median				1.9	10.48			

HBeAg, Hepatitis B e antigen; LC, liver cirrhosis; CH, chronic hepatitis; IFN, interferon; ALT, alanine aminotransferase, times upper normal limit ($\times$ UNL); good, response at the sixth month; late, response after the sixth month; poor, no response, or breakthrough (see text); mixed, mixed population containing Y(M/V/I)DD; PM, persistent mutation; RT, YMDD re-takeover (see text); NA, not applicable for responder patients

*Sequence determined in just one sample

^bSequence determined only during therapy

Results

Baseline characteristics of the patients

Clinical profiles and baseline data of the 20 HBeAg-positive and the 12 HBeAg-negative patients are shown in Tables 1 and 2, respectively. Viral load exponent medians were $10^{10.48}$ and $10^{4.48}$, and means were $10^{9.53 \pm 2.28}$ and $10^{5.65 \pm 2.41}$ in HBeAg-positive and -negative groups, respectively ($P = 0.0002$; Mann-Whitney test). Concerning the patients, ethnic groups, 5 (25%) of the 20 HBeAg-positive and 1 of the 12 (8%) HBeAg-negative patients were of Asian ancestry; all the others were White ($P = 0.37$; Fisher's exact test, two-tailed).

At entry, 19 (59.4%) patients had low levels of ALT ($<2 \times$ UNL), 6 (18.8%) had intermediate levels ($2-4 \times$ UNL), and 7 (21.9%) had high levels (greater than $4 \times$ UNL). Mean ALT levels in the HBeAg-positive and -negative groups were $3.02 \pm 2.57 \times$ UNL and $2.21 \pm 2.60 \times$ UNL, respectively ($P = 0.02$; Mann-Whitney test).

Tolerance to lamivudine

No evidence of side effects caused by lamivudine was observed in our patients, even in those with liver cirrho-

sis. A small asymptomatic amylase elevation (up to $2.5 \times$ UNL), but without clinical manifestations of pancreatitis, was observed in 12 patients.

HBV viremia during different periods of lamivudine therapy

At the sixth month of therapy, negative results by EPD PCR (considered by us as response to lamivudine) were observed in 19 (59.4%) of the 32 patients (Fig. 1). With one exception, these negative results were maintained thereafter. The only exception was observed in a male patient (no. 16) with high baseline viremia ($10^{10.48}$ genomes/ml) and low viremia ($10^{3.48}$ genomes/ml) at the sixth month, but who was negative at the ninth month and subsequently, as determined by PCR. This patient was considered a late responder (Table 1). As seen in Figs. 2-4, a decrease in HBV DNA levels was observed at the third month of therapy, even in nonresponder patients. Of the 13 (40.6%) nonresponder patients, all but 1 showed a positive PCR at the sixth month (Fig. 3). The only exception was observed in a female patient (no. 2; Table 1), with negative PCR result at the sixth and twelfth months, but who was positive at 18 months

Table 2. Clinical profiles and baseline data of the 12 HBeAg-negative patients, and response to lamivudine

Patient	Age (years)/ Sex	Diagnosis	Previous IFN therapy	ALT (IU/l) ($\times$ UNL)	HBV DNA, log (genomes/ml)	Response to lamivudine
21	48/M	LC	Yes	1.1	3.48	Good
22	46/M	LC	Yes	7.2	8.48	Good
23	27/M	CH	Yes	0.5	4.48	Good
24	43/M	LC	Yes	1.5	3.48	Good
25	26/M	CH	Yes	0.6	8.48	Good
26	67/M	CH	No	6.0	6.48	Good
27	57/M	LC	Yes	0.8	6.48	Good
28	72/M	LC	No	6.2	10.48	Good
29	40/M	CH	Yes	0.6	4.48	Good
30	46/M	CH	No	0.6	4.48	Good
31	72/M	LC	Yes	0.8	3.48	Good
32	33/M	LC	Yes	0.6	3.48	Good
Mean				2.21	5.65	
SD				2.60	2.41	
Median				0.7	4.48	

LC, liver cirrhosis; CH, chronic hepatitis; ALT, times upper normal limit ($\times$ UNL); good, PCR negatvation in the sixth month

Fig. 1. Response to lamivudine (negative polymerase chain reaction [PCR] at 6 months) according to hepatitis B e antigen (HBeAg) status. *Three of the 12 responder patients (25%) presented with high viremia ($>10^{1.48}$ genomes/ml). **In 5 of the 12 patients (41.7%), mutation was detected between 9 and 12 months after the beginning of therapy and in the other 7 patients (58.3%), mutation was detected between 13 and 24 months after the beginning of therapy (see Fig. 5). Pos, Positive; neg, negative

Fig. 2. Hepatitis B virus (HBV) DNA levels in seven hepatitis B e antigen (HBeAg)-positive responders during lamivudine therapy (dots). Asterisk shows late responder

Fig. 3. HBV DNA levels in 13 HBeAg-positive nonresponder patients, before ($t = 0$), during (dots) and after (circles) lamivudine therapy. Lamivudine was not withdrawn in 3 patients

Fig. 4. HBV DNA levels in 12 HBeAg-negative patients during lamivudine therapy. Crosses indicate temporary interruption of lamivudine therapy

and afterwards. This patient was considered a non-responder. Transient negative PCR results were also observed in 2 other nonresponder patients. All 13 nonresponder patients were HBeAg-positive.

In ten nonresponder patients, lamivudine was withdrawn at or after the twelfth month of therapy because of the presence of resistance-associated mutations (Fig. 3). Lamivudine was maintained in the other three nonresponder patients. Viral load tended to be lower in patients on therapy and stabilized at levels lower than the pretreatment ones, despite the presence of resistant mutants. As shown in Fig. 3, at the twenty-fourth month, 6 nonresponder patients from whom lamivudine was withdrawn showed a viral load ranging from $10^{10.48}$ to $10^{12.48}$, while viral load ranged from $10^{4.48}$ to $10^{6.48}$ in the four other nonresponder patients who were still receiving therapy at that time ($P = 0.01$; Friedman's test).

HBeAg/anti-HBe system

Serum HBeAg at baseline was detected in 20 (62.5%) out of the 32 patients (Fig. 1). All HBeAg-negative patients did respond ($P = 0.00045$; Fisher's exact test, two-tailed). Response was also observed in 7 of the 20 (35%) HBeAg-positive patients, preceding HBeAg negatization. All these 7 patients showed HBeAg-to-anti-HBe seroconversion, which took place at the third to the thirtieth month (mean, 14.1 ± 11.96 months). Thus, cumulative HBeAg seroconversion was observed in 4 of 20 HBeAg-positive patients (20%) in the first year, 5/20 (25%) in the second year, and 7/20 (35%) during the third year. Negative PCR results persisted during lamivudine therapy in all patients with HBeAg-to-anti-HBe seroconversion. In 4 of the 7 patients, ALT levels became normal and, in 3, they varied from 1.5 to $2 \times \text{UNL}$.

Disappearance of HBeAg was observed in 5 of the 13 nonresponder patients, including 4 with seroconversion to anti-HBe. However, this phenomenon was transient (2 to 6 months) and occurred simultaneously with a sharp decrease in the HBV DNA levels. In 1 patient in this group (no. 18), seroconversion to anti-HBe was detected despite the return of HBV DNA to a level similar to the baseline level, and the appearance of a mixed population of mutants (see below).

Serum alanine aminotransferase (ALT) activity

The mean baseline ALT level in HBeAg-positive responder patients ($4.67 \pm 2.93 \times \text{UNL}$) was significantly higher than that in HBeAg-positive nonresponder patients ($2.12 \pm 1.93 \times \text{UNL}$) and in anti-HBe-positive patients ($2.21 \pm 2.60 \times \text{UNL}$) ($P = 0.013$; Kruskal-Wallis test). In anti-HBe-positive patients, mean ALT level decreased to $0.75 \pm 0.22 \times \text{UNL}$ after 3 months of therapy. All patients with previously elevated ALT the levels (ranging from 1.5 to $7.2 \times \text{UNL}$) had normal levels after 3 months of treatment. In HBeAg-positive patients, mean ALT levels in nonresponder and responder patients decreased to 2.13 ± 1.07 and $1.24 \pm 0.95 \times \text{UNL}$, respectively, after 3 months of treatment. No ALT flare was observed after the third month of lamivudine therapy, when YMDD mutation tends to appear in resistant patients.

HBV DNA polymerase gene mutations

Sequencing of the YMDD motif was carried out in the 13 nonresponder patients. YMDD mutation (codon 552) was detected in 12 of them. Methionine changes to valine (YVDD) or isoleucine (YIDD) were detected in 9 and 2 patients, respectively (Figs. 1 and 5). In 1 patient (no. 18), YVDD, YIDD, and YMDD strains were detected together in the twenty-third month of therapy. In only 1 patient (no. 5), was no mutation detected, despite nonresponse to continued lamivudine therapy. Interestingly, viremia levels in this patient stabilized at $10^{4.48}$ during the last 18 months of follow up. Mutation at codon 528 (L528M) was also observed in 9 of these 13 nonresponder patients, including 2 patients with the YIDD substitution. As shown in Fig. 5, mutations appeared from the ninth to the twenty-fourth month. Cumulatively, these mutations appeared in 5 (25%) and 12 (60%) of the 20 HBeAg-positive patients in the first and the second years of therapy, respectively. No mutations associated with resistance appeared thereafter.

Lamivudine was withdrawn in ten patients with the YMDD mutation. Resistance associated mutations persisted in 4 of them, within follow-up periods of 3 to 14 months (Fig. 5). In the other six patients, re-

482

Fig. 5. Schematic representation of HBV DNA polymerase gene sequencing and time of detection of mutation in nonresponder patients. Patient no. 5 is not shown, as no mutation was observed. Asterisks indicate ALT flare after lamivudine (LAM) withdrawal

takeover by the wild type was observed 6 to 18 months (mean, 10.50 ± 4.51 months) after lamivudine withdrawal.

Treatment response in cirrhotic patients

Lamivudine was given to 14 cirrhotic patients, and 10 (71.4%) of them showed virological response. Serum HBeAg was detected in 7 (50%) patients, including 3 with virological response. Response to lamivudine was not significantly different when compared with that in noncirrhotic patients (9/18; 50%; $P = 0.39$; χ^2 test, Yates corrected).

Response to lamivudine in patients previously treated with IFN- α and in "naïve" patients

Comparison of lamivudine response in patients with (IFN group) and without ("naïve") previous IFN therapy showed viral clearance in 12/19 patients (63%) in the IFN group and in 7/13 (54%) in the naïve group ($P = 0.87$; χ^2 test, Yates corrected). Virological response to lamivudine was detected in all 9 (100%) patients who had shown HBeAg seroconversion (partial resistance) during IFN treatment, but in only 3 (30%) of 10 patients

L.C. da Silva et al.: Long-term high-dose lamivudine therapy

who had shown complete resistance to IFN ($P = 0.003$; Fisher's exact test, two-tailed).

HBsAg clearance

Clearance of HBsAg with anti-HBs seroconversion was observed in two patients. One of them (no. 23) had presented partial resistance to previous IFN therapy. HBsAg became undetectable in the eighteenth month of lamivudine therapy, and seroconversion was observed in the twenty-fourth month. Lamivudine was withdrawn in the twenty-seventh month without relapse. The other patient (no. 19) was HBeAg-positive, with a high viral load. He showed persistent HBV DNA and was HBeAg-positive in the sixth month. In the sixteenth month, the YVDD mutation was detected; despite the interruption of lamivudine in the eighteenth month, viremia levels decreased slowly, up to the ninth month after lamivudine withdrawal, when re-takeover by the wild type (YMDD) and HBeAg seroconversion were observed. Five and eight months later, disappearance of serum HBsAg and seroconversion to anti-HBs, respectively, were observed. This patient did not present with ALT flare during re-takeover.

Discussion

Despite the benefits of IFN- α therapy,³⁵⁻³⁶ it remains a far from ideal treatment for chronic hepatitis B. Furthermore, it may be associated with transient flares in serum transaminase levels and exacerbation of hepatitis. The duration of antiviral therapy to eradicate HBV would depend on the complete inhibition of new HBV DNA synthesis and depletion of the existing cccDNA pool.^{37,38} By decreasing viremia, lamivudine may also reverse the reduced T-cell responsiveness to hepatitis B viral antigens observed in patients with chronic infection.³⁹ Our data support the clinical role of prolonged treatment, i.e., for up to 3 years. No relevant side effects appeared during follow up. A decrease in HBV DNA was observed in the third month of therapy in all patients. HBV DNA was undetectable after 6 months of treatment in all but one responder patient and remained negative afterwards. Nonresponder patients also benefited from the therapy, as HBV DNA levels remained lower after long-term therapy, despite the presence of resistant mutants.

Our primary efficacy endpoint was the undetectability of serum HBV DNA by PCR, with a detection limit of 300 genomes per mL, in the sixth month. According to this criterion, response to lamivudine was observed in 19 (59.4%) of our 32 patients; 7 (35%) of the 20 who were HBeAg-positive and 12 (100%) of the 12 who were HBeAg-negative. HBeAg-positive patients

showed a decrease of viral load, but rarely a negative PCR result in the third month (3 patients). This is in sharp contrast with previously described findings^{2,5} of undetectable levels of HBV DNA in nearly all patients treated within a median of 4 weeks. However, these authors utilized a commercial hybridization assay with a detection limit of approximately 1.6 pg (10^6 genomes) per ml. The role of a sensitive method for HBV DNA quantitation was well illustrated by Lau et al.,⁹ who showed that resistance can occur particularly in patients in whom DNA persists at levels greater than 1000 copies/ml.

In all but two of our responder patients, lamivudine therapy was maintained for more than 2 years and is still continuing. In these two patients, low viremia was again observed after 16 and 26 months of lamivudine therapy when they decided to interrupt the medication by themselves; a subsequent response was seen after reinstitution of therapy. These findings show that a negative PCR result (with a sensitivity of 300 to 400 copies/ml) may again become positive if the long-term therapy with lamivudine is discontinued. On the other hand, no case of lamivudine resistance caused by the YMDD mutation was observed after a 2-year course of lamivudine in the patients who responded to treatment.

Primary efficacy endpoints have varied, some authors referring to an improvement in the histologic activity index,³⁴ others to HBeAg seroconversion,¹⁵ and others to undetectable DNA.^{2,5,15,40} We did not include histologic changes as an efficacy endpoint, because only 12 patients agreed to a repeat biopsy after treatment, but it has been shown that histological progression can be prevented in patients with chronic hepatitis B who are treated with lamivudine.³

Our study with lamivudine included patients with mild degrees of chronic hepatitis and patients with compensated (Child-Pugh A) and decompensated cirrhosis (Child-Pugh B). Similar to observations reported by others,^{4,15} we found that four patients with liver cirrhosis had normal baseline levels of ALT at entry. Lamivudine therapy was safe and effective in the treatment of cirrhotic patients. Lamivudine efficacy was similar in patients with and without cirrhosis (71% and 50%, respectively). Four cirrhotic patients (two HBeAg-positive and two HBeAg-negative) showed moderate signs of liver failure. PCR negativation was observed in three of them and tolerance to lamivudine was equally good in all three. Clinical improvement in patients with decompensated liver disease after treatment with lamivudine was also described by others.^{10-12,41}

Lamivudine can suppress HBV replication in children with chronic hepatitis B.⁴² Only two children (9 and 11 years old) were included in this trial, because they belonged to a family of Japanese descent with a

strong vertical transmission history, but they did not respond to lamivudine.

HBeAg clearance, in all instances occurring with anti-HBe seroconversion, was observed in 7 (35%) out of the 20 HBeAg-positive patients, occurring in periods varying from 3 to 30 months. This finding reinforces the role of serum HBV DNA negativation by PCR as a primary endpoint, and the need for prolonged lamivudine therapy, for at least more than 30 months, in some patients. The median time to loss of HBeAg was 60 weeks, higher than that previously published.¹⁵ Responder patients with anti-HBe seroconversion were also maintained with therapy because of our schedule of a 3-year course of therapy.

The high degree of response (100%) in our 12 HBeAg-negative patients may be explained by the initial low viral load ($10^{4.48}$ genomes/ml) in 9 of these patients. Three patients with high viral load probably harbored a precore mutant HBV. This was confirmed by sequencing in patient no. 28 (data not shown). Another report showed a complete response after 1 year of lamivudine treatment (100mg daily) in 38 (63%) of 60 HBeAg-negative patients.⁴³ It is worth mentioning that these authors detected YMDD variant HBV in 27% of the patients at week 52. Further studies involving a larger number of HBeAg-negative patients should be carried out to evaluate whether higher doses are more effective in this population. It is an open question whether HBsAg clearance can be observed in sustained responders to lamivudine.³⁷ We observed two patients with HBsAg clearance, including one with YVDD mutation, after lamivudine withdrawal.

Baseline serum ALT levels were higher in our HBeAg-positive patients who responded to lamivudine as compared with those in nonresponder patients. ALT levels seem to be the strongest determinant for HBeAg seroconversion during lamivudine therapy.⁴⁴ During lamivudine therapy, only 3 of our HBeAg-positive patients showed ALT values higher than $5 \times \text{UNL}$, but this occurred only during the first 3 months when the YMDD mutation is not likely to appear, in contrast to other data published previously.¹⁷ ALT elevations during lamivudine therapy were also observed by others.^{45,46} Liaw et al.¹⁷ detected HBeAg seroconversion in 8 of 13 patients with YMDD mutations, while, in our study, HBeAg seroconversion was observed in only 2 of 12 (16.7%) patients with mutations. In 4 other patients, HBeAg seroconversion was temporary (2 to 6 months) and occurred simultaneously with a decrease in HBV DNA viral load.

Sequencing of the YMDD motif in nonresponder patients allowed us to detect a methionine-to-valine substitution (YVDD) in 9 patients and a methionine-to-isoleucine (YIDD) mutation in 2 patients, and mixed mutant forms in 1 patient. Substitution of methionine

for leucine at residue 528 was also found in most patients, including 2 patients with the YIDD mutation. In other reports,^{17,47} L528M was only found in YVDD mutants. YMDD mutations were detected in 5 (25%) of our 20 HBeAg-positive patients in the first year, and cumulatively in 12 (60%) of these 20 patients in the second year. No mutation appeared during the third year in 17 patients studied for more than 2 years. In 10 of the 12 patients with the YMDD mutation, lamivudine was withdrawn, and the mutant forms were replaced by the wild-type virus (re-takeover) in 6 patients 6 to 17 months after lamivudine withdrawal. Re-takeover was previously reported as occurring 3 and 4 months after cessation of therapy.²⁸ In the other 4 patients with the YMDD mutation in whom lamivudine was withdrawn, who were followed from 3 to 8 months, re-takeover has not been observed so far. According to some authors, the YVDD/YIDD variants present reduced replication ability when compared with the wild-type HBV.^{26,48} A lower viral load was observed in our resistant patients receiving lamivudine.

Comparison of lamivudine response in patients with and without previous therapy with IFN showed similar lamivudine efficacy. All nine patients who had presented HBeAg negativation during or after IFN responded to lamivudine, whereas such response was observed in only three (30%) of the ten patients who were persistently HBeAg-positive after IFN.

Our schedule was based on the administration of 150mg every 12h for 12 months and 150mg a day thereafter. Only decompensated cirrhotic patients received 150mg daily from the beginning, because the development of ascites may lead to some degree of renal dysfunction. A 24-week dose-ranging study showed that 100mg of lamivudine daily should be as effective as higher doses.^{4,5} This conclusion was based on the observation that HBV DNA levels became undetectable by liquid hybridization assay in the 100-mg and 300-mg dose groups. However, quantification of HBV DNA levels by EPD PCR suggests a more profound drop in HBV DNA levels in the 300-mg group.⁴⁹ Lamivudine doses of more than 100mg daily may impair viral replication more rapidly than lower doses, delaying the development of viral mutations.⁷ Similar efficacy with both doses, but a shorter time to HBV DNA suppression with the 300-mg dose, was also suggested by others.^{1,6}

The benefits of treatment duration of more than 12 months should be weighed against the potential harm of increasing the risks of developing drug resistance.³⁷ We observed drug resistance in 13 of our 20 HBeAg-positive patients, but in 7 of these patients, HBeAg seroconversion was detected, at different periods, ranging from 3 to 30 months. It is worth mentioning that HBsAg clearance occurred in 2 patients just 30 months after they started on lamivudine.

In conclusion, long-term therapy with lamivudine was well tolerated, and no significant side effects were observed. Maintenance of therapy in responder patients was associated with continued effects on HBV replication, as monitored by highly sensitive methods. HBeAg-to-anti-HBe seroconversion was found after different periods in all responder patients. HBsAg clearance and anti-HBs seroconversion were found occasionally. Nonresponder patients characteristically showed the YMDD mutation up to the second year of lamivudine therapy.

References

- Wong WWS, Tyrrell DLJ. Treatment of hepatitis B virus with lamivudine. In: Schinazi RF, Sommadossi J-P, Tomas HC, editors. Therapies for viral hepatitis. London: International Medical Press; 1998. p. 353-63.
- Dienstag JL, Perrillo RP, Schiff ER, Bartholomey M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. *N Engl J Med* 1995;333:1657-61.
- Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, et al. A 1-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998;339:61-8.
- Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999;341:1256-63.
- Nevens F, Main J, Honkoop P, Tyrrell DL, Barber J, Sullivan MT, et al. Lamivudine therapy for chronic hepatitis B: a 6-month randomized dose-ranging study. *Gastroenterology* 1997;113:1258-63.
- Zeuzem S, de Man RA, Honkoop P, Roth WK, Schalm SW, Schmidt JM. Dynamics of hepatitis B virus infection in vivo. *J Hepatol* 1997;27:431-6.
- Honkoop P, De Man RA, Niesters HGM, Main J, Nevens F, Thomas HC, et al. Quantitative hepatitis B virus DNA assessment by the limiting dilution polymerase chain reaction in chronic hepatitis B patients: evidence of continuing viral suppression with longer duration and higher dose of lamivudine therapy. *J Viral Hepatitis* 1998;5:107-12.
- Bartholomeusz A, Schinazi RF, Locardinini AS. Significance of mutations in the hepatitis B virus polymerase selected by nucleoside analogues and implications for controlling chronic disease. *Viral Hepatitis Rev* 1998;4:167-87.
- Lau DTY, Doo E, Ghany MG, Kleiner DE, Park Y, Condreay L, et al. Lamivudine for chronic hepatitis B with typical and atypical serology (abstract). *Hepatology* 1997;26:429.
- Villeneuve JP, Condreay LD, Willems B, Pomier G, Pilon D, Raymond G, Willems B. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. *Hepatology* 2000;31:207-10.
- Bain VG, Kneteman NM, Ma MM, Gutfreund K, Shapiro JA, Fischer K, et al. Efficacy of lamivudine in chronic hepatitis B patients with active viral replication and decompensated cirrhosis undergoing liver transplantation. *Transplantation* 1996;62:1456-62.
- Van Thiel DH, Friedlander L, Kania RJ, Molloy PJ, Hassanein T, Wahlstrom E, Faruki H. Lamivudine treatment of advanced and decompensated liver disease due to hepatitis B. *Hepatogastroenterology* 1997;44:808-12.
- Al Faraidy K, Yoshida EM, Davis JE, Vartanian RK, Anderson FH, Steinbrecher UP. Alteration of the dismal natural history of fibrosing cholestatic hepatitis secondary to hepatitis B virus with the use of lamivudine. *Transplantation* 1997;64:926-8.

14. Chan TM, Wu PC, Li FK, Lai CL, Cheng IK, Lai KN. Treatment of fibrosing cholestatic hepatitis with lamivudine. *Gastroenterology* 1998;115:177-81.
15. Dienstag JL, Schiff ER, Mitchell M, Gitlin N, Lissos T, Condreay L, et al. Extended lamivudine retreatment for chronic hepatitis B: maintenance of viral suppression after discontinuation of therapy. *Hepatology* 1999;30:1077-82.
16. Honkoop P, De Man RA, Niesters HGM, Schalm SJ. Lamivudine withdrawal hepatitis flare successfully treated with lamivudine in patients with chronic hepatitis B (abstract). *Hepatology* 1997;26:429.
17. Liaw YF, Chien RN, Yeh CT, Tsai SL, Chu CM. Acute exacerbation and hepatitis B virus clearance after emergence of YMDD motif mutation during lamivudine therapy. *Hepatology* 1999;30:567-72.
18. Chen DS. Natural history of chronic hepatitis B virus infection: new light to an old story. *J Gastroenterol Hepatol* 1993;8:470-5.
19. Da Silva LC, Madruga CLA, Carrilho FJ, Pinho JRR, Saez-Alquezar A, Santos C, et al. Spontaneous hepatitis B surface antigen clearance in a long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with hepatitis C and D virus replication. *J Gastroenterol*. 1996;31:696-701.
20. Han S-HB, Martin P. Successful antiviral therapy and prognosis in cirrhosis due to hepatitis B (editorial). *Am J Gastroenterol* 1998;93:859-60.
21. Nitsuma H, Ishii M, Miura M, Kobayashi K, Toyota T. Low level hepatitis B viremia detected by polymerase chain reaction accompanies the absence of HBe antigenemia and hepatitis in hepatitis B virus carriers. *Am J Gastroenterol* 1997;92:119-23.
22. Honkoop P, Niesters HG, De Man RA, Osterhaus AD, Schalm SJ. Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. *Hepatology* 1997;26:1393-5.
23. Lin R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, Harrison TJ. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996;24:711-3.
24. Lai CL, Liaw YF, Leung NWY, Deslauriers M, Barnard J, Sanathaman L, et al. Genotypic resistance to lamivudine, placebo-controlled multicentre study in Asia of lamivudine therapy for chronic hepatitis B infection: incidence, kinetics of emergence and correlation with disease parameters (abstract). *Hepatology* 1997;26:259.
25. Da Silva LC, Pinho JRR, Sitnik R, Fonseca LEP, Carrilho FJ. Predictive factors for response to lamivudine in chronic hepatitis B. *Rev Inst Med Trop S Paulo* 2000;42:179-86.
26. Melegari M, Scaglioni PP, Wands JR. Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. *Hepatology* 1998;27:628-33.
27. Aye TT, Bartholomew A, Shaw T, Bowden S, Breschkin A, McMillan J, et al. Hepatitis B virus polymerase mutations during antiviral therapy in a patient following liver transplantation. *J Hepatol* 1997;26:1148-53.
28. Chayama K, Suzuki Y, Kobayashi M, Kobayashi M, Tsubota A, Hashimoto M, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. *Hepatology* 1998;27:1711-6.
29. Kaneko S, Feinstone SM, Miller RH. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J Clin Microbiol* 1989;27:1930-3.
30. Kaneko S, Miller RH, Di Bisceglie AM, Feinstone SM, Hoofnagle JH, Purcell RH. Detection of hepatitis B virus DNA in serum by polymerase chain reaction. Application for clinical diagnosis. *Gastroenterology* 1990;99:799-804.
31. Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989;339:237-8.
32. Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, Harrison TJ. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* 1996;24:711-3.
33. Tipples G, Ma M, Fischer K, Bain V, Kneteman N, Tyrrell D. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. *Hepatology* 1996;24:714-7.
34. Bartholomew MM, Jansen RW, Jeffers LJ, Reddy KR, Johnson LC, Bunzendahl H, et al. Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation. *Lancet* 1997;349:20-2.
35. Hoofnagle JH, Di Bisceglie AM. The treatment of chronic viral hepatitis. *N Engl J Med* 1997;336:347-56.
36. Wong DKH, Cheng AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Int Med* 1993;119:312-23.
37. Lok ASF. Treatment of chronic hepatitis B virus infection. In: Arroyo V, Bosch J, Bruguera M, Rodes J, Tapias JMS, editors. *Treatment of liver diseases*. Barcelona: Masson; 1999. p. 261-71.
38. Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H. Viral dynamics in hepatitis B virus infection. *Proc Natl Acad Sci USA* 1996;93:4398-402.
39. Boni C, Bertolotti A, Penna A, Cavalli A, Pilli M, Urbani S, et al. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* 1998;102:968-75.
40. Jarvis B, Faulds D. Lamivudine. A review of its therapeutic potential in chronic hepatitis B. *Drugs* 1999;58:101-41.
41. Sponseller CA, Bacon BR, Di Bisceglie AM. Clinical improvement in patients with decompensated liver disease caused by hepatitis B after treatment with lamivudine. *Liver Transpl* 2000;6:715-20.
42. Sokal EM, Roberts EA, Mieli-Vergani G, McPhillips P, Johnson M, Barber J, et al. A dose-ranging study of the pharmacokinetics, safety, and preliminary efficacy of lamivudine in children and adolescents with chronic hepatitis B. *Antimicrob Agents Chemother* 2000;44:590-7.
43. Tassopoulos NC, Volpes R, Pastore G, Heathcote J, Buti M, Goldin RD, et al. Efficacy of lamivudine in patients with hepatitis B e antigen-negative/hepatitis B virus DNA-positive (precore mutant) chronic hepatitis B. Lamivudine Precore Mutant Study Group. *Hepatology* 1999;29:889-96.
44. Chien RN, Liaw YF, Atkins M. Pretherapy alanine transaminase level as a determinant for hepatitis B e antigen seroconversion during lamivudine therapy in patients with chronic hepatitis B. Asian Hepatitis Lamivudine Trial Group. *Hepatology* 1999;30:770-4.
45. Atkins M, Hunt CM, Brown N. Clinical significance of YMDD mutant hepatitis B virus (HBV) in a large cohort of lamivudine treated hepatitis B patients (abstract). *Hepatology* 1998;28:319.
46. Lung N, Dienstag J, Schiff ER. Clinical safety profile of lamivudine treatment in a large cohort of hepatitis B patients (abstract). *Hepatology* 1998;28:587.
47. Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, et al. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998;27:1670-7.
48. Ono-Nita SK, Kato N, Shiratori Y, Masaki T, Lan KH, Carrilho FJ, Omata M. YMDD motif in hepatitis B virus DNA polymerase influences on replication and lamivudine resistance: a study by in vitro full-length viral DNA transfection. *Hepatology* 1999;29:939-45.
49. De Man RA, Heijtkink RA, Niesters HG, Schalm SW. New developments in antiviral therapy for chronic hepatitis B infection. *Scand J Gastroenterol Suppl* 1995;212:100-4.

- 8- Luiz Caetano da Silva, Maria Luiza da Nova, Suzane Kioko Ono-Nita, João Renato Rebello Pinho, Roberta Sitnik, Vera Aparecida dos Santos, Flair José Carrilho. Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic Hepatitis B. Revista do Instituto de Medicina Tropical de São Paulo, 51(5):261-268, September-October, 2009**

Rev. Inst. Med. trop. S. Paulo
51(5):261-268, September-October, 2009
doi: 10.1590/S0036-46652009000500005

SIMULTANEOUS QUANTITATION OF SERUM HBV DNA AND HBeAg CAN DISTINGUISH BETWEEN SLOW AND FAST VIRAL RESPONSES TO ANTIVIRAL THERAPY IN PATIENTS WITH CHRONIC HEPATITIS B

Luiz Caetano DA SILVA, Maria Luiza da NOVA, Suzane Kioko ONO-NITA, João Renato Rebello PINHO, Roberta SITNIK, Vera Aparecida dos SANTOS & Flair José CARRILHO

SUMMARY

Background: The quantitation of serum HBeAg is not commonly used to monitor viral response to therapy in chronic hepatitis B. **Methods:** In this study, 21 patients receiving varying therapies were followed and their viral response monitored by concomitant viral load and HBeAg quantitation in order to study the meaning and the kinetics of both parameters. **Results:** It was possible to distinguish between three different patterns of viral response. The first was characterized by a simultaneous decrease in serum HBV DNA and HBeAg. The second pattern was characterized by a decrease in serum HBeAg but persistent detection of HBV DNA. The third pattern was characterized by undetectable HBV DNA with persistent HBeAg positivity, which points to a non-response (Pattern III-B) except when HBeAg levels showed a slow but steady drop, characterizing a "slow responder" patient (Pattern III-A). **Conclusions:** The first pattern is compatible with a viral response. A long-term HBeAg seropositivity with a slow and persistent decrease (Pattern III-A) is also compatible with a viral response and calls for a prolongation of anti-viral treatment.

KEYWORDS: Hepatitis B; Serological markers; Treatment response; Treatment follow-up.

INTRODUCTION

The management of chronic hepatitis B has been the subject of many reviews over the last few years^{1,11,19,23,24}. Updates to the recommendations for monitoring patients infected with hepatitis B virus (HBV) were recently published^{20,25}, and in them, as well as recent publications on this subject, serum Hepatitis e antigen (HBeAg) is considered only qualitatively with no evaluation of its expression (quantitation) as a tool for monitoring patients undergoing treatment^{20,25}.

According to KEEFE *et al.*²⁰, the quantitation of HBeAg has not been widely used, is expensive, and available data about the predictive value of HBeAg serostatus remains limited. The same authors mention that approximately one third of patients with serum HBV DNA levels lower than 10^4 copies will seroconvert, suggesting a close relationship between HBV DNA suppression and the likelihood of seroconversion. HBeAg-positive chronic hepatitis B, considered as the classical form, is characterized by a stable high level viremia (10^7 - 10^{10} HBV copies/mL), whereas HBeAg-negative hepatitis B is characterized by a less stable and severe viremia, or less than 10^6 copies/mL²⁰.

Several different types of quantitative assays based on signal or target amplification are available for viral DNA, including high sensitivity

polymerase chain reaction (PCR)-based methods that measure serum HBV DNA levels with a wide dynamic range^{1,25}. Nowadays, real-time PCR assays are preferably used²⁵.

The maximum HBV DNA viral load detected in patients with chronic HBV is not well established and may depend on the quantitative technique used to measure it. Thus, CORDEN *et al.*⁵ measured HBV DNA with the Chiron Amplicor HBV DNA Monitor Assay and found that the viral load was between 4×10^5 and 1.2×10^9 copies/mL. In HBeAg-positive patients, they found HBV DNA levels of up to $10^{9.5}$ copies/mL. By using in-house end-point dilution PCR based on Kaneko's method^{17,18}, our group found levels of up to 3×10^{12} copies/mL^{6,8}. Also, using real-time PCR, HO *et al.*¹⁴ found median levels of 1.5×10^7 copies/mL, and JARDI *et al.*¹⁶ found median a serum HBV DNA viral load of 9.2×10^8 copies/mL (range 2.9×10^5 - 4.8×10^{11}).

For HBeAg-positive chronic hepatitis B patients, the main goals for treatment are HBeAg loss, seroconversion to anti-HBe, normalized alanine aminotransferase (ALT) levels, and the suppression of viremia to undetectable levels²⁵. However, it has been consistently observed that lamivudine (LAM) or adefovir dipivoxil (ADV) produces a rapid and dramatic decrease in viremia, but a lower proportion of patients exhibit serum HBeAg loss³⁰. This important aspect was also emphasized by

University of São Paulo School of Medicine, Department of Gastroenterology, Hepatology Branch.

List of abbreviations used: ADV - Adefovir dipivoxil; ALT - Alanine aminotransferase; HBV - Hepatitis B virus; IFN - Interferon; LAM - Lamivudine; PEG-IFN - Pegylated interferon.

Correspondence to: Prof. Luiz Caetano da Silva, IMT2, Av. Dr. Enéas de Carvalho Aguiar 500, 05403-000 São Paulo, SP, Brasil. Tel/fax: 55-11-3064-5132, e-mails: LCDS - lucadasi@bol.com.br, MLDN - marialuizanova@gmail.com, SKON - skon@usp.br, JRRP - jrpinho@usp.br, RS - rositnik@hotmail.com, VADS - vera@usp.br, FJC - fjcarrilho@usp.br

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; SITNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 51(5): 261-268, 2009.

NEUMANN²⁶. It is worth mentioning that the production of HBeAg is not dependent on the formation of HBV DNA, but it reflects the level of HBV core/precure gene expression in the liver²⁴.

The role of HBeAg quantitation has already been addressed by some authors^{13,28}. PERILLO *et al.*²⁸ found that the baseline HBeAg concentration is the best independent predictor of a patient's response to interferon-alpha (IFN- α) when compared to baseline HBV DNA levels. Furthermore, it was observed, in this study and another^{12,13}, that a steep decline in the HBeAg concentration during the first weeks of therapy was predictive of an antiviral response. A long-term study on the serial quantitation of HBeAg demonstrated that a high serum level (> 100 IU/mL) at week 24 of therapy with peginterferon alpha-2a (Peg-IFN- α 2a) had an excellent negative predictive value; less than 5% of patients achieved HBeAg seroconversion at week 72¹⁰.

During LAM therapy, different types of changing patterns have been observed in relation to levels of HBeAg pretreatment, and they have been categorized into three groups: "decrecendo" ("falling"), "decrecendo-crescendo" ("falling-rising"), and "no changing" or "fluctuating"^{27,28}. These patterns determined by serial monitoring during LAM therapy allowed the prediction of the treatment response as well as early recognition of a viral breakthrough. However, a simultaneous determination of HBV DNA was not performed.

It is worth mentioning that, in HBeAg-positive patients, entecavir was found to be more potent than LAM and resulted in undetectable HBV DNA by PCR in 67% of patients at week 48, compared to 36% of patients treated with LAM. However, the HBeAg seroconversion rate was similar in the two groups, 21% and 18%. Despite these interesting and valuable observations, serial quantitative determinations of serum HBeAg have not been routinely carried out during the last few years and have not been mentioned in some recent recommendations^{20,25}.

Using serial quantitative PCR for HBV DNA and a simultaneous quantitative HBeAg assay, we previously observed an interesting dissociation between HBV DNA and HBeAg in some patients. Our aim is to show that a simultaneous decrease in HBV DNA and HBeAg levels, in addition to a slow but stepwise decreasing pattern of HBeAg levels in some patients, may predict the negative HBeAg response despite a very prolonged positive response during antiviral therapy. This is particularly important because serum HBV DNA levels may decrease to undetectable levels soon after initiating therapy with LAM or other drugs^{8,26,30,34} and can not be used further for monitoring the treatment response.

PATIENTS AND METHODS

Patients: Twenty-one HBeAg-positive patients with chronic hepatitis B were enrolled in the study (Table 1). Sixteen patients received LAM and five patients received IFN- α plus LAM (Table 2).

In this phase of the study, we detected some patterns of the viral response (described below) in 11 of the 16 patients given LAM and in four of the five patients given IFN- α plus LAM.

Nine patients who did not respond to antiviral therapy, and one who presented with a relapse after LAM withdrawal, were retreated as shown

Table 1
Demographic and baseline data for naïve patients (first phase)

Sex (M/F)	16/5
Age (years)	9 - 65 ($\pm$ 38)
Caucasians/Asians	14/7
HBV DNA (log)	5.41 - 12.48 ($\pm$ 9.26)
ALT (xUNL)	0.7 - 8.8 ($\pm$ 3.24)
Chronic hepatitis/Liver cirrhosis	16/5
First therapy	No. of patients
Lamivudine (LAM)	16
IFN + LAM	5
Follow-up (months)	34 - 133 ($\pm$ 91)

in Table 3. Three patients (5, 6, and 13) had to receive another series of therapy after the second trial.

Patient ages varied from nine to 65 years (mean 38 yr) and they were studied during a period of 34 to 133 months (Table 1). All patients had Hepatitis B surface antigen (HBsAg) in their serum for at least six months and detectable HBV DNA. Patients were excluded if they were also infected with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus (HIV). Patients who withdrew prematurely from the study or had only minimal lesions (inactive HBsAg carrier state) were also excluded. All patients gave informed consent for the study, which was approved by the Institutional Review Board (Hospital das Clínicas - University of São Paulo School of Medicine).

Treatment schedules and primary endpoints: Sixteen patients received monotherapy with oral LAM at a dose varying from 150 mg to 300 mg per day for 12 months and 150 mg/day thereafter as previously described⁸. Three out of four patients with liver cirrhosis received 150 mg/day from the beginning (Table 2). Therapy with IFN- α (3 to 5 MU three times a week) plus LAM (150 mg/day) was initially given to four patients (Table 2). Another patient was submitted to combined therapy with Peg-IFN- α 2b and LAM because of high serum levels of HBV DNA and HBeAg and a slight increase in ALT. In our experience, patients with such baseline values are prone to develop resistance to monotherapy with LAM^{6,9}. Such patients were included in the IFN-related group (Table 2). Overall, 21 series of antiviral therapy were used during this period (Table 2).

Nine LAM-resistant patients with mutations in the YMDD motif of polymerase gene, six with M204V, two with M204I, and one patient with both, were retreated (Table 3). Adefovir (10 mg/day) was given to three patients as a second series and one patient as a third series (Table 3). Pegylated interferon (Peg-IFN) was given to five patients, four received Peg-IFN- α 2b (1.5 μ g/kg of body weight) and one patient received Peg-IFN- α 2a (180 μ g), all in association with LAM. Finally, tenofovir was given at a dose of 300 mg/day to four unresponsive patients as a rescue drug.

As for the two patients who experienced a relapse after withdrawal of LAM, one (patient 2) presented with a spontaneous absence of serum

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; STNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 51(5): 261-268, 2009.

Table 2

Demographic, baseline data, and patterns of HBV DNA and HBeAg responses to initial therapy with lamivudine (LAM) in 16 patients or interferon-alpha (IFN- α) plus LAM in five other patients with chronic hepatitis B (CH) or liver cirrhosis (LC)

Patient/ Diagnosis	Age/Sex	HBV DNA (log)	ALT* (xUNL)	First therapy	Viral response (VR) / (Mutation)**	Pattern	Follow-up (months)	Outcome
1/CH	37/M	10.48	4.5	LAM	VR / (NO)	I	102	Seroconversion HBsAg/anti-HBs
2/CH	37/F	6.48	8.8	LAM	VR / (NO)	I	125	Relapse Re-treatment
3/CH	45/M	8.63	2.3	LAM	VR / (M204V) (late)	I	125	Breakthrough (late) Re-treatment
4/LC	65/M	12.48	6.9	LAM	VR / (NO)	I	109	Seroconversion HBsAg/anti-HBs
5/CH	48/M	8.48	1.7	LAM	BT / (M204V)	II	109	Breakthrough Re-treatment
6/LC	52/M	8.48	1.5	LAM	BT / (M204V/I)	II	108	Breakthrough Re-treatment
7/LC	42/M	10.48	3.1	LAM	VR / (NO)	IIIA	90	Seroconversion HBeAg/anti-HBe
8/LC	59/M	7.96	8.7	LAM	VR / (NO)	IIIA	48	Seroconversion HBeAg/anti-HBe
9/CH	62/M	12.48	1.7	LAM	VR / (NO)	IIIA	83	VR with relapse Re-treatment
10/CH	9/F	8.48	3.3	LAM	BT / (M204I)	IIIB	37	Breakthrough. Drop out
11/CH	34/M	5.41	2.0	LAM	BT / (M204I)	IIIB	95	Breakthrough Re-treatment
12/CH	11/M	10.48	0.7	LAM	NR / (M204I)	NR	133	Re-treatment
13/CH	30/M	8.48	2.9	LAM	NR / (M204V)	NR	117	Re-treatment
14/CH	43/M	10.48	1.7	LAM	NR / (M204V)	NR	86	Re-treatment
15/CH	44/M	10.48	1.2	LAM	NR / (M204V)	NR	119	Re-treatment
16/CH	49/M	9.48	8.0	LAM	NR / (M204V)	NR	103	Breakthrough Re-treatment
17/CH	29/M	12.48	1.5	IFN+LAM	VR / (NO)	I	85	Seroconversion HBeAg/anti-HBe
18/CH	21/M	6.48	4.2	IFN+LAM	VR / (NO)	I	34	Seroconversion HBeAg/anti-HBe
19/CH	32/F	7.60	2.0	Peg+LAM ***	VR / (NO)	IIIA	72	Seroconversion HBeAg/anti-HBe
20/LC	11/F	6.40	0.7	IFN+LAM	VR / (NO)	IIIA	62	Seroconversion HBeAg/anti-HBe
21/CH	12/M	8.70	0.7	IFN+LAM	NR / (NO)	NR	60	Under treatment. Steady drop of HBV DNA and HBeAg

HBV, Hepatitis B virus; HBeAg, Hepatitis e antigen. * XUNL = upper normal limit; ** VR = viral response (PCR negatvation); NR = non-response; *** see text.

HBeAg but had a persistence of HBV DNA during the non-treatment period and received a second series of LAM with subsequent clearance of the HBV DNA. The other patient (patient 9) was treated with Peg-IFN- α 2b plus LAM and presented with an HBsAg/anti-HBs seroconversion. Finally, another patient non-responder to LAM (patient 16) received LAM associated to lobucavir as a rescue drug (withdrawn from the market).

As shown in Table 3, more than two therapeutic series had to be used in three patients (patients 5, 6, and 13). Overall, 15 series of antiviral therapy were used in the retreatment, resulting in a total number of 36 therapeutic series for the two phases. The primary endpoints were an inability to detect HBV DNA and the loss of HBeAg with seroconversion to anti-HBe.

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; SITNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 51(5): 261-268, 2009.

Table 3
Patterns of HBV DNA and HBeAg responses in chronic hepatitis B patients previously resistant to lamivudine (LAM)* and submitted to a second or a third anti-viral therapy

Patient **	Type of previous resistance	Antiviral therapy (re-treatment)	Pattern of response***	Outcome
2/CH	Relapse	Lamivudine****	I	HBeAg/anti-HBe seroconversion
3/CH	M204V	Adefovir	NR	Late HBVDNA negatization and late HBeAg/anti-HBe seroconversion
5/CH	M204V	PegIFN- α 2b+Lamivudine	I	Viral response with relapse
		Adefovir	IIIB	HBeAg-non-response
		Tenofovir	IIIA	Profound drop of HBeAg
6/LC	M204V/M204I	PegIFN- α 2b+Lamivudine	NR	HBVDNA persistently positive
		Tenofovir	***	Clearance of HBVDNA (See table 2)
9/CH	Relapse	PegIFN- α 2b+Lamivudine	I	HBsAg/anti-HBs seroconversion
11/CH	M204I	Adefovir	IIIB	Breakthrough
12/CH	M204I	Tenofovir	IIIA	Under treatment
13/CH	M204V	PegIFN- α 2b+Lamivudine	I	VR with relapse
		Tenofovir	I	Under treatment
14/CH	M204V	Adefovir	I	Under treatment
15/CH	M204V	PegIFN- α 2a+Lamivudine	I	HBeAg/anti-HBe seroconversion
16/CH	M204V	Lamivudine+Lobucavir	I	HBsAg/anti-HBs seroconversion

HBV, Hepatitis B virus; HBeAg, Hepatitis e antigen. * See Table 1; ** No. according to Table 2; *** NR = non-response; undetectability of HBeAg just before tenofovir; **** See text.

Laboratory methods: Serum samples were available for study at the following time points: initial screening ("baseline"), every three to six months during therapy, and after the completion of therapy. The levels of ALT, HBV DNA, and HBeAg were available at each time point. The serum ALT levels were tested using commercially available assays (Abbott Laboratories). Results are expressed as values times the upper normal limit (x UNL). The semi-quantitation of serum HBeAg was performed prospectively and simultaneously with the determination of serum HBV DNA. Because no commercial assay was available for measuring the HBeAg concentration, a micro-particle enzyme immunoassay (MEIA, AxSYM HBe 2.0, Abbott Laboratories, Abbott Park, IL) was used¹³. The assay is based on two different monoclonal antibodies, and its reference preparation for quantitation of HBeAg makes use of purified recombinant HBeAg as a control. Results are expressed as an index (sample to cutoff = S/CO) luminescent values ratio²⁷.

In 17 patients, the quantitation of HBV DNA was performed by in-house PCR based on Kaneko's method^{17,38} and as previously described^{6,9}. The sensitivity of the method was reported to be 3×10^2 copies/mL, and its dynamic range went up to 3×10^{12} copies/mL. Quantitative results were estimated based on end-point dilutions. In the last four patients (patients 18 to 21), the serum HBV DNA was determined by the Cobas Amplicor HBV Monitor Test (Roche Diagnostics, Branchburg, NJ) with a linear range from 4×10^2 (lower limit of detection) to 4×10^7 copies/mL²⁷. We have recorded the HBV-DNA levels in copies/mL instead of IU/mL as recently reported⁹.

A virologic response was defined as a decrease in serum HBV DNA to undetectable levels by PCR and a loss of HBeAg²⁵. Patients who showed neither of these serological features were considered to be non-responders, and those who presented with only one type of response (HBV DNA or HBeAg) were classified according to the patterns as described below. A sustained response was defined as the persistence of a virologic response for six months after the discontinuation of therapy²⁵. The clearance of HBeAg and HBsAg was defined as the absence of the particular antigen in two consecutive samples at least one month apart. Viral polymerase, precore/core, and surface genes were sequenced to determine mutations in the YMDD domain as previously described^{9,32}.

Liver biopsies: Liver biopsy was performed with a Tru-cut (Baxter Health Care, Deerfield, IL, USA) needle in 20 out of 21 patients. The exception was a cirrhotic patient with ascitis and very low platelet counts.

RESULTS

Demographic and baseline characteristics of patients: The demographics, baseline data, and patterns of initial therapeutic response for the patients studied are shown in Tables 1, 2, and 3. Changes in the HBeAg/anti-HBe system were observed in 15/21 patients, and the behavior of serum HBeAg expression during a long-term follow-up will be further detailed.

As shown in Table 2, a relapse after withdrawal, viral breakthrough,

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; SITNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 51(5): 261-268, 2009.

or non-response was detected in 13/21 (61.9%) patients and in 12/16 (75.0%) patients receiving monotherapy with LAM. Sequencing the YMDD motif in non-responders receiving LAM revealed the M204V mutation in six patients, the M204I mutation in three, and a mixed M204V/I mutation in one patient (Table 2). All but one patient (patient 10) were submitted to re-treatment as stated in Methods (Table 3).

Simultaneous analysis of HBV DNA and HBeAg and characterization of decay patterns: The main goal of our study was to describe the response of HBV to treatment by comparing the patterns of HBV DNA and HBeAg decreases. The results from treated and retreated patients were analyzed together. Overall, 36 series of therapy were obtained.

Three different response patterns could be characterized in our patients: Pattern I was characterized by a simultaneous decrease in serum HBV DNA and HBeAg levels until they were absent within a period of six months (Fig. 1A). If the absence of HBV DNA was followed by the

loss of HBeAg in a maximum period of six months, the pattern was still considered as Pattern I.

A concomitant decrease in HBV DNA and HBeAg was observed in 13/36 (36.1%) series as shown in Tables 2 and 3. An example of this pattern is shown in Fig. 1A for patient 15, who had previous resistance to LAM due to the M204V mutation and an excellent response to a combined therapy with Peg-IFN- α 2a (180 μ g per week) and LAM. Pattern I during LAM or interferon plus LAM was detected in 4/16 patients (15%) and 6/10 patients (60%), respectively ($p = 0.10$, two-tailed Fisher exact test).

Pattern II was characterized by the persistence of viremia (HBV DNA levels above 1,000 copies/mL) simultaneous with undetectable HBeAg (Fig. 1B) for a period greater than six months.

This pattern was observed in 2/36 (5.6%) series. In one patient (patient 5), this pattern was detected during 10 months of therapy with

Fig. 1 - Viral kinetics in chronic hepatitis B patients. (A) Patient 15, 44 year old male previously resistant to lamivudine (LAM) with a M204V mutation. He exhibited a simultaneous decrease leading to undetectable HBV DNA and HBeAg levels (Pattern I) and sustained HBeAg/anti-HBe seroconversion after the administration of combined therapy (Peg-IFN- α 2a + LAM). (B) Patient 5, 48 year old male exhibiting a rapid decrease in HBeAg levels but persistent HBV DNA (Pattern II) followed by a breakthrough during therapy. (C) Patient 19, 32 year old female who was treatment-naïve and exhibited a rapid decrease in viremia, a slow decrease in HBeAg (still positive at 24 months), and eventual HBeAg/anti-HBe seroconversion at month 26 (Pattern III-A). (D) Patient 11, 34 year old male with persistent levels of HBeAg despite the loss of HBV DNA shown by PCR. He exhibited a breakthrough during LAM administration (Pattern III-B can be observed in both series of therapy). LD (limit of detection) for HBV DNA (*) and HBeAg (**). # "Decrescendo-crescendo" ("falling-rising") pattern of HBeAg levels*, ** = see Fig. 1A. Hatched bars represent serum HBV DNA levels above LD and open bars represent HBV DNA levels below LD. Open circles represent HBeAg+, filled circles represent HBeAg-. HBV, Hepatitis B virus; HBeAg, Hepatitis e antigen.

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; SITNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 51(5): 261-268, 2009.

LAM, but HBeAg became positive thereafter (Fig. 1B). In another patient (patient 6), a loss of HBeAg was observed during therapy with LAM and persisted to be negative throughout despite the high levels of HBV DNA over three years. However, a clearance of serum HBV DNA was detected three months after the administration of tenofovir.

Pattern III was characterized by the persistence of detectable HBeAg despite the absence of HBV DNA for a period greater than six months. In some of these patients, a slow but steady decline of HBeAg was observed and was referred to as Pattern III-A (Fig. 1C).

In other patients, HBeAg decreased followed by an increase in serum levels, or it did not change at all, despite the absence of HBV DNA and was referred to as Pattern III-B (Fig. 1D).

The absence of HBV DNA preceding the loss of HBeAg for more than six months was observed in 11/36 therapeutic series (30.6%).

Pattern III-A was observed in seven patients. Prolonged periods of HBeAg positivity with continuously decreasing levels indicate a good, but slow, viral response (Fig. 1C). One patient (No. 19) with chronic hepatitis B, very high viremia (above 4×10^7 copies/mL by the Amplicor Monitor technique), and a high level of HBeAg was submitted to a combined therapy with Peg-IFN- $\alpha 2b$ and LAM. As seen in Fig. 1C, HBV DNA became undetectable at the third month. A slow but steady decrease of HBeAg was observed.

Pattern III-B was observed in the other four patients, all of them presenting a viral relapse (Fig. 1D). As seen in Tables 2 and 3, HBeAg/anti-HBe seroconversion was detected in 5/6 patients who exhibited Pattern III-A, whereas no viral response was seen in the four patients exhibiting Pattern III-B.

In non-responder patients with persistent serum HBV DNA and HBeAg, and in patients presenting with Pattern III-B, the levels of HBeAg varied widely either in a "falling-rising" ("decrecendo-crescendo") pattern or with fluctuating values^{27,31}.

The small number of patients in each group did not allow a statistical comparison among the different patterns.

In one patient, all three patterns of viral response were observed, as seen in Fig. 2.

The ALT levels varied widely independently of the described patterns and were not included in this analysis.

DISCUSSION

Based on the simultaneous quantitative determinations of serum HBV DNA and HBeAg, our results suggest that the HBV kinetics can vary widely, depending on the antiviral drugs used for the treatment of HBeAg-positive patients with chronic hepatitis B. The kinetics of HBV have been investigated, but some problems still need clarification^{22,26}.

Our results suggest that patients with positive HBeAg levels are less responsive to LAM or adefovir than combined therapy with interferon and LAM or tenofovir. Thus, Pattern I, which is characterized by the simultaneous decrease of HBV DNA and of HBeAg in a maximum

Fig. 2 - The appearance of four different patterns in the same patient during different series of therapy. Patient 5, 48 year old male presented initially with persistence of HBV DNA under lamivudine (LAM) treatment despite the temporary clearance of HBeAg (Pattern II). Patterns II, I, III-B, and III-A emerged during LAM, Peg-IFN- $\alpha 2b$ +LAM, adefovir, and tenofovir treatment (arrows), respectively. HBV, Hepatitis B virus; HBeAg, Hepatitis B antigen.

period of six months, is more frequently found during treatment with interferon plus LAM or during tenofovir treatment. This pattern presents a favorable evolution in most cases, though a relapse or breakthrough is occasionally found. Pattern II was detected in very few cases and must be investigated with a larger number of patients. In our patients a breakthrough was found.

Our patients were followed for periods of time varying from 34 months to 133 months. Such long periods allowed us to observe that different patterns of serum HBeAg changes can be present in the same patient, and it allowed us to note how unpredictable the outcomes of patients on and off antiviral therapy are⁷. Despite the role of HBV DNA quantitation, the rapid and dramatic decrease in viremia after the introduction of nucleoside analogues reinforces the necessity to quantify HBeAg levels during the period of HBeAg positivity as an additional marker for monitoring responses. In some patients, a progressive drop in HBeAg levels may be paralleled with an HBV DNA decrease (Pattern I), but in other patients, the decrease of HBeAg is steady and slow, pointing to a late response (Pattern III-A).

A progressive decrease in serum HBeAg has been referred as a "decrecendo" ("falling") pattern and a decrease in HBeAg followed by a return to high levels as a "decrecendo-crescendo" ("falling-rising") pattern^{27,31}. The first pattern is predictive of a viral response, whereas most of the patients who fail to show a continuous decrease in HBeAg levels present with a viral breakthrough or non-response. The characteristics of our study do not allow for a close comparison with the above-mentioned patterns.

Possibly an immune-modulating protein, HBeAg is a nonstructural secreted protein translated from HBV e (precore and core) mRNA. On the other hand, HBcAg, the major protein of the HBV capsid, is translated from HBV pregenomic mRNA³¹. Since HBeAg and HBcAg share a sequence consisting of 149 amino acids, they are collectively called Hepatitis B core-related antigens. The measurement of these antigens would be particularly useful for monitoring the decline of viral

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; SITNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. Trop. S. Paulo*, 51(5): 261-268, 2009.

translation activity. It would also allow for a comparison of the efficacies of potent antiviral drugs when a large proportion of patients have HBV DNA below the detection threshold²¹. ROKUHARA *et al.*²⁸ reported that five out of six patients treated with LAM for six to eight months still had detectable levels of core-related antigen. A similar result was also seen in another study³⁴ after 48 weeks of nucleoside analogue therapy; 65% of the patients who had undetectable serum HBV DNA (< 300 copies/mL) still had detectable core-related antigen.

Persistent and steady levels of HBeAg for a prolonged period of time, despite the absence of HBV DNA (Pattern III-B) may indicate some degree of resistance and herald a breakthrough, as was observed in some of our patients. This observation led us to switch adefovir for tenofovir in one patient, and a sharp decrease of HBeAg was observed (Fig. 2). On the other hand, a progressive decrease in HBeAg points to a favorable response, even when the persistence of HBeAg is as long as 26 months of therapy. As seen in Fig. 1C, such a long-term persistence might lead to a withdrawal of the combined therapy of Peg-IFN and LAM. As could be predicted by the trend of Pattern III-A in these patients, the loss of HBeAg and seroconversion to anti-HBe was a late event during this therapy.

Previous results⁶ had also showed that a non-response to LAM is mainly observed in patients with high viremia, and most of them were positive for HBeAg. This correlation was so significant that a proportion between the probability of non-response and an increase in the logarithm of genome copies per mL could be established. The above-mentioned differences in viral dynamics are not surprising as nucleotide analogues produce a decrease in viral load mainly through a block of viral replication, whereas interferon acts directly through some intracellular antiviral pathways and indirectly through a modulation of the immune system.

Daily LAM doses of 300 mg may lead to an earlier³⁵ and more profound suppression of HBV DNA³⁵. In a previous paper, we showed a rapid decrease in serum HBV DNA in 27 out of 29 patients (93%) whose quantitation was performed after 12 weeks of LAM treatment⁶. This response was demonstrated by three different quantitative methods: endpoint dilution, Amplicor-Monitor (Roche), and branched DNA. The endpoint dilution method using 10-fold serial dilutions was also used by YOTSUYANAGI *et al.*³⁵ Our data suggests that combination therapy with Peg-IFN and LAM usually produces a concomitant decrease of HBeAg and HBV DNA, whereas monotherapy with LAM or adefovir may produce a rapid decrease in HBV DNA, but result in a slow, or lack of, decrease of HBeAg in some patients.

Studies in patients with chronic hepatitis B have reported conflicting results regarding the benefits of combining IFN- α or Peg-IFN and LAM, and the role of such combinations in chronic hepatitis B treatment requires further clarification. According to TER BORG *et al.*³², the HBV DNA decline after 52 weeks of therapy was significantly higher in patients undergoing combined therapy (Peg-IFN plus LAM) compared to those undergoing monotherapy with Peg-IFN.

The most important limitation of this study relates to the small number of patients and therapeutic series. Despite these limitations, our study provides some evidence that the decrease in serum HBV DNA accompanied by a steady decrease in HBeAg (Pattern I), even when it is

slow (Pattern III-A), points to a favorable outcome. As was also recently proposed for hepatitis C patients², the use of customized treatment for each patient may increase the likelihood of a response, and following up on HBV DNA and HBeAg levels helps in choosing the best treatment for each patient as well in predicting treatment response. It must be emphasized that stopping rules based on HBV DNA determination for Peg-IFN treatment could not be established¹⁰. Furthermore, our data suggests that the period of 48 weeks for the discontinuation of Peg-IFN may be too short in some patients.

In conclusion, three different patterns can be observed for HBV DNA and HBeAg levels over time when quantitative PCR and HBeAg (MEIA AxSYM) techniques are used simultaneously. A more detailed study with different therapy schedules and using both methods in a large number of patients with chronic hepatitis B is urgently needed.

RESUMO

A quantificação simultânea do DNA VHB e do AgHBe séricos podem distinguir entre resposta viral lenta e rápida à terapêutica anti-viral em pacientes com hepatite crônica B

Introdução: A quantificação do AgHBe sérico não é habitualmente utilizada para monitorizar a resposta viral ao tratamento da hepatite crônica B. **Métodos:** Neste estudo, 21 pacientes sob tratamento com diferentes terapias foram acompanhados e a resposta viral monitorizada pela quantificação concomitante da carga viral e do AgHBe a fim de investigar o significado e a cinética de ambos os parâmetros. **Resultados:** Distinguíram-se três diferentes padrões de resposta viral. O primeiro caracterizou-se pela redução simultânea do HBV DNA e AgHBe séricos. O segundo padrão caracterizou-se por uma redução do AgHBe porém com detecção persistente do HBV DNA. O terceiro padrão caracterizou-se por HBV DNA indetectável com positividade persistente do AgHBe, sugerindo ausência de resposta (Padrão III-B), exceto quando os níveis de AgHBe mostraram uma queda lenta porém persistente, caracterizando um "respondedor lento" (Padrão III-A). **Conclusões:** O primeiro padrão é compatível com resposta viral. Uma seropositividade prolongada do AgHBe porém com uma redução lenta e persistente (Padrão III-A) é também compatível com resposta viral, sugerindo o prolongamento do tratamento anti-viral.

COMPETING INTERESTS

The authors declare that they have no competing interests.

LCDS conceived of the study, participated in its design and coordination, and drafted the manuscript. MLDN participated in its design and drafted the manuscript. SKON participated in its design and drafted the manuscript. JRRP participated in its design and drafted the manuscript. RS participated in drafting the manuscript. VADS carried out the immunoassays, and FJC participated in its design and drafted manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This study was supported in part by research grants from the Alves de Queirós Family Fund for Research and CNPq.

DA SILVA, L.C.; NOVA, M.L.; ONO-NITA, S.K.; PINHO, J.R.R.; SITNIK, R.; SANTOS, V.A. & CARRILHO, F.J. - Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 51(5): 261-268, 2009.

REFERENCES

- ALBERTI, A. - Can serum HBV-DNA be used as a primary end point to assess efficacy of new treatments for chronic hepatitis B? *Hepatology*, 38: 18-20, 2003.
- BUTI, M.; CASADO, M.A. & ESTEBAN, R. - Evaluating the cost of sustained virologic response in naïve chronic hepatitis C patients treated with a la carte. *Aliment. Pharmacol. Ther.*, 26: 705-716, 2007.
- CHANG, T.T.; GISH, R.G.; DE MAN, R. *et al.* - A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *New Engl. J. Med.*, 354: 1001-1010, 2006.
- CONJEEVARAM, H.S. & LOK, A.S. - Management of chronic hepatitis B. *J. Hepatol.*, 38 (suppl. 1): S90-S103, 2003.
- CORDEN, S.; BALLARD, A.L.; IIAZ, S. *et al.* - HBV DNA levels and transmission of hepatitis B by health care workers. *J. clin. Virol.*, 27: 52-58, 2003.
- DA SILVA, L.C.; DA FONSECA, L.E.; CARRILHO, F.J. *et al.* - Predictive factors for response to lamivudine in chronic hepatitis B. *Rev. Inst. Med. trop. S. Paulo*, 42: 189-196, 2000.
- DA SILVA, L.C.; ONO-NITA, S.K.; PINHO, J.R. & CARRILHO, F.J. - Variable and unpredictable outcome in chronic hepatitis B (CHB) patients during and after therapy with high daily doses of lamivudine (LAM). *Hepatology*, 36: 638A, 2002.
- DA SILVA, L.C.; PINHO, J.R.; SITNIK, R.; DA FONSECA, L.E. & CARRILHO, F.J. - Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *J. Gastroenterol.*, 36: 476-485, 2001.
- DIENSTAG, J.L.; PERRILLO, R.P.; SCHIFF, E.R. *et al.* - A preliminary trial of lamivudine for chronic hepatitis B infection. *New Engl. J. Med.*, 333: 1657-1661, 1995.
- FRIED, M.W.; PRATVISUTH, T.; LAU, G.K. *et al.* - HBeAg and hepatitis B virus DNA as outcome predictors during therapy with peginterferon alpha-2a for HBeAg-positive chronic hepatitis B. *Hepatology*, 47: 428-434, 2008.
- FUNG, S.K. & LOK, A.S. - Treatment of chronic hepatitis B: who to treat, what to use, and for how long? *Clin. Gastroenterol. Hepatol.*, 2: 839-848, 2004.
- HEIJTINK, R.A.; JANSSEN, H.L.; HOP, W.C.; OSTERHAUS, A.D. & SCHALM, S.W. - Interferon-alpha therapy in chronic hepatitis B: early monitoring of hepatitis B e antigen may help to decide whether to stop or to prolong therapy. *J. viral Hepatol.*, 7: 382-386, 2000.
- HEIJTINK, R.A.; KRUINING, J.; HONKOOP, P. *et al.* - Serum HBeAg quantitation during antiviral therapy for chronic hepatitis B. *J. med. Virol.*, 53: 282-287, 1997.
- HO, S.K.; YAM, W.C.; LEUNG, E.T. *et al.* - Rapid quantification of hepatitis B virus DNA by real-time PCR using fluorescent hybridization probes. *J. med. Microbiol.*, 52: 397-402, 2003.
- HONKOOP, P.; DE MAN, R.A.; NIESTERS, H.G. *et al.* - Quantitative hepatitis B virus DNA assessment by the limiting-dilution polymerase chain reaction in chronic hepatitis B patients: evidence of continuing viral suppression with longer duration and higher dose of lamivudine therapy. *J. viral Hepatol.*, 5: 307-312, 1998.
- JARDI, R.; RODRIGUEZ, F.; BUTI, M. *et al.* - Quantitative detection of hepatitis B virus DNA in serum by a new rapid real-time fluorescence PCR assay. *J. viral Hepatol.*, 8: 465-471, 2001.
- KANEKO, S.; FEINSTONE, S.M. & MILLER, R.H. - Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. clin. Microbiol.*, 27: 1930-1933, 1989.
- KANEKO, S.; MILLER, R.H.; DI BISCEGLIE, A.M. *et al.* - Detection of hepatitis B virus DNA in serum by polymerase chain reaction. Application for clinical diagnosis. *Gastroenterology*, 99: 799-804, 1990.
- KEEFE, E.B.; DIETERICH, D.T.; HAN, S.H. *et al.* - A treatment algorithm for the management of chronic hepatitis B virus infection in the United States. *Clin. Gastroenterol. Hepatol.*, 2: 87-106, 2004.
- KEEFE, E.B.; ZEUSZEM, S.; KOFF, R.S. *et al.* - Report of an international workshop: roadmap for management of patients receiving oral therapy for chronic hepatitis B. *Clin. Gastroenterol. Hepatol.*, 5: 890-897, 2007.
- KIMURA, T.; OHNO, N.; TERADA, N. *et al.* - Hepatitis B virus DNA-negative Dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J. Biol. Chem.*, 280: 21713-21719, 2005.
- LEWIN, S.R.; RIBEIRO, R.M.; WALTERS, T. *et al.* - Analysis of hepatitis B viral load decline under potent therapy: complex decay profiles observed. *Hepatology*, 34: 1012-1020, 2001.
- LOK, A.S.; HEATHCOTE, E.J. & HOFFNAGLE, J.H. - Management of hepatitis B: 2000-summary of a workshop. *Gastroenterology*, 120: 1828-1853, 2001.
- LOK, A.S. & McMAHON, B.J. - Chronic hepatitis B: update of recommendations. *Hepatology*, 39: 857-861, 2004.
- LOK, A.S. & McMAHON, B.J. - Chronic hepatitis B. *Hepatology*, 45: 507-539, 2007.
- NEUMANN, A.U. - Hepatitis B viral kinetics: a dynamic puzzle still to be resolved. *Hepatology*, 42: 249-254, 2005.
- PARK, N.H.; SHIN, I.W.; PARK, J.H. *et al.* - Monitoring of HBeAg levels may help to predict the outcomes of lamivudine therapy for HBeAg positive chronic hepatitis B. *J. viral Hepatol.*, 12: 216-221, 2005.
- PERRILLO, R.; MIMMS, L.; SCHECHTMAN, K.; ROBBINS, D. & CAMPBELL, C. - Monitoring of antiviral therapy with quantitative evaluation of HBeAg: a comparison with HBV DNA testing. *Hepatology*, 18: 1306-1312, 1993.
- ROKHARA, A.; TANAKA, E.; MATSUMOTO, A. *et al.* - Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. *J. viral Hepatol.*, 10: 324-330, 2003.
- SHAW, T.; BOWDEN, S. & LOCARNINI, S. - Chemotherapy for hepatitis B: new treatment options necessitate reappraisal of traditional endpoints. *Gastroenterology*, 123: 2135-2140, 2002.
- SHIN, I.W.; PARK, N.H.; JUNG, S.W. *et al.* - Clinical significance of hepatitis B e antigen level measurement during long-term lamivudine therapy in chronic hepatitis B patients with e antigen positive. *Wld J. Gastroenterol.*, 12: 6693-6698, 2006.
- SITNIK, R.; PINHO, J.R.; BETTOLINI, D.A. *et al.* - Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J. clin. Microbiol.*, 42: 2455-2460, 2004.
- TER BORG, M.J.; VAN ZONNEVELD, M.; ZEUSZEM, S. *et al.* - Patterns of viral decline during PEG-interferon alpha-2b therapy in HBeAg-positive chronic hepatitis B: relation to treatment response. *Hepatology*, 44: 721-727, 2006.
- WONG, D.K.; TANAKA, Y.; LAI, C.L. *et al.* - Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J. clin. Microbiol.*, 45: 3942-3947, 2007.
- YOTSUYANAGI, H.; YASUDA, K.; IINO, S. *et al.* - Persistent viremia after recovery from self-limited acute hepatitis B. *Hepatology*, 27: 1377-1382, 1998.

Received: 23 March 2009

Accepted: 28 September 2009

- 9- Mendes-Correa MC, Pinho JR, Gomes-Gouvea MS, da Silva AC, Guastini CF, Martins LG, Leite AG, Silva MH, Gianini RJ, Uip DE. Predictors of HBeAg status and hepatitis B viraemia in HIV-infected patients with chronic hepatitis B in the HAART era in Brazil. BMC Infectious Diseases 2011 Sep 20; 11: 247.**

RESEARCH ARTICLE

Open Access

Predictors of HBeAg status and hepatitis B viraemia in HIV-infected patients with chronic hepatitis B in the HAART era in Brazil

Maria Cassia Mendes-Correa^{1,2*}, João RR Pinho³, Michele S Gomes-Gouveia³, Adriana C da Silva², Cristina F Guastini², Luiz G Martins⁴, Andréa G Leite^{1,2}, Mariliza H Silva⁴, Reinaldo J Gianini⁵ and David E Uip¹

Abstract

Background: HBV-HIV co-infection is associated with an increased liver-related morbidity and mortality. However, little is known about the natural history of chronic hepatitis B in HIV-infected individuals under highly active antiretroviral therapy (HAART) receiving at least one of the two drugs that also affect HBV (TDF and LAM). Information about HBeAg status and HBV viremia in HIV/HBV co-infected patients is scarce. The objective of this study was to search for clinical and virological variables associated with HBeAg status and HBV viremia in patients of an HIV/HBV co-infected cohort.

Methods: A retrospective cross-sectional study was performed, of HBsAg-positive HIV-infected patients in treatment between 1994 and 2007 in two AIDS outpatient clinics located in the São Paulo metropolitan area, Brazil. The baseline data were age, sex, CD4 T+ cell count, ALT level, HIV and HBV viral load, HBV genotype, and duration of antiretroviral use. The variables associated to HBeAg status and HBV viremia were assessed using logistic regression.

Results: A total of 86 HBsAg patients were included in the study. Of these, 48 (56%) were using combination therapy that included lamivudine (LAM) and tenofovir (TDF), 31 (36%) were using LAM monotherapy, and 7 patients had no previous use of either one. Duration of use of TDF and LAM varied from 4 to 21 and 7 to 144 months, respectively. A total of 42 (48.9%) patients were HBeAg positive and 44 (51.1%) were HBeAg negative. The multivariate analysis revealed that the use of TDF for longer than 12 months was associated with undetectable HBV DNA viral load (serum HBV DNA level < 60 IU/ml) ($p = 0.047$). HBeAg positivity was associated with HBV DNA > 60 IU/ml ($p = 0.001$) and ALT levels above normality ($p = 0.038$).

Conclusion: Prolonged use of TDF containing HAART is associated with undetectable HBV DNA viral load. HBeAg positivity is associated with HBV viremia and increased ALT levels.

Keywords: hepatitis B virus, HIV, virological outcome, tenofovir

Background

Hepatitis B virus (HBV) and human immunodeficiency virus (HIV) co-infection is a frequent event because both viruses share the same routes of transmission. Studies conducted in North America, Europe, and Australia have shown that between 5% and 10% of all HIV-infected patients are also infected with HBV [1]. In Brazil, this prevalence ranges from 1.6% to 8.5% [2-8].

HIV infection has a negative impact in all phases of the natural history of hepatitis B, leading to increased rates of persistent infection, higher HBV DNA levels, and lower rates of hepatitis B and antigen loss [9-11]. Progression of fibrosis, development of cirrhosis, and liver-related mortality are more frequent among HIV-HBV co-infected patients than among individuals infected with either one alone [9-11].

However, it is still unknown what kind of impact long-term use of the highly active antiretroviral therapy (HAART), which includes lamivudine (LAM) and

* Correspondence: cassiamc@uol.com.br

¹Infectious Diseases Research Unit, ABC Medical School, Santo André, Brazil
Full list of author information is available at the end of the article

© 2011 Mendes-Correa et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

tenofovir (TDF) (two drugs that are also active against HBV), may have on the natural history of chronic hepatitis B in co-infected individuals. The impact of the use of antiretroviral therapy in HBeAg status and HBV viremia has rarely been investigated among HIV-infected patients receiving highly active antiretroviral therapy.

Therefore, the aim of the present study was to evaluate clinical and virological variables that may influence HBeAg status and HBV viraemia in a HIV/HBV co-infected cohort followed up in São Paulo Metropolitan area, Brazil, that were under antiretroviral therapy which included anti-HBV drugs, such as lamivudine (LAM) and tenofovir (TDF).

Methods

Study Design

A retrospective cross-sectional study was conducted in two AIDS outpatient clinics located in the São Paulo metropolitan area, Brazil. HIV-HBV co-infected patients were included in the study.

Patients

This retrospective study evaluated the medical records of all HIV patients under treatment between November 1994 and November 2007 in an attempt to identify patients who were also infected with HBsAg. Clinical, demographic, and serological data contained in the medical records of HIV patients who had anti-HIV antibodies confirmed by Western blot and positive HBsAg serum for longer than 6 months were analyzed.

The data collected at the time a serum sample was drawn were: age, sex, CD4 T-cell count, use of antiretroviral therapy, plasma HIV-RNA level, TDF and LAM therapy and time of use of these drugs, HBeAg status, HBV viral load, HBV genotype, presence of hepatitis C infection and hepatitis D super-infection, presence of advanced liver disease, alanine aminotransferase (ALT) level, and compliance with therapy.

Antiretroviral therapy (including TDF and LAM) usage was defined as the use of any antiretroviral medication for longer than 6 months at any time prior to the time a serum sample was drawn. Compliance to therapy was assessed by the assistant physician. Patients were considered compliant when their medical records indicated that they had used at least 80% of the medication prescribed by their doctor.

The Local Ethics Research Boards approved this study. These were the "Comissão de Bioética-CoBi, do HCFMUSP" (Ethics Commission-Hospital das Clínicas, São Paulo University Medical School) and "Comissão de Ética da FMABC" (Ethics Commission-ABC Foundation Medical School). All patients who participated in the study signed an informed consent form.

Laboratory Testing

Hepatitis B serological testing was performed according to standard procedures and using licensed assays, including HBsAg, total anti-HBc, anti-HBs, HBeAg, and anti-HBe.

Absolute quantification of HBV DNA was carried out by an in-house real-time polymerase chain reaction (PCR) [12] assay using primers capable of amplifying HBV genotypes A to H (28 samples), or by the commercially available COBAS Amplicor HBV Monitor Test assay (Roche Molecular Systems, Pleasanton, CA, USA) according to the manufacturer's instructions (58 samples), as previously described [13,14].

HBV-positive DNA was further amplified and followed by sequencing of the 737 bp PCR fragment covering amino acids 48 to 288 to determine genotypes and antiviral resistance. We used primers FHBS1, FHBS2, RADE1M, and RADE2M described in previous studies to amplify a larger informative region of the Polymerase gene [15,16].

Hepatitis C virus infection was defined by the presence of anti-HCV antibodies (ELISA, 3rd generation). Current HCV infection was confirmed by PCR.

Hepatitis Delta virus (HDV) co-infection was defined by the presence of positive anti-HDV antibodies by ELISA. Current HDV infection was confirmed by PCR, and HDV genotype was determined by sequencing and phylogenetic analysis, as previously reported [16].

Alanine transaminase (ALT) level was examined at baseline and on at least two separate occasions during the 12 months before baseline. A normal ALT level was considered when at least three consecutive normal ALT results were obtained during a follow-up of 12 months.

Statistical Analysis

Statistical analyses were carried out using Stata software (Stata Corp., College Station, TX, USA). We initially conducted an analysis of frequency distribution for each selected variable. Continuous parameters were compared between groups using Student's t-test, except for continuous variables without normal distributions or those for small groups, in which case the parameters were compared using the Mann-Whitney U-test. For categorical variables, the Pearson χ^2 or Fisher's exact test were used according to group size. Differences were considered statistically significant when $p < 0.05$. Multivariate logistic regression analysis was performed to examine the association of several variables and HBeAg status and HBV viremia.

Results

A total of 3,259 patients from the two AIDS outpatient clinics with confirmed HIV infection and serological markers for hepatitis B were included. Among them,

154 (4.7%) were HBsAg-positive. Of these 154, only 86 for whom blood samples were available were included in the study.

The main characteristics of the study population are shown in Table 1. Most of the patients were males (96.5%) and the mean age was 42 years. Patients were analyzed according to their risk factors for hepatitis B infection. Sixty-four (74.4%) were men who had sex with men and 16 (18.6%) were intravenous drug users. Seventeen patients (19.7%) presented clinical evidence of advanced liver disease (splenomegaly, ascites, and esophageal varices). Nine patients (10.4%) were not compliant to the therapy.

Among the 86 patients, 81 (94.1%) declared previous use of antiretroviral therapy. For the two drugs that also

work against HBV (LAM and TDF), 79 (91.8%) declared previous use of the former and 48 (55.8%) of the latter. At the baseline, 31 (36%) and 48 (56%) of them were under LAM monotherapy or combination therapy of both drugs, respectively. All patients under TDF monotherapy were changed to combination therapy with LAM. Time of exposure to TDF and LAM varied from 4 to 21 and 7 to 144 months, respectively.

Among the 34 patients with detectable HBV viremia, 5 had never received any anti-retroviral treatment, 29 had used LAM, and 13 had also used TDF (LAM + TDF). Sixteen patients with detectable HBV viremia were on LAM monotherapy and among them, eight patients presented LAM-resistant mutations. Thirteen patients under LAM + TDF presented detectable HBV viremia and among them, LAM-resistant mutations were detected in five patients. Among the 34 patients with HBV detectable viremia, no TDF-resistant mutations were observed.

Forty-two patients (48.9%) were HBeAg positive and 44 (51.1%) were HBeAg negative. Baseline plasma HBV viral load was above 60 UI/mL among 34 (39.5%) patients and below 60 UI/mL among 50 (58.1%) out of 86 patients.

Among the 34 patients with detectable HBV DNA, HBV genotype distribution was: A-21 (61.8%); D-5 (14.7%); G-5 (14.7%); F-3 (8.8%). Genotypes B, C, and E were not detected. Electropherogram analysis did not show any evidence for the presence of mixed infections with different genotypes.

Presence of anti-HCV was observed in 20 out of 86 patients (23.2%) and HCV-RNA was confirmed in 18 patients.

Only one (1.1%) anti-HDV positive patient was identified. Current HDV infection was confirmed by PCR and the genotype of this case was determined by phylogenetic analysis of the sequence as HDV-1.

The mean CD4 T-cell count of the 86 patients was 545 ($\pm$ 250) cells/ μ l. Eighty-five percent of patients (73/86) had baseline HIV RNA < 400 copies/mL. A total of 37% of patients (32/86) had elevated ALT values. ALT levels were on average twice the upper normal limit.

In univariate analysis, presence of HBeAg was associated with HBV-DNA viral load > 60 IU/mL (p < 0.001) and ALT level > 1.5 times the upper normal limit (p = 0.002) (Table 2). After multivariate analysis, presence of HBeAg remained associated with the same variables: HBV-DNA > 60 UI/mL (OR 5.442891; 95% CI 1.92026-15.42763; p = 0.001); and ALT > 1.5 $\times$ ULN (OR 3.970025; 95% CI 1.077474-14.62782; p = 0.038).

For HBV viral load, the univariate analysis showed that HBV-DNA < 60 IU/mL was associated to previous exposure to either LAM or TDF (p = 0.005), exposure to TDF (p = 0.005), exposure to TDF for longer than 12 months

Table 1 Main baseline characteristics of 86 HIV/AgHBs co-infected patients

Variables	n
Male gender (%)	83 (96.5%)
Mean age (years)	42 ($\pm$ 7.2)
Mode of HIV transmission	
- Men who have unprotected sex with men (%)	64 (74.4%)
- Exchange of needles among intravenous drug users (%)	16 (18.6%)
Clinical Variables	
- Advanced hepatic disease	17 (19.7%)
- Non adherence to treatment	09 (10.4%)
Treatment Given-HAART	
- Previous use of antiretroviral therapy	81 (94.1%)
- Patients under LAM + TDF combination therapy	48 (56.0%)
- Patients under LAM monotherapy	31 (36.0%)
- Previous use of either LAM or TDF	81 (94.1%)
- Previous use of LAM	79 (91.8%)
- Previous use of TDF	48 (55.8%)
- Time of use of TDF (months)	4-21
- Time of use of LAM (months)	7-144
Laboratory Variables	
HBeAg positive	42 (48.9%)
HBV-DNA < 60 UI/mL	50 (58.1%)
HBV-DNA > 60 UI/mL	34 (39.5%)
HBV-DNA unknown	02 (2.32%)
HBV Genotype	
- genotype A	21
- genotype D	05
- genotype G	05
- genotype F	03
- CD4 cell count, median cells/ μ l	545 ($\pm$ 250)
- Baseline HIV RNA < 400 copies/mL	73 (85%)
ALT level, normal	54 (62.7%)
- ALT level > 1.5 $\times$ ULN†	32 (37.2%)
- Anti-HCV positive	20 (23.2%)
- Anti-HDV positive	01 (1.1%)

ULN†-Upper Limit of Normality; LAM = lamivudine; TDF = tenofovir

Table 2 Predictors of HBeAg status among patients with HIV-HBV co-infection: Univariate analyses

	HBeAg Non-reagent	HBeAg Reagent	OR	95%CI	p
Sex					0.59
female	2 (66.67%)	1 (33.33%)	1.00		
male	42(50.60%)	41 (49.40%)	1.95	0.17-22.37	
Age					0.16
<= 42 years	18 (43.99%)	23 (56.19%)	1.00		
> 42 years	26 (59.09%)	18 (40.91%)	0.54	0.22-1.28	
CD4					0.54
< 350 cels/ μ l	12 (46.15%)	14 (53.85%)	1.00		
> 350 cels/ μ l	32 (53.33%)	28 (46.67%)	0.75	0.29-1.88	
Use of ARV					0.18
no	1(20%)	4 (80%)	1.00		
yes	43 (53.09%)	38 (46.91%)	0.22	0.02-2.06	
HBV Genotype					0.68
A	5 (23.89%)	16 (78.19%)	0.58	0.1-3.2	
Non-A	2 (19.39%)	11 (84.6%)	1.00		
Previous use of LAM					0.64
no	3 (42.89%)	4 (57.19%)	1.00		
yes	41(51.99%)	38 (48.10%)	0.69	0.14-3.31	
Previous use of TDF					0.26
no	22 (57.89%)	16 (42.11%)	1.00		
yes	22 (45.89%)	26 (54.19%)	1.62	0.68-3.83	
Time of TDF use > 12 months					0.73
no	6 (50%)	6 (50%)	1.00		
yes	16 (44.49%)	20 (55.59%)	1.25	0.33-4.62	
HBV-DNA					< 0.001
< 60 IU/mL	34(40.99%)	15(18.9%)	1.00		
> 60 IU/mL	08(9.69%)	26(31.39%)	21.52	4.58-100.98	
ALT Level					0.002
Normal	39(46.99%)	24(28.99%)	1.00		
> 1.5 $\times$ ULN	4(4.89%)	16(19.2%)	6.66	1.99-22.28	
Compliance to therapy					0.26
yes	41 (53.25%)	36 (46.75%)	1.00		
no	3 (33.33%)	6 (66.66%)	2.27	0.53-9.77	

ULN-Upper Limit of Normality

($p = 0.047$), normal ALT levels ($p = 0.004$), and compliance to therapy ($p = 0.033$) (Table 3). After multivariate analysis, HBV-DNA < 60 IU/ml was only associated to use of TDF for longer than 12 months ($p = 0.047$) (OR 0.24; 95% CI 0.059-0.979463; $p = 0.047$).

Discussion

Our cross-sectional study evaluated the factors associated to HBeAg status and hepatitis B viremia in HIV-HBV co-infected patients in São Paulo, Brazil. Among 86 HIV/HBV co-infected patients with prior use of either LAM or TDF (or both), we showed that individuals receiving combination therapy with prolonged use of TDF were significantly more likely to have

undetectable HBV DNA. We also found that presence of HBeAg was associated with detectable HBV viremia (HBV DNA > 60 IU/ml) and increased ALT levels. On the other hand, prolonged TDF use was not associated to HBeAg seroconversion.

In HBV mono-infected patients, successful HBV therapy can reduce the risk of developing complications related to liver disease [17,18]. Recent recommendations advocated undetectable HBV DNA as a treatment goal in HBV mono-infected patients and co-infected patients [19-23]. Reaching undetectable HBV DNA also minimizes the risk for developing drug-resistant HBV, which is one of the most undesirable complications of hepatitis B treatment [19].

Table 3 Predictors of HBV-DNA viremia among patients with HIV-HBV co-infection: Univariate analyses

	Plasma HBV DNA < 60 UI/mL	Plasma HBV DNA > 60 UI/mL	OR	95% CI	p
Sex					0.79
female	2 (6.6, 67%)	1 (3.3, 33%)	1.00		
male	48 (59.26%)	33 (40.74%)	1.37	0.11-15.79	
Age					0.41
< = 42	22 (55%)	18 (45%)	1.00		
> 42	27 (62.79%)	16 (37.21%)	0.72	0.30-1.74	
CD4					0.47
< 350 cels/ μ l	14 (53.85%)	12 (46.15%)	1.00		
> 350 cels/ μ l	36 (62.07%)	22 (37.93%)	0.71	0.27-1.81	
Under HAART*					0.005
yes	50 (63.29%)	29 (36.71%)			
no	2 (0.3%)	0 (0.0%)			
HBV Genotype					
A	4 (20%)	16 (80%)	0.36	0-2.9	0.63
Non-A	1 (8.3%)	11 (91.6%)	1.00		
Previous exposure to LAM					0.10
no	2 (8.57%)	5 (71.43%)	1.00		
yes	48 (62.34%)	29 (37.66%)	0.24	0.04-1.32	
Under TDF					0.005
no	15 (17.8%)	21 (25.16%)	1.00		
yes	35 (41.6%)	13 (15.4%)	0.26	0.10-0.66	
Use of TDF > 12 months					0.047
no	21 (25%)	27 (32.1%)	1.00		
yes	29 (34.5%)	7 (8.2%)	0.24	0.059-0.97	
ALT level					0.004
Normal	44 (53.6%)	19 (23.1%)	1.00		
> 1.5 ULN**	6 (7.3%)	13 (15.7%)	5.01	1.65-15.17	
Compliance with therapy					0.033
no	0 (0.0%)	0 (0.0%)	10.5	1.20-91.71	
yes	49 (63.64%)	28 (36.36%)	1.00		

Under HAART*: Previous use of either lamivudine (LAM) or tenofovir (TDF)

ULN**: Upper Limit of Normality

The Brazilian AIDS treatment program provides free access to highly active antiretroviral therapy for all persons living with HIV/AIDS who seek treatment. The fact that LAM has been used at large in the country as anti-HIV therapy since the mid-1990s means that a great number of HIV patients in Brazil who are also infected with HBV, have received this drug for many years. This situation provides an opportunity to investigate the effects that this drug, and also TDF, have on HBV when used for long periods by HIV-HBV co-infected patients.

Most patients included in our study had previously used antiretroviral therapy, which included LAM (91.8%). All patients receiving TDF had previous use of LAM. Among our group of patients, time of exposure to TDF and LAM varied from 4 to 21 and 7 to 144 months, respectively. Nevertheless, only the prolonged use of TDF (more than 12 months) was associated with undetectable HBV viral load.

In patients already using LAM, the use of TDF can eventually lead to a slower decrease in HBV viremia [24-27]. This probably explains why we only observed association between undetectable HBV viremia and prolonged TDF use. Among our patients, the use of TDF was typically associated with undetectable or low HBV-DNA level. Our results are in accordance with previous studies that addressed the same question [27-29].

Current guidelines recommend the use of TDF and emtricitabine (FTC), or TDF and LAM, as the nucleos(t)ide reverse transcriptase inhibitor (NRTI) backbone of antiretroviral regimens in HIV/HBV co-infected individuals. The benefit of a combination therapy in which TDF is included over its use as a monotherapy for HBV has not been definitively demonstrated in any patient population, including HBV/HIV co-infected patients. More studies are needed to determine whether TDF maintains its efficacy for longer periods.

We were able to determine the HBV genotypes in 34 of the patients with detectable HBV viremia. Among them, the HBV genotype distribution was as follows: A-21 (61.8%); D-5 (14.7%); G-5 (14.7%); F-3 (8.8%). Genotypes B, C, and E were not detected. Genotypes B and C are typical from Asian descendants living in Brazil although we do not have any patient with this ancestry in our study, while genotype E has only been found in Brazil in African immigrants [30].

Our findings suggest that the risk for virological non-response is not associated to HBV genotype, as previously suggested by other studies [31]. This may be explained by the fact that data on HBV genotype distribution were not available before patients started the therapy. Therefore the genotype distribution reflects that among patients under the use of LAM or TDF and presenting detectable viremia, which may not reflect the genotype distribution of all patients included in our study.

In our population, five cases of genotype G were identified. This genotype is uncommon in Brazil.

The presence of genotype G has been associated by some authors with a sexual transmission among males [32]. Indeed, the five patients in our study carrying this genotype were homosexual males. Additional studies with larger sample populations that analyze HBV genotypes in individuals with different sexual behaviors will be enlightening.

Among the 86 HBeAg-positive patients, 42 (48.8%) were HBeAg positive and 44 (51.1%) were HBeA negative. These results agree with other studies also describing a predominance of cases of patients with HBeAg positivity among HIV-HBV co-infected patients [29,31,33,34]. Because only a few genotypes were not A genotypes we were unable to analyze the distribution of HBeAg seropositivity in the sera of individuals carrying different HBV genotypes.

Our data showed an association between HBeAg positivity and HBV-DNA viremia. Several studies have already addressed this question in HIV-HBV co-infected patients. Our study confirms this data [34,35].

In HBeAg-positive chronic hepatitis B, HBeAg and HBV DNA levels are supposed to follow parallel changes in their levels as they both indicate viral replication. It is noteworthy that despite the fact that in our study prolonged use of TDF was associated with undetectable HBV DNA, HBeAg was still detectable in most patients. This finding reflects the initial effects of TDF in the replicating viral DNA. Its efficacy in decreasing HBeAg and even HBsAg levels takes longer, as it reflects a decrease in the translation of viral mRNAs that depends on decreasing levels of the viral DNA in the hepatocyte nuclei, especially the cccDNA.

Among mono-infected HBeAg-positive patients, HBe seroconversion rates are of the order of 20%, after one year of TDF use. Among co-infected patients on TDF use, higher rates of seroconversion have been shown in some cohorts (33%-36%), but only in antiretroviral-naïve individuals and not when TDF was introduced as a second-line agent. This might be a consequence of immune reconstitution and acquisition of either enhanced pathogen-specific innate or adaptive immune responses to HAART [36-39].

Most of our patients had previous use of TDF as a second-line therapy. Therefore it is reasonable to suppose that for this group of patients a longer follow-up would be necessary in order to observe HBeAg seroconversion. We have not applied in this study quantitative assays for HBe or HBsAg that would allow us to follow their levels during the treatment.

In our study, HBeAg positivity was also associated with ALT levels 1.5 times the upper limit of normality. In patients with HIV-HBV co-infection, little is known about the association between HBeAg positivity and ALT levels. Previous studies, mostly in the pre-HAART era, have suggested that liver damage (assessed by ALT levels) is less severe in persons with HIV infection and active HBV replication [40,41]. Nevertheless, the natural histories of HIV and HBV infections have been touched by the advent of HAART.

These multidrug regimens changed the course of liver disease in co-infected patients. The primary mechanism of HBV-associated liver damage is cell-mediated immune response. In the present study, most patients were on HAART, which most likely resulted in at least partial immunological reconstitution. At baseline, the mean CD4+ T-cell count of the patients was 545 cells/mm³. Most of the patients (91.8%) had previous experience with LAM and resistance mutations were frequently found among patients. We wonder whether the rise in ALT levels is linked to the partially reconstituted immune status of these patients.

ALT is considered a marker of hepatic necrosis and inflammation. As stated above, our study had a cross-sectional design, precluding analysis of serially tested specimens and precluding determination of the evolution of ALT variations over time. It would also be reasonable to suppose that for this group of patients a longer follow-up would be necessary in order to observe ALT normalization, assuming that patients would be adherent to treatment. Rises in ALT in patients with HIV have multiple etiologies, including antiretroviral therapy, alcohol and other drug use, as well as opportunistic infections. No particular cause has been identified in the present study although none of these etiologies can be ruled out either.

It is noteworthy that the presence of anti-HCV was also observed in 20 out of 86 patients and HCV-RNA was confirmed in 18 patients. HCV co-infection may also have contributed to elevation in ALT observed in this group of patients.

Furthermore, hepatitis delta virus (HDV) was found in only one out of 86 patients analyzed. The prevalence of anti-delta antibodies in HIV patients with detectable HBsAg ranges from 15% to 50%, depending on geographical region and risk group category [42]. In Brazil, HDV is endemic in the northern part of the country (Amazon Basin) and is only exceptionally found outside this area [43]. This fact probably explains why only one patient with hepatitis delta was identified. It is interesting to note that the genotype of this case was determined by phylogenetic analysis of the sequence as HDV-1.

This study has some limitations. First, it has a cross-sectional design, precluding analysis of serially tested specimens and precluding determination of the evolution of serological markers (in particular to follow the evolution of HBsAg status) or HBV viral load before antiviral therapy usage. HAART status and HBV characteristics were assessed at a single time point (at baseline) and data associated to HBV and HIV prior to HAART initiation were not uniformly acquired. Thus, we cannot rule out that the associations we observed were already present before these drugs were introduced. Second, compliance with therapy was not confirmed. Finally, we believe that follow-up of this cohort will be needed to determine if the associations observed in our study regarding HBsAg status and HBV viremia will remain over time and to determine factors that might be associated with the development of TDF resistance.

Conclusions

In summary, prolonged use of TDF was able to control HBV replication in most HIV-HBV co-infected patients. The prescription of HAART containing anti-HBV drugs, particularly TDF, had a positive impact on the virological outcome of hepatitis B in HIV-HBV co-infected patients, regardless of previous LAM treatment.

Non-financial Competing interests

The authors declare that there are no non-financial competing interests (political, personal, religious, ideological, academic, intellectual, commercial, or any other) to declare in relation to this manuscript.

Acknowledgements

This work was supported in part by grants from FAPESP (Fundação para o Amparo à Pesquisa do Estado de São Paulo) 2006/54332-0 and 2008/57146-9.

We want to particularly acknowledge the patients in this study for their participation.

This study was presented as a poster at the 5th International Workshop on HIV and Hepatitis Co-Infection, 2009, Lisbon. *Reviews in Antiviral Therapy*, 2009, **4**, p. 50-50.

Author details

¹Infectious Diseases Research Unit, ABC Medical School, Santo André, Brazil.

²Department of Infectious Diseases Medical School, University of São Paulo, São Paulo, Brazil.

³Tropical Medicine Institute and Department of Gastroenterology, Medical School, University of São Paulo, São Paulo, Brazil.

⁴AIDS Outpatient Clinic, São Bernardo do Campo, São Paulo, Brazil.

⁵Laboratory of Medical Investigation in Epidemiology and Statistics, Medical School, University of São Paulo, São Paulo, Brazil.

Authors' contributions

MCMC and DEU designed the study. ACS, JRRP, and MSGG did most of the laboratory work. CFG, LGM, AGL, and MHS provided and checked the clinical data for patients. MCMC, CFG, LGM, AGL, and MHS also participated in data collection.

MCMC and JRRP wrote the manuscript. RJG participated in the design of the study and performed the statistical analysis. All authors reviewed the draft and approved the final version.

Competing interests

The authors declare that they have no competing interests.

Financial Competing interests

In the past five years the authors did not receive any reimbursements, fees, funding, or salary from any organization that may in any way gain or lose financially from the publication of this manuscript, either now or in the future. The authors also declare that there is no organization financing this manuscript (including the article-processing charge). The authors declare that do not hold any stocks or shares in an organization that may in any way gain or lose financially from the publication of this manuscript, either now or in the future. The authors declare that they are not currently applying for any patents relating to the content of the manuscript.

Received: 22 February 2011 Accepted: 20 September 2011

Published: 20 September 2011

References

1. Thio CL: Hepatitis B and human immunodeficiency virus coinfection. *Hepatology* 2009, **49**(5 Suppl):S138-145.
2. Mendes-Correa MC, Barone AA, Cavaleiro N, Tengan FM, Gastini C: Prevalence of hepatitis B and C in the sera of patients with HIV infection in São Paulo, Brazil. *Revista Instituto Medicina Tropical São Paulo* 2000, **42**:81-85.
3. Monteiro MR, do Nascimento MM, Passos AD, Figueiredo JF: Seroepidemiological survey of hepatitis B virus among HIV/AIDS patients in Belém do Pará-Brazil. *Revista Sociedade Brasileira Medicina Tropical* 2004, **37**:27-32.
4. Souza MG, Passos AD, Machado AA, Figueiredo JF, Esmeraldino LE: HIV and hepatitis B virus co-infection: prevalence and risk factors. *Revista Sociedade Brasileira Medicina Tropical* 2004, **37**:391-395.
5. Tovo CV, Dos Santos DE, de Mattos AZ, de Almeida PR, de Mattos AA, Santos BR: Ambulatory prevalence of hepatitis B and C markers in patients with human immunodeficiency virus infection in a general hospital. *Arquivos de Gastroenterologia* 2006, **43**:73-76.
6. Zago AM, Machado TF, Casarim FL, Miranda AE: Prevalence and risk factors for chronic hepatitis B in HIV patients attended at a sexually-transmitted disease clinic in Vitória, Brazil. *Brazilian Journal of Infectious Diseases* 2007, **11**:475-478.
7. Oliveira LH, Silva R, Xavier BL, Cavalcanti SM: Hepatitis B infection among patients attending a sexually transmitted diseases clinic in Rio de Janeiro, Brazil. *Memórias Instituto Oswaldo Cruz* 2001, **96**(5):635-640.
8. Portelinha Filho AM, do Nascimento CJ, Tannousi TN, Ascencio EL, Bonfim R, Dandrea LA, Prestes-Carneiro LE: Sero-prevalence of HBV, HCV and HIV co-infection in selected individuals from state of São Paulo, Brazil. *Memórias Instituto Oswaldo Cruz* 2009, **104**:960-963.
9. Puoti M, Torti C, Bruno R, Filice G, Carosi G: Natural history of chronic hepatitis B in coinfected patients. *Journal of Hepatology* 2006, **44**:S65-S70.

10. Konopnicki D, Mocroft A, de Wit S, Antunes F, Ledergerber B, Katlama C, Zilmer K, Vella S, Kirk Q, Lundgren JD: Hepatitis B and HIV: prevalence, AIDS progression, response to highly active antiretroviral therapy and increased mortality in the EuroSIDA cohort. *AIDS* 2005, 19:593-601.
11. Thio CL, Seiberg EC, Skolasky RJ Jr, Phair J, Visscher B, Muñoz A, Thomas DL: HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicentre Cohort Study (MACS). *Lancet* 2002, 360:1921-1926.
12. Strik R, Paes A, Manguera CP, Pinho JR: A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Revista do Instituto de Medicina Tropical de São Paulo* 2010, 52(3):119-24.
13. Mendes-Correa MC, Pinho JR, Locamini S, Yuen L, Strik R, Santana RA, Gomes-Gouveia MS, Leite OM, Martins LG, Silva MH, Ganini RJ, Uip DE: High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. *Journal of Medical Virology* 2010, 82(9):1481-1488.
14. Silva AC, Spina AM, Lemos MF, Oba IT, Gustinini Cde F, Gomes-Gouveia MS, Pinho JR, Mendes-Correa MC: Hepatitis B genotype G and high frequency of lamivudine-resistance mutations among human immunodeficiency virus/hepatitis B virus co-infected patients in Brazil. *Memorias do Instituto Oswaldo Cruz* 2010, 105(6):770-778.
15. Strik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Camilo FJ: Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *Journal of Clinical Microbiology* 2004, 42(6):2455-2460.
16. Gomes-Gouveia MS, Soares MC, Bensabath G, de Carvalho-Mello IM, Brito EM, Souza OS, Queiroz AT, Camilo FJ, Pinho JR: Hepatitis B and Delta virus genotypes in Outbreaks of fulminant Hepatitis (Labrea black fever) in the Western Brazilian Amazon region. *Journal of General Virology* 2009, 90(11):2638-2643.
17. Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ: Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006, 130:678-686.
18. Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH: Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *Journal of American Medical Association* 2006, 295:65-73.
19. European Association for the Study of the Liver: EASL clinical practice guidelines: management of chronic hepatitis B. *Journal of Hepatology* 2009, 50:227-242.
20. Lok AS, McMahon BJ: Chronic hepatitis B: update 2009. *Hepatology* 2009, 50(3):661-662.
21. Iser DM, Lewin SR: Future directions in the treatment of HIV-HBV co-infection. *HIV Therapy* 2009, 3(4):405-415.
22. Mendes-Correa MC, Núñez M: Management of HIV and hepatitis virus co-infection. *Expert Opinion on Pharmacotherapy* 2010, 11(15):2497-2516.
23. Lacombe K, Boyd A, Goulet J, Lavocat F, Girard PM, Zoulim F: Drug-resistant and immune-escape HBV mutants in HIV-infected hosts. *Antiviral Therapy* 2010, 15:493-497.
24. Lada O, Gervais A, Branger M: De novo combination therapy of tenofovir disoproxil fumarate (TDF) plus lamivudine (LAM) or TDF plus emtricitabine (FTC) is associated with early virologic response in HIV/HBV co-infected patients. *59th Annual Meeting of the American Association for the Study of Liver Diseases* San Francisco, CA; 2008, [Abstract 902].
25. Turra P, Bottechia M, Sheldon J, Soriano V: Prior lamivudine (LAM) failure may delay time to complete HBV-DNA suppression in HIV patients treated with tenofovir plus LAM. *59th Annual Meeting of the American Association for the Study of Liver Diseases* San Francisco, CA; 2008, [Abstract 967].
26. Lacombe K, Goulet J, Boyd A, et al: HBV blippers and rebounders under treatment with tenofovir in HIV/HBV co-infection. *Conference on Retrovirus and Opportunistic Infections* San Francisco, CA; 2009, [Abstract 100].
27. Alvarez-Uria G, Raccifile L, Vilar J: Long-term outcome of tenofovir disoproxil fumarate use against hepatitis B in an HIV-coinfected cohort. *HIV Medicine* 2009, 10(5):269-273.
28. Benhamou Y, Fleury H, Trimolet P, Pellegrin I, Urbini R, Katlama C, Rozenbaum W, Le Teuff G, Tyklesinski A, Pketty C: Anti-hepatitis B virus efficacy of tenofovir disoproxil fumarate in HIV-infected patients. *Hepatology* 2006, 43(3):548-555.
29. Quins-Roldan E, Calabrese A, Lapadula G, Tirelli V, Costarelli S, Cologni G, Zaiton S, Puoti M, Carosi G, Torti C: Evidence of long-term suppression of hepatitis B virus DNA by tenofovir as rescue treatment in patients co-infected by HIV. *Antiviral Therapy* 2008, 13(3):341-348.
30. Strik R, Sette H Jr, Santana RA, Menezes LC, Graça CH, Dastoli GT, Silbert S, Pinho JR: Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. *Brazilian Journal of Medical and Biological Research* 2007, 40(12):1689-1692.
31. Jai Jai MK, Comanor L, White C, Kipris P, Elkin C, Leung K, Ocampo A, Attar N, Kiser P, Lee WM: Treatment of hepatitis B with lamivudine and tenofovir in HIV/HBV co-infected patients: factors associated with response. *Journal of Viral Hepatitis* 2007, 14(3):176-182.
32. Sanchez LV, Tanaka Y, Maldonado M, Mizokami M, Panduro A: Difference of hepatitis B virus genotype distribution in two groups of Mexican patients with different risk factors. High prevalence of genotype H and G. *Intervirology* 2007, 50:9-15.
33. Lee T, Núñez M: Longer duration of HBV-active antiretroviral therapy is linked to favorable virological outcome in HIV-HBV co-infected patients. *HIV Clin Trials* 2009, 10(3):153-159.
34. Piroth L, Sene D, Pol S, Godelet I, Lacombe K, Marthas B, Rey D, Loutau-Ratti V, Bergmann JF, Pialoux G, Gervais A, Lascaux-Combe C, Carat F, Cacoub P: Epidemiology, diagnosis and treatment of chronic hepatitis B in HIV-infected patients (EPHB 2005 STUDY). *AIDS* 2007, 21(10):1323-1331.
35. Matthews GV, Seaberg E, Dore GJ, Bowden S, Lewin SR, Sasseus J, Marks P, Goodman Z, Philp FH, Tang Y, Locamini S, Thio CL: Combination HBV therapy is linked to greater HBV DNA suppression in a cohort of lamivudine-experienced HIV/HBV co-infected individuals. *AIDS* 2009, 23(13):1707-1715.
36. Schmutz G, Nelson M, Lutz T, Sheldon J, Bruno R, von Boemmel F, Hoffmann C, Rockstroh J, Stöhr A, Wolf E, Soriano V, Berger F, Berg T, Carlebach A, Schwarze-Zander C, Schürmann D, Jaeger H, Mauss S: Combination of tenofovir and lamivudine versus tenofovir after lamivudine failure for therapy of hepatitis B in HIV-coinfection. *AIDS* 2006, 20:1951-1954.
37. Avihingsanon A, Lewin SR, Kerr S, Chang JJ, Piyawat K, Napissanan N, Matthews GV, Dore GJ, Bowden S, Lange J, Ruxrungtham K: Efficacy of tenofovir disoproxil fumarate/emtricitabine compared with emtricitabine alone in antiretroviral-naïve HIV-HBV coinfection in Thailand. *Antiviral Therapy* 2010, 15(6):917-922.
38. Matthews GV, Avihingsanon A, Lewin SR, Amin J, Berikimiri R, Petchasirap P, Marks P, Sasseus J, Cooper DA, Bowden S, Locamini S, Ruxrungtham K, Dore GJ: A randomized trial of combination hepatitis B therapy in HIV/HBV coinfecting antiretroviral naïve individuals in Thailand. *Hepatology* 2008, 48(4):1062-1069.
39. Peters MG, Andersen J, Lynch P, Liu T, Alston-Smith B, Brosgart CL, Jacobson JM, Johnson VA, Pollard RB, Rooney JF, Sherman KE, Swindells S, Polisky B: Randomized controlled study of tenofovir and adefovir in chronic hepatitis B virus and HIV infection: ACTG A5127. *HEPATOLOGY* 2006, 44:1110-1116.
40. Bodsworth N, Donovan B, Nightingale BN: The effect of concurrent human immunodeficiency virus infection on chronic hepatitis B: a study of 150 homosexual men. *Journal of Infectious Diseases* 1989, 160:577-582.
41. Bonacini M, Govindarajan S, Redeker AG: Human immunodeficiency virus infection does not alter serum transaminases and hepatitis B virus (HBV) DNA in homosexual patients with chronic HBV infection. *American Journal of Gastroenterology* 1991, 86(5):570-573.
42. Soriano V, Vispo E, Labarga P, Medrano J, Barreiro P: Viral hepatitis and HIV co-infection. *Antiviral Research* 2010, 85(1):303-315.
43. Gomes-Gouveia MS, Pereira Soares Mdo C, Guedes de Carvalho Mello IM, Brito EM, Pereira Moia Lde J, Bensabath G, Nunes HM, Camilo FJ, Pinho JR: Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin. *Acta Tropica* 2008, 106:149-155.

Pre-publication history

The pre-publication history for this paper can be accessed here:
<http://www.biomedcentral.com/1471-2334/11/247/prepub>

doi:10.1186/1471-2334-11-247

Cite this article as: Mendes-Correa et al: Predictors of HBeAg status and hepatitis B viraemia in HIV-infected patients with chronic hepatitis B in the HAART era in Brazil. *BMC Infectious Diseases* 2011 11:247.

- 10- Sitnik R, Pinho JRR, Bertolini DA, Bernardini AP, da Silva LC & Carrilho FJ. Hepatitis B virus genotypes and precore and core mutations in Brazilian patients. Journal of Clinical Microbiology 42 (6): 2455-2460, 2004.**

Hepatitis B Virus Genotypes and Precore and Core Mutants in Brazilian Patients

Roberta Sitnik,¹ João Renato Rebello Pinho,^{1,2*} Dennis Armando Bertolini,²
 Antonio Plinio Bernardini,¹ Luiz Caetano da Silva,³ and Flair José Carrilho³

Laboratório Bioquímico Jardim Paulista,¹ Serviço de Virologia, Instituto Adolfo Lutz,² and Departamento de Gastroenterologia, Faculdade de Medicina da Universidade de São Paulo,³ São Paulo, Brazil

Received 27 June 2003/Returned for modification 8 February 2004/Accepted 2 March 2004

A method for genotyping hepatitis B virus by partial HBsAg gene sequencing with primers common to all known genotypes was developed. Mutations related to anti-HBs resistance are also detected with this method. Samples from 103 Brazilian patients were analyzed. Precore and core region of these viruses were also sequenced in 101 patients. Genotypes A, B, C, D, and F were found with frequencies of 49.5, 2.9, 13.6, 24.3, and 9.7%, respectively. Genotypes B and C were found only in Asian patients, whereas genotypes A, D, and F were more common in patients without an Asian background. Precore mutants were found in 32 (31.7%) of 101 patients, with a higher frequency in those infected with genotype D (22 of 25 [88.0%]). Analysis of nucleotide 1858 showed presence of thymine in all patients with genotypes B, C, and D and in a few patients with genotypes A (10.0%) and F (30.0%), who showed more frequently the presence of cytosine. This nucleotide was closely related to the presence of precore mutants. Mutations in the basal core promoter were found in 64 of 101 (63.4%) samples. These mutations were more frequent in patients infected with genotype F (90.0%) and less frequent in patients infected with genotype B (33.3%). Deletions in this region were found in two genotype C-infected patients.

Hepatitis B virus (HBV) infection is very common worldwide, and more than 350 million people are chronic carriers (22). HBV infection is associated with different clinical pictures and leads to chronic carrier state in 5 to 10% patients infected in adult life and 85 to 90% of those infected in infancy (13).

HBV was formerly classified into four different subtypes that were afterward subdivided according to the antigenic determinants of HBsAg in *adw* (*adw2* and *adw4*), *ayw* (*ayw1*, *ayw2*, *ayw3*, and *ayw4*), *adr* (*adrq* and *adrq*), and *ayr* (33). Subtype *a* is common to the majority of viruses and is related to a neutralizing epitope. Divergence of the complete genome in a same subtype is ca. 8%, similar to the one found between different subtypes (38).

Another classification reflecting the phylogenetic origin of the virus isolates was later proposed dividing HBV into six genotypes (36–38), designated A to F. These genotypes were differentiated by a sequence divergence in the entire genome exceeding 8%. Norder et al. (35) demonstrated that HBV genotyping could also be performed by sequencing the S gene. Recently, seventh and eighth genotypes were reported: genotype G, which has an insertion of 36 nucleotides (nt) in the core gene and was discovered in France and United States (49), and genotype H, which was found in Nicaragua, Mexico, and California and has probably split off from genotype F within the New World (2). Previous studies have shown the geographical distribution of HBV genotypes worldwide but the results are still incomplete. Few data are reported from Brazil, where genotypes A, D, and F have been found in Rio de Janeiro, Goiás, and Santa Catarina states (8, 32, 52).

HBV vaccination, anti-HBV hyperimmunoglobulin (HBIG), and anti-HBs monoclonal antibody may induce resistant mutations, as previously shown by other researchers (5–7, 11, 40–42, 46, 53). These mutations are especially common in regions where HBV is endemic and where widespread anti-HBV vaccination have already been established and in patients undergoing liver transplantation due to hepatitis B who are using specific prophylaxis with HBIG or anti-HBs monoclonal antibodies.

Precore mutants were first described in Italy (4) related to cases showing negative HBeAg but with detectable HBV-DNA, HBsAg, anti-HBe, and anti-HBc. These strains have mutations in the nt 1896 (G→A) and 1899 (C→A) in the precore region, leading to a stop codon that prevents HBeAg synthesis. Precore region also contains the ϵ encapsidation signal, a secondary structure maintained by hydrogen bonds among bases in the same strand. Precore mutations are especially rare in genotypes that usually contain C at position 1858, since this nucleotide pairs with the nt 1896 in the secondary structure of ϵ encapsidation sign (23). Precore mutants have been associated with more severe hepatitis B evolution. This effect was related to the absence of the immune response modulator role from HBeAg and to alterations in the binding of the ϵ signal, leading to more efficient viral replication (50), but the overall pathogenic meaning of these mutations remains unclear (15, 28, 39). Since HBV genotypes are not uniformly distributed around the world, precore mutants also have a particular distribution, being more frequent in geographic regions such as Asia and the Mediterranean basin, where genotypes B, C, or D are predominant, and rare in North America and Europe, where genotype A is commonly found (24, 45). In South America, the frequency of this mutation is poorly studied, but Castro et al. (9) found a low frequency of precore

* Corresponding author. Mailing address: Serviço de Virologia, Instituto Adolfo Lutz, Av. Brigadeiro Luiz Antonio, 4701, 01401-002 São Paulo, SP, Brazil. Phone: 55-11-3885-7955. Fax: 55-11-3885-7955. E-mail: jrrpinho@terra.com.br.

TABLE 1. Partial S-gene sequences utilized in the alignment carried out to distinguish different HBV genotypes

Genotype	Sequence (GenBank accession no.)
A.....	X75666, X75669, X65258, M54898, J02205, X51970, and M74498
B.....	X75660, D23677, D23678, D00329, D00330, D00331, M54923, S74815, and S74867
C.....	X75667, X75665, X75792, X75656, X01587, D23680, D23682, D23681, D16665, S62754, S75184, S81945, S81946, and U19777
D.....	X75668, X75662, M32138, X77309, X65259, X59795, X68292, X77308, J02202, V01460, X02496, and X77310
E.....	X75657, X75664, and L24071
F.....	X75658, X75661, and X69798
G.....	AF369533 and AF160501
H.....	U91819, U91827, and AY090460

mutations (7 of 29 [24%]) in anti-HBe-positive Brazilian carriers.

HBsAg, anti-HBe, and HBV-DNA positive serological profile can also be related to mutations affecting the basal core promoter (BCP), especially A1762T and G1764A. These mutations have been associated with active hepatitis, severe disease after liver transplantation, development of hepatocellular carcinoma, and fulminant hepatitis (3, 51).

Recently, several methods based on sequencing have been described that allow determination of viral features related to prognosis and therapeutic response, such as genotype, precore and BCP mutants, and drug resistance.

We describe here a new set of primers for determining HBV genotypes with the same reagents by nested PCR and automated DNA sequencing. We also verified the frequency of HBV genotypes and precore and BCP mutations in Brazilian patients.

MATERIALS AND METHODS

Patients. We studied 103 samples from HBV chronic carriers (determined by two consecutive HBsAg-positive results from samples obtained at least 6 months apart) from different Brazilian regions that were sent to Laboratório Bioquímico Jardim Paulista, São Paulo, Brazil, from 1998 to 2001. Serum samples were stored at -20°C and thawed immediately before use. The Ethical Committee

from the University of São Paulo School of Medicine approved this protocol (research protocol 145/00).

Detection of HBV-DNA by PCR. Extraction and amplification of HBV-DNA were carried out by nested PCR as described by Kaneko et al. (16). All reagents utilized were obtained from Life Technologies do Brazil, São Paulo, Brazil.

Analysis of HBV sequences from different genotypes for primer design. For HBV genotyping, we developed a system amplifying a 417-bp fragment covering a region of S gene with marked heterogeneity among genotypes, flanked by a conserved region to allow equal amplification of them. In this way, an alignment was made with S gene sequences from the different genotypes already described. We used the MegAlign program from Lasergene package (DNASTAR, Inc., Madison, Wis.) to align 49 sequences corresponding to the gene coding for HBsAg of different HBV isolates obtained from GenBank and corresponding to the known HBV genotypes (Table 1). Alignment was carried out by using the CLUSTAL method (14) and the weight residues table PAM250.

Selected primers corresponded to conserved regions among the different genotypes, surrounding heterogeneous regions between them, allowing the distinction of HBV genotypes, as shown in Fig. 1. The selected region for amplification also covers the amino acid loop corresponding to *a*, *d/y*, and *w/r* specificities and mutations related to HBIG, anti-HBs monoclonal antibody, and vaccine resistance. Selected primers were as follows: FHBS1, 5'-GAG TCT AGA CTC GTG GTG GAC TTC-3'; FHBS2, 5'-CGT GGT GGA CTT CTC TCA ATT TTC-3'; RHBS1, 5'-AAA TKG CAC TAG TAA ACT GAG CCA-3'; and RHBS2, 5'-GCC ARG AGA AAC GGR CTG AGG CCC-3'. The primer positions in the HBV genome (strain HBVADW; GenBank accession number V00866) were as follows: HBS1F (positions 244 to 267), HBS2F (positions 255 to 278), HBS2R (positions 648 to 671), and HBS1R (positions 668 to 691).

HBsAg gene partial amplification. Samples with detectable HBV-DNA were also subjected to S-gene partial amplification for HBV genotyping by sequencing. Serum samples were treated as described above and subjected to two rounds of amplification with sequentially outer (FHBS1 and RHBS1) and inner (FHBS2 and RHBS2) primers. The amplification conditions for the two rounds of the nested PCR were as follows: initial denaturation at 94°C for 20 s, followed by 30 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, with a final extension step at 72°C for 1 min in a PTC-200 Thermocycler (MJ Research, Watertown, Mass.).

Detection of BCP and precore mutants. For the detection of BCP and precore mutants, HBV-DNA-positive samples were amplified by using the primers described by Takahashi et al. (51).

Sequencing reaction. PCR products were subjected to cycle sequencing reactions as described previously (48) by using the second-round primers and the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.). After purification, samples were denatured and loaded in a 5% Long Ranger 6 M urea gel (Long Ranger gel solution; FMC) in an automated ABI Prism 377 DNA sequencer (Applied Biosystems).

Sequence analysis. Genotyping, BCP, and precore mutant analysis were carried out by sequence comparison with other known sequences from different HBV genotypes described previously and were aligned as described above. For this purpose, EditSeq and MegAlign programs from the DNASTAR package (LaserGene, Inc.) were used.

FIG. 1. Schematic representation of the HBsAg coding region (amino acids 50 to 163) corresponding to the region amplified for genotyping. GenBank accession number sequence X75666 is shown in detail. For each known HBV genotype, two sequences are shown, and only amino acids differing from X75666 are indicated. Dots indicate that the same amino acid was observed in the sequence. Mutations specific for HBV subtypes: 122 (K = d, R = y), 160 (K = w, R = r), 127 (L = w/f), and 140 (S = w/f). Mutations related to resistance to HBIG, anti-HBs monoclonal antibody, or vaccination: 107 (Y), 110 (V), 111 (T), 114 (R), 115 (K or S), 116 (S), 117 (R), 118 (A, V, or S), 120 (E, T, S, or N insertion), 121 (T or R insertion), 122 (NT insertion or RA insertion), 123 (N or A or NSTGPGCTT insertion), 124 (R or Y), 125 (A), 126 (S, N, or A), 127 (S or T), 129 (H), 130 (R or N), 131 (S or I), 132 (P), 133 (T, I, or I), 134 (L, I, S, or N), 135 (S), 137 (Y), 138 (Y or S), 140 (R, I, and P), 141 (E), 142 (S), 143 (W, R, M, or L), 144 (H, N, A, or E), 145 (R, K, or A), 146 (S), 147 (R), 148 (I), 149 (R or W), 154 (T or W), 155 (Y or P), 156 (L), and 157 (R).

Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 2.1 (21). Sequences were aligned by using CLUSTAL X version 1.81 (54) and then analyzed by using the neighbor-joining method by a distance matrix calculated according to the Kimura two-parameter model (19). Woolly monkey hepatitis B virus (GenBank accession number AF046996) was utilized as an outgroup. A bootstrap test of phylogeny was performed with 1,000 replicates, and values ≥ 67 are indicated on the branches.

Statistical analysis. For statistical analysis, we used the EpiInfo program (version 6.04; Centers for Disease Control and Prevention, Atlanta, Ga., and World Health Organization, Geneva, Switzerland).

GenBank accession numbers. Sequences from the S and preC/C genes from Brazilian cases were deposited in the GenBank database under accession numbers AY323306 to AY323408 and AY329494 to AY329594, respectively.

RESULTS

HBsAg gene partial amplification analysis. As shown in the phylogenetic analysis (Fig. 2), genotypes A, B, C, D, and F were found among our samples. The frequency of genotypes in the 103 patients is shown in Fig. 3. The following subtypes were found: *adw*, *ayw*, *adr*, and *adw4*. Genotypes A and B had only subtype *adw*, and genotype F had only *adw4*. Genotype C showed subtype *adr* more frequently, with one patient infected with subtype *adw*. Genotype D samples were all subtype *ayw* except for one (*adw*). In two Asian patients, mutations in the epitope "a" region were found. One genotype B sample had a T126S mutation, previously associated HBV resistance, and the other one (genotype C) had a T118A mutation, which was associated with vaccine resistance.

Genotypes, age, gender, and ethnicity. The gender, age mean, and ethnicity ranges according to genotypes can be seen in Table 2. Most of the patients studied had a Western ethnicity ($n = 83$, 80.6%). Genotype A was found mainly in such patients (94.1%), and genotypes D and F were exclusive to them. The only three Asian patients infected with genotype A belonged to the same family (father, mother, and son). Among the 20 (19.4%) Asian patients, 17 (85.0%) had genotypes B or C. None of non-Asian patients had genotype B or C. Statistical analysis showed that infection with genotypes B and C is significantly more frequent among Asian patients in our population ($P = 0.01$ and $P < 0.0001$, respectively).

Genotypes, precore and BCP mutants, and nt 1858 analysis. In 101 patients, we also analyzed the BCP and precore region of HBV. In the other two patients, it was not possible to amplify this region. The frequency of mutants in these regions among the different genotypes, as well as the results of the nt 1858 analysis, are shown in Table 2. Precore mutants were more frequent in patients infected with genotype D virus ($P < 0.0001$).

Analysis of nt 1858 showed the presence of thymine in all patients with genotypes B, C, and D and in a few patients with genotypes A (5 of 50 [10.0%]) and F (3 of 10 [30.0%]). This nucleotide was closely related to the presence of precore mutants ($P < 0.0001$) since, of 49 isolates with nt 1851, 32 (65.3%) also had the stop codon mutation at nt 1896, whereas none of the isolates with a cytosine at nt 1858 showed this mutation. All patients with genotypes A and F that had a mutation in the precore region also had a thymine at position 1858.

BCP mutants were less frequent among genotype B-infected patients (33.3%) and more frequent among genotype F-infected patients (90.0%), but no statistical significant difference was found. Interestingly, in two patients infected with HBV

FIG. 2. Neighbor-joining tree of a 339-nt fragment of HBV-DNA (S gene) from isolates obtained in the studied population with woolly monkey hepatitis virus (GenBank accession number AF046996) used as an outgroup.

genotype C, we found deletions in the BCP encompassing the studied mutation. These deletions extended from nt 1760 to 1770 in one patient and from nt 1760 to 1769 in the other.

DISCUSSION

HBV genotyping has been extensively analyzed recently. Better responses to treatment have been reported for genotypes A and B compared to genotypes D and C, respectively (44, 47, 56). On the other hand, evolution to chronic hepatitis or to more severe diseases such as hepatocellular carcinoma has been related to genotypes A and C, respectively (12, 30, 43,

FIG. 3. HBV genotype distribution in Brazilian patients ($n = 103$).

44). Few data regarding genotype F have been reported, but death related to liver disease was more frequently associated with genotype F than with genotypes A or D in one study (47).

The genotyping method described here has the advantage of being very fast and straightforward and uses the same reagents to amplify all known HBV genotypes. It also allows analysis of subtype and other mutations, such as in the epitope *a* region of the same sequence. Other methods for HBV genotyping, such as restriction length fragment polymorphism analysis (24, 25, 29) or line probe assay (52), can distinguish the different genotypes, but they do not allow detection of subtypes or other point mutations specific to vaccine or gamma globulin resistance.

When we used this genotyping method in Brazilian patients, we detected genotypes A, B, C, D, and F. Genotype A was the most common and was found among 49.5% patients. Moraes et al. (32) found genotypes A, D, and F in Rio de Janeiro, Brazil. These same genotypes were also described in populations from Goiânia and Santa Catarina (8, 52). To our knowledge, the present study is the first time that genotypes B and C have been detected in Brazil, probably due to the low percentage of Asian subjects in the Brazilian states studied previously, although one of our patients infected with genotype C virus was from Rio de Janeiro.

A total of 85% of Asian patients had genotype B or C. Only three of these patients had genotype A, and all three individuals belonged to the same family. Several studies carried out in Eastern Asia have shown that genotypes B and C are the most common in this region. It was also reported that patients infected with these genotypes have a worse prognosis with a higher frequency of progression to cirrhosis and hepatocellular

carcinoma (17). In China, Ding et al. (12) showed that genotype C infections were more severe than genotype B infections. Similar results have also been found in other studies in China and Japan, and it has been hypothesized that the higher frequency of BCP mutants in genotype C could be associated with the higher pathogenicity of this genotype (18, 44). Studies addressing the evolution of hepatitis B and the viral genotypes should also be carried out among Brazilian patients.

Genotypes E, G, and H were not found in our study population. Genotype E has only been found in some regions of Africa (31), and only one study outside Africa found a Haitian child infected with this genotype in Belgium (26). Genotype G has been found in ca. 10% of studied cases from France and United States (49). Genotype H seems to be a divergent clade from genotype F and was described after our primer selection and sample processing, but a later analysis with three sequences from GenBank (U91819, U91827, and AY090460) showed that this method is also able to distinguish this new genotype.

Although there are many African descendants in Brazil due to slaves being brought from Africa from the 16th to the 19th centuries, we did not find genotype E in our sample. There were few patients of known African descent in the studied population. Other studies in Brazilian states with a higher percentage of African descendants, such as Bahia, should be carried out to better evaluate this issue.

Different subtypes were also found in our samples. As expected, it would not be possible to classify the genotypes only based on that.

Mutations related to epitope *a* recognition were found in only two patients. Mutation T126S has already been described and associated with vaccination resistance (40, 55). Mutation T118A, on the other hand, is outside the main region of epitope *a* and was related to HBIG resistance (11). Unfortunately, we were not able to obtain more information about the progress of the disease in these two patients, but it is noteworthy that both had genotypes common to Asiatic regions (43). The finding of these mutations among our cases highlights the need for a continued surveillance to address the appearance of such cases and to better analyze their real meaning.

Precore mutants had a low frequency in our population (31.7%). Such mutants were found more frequently in patients infected with genotype D, with intermediate frequency in pa-

TABLE 2. HBV genotypes and their relationships to patients and viral features^a

HBV genotype	Patient feature			BCP and precore region mutations (no. [%])					
	No. male (%)	Mean age (yr) (SD)	No. Asian (%)	BCP		nt 1858		Precore region	
				WT	Mutant	C	T	WT	Mutant
A	35 (68.6)	40.7 (11.6)	3 (5.9)	19 (38.0)	31 (62.0)	45 ^c (90.0)	5 (10.0)	46 (92.0)	4 (8.0)
B	3 (100)	49.0 (9.87)	3 (100) [†]	2 (66.7)	1 (33.3)	0	3 (100)	2 (66.7)	1 (33.3)
C	9 (64.3)	33.5 (16.5)	14 (100) [*]	3 (23.1)	10 ^b (76.9)	0	13 (100)	10 (76.9)	3 (23.1)
D	19 (76.0)	51.1 (12.9)	0	12 (48.0)	13 (52.0)	0	25 (100)	3 (12.0)	22 (88.0)
F	10 (100)	33.0 (7.12)	0	1 (10.0)	9 (90.0)	7 (70.0)	3 (30.0)	8 (80.0)	2 (20.0)
Total	76 (73.8)	42.0 (13.9)	20 (19.4)	37 (36.6)	64 (63.4)	52 (51.5)	49 (48.5)	69 (68.3)	32 (31.7)

^a *, $P < 0.0001$; [†], $P = 0.01$. WT, wild type.

^b Includes two cases with a deletion in the BCP region.

^c Includes two cases with an adenine at nt 1858.

tients infected with genotypes B and C, and at a very low frequency in genotype A- and F-infected patients. The occurrence of this mutation is dependent upon the nucleotide (cytosine or thymine) at position 1858, which forms a base pair with nt 1896 in the pregenomic RNA loop at the ϵ encapsidation sign. A thymine at position 1858 is particularly common in genotype D viruses. The presence of a cytosine at position 1858 precludes the G-to-A mutation at nt 1896, since this would destabilize the stem-loop structure of the RNA encapsidation signal (27). Interactions of this encapsidation signal with the viral DNA polymerase is an essential step in the viral replication cycle, and it has been hypothesized that the increased strength of the guanine-to-cytosine base pairing found in precore mutants would implicate in a stronger ϵ encapsidation sign, allowing a more efficient replication of these strains. Genotype A usually shows a cytosine at this position (23), whereas genotype F may present thymine or cytosine (1). Our results from nt 1858 analysis corroborate with these data, since we also have found the presence of cytosine only in genotypes A and F, which showed a low frequency of precore mutants.

The frequency of this mutation varies widely around the world. Castro et al. (9) studied Brazilian patients and found the precore stop codon mutation in only 24% of them—a result similar to ours. These authors also demonstrated the higher frequency of the stop codon mutation at nt 1896 in the isolates that had T1858. On the other hand, the prevalence of this mutation in China was 86% (10). This discrepancy may reflect differences in genotype distributions in each studied population, since genotype A and D viruses are common in Brazilian populations, whereas in China genotypes B and C are more common. Furthermore, in our population, the frequencies of precore mutants were low even in genotype B- and C-infected patients (33.3 and 23.1%, respectively).

The BCP mutation was found in 63.4% of our patients and was present in all genotypes. BCP mutants frequency ranged from 33.3% in genotype B to 90.0% in genotype F. Changes at positions 1762 and 1764 have been described to be especially common in patients with C1858 (24), but we did not find any correlation between them. Among our patients, the frequency of BCP mutants was high in genotypes A and F patients from whom C1858 is especially common, but genotype C- and D-infected patients, in whom T1858 is common, have similar frequencies. On the other hand, other authors have reported that the presence of these mutants is not related to the presence of precore mutants (9). This point should be further analyzed in our population.

Two genotype C-infected patients in the present study had a deletion in this region. Other authors have reported a number of point mutations, as well as deletions and insertions of nucleotides in the precore region leading to initiation failure or premature termination. The positions of these mutations varied and were normally restricted from 8 to 21 nt, flanking AT-rich regions and overlapping transcription factor binding sites (10, 20, 34). An 8-bp deletion from nt 1768 to 1775 was also found in precore promoter region before exacerbation, but in a later sample this deletion was no longer found (34). The deletions reported here are different from the ones described previously, but they were always in the same region.

In conclusion, we have developed a method for HBV genotyping that can amplify isolates from all of the HBV genotypes

found in Brazil. Furthermore, we have characterized by this approach the HBV genotypes in our population and their relationship to precore and BCP mutants. We have shown that genotypes E, G, and H were not found in Brazil until now. Genotype A was the most common in our study population. Genotypes A, D, and F are found in non-Asian subjects, whereas genotypes B and C are found among Asian patients. Precore mutants are more common among genotype D-positive patients, whereas BCP mutants were present in all of the genotypes, with a higher frequency among genotype F-infected patients.

ACKNOWLEDGMENTS

We are deeply indebted to Simone Campioto, Marcilio F. Lemos, and Rúbia A. Santana for technical assistance during the development of this study and to André C. Lyra for help in handling the MEGA2 package.

This study was supported by Laboratório Bioquímico Jardim Paulista, CAPES, and FAPESP (99/09551-0). Roberta Sitnik and Dennis Armando Bertolini were Ph.D. students from Faculdade de Medicina de Universidade de São Paulo and Escola Paulista de Medicina, respectively.

REFERENCES

1. Arauz-Ruiz, P., H. Norder, K. A. Visona, and L. O. Magnius. 1997. Genotype F prevails in HBV-infected patients of Hispanic origin in Central America and may carry the precore stop mutant. *J. Med. Virol.* 51:305-312.
2. Arauz-Ruiz, P., H. Norder, B. H. Robertson, and L. O. Magnius. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J. Gen. Virol.* 83:2059-2073.
3. Baumert, T. F., S. A. Rogers, K. Hasegawa, and T. J. Liang. 1996. Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J. Clin. Invest.* 98:2268-2276.
4. Brunetto, M. R., M. Stemler, F. Bonino, F. Schodel, F. Olivieri, M. Rizzetto, G. Verme, and H. Will. 1990. A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B. *J. Hepatol.* 10:258-261.
5. Carman, W. F., J. Korula, L. Wallace, R. MacPhee, L. Mims, and R. Decker. 1995. Fulminant reactivation of Hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. *Lancet* 345: 1406-1407.
6. Carman, W. F., H. C. Thomas, A. J. Zuckerman, and T. J. Harrison. 1998. Molecular variants of hepatitis B virus, p. 141-177. In A. J. Zuckerman and H. C. Thomas (ed.), *Viral hepatitis*, 2nd ed. Churchill Livingstone, London, United Kingdom.
7. Carman, W. F., A. Owsianka, L. A. Wallace, B. C. Dow, and D. J. Mutimer. 1999. Antigenic characterisation of pre- and post-liver transplant hepatitis B surface antigen sequences from patients treated with hepatitis B immune globulin. *J. Hepatol.* 31:195-201.
8. Carrilho, F. J., C. R. Moraes, J. R. R. Pinho, R. Moreira, D. Bertolini, I. M. Mello, S. Gatura, L. Bassit, R. A. Cardoso, L. C. Da Silva, and A. A. Laudanna. 2001. Hepatitis B virus infection in haemodialysis centers from Santa Catarina State, Southern Brazil: predictive factors for infection and molecular epidemiology. *Hepatology* 34(Pt. 4):612A.
9. Castro, L., C. Niel, and S. A. Gomes. 2001. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. *BMC Microbiol.* 1:10-18.
10. Chan, H. L. Y., M. Hussain, and A. S. Lok. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B antigen seroconversion. *Hepatology* 29:976-984.
11. Chong-Jin, O., C. Wei Ning, K. Shiuan, and G. L. Keow. 1999. Identification of hepatitis B surface antigen variants with alterations outside the "a" determinant in immunized Singapore infants. *J. Infect. Dis.* 179:259-263.
12. Ding, X., M. Mizokami, G. Yao, B. Xu, E. Orito, and M. Nakanishi. 2001. Hepatitis B virus genotype distribution among chronic hepatitis B virus carriers in Shanghai, China. *Intervirology* 44:43-47.
13. Gust, L., and S. Crowe. 1986. The global importance of viral hepatitis. *Clin. Trop. Med. Commun. Dis.* 1:281-301.
14. Higgins, D. G., and P. M. Sharp. 1988. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* 73:237-244.
15. Hsu, H. Y., M. H. Chang, C. Y. Lee, K. H. Hsieh, Y. H. Ni, P. J. Chen, and D. S. Chen. 1995. Precore mutant of hepatitis B virus in childhood fulminant hepatitis B: an infrequent association. *J. Infect. Dis.* 171:776-781.
16. Kaneko, S., S. M. Feinstone, and R. H. Miller. 1989. Rapid and sensitive

- method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. Clin. Microb.* 27:1930-1933.
17. Kao, J. H., P. J. Chen, M. Y. Lai, and D. S. Chen. 2000. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 118:554-559.
 18. Kao, J. H., N. H. Wu, P. J. Chen, M. Y. Lai, and D. S. Chen. 2000. Hepatitis B genotypes and the response to interferon therapy. *J. Hepatol.* 33:998-1002.
 19. Kimura, M. 1980. A simple method for estimating evolutionary rates of bases substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-120.
 20. Kramvis, A., and M. C. Kew. 1999. The core promoter of hepatitis B virus. *J. Viral Hepat.* 6:415-427.
 21. Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 17:1244-1245.
 22. Lee, W. M. 1997. Hepatitis B virus infection. *N. Engl. J. Med.* 337:1733-1745.
 23. Li, J. S., S. P. Tong, Y. M. Wen, L. Vitvitski, Q. Zhang, and C. Trepo. 1993. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J. Virol.* 67:5402-5410.
 24. Lindh, M., A. S. Andersson, and A. Gusdal. 1997. Genotypes, nt1858 variants, and geographic origin of hepatitis B virus: large-scale analysis using a new genotyping method. *J. Infect. Dis.* 175:1285-1293.
 25. Lindh, M., J. E. Gonzalez, G. Norkrans, and P. Horal. 1998. Genotyping of hepatitis B virus by restriction pattern analysis of a Pre-S amplicon. *J. Virol. Methods* 72:163-174.
 26. Liu, H. F., E. Sokal, and P. Goubau. 2001. Wide variety of genotypes and geographic origins of hepatitis B virus in Belgian children. *J. Pediatr. Gastroenterol. Nutr.* 32:244-247.
 27. Lok, A. S., U. Akarca, and S. Greene. 1994. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc. Natl. Acad. Sci. USA* 91:4077-4081.
 28. Lok, A. S., E. J. Heathcote, and J. H. Hoofnagle. 2001. Management of hepatitis B: summary of a workshop. *Gastroenterology* 120:1828-1853.
 29. Magnus, L. O., and H. Norder. 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S gene. *Intervirology* 38:24-34.
 30. Mayer, C., A. Mantegani, and P. C. Frei. 1999. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J. Viral Hepat.* 6:299-304.
 31. Mizokami, M., T. Nakano, E. Orito, Y. Tanaka, H. Sakugawa, M. Mukaido, and B. H. Robertson. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett.* 450:66-71.
 32. Moraes, M. T., A. S. Gomes, and C. Niel. 1996. Sequence analysis of the pre-S/S gene of hepatitis B virus strains genotypes A, D, and F isolated in Brazil. *Arch. Virol.* 114:1767-1773.
 33. Neurath, A. R., and Y. Thanavala. 1990. Hepadnaviruses, p. 403-458. In M. H. V. Van Regenmortel and A. R. Neurath (ed.), *Immunochimistry of viruses. II. The basis for serodiagnosis and vaccines*. Elsevier, Amsterdam, The Netherlands.
 34. Nishizono, A., K. Kohno, Y. Takita-Sonoda, M. Hiraga, H. Terao, T. Fujioka, M. Nasu, and K. Mifune. 1997. Sequential analyses of the mutations in the core upstream and precore regions of hepatitis B virus genome in anti-HBe positive-carriers developing acute exacerbation. *J. Med. Virol.* 53:266-272.
 35. Norder, H., B. Hammas, S. Lofdal, A. M. Courouce, and L. O. Magnus. 1992. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J. Virol.* 73:1201-1208.
 36. Norder, H., A. M. Courouce, and L. O. Magnus. 1992. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J. Gen. Virol.* 73:3141-3145.
 37. Norder, H., A. M. Courouce, and L. O. Magnus. 1994. Complete genomes, phylogenetic relatedness and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 198:489-503.
 38. Okamoto, H., F. Tsuda, H. Sakugawa, R. I. Sastroewignjo, M. Imai, Y. Miyakawa, and M. Mayumi. 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. Gen. Virol.* 69:2575-2583.
 39. Okamoto, H., S. Yotsumoto, Y. Akahane, T. Tamanaka, Y. Miyazaki, Y. Sugai, F. Tsuda, T. Tanaka, Y. Miyakawa, and M. Mayumi. 1990. Hepatitis B viruses with precore regions defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J. Virol.* 64:1298-1303.
 40. Okamoto, H., K. Yano, Y. Nozaki, A. Matsui, H. Miyazaki, K. Yamamoto, F. Tsuda, A. Machida, and S. Mishiho. 1992. Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis-B immune globulin and vaccine. *Pediatr. Res.* 32:264-268.
 41. Oon, C. J., K. L. Tan, T. Harrison, and A. Zuckerman. 1996. Natural history of hepatitis B surface antigen mutants in children. *Lancet* 348:1524.
 42. Oon, C. J., and W. N. Chen. 1998. Current aspects of hepatitis B surface antigen mutants in Singapore. *J. Viral Hepatitis* 5(Suppl. 2):17-23.
 43. Orito, E., T. Ichida, H. Sakugawa, M. Sata, N. Horiike, K. Hino, K. Okita, T. Okanoue, S. Iino, E. Tanaka, K. Suzuki, H. Watanabe, S. Hige, and M. Mizokami. 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 34:590-594.
 44. Orito, E., M. Mizokami, H. Sakugawa, K. Michitaka, K. Ishikawa, T. Ichida, T. Okanoue, H. Yotsuyanagi, S. Iino, et al. 2001. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. *Hepatology* 33:218-223.
 45. Rodriguez-Frias, F., M. Buti, R. Jardi, M. Cotrina, L. Viladomiu, R. Esteban, and J. Guardia. 1995. Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. *Hepatology* 22:1641-1647.
 46. Rodriguez-Frias, F., M. Buti, R. Jardi, V. Vargas, J. Quer, M. Cotrina, M. Martell, R. Esteban, and J. Guardia. 1999. Genetic alterations in the S gene of hepatitis B virus in patients with acute hepatitis B, chronic hepatitis B, and hepatitis B liver cirrhosis before and after liver transplantation. *Liver* 19:177-182.
 47. Sanchez-Tapias, J. M., J. Costa, A. Mas, M. Bruguera, and J. Rodes. 2003. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 125:1559-1560.
 48. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
 49. Stuyver, L., S. De Gendt, C. Van Geyt, F. Zoulim, M. Fried, R. F. Schinazi, and R. Rossau. 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.* 81:67-74.
 50. Su, H., and J. K. Yee. 1992. Regulation of hepatitis B virus gene expression by its two enhancers. *Proc. Natl. Acad. Sci. USA* 89:2708-2712.
 51. Takahashi, K., K. Aoyama, N. Ohno, K. Iwata, Y. Akahane, K. Baba, H. Yoshizawa, and S. Mishiho. 1995. The pre core/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and easy method for detection. *J. Gen. Virol.* 76:3159-3164.
 52. Teles, S. A., R. M. Martins, B. Vanderborght, L. Stuyver, A. M. Gaspar, and C. F. Yoshida. 1999. Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients. *Artif. Organs* 23:1074-1078.
 53. Theamboonlers, A., V. Chongsrisawat, P. Jantaradsamee, and Y. Poovorawan. 2001. Variants within the "a" determinant of HBs gene in children and adolescents with and without hepatitis B vaccination as part of Thailand's Expanded Program on Immunization (EPI). *Tohoku J. Exp. Med.* 193:197-205.
 54. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24:4876-4882.
 55. Tong, C. Y. W. 2000. Genetic variations of hepatitis B virus. *Curr. Opin. Infect. Dis.* 13:481-487.
 56. Zhang, X., F. Zoulim, F. Habersetzer, S. Xiong, and C. Trepo. 1996. Analysis of hepatitis B virus genotypes and pre-core region variability during interferon treatment of HBe antigen negative chronic hepatitis B. *J. Med. Virol.* 48:8-16.

- 11- Michele S Gomes-Gouvêa, Ariana C Ferreira, Rosangela Teixeira, José R Andrade, Adalgisa SP Ferreira, Lena MF Barros, Rosamar EF Rezende, Ana CS Santos Nastri, Andrea GB Leite, Leonora Z Piccoli, Josiane Galvan, Simone RSS Conde, Manoel CP Soares, Dimas A Kliemann, Dennis A Bertolini, Aline SO Kunyoshi, André C Lyra, Marcio K Oikawa, Luciano V de Araújo, Flair J Carrilho, Maria CJ Mendes-Corrêa, João R Rebello Pinho. HBV carrying drug-resistance mutations in chronically infected treatment-naive patients. Antiviral Therapy 2015 Jan 27 doi: 10.3851/IMP2938**

HBV carrying drug-resistance mutations in chronically infected treatment-naïve patients

Michele S Gomes-Gouvêa, Ariana C Ferreira, Rosangela Teixeira, José R Andrade, Adalgisa SP Ferreira, Lena MF Barros, Rosamar EF Rezende, Ana CS Santos Nastro, Andrea GB Leite, Leonora Z Piccoli, Josiane Galvan, Simone RSS Conde, Manoel CP Soares, Dimas A Kliemann, Dennis A Bertolini, Aline SO Kunyoshi, André C Lyra, Marcio K Oikawa, Luciano V de Araújo, Flair J Carrilho, Maria CJ Mendes-Corrêa, João R Rebello Pinho

Antiviral Therapy 2015; 10.3851/IMP2938

Submission date 14th May 2014
Acceptance date 2nd October 2014
Publication date 27th January 2015

This provisional PDF matches the article and figures as they appeared upon acceptance. Copyedited and fully formatted PDF and full text (HTML) versions will be made available soon.

For information about publishing your article in *Antiviral Therapy* go to <http://www.intmedpress.com/index.cfm?pid=12>

Publication: Antiviral Therapy, Type: Original article
DOI: 10.3851/IMP2938

Original article

HBV carrying drug-resistance mutations in chronically infected treatment-naïve patients

Michele S Gomes-Gouvêa^{1*}, Ariana C Ferreira¹, Rosângela Teixeira², José R Andrade², Adalgisa SP Ferreira³, Lena MF Barros³, Rosamar EF Rezende⁴, Ana CS Santos Nasti^{1,5}, Andrea GB Leite⁶, Leonora Z Piccoli⁶, Josiane Galvan⁶, Simone RSS Conde⁷, Manoel CP Soares⁸, Dimas A Kliemann⁹, Dennis A Bertolini¹⁰, Aline SO Kunyoshi¹⁰, André C Lyra¹¹, Marcio K Oikawa¹², Luciano V de Araújo¹³, Flair J Camilho¹, Maria CJ Mendes-Corrêa^{5†}, João R Rebello Pinho^{1†}

¹Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, Brazil

²Department of Internal Medicine, Medical School, Federal University of Minas Gerais, Minas Gerais, Brazil

³Center of Clinical Research, Federal University of Maranhão, Maranhão, Brazil

⁴Municipal Secretary of Health, Ribeirão Preto, São Paulo, Brazil

⁵Department of Infectious Diseases, Medical School, University of São Paulo, São Paulo, Brazil

⁶Serviço Municipal de Infectologia do Centro Especializado de Saúde de Caxias do Sul, Rio Grande do Sul, Brazil

⁷Fundação Santa Casa de Misericórdia do Pará, Pará, Brazil

⁸Serviço de Hepatologia do Instituto Evandro Chagas, Pará, Brazil

⁹Hospital Nossa Senhora da Conceição, Serviço de Infectologia, Porto Alegre, Rio Grande do Sul, Brazil

¹⁰Laboratório de Imunologia Clínica, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Paraná, Brazil

¹¹Division of Gastroenterology & Hepatology, Department of Medicine, Federal University of Bahia, Bahia, Brazil

¹²Universidade Federal do ABC - Centro de Matemática, Computação e Cognição, São Paulo, Brazil

¹³Escola de Artes, Ciências e Humanidades- Universidade de São Paulo, São Paulo, Brazil

*Corresponding author e-mail: gomesmic@yahoo.com.br

†These two authors share senior authorship

Abstract

Background: Nucleos(t)ide analogue (NA) treatment causes selection pressure for hepatitis B virus strains carrying mutations conferring NA resistance. Drug-resistance mutations occur in the reverse transcriptase (RT) region of the HBV polymerase gene and spontaneously arise during

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

viral replication. These mutations can also alter the HBs protein and in some cases reduce binding to HBs antibodies. The spread of NA-resistant HBV may impact the efficacy of antiviral treatment and hepatitis B immunization programs. In this study, we used direct sequencing to assess the occurrence of HBV carrying known mutations that confer NA resistance in the largest cohort of treatment-naïve patients with chronic hepatitis B (CHB) to date.

Methods: HBV DNA was sequenced in 702 patients and the RT region of these samples was subjected to mutational analysis.

Results - There was high genetic variability among the HBV samples analyzed: A1 (63.7%); D3 (14.5%); A2 (3.3%); A3 (0.1%); B1 (0.1%); B2 (0.1%); C2 (0.9%); D1 (0.9%); D2 (4.6%); D4 (5.1%); D unclassified subgenotype (0.7%); E (0.6%); F2a (4.6%); F4 (0.4%) and G (0.4%). HBV strains harboring mutations conferring NA resistance alone or combined with compensatory mutations were identified in 1.6% (11/702) of the patients.

Conclusions: There is a low frequency of HBV NA-resistant strains as major population of HBV quasispecies among CHB treatment-naïve patients in Brazil. These findings suggest that the spread and natural selection of drug-resistant HBV is an uncommon event and/or most of these strains remain unstable in the absence of NA selective pressure.

Accepted 2 October 2014, published online 27 January 2015

Running head: HBV with drug-resistance mutations in treatment-naïve patients

Introduction

Although universal hepatitis B vaccination has lowered the prevalence of hepatitis B virus (HBV) over the past 30 years, it still remains a serious health threat in many regions worldwide [1], where chronic hepatitis B (CHB) patients have higher risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) [2]. Global prevalence of HBV infection varies considerably and is divided into high ($\geq 8\%$), intermediate (2–7%), and low ($< 2\%$) endemic areas [1]. A recent Brazilian epidemiological study found low (0.37%) prevalence of HBV infection in the capital cities of Northeastern and Central-Western Brazilian states [3]. Nevertheless, prevalence of HBV is non-uniform, as infection is hyperendemic in some inland areas of many Brazilian states [1]. The Brazilian Ministry of Health estimates that approximately 600,000 people are chronically infected in Brazil [4].

Treatments have been developed and approved for CHB that significantly reduce morbidity and mortality, including two types of interferon (conventional and pegylated- $\alpha 2a$) and five nucleos(t)ide analogues (NA): lamivudine (LAM), telbivudine (TBV), entecavir (ETV), adefovir (ADV), and tenofovir (TDF) [2]. Until 2009 in Brazil lamivudine and interferon- α were the only drugs provided by the Brazilian government for CHB treatment. In 2010, nucleos(t)ide analogues (NAs) ADV, ETV, and TDF were incorporated when the Brazilian Ministry of Health also released national guidelines for CHB treatment [5].

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

NAs competitively bind to the HBV polymerase to inhibit its activity, suppressing replication, but development of drug resistance during long-term therapy is a common problem [6]. NA treatment causes selection pressure for HBV strains carrying mutations conferring drug resistance, which is associated with treatment failure [6]. Drug-resistance mutations occur in the reverse transcriptase (RT) region of the HBV polymerase gene and spontaneously arise during viral replication. Sequential monotherapy can select for multidrug-resistant HBV strains with mutations in the polymerase gene that can also modify the amino acid sequence of the HBs protein (HBsAg) [6]. Some of these mutations in HBsAg reduce binding to HBs antibodies from hepatitis B vaccination, bypassing the neutralizing activity of these antibodies (antibody escape) and successfully infecting HBV-vaccinated individuals. The infectivity of LAM-resistant mutant rtV173L+rtL180M+rtM204V (producing sE164D and sI195M in HBsAg) was demonstrated in vaccinated chimpanzees, thus the spread of HBV-resistant mutants with potential HBs antibody escape may affect antiviral treatment efficacy and hepatitis B immunization programs [7].

HBV with drug resistance mutations has been identified in different countries among cases of acute infection and CHB patients who have never undergone NA treatment [8–26]. In Brazil were reported that among CHB patients undergoing NA treatment with LAM monotherapy (66.7%) or combined with TDF, ADV, or ETV (14.7%), 54% were infected with HBV strains harboring drug resistance mutations and most (55.6%) exhibited viral loads above 10^5 copies/mL [27]. The extent to which these HBV strains have spread and their transmission in our country are still unknown. Therefore in this study we assessed the occurrence of HBV with known mutations conferring NA resistance in a large cohort of CHB treatment-naïve patients by direct sequencing.

Patients and methods

Patients

This study included serum samples isolated from 779 CHB patients who were followed at eight centers located in different Brazilian regions (Figure 1). The samples were collected between 2006 and 2011 and all patients were HBsAg positive for at least 6 months prior to inclusion in the study, tested negative for human immunodeficiency virus (HIV) and had never been exposed to any anti-HBV drugs.

This study was approved by the Ethics Committee of Faculdade de Medicina da Universidade de São Paulo under protocol number 036/10.

Hepatitis B e antigen (HBeAg) and its antibodies (anti-HBe) were tested by enzyme-linked immunosorbent assay (ELISA) using commercial kits (DiaSorin, Italy).

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

HBV DNA extraction and quantification

HBV DNA was extracted from 200 µL of serum samples using a QiaAmp DNA mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and the extracted DNA was eluted in 50 µL of elution buffer. HBV DNA was detected and quantified by real-time PCR assay [28].

HBV polymerase gene (RT region) characterization

We characterized the HBV polymerase sequences in all of the samples with detectable HBV DNA. Initially, a fragment (1,306 bp) of HBV polymerase covering the entire RT region (344 amino acids) was amplified by nested PCR. Platinum Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA) and the following primers were used in the PCR amplifications: PS3132F (5' CCT CCY GCH TCY ACC AAT CG 3'; nt 3132 – 3151) and 2920RM (5' ACG TCC CKG GHA GRA TCC AG 3'; nt 1417 – 1398) for the first round; and PS3201F (5' CAY CCH CAG GCM ATG CAG TGG 3'; nt 3201 – 3221) and P1285R (5' CWA GGA GTT CCG CAG TAT GG 3'; nt 1285 – 1266) for the second round. There were 35 cycles for both rounds, as follows: 94 °C for 30 seconds, 48 °C (first round) or 54 °C (second round) for 30 seconds, and 72 °C for 1 minute and 40 seconds. The amplified PCR products were purified using an ExoSAP-IT PCR Clean-up kit (GE Healthcare, Buckinghamshire, UK) and then sequenced using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Three inner and partially overlapping sequences were obtained in forward and reverse directions (six total) using the following three primer pairs: 1 - PS3201F and HBV477R (5' GGA CAV ACG GGC AAC ATA CCT T 3'; nt 477 – 456); 2 - L372 (5' - TCG YTG GAT GTR TCT GCG GCG TTT TAT - 3'; nt 370 – 396) and RADE2M (5' - TGR CAN ACY TTC CAR TCA ATN GG - 3'; nt 989 – 970); 3 - P781F (5' GAR TCC CTT TWT RCC KCT RTT ACC 3'; nt 781 – 804), and P1285R. We sequenced the DNA using an automated ABI 3500 DNA Sequencer (Applied Biosystems).

Genotyping and HBV polymerase sequence analysis

The quality of each electropherogram was evaluated using the Phred-Phrap software, and consensus sequences were obtained by aligning six sequences per patient using the web-based CAP3 software available on the Embrapa website (<http://asparagin.cenargen.embrapa.br/phph/>).

The sequences were aligned and edited using the BioEdit software (v. 7.0.8) and the integrated CLUSTALW program. HBV genotypes and subgenotypes were determined by phylogenetic analysis using published sequences available in the GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>) as reference sequences.

The analysis of mutations in the HBV polymerase RT region was limited to amino acids from domains A to E, including those located between domains B–C and C–D, where there are mutations associated with NA resistance. Each amino acid in the polymerase RT

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

region was numbered following the previously standardized genotype-independent numbering system [29].

For this analysis, the nucleotide sequences were translated to amino acids and the presence of specific mutations previously associated with NA resistance was investigated (mutations described in additional file 1). These mutations were classified as mutations conferring total or partial NA resistance; compensatory mutations; mutations suggested as potentially involved in adefovir or entecavir resistance. Moreover other amino acid substitutions in RT sequences were identified by comparing with the genotype/subgenotype-matched consensus sequence that was created considering the most frequent amino acid (> 70%) observed in the alignment of the HBV sequences obtained in this study and the reference sequences published in GenBank (alignment available from the authors on request) according with viral genotype/subgenotype. The number of sequences from GenBank used to create this alignment was: A1= 69, A2=51, A3=6, B1=17, B2=29, C2=21, D1=35, D2=22, D3=48, D4=36, E=17, F2a=18, F4=10, G=14.

Results

Virological characteristics of the study patients

HBV DNA was detectable in 741 (95.1%) treatment-naïve CHB patients analyzed and RT region was successfully amplified and/or sequenced in 702 of these samples, which were subjected to mutational analysis. These patients were from different regions of Brazil (Figure 1).

The mean HBV DNA level among these 702 samples was $3.8 \log_{10}$ IU/mL ($1.7 - 7.9 \log_{10}$ IU/mL). It was not possible to accurately measure the viral load in 60 samples because the HBV DNA levels were higher than the linear range of the assay ($> 8 \log_{10}$ UI/mL) and these samples were not considered for calculating this mean.

Among the patients with detectable HBV DNA, from 678 we had enough serum samples to determine HBeAg/anti-HBe serological status. HBeAg was negative and anti-HBe was positive in 84.1% (570/678) of the patients.

There was high genetic variability in the HBV samples analyzed in this study; seven genotypes and twelve subgenotypes were identified. HBV/A1 was the most frequent (63.7%, 447/702), followed by HBV/D3 (14.5%, 102/702). The remaining genotypes and subgenotypes were identified and their frequencies were as follows: A2 (3.3%, 23/702), A3 (0.1%, 1/702), B1 (0.1%, 1/702), B2 (0.1%, 1/702), C2 (0.9%, 6/702), D1 (0.9%, 6/702), D2 (4.6%, 32/702), D4 (5.1%, 36/702), D unclassified subgenotype (0.7%, 5/702), E (0.6%, 4/702), F2a (4.6%, 32/702), F4 (0.4%, 3/702), and G (0.4%, 3/702).

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

RT sequence variability

Based on the constructed genotype/subgenotype consensus sequences, 39% (276/702) of the samples had HBV with at least one mutation in the polymerase RT region. Most of these samples (66.3%, 183/276) had strains harboring mutations in only one of the RT domains or interdomains here analyzed. Mutations were observed in 56 of the 107 positions analyzed and among the regions examined, the C–D interdomain (amino acids 211–229) was most variable with mutations in 14 (73.7%) of its 19 positions

Table 1 and additional file 2 display the mutations found at these positions and their frequencies among the HBV strains characterized in this study. Considering all of the positions containing mutations, five (rt194, rt207, rt221, rt238, and rt253) exhibited higher polymorphism with four to six different mutations (Table 1 and additional file 2). In half of the positions (50%; 28/56), only one type of mutation was observed; 23.2% (13/56) exhibited two mutation types, and 5.4% (3/56) exhibited three mutation types (Table 1 and additional file 2). In 21 out of these 56 mutated positions, the mutations were associated with total (rt181, rt184, rt202, rt204, and rt250) or partial NA resistance (rt194); compensatory functions (rt80, rt173, rt180, and rt207); potentially related with adefovir resistance (rt84, rt85, rt214, rt215, rt217, rt218, rt233, rt237, rt238, and rt248), or entecavir resistance (rt219) (Table 1).

Nucleotide analogue-related resistance mutations

In this study, we identified HBV strains harboring mutations that confer NA resistance in 1.6% (11/702) of the patients. Moreover, some of these strains also had compensatory mutations. Table 2 shows the resistance mutations observed in these samples, alterations in the corresponding codon of the HBs, HBV DNA levels, subgenotype, and the NA resistance profile. HBV DNA with compensatory mutations alone (rtL180M/ rtV173L + rtL180M/ rtV207I) were found in 0.4% (3/702) of the patients.

Isolates harboring mutations potentially associated with adefovir resistance (rtS85A, rtL217R, rtI233V, rtN238T, rtN238D, rtN248H, rtV214A, rtQ215S) were identified in 7.7% (54/702) of the patients. Among these adefovir-related mutations, rtQ215S was the most frequent (13/54), followed by rtI233V (11/54). rtL217R was not considered a mutation in HBV/A2 cases because arginine (R) in this position is characteristic of HBV/A2 wild-type strain.

HBV strains with the rtS219A mutation were identified in 2.6% (16/702) of the patients. This mutation has been proposed as potentially related to entecavir resistance [30].

HBV samples from 7.3% (51/702) of the patients contained other type of amino acid substitutions at positions where previously described mutation related or potentially related with NA resistance have been found: rt84, rt181, rt194, rt214, rt215, rt218, rt219, rt233, rt237, rt238, and rt248 (Table 1).

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

Discussion

Using the largest sample size to date (n=702), we investigated the occurrence of HBV with NA resistance mutations among CHB treatment-naïve patients. To our knowledge, this is the largest published study analyzing resistance mutations throughout the world.

These patients were from different geographic regions in Brazil. Fifteen genotypes or subgenotypes were found among the patients, as follows: most (63.7%) of the strains were characterized as HBV/A1, followed by HBV/D3 (14.5%) subgenotype. HBV/A2, D2, D4, and F2a each had approximate frequencies ranging from 3.3 to 5.1% and the other strains identified (A3, B1, B2, C2, D1, D unclassified subgenotype, E, F4, and G) made up the remaining <5%. These results corroborate previously published data on HBV genotype distribution in Brazil and therefore confirm that the samples included in this study are representative of the HBV strains circulating within this country [31,32]. We also show that there is high genetic variability among HBV genotypes circulating in Brazil.

In the present study, we used population sequencing (direct PCR sequencing) to characterize the polymerase gene of the HBV strains, which detects the major population of HBV quasispecies [6,29]. Our results show that the presence of HBV strains harboring mutations in the polymerase RT region is frequent among treatment-naïve patients, as 39% (276/702) of them were infected by HBV with at least one mutation. On the other hand, patients infected by HBV strains with mutations that impact NA resistance are less frequent. Only 1.6% (11/702) of the patients had HBV strains with mutations associated with NA resistance alone (rtS202G, rtM204V/I, rtA194T, rtM250I, rtA181T/S, and rtT184S) or together with compensatory mutations (rtL80I, rtV173L, rtL180M, and rtV207I). The mutation patterns in the HBV strains in nine of these patients confer total or partial resistance to lamivudine, adefovir, entecavir, or tenofovir (Table 2). We identified mutations (rtT184S or rtM250I) related with entecavir resistance in two patients [33], which when combined with the presence of rtM204V/I has clinical effects, but this mutation was not observed in these cases. Whether these patients would develop resistance to entecavir if they were treated with this drug remains unknown.

Recent studies using population sequencing with HBV treatment-naïve patients from different countries have described frequencies of HBV strains with drug resistance mutations (NA resistance) ranging from 0 to 5.1%, only considering mutations with clear clinical significance [8–15,17–23,26]. Just one study in South Africa that analyzed the presence of lamivudine-resistance mutations (at the YMDD motif) in samples from untreated HBV mono-infected and HBV/HIV co-infected patients found a higher frequency of drug-resistant HBV strains: 20% in mono-infected and 50% in co-infected patients [34]. We may suggest that the presence of HBV drug resistance mutations is very different in countries with high HIV prevalence compared to those where this infection is less frequent. On the other hand, Thio and collaborators verified that the mutation pattern found in most samples tested in the South

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

African study was identical; suggesting that cross contamination may explain the high prevalence found for lamivudine-resistant strains. In this other study, analyzing 115 samples from HIV-HBV co-infected patients from 11 countries from Africa, Asia and Americas, they did not find any case with NA resistance mutations [35].

HBV DNA replication involves reverse transcription of an intermediate RNA, allowing for mismatches to occur because HBV polymerase lacks a proofreading function. NA treatment causes selection pressure for NA-related mutations during this process [6]. However, HBV strains harboring resistance mutations in untreated CHB patients may spontaneously arise and become dominant via other selection pressures (e.g., immunological pressure exerted on the overlapping surface antigen expressing gene) or may be directly acquired as the infecting strain from a patient under antiviral treatment. A study using pyrosequencing characterized the HBV population in NA-treated patients (86.4% LAM and 13.6% LAM + ADV or TDF) and found that the wild-type virus remained undetected in 76% of the patients carrying drug-resistant strains [36]. These results suggest that HBV carriers undergoing NA treatment can be sources of infection for susceptible individuals, transmitting exclusively HBV strains that are drug-resistant mutants. The ability to transmit HBV with rtV173L+rtL180M+rtM204V mutations that confer LAM resistance was demonstrated in a chimpanzee model and in other studies reporting acute infection with drug-resistant HBV strains [7,16,24,25].

The selection of compensatory mutations improves the replication in resistant viruses and establishes resistance mutations in the HBV genetic archive in the hepatocyte nuclei as covalent closed circular DNA (cccDNA) [37], explaining how resistant strains may be maintained and transmitted to other patients. The higher replication levels of these resulting HBV strains in patients undergoing continued therapy might increase the viral load, favoring transmission of resistant strains with higher fitness and increasing the odds of perpetuating them within those newly-infected.

Nevertheless, in the absence of selective pressure, HBV strains harboring resistance mutations seem to persist for a shorter time, explaining their lower frequency even as minor population in non-NA-treated patients. Margeridon-Thermet and collaborators demonstrated that HBV lamivudine-resistant strains showed shorter persistence after lamivudine withdrawal and the likelihood to detect a lamivudine resistance mutation was inversely associated with the time span after lamivudine discontinuation [38].

In untreated patients, drug-resistant HBV variants may affect the efficacy and outcome of NA treatment since its introduction. Despite their low frequency, it is important to consider that primary treatment failure may result from pre-existing drug-resistant HBV variants, which spread in the patients can be continually assessed.

Monitoring the spread of these mutants is also important because of their ability to infect hepatitis B vaccinated individuals. Some nucleotide mutations in the overlapping genes S and POL can alter the amino acids in polymerase and HBs proteins. These changes occur

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

more frequently at the carboxy-terminus of HBsAg, some of them leading to altered viral secretion or infectivity, and HBs antibody escape [6,39]. Among the HBV strains harboring NA resistance and/or compensatory mutations identified in this study, we found mutations in the HBs protein (sE164D, sW172STOP, sI195M, sW196S, sW196STOP, sM198I, and sW199L) that are related to reduced binding to HBs antibodies [40].

Adefovir resistance is mainly mediated by mutations rtN236T or rtA181V/T [29], but several other mutations (rtL80V/I, rtV84M, rtS85A, rtV214A, rtQ215S, rtL217R, rtE218G, rtI233V, rtP237H, rtN238T/D/R, rtT240Y, and rtN248H) have also been suggested as potentially involved [41–46]. Seven (rtS85A, rtV214A, rtQ215S, rtL217R, rtI233V, rtN238T, rtN238D, and rtN248H) of these 14 mutations were found in HBV strains isolated from treatment-naïve patients included in this study. HBV strains harboring any of these mutations were found in 7.7% (54/702) of the patients and among them the mutations rtQ215S and rtI233V occurred more frequently, 1.9% and 1.6%, respectively. Other studies have found these mutations in treatment-naïve patients and some are suggested to represent natural polymorphisms common in some genotypes [47–49]. The rtL217R mutation is characteristic of HBV/A2 subgenotype and alterations at rt215 and rt233 positions seem more common among HBV genotype D [47,50]. In our study, we also observed that HBV genotype D sequences exhibited more frequent alterations at these positions and at others related to adefovir resistance, i.e., positions rt214 and rt238. Finally, other clinical studies have demonstrated that response to adefovir treatment remained unaffected by the presence of some of these mutations at baseline, and *in vitro* tests showed that they neither impair viral replication efficiency nor susceptibility to adefovir [47,48,51]. As adefovir, a less potent drug, was commonly used in North America and Europe, where genotype D was very frequent, it is not surprising that many mutations were initially described and most likely only represent viral polymorphisms.

Additionally we observed different amino acid substitutions at positions rt84, rt181, rt194, rt214, rt215, rt218, rt219, rt233, rt237, rt238, and rt248 than those usually described (Table 1): fifty-one (7.3%) patients were infected with HBV strains harboring some uncommon mutations at these amino acid positions.

The role of the aforementioned mutations in NA treatment failure remains unclear and needs to be clarified, considering the genetic background of specific strains. Some studies have shown that individual mutations, although fundamentally crucial, are not enough to explain varied therapy results [52]. Similarly, the role of the rtS219A mutation in entecavir resistance must be further evaluated because it was found in HBV strains from 2.6% of the treatment-naïve patients included in our study.

In summary, this study showed that HBV strains harboring resistance mutations can comprise the major population of HBV quasispecies in treatment-naïve patients. In Brazil, there is a very low frequency of untreated patients who are infected with these strains. These

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

findings suggest that the spread and natural selection of drug-resistant HBV is an uncommon event and/or most of these strains remain unstable in the absence of NA selective pressure.

This study generates important information about the characteristics of RT sequences of HBV strains circulating in some Brazilian regions among treatment-naïve patients. This information will be useful in evaluating the treatment guidelines and determining if the mutations identified in a specific HBV sequence isolated from a CHB patient are common polymorphisms or represent potentially new drug resistance mutations.

Acknowledgement statement

This work was supported by FAPESP (São Paulo Research Foundation): 2010/50081-9 and 2010/51208-2 and Alves de Queiroz Family Fund for Research

Disclosure statement

The authors declare no competing interests.

References

1. Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: a global overview. *Clin Liver Dis* 2010; 14:1–21 [vii].
2. Lok AS, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; 50:661–662.
3. Pereira LM, Martelli CM, Merchan-Hamann E, et al. Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am J Trop Med Hyg* 2009; 81:240–247.
4. Ministério da Saúde, Departamento de DST, Aids e Hepatites Virais Secretaria de Vigilância em Saúde. HEPATITES VIRAIS NO BRASIL: SITUAÇÃO, AÇÕES. *Agenda* 2011.
5. Ministério da Saúde, Departamento de DST, Aids e Hepatites Virais. Protocolo Clínico e Diretrizes Terapêuticas para o Tratamento da Hepatite Viral Crônica B e Coinfecções. 2011.
6. Zoulim F, Locamini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009; 137:1593–1608 e1591–1592.
7. Kamili S, Sozzi V, Thompson G, et al. Efficacy of hepatitis B vaccine against antiviral drug-resistant hepatitis B virus mutants in the chimpanzee model. *Hepatology* 2009; 49:1483–1491.
8. Alvarez Estevez M, Chueca-Porcuna N, Guillot-Suay V, Pena-Monje A, Garcia-Garcia F, Garcia-Garcia F. [Low prevalence of hepatitis B virus primary drug resistance in Southern Spain]. *Enferm Infecc Microbiol Clin* 2013; 31:520–522.
9. Han Y, Huang LH, Liu CM, et al. Characterization of hepatitis B virus reverse transcriptase sequences in Chinese treatment naïve patients. *J Gastroenterol Hepatol* 2009; 24:1417–1423.
10. Ismail AM, Samuel P, Eapen CE, Kannangai R, Abraham P. Antiviral resistance mutations and genotype-associated amino acid substitutions in treatment-naïve hepatitis B virus-infected individuals from the Indian subcontinent. *Intervirology* 2012; 55:36–44.
11. Jardi R, Rodriguez-Frias F, Schaper M, et al. Hepatitis B virus polymerase variants associated with entecavir drug resistance in treatment-naïve patients. *J Viral Hepat* 2007; 14:835–840.
12. Li XG, Liu BM, Xu J, Liu XE, Ding H, Li T. Discrepancy of potential antiviral resistance mutation profiles within the HBV reverse transcriptase between nucleos(t)ide analogue-untreated and -treated patients with chronic hepatitis B in a hospital in China. *J Med Virol* 2012; 84:207–216.

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

13. Liu BM, Li T, Xu J, *et al.* Characterization of potential antiviral resistance mutations in hepatitis B virus reverse transcriptase sequences in treatment-naïve Chinese patients. *Antiviral Res* 2010; 85:512–519.
14. Mantovani N, Cicero M, Santana LC, *et al.* Detection of lamivudine-resistant variants and mutations related to reduced antigenicity of HBsAg in individuals from the cities of Santos and Sao Paulo, Brazil. *Viral J* 2013; 10:320.
15. Mohebbi SR, Amini-Bavil-Olyaei S, Zali N, *et al.* Characterization of hepatitis B virus genome variability in Iranian patients with chronic infection, a nationwide study. *J Med Virol* 2012; 84:414–423.
16. Morando F, Rosi S, Fasolato S, *et al.* Severe acute hepatitis B in a treatment-naïve patient with antiviral drug resistant mutations in the polymerase gene. *J Med Virol* 2013; 85:210–213.
17. Nguyen MH, Garcia RT, Trinh HN, *et al.* Prevalence of hepatitis B virus DNA polymerase mutations in treatment-naïve patients with chronic hepatitis B. *Aliment Pharmacol Ther* 2009; 30:1150–1158.
18. Pollicino T, Isgro G, Di Stefano R, *et al.* Variability of reverse transcriptase and overlapping S gene in hepatitis B virus isolates from untreated and lamivudine-resistant chronic hepatitis B patients. *Antivir Ther* 2009; 14:649–654.
19. Salpini R, Svicher V, Cento V, *et al.* Characterization of drug-resistance mutations in HBV D-genotype chronically infected patients, naïve to antiviral drugs. *Antiviral Res* 2011; 92:382–385.
20. Sayan M, Akhan SC. Antiviral drug-associated potential vaccine-escape hepatitis B virus mutants in Turkish patients with chronic hepatitis B. *Int J Infect Dis* 2011; 15:e722–e726.
21. Sayan M, Akhan SC, Meric M. Naturally occurring amino-acid substitutions to nucleos(t)ide analogues in treatment naïve Turkish patients with chronic hepatitis B. *J Viral Hepat* 2010; 17:23–27.
22. Sayan M, Senturk O, Akhan SC, Hulagu S, Cekmen MB. Monitoring of hepatitis B virus surface antigen escape mutations and concomitantly nucleos(t)ide analog resistance mutations in Turkish patients with chronic hepatitis B. *Int J Infect Dis* 2010; 14 Suppl 3:e136–e141.
23. Singla B, Chakraborti A, Shama BK, *et al.* Hepatitis B virus reverse transcriptase mutations in treatment Naïve chronic hepatitis B patients. *J Med Virol* 2013; 85:1155–1162.
24. Trevino A, Soriano V, Madejon A, *et al.* Short communication: transmission of hepatitis B viruses with lamivudine resistance mutations in newly diagnosed HIV individuals. *AIDS Res Hum Retroviruses* 2009; 25:1273–1276.
25. Xu Z, Liu Y, Xu T, *et al.* Acute hepatitis B infection associated with drug-resistant hepatitis B virus. *J Clin Virol* 2010; 48:270–274.
26. Zheng J, Zeng Z, Zhang D, Yu Y, Wang F, Pan CQ. Prevalence and significance of Hepatitis B reverse transcriptase mutants in different disease stages of untreated patients. *Liver Int* 2012; 32:1535–1542.
27. Mello FC, Fernandes CA, Gomes Sde A. Antiviral therapy against chronic hepatitis B in Brazil: high rates of lamivudine resistance mutations and correlation with HBV genotypes. *Mem Inst Oswaldo Cruz* 2012; 107:317–325.
28. Sitnik R, Paes A, Mangueira CP, Pinho JRR. A Real-Time Quantitative Assay for Hepatitis B DNA Virus (Hbv) Developed to Detect All Hbv Genotypes. *Rev Inst Med Trop Sao Paulo* 2010; 52:119–124.
29. Lok AS, Zoulim F, Locamini S, *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; 46:254–265.

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

30. Karatayli E, Karatayli SC, Cinar K, *et al.* Molecular characterization of a novel entecavir mutation pattern isolated from a multi-drug refractory patient with chronic hepatitis B infection. *J Clin Virol* 2012; 53:130–134.
31. Mello FC, Souto FJ, Nabuco LC, *et al.* Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol* 2007; 7:103.
32. Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Carrilho FJ. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 2004; 42:2455–2460.
33. Tenney DJ, Levine SM, Rose RE, *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob Agents Chemother* 2004; 48:3498–3507.
34. Selabe SG, Lukhwareni A, Song E, Leeuw YG, Burnett RJ, Mphahlele MJ. Mutations associated with lamivudine-resistance in therapy-naïve hepatitis B virus (HBV) infected patients with and without HIV co-infection: implications for antiretroviral therapy in HBV and HIV co-infected South African patients. *J Med Virol* 2007; 79:1650–1654.
35. Thio CL, Smeaton L, Saulynas M, *et al.* Characterization of HIV-HBV coinfection in a multinational HIV-infected cohort. *AIDS* 2013; 27:191–201.
36. Mello FC, Lago BV, Lewis-Ximenez LL, Fernandes CA, Gomes SA. Detection of mixed populations of wild-type and YMDD hepatitis B variants by pyrosequencing in acutely and chronically infected patients. *BMC Microbiol* 2012; 12:96.
37. Delaney WET, Yang H, Westland CE, *et al.* The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 2003; 77:11833–11841.
38. Margeridon-Thermet S, Svarovskaia ES, Babrzadeh F, *et al.* Low-level persistence of drug resistance mutations in hepatitis B virus-infected subjects with a past history of Lamivudine treatment. *Antimicrob Agents Chemother* 2013; 57:343–349.
39. Yeh CT. Development of HBV S gene mutants in chronic hepatitis B patients receiving nucleotide/nucleoside analogue therapy. *Antivir Ther* 2010; 15:471–475.
40. Torresi J, Earnest-Silveira L, Deliyannis G, *et al.* Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. *Virology* 2002; 293:305–313.
41. Bartholomeusz A, Locamini SA. Antiviral drug resistance: clinical consequences and molecular aspects. *Semin Liver Dis* 2006; 26:162–170.
42. Lee YS, Chung YH, Kim JA, *et al.* rtL180M mutation of hepatitis B virus is closely associated with frequent virological resistance to adefovir dipivoxil therapy. *J Gastroenterol Hepatol* 2012; 27:300–305.
43. Liu LJ, Wang JH, Du SC, Tian JH, Yang RF, Wei L. rtE218G, a novel hepatitis B virus mutation with resistance to adefovir dipivoxil in patients with chronic hepatitis B. *J Viral Hepat* 2010; 17 Suppl 1:66–72.
44. Qin B, Pei RJ, He TT, *et al.* Polymerase mutations rtN238R, rtT240Y and rtN248H of hepatitis B virus decrease susceptibility to adefovir. *Chin Sci Bull* 2013; 58:1760–1766.
45. Schildgen O, Olotu C, Funk A, *et al.* Selection and counterselection of the rtI233V adefovir resistance mutation during antiviral therapy. *J Clin Microbiol* 2010; 48:631–634.
46. Sheldon J, Rodes B, Zoulim F, Bartholomeusz A, Soriano V. Mutations affecting the replication capacity of the hepatitis B virus. *J Viral Hepat* 2006; 13:427–434.
47. Amini-Bavil-Olyaei S, Herbers U, Mohebbi SR, *et al.* Prevalence, viral replication efficiency and antiviral drug susceptibility of rtQ215 polymerase mutations within the hepatitis B virus genome. *J Hepatol* 2009; 51:647–654.
48. Borroto-Esoda K, Miller MD, Arterburn S. Pooled analysis of amino acid changes in the HBV polymerase in patients from four major adefovir dipivoxil clinical trials. *J Hepatol* 2007; 47:492–498.

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

49. De Maddalena C, Giambelli C, Tanzi E, *et al.* High level of genetic heterogeneity in S and P genes of genotype D hepatitis B virus. *Virology* 2007; 365:113–124.
50. Bottecchia M, Madejon A, Sheldon J, Garcia-Samaniego J, Barreiro P, Soriano V. Hepatitis B virus genotype A2 harbours an L217R polymorphism which may account for a lower response to adefovir. *J Antimicrob Chemother* 2008; 62:626–627.
51. Curtis M, Zhu Y, Borroto-Esoda K. Hepatitis B virus containing the I233V mutation in the polymerase reverse-transcriptase domain remains sensitive to inhibition by adefovir. *J Infect Dis* 2007; 196:1483–1486.
52. Khudyakov Y. Coevolution and HBV drug resistance. *Antivir Ther* 2010; 15:505–515.

Figure legends

Figure 1 – Sample contribution (%) according to Brazilian states.

Publication: Antiviral Therapy; Type: Original article

DOI: 10.3851/IMP2938

Table 1- Frequency of patients infected with HBV strains harboring NA resistance mutations in RT region of polymerase among 702 treatment-naïve CHB studied

RT Region	Position	Wild-type aa	Mutation*	N	Frequency (%)
Domain A (aa 75 - 91)	80	L	<u>I</u>	1	0.1
	84	V	I	1	0.1
	85	S	A	2	0.3
Domain B (aa 163 - 189)	173	V	<u>L</u>	2	0.3
	180	L	<u>M</u>	5	0.7
	181	A	<u>I</u>	1	0.1
			S	1	0.1
	184	T	<u>S</u>	1	0.1
Interdomain B - C (aa 190 - 199)	194	A	<u>I</u>	2	0.3
			V	1	0.1
			S	1	0.1
			G	1	0.1
			M	1	0.1
Domain C (aa 200 - 210)	202	S	<u>G</u>	2	0.3
	204	M	<u>V</u>	3	0.4
			<u>I</u>	2	0.3
	207	V/L*	M	4	0.6
			I	2	0.3
Interdomain C-D (aa 211 - 229)	214	V	A	8	1.1
			E	6	0.9
			G	2	0.3
	215	Q	H	18	2.6
			S	13	1.9
			P	4	0.6
	218	E	D	4	0.6
	219	S	A	16	2.3
			T	1	0.1
	229	L	M	7	1.0
Domain D (aa 230 - 241)			V	3	0.4
			V/D	1	0.1
	233	I	V	11	1.6
			L	1	0.1
	237	P/T*	A	1	0.1
	238	N/H [§] /S*	T	7	1.0

Publication: Antiviral Therapy; Type: Original article
DOI: 10.3851/IMP2938

			A	1	0.1
			E	1	0.1
			D	1	0.1
Domain E (aa	248	N/H [§]	Q	5	0.7
247 - 257)	250	M	<u>I</u>	1	0.1

*Mutations are shown according to their role described in additional file 1: mutations conferring total or partial NA resistance (**bold and underlined**); compensatory mutations (underlined); mutations suggested as potentially involved in adefovir or entecavir resistance (**bold**). The other mutations represent new mutation patterns observed at these positions that were not related with resistance.

* Wild-type aa in F2a and F4 sequences; [§] Wild-type aa in B1 and B2 sequences; [§] Wild-type aa in B1, B2, F2a and F4 sequences.

Table 2 – Virological and resistance profile of patients infected with hepatitis B virus (HBV) strains harboring mutations conferring resistance to nucleo(s)ide analogue (NA) treatment

Samples	Viral load (log ₁₀ UI/ml)	Subgenotype	Resistance mutation in HBV polymerase RT region	HBsAg corresponding changes	HBeAg/ Anti-HBe status	Resistance profile			
						LMV	ADV	ETV	TDF
MA_8052	2.3	D3	rtL180M + rtS202G + rtM204V	sl195M	Neg/Pos	R	S	R	S
MA_8075	2.3	D3	rtL180M + rtS202G + rtM204V	sl195M	Neg/Pos	R	S	R	S
MA_8130	2.7	A1	rtA194T	no change in HBsAg	Neg/Pos	I	S	S	I
MG_206	4.2	F2a	rtL180M + rtM204I	sW196S	Pos/Neg	R	S	I	S
PA_16726	3.9	F2a	rtM250I	after the end of HBsAg	Neg/Pos	S	S	S	S
PA_16727	ND	A1	rtL180M + rtM204V + rtV207I	sl195M + sM198I + sW199L	Neg/Pos	R	S	I	S
PA_17071	6.0	A1	rtA181T + rtD205N	sW172STOP + sW196STOP	Neg/Pos	R	R	S	I
RP_54	2.2	D3	rtA181S	sW172F	Neg/Pos	R	R	S	I
SP_125	8.3	D2	rtL80I + rtM204I	sW196L	Pos/Neg	R	S	I	S
SP_15	2.7	F2a	rtA194T	no change in HBsAg	Neg/Pos	I	S	S	I
SP_81	3.3	A1	rtT184S	sL176V	Neg/Pos	S	S	S	S

Abbreviations:

S= sensitive; R= resistant; I= intermediary; ND= not determined

Additional file 1 – Mutations previously associated with nucleos(t)ide analogues resistance

RT region (Domain)	Position	Nucleos(t)ide analogues resistance			
		Lamivudine	Adefovir	Entecavir	Tenofovir
A	80	L80V/I	L80V/I		
	84		V84M		
	85		S85A		
B	169			I169T*	
	173	V173L			
	177				P177G
	180	L180M/C	L180M		
	181	A181T/V/S	A181T/V/S		A181T/V/S
	184			T184G/A/I/S/C/L/F/M*	
Interdomain B-C	194	A194T			A194T
C	200	A200V			
	202			S202G/I/C/A*	
	204	M204V/I/S		M204V/I/S	
	207	V/F/L/M207I			
Interdomain C-D	214	V214A	V214A		
	215	Q215S/ Q215S	Q215S		
	217	L217P	L217R		
	218		E218G		
	219			S219A	
	229	L229F			
D	233		I233V		
	236		N236T		N236T
	237		P237H		
	238		N238T/D/R		
	240		T240Y		
E	245			Y245H	
	248		N248H		
	249				P249A
	250			M250V/I/L/C*	

Mutations are highlight according its role in nucleos(t)ide analogues resistance:

Red: mutations conferring total or partial NA resistance;

Green: compensatory mutations;

Blue: mutations suggested as potentially involved in adefovir or entecavir resistance

Additional file 2 - Mutations not associated with nucleo(s)tide analogue (NA) resistance in hepatitis B virus (HBV) polymerase gene (RT region – domains A–E and interdomains B–C, C–D) from HBV DNA samples isolated from 702 treatment-naïve chronic hepatitis B (CHB) patients.

RT Region	Position	HBV/A1 (N=447)	HBV/A2 (N=23)	HBV/D2 (N=32)	HBV/D3 (N=102)	HBV/D4 (N=36)	HBV/F2a (N=32)	Total (702)
Domain A (aa 75 - 91)	76	N D ^{1.8}	N	N D ^{3.1}	N D ^{7.8}	N	N	N D ^{2.7}
	78	S	S	S T ^{3.1}	S	S Y ^{2.8}	S T ^{8.3}	S T ^{0.4} Y ^{0.1}
	79	W V ^{0.2}	W	W	W	W	W	W V ^{0.1}
	88	F	F	F	F	F Y ^{2.8}	F	F Y ^{0.1}
	91	I	I	L I ^{15.6}	L I ^{23.5}	L	L	I L
Domain B (aa 163 - 189)	163	I	V	I	I V ^{3.9}	I V ^{8.3}	I	I V
	164	L M ^{1.1}	L	L	L M ^{5.9}	L M ^{8.3}	L	L M
	175	L I ^{0.2}	L	L	L	L	L	L I ^{0.1}
	183	F L ^{0.7}	F	F	F	F	F	F L ^{0.4}
	185	S G ^{0.2}	S	S G ^{8.3}	S G ^{1.0}	S	S	S G ^{0.6} T ^{0.1}
	186	A S ^{0.4} T ^{0.2}	A	A	A S ^{2.0}	A	A	A S ^{0.6} T ^{0.1}
	187	I L ^{1.8} V ^{0.7}	I L ^{4.3}	I L ^{8.3}	I L ^{2.9} V ^{2.9}	I	I	I L ^{2.1} V ^{0.9}
	188	C Y ^{0.2}	C	C	C	C	C	C Y ^{0.1}
Interdomain B - C (aa 190 - 199)	190	V	V	V	V M ^{1.0}	V	V M ^{3.1}	V M ^{0.3}
	191	V I ^{0.7}	V I ^{8.7}	V	V	V	V	V I ^{0.9}
	197	H N ^{0.2}	H	H	H	H	H	H N ^{0.1}
	199	L V ^{0.2}	L	L V ^{3.1}	L	L	L	L V ^{0.3}

First column shows the RT region analyzed; column 2 shows the positions where mutations were found; columns 3–8 show the amino acid found at each position according to genotype or subgenotype more frequent in this study and the superscripts represent the frequency in percentage of each mutation; column 9 shows the amino acid at each position and the overall frequency in percentage for each mutation (superscripts). The consensus amino acids for each genotype or subgenotype are bolded on the first line of each position and the mutations are shown in black.

Additional file 2 – Cont.

RT Region	Position	HBV/A1 (N=447)	HBV/A2 (N=23)	HBV/D2 (N=32)	HBV/D3 (N=102)	HBV/D4 (N=36)	HBV/F2a (N=32)	Total (702)
Domain C (aa 200 - 210)	205	D N ^{0.2}	D	D	D	D	D	D N ^{0.1}
	209	L	L	L	L	L	L	L V ^{0.1}
Interdomain C-D (aa 211 - 229)	211	A T ^{0.4}	A	A T ^{6.3}	A T ^{2.0}	A	A	A T ^{1.0}
	212	K R ^{1.1}	K	K	K N ^{2.9}	K	K	K R ^{0.7} N ^{0.4}
	213	S T ^{0.9}	S T ^{4.3}	S T ^{6.3}	S T ^{1.0}	S T ^{5.6}	S	S T ^{1.7}
	220	L I ^{0.4}	L	L	L I ^{1.0}	L	L	L I ^{0.6}
	221	Y F ^{0.7} C ^{0.2}	Y	F Y ^{6.3}	F Y ^{5.9} S ^{1.0}	F Y ^{2.8}	Y	F Y ^{0.1} S ^{0.1} C ^{0.1}
	222	T A ^{0.2} S ^{0.2}	T	T S ^{3.1}	T S ^{1.0}	T	T	T A ^{0.4} S ^{0.4}
	225	T S ^{0.2}	T A ^{4.3}	T	T S ^{2.0}	T	T	T S ^{0.4} I ^{0.1} A ^{0.1}
	226	N T ^{0.4}	N	N	N	N	N	N T ^{0.3}
Domain D (aa 230 - 241)	231	L	L	L	L	L	V	L V
	234	H Q ^{0.7}	H	H	H N ^{1.0}	H	H	H Q ^{0.4} N ^{0.1}
	235	L	L	L	L I ^{2.9}	L	L	L I ^{0.6}
	241	K Q ^{0.4} R ^{0.2}	K	K	K R ^{2.0}	K	K	K R ^{0.4} Q ^{0.3}
Domain E (aa 247 - 257)	253	V I ^{12.8} A ^{0.4} T ^{0.4}	I	V S ^{3.1}	V I ^{1.0}	V	V	V I ^{0.3} A ^{0.3} T ^{1.1} E ^{0.1} S ^{0.1}
	256	S	S	C S ^{3.1}	C S ^{10.8} G ^{3.9}	C S ^{5.6} G ^{2.8}	S	S C ^{0.9} G ^{0.9}
	257	W	W	Y H ^{3.1} F ^{3.1} Q ^{3.1}	Y H ^{9.8}	W	W	W Y ^{1.6} H ^{0.3} F ^{0.1} Q ^{0.1}

- 12- Maria Cássia Mendes-Correa, JOÃO RENATO REBELLO PINHO, Roberta Sitnik, Rúbia Anita Ferraz Santana, Michele Mesquita Gomes-Gouvêa, Olavo M. Leite, Luiz G. Martins, Andrea G. Leite, Mariliza H. Silva, R.J.Gianini, David E. Uip. High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. Journal of Medical Virology, 2010. Sep; 82 (9):1481-8**

High Frequency of Lamivudine Resistance Mutations in Brazilian Patients Co-Infected With HIV and Hepatitis B

M.C. Mendes-Correa,^{1*} J.R.R. Pinho,^{2,3} S. Locarnini,⁴ L. Yuen,⁴ R. Sitnik,² R.A.F. Santana,² M.S. Gomes-Gouveia,³ O.M. Leite,¹ L.G. Martins,⁵ M.H. Silva,⁵ R.J. Gianini,⁶ and D.E. Uip¹

¹Infectious Diseases Research Unit, ABC Foundation-Medical School, São Paulo, Brazil

²Hospital Israelita Albert Einstein, São Paulo, Brazil

³Tropical Medicine Institute and Department of Gastroenterology, Medical School, University of São Paulo, São Paulo, Brazil

⁴Victorian Infectious Diseases Reference Laboratory, North Melbourne, Victoria, Australia

⁵Clinica Municipal de Especialidades Médicas, São Bernardo do Campo, Brazil

⁶Laboratory of Medical Investigation in Epidemiology and Statistics, Medical School, University of São Paulo, São Paulo, Brazil

This study analyzed the genotype distribution and frequency of lamivudine (LAM) and tenofovir (TDF) resistance mutations in a group of patients co-infected with HIV and hepatitis B virus (HBV). A cross-sectional study of 847 patients with HIV was conducted. Patients provided blood samples for HBsAg detection. The load of HBV was determined using an "in-house" real-time polymerase chain reaction. HBV genotypes/subgenotypes, antiviral resistance, basal core promoter (BCP), and precore mutations were detected by DNA sequencing. Twenty-eight patients with co-infection were identified. The distribution of HBV genotypes among these patients was A (n = 9; 50%), D (n = 4; 22.2%), G (n = 3; 16.7%), and F (n = 2; 11.1%). Eighteen patients were treated with LAM and six patients were treated with LAM plus TDF. The length of exposure to LAM and TDF varied from 4 to 216 months. LAM resistance substitutions (rtL180M + rtM204V) were detected in 10 (50%) of the 20 patients with viremia. This pattern and an accompanying rtV173L mutation was found in four patients. Three patients with the triple polymerase substitution pattern (rtV173L + rtL180M + rtM204V) had associated changes in the envelope gene (sE164D + sI195M). Mutations in the BCP region (A1762T, G1764A) and in the precore region (G1896A, G1899A) were also found. No putative TDF resistance substitution was detected. The data suggest that prolonged LAM use is associated with the emergence of particular changes in the HBV genome, including substitutions that may elicit a vaccine escape phenotype. No putative

TDF resistance change was detected after prolonged use of TDF. *J. Med. Virol.* 82:1481–1488, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: hepatitis B; resistance mutations; lamivudine; tenofovir; HIV

INTRODUCTION

Studies in North America, Europe, and Australia have shown that between 5% and 10% of patients with HIV are co-infected with the hepatitis B virus (HBV) [Soriano et al., 2008]. In Brazil, the prevalence of co-infection with these viruses ranges from 3.4% to 8.5% [Mendes-Correa et al., 2000; Oliveira et al., 2001; Monteiro et al., 2004; Souza et al., 2004; Braga et al., 2006; Tovo et al., 2006; Zago et al., 2007].

Brazil's AIDS treatment program has been cited widely as the developing world's largest and most successful AIDS treatment program. The program guarantees free access to highly active antiretroviral therapy (HAART) for all persons living with HIV/AIDS in need of treatment. In 2006, of the estimated 600,000 individuals with HIV/AIDS in Brazil, 180,000 were treated with antiretroviral drugs and had access to

*Correspondence to: M.C. Mendes-Correa, Rua Capote Valente, n. 432-cj.145, 05409-001 São Paulo, Brazil.
E-mail: cassiamc@uol.com.br

Accepted 20 April 2010

DOI 10.1002/jmv.21845

Published online in Wiley InterScience
(www.interscience.wiley.com)

comprehensive medical care, CD4+ lymphocyte counts, viral loads, and genotyping [Greco and Simão, 2007].

Given the widespread use of lamivudine (LAM) for anti-HIV therapy since the mid-1990s, many Brazilian patients with HIV and HBV co-infection have received prolonged LAM therapy. LAM is a nucleoside analog that has both anti-HIV and anti-HBV activity. Tenofovir (TDF), a nucleotide analog, is also active against HBV and HIV and has been introduced more recently for treatment of HIV in Brazil.

The consequences of drug resistance-associated substitutions in the evolution and treatment of HBV-related liver disease are under study. The clinical relevance of hepatitis B genotypes is not completely clear at present. A few studies have suggested that HBV genotypes and subgenotypes may influence the clinical outcome, prognosis, and response to interferon treatment in some parts of the world [Cao, 2009].

The aims of the present study were to analyze the genotype distribution and frequency of LAM and TDF-associated resistance changes in a group of patients with HIV and HBV co-infection, and then to analyze the predictors of LAM and TDF resistance mutations including the HBeAg genotype and phenotype among these patients.

MATERIALS AND METHODS

Patients

A cross-sectional study was performed at an HIV/STD Referral Outpatient Clinic in São Bernardo do Campo (São Paulo State, Brazil) to identify patients with HIV and HBV co-infection.

All patients with HIV followed at this Institution were included. From November 2006 to November 2007, medical records were reviewed to identify patients with HBsAg. Patients were selected if they had both anti-HIV antibodies confirmed by Western blot and persistent serum HBsAg for >6 months during follow-up at the Institution. Identified patients were then invited to come for an interview, and a blood sample was collected for additional hepatitis B serological testing and virological analysis.

Medical records were reviewed to analyze clinical, demographic, and serological characteristics. Data were obtained on age, sex, CD4+ T-cell count (current and nadir), use of antiretroviral therapy, plasma HIV-RNA level, TDF and LAM therapy, and duration of exposure to these drugs, HBeAg reactivity, HBV viral load, HBV genotype, alanine aminotransferase (ALT) level, LAM or TDF resistance-associated mutations, and compliance with therapy.

The use of antiretroviral therapy (including TDF or LAM) was defined as the use of any antiretroviral medication for >6 months, any time prior to baseline. Compliance with therapy was assessed by the assistant physician: Patients were considered compliant when their medical records indicated that they had used at least 80% of the medication prescribed during scheduled medical visits.

The Local Ethics Research Board approved the study protocol. All patients who participated in the study signed an informed consent form.

Laboratory Testing

Hepatitis B serological testing was performed according to standard procedures and using licensed assays. Serological markers for HBV included HBsAg, total anti-HBc, anti-HBs, HBeAg, and anti-HBe.

ALT level was examined at baseline and on at least at two separate occasions during the 12 months before baseline. The mean ALT level was calculated using the sum of all ALT values. A normal ALT level was defined as at least three consecutive normal ALT determinations during a follow-up of 12 months.

HBV-DNA was extracted from plasma collected in PPT tubes using the QiaAmp DNA minikit (Qiagen, Hilden, Germany). HBV viral load was determined by real-time polymerase chain reaction (PCR) assay using primers that equally amplified HBV genotypes A–H. The PCR assay had a detection limit of 50 IU/ml and viral loads were quantified up to 1,000,000 IU/ml. HBV-DNA quantitation was standardized using reference plasmas developed by BBI Diagnostics (Boston, MA), namely, PHD801.

The design of the PCR primers and probes meant that they equally amplified all known HBV genotypes. An alignment with the sequences of all HBV genotypes described in a previous study of Brazilian patients was carried out and a region of the S gene that is well-conserved among various genotypes was selected [Sitnik et al., 2004]. PCR primers and probes were then designed using Primer Express software (Applied Biosystems, Foster City, CA). The primers had previously been used for HBV detection in liver tissue [Alencar et al., 2008].

Amplification was performed in a 50 µl reaction mixture containing 1× TaqMan Universal PCR Master Mix, 20 µM of each primer and probe, and 5 µl of extracted DNA. All reactions were performed in duplicate. The TaqMan Exogenous Internal Positive Control Reagents kit with 1× IPC Mix (primers and TaqMan probe labeled with VIC) and 1× IPC DNA was used as an internal control. All reagents were obtained from Applied Biosystems.

Absolute quantification of HBV-DNA was performed with an ABI PRISM 7500 Real-Time PCR machine. A high-HBV-DNA viral load was defined as a value $\geq 20,000$ IU/ml for HBeAg-positive patients and $\geq 2,000$ IU/ml for HBeAg-negative patients. Low-HBV-DNA was defined as a value $< 20,000$ IU/ml for HBeAg-positive patients and $< 2,000$ IU/ml for HBeAg-negative patients.

Samples that were positive for HBV-DNA were further amplified, and a 720 bp PCR fragment covering amino acids 48–288 in the polymerase gene was sequenced to determine genotypes and antiviral resistance. We used primers FHBS1 and FHBS2 as described in a previous study [Sitnik et al., 2004] and designed new

ones (RADE1M and RADE2M) to amplify a larger and more informative region of the Polymerase gene [Gomes-Gouveia et al., 2009]. The primers were selected to amplify a region of HBV that allowed distinction of all HBV genotypes, detection of all important changes for antiviral drug resistance in the polymerase gene, and detection of substitutions in the surface gene related to resistance to HBIG and anti-HBs monoclonal antibody or vaccination in the HBsAg coding region. This sequence was obtained in 11 patients who were then analyzed further. In the case of patients 1, 2, 10, 14, 21, 24, and 25 (Table I), a shorter region of the polymerase gene was sequenced and it was not possible to determine the specific changes in the major immune dominant region of HBsAg (epitope *a*). In the case of patients 12 and 20 (Table I), it was possible to analyze only HBV viral load and genotype; these two patients were included retrospectively.

Precore/core regions were also amplified and sequenced to determine the presence of precore and basal core promoter (BCP) mutations from a 453 bp PCR fragment covering nucleotides 1,672–2,125 which included the major known BCP and precore mutations [Takahashi et al., 1995].

A phylogenetic analysis of the partial surface/polymerase gene sequence from 93 HBV isolates, including 18 sequences obtained in this study and 73 sequences from the Genbank/EMBL, representing the 8 HBV genotypes and their subgenotypes was then carried out. The tree was constructed by the neighbor-joining method with the distance matrix calculated with the Kimura 2-parameter method using MEGA program (version 4) [Tamura et al., 2007]. The bootstrap test of phylogeny was performed with 1,000 replications and values ≥ 70 are indicated on the branches.

Statistical Analysis

Statistical analyses were carried out using Stata software (Stata Corp., College Station, TX). We initially conducted an analysis of frequency distribution for each selected variable. Continuous parameters were compared between groups using Student's *t*-test, except for continuous variables without normal distributions or those for small groups, in which case the parameters were compared using the Mann-Whitney *U*-test. For categorical variables, the Pearson χ^2 or Fisher's exact test was used if the group was small.

Differences were considered statistically significant when $P < 0.05$.

RESULTS

A total of 847 patients with HIV were included. Initially, 34 (4%) patients with HBsAg were identified. The majority of the patients were male (91%) and the median age was 44 years. Patients were analyzed according to their risk factors for hepatitis B infection: Twenty-two (64.7%) were men who have sex with men; 11 (32.3%) injected drugs; only 1 (2.9%) was a hetero-

sexual contact of a patient with HIV. Among these 34 patients, 5 experienced HBsAg seroconversion and 1 died during clinical follow-up. These 6 patients were excluded from the study as it was not possible to characterize further their HBV status.

Plasma specimens were obtained from the remaining 28 patients, comprising the study population that underwent further more extensive virological characterization. Of these 28 patients, 24 (85.7%) were taking HAART. The remaining 4 patients were not taking any other antiviral medication.

The mean CD4+ T-cell count of the 28 patients was 556 (range: 231–1417 cells/mm³), while the mean nadir CD4+ T-cell count was 289 (range: 55–780 cells/mm³). According to data collected from the medical charts, all 24 patients taking HAART were compliant with anti-retroviral therapy.

Fifty-seven per cent of patients (16/28) had abnormal ALT values. ALT levels were on average twice the upper normal limit.

Sixteen patients were HBeAg-positive whilst 12 were HBeAg-negative.

Statistical analysis revealed HBeAg positivity was associated with an HBV-DNA viral load $>20,000$ IU/ml ($P < 0.05$), an ALT level >1.5 times the upper limit of normal ($P < 0.05$), and a CD4+ T-cell count <350 cells/mm³ ($P < 0.05$).

Among the 24 patients on antiretroviral therapy, 18 were using LAM (monotherapy for HBV) and 6 patients were using LAM plus TDF. The duration of exposure to LAM varied from 18 to 216 months. The median time on LAM was 74 months. The time of exposure to TDF varied from 4 to 21 months.

Considering only the drugs effective for treatment of HBV, 6 (33.3%) of the 18 patients treated with LAM but not with TDF had HBV-DNA below the limit of detection (<50 IU/ml). Among patients treated with LAM plus TDF, 2 had HBV-DNA below the limit of detection. Prolonged use of LAM and TDF was associated with a low-HBV viral load ($P < 0.05$).

HBV genotypes were determined for 18 patients (of the 20 patients with viremia). The distribution of HBV genotypes among these patients was A ($n=9$; 50%), D ($n=4$; 22.2%), G ($n=3$; 16.7%), and F ($n=2$; 11.1%; Table I). A phylogenetic tree was constructed that included only cases submitted to the larger surface and polymerase overlapping genetic sequences. It was possible to obtain 498–536 bp good quality sequences from these cases. The two genotype F samples belonged to subgenotype F2a. In other cases, there was insufficient sequence to determine HBV subgenotypes (Fig. 1). Patient 26 demonstrated mixed sequence indicating that this individual was probably infected by two different genotypes (A and G). In the phylogenetic tree, this case was classified as genotype G.

Polymerase region sequencing was performed on 18 of the 20 patients who were viremic. In the case of patients 12 and 20, it was possible to determine HBV viral load and genotype only retrospectively. LAM resistance substitutions were detected in 9 (50%) out of 18 patients

TABLE I. Main HBV Features of the 28 Patients Coinfected With HBV/HIV

Pt.	HBV (IU/ml)	Genotype	Lamivudine			Tenofovir			Mutations polymerase	HBsAg	PreCore/BCP
			Use	Months	Use	Months	Use	Months			
1	>1 × 10 ⁶	G	No	NA	No	NA	Absent	ND	ND	ND	
2	60	ND	Yes	72	Yes	18	Absent	ND	ND	ND	
3	ULD	ND	Yes	60	No	NA	ULD	ND	ND	ND	
4	ULD	ND	Yes	72	Yes	4	ULD	ND	ND	ND	
5	>1 × 10 ⁶	F	Yes	18	No	NA	L180M, M204V, V207L	sP127I, sI195M	AI1762T, G1764A	Absent	
6	>1 × 10 ⁶	A	No	NA	No	NA	Absent	Absent	Absent	ND	
7	ULD	ND	Yes	144	No	NA	ULD	ND	ND	ND	
8	47 × 10 ³	A	Yes	60	No	NA	L180M, M204V	sI195M	Absent	Absent	
9	ULD	ND	Yes	40	No	NA	ULD	ND	ND	ND	
10	>1 × 10 ⁶	A	Yes	96	No	NA	L180M, M204V	ND	ND	ND	
11	ULD	ND	Yes	84	Yes	12	ULD	ND	ND	ND	
12	>1 × 10 ³	D	Yes	216	No	NA	ULD	ND	ND	ND	
13	>1 × 10 ⁶	D	Yes	118	No	NA	V173L, L180M, M204V	sT118V, sP127T, sA128V, sE164D, sI195M	G1896A, G1899A	ND	
14	70	A	Yes	72	Yes	21	Absent	ND	ND	ND	
15	>1 × 10 ⁶	A	Yes	72	No	NA	V173L, L180M, M204V	sE164D, sI195M	Absent	Absent	
16	>1 × 10 ⁶	D	Yes	NA	No	NA	V173L, L180M, M204V	sT118V, sP127T, sA128V, sF134N, sE164D	Absent	Absent	
17	600	G	Yes	72	Yes	15	Absent	Absent	Absent	Absent	
18	60	F	No	NA	No	NA	V207L	sA159R	Absent	Absent	
19	ULD	ND	Yes	60	No	NA	ULD	ND	ND	ND	
20	>1 × 10 ⁶	A	Yes	60	No	NA	ND	ND	ND	ND	
21	>1 × 10 ⁶	A	Yes	84	No	NA	L180M, M204V	ND	ND	ND	
22	107.8	A	Yes	123	Yes	7	L180M, M204V	Absent	Absent	Absent	
23	>1 × 10 ⁶	D	Yes	29	No	NA	V173L, L180M, M204V/S, P237H	sT118V, sP127T, sA128V, sE164D, sI195M	Absent	Absent	
24	>1 × 10 ⁶	A	No	NA	No	NA	Absent	ND	ND	ND	
25	779	ND	Yes	68	No	NA	L180M, M204V	ND	ND	ND	
26	>1 × 10 ⁶	G	Yes	46	No	NA	Absent	Absent	Absent	Absent	
27	ULD	ND	Yes	72	No	NA	ULD	ND	ND	ND	
28	ULD	ND	Yes	46	No	NA	ULD	ND	ND	ND	

Pt, patient; ULD, under the limit of detection (< 50 IU/ml); ND, not done.

Lamivudine Resistance Mutations in Brazil

1485

Fig. 1. Phylogenetic analysis of the partial surface/polymerase gene sequence from 86 HBV isolates, including 11 sequences obtained in this work (highlighted with a black triangle) and 73 sequences from the Genbank/EMBL representing the 8 HBV genotypes (A–F). The tree was constructed by the neighbor-joining method with the distance matrix calculated with the Kimura 2-parameter method using MEGA program (version 4). Bootstrap test of phylogeny was performed with 1,000 replications and values ≥ 70 are indicated on the branches.

managed with LAM monotherapy and in 1 patient on combination therapy with LAM and TDF. The most frequently found substitution pattern was rtL180M + rtM204V ($n=10$). This pattern was found with an accompanying rtV173L change in four individuals.

Among patients undergoing treatment with LAM plus TDF, no putative TDF resistance change was found. LAM resistance changes (rtL180M + rtM204V) were detected in only one of these patients. HBV antiviral

drug resistance was associated with LAM therapy and antiretroviral therapy ($P < 0.05$).

It was possible to determine the HBsAg coding gene sequence in only 11 patients. Analysis of envelope substitutions was performed and seven changes in the envelope gene sequence were detected (Table I).

The triple polymerase mutation (rtV173L + rtL180M + rtM204V) found in the HBV from three patients, resulted in the LAM-associated changes sE164D + sI195M in the HBsAg protein.

In the case of seven patients (1, 2, 10, 14, 21, 24, and 25; Table I), a shorter region of the polymerase gene was sequenced and it was not possible to determine the mutations in the major immune dominant region of HBsAg (epitope a).

BCP and precore region sequencing was also carried out in the 11 patients who were tested for envelope mutations (Table I). Only 2 patients both HBsAg-positive, had mutations in their HBV-DNA, one in the BCP (A1762T and G1764A) and the other in the precore region (G1896A and G1899A).

DISCUSSION

The results from this study demonstrated that prolonged LAM use was associated with the emergence of various mutations in the HBV genome. This high degree of mutational emergence, leading to drug resistance, has already been described by studies in other parts of the world [Benhamou et al., 1999; Wolters et al., 2002], but has not been described previously in Brazil. Among the 18 patients who were on LAM monotherapy, LAM resistance substitutions were detected in 9 (50%) patients. The most frequent resistance-associated change pattern was rtL180M + rtM204V. This pattern was found with an accompanying mutation rtV173L in four individuals.

TherV173L + rtL180M + rtM204V triple change has been previously described in patients with HIV and HBV co-infection [Cooley et al., 2003; Delaney et al., 2003; Santos et al., 2004; Matthews et al., 2006; Sheldon et al., 2007; Bottecchia et al., 2008; Iacomi et al., 2009]. The mutations associated with these changes result in amino acid substitutions at positions 164 and 195 (sE164D plus sI195M) of the HBsAg protein. This is a direct result of the overlapping reading frames of the envelope and polymerase genes. In vitro anti-HBs binding assays of HBsAg with this triple mutant have demonstrated significantly reduced affinity for anti-HBs antibody, with an effect comparable to that of hepatitis B immunoglobulin vaccine escape mutations, such as sG145R [Torres, 2002; Torres et al., 2002; Cooley et al., 2003; Matthews et al., 2006].

The real epidemiologic impact of this variant needs to be clarified especially in the setting of transmission. Further studies are required to determine the clinical and public health relevance of these findings, as well as the importance of the other changes identified in the envelope protein of HBV as a consequence of LAM therapy.

Among patients undergoing treatment with LAM plus TDF, putative TDF-associated resistance changes were not detected. The use of TDF was typically associated with undetectable or low-HBV-DNA level.

Interestingly, it was possible to collect new blood samples from the four patients with detectable HBV-DNA using TDF and LAM. Three of these (patients 14, 17, and 22) had undetectable HBV-DNA 6 and 12 months after they had been included in the study. In patients already using LAM, the use of TDF can eventually lead to a slower decrease in HBV viremia [Lada et al., 2008; Tuma et al., 2008; Lacombe et al., 2009]. This probably explains the presence of detectable HBV viremia in the above-mentioned patients at baseline, because at baseline they were being treated for a relatively short time with TDF, after being treated with LAM for a longer period.

The data obtained during this study also showed that one of the patients had precore mutations G1896A and G1899A. Another one showed the classical BCP mutations A1762T and G1764A. The impact of these mutations on hepatitis B outcome is currently unclear [Fang et al., 2008; Poustchi et al., 2008; Cassino et al., 2009]. It has been suggested that BCP and precore mutations could enhance the replication efficacy of LAM-resistant mutations [Poustchi et al., 2008]. The current findings support this, as both patients showed elevated HBV viral loads.

Among the 28 patients with HBsAg, 16 were HBeAg-positive and 12 were HBeAg-negative. Our data showed an association between a high-HBV-DNA level and HBeAg reactivity. Several studies have already addressed this question in patients coinfected with HIV and HBV, and our studies confirm this [Alberti et al., 2005; Matthews et al., 2009; Núñez et al., 2006]. In our study, HBeAg reactivity was associated with ALT levels 1.5 times the upper limit of normality.

In patients with HIV and HBV co-infection, little is known about the association between HBV viremia, LAM resistance, and ALT levels. Cooley et al. [2003] analyzed a group of patients with HIV and HBV and observed significantly elevated HBV viral loads and serum ALT in the setting of LAM-resistant HBV. Previous studies, mostly in the pre-HAART era, have suggested that liver damage, as assessed by ALT levels, is less severe in persons with HIV infection and active HBV replication [Bodsworth et al., 1989; Bonacini et al., 1991]. Nevertheless, the natural histories of HIV and HBV have been altered with the advent of HAART. These multidrug regimens have changed the course of liver disease in patients with co-infection. The primary mechanism of HBV-associated liver damage is the cell-mediated immune response. In the present study, the majority of patients were on HAART, which most likely resulted in at least partial immunologic reconstitution. At baseline, the mean CD4⁺ T-cell count of the patients was 556 cells/mm³. The nadir CD4⁺ T-cell count was 289 cells/mm³. Most of the patients (87.5%) were undergoing treatment with LAM and resistance substitutions were found frequently in these patients. We speculate

that HBV drug resistance to LAM could lead to the rebound of HBV-DNA, and consequently cause persistent or intermittent elevation of ALT levels linked to the partially reconstituted immune status of the patient.

Rises in ALT in patients with HIV have multiple etiologies, including antiretroviral therapy, alcohol and other drug use, and opportunist infections. No particular cause was identified in the present study but it was not possible to exclude completely a possible contribution of such causes in the elevation in ALT observed in the study group described here.

The data also revealed that HBeAg reactivity was associated with CD4⁺ T-cell counts <350 cells/mm³ ($P < 0.05$). Some investigators have suggested that the ability to clear HBsAg and HBeAg is dependent upon CD4⁺ T-cell count [Bodsworth et al., 1989]. The present results support this contention and suggest that immunosuppression in patients with more advanced disease and low CD4⁺ T-cell counts may play a role in preventing HBeAg seroconversion.

Previous studies of the distribution of HBV genotypes in Brazil have shown that the most frequent HBV genotypes are A and D and this was confirmed by the present study. Three cases of genotype G were identified noting that the presence of genotype G has been described in Brazil, but it is uncommon [Lobato et al., 2006; Bottecchia et al., 2008]. The two cases of genotype F were classified as to subgenotype F2a, which was previously identified in Amerindians from Venezuela [Devesa et al., 2008]. Subgenotype F2 has been identified throughout Brazil, except in the South Region, and a few cases of subgenotype F1 have also been described [Mello et al., 2007; Gomes-Gouvêa et al., 2009].

The study has some limitations. First, it has a cross-sectional design, precluding analysis of serially tested specimens and precluding determination of the evolution of the mutations over time. Second, it was not possible to determine whether any specific substitutions were present in patients prior to the use of LAM. Third, compliance with therapy was not confirmed.

In conclusion, this study confirmed that LAM resistance may be frequent among patients with HIV and HBV co-infection and that the incidence of the rtV173L + rtL180M + rtM204V triple substitution is not uncommon in this setting. The data also showed that prolonged use of LAM and TDF was associated with low-HBV viral load.

The results reinforce the approach that it is important to use potent and specific HBV antiviral therapy that is able to reduce rapidly viral DNA levels and to minimize the risk of development of HBV resistance. The use of a potent antiviral drug with a high-genetic barrier or the adoption of combination therapy would be appropriate strategies that should reduce the risk of emergence of antiviral resistance.

REFERENCES

- Alberti A, Clumeck N, Collins S, Gerlich W, Lundgren J, Páll G, Reiss P, Thiebaut R, Weiland O, Yadanapanah Y, Zeuzem S. EOC Jury.

Lamivudine Resistance Mutations in Brazil

1487

2005. Short statement of the first European consensus conference on the treatment of chronic hepatitis B and C in HIV co-infected patients. *J Hepatol* 42:615–624.
- Alencar RS, Gomes MM, Sitnik R, Pinho JR, Malta FM, Mello IM, Mello ES, Bacchella T, Machado MC, Alves VA, Carrilho FJ. 2008. Low occurrence of occult hepatitis B virus infection and high frequency of hepatitis C virus genotype 3 in hepatocellular carcinoma in Brazil. *Braz J Med Biol Res* 41:235–240.
- Benhamou Y, Bochet M, Thibault V, Di Martino V, Caumes E, Briceau F, Opolon P, Katlama C, Poyard T. 1999. Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. *Hepatology* 30:1302–1306.
- Bodeworth N, Donovan B, Nightingale BN. 1989. The effect of concurrent human immunodeficiency virus infection on chronic hepatitis B: A study of 150 homosexual men. *J Infect Dis* 160:577–582.
- Bonacini M, Govindarajan S, Redeker AG. 1991. Human immunodeficiency virus infection does not alter serum transaminases and hepatitis B virus (HBV) DNA in homosexual patients with chronic HBV infection. *Am J Gastroenterol* 86:570–573.
- Bottechia M, Souto FJ, O KM, Amendola M, Brandão CE, Niel C, Gomes SA. 2008. Hepatitis B virus genotypes and resistance mutations in patients under long-term lamivudine therapy: Characterization of genotype G in Brazil. *BMC Microbiol* 8:11.
- Braga WS, da Costa Castilho M, dos Santos IC, Moura MA, Segurado AC. 2006. Low prevalence of hepatitis B virus, hepatitis D virus and hepatitis C among patients with human immunodeficiency virus. *Rev Soc Bras Med Trop* 39:519–522.
- Cao GW. 2009. Clinical relevance and public health significance of hepatitis B virus genomic variations. *World J Gastroenterol* 15:5761–5769.
- Cassino L, Laufer N, Salomon H, Campos R, Quarleri J. 2009. Hepatitis B precore/core promoter mutations in isolates from HBV-monoinfected and HIV-HBV coinfected patients: A 3-yr prospective study. *J Clin Virol* 46:354–359.
- Cooley L, Ayres A, Bartholomew A, Lewin S, Crowe S, Mijch A, Locarnini S, Sasadeusz J. 2003. Prevalence and characterization of lamivudine-resistant hepatitis B virus mutations in HIV-HBV co-infected individuals. *AIDS* 17:1649–1657.
- Delaney WE IV, Yang H, Westland CE, Das K, Arnold E, Gibbs CS, Miller MD, Xiong S. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 77:11833–11841.
- Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, Duarte MC, Pohlert F, Gutierrez MF, Botto C, Pujol FH. 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol* 80:20–26.
- Fang ZL, Sahin CA, Dong BQ, Ge LY, Wei SC, Chen QY, Fang KX, Yang JY, Wang XY, Harrison TJ. 2008. HBV A1762T, G1764A mutations are a valuable biomarker for identifying a subset of male HBsAg carriers at extremely high risk of hepatocellular carcinoma: A prospective study. *Am J Gastroenterol* 103:2254–2262.
- Gomes-Gouveia MS, Soares MC, Bensabath G, de Carvalho-Mello IM, Brito EM, Souza OS, Queiroz AT, Carrilho FJ, Pinho JR. 2009. Hepatitis B and Delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the Western Brazilian Amazon region. *J Gen Virol* 90:2638–2643.
- Groen DB, Simão M. 2007. Brazilian policy of universal access to AIDS treatment: Sustainability challenges and perspectives. *AIDS* 21:S37–S45.
- Iacomi F, Vincenti D, Vairo F, Solimone M, Mariano A, Piselli P, Puro V, Capobianchi MR, Antonucci G. 2009. Effect of HIV co-infection on mutation patterns of HBV in patients with lamivudine-resistant chronic hepatitis B. *J Med Virol* 81:1151–1156.
- Lacombe K, Gozlan J, Boyd A, Bonnard P, Molina J-M, Mialhes P, Lascoux-Combe C, Zoulim F, Pacanowski J, Girard P-M. 2009. HBV blippers and rebounders under treatment with tenofovir in HIV/HBV co-infection. Conference on Retrovirus and Opportunistic Infection (San Francisco). Abstract 100.
- Lada O, Gervais A, Branger M. 2008. De novo combination therapy of tenofovir disoproxil fumarate (TDF) plus lamivudine (LAM) or TDF plus emtricitabine (FTC) is associated with early virologic response in HIV/HBV co-infected patients. 59th Annual Meeting of the American Association for the Study of Liver Diseases (San Francisco). Abstract 922.
- Lobato C, Tavares-Neto J, Rios-Leite M, Trepo C, Vitvitski L, Parvaz P, Zoulim F, D'Oliveira A, Jr., Paraná R. 2006. Intrafamilial prevalence of hepatitis B virus in Western Brazilian Amazon region: Epidemiologic and biomolecular study. *J Gastroenterol Hepatol* 21:863–868.
- Matthews GV, Bartholomew A, Locarnini S, Ayres A, Sasadeusz J, Seaberg E, Cooper DA, Lewin S, Dore GJ, Thio CL. 2006. Characteristics of drug resistant HBV in an international collaborative study of HIV-HBV infected individuals on extended lamivudine therapy. *AIDS* 20:863–870.
- Matthews GV, Seaberg E, Dore GJ, Bowden S, Lewin SR, Sasadeusz J, Marks P, Goodman Z, Philp FH, Tang Y, Locarnini S, Thio CL. 2006. Combination HBV therapy is linked to greater HBV DNA suppression in a cohort of lamivudine-experienced HIV/HBV coinfected individuals. *AIDS* 23:1707–1715.
- Mello FC, Souto FJ, Nabuco LC, Villela-Nogueira CA, Coelho HS, Franz HC, Saraiva JC, Virgolino HA, Motta-Castro AR, Melo MM, Martins RM, Gomes SA. 2007. Hepatitis B virus genotypes circulating in Brazil: Molecular characterization of genotype F isolates. *BMC Microbiol* 7:103.
- Mendes-Corrêa MC, Barone AA, Cavalcante N, Tengan FM, Guastini C. 2000. Prevalence of hepatitis B and C in the sera of patients with HIV infection in São Paulo, Brazil. *Rev Inst Med Trop São Paulo* 42:81–85.
- Monteiro MR, do Nascimento MM, Paves AD, Figueiredo JF. 2004. Seroepidemiological survey of hepatitis B virus among HIV/AIDS patients in Belém, Pará-Brazil. *Rev Soc Bras Med Trop* 37:27–32.
- Núñez M, Ramos B, Díaz-Pollán R, Camino N, Martín-Carbonero I, Barreiro P, González-Lahoz J, Soriano V. 2006. Virological outcome of chronic hepatitis B virus infection in HIV-coinfected patients receiving anti-HBV active antiretroviral therapy. *AIDS Res Hum Retroviruses* 22:842–848.
- Oliveira LH, Silva IR, Xavier BL, Cavalcanti SM. 2001. Hepatitis B infection among patients attending a sexually transmitted diseases clinic in Rio de Janeiro, Brazil. *Mem Inst Oswaldo Cruz* 96:635–640.
- Poustchi H, Mohamadkhani A, Bowden S, Montazeri G, Ayres A, Revill P, Farrell GC, Locarnini S, George J, Malekzadeh R. 2008. Clinical significance of precore and core promoter mutations in genotype D hepatitis B-related chronic liver disease. *J Viral Hepat* 15:753–760.
- Santos EA, Sucupira MVF, Araujo J, Gomes SA. 2004. Hepatitis B virus variants in a HIV-HBV co-infected patient at different periods of antiretroviral treatment with and without lamivudine. *BMC Infect Dis* 4:29.
- Sheldon J, Ramos B, Garcia-Samaniego J, Rios P, Bartholomew A, Romero M, Locarnini S, Zoulim F, Soriano V. 2007. Selection of hepatitis B virus (HBV) vaccine escape mutants in HBV-infected and HIV/HBV-coinfected patients failing antiretroviral drugs with anti-HBV activity. *J Acquir Immune Defic Syndr* 46:279–282.
- Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, da Silva LC, Carrilho FJ. 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 42:2455–2460.
- Soriano V, Puoti M, Peters M, Benhamou Y, Sulkowski M, Zoulim F, Mauss S, Rockstroh J. 2006. Care of HIV patients with chronic hepatitis B: Updated recommendations from the HIV-Hepatitis B Virus International Panel. *AIDS* 22:1399–1410.
- Souza MG, Passos AD, Machado AA, Figueiredo JF, Emeraldino LE. 2004. HIV and hepatitis B virus co-infection: Prevalence and risk factors. *Rev Soc Bras Med Trop* 37:391–395.
- Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishihiro S. 1995. The pre core/core promoter mutant (T1762A1764) of hepatitis B virus: Clinical significance and easy method for detection. *J Gen Virol* 76:3159–3164.
- Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599.
- Torres J. 2002. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol* 25:97–106.
- Torres J, Earnest-Silveira L, Deliyannis G, Edgton K, Zhuang H, Locarnini SA, Fyfe J, Sozzi T, Jackson DC. 2002. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. *Virology* 293:305–313.
- Tovo CV, Dos Santos DE, de Mattos AZ, de Almeida PR, de Mattos AA, Santos BR. 2006. Ambulatory prevalence of hepatitis B and C

1488

Mendes-Correa et al.

- markers in patients with human immunodeficiency virus infection in a general hospital. *Arq Gastroenterol* 43:73-76.
- Tuma P, Bottecchia M, Sheldon J, Soriano V. 2008. Prior lamivudine (LAM) failure may delay time to complete HBV-DNA suppression in HIV patients treated with tenofovir plus LAM. 59th Annual Meeting of the American Association for the Study of Liver Diseases (San Francisco). Abstract 967.
- Wolters LM, Niesters HG, Hansen BE, van der Ende ME, Kroon FP, Richter C, Brinkman K, Meenhout PL, de Man RA. 2002.

- Development of hepatitis B virus resistance for lamivudine in chronic hepatitis B patients co-infected with the human immunodeficiency virus in a Dutch cohort. *J Clin Virol* 24:173-181.
- Zago AM, Machado TF, Cazarim FL, Miranda AE. 2007. Prevalence and risk factors for chronic hepatitis B in HIV patients attended at a sexually-transmitted disease clinic in Vitoria, Brazil. *Braz J Infect Dis* 11:475-478.

- 13- Adriana Cristina da Silva, Ângela Maria Miranda Spina, Marcílio Figueiredo Lemos, Isabel Takano Oba, Cristina de Fátima Guastini, Michele Soares Gomes-Gouvêa, JOÃO RENATO REBELLO PINHO, Maria Cássia Jacintho Mendes-Correa. Hepatitis B genotype G and high frequency of lamivudine resistance mutations among HIV/Hepatitis B virus co-infected patients in Brazil. Memórias do Instituto Oswaldo Cruz, 2010 Sep. 105 (6): 770 -778**

Hepatitis B genotype G and high frequency of lamivudine-resistance mutations among human immunodeficiency virus/hepatitis B virus co-infected patients in Brazil

Adriana Cristina da Silva^{1,2}, Ângela Maria Miranda Spina², Marcílio Figueiredo Lemos², Isabel Takano Oba², Cristina de Fátima Guastini³, Michele Soares Gomes-Gouvêa³, João Renato Rebello Pinho^{3,4}, Maria Cássia Jacintho Mendes-Correa^{1,4}

¹Departamento de Doenças Infecciosas e Parasitárias, Hospital das Clínicas ²Departamento de Gastroenterologia, Laboratório de Gastroenterologia e Hepatologia Tropical, Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil ³Laboratório de Hepatites Virais, Instituto Adolfo Lutz, São Paulo, SP, Brasil

⁴Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil

In this study, we evaluated the hepatitis B virus (HBV) genotype distribution and HBV genomic mutations among a group of human immunodeficiency virus-HBV co-infected patients from an AIDS outpatient clinic in São Paulo. HBV serological markers were detected by commercially available enzyme immunoassay kits. HBV DNA was detected using in-house nested polymerase chain reaction and quantified by Cobas Amplicor. HBV genotypes and mutations in the basal core promoter (BCP)/pre-core/core regions and surface/polymerase genes were determined by sequencing. Among the 59 patients included in this study, 55 reported prior use of lamivudine (LAM) or tenofovir. HBV DNA was detected in 16/22 patients, with a genotype distribution of A (n = 12, 75%), G (n = 2, 13%), D (n = 1, 6%) and F (n = 1, 6%). The sequence data of the two patients infected with genotype G strongly suggested co-infection with genotype A. In 10 patients with viremia, LAM-resistance mutations in the polymerase gene (rtL180M + rtM204V and rtV173L + rtL180M + rtM204V) were found, accompanied by changes in the envelope gene (sI195M, sW196L and sI195M/sE164D). Mutations in the BCP and pre-core regions were identified in four patients. In conclusion, genotype G, which is rarely seen in Brazil, was observed in the group of patients included in our study. A high prevalence of mutations associated with LAM-resistance and mutations associated with anti-HBs resistance were also found among these patients.

Key words: hepatitis B - HIV - genotypes - genotype G - mutation - lamivudine

Chronic hepatitis B virus (HBV) infection is an important public health problem affecting around 360 million people worldwide (Shepard et al. 2006). In Brazil, the prevalence of HBV carriers is difficult to assess because of the size and the cultural and socio-economic diversity of Brazil. The prevalence of the HBsAg was estimated at less than 1% in the Northeast and Central-West Regions (Pereira et al. 2009) and around 1% in the municipality of São Paulo (Focaccia et al. 1998). In the Brazilian Amazon Region, a higher prevalence was estimated to be 3.3-20.6% in some specific populations, such as certain indigenous groups (Braga et al. 2001, 2005, Tavares-Neto et al. 2004).

Among patients co-infected with the human immunodeficiency virus (HIV), HBV prevalence in Brazil varies from 1.6-8.5% (Mendes-Correa et al. 2000, Monteiro et al. 2004, Souza et al. 2004, Tovo et al. 2006, Zago et al. 2007, Portelinha Filho et al. 2009). HIV co-infection modifies hepatitis B progression by decreasing

the anti-HBV immune response after acute HBV infection, thereby increasing the risk of chronic infection (Benhamou 2007). Furthermore, higher HBV DNA levels and severe forms of hepatic disease, such as cirrhosis and hepatocellular carcinoma, have been observed in patients with HBV/HIV co-infection (Bonacini et al. 2004, Soriano et al. 2008).

Lamivudine (LAM) is the most common drug utilized for hepatitis B treatment in patients co-infected with HIV, as it inhibits both HIV and HBV viral replication. LAM has a high tolerance and proven efficacy and is part of a highly effective antiretroviral therapy. LAM inhibits HBV replication in up to 86% of patients co-infected with HIV/HBV. Nevertheless, its prolonged use may result in resistance mutations in the HBV genome. In patients infected with HBV only, resistance to LAM progressively increases at an annual rate of 14-32% and in patients co-infected with HIV, HBV resistance to LAM can rapidly appear (Benhamou et al. 1999). After four years of continuous use in co-infected patients, HBV resistance to LAM can be detected in up to 90% of this population (Soriano et al. 2009).

Data on HBV genotype distribution and LAM-resistance among HIV co-infected patients are lacking in Brazil (Santos et al. 2004, Sucupira et al. 2006, Bottechia et al. 2008). Thus, in this study, we evaluated the HBV genotype distribution and presence of clinically relevant HBV mutations among HIV co-infected

Financial support: FAPESP

+ Corresponding author: cassiamc@uol.com.br

Received 6 January 2010

Accepted 5 July 2010

online | memorias.ioc.fiocruz.br

patients receiving treatment in the municipality of São Paulo. The following mutations were studied: (i) LAM-resistance-associated mutations in the polymerase gene, (ii) mutations in the overlapping surface antigen gene, (iii) basal core promoter (BCP) mutations and (iv) pre-core mutations. The presence of these mutations was analyzed in conjunction with HBV genotypes and other selected clinical and demographic variables.

PATIENTS, MATERIALS AND METHODS

Patients were selected among those regularly followed up by the AIDS Outpatient Clinic of the Clinics Hospital, University of São Paulo School of Medicine (HC-FMUSP), between May 2006-May 2007.

Electronic database search and analysis of medical records - We searched for information about HBsAg-reactive patients using an electronic database query and performed a serological profile analysis to identify HBsAg-positive patients under regular treatment during the time frame cited above.

The following clinical and demographic variables were also considered for analysis: gender, age, HIV or HBV risk factors, evidence of advanced hepatic disease, adherence to treatment regimen, antiviral drug use [LAM, tenofovir (TDF), entecavir (ETV) or interferon (IFN)], CD4⁺ T cell count, liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP)] and histological alterations in liver biopsies.

Blood collection - After informed consent was obtained from patients that joined the study, a blood sample was collected for HBV serological and molecular biological tests.

Serology - A micro-particle enzyme immunoassay (MEIA) (AxSYM, Abbott Laboratories, Abbott Park, IL, USA) was used to detect hepatitis B serological markers (HBsAg, total anti-HBc, anti-HBs, HBeAg and anti-HBe) at the central laboratory of the HC-FMUSP.

Molecular biology assays - Molecular biology assays were performed at the Laboratory of Viral Hepatitis of the Instituto Adolfo Lutz and at the Laboratory of Tropical Hepatology and Gastroenterology of the Tropical Medicine Institute, Department of Gastroenterology, HC-FMUSP.

HBV DNA detection by nested polymerase chain reaction (PCR) - HBV DNA was detected by nested PCR as previously described (Pinho et al. 1993). Briefly, serum samples (10 µL) were denatured with 2.5 µL of 0.5 M NaOH and incubated at 37°C for 1 h. Samples were neutralized with 2.5 µL of 0.5 M HCl. For the first round, 1 µM of each primer (1763 and 2032R) was added to the mixture. Amplification was carried out by 25 cycles at 94°C for 1 min, 42°C for 1 min and 72°C for 2 min, followed by an extension step at 72°C for 5 min. In the second round, a 1:10 dilution of the first round product was amplified by primers 1778 and 2017 using the same conditions as above. Amplified products from the second round were electrophoresed in a 2% agarose gel, stained with ethidium bromide and visualized under ultra-violet light.

HBV DNA quantification - HBV DNA levels in serum were analyzed by the Cobas Amplicor HBV Monitor Test assay (Roche Molecular Systems, Pleasanton, CA, USA) according to the manufacturer's instructions. Briefly, DNA was isolated from 100 µL of serum by polyethylene glycol precipitation, followed by virion lysis and neutralization. A known quantity of an internal quantification standard (QS) was introduced into each sample and used throughout the molecular assay. A 104-bp segment of the highly conserved pre-core region was amplified using one biotinylated primer (HBV-104UB) and one non-biotinylated primer (HBV-104D). The QS was amplified with the same primers as target HBV, generating a 104-bp amplicon. The Cobas Amplicor instrument automatically performed PCR amplification, hybridization and detection. According to the manufacturer's package insert, the Cobas Amplicor HBV Monitor test shows linearity from 2.0×10^2 (lower detection limit) to 2.0×10^6 HBV DNA copies/mL.

Amplification of surface/DNA polymerase (S/P) and BCP/pre-core/core regions - HBV DNA was extracted from 100 µL of serum by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi 1987). A 476-nucleotide fragment (nt 255-731) of the overlapping S/P genes was analyzed to determine the HBV genotype and the presence of drug resistance mutations. This S/P fragment was amplified by nested PCR using a set of primers previously described (Da Silva et al. 2001, Sitnik et al. 2004). Primers FHBS1 and L840 and primers FHBS2 and 3'LAM2 were used for the first and second rounds, respectively. Amplifications were carried out for 35 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, with a final extension step of 5 min at 72°C.

A 306-nucleotide fragment (nt 1653-1959) of the BCP/pre-core/core regions was analyzed to identify mutations related to HBeAg expression and/or viral replication, such as those found in the BCP (A1762T and G1764A) and pre-core (G1896A and C1899A) regions. The set of primers described by Takahashi et al. (1995) was used and includes the following: primers EP1.1 and EP1.2 for the first round and primers EP2.1 and EP 2 for the second round of PCR. The cycling temperatures were 1 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 56°C and 40 s at 72°C, with a final extension step of 5 min at 72°C.

Positive and negative controls were included in each set of reactions. The nested PCR products were electrophoresed in a 2% agarose gel.

Sequencing - Sequencing was performed with dideoxynucleotides (ddNTPs) and fluorescent markers using the ABI Prism® BigDye™ Terminator kit (Applied Biosystems, Foster City, CA, USA) with the same primers used in the second round of PCR according to manufacturer's instructions. Samples were sequenced on an Automatic Sequencer ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA). Both sense and antisense strands were analyzed.

Sequence analysis and genotyping - Nucleotide sequences of both regions were validated using the Phred-Phrap software and consensus sequences were obtained by alignment of both strands (sense and antisense) using

the CAP3 DNA sequence assembly programme (Ewing et al. 1998, Ewing & Green 1998, Huang & Madan 1999).

Nucleotide sequences were compared with previously reported sequences of the different HBV genotypes (A-H) obtained from GenBank (ncbi.nlm.nih.gov/). Sequences were aligned using CLUSTAL_X (Thompson et al. 1997) and the alignments were visually inspected and edited using BioEdit 7.0.5.3 (Hall 1999).

HBV genotypes were determined by phylogenetic analysis using the distance method (neighbor joining) implemented in Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (megasoftware.net/) (Tamura et al. 2007). Robustness of the phylogenetic groups was evaluated using 1,000 bootstrap replicates. MEGA version 4.0 was used to translate the sequences and analyze their amino acid sequences. To identify the mutations in the S/P and BCP/pre-core/core regions, HBV sequences from this study were compared with a sequence from wild type HBV.

Nucleotide sequence data were deposited in the GenBank database under the following accession: GU968704-GU968714 (BCP/pre-core/core regions) and GU968715-GU968715 (S/P region).

Statistical analysis - Initially, a descriptive analysis of the study variables was performed. The co-infection prevalence rate and the 95% confidence interval (CI 95%) were calculated using binomial distribution. To compare the proportion presented in the association tables, Chi-square or Fisher's exact statistic tests were applied (Kirkwood 2003). Regarding age and number of CD4⁺ T cells in peripheral blood, estimates were made to determine the central tendency and dispersion measurements. We verified that these variables were not normally distributed by evaluation with the non-parametric Kolmogorov-Smirnov test and, therefore, the median within the minimum and maximum values was used. To compare medians, the non-parametric Mann-Whitney test was used (Wilcoxon sum of ranks test). A significance level of 5% was considered for all statistical tests. We used Excel to construct tables and figures and Stata (version 9.0) for statistical analysis.

Ethics - The study was approved by the Research Ethical Committee of HC-FMUSP and by the Research Ethical Committee of Instituto Adolfo Lutz.

TABLE I
Epidemiological, clinical and laboratory results of 59 HBsAg-reactive patients
co-infected with human immunodeficiency virus

Demographic and epidemiological variables	n	%
Males	58	98.3
Age (mean $\pm$ standard deviation) (years)	-	44.8 $\pm$ 7.2
Men who have sex with men	42	71.1
Intravenous drug use	05	8.5
Clinical variables	n	%
Advanced hepatic disease	10	16.9
Non adherence to treatment	05	8.5
Previous antiretrovirals use	59	100
Previous LAM use	56	95
Previous TDF use	43	72.9
Previous ENT use	04	6.8
Previous IFN use	09	15.2
Previous LAM, TDF, ENT or IFN use	55	93.2
Laboratory variables	n	%
HBsAg reactivity	31	52.5 (CI 95: 39.1-65.7)
Viremia (HBV DNA $\geq$ 60 IU/mL) ^a	22	37.3
Mean CD4 ⁺ T-cells count (range)	538	16-1200
Liver fibrosis presence at histology $\geq$ 2	14	23.7
ALT $>$ 1.5 x ULN	16	27
GGT $>$ 1.5 x ULN	15	25.4
ALP $>$ 1.5 x ULN	07	11.9

a. quantified by Cobas Amplicor; ALP: alkaline phosphatase ALT: alanine aminotransferase; ENT: entecavir; GGT: gamma-glutamyl transferase; HBV: hepatitis B virus; IFN: interferon; LAM: lamivudine; TDF: tenofovir; ULN: upper limit of normality.

RESULTS

Patients - A total of 2,412 patients from the AIDS Outpatient Clinic of the HC-FMUSP with confirmed HIV infection and serological markers for hepatitis B were initially enrolled in our study. Among the 2,412 identified patients, 120 (4.9%; CI 95%: 4.2-5.9) were HBsAg-reactive. Only 74 patients actually participated in the study. Of these, 59 remained HBsAg-reactive and were analyzed for HBV DNA quantification and sequencing.

Clinical and epidemiological features - Clinical and epidemiological features of the 59 HBsAg-reactive patients are shown in Table I. Among the 59 patients, previous TDF usage ranged from 1-34 months, while for LAM it ranged from 6-96 months.

Characteristics of HBeAg and the HBV viral load - Among the 59 HBsAg-reactive patients, 22 (37.3%) showed positive viremia (HBV DNA) either by in-house nested PCR or by Cobas Amplicor. Among the 59 HBsAg-reactive patients, 31 were HBeAg-reactive (52.5%; CI 95%: 39.1-65.7).

Most (86.4%) of the patients with detectable HBV DNA were HBeAg-reactive, while only 10.8% of the non-reactive HBeAg patients showed detectable HBV DNA. We observed a statistically significant ($p < 0.001$) association between HBeAg reactivity and positive viremia (HBV DNA) (Table II).

HBV genotypes by phylogenetic analysis of the S/P region and the BCP/pre-core/core regions - Among the 22 patients with detectable HBV DNA, we determined the HBV genotype in 16 patients. The phylogenetic tree analysis (Fig. 1) showed the following genotypes: A: 12 (75%), D: one (6.25%), F: one (6.25%) and G: two (12.50%).

Analysis of the amino acid sequences of the HBsAg coding region from HBV genotype G from patients 6 and 14 is shown in Fig. 2. For patient 14, some polymorphic sites were identified, suggesting that this patient harbored a heterogeneous population of virus.

TABLE II

Distribution of hepatitis B virus (HBV) according to HBeAg seropositivity in patients co-infected with human immunodeficiency virus and HBV

	HBeAg	
	negative	positive
Viral load	n (%)	n (%)
< 60	25 (89.3)	12 (38.7)
60-999	1 (3.6)	7 (22.6)
1,000-1,999	1 (3.6)	1 (3.2)
2,000-19,999	1 (3.6)	1 (3.2)
20,000	-	10 (32.3)
Total	28 (100)	31 (100)

$p < 0.001$.

The sequence analysis of the BCP/pre-core/core regions is shown in Table III. We observed the presence of two BCP mutations, A1762T and G1764A, in two patients and only G1764A in another two. Only one patient also showed a pre-core G1896A mutation. This was the only genotype D case in our study population.

A good quality sequence for the BCP/pre-core/core regions was not obtained for patient 14. These regions show the hallmark characteristics of genotype G (translational stop codons at amino acids 2 and 28 of the pre-core region and a 12 amino acid insert in the amino-terminal part of the HBcAg). The low quality sequence could be explained by the fact that this patient harbored both A and G genotypes due to the insertion characteristic of genotype G present in some of the circulating viruses.

For patient 6, the S/P sequence was associated with other genotype G sequences in the phylogenetic tree, but the BCP/pre-core/core region sequence was characteristic of HBV genotype A and did not show any of the genotype G hallmarks.

HBV mutations in the S/P region - Mutations found in the surface and polymerase genes are also shown in Table III. A mutation at codon 204 of the reverse transcriptase of the HBV polymerase gene was observed; nine patients had a methionine-to-valine substitution (rtM204V) and one had a methionine-to-isoleucine substitution (rtM204I). All of these patients also had an as-

Fig. 1: phylogenetic tree of hepatitis B virus (HBV) surface/DNA polymerase (S/P) region. Neighbor joining analysis was carried out with other sequences from different HBV genotypes previously described using MEGA4 software. Sequences obtained from this study are marked with black triangles and those obtained from the GenBank are designated by their accession number followed by the country of origin. Bootstrap values obtained with 1,000 replicates are shown in the nodes (only values > 70% are shown). Genotypes are indicated at the corresponding tree branches.

	53	63	73	83	93	103	113	123
G_AB056513_USA	SQSP7SMISP	ISCPPTCPGY	RWMLRRFII	FLVILLGLI	FLVLLDYQG	MLPVCPLIPG	SSTTSTGPKR	TCTTBAQGN
G_AB056515_USA								
G_AB064313_USA								
G_AF05706_Germany								
G_AP007264_Japan								
G_DQ207798_Germany								
G_EF664097_Brazil								
G_EF664098_Brazil								
G_EF664099_Brazil								
Patient_6								
Patient_14								
A_AB116092_Philippines								
A_AB116091_Philippines								
	133	143	153	163	173	183	193	203
G_AB056513_USA	MYPSCCCTRP	SDGNCTCIPI	PSMAFARYL	MEWASVRFSW	LSLLVFPVQM	FVGLSPTVWL	SAIWMWYMG	PNLYNLSL
G_AB056515_USA								
G_AB064313_USA								
G_AF05706_Germany								
G_AP007264_Japan								
G_DQ207798_Germany								
G_EF664097_Brazil								
G_EF664098_Brazil								
G_EF664099_Brazil								
Patient_6								
Patient_14								
A1_AB116092_Philippines								
A1_AB116091_Philippines								

Fig. 2: amino acid sequences from partial nucleotide sequence of HBsAg (S) gene from two hepatitis B virus (HBV) genotype G in HBV/ human immunodeficiency virus co-infected Brazilian patients (shown in the grey box) and from other HBV genotype G and A sequences previously reported, retrieved from GenBank. Interrogation marks in patient 14 indicate ambiguities in nucleotide sequence representing characteristics of both genotypes (G and A). Dashes indicate identity to the sequence AB056513 and changed amino acids are indicated by the appropriate single letter code.

sociated leucine-to-methionine substitution at position 180 (rtL180M). Along with the rtL180M and rtM204V mutations, three patients also showed a leucine-to-valine substitution (rtL80V) and two patients had a valine-to-leucine substitution (rtV173L) in the polymerase gene.

Some mutations in the HBV polymerase region implied changes in the following envelope HBsAg gene codons: sI195M (7 patients), sI195M/sE164D (2 patients) and sW196L (1 patient).

Association between HBV genotypes and selected variables - To analyze the association between HBV genotypes and selected variables, we separated the patients into "genotype A" and "non-genotype A" groups because of the small number of cases and low representation of the other genotypes. We did not observe any statistically significant associations between HBV genotypes and the selected clinical or epidemiological variables.

Association between mutations in the HBV polymerase region and selected variables - The only observed association with statistical significance occurred between the presence of HBV mutations and use of LAM ($p = 0.04$). However, statistically significant associations were not observed when the presence or absence of mutations in the polymerase region was compared to other clinical or epidemiological variables.

DISCUSSION

The development of resistance to antiviral medications is a great challenge for the treatment of hepatitis B. In our study, the presence of mutations in the HBV genome associated with LAM-resistance was observed in 10

of the 22 patients with detectable viremia. Among these, all were using LAM and seven were also using TDF. The duration of TDF usage varied from 1-34 months, while the use of LAM ranged from 6-96 months.

TDF and LAM are highly effective medications for decreasing HBV viremia in patients infected only with HBV (Marcellin et al. 2008) and in patients co-infected with HIV (Matthews et al. 2008). Nevertheless, in patients already using LAM, the use of TDF can eventually lead to a slower decrease in HBV viremia. In some cases, viral DNA can still be detected for longer periods, sometimes even after 24 months of continued therapy with both medications (Lada et al. 2008, Tuma et al. 2008). This fact probably explains the presence of detectable levels of HBV in patients using LAM and TDF because all patients using TDF had a prolonged use of LAM monotherapy.

Another possible explanation for the persistence of HBV viremia in our study population is the non-adherence to the prescribed medications. For instance, we observed that five of the 59 patients included were not conforming to the antiviral treatment regimen as recommended in their prescriptions.

Considering the mutations observed in the reverse transcriptase region of the polymerase gene, rtM204V was found in nine patients while rtM204I was found in only one patient. This patient with rtM204I also showed the rtL180M mutation and the sW196L mutation in the HBsAg overlapping gene. There were three identified mutation patterns in the rtM204V mutation: rtL180M + rtM204V (1 patient), rtL180M + rtT184S/L + rtM204V (3 patients), rtL80V + rtL180M + rtM204V (3 patients)

TABLE III
Features related to the infection with hepatitis B virus (HBV) in 22 HBsAg-reactive patients with viremia (HBV DNA positive)

Patient number	HBsAg status	Viral load (IU/mL) ^a	HBV genotype and subtype	LAM-resistance mutations in the polymerase gene	HBsAg gene mutations	BCP, pre-core and core mutations	Use LAM-TDF
1	R	82	ND	ND	ND	ND	yes - yes
2	NR	131	ND	ND	ND	ND	yes - yes
3	R	> 38,000	A (adw2)	rtL180M and rtM204V	sI195M	ND	yes - yes
4	R	> 38,000	A (adw2)	rtL180M and rtM204V	sI195M	G1764A	yes - no
5	NR	11,100	A (adw2)	absent	absent	absent	yes - no
6	R	> 38,000	A-G (adw2)	absent	absent	absent	yes - yes
7	R	> 38,000	A (adw2)	absent	absent	A1762T and G1764A	yes - no
8	R	> 38,000	D (ayw3)	absent	absent	A1762T, G1764A and G1896A	yes - yes
9	R	79	ND	ND	ND	ND	yes - yes
10	NR	1,350	ND	ND	ND	ND	yes - no
11	R	238	ND	ND	ND	ND	yes - yes
12	R	> 38,000	A (adw2)	rtL80V, rtL180M and rtM204V	sI195M	G1764A	yes - yes
13	R	> 38,000	F (adw4)	absent	absent	absent	no - no
14	R	17,400	A - G (adw2)	rtV173L, rtL180M and rtM204V	sI195M/sE164D	ND	yes - yes
15	NR	265	A (adw2)	rtL180M and rtM204V	sW196L	ND	yes - no
16	R	38,000	A (adw2)	rtL80V, rtL180M and rtM204V	sI195M	absent	yes - no
17	R	> 38,000	A (adw2)	rtV173L, rtL180M and rtM204V	sI195M/sE164D	absent	yes - yes
18	R	75	ND	ND	ND	ND	yes - yes
19	R	1,130	A (adw2)	rtL180M and rtM204V	sI195M	ND	yes - yes
20	R	> 38,000	A (adw2)	ND	ND	ND	yes - yes
21	R	164	A (adw2)	rtL180M and rtM204V	sI195M	ND	yes - yes
22	R	905	A (adw2)	rtL80V, rtL180M and rtM204V	I195M	absent	yes - yes

^a Cobas Amplicor HBV quantitative assay in IU/mL; BCP: basal core promoter; LAM: lamivudine; ND: not determined; NR: non-reactive; R: reactive; TDF: tenofovir.

and rtV173L + rtL180M + rtM204V (2 patients). The sI195M mutation in the HBsAg surface gene reading frame, which is associated with rtL180M, was present in all patients. More importantly, it is known that the mutation pattern, rtV173L + rtL180M + rtM204V (seen in 2 patients), is associated with sE164D + sI195M in the HBsAg gene (Torresi et al. 2002). HBV strains carrying these mutations have a reduced capability to bind to HBsAg antibodies (anti-HBs) *in vitro*. This feature is shared with the classical vaccine-escape strain (harboring mutation sG145R) (Torresi et al. 2002). We do not know whether the HBV strains that showed decreased antigenicity could escape the neutralizing action of the anti-HBs antibodies induced by vaccination and cause infection in previously vaccinated individuals (Torresi et al. 2002, Matthews et al. 2006).

Various studies in HBV-HIV co-infected patient populations have evaluated the presence and clinical importance of these mutations (Matthews et al. 2006, Soriano et al. 2008), but in Brazil few studies have evaluated this matter (Santos et al. 2004, Sucupira et al. 2006, Bottecchia et al. 2008, Mendes-Correa et al. 2008). If these mutations are indeed associated with vaccine escape, they would have an important impact on public health. Indeed, larger and additional population studies are needed to evaluate the presence of these mutations and their ability to spread in the general population or in high-risk groups, especially in immunocompromised patients, such as HIV-positive individuals.

It is noteworthy that the four patients in our population who showed BCP and/or pre-core mutations were HBeAg-reactive. This mutation pattern is commonly observed in HBeAg-non-reactive patients. This finding could indicate that these patients probably have some minor viral strains that are not mutated in this region and thus maintain the HBeAg-reactive status.

In our sample population, most of the patients with detectable HBV DNA (86.4%) were HBeAg-reactive. Most of them were males who had sex with multiple male partners without protection. Our data also showed association between HBeAg reactivity and HBV viremia above 20,000 IU/mL ($p < 0.001$). These results are in accordance with similar results found in patients infected with HBV only (Lok & McMahon 2009) and a few groups have evaluated this finding among HIV co-infected patients (Soriano et al. 2008, Matthews et al. 2009). These data are relevant for the studied population and have practical importance, especially in instances where the determination of the HBV viral load is not available.

We were able to determine the HBV genotypes in 16 of the patients with detectable HBV viremia. Among them, the HBV genotype distribution was as follows: A ($n = 12$; 75%), G ($n = 2$; 13%), D ($n = 1$; 6%) and F ($n = 1$; 6%). In relation to the identified genotypes, it is important to stress that the predominance of genotype A in this population should be carefully analyzed. We can only conclude that among the patients with detectable viremia, genotype A was predominant. It is possible that other genotypes predominate among the patients whose genotypes we were unable to determine.

In Brazil, the presence of genotype G has been rarely seen (Lobato et al. 2006, Bottecchia et al. 2008, Mendes-Correa et al. 2008). Bottecchia et al. (2008) identified genotype G in a patient infected with HBV only and in two patients co-infected with HIV. The current study is the second to identify the genotype among HIV co-infected patients. The presence of genotype G has been associated by some authors with a history of homosexual transmission among males (Sánchez et al. 2007). The two patients in our study with this genotype were homosexual males, which supports the hypothesis that the transmission of HBV-G could somehow be associated with this sexual behavior. Additional studies with larger sample populations that analyze HBV genotypes in partners with different sexual behaviors are needed to demonstrate this association.

HBV genotype G has been associated with the presence of two stop codons in the pre-core region, which could prevent HBeAg production (Kato et al. 2002). The two infected patients with genotype G were positive for HBeAg, but as genotype G does not produce this antigen, a co-infection with another genotype is possible.

Our sequence data strongly suggest co-infection with genotype A or infection with a recombinant virus between genotypes A and G. For patient 6, the S/P sequence was clearly associated with other genotype G sequences in the phylogenetic tree. The BCP/pre-core/core region sequence was characteristic of HBV genotype A and did not show any of the genotype G hallmarks. For patient 14, we found the presence of two peaks in the chromatogram at some positions, meaning that two different nucleotides were found. Such ambiguities do not indicate background in the chromatograms, as they had good quality according to the Phred Phrap programme. To fully resolve this question, cloning, end point dilution PCR or specific PCR to detect HBV genotypes would have to be performed.

The BCP mutations, A1762T and G1764A, were found in two patients and the G1764A mutation only in another two. Only one patient showed a pre-core G1896A mutation. This was the only patient with genotype D in our study group; pre-core mutations are more frequent in this genotype. Interestingly, all of these patients were HBeAg-reactive.

Nonetheless, our study presents some limitations. First, it was a cross-sectional study, which did not permit serial analysis of the samples or demonstration of the evolutionary emergence of mutations. Second, it was not possible to evaluate the presence of these mutations in all patients, particularly before antiviral therapy usage. Finally, the lack of association between mutations and genotypes observed in this study may be due to the small number of patients studied.

In conclusion, among patients co-infected with HIV and HBV under antiviral treatment, a high prevalence of mutations associated with LAM-resistance was observed in the DNA polymerase gene of HBV. Some mutations linked to vaccine escape mutations were found in the HBsAg coding gene, which could have an impact on public health if confirmed further. An association

between the presence of HBeAg and a high viral load for HBV was also observed. The genotype G, which is rarely seen in Brazil, was also found in two patients, with strong serological and molecular data suggesting co-infection or recombination with genotype A.

REFERENCES

- Benhamou Y 2007. Hepatitis B in the HIV-coinfected patient. *J Acquir Immune Defic Syndr* 45 (Suppl. 2): S57-65.
- Benhamou Y, Bochet M, Thibault V, Di Martino V, Caumes E, Briceau F, Opolon P, Katlama C, Poyard T 1999. Long-term incidence of hepatitis B virus resistance to lamivudine in human immunodeficiency virus-infected patients. *Hepatology* 30: 1302-1306.
- Bonacini M, Louie S, Bzowej N, Wohl AR 2004. Survival in patients with HIV infection and viral hepatitis B or C: a cohort study. *AIDS* 18: 2039-2045.
- Bottechia M, Souto FI, O KM, Amendola M, Brandão CE, Niel C, Gomes SA 2008. Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *BMC Microbiol* 22: 8-11.
- Braga WS, Brasil LM, de Souza RA, Castilho M da C, da Fonseca JC 2001. The occurrence of hepatitis B and delta virus infection within seven Amerindian ethnic groups in the Brazilian western Amazon. *Rev Soc Bras Med Trop* 34: 349-355.
- Braga WS, Silva EB, Souza RA, Tosta CE 2005. Seroprevalence of hepatitis B and malaria infection in Lábrea, Brazilian western Amazon: estimates of coinfection rates. *Rev Soc Bras Med Trop* 38: 218-223.
- Chomczynski P, Sacchi N 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.
- Da Silva LC, Pinho JR, Sitnik R, Da Fonseca LE, Carrilho FJ 2001. Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *J Gastroenterol* 36: 476-485.
- Ewing B, Green P 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8: 186-194.
- Ewing B, Hillier L, Wendt MC, Green P 1998. Base-calling of automated sequencer traces using phred I. Accuracy assessment. *Genome Res* 8: 175-185.
- Focaccia R, da Conceição OJ, Sette H Jr, Sabino E, Bassit L, Nitrini DR, Lomar AV, Lorenço R, Vieira De Souza F, Kiffer CR, Santos EB, Gonzales MP, Sáez-Alquézar A, Riscal JR, Fischer D 1998. Estimated prevalence of viral hepatitis in the general population of the municipality of São Paulo, measured by a serologic survey of a stratified, randomized and residence-based population. *Braz J Infect Dis* 2: 269-284.
- Hall TA 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* 41: 95-98.
- Huang X, Madan A 1999. CAP3: a DNA sequence assembly program. *Genome Res* 9: 868-877.
- Kato H, Orito E, Gish RG, Suguchi F, Suzuki S, Ueda R, Miyakawa Y, Mizokami M 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). *J Virol* 76: 6131-6137.
- Kirkwood BR 2003. Univariate analysis and chi-square test. In BR Kirkwood, JAC Sterne. *Essential medical statistics*, Blackwell Science, Malden, 501 pp.
- Lada O, Gervais A, Branger M, Peytavin G, Collin G, Fraqueiro G, Moucari R, Males S, Martinot-Peignoux M, Matheron S, Marcellin P 2008. De novo combination therapy of tenofovir disoproxil fumarate (TDF) plus lamivudine (LAM) or TDF plus emtricitabine (FTC) is associated with early virologic response in HIV/HBV co-infected patients. *Hepatology* 48: 720A.
- Lobato C, Tavares-Neto J, Rios-Leite M, Trepo C, Vitvitski L, Parvaz P, Zoulim F, D'Oliveira A Jr, Paraná R 2006. Intrafamilial prevalence of hepatitis B virus in Western Brazilian Amazon Region: epidemiologic and biomolecular study. *J Gastroenterol Hepatol* 21: 863-868.
- Lok AS, McMahon BJ 2009. Chronic hepatitis B: update 2009. *Hepatology* 50: 661-662.
- Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z, Germanidis G, Lee SS, Flisiak R, Kaita K, Manns M, Kotzev I, Tchernev K, Buggisch P, Weibert F, Kordas OO, Shiffman ML, Trinh H, Washington MK, Sorbel J, Anderson J, Snow-Lampart A, Mondou E, Quinn J, Rousseau F 2008. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 359: 2442-2455.
- Matthews GV, Avihingsanon A, Lewin SR, Amin J, Rerknimitr R, Petcharapir P, Marks P, Sasadeusz J, Cooper DA, Bowden S, Locarnini S, Ruxrungtham K, Dore GJ 2008. A randomized trial of combination hepatitis B therapy in HIV/HBV coinfecting antiretroviral naïve individuals in Thailand. *Hepatology* 48: 1062-1069.
- Matthews GV, Bartholomeusz A, Locarnini S, Ayres A, Sasadeusz J, Seaberg E, Cooper DA, Lewin S, Dore GJ, Thio CL 2006. Characteristics of drug resistant HBV in an international collaborative study of HIV-HBV-infected individuals on extended lamivudine therapy. *AIDS* 20: 863-870.
- Matthews GV, Seaberg E, Dore GJ, Bowden S, Lewin SR, Sasadeusz J, Marks P, Goodman Z, Philp FH, Tang Y, Locarnini S, Thio CL 2009. Combination HBV therapy is linked to greater HBV DNA suppression in a cohort of lamivudine-experienced HIV/HBV coinfecting individuals. *AIDS* 23: 1707-1715.
- Mendes-Correa MC, Barone AA, Cavalheiro N, Tengan FM, Guastini C 2000. Prevalence of hepatitis B and C in the sera of patients with HIV infection in São Paulo, Brazil. *Rev Inst Med Trop São Paulo* 42: 81-85.
- Mendes-Correa MC, Pinho JR, Leite OM, Martins LG, Leite AG, Silva MH, Sitnik R, Uip DE 2008. Hepatitis B genotype distribution and high frequency of lamivudine resistance mutations in Brazilian HIV/hepatitis B virus co-infected patients. *Hepatology* 48: 538A.
- Monteiro MR, do Nascimento MM, Passos AD, Figueiredo JF 2004. Seroepidemiological survey of hepatitis B virus among HIV/AIDS patients in Belém, Pará-Brazil. *Rev Soc Bras Med Trop* 37 (Suppl. 2): 27-32.
- Pereira LM, Martelli CM, Merchán-Hamann E, Montarroyos UR, Braga MC, de Lima ML, Cardoso MR, Turchi MD, Costa MA, de Alencar LC, Moreira RC, Figueiredo GM, Ximenes RA 2009. Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am J Trop Med Hyg* 81: 240-247.
- Pinho JR, Santos CA, Gonzalez CL, Bassit L, Barreto CC, Sáez-Alquézar A, França AV, Carrilho FJ, Fonseca LE, Chamone DA, da Silva LC 1993. Detection of hepatitis B virus DNA by the polymerase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev Inst Med Trop São Paulo* 35: 515-520.
- Portelinha Filho AM, do Nascimento CU, Tannouri TN, Troiani C, Ascêncio EL, Bonfim R, D'Andrea LAZ, Prestes-Carneiro LE 2009. Seroprevalence of HBV, HCV and HIV co-infection in selected individuals from state of São Paulo, Brazil. *Mém Inst Oswaldo Cruz* 104: 960-963.

- Sánchez LV, Tanaka Y, Maldonado M, Mizokami M, Panduro A 2007. Difference of hepatitis B virus genotype distribution in two groups of Mexican patients with different risk factors. High prevalence of genotype H and G. *Intervirology* 50: 9-15.
- Santos EA, Sucupira MV, Arabe J, Gomes SA 2004. Hepatitis B virus variants in an HIV-HBV co-infected patient at different periods of antiretroviral treatment with and without lamivudine. *BMC Infect Dis* 4: 29.
- Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP 2006. Hepatitis B virus infection: epidemiology and vaccination. *Epidemiol Rev* 28: 112-125.
- Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Carrilho FJ 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 42: 2455-2460.
- Soriano V, Puoti M, Peters M, Benhamou Y, Sulkowski M, Zoulim F, Mauss S, Rockstroh J 2008. Care of HIV patients with chronic hepatitis B: updated recommendations from the HIV-Hepatitis B Virus International Panel. *AIDS* 22: 1399-1410.
- Soriano V, Tuma P, Vispo E, Labarga P, Fernández JV, Medrano J, Barreiro P 2009. Hepatitis B in HIV patients: what is the current treatment and what are the challenges? *J HIV Ther* 14: 13-18.
- Souza MG, Passos AD, Machado AA, Figueiredo JF, Esmeraldino LE 2004. HIV and hepatitis B virus co-infection: prevalence and risk factors. *Rev Soc Bras Med Trop* 37: 391-395.
- Sucupira MV, Mello FCA, Santos EA, Niel C, Rolla VC, Arabe J, Gomes SA 2006. Patterns of hepatitis B virus infection in Brazilian human immunodeficiency virus infected patients: high prevalence of occult infection and low frequency of lamivudine resistant mutations. *Mem Inst Oswaldo Cruz* 101: 655-660.
- Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishiro S 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 76: 3159-3164.
- Tamura K, Dudley J, Nei M, Kumar S 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596-1599.
- Tavares-Neto J, Almeida D, Soares MC, Uchoa R, Viana S, Darub R, Farias E, Rocha G, Vitvitski L, Paraná R 2004. Seroprevalence of hepatitis B and C in the Western Brazilian Amazon Region (Rio Branco, Acre): a pilot study carried out during a hepatitis B vaccination program. *Braz J Infect Dis* 8: 133-139.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG 1997. The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876-4882.
- Torres J 2002. The virological and clinical significance of mutations in the overlapping envelope and polymerase genes of hepatitis B virus. *J Clin Virol* 25: 97-106.
- Tovo CV, Dos Santos DE, de Mattos AZ, de Almeida PR, de Mattos AA, Santos BR 2006. Ambulatory prevalence of hepatitis B and C markers in patients with human immunodeficiency virus infection in a general hospital. *Arg Gastroenterol* 43: 73-76.
- Tuma P, Bottecchia M, Sheldon J, Medrano J, Vispo E, Madejón A, Martín-Carbonero L, Barreiro P, Soriano V 2008. Prior lamivudine (LAM) failure may delay time to complete HBV-DNA suppression in HIV patients treated with tenofovir plus LAM. *Hepatology* 48: 740A.
- Zago AM, Machado TF, Cazarim FL, Miranda AE 2007. Prevalence and risk factors for chronic hepatitis B in HIV patients attended at a sexually-transmitted disease clinic in Vitória, Brazil. *Braz J Infect Dis* 11: 475-478.

- 14- Mónica Viviana Alvarado-Mora, Rúbia Anita Ferraz Santana, Roberta Sitnik, Paulo Roberto Abrão Ferreira, Cristovão Luís Pitangueira Manguiera, Flair José Carrilho, João Renato Rebello Pinho. Full-length genomic sequence of hepatitis B virus genotype C2 isolated from a native Brazilian patient. Memórias do Instituto Oswaldo Cruz, Rio de Janeiro, Vol. 106(4): 495-498, June 2011**

Full-length genomic sequence of hepatitis B virus genotype C2 isolated from a native Brazilian patient

Mônica Viviana Alvarado-Mora¹*, Rúbia Anita Ferraz Santana², Roberta Sitnik², Paulo Roberto Abrão Ferreira³, Cristovão Luís Pitangueira Manguiera², Flair José Carrilho¹, João Renato Rebello Pinho^{1,2}

¹Laboratório de Gastroenterologia e Hepatologia Tropical, Departamento de Gastroenterologia, Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar 500 prédio IMT2 2º andar, 05403-000 São Paulo, SP, Brasil ²Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil

³Universidade Federal de São Paulo, São Paulo, SP, Brasil

The hepatitis B virus (HBV) is among the leading causes of chronic hepatitis, cirrhosis and hepatocellular carcinoma. In Brazil, genotype A is the most frequent, followed by genotypes D and F. Genotypes B and C are found in Brazil exclusively among Asian patients and their descendants. The aim of this study was to sequence the entire HBV genome of a Caucasian patient infected with HBV/C2 and to infer the origin of the virus based on sequencing analysis. The sequence of this Brazilian isolate was grouped with four other sequences described in China. The sequence of this patient is the first complete genome of HBV/C2 reported in Brazil.

Key words: hepatitis B virus - genotype C2 - China - Bayesian analyses - complete genome - Brazilian patient

Hepatitis B virus (HBV) infection is a public health problem; approximately two billion people have been exposed to HBV and more than 300 million are chronically infected with this virus (Grimm et al. 2011). HBV is the prototype member of the Hepadnaviridae, a family of hepatotropic DNA viruses. The virus has a 42 nm diameter viral particle and an extremely compact partially double-stranded circular genome (Dane et al. 1970). HBV is classified into eight genotypes (A-H) based on inter-group divergence of the entire genomic nucleotide sequence (Kramvis et al. 2008). Recently, an additional HBV genotype (I) was proposed (Yu et al. 2010).

HBV genotypes and subgenotypes have distinct geographical distributions and are associated with the severity of liver diseases in different populations. Genotype A is distributed globally and is the main genotype found in Europe, North America, Africa and India. Genotype D is mainly found in the Middle East and the Mediterranean Basin. Genotype E was originally reported in Africa (Kramvis & Kew 2007), while genotype G was initially reported in Europe and North America (Stuyver et al. 2000). HBV genotype F is found in Central and South America, particularly in indigenous populations in South America (Devesa & Pujol 2007, Alvarado-Mora et al. 2011). Genotype H is frequent in Central and North America (Arauz-Ruiz et al. 2002) and is very closely related to genotype F (Alvarado-Mora et al. 2011). Recently, HBV genotype I was described in northwestern China, Vietnam and Laos (Yu et al. 2010).

The most frequent HBV genotypes in Asia are B and C. Genotype C is associated with more aggressive liver disease and poorer response to antiviral therapy compared to genotype B (Chu et al. 2002). Genotype C is associated with hepatocellular carcinoma (HCC) in older patients (Yu et al. 2005), while genotype B is associated with HCC in younger people and with acute hepatitis B in adults (Ni et al. 2004).

Genotype C is classified into 10 subgenotypes that differ in their geographical distribution: C1 is found in Thailand, Vietnam and Myanmar (South Asia), C2 is found in Japan and China (Far East Asia), C3 is found in New Caledonia and Polynesia, C4 is found in Australian Aborigines and C5 is confined to the Philippines and Vietnam (Ni et al. 2004, Chan et al. 2005, Kramvis et al. 2008). The subgenotypes C3-C10 are also found in Indonesia (Mulyanto et al. 2009, 2010).

The most common genotype in Brazil is genotype A, followed by genotypes D and F. The North, Northeast and Southeast Regions have a higher frequency of genotype A, while genotype D is the most frequent in the South Region (Mello et al. 2007). In some regions, genotypes B and C are also detected at low prevalences, reflecting the presence of Asian descendants within the populations of these regions (Sitnik et al. 2004). The aim of this study was to determine the complete genome sequence of an HBV subgenotype C2 isolate from a native Brazilian patient and to infer the origin of this virus.

The patient was a 54-year-old Caucasian female who was born in state of São Paulo (SP), Brazil. She was unemployed at the time of the study (but worked in Ota, Gunma, Japan, from 2000-2009) and had been diagnosed with chronic hepatitis B in Japan, at which time antiviral treatment was started. No other comorbidities or relevant data on her clinical history were reported and her physical examination was normal. At that time, she had the following clinical assay results: aspartate

Financial support: FAPESP (2007/53457-7, 2008/50461-6), CNPq, IIRS/HIAE

* Corresponding author: monica.viviana@usp.br

Received 4 February 2011

Accepted 9 May 2011

transaminase (AST) 73 IU/mL, alanine transaminase (ALT) 93 IU/mL, HBsAg (+), HBeAg (+) and anti-HBc (+). She received lamivudine 150 mg/day from August 2005–October 2008. As HBV DNA was always detected, the treatment was changed to adefovir 10 mg/day. At that point, her clinical assay results were as follows: anti-HCV (-), anti-HIV (-), HBeAg (+), AST 37 IU/mL, ALT 32 IU/mL, HBV DNA 13,900,000 IU/mL (log 6.14) and genotype C; no resistance-related mutations were found in the DNA polymerase coding region. We started lamivudine plus tenofovir in June 2010 and HBV DNA became undetectable in September 2010. All procedures followed were in accordance with the ethical standards.

The sample collected when the patient arrived in Brazil was processed to amplify the complete HBV genome. HBV DNA extraction was conducted from 100 µL of serum using the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski & Sacchi 1987). Amplification of the whole HBV genome was performed with the P1 and P2 primers described previously, with slight modifications (Gunther et al. 1995). The sample was submitted to DNA amplification by polymerase chain reaction (PCR) (Gomes-Gouveia 2005).

Sequencing was performed in an ABI Prism® 3100 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA) (Sanger et al. 1977) using dideoxy nucleoside triphosphates containing fluorescent markers (Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction kit, Applied Biosystems, Foster City, CA, USA). For sequencing, we used 18 primers internal to the PCR fragment, generating sequences around 300–500 bp in length. The primers were as follows: PS3076F (Stuyver et al. 2000), RHBS2 (Sitnik et al. 2004), P1.2, P2.2 (Gunther et al. 1995), 582R, LAMIF, 2817R, P184, P192, P194, P197, P1193R, P970F, 2029R, X1577F (Gomes-Gouveia 2005), EP2.2 (Takahashi et al. 1995), PS3216R (Norder et al. 1994) and 5LAM5 (Da Silva et al. 2000). The quality of each electropherogram was evaluated using Phred-Phrap software (Ewing et al. 1998) and consensus sequences were obtained by alignment of both sequenced strands using CAP3 software available from the Electropherogram quality analysis web page (asparagin.cenargen.embrapa.br/phph/).

The isolated complete genome was aligned with other previously reported complete genome sequences ($n = 192$) using ClustalX software (Thompson et al. 1997) and was edited with the SE-AL software (available from: tree.bio.ed.ac.uk/software/seal/). The Bayesian Markov chain Monte Carlo simulation implemented in BEAST v1.4.8 (Drummond & Rambaut 2007) was applied to obtain the best possible estimates using both the relaxed uncorrelated log_{normal} and exponential molecular clock models and using the model of nucleotide substitution (general time-reversible + gamma + proportion invariant). The molecular clock that best fits the data was chosen by Bayes factor comparison. After 10 million generations, the maximum clade credibility (MCC) tree was obtained by summarising 10,000 substitution trees and was then modified using a 10% burn-in using Tree Annotator v1.5.3 (Drummond & Rambaut 2007). Phylogenetic trees were visualised and midpoint rooted in FigTree v1.2.2 (tree.bio.ed.ac.uk/software/figtree/).

The complete HBV genome was successfully amplified. The sequence was analysed for all reported mutations of antiviral resistance and it did not show any mutation-related response. In addition, the patient harboured a virus with a precore mutation at position 1896 (G-A), but no mutation at position 1899; this patient's virus also lacked basal core promoter mutations. Once the MCC tree was obtained, the Brazilian isolate sequence grouped within genotype C, subgenotype HBV/C2, along with four other sequences from China (Figure). This cluster was supported by an *a posteriori* probability of approximately 0.83. Sequence was deposited in GenBank under the accession HQ622095.

In Brazil, there is a highly miscegenated population and the HBV genotype distribution pattern in Brazil is different from that of other Latin American countries, with genotypes A, D and F being the most prevalent among HBV carriers in Brazil (Devesa & Pujol 2007, Mello et al. 2007, Santos et al. 2010). In the Southeast and South Regions, other HBV genotypes have been found that reflect the migrant origin of the population. In a previous study performed with chronic HBV carriers from different Brazilian regions, it was reported that genotype C was found in 13.2% of the patients, all of whom had an Asian background (Sitnik et al. 2004). In another study in Campinas, SP, the presence of the genotype C (3%) was reported in two Asian descendants and also in two Caucasians (Tonetto et al. 2009). In the Brazilian Amazon Region, one case of genotype C was reported among 44 patients (de Oliveira et al. 2008). Finally, in another study carried out in the states of Rio de Janeiro and Mato Grosso, Brazil, one case of genotype C was found (Bottecchia et al. 2008). In conclusion, the presence of genotype C in the Brazilian population is very low and most cases are restricted to the Asian population living in the country or to those individuals with some contact with Asian people. In addition, because only a short HBV genome region was amplified in each of these previous studies, it is not possible infer the specific origin of each sequence.

HBV infection is highly prevalent in China. A previous study verified that genotypes B and C are the predominant genotypes in China (Zhu & Dong 2009). The two major subgenotypes of genotype C HBV are subgenotype C1 (predominant in Southeast Asia) and C2 (predominant in East Asia). In an interesting study, it was reported that in China the frequency of HBV genotype B tends to decrease gradually and that the prevalence of genotype C increases with increasing latitude (Zhu & Dong 2009). Furthermore, genotype C prevails in the northern provinces of China, while genotype B is more prevalent in southern China (Zeng et al. 2005). Likewise, there are significant differences in geographical distribution among the patients with genotypes B and C in Japan (Orito et al. 2001).

The complete HBV genome reported in this study grouped with other sequences from China. Based on the demographic and clinical information for this patient, we inferred that she may have acquired the infection during the period that she lived in Japan (9 years), although the origin of this sequence does not appear to be Japan. The

The maximum clade credibility tree was estimated by Bayesian analysis of 192 complete genomes of hepatitis B virus (HBV) strains. The posterior probabilities (> 0.80) of the key nodes are depicted above the respective nodes. The HBV/C2 isolate from the Brazilian patient is represented in red and was analyzed together with other worldwide strains. The collapsed clades correspond to the other genotypes of HBV.

patient did not report any other information about the possible mode of transmission that could have aided us in making more inferences about this case. Phylogenetic analysis showed that within the group of genotype C2, the sequences previously reported from different countries (China, Japan, Korea, Taiwan, Thailand, Uzbekistan) are interspersed. The sequence from this Brazilian case grouped with four other sequences described in China, raising the possibility that someone who had direct or indirect contact with people living in China might have infected our patient. It was not possible to determine the specific region of China from which this sequence could have originated because there is no specific publication about these sequences and we could not determine from which country these sequences were collected. The sequence of our patient is the first complete genome sequence of HBV subgenotype C2 reported in Brazil and it is noteworthy that this patient was not of Asian descent.

In conclusion, our results increase the understanding of the dynamics of HBV and demonstrate the application of the Bayesian inference to determine the origin of an infection. We hypothesize that the native Brazilian patient was infected with an HBV/C2 strain that is prevalent in China when she lived in Japan.

REFERENCES

- Alvarado-Mora MV, Romero CM, Gomes-Gouveia MS, Gutierrez MF, Botelho L, Carrilho FJ, Pinho JR. 2011. Molecular characterization of the hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol* 11: 103-108.
- Arauz-Ruiz P, Norder H, Robertson BH, Magnus LO. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83: 2059-2073.
- Bottecchia M, Souto FJ, O KM, Amendola M, Brandão CE, Niel C, Gomes SA. 2008. Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *J Gen Virol* 8: 11.

- Chan HL, Tsui SK, Tse CH, Ng EY, Au TC, Yuen L, Bartholomewsz A, Leung KS, Lee KH, Locarnini S, Sung JJ 2005. Epidemiological and virological characteristics of 2 subgroups of hepatitis B virus genotype C. *J Infect Dis* 191: 2022-2032.
- Chomczynski P, Sacchi N 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159.
- Chu CJ, Hussain M, Lok AS 2002. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 122: 1756-1762.
- Dane DS, Cameron CH, Briggs M 1970. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* 1: 695-698.
- Da Silva LC, da Fonseca LE, Carrilho FJ, Alves VA, Sitnik R, Pinho JR 2000. Predictive factors for response to lamivudine in chronic hepatitis B. *Rev Inst Med Trop São Paulo* 42: 189-196.
- de Oliveira CM, Farias IP, Ferraz da Fonseca JC, Brasil LM, de Souza R, Astolfi-Filho S 2008. Phylogeny and molecular genetic parameters of different stages of hepatitis B virus infection in patients from the Brazilian Amazon. *Arch Virol* 153: 823-830.
- Devesa M, Pujol FH 2007. Hepatitis B virus genetic diversity in Latin America. *Virus Res* 127: 177-184.
- Drummond AJ, Rambaut A 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7: 214.
- Ewing B, Hillier L, Wendt MC, Green P 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8: 175-185.
- Gomes-Gouveia MS 2005. *Characterization of complete genomes of the hepatitis B virus of different genotypes isolated in Brazil*. Secretaria da Saúde/Coordenação de Controle de Doenças/Programa de Pós-Graduação em Ciências para obtenção do grau de Mestre, São Paulo, 130 pp.
- Grimm D, Thimme R, Blum HE 2011. HBV life cycle and novel drug targets. *Hepatol Int* 5: 644-653.
- Gunther S, Li BC, Miska S, Kruger DH, Meisel H, Will H 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 69: 5437-5444.
- Kramvis A, Arakawa K, Yu MC, Nogueira R, Stram DO, Kew MC 2008. Relationship of serological subtype, basic core promoter and precore mutations to genotypes/subgenotypes of hepatitis B virus. *J Med Virol* 80: 27-46.
- Kramvis A, Kew MC 2007. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res* 37 (Suppl): S9-S19.
- Mello FC, Souto FJ, Nabuco LC, Villela-Nogueira CA, Coelho HS, Franz HC, Saraiva JC, Virgolino HA, Motta-Castro AR, Melo MM, Martins RM, Gomes SA 2007. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol* 7: 103.
- Mulyanto, Depamede SN, Surayah K, Tjahjono AA, Jirintai, Nagashima S, Takahashi M, Okamoto H 2010. Identification and characterization of novel hepatitis B virus subgenotype C10 in Nusa Tenggara, Indonesia. *Arch Virol* 155: 705-715.
- Mulyanto, Depamede SN, Surayah K, Tsuda F, Ichiyama K, Takahashi M, Okamoto H 2009. A nationwide molecular epidemiological study on hepatitis B virus in Indonesia: identification of two novel subgenotypes, B8 and C7. *Arch Virol* 154: 1047-1059.
- Ni YH, Chang MH, Wang KJ, Hsu HY, Chen HL, Kao JH, Yeh SH, Jeng YM, Tsai KS, Chen DS 2004. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 127: 1733-1738.
- Norder H, Courouce AM, Magnus LO 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 198: 489-503.
- Orito E, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M 2001. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 34: 590-594.
- Sanger F, Nicklen S, Coulson AR 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463-5467.
- Santos AO, Alvarado-Mora MV, Botelho L, Vieira DS, Pinho JR, Carrilho FJ, Honda ER, Salcedo JM 2010. Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virol J* 7: 315.
- Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Carrilho FJ 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 42: 2455-2460.
- Struyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 81: 67-74.
- Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishihiro S 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 76: 3159-3164.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25: 4876-4882.
- Tonetto PA, Goncalves NS, Fais VC, Vigani AG, Goncalves ES, Feltrin A, Goncalves FL Jr 2009. Hepatitis B virus: molecular genotypes and HBeAg serological status among HBV-infected patients in the Southeast of Brazil. *BMC Infect Dis* 9: 149.
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ, Xia NS 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "T". *PLoS ONE* 5: e9297.
- Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ 2005. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *J Natl Cancer Inst* 97: 265-272.
- Zeng G, Wang Z, Wen S, Jiang J, Wang L, Cheng J, Tan D, Xiao F, Ma S, Li W, Luo K, Naoumov NV, Hou J 2005. Geographic distribution, virologic and clinical characteristics of hepatitis B virus genotypes in China. *J Viral Hepat* 12: 609-617.
- Zhu CT, Dong CL 2009. Characteristics of general distribution of hepatitis B virus genotypes in China. *Hepatobiliary Pancreat Dis Int* 8: 397-401.

- 15- Alvarado-Mora MV, Botelho-Lima LS, Santana RA, Sitnik R, Ferreira PA, do Amaral Mello F, Mangueira CP, Carrilho FJ, Rebello Pinho JR. Distribution of hepatitis B virus subgenotype F2a in São Paulo, Brazil. BMC Res Notes. 2013**

SHORT REPORT

Open Access

Distribution of hepatitis B virus subgenotype F2a in São Paulo, Brazil

Mônica V Alvarado-Mora^{1*}, Livia S Botelho-Lima¹, Rubia A Santana², Roberta Sitnik², Paulo Abrão Ferreira³, Francisco do Amaral Mello⁴, Cristovão P Manguiera², Flair J Camilho¹ and João R Rebello Pinho^{1,2}

Abstract

Background: HBV genotype F is primarily found in indigenous populations from South America and is classified in four subgenotypes (F1 to F4). Subgenotype F2a is the most common in Brazil among genotype F cases. The aim of this study was to characterize HBV genotype F2a circulating in 16 patients from São Paulo, Brazil. Samples were collected between 2006 and 2012 and sent to Hospital Israelita Albert Einstein. A fragment of 1306 bp partially comprising HBsAg and DNA polymerase coding regions was amplified and sequenced. Viral sequences were genotyped by phylogenetic analysis using reference sequences from GenBank (n=198), including 80 classified as subgenotype F2a. Bayesian Markov chain Monte Carlo simulation implemented in BEAST v.1.5.4 was applied to obtain the best possible estimates using the model of nucleotide substitutions GTR+G+I.

Findings: It were identified three groups of sequences of subgenotype F2a: 1) 10 sequences from São Paulo state; 2) 3 sequences from Rio de Janeiro and one from São Paulo states; 3) 8 sequences from the West Amazon Basin.

Conclusions: These results showing for the first time the distribution of F2a subgenotype in Brazil. The spreading and the dynamic of subgenotype F2a in Brazil requires the study of a higher number of samples from different regions as it is unfold in almost all Brazilian populations studied so far. We cannot infer with certainty the origin of these different groups due to the lack of available sequences. Nevertheless, our data suggest that the common origin of these groups probably occurred a long time ago.

Keywords: Hepatitis B, Brazil, Bayesian analysis, Subgenotype F2a

Findings

Despite the availability of an effective vaccine, more than 350 million people worldwide are chronically infected with hepatitis B virus (HBV), and many people have developed severe liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC) [1]. HBV has a partially double-stranded DNA genome composed of approximately 3.2 kb, with four overlapping open reading frames (ORF): preS/S, precore/core, X and polymerase) [2]. As a result of its unusual mechanism of replication by reverse transcription and the lack of proofreading activity of this enzyme, it displays a highly heterogeneous sequence diversity.

Nine HBV genotypes (HBV/A to HBV/I) and a controversial HBV/J have been reported based on the differences in full-length genome sequences [3]. Genotype A (A1 to A7) is highly prevalent in sub-Saharan Africa (subgenotype A1), Northern Europe (subgenotype A2), and Western Africa (subgenotype A3). Furthermore, both A4 and potential A5 strains were found in sub-Saharan Africa (Mali, Gambia and Nigeria) [4,5]. Recently, A5 was also found in African descendants in Haiti [6]. Proposed subgenotype A6 include strains from African-Belgian patients originating from Congo and Rwanda [7].

HBV/B and HBV/C genotypes are common in Asia. Currently, genotype B is divided in subgenotypes B1 to B6. Among them, B1 is found mainly in Japan, B2-5 in East Asia, and B6 in indigenous populations living in the Arctic, such as Alaska, Northern Canada and Greenland [8]. HBV genotype C (C1 to C5) is found throughout the eastern and southeastern portions of Asia and the Pacific islands (Micronesia, Melanesia, and Polynesia), as well

* Correspondence: monica.viviana@usp.br

¹Laboratory of Tropical Gastroenterology and Hepatology, Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, SP, Brazil

Full list of author information is available at the end of the article

© 2013 Alvarado-Mora et al.; licensee BioMed Central Ltd. This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

as in immigrants from these areas in the United States, Europe, Australia, New Zealand [9] and Brazil [10]. HBV/D with subtypes D1–D5 is prevalent in the Mediterranean region expanding through South Asia up to India [8]. HBV/E is restricted to West Africa [11] but some studies reported the presence of this genotype in South America [12] and India [13]. This genotype is characterized by a low genetic diversity among strains from throughout the world [14]. Genotype G has been reported in USA and France [15]. HBV/F and HBV/H genotypes are found in the Americas, with genotype F described from Alaska to Argentina, while genotype H has been mainly described in Central America and Mexico [16].

HBV genotypes F and H are considered indigenous to the American continent [15,17,18]. An HBV strain isolated from woolly monkey (a New World monkey) is

closer to genotype F but with a noteworthy phylogenetic distance from other strains of HBV [19]. Several strains of genotype F have been isolated in different countries in America, especially among Amerindian population and four subgenotypes have been described with genetic divergence among 4.3–6.1% [20].

HBV genotypes and subgenotypes have distinct geographical distribution and it is currently discussed if they are associated with different prognosis considering response to antiviral treatment and the severity of liver disease in different populations. Nevertheless, global human migrations affect the pattern of HBV genotypes distribution, introducing genotypes differing from those found in the original inhabitants.

HBV genotypes A, D and F are the most frequent in São Paulo [21], but there are not any published data on subgenotypes found there. It was previously reports that

subgenotype F2 is the most frequent in Brazil [22], particularly subgenotype F2a, as latter subclassified by Devesa et al. [20]. In this study, we compare HBV subgenotype F2a sequences recently obtained in São Paulo from chronic hepatitis B patients with other sequences previously described around the world with the aim to determine the origin of subgenotype F2a circulating in São Paulo, Brazil.

Nineteen samples from chronically infected patients (seventeen male and two female) with HBV subgenotype F2a were obtained between 2006 to 2012 at Albert Einstein Diagnostic Medicine. The study was approved by the research Ethics committee of Albert Einstein Hospital (CEP/Einstein No 10/1293) and an informed consent for participation in the study was obtained from each patient.

These samples were initially subgenotyped using a system developed in the Clinical Laboratory [23] and were then selected for a longer fragment (1306 bp) amplification using other previously described primers [18].

For viral DNA extraction from 200 µl serum, it was used a commercial available kit (QIAamp mini, QIAGEN, Germany), according to the manufacturer's instructions. Precipitated DNA was resuspended in 200 µl of elution buffer and stored at -20°C until use. To avoid false-positive results, strict procedures proposed for nucleic acid amplification diagnostic techniques were followed [24]. The amplified DNA was purified using ChargeSwitch® PCR Clean-Up kit (Life Technologies, São Paulo, Brazil). Sequencing was performed in an ABI 3500 Genetic Analyzer (Life Technologies, Foster City, CA, USA) using dideoxynucleoside triphosphates (ddNTPs) containing fluorescent markers (Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction kit - Life Technologies, Foster City, CA, USA). The quality of each electropherogram was evaluated using the Phred-Phrap software [25] and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available at the web page Electropherogram quality analysis Phred (<http://www.biomol.unb.br/phph/>).

HBV sequences were aligned with other previously reported genome sequences (n = 198) using ClustalX software [26] and was edited with the SE-AL software (available from: tree.bio.ed.ac.uk/software/seal/). The Bayesian Markov chain Monte Carlo simulation implemented in BEAST v.1.4.8 [27] was applied to obtain the best possible estimates using both the relaxed uncorrelated lognormal and exponential molecular clock models and using the model of nucleotide substitution (general time-reversible + gamma + proportion invariant). The molecular clock that best fits the data was chosen by Bayes factor comparison. After 20 million generations, the maximum clade credibility (MCC) tree was obtained by summarizing 20,000 substitution trees and was then modified using a 10% burn-in using Tree Annotator v.1.5.3 [27]. Phylogenetic trees were visualized and midpoint rooted in FigTree v1.2.2 (tree.bio.ed.ac.uk/software/figtree/).

A phylogenetic tree constructed with the 1,306 bp sequences partially comprising HBsAg and DNA polymerase coding regions (S/POL) (n = 198) is shown in Figure 1, including the 19 new sequences obtained by our group from São Paulo state. Bayesian analyses were performed to try to determine the grouping of the genotype F2a sequences available. The complete dataset included 71 sequences from Brazil: North Region (3 AP - Amapá State, 5 RO - Rondônia State, 26 AM - Amazonas State, including 5 from Manaus, the capital city, 5 from the Apurinã people and 16 WAB from Western Amazon Basin (Apurinã and WAB samples were obtained from riverside communities living near Purus River); Northwest Region (5 PE -

Pernambuco State); Southeast Region (11 RJ - Rio de Janeiro State and 19 SP - São Paulo State) and Center West Region (2 MT - Mato Grosso State) (Figure 2).

The phylogenetic tree showed three groups containing sequences from the same state that grouped with a high a posteriori probability: a branch of sequences from the WAB, including Apurinã samples, all from the North Region and two branches of sequences from the Southeastern regions, one containing mostly sequences from Rio de Janeiro State and another with 10 sequences from São Paulo State. The other sequences did not show a pattern organized by Brazilian states or regions.

Currently, subgenotype F2a has been mostly found in Venezuela and it has been considered as originating from the Amazon region, this subgenotype has been spreading through the different entries in different regions so diverse.

We used all of genotype F sequences available in the GenBank and applied a coalescent-based approach. Ten HBV F2a sequences of patients from São Paulo were grouped in one cluster. It was not found specific relation or similar characteristics among these 10 patients when compared with the other nine sequences from São Paulo.

Since most of the subgenotype F2a sequences are from Brazil and it is not reported from which state each sequence was obtained, it was not possible to infer its routes of spread in this work. The spreading and the dynamic of subgenotype F2a in Brazil requires the study of a higher number of samples from different regions as it is unfold in almost all Brazilian populations studied so far. However, these results showing for the first time the distribution of F2a subgenotype in Brazil.

In conclusion, we identified three groups of sequences of subgenotype F2a: 1) 10 sequences from São Paulo state; 2) 3 sequences from Rio de Janeiro and one from São Paulo states; 3) 8 sequences from the West Amazon Basin. We cannot infer with certainty the origin of these different groups due to the lack of available sequences. Nevertheless, our data suggest that the common origin of these groups probably occurred a long time ago. In this sense, more studies should be carried out with HBV infected populations in our country, particularly with indigenous populations in other regions of our country, particularly in the regions where the indigenous populations were common at the time of the beginning of Brazil's colonization by European populations.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

MVAM participated in the design of the study and drafted the manuscript, conducted the sequencing process and phylogenetic analysis. LSBL and RAS participated in the PCR amplification and sequencing process. RS, PAF, CPM and FJC participated in the design of the study. FAM participated with some previously published data of Brazilian sequences. JRRP participated in the design of the study and the elaboration of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work has been supported by CNPq and by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP 2011/00562-0 and 2008/00461-6, HCRMUSP, FFM, Alves de Queiroz Family Fund for Research and IRS-SB/BAE. These supported institutions provided the found to develop the DNA amplification, sequencing and phylogenetic analysis.

Author details

¹Laboratory of Tropical Gastroenterology and Hepatology, Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, SP, Brazil. ²Albert Einstein Diagnostic Medicine, São Paulo, SP, Brazil. ³Federal University of São Paulo – UNFESP, São Paulo, SP, Brazil. ⁴Oswaldo Cruz Foundation, FIOCRUZ, Rio de Janeiro, Brazil.

Received: 8 February 2013 Accepted: 17 October 2013

Published: 21 October 2013

References

1. Tiollais P, Poullet C, Dejean A: The hepatitis B virus. *Nature* 1985, **317**:489–495.
2. Seeger C, Mason WS: Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000, **64**:51–68.
3. Lin CL, Kao JH: The clinical implications of hepatitis B virus genotype: recent advances. *J Gastroenterol Hepatol* 2011, **26**(Suppl 1):123–130.
4. Hannoun C, Soderstrom A, Norrman G, Lindh M: Phylogeny of African complete genomes reveals a West African genotype A subtype of hepatitis B virus and relatedness between Somali and Asian A1 sequences. *J Gen Virol* 2005, **86**:2163–2167.
5. Ollinger CM, Venard V, Njaye M, Oyeleke AO, Maiga I, Kemp AJ, Omlibu SA, le Faou A, Muller CP: Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 2006, **87**:1163–1173.
6. Adermach IE, Nolte C, Pape JW, Muller CP: Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg Infect Dis* 2009, **15**:1222–1228.
7. Pourkarim MR, Lemey P, Amini-Bavil-Olyaee S, Mares P, Van Ranst M: Novel hepatitis B virus subgenotype A6 in African-Belgian patients. *J Clin Virol* 2010, **47**:93–96.
8. McMahon BJ: The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatology* 2009, **3**:334–342.
9. Chu CJ, Keeffe EB, Han SH, Penttilä RP, Min AD, Soldavila-Pico C, Carey W, Brown RS Jr, Luketic VA, Terrault N, Lok AS: Prevalence of HBV precore/core promoter variants in the United States. *Hepatology* 2003, **38**:619–628.
10. Alvarado-Mora MV, Santana RA, Strik R, Ferreira PR, Manguerra CL, Camillo FJ, Pinho JR: Full-length genomic sequence of hepatitis B virus genotype C2 isolated from a native Brazilian patient. *Mem Inst Oswaldo Cruz* 2011, **106**:495–498.
11. Kramvis A, Kew MC: Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatology* 2007, **37**:59–519.
12. Alvarado-Mora MV, Romano CM, Gomes-Gouveas MS, Gutierrez MF, Camillo FJ, Pinho JR: Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol* 2010, **91**:501–508.
13. Singh J, Dickens C, Pahal V, Kumar R, Chaudhary R, Kramvis A, Kew MC: First report of genotype e of hepatitis B virus in an Indian population. *Intervirology* 2009, **52**:235–238.
14. Mulders MN, Venard V, Njaye M, Edoh AP, Bola Oyeleke AO, Kehinde MO, Muyembe-Tamfum JJ, Ndiye YK, Maiga I, Ammerlaan W, et al: Low Genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 2004, **190**:400–408.
15. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000, **81**:67–74.
16. Arauz-Ruiz P, Norder H, Robertson BH, Magnus LO: Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002, **83**:2059–2073.
17. Devesa M, Pujol FH: Hepatitis B virus genetic diversity in Latin America. *Virus Res* 2007, **127**:177–184.

18. Alvarado-Mora MV, Romano CM, Gomes-Gouveas MS, Gutierrez MF, Botelho I, Camillo FJ, Pinho JR: Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol* 2011, **11**:103–108.
19. Lanford RE, Chavez D, Brasly NM, Burns RB 3rd, Rico-Hesse R: Isolation of a hepatitis B virus from the woolly monkey, a New World primate. *Proc Natl Acad Sci USA* 1998, **95**:5757–5761.
20. Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, Duarte MC, Poblete F, Gutierrez MF, Botto C, Pujol FH: Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol* 2008, **80**:20–26.
21. Strik R, Pinho JR, Benolli DA, Bernardini AP, Da Silva LC, Camillo FJ: Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 2004, **42**:2455–2460.
22. Mello FC, Souto FJ, Nabuco LC, Vilela-Nogueira CA, Coelho HS, Franz HC, Santana JC, Virgolino HA, Motta-Castro AR, Melo MM, et al: Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol* 2007, **7**:103.
23. Strik R, Santana RA, Oyakawa L, Manguerra CL, Pinho JR: Molecular characterization of hepatitis B virus: a six years follow-up of genotypes and subgenotypes distribution. *J Mol Diagn* 2012, **14**(9):675–676.
24. Kwok S, Higuchi R: Avoiding false positives with PCR. *Nature* 1989, **339**:237–238.
25. Ewing B, Hillier L, Wendt MC, Green P: Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 1998, **8**:175–185.
26. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997, **25**:4876–4882.
27. Drummond AJ, Rambaut A: BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007, **7**:214.

doi:10.1186/1756-0500-6-423

Cite this article as: Alvarado-Mora et al.: Distribution of hepatitis B virus subgenotype F2a in São Paulo, Brazil. *BMC Research Notes* 2013 6:423.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

- 16- Rosamar E. F. Rezende, Benedito A. L. Fonseca, Leandra N. Z. Ramalho, Sérgio Zucoloto, JOÃO RENATO R. PINHO, Dennis A. Bertolini, Ana L. C. Martinelli. The precore mutation is associated with severity of liver damage in chronic Hepatitis B. Journal of Clinical Virology 32(1): 53 – 59 (2005).**

The precore mutation is associated with severity of liver damage in Brazilian patients with chronic hepatitis B

Rosamar E.F. Rezende^{a,*}, Benedito A.L. Fonseca^a, Leandra N.Z. Ramalho^b, Sérgio Zucoloto^b, João Renato R. Pinho^c, Dennis A. Bertolini^c, Ana L.C. Martinelli^a

^a Department of Medicine, School of Medicine of Ribeirão Preto, University of São Paulo, FMRP-USP, 14048-900, Ribeirão Preto, SP, Brazil

^b Department of Pathology, School of Medicine of Ribeirão Preto, University of São Paulo, FMRP-USP, Ribeirão Preto, Brazil

^c Virology Branch, Instituto Adolfo Lutz, São Paulo, Brazil

Received in revised form 1 August 2004; accepted 1 August 2004

Abstract

Background: The clinical relevance of the G1896A precore mutation in chronic hepatitis B is still poorly understood.

Objectives: To assess the frequency of G1896A precore mutation in Brazilian patients with chronic hepatitis B, as well as its relation to the viral genotype, serum HBV-DNA levels and liver damage.

Study design: Fifty chronic hepatitis B patients (29 HBeAg-negative and 21 HBeAg-positive) were studied. HBV-DNA was quantified by the Amplicor HBV Monitor test and precore region and S gene were amplified and submitted to automatic sequencing. The histological activity index (HAI), degrees of hepatic fibrosis and distribution of core antigen (HBcAg) in hepatocytes were determined.

Results: Precore mutation occurred in 1/21 (4.8%) HBeAg-positive patients and in 17/29 (58.6%) HBeAg-negative ($p < 0.0001$). Genotype D was identified in 56.5%, genotype A in 41.3%, and genotype F in 2.2%. The frequency of genotypes D and A, as well as serum levels of ALT and HBV-DNA were similar in patients infected with wild type and with precore mutant. Patients infected with precore mutant presented a higher frequency of moderate/severe HAI ($p: 0.03$) and moderate/severe fibrosis and cirrhosis ($p: 0.03$) than those infected with wild type. There was no association between G1896A mutation and cytoplasmic expression of HBcAg.

Conclusions: Precore mutation was frequent among Brazilian subjects with chronic hepatitis B and its presence was associated with greater severity of liver disease.

© 2004 Elsevier B.V. All rights reserved.

Keywords: Hepatitis B virus; HBV genotypes; G1896A precore mutation; Severity of liver disease; Chronic hepatitis

1. Introduction

The integrity of the host immunological system, viral replication and probably the genetic heterogeneity of the hepatitis B virus (HBV), including genotypes and mutations, play an important role in the determination of the natural

history of hepatitis B (Fattovich et al., 1991; Lindh et al., 2000).

The major mutation of the precore region of the HBV is that of codon 28, position of the 1896 nucleotide (G1896A), resulting from the exchange of a guanine to adenine, which promotes a TAG stop codon leading to premature interruption of the translation of the HBe antigen (Brunetto et al., 1989; Carman et al., 1989).

The prevalence of the precore mutation is variable, being high in the Mediterranean region and in the Far East and rare in the US and northern Europe (Hadziyannis and Vassilopoulos, 2001). In Brazil, the prevalence of this mu-

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis B virus; HBcAg, core antigen; HCV, hepatitis C virus; HAI, histological activity index; HIV, human immunodeficiency virus; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +55 16 602 2456; fax: +55 16 633 1144.

E-mail address: rosamarrezende@uol.com.br (R.E.F. Rezende).

1386-6532/\$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jcv.2004.08.001

tation is still unknown. A previous study in which hepatitis B virus DNA was detected by the polymerase chain reaction (PCR) in anti-HBe positive Brazilian patients with chronic hepatitis B suggested that infection by viral variant precore mutations could occur in our country (Pinho et al., 1993).

The variability of infection with the G1896A precore mutant of HBV in different geographic areas is related to the distribution of HBV genotypes, being more frequent in patients infected with the B, C, D and E genotypes and rare in patients infected with the A genotype (Hadziyannis and Vassilopoulos, 2001; Li et al., 1993; Lindh et al., 1995; Lok et al., 1994).

There are many doubts about the clinical significance of the G1896A precore mutation, with reports of its association with severe chronic hepatitis (Brunetto et al., 1990) and fulminant hepatitis (Bahn et al., 1995; Liang et al., 1991; Omata et al., 1991; Terazawa et al., 1991), and of its occurrence in inactive HBV carriers (Niituma et al., 1995; Okamoto et al., 1990; Tur-Kaspa et al., 1992).

The objectives of the present study were to assess the frequency of the G1896A precore mutation in Brazilian patients with chronic hepatitis B, to investigate its effect on the severity of liver damage and its relation to different HBV genotypes and serum HBV-DNA levels.

2. Patients and methods

2.1. Patient series

The study was conducted prospectively on 50 Brazilian patients followed up at the Hepatitis Outpatient Clinic of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo (HC-FMRP-USP), with chronic hepatitis B, i.e. with serum positivity for HBsAg for a period of more than 6 months and with evidence of liver damage determined by a liver biopsy showing chronic hepatitis or by persistent or intermittent elevation in serum ALT levels. The patients were selected at random and studied before antiviral treatment during the period from March 1999 to March 2002. All patients were white and came from the Southeast region of the country. Only two patients had a history of decompensated liver disease, due to upper digestive hemorrhage secondary to the rupture of esophageal varices associated with ascites in one, and to ascites complicated by spontaneous bacterial peritonitis in the other. No patient had hepatocellular carcinoma. Serum collected before antiviral treatment and stored at -20°C was available for all patients. Patients with hepatitis C virus (HCV) or human immunodeficiency virus (HIV) co-infection, with a history of alcohol ingestion of more than 40 g ethanol/day, with autoimmune hepatitis or drug-induced hepatitis were excluded. The study was conducted according to the ethical norms of the 1975 Helsinki Declaration and was analyzed and approved by the Ethics Committee of the institution. The patients included in the study signed a term of informed consent.

2.2. Clinical evaluation and determination of alanine aminotransferase (ALT)

Data concerning age, sex and risk factors for HBV infection were obtained and the time of HBV infection was estimated for patients with risk factors of blood transfusion and vertical transmission. Mean serum ALT levels were calculated after measurements on at least three occasions during a 6 month period preceding the liver biopsy (47 patients) or starting from the beginning of clinical follow-up for patients who were not submitted to a biopsy (three patients).

2.3. Serologic tests

A search for serologic markers of HBV (HBsAg, anti-HBc total, HBeAg, and anti-HBe), hepatitis C virus (anti-HCV) and human immunodeficiency virus type 1 and 2 (anti-HIV) was carried out by enzyme immunoassay (ELISA, Abbott Laboratories, Chicago, IL, USA).

2.4. Quantification of serum HBV-DNA

Serum HBV-DNA was quantified in 48/50 patients using the Amplicor HBV Monitor Test kit (Roche Diagnostic Systems, Branchburg, NJ, USA). The detection limits of this method range from 4×10^2 to 4×10^7 copies/ml.

2.5. HBV genotyping and detection of mutations in the precore region

HBV-DNA was extracted using the commercial QIAamp blood kit (Qiagen, Hilden, Germany) and a 200 μl serum aliquot according to manufacturer instructions. The S gene was amplified by nested PCR and HBV genotypes were determined by automatic sequencing (ABI Prism 377 Genetic Analyzer-PE Applied Biosystem, Foster City, CA, USA) as previously described (Sitnik et al., 2000). Mutation of codon 28 and genetic variability of the nucleotide at position 1858 (codon 15) of the precore region were determined by nested PCR according to a previously described method (Kramvis et al., 1998). Fragments of 204 base pairs (bp) were obtained by nested PCR and visualized on 1.5% agarose gel, corresponding to the position of nucleotides between 1763 and 1966. Automatic sequencing was carried out (ABI Prism 310 Genetic Analyzer-PE Applied Biosystem, Foster City, CA, USA) using the reagents of the ABI PRISM Big DyeTM Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA). All PCR procedures were accompanied by a negative control. The EditSeg and Megalign programs of the Lasergene software (DNASTar, Inc., Madison, WI, USA) and the PolyPhred software version 4.0 (University of Washington, Nickerson Group, USA) were used for analysis of the data obtained by automatic sequencing and the sequences obtained were compared to the HBV sequences of GenBank.

2.6. Liver histology and immunohistochemistry for the detection of HBcAg

Forty-seven patients were submitted to an ultrasound-guided percutaneous liver biopsy with a 14G Tru-Cut needle under local anesthesia. Analysis of liver histology and of immunohistochemistry was carried out by a single experienced pathologist of our service in a blinded fashion. The degree of fibrosis and the histological activity index (HAI) were assessed according to the classification of Knodell et al. (1981) modified by Desmet et al. (1994). Anti-HBc mouse primary monoclonal antibodies (DAKO A/S, Glostrup, Denmark) diluted 1:100 were used for the detection of the core antigen in liver. Standard light microscopy at 40 $\times$ magnification was used to determine the localization of HBc antigen positivity in the nucleus and cytoplasm of the hepatocytes, defined as N: nucleus, C: cytoplasm, and NC: nucleus and cytoplasm.

2.7. Statistical analysis

Proportions were compared by the exact Fisher test or by the chi-square test when recommended (expected frequency higher than five). Quantitative variables were analyzed by the nonparametric Mann–Whitney test. Values of 4×10^2 and 4×10^7 copies/ml were attributed when the serum levels of HBV-DNA were below or above the detection limit of the method employed, respectively. The level of significance was set at $p < 0.05$.

3. Results

3.1. Demographic and epidemiological characteristics of the patients

The patients were first classified into two groups according to the results of the HBeAg/anti-HBe serologic markers. During an observation period of at least 6 months, without receiving specific antiviral treatment, 21 patients presented HBeAg-positive/anti-HBe-negative serologic markers and 29 patients presented HBeAg-negative/anti-HBe-positive markers. The

Table 2

Distribution of patients infected with the wild type and the G1896A precore mutant variant according to HBV genotype

Genotype	Wild	G1896A precore mutant	p-value
A	14/29 (48.3%)	5/17 (29.4%)	0.3
D	15/29 (51.7%)	11/17 (64.7%)	0.3
F	–	1/17 (5.9%)	–

two groups were similar in terms of age, sex, risk factors, and estimated time of HBV infection (Table 1).

3.2. Frequency of mutations in the precore region

All 50 patients in the study presented amplification of the precore region by nested PCR. No false-positive PCR result was observed in the negative controls. The precore mutation of codon 28, G1896A, was detected in 18/50 (36%) patients and was more frequent in HBeAg-negative patients ($p < 0.0001$). The G1896A mutation occurred in 1/21 (4.8%) patients of the HBeAg-positive group (mixed sequence of wild type + precore mutant) and in 17/29 (58.6%) patients of the HBeAg-negative group (11/29 (37.9%) with mixed sequence of wild type + precore mutant and 6/29 (20.7%) with pure sequence precore mutant).

3.3. HBV genotypes and precore G1896A mutation

In four patients (three infected with the wild type and one infected with the precore mutant variant), identification of the HBV genotype was not possible because of difficulty in S gene amplification. The following genotypes were detected: genotype D in 26/46 (56.5%) cases, genotype A in 19/46 (41.3%) cases, and genotype F in 1/46 (2.2%). The frequency of genotype A and D distribution was similar for patients infected with the wild type and patients infected with the G1896A precore mutant variant (Table 2).

The presence of the thymine nitrogen base in the position of nucleotide 1858 was more frequent in genotype D ($p: 0.0005$). Thymine was observed in the position of nucleotide 1858 in 22/26 (84.6%) cases and cytosine in 4/26 (15.4%) cases of genotype D. In genotype A, thymine was

Table 1

General characteristic of patients with chronic hepatitis B distributed according to the presence or absence of HBe antigen in serum

	HBeAg (+)	HBeAg (–)	p-value
No of patients	21	29	
Age (years) Mean $\pm$ S.D. (range)	33.2 $\pm$ 13.2 (14–62)	34.1 $\pm$ 11.6 (15–59)	0.7
Sex (M/F)	16/5	25/4	0.4
Risk factors:			
Vertical	6 (28.6%)	12 (41.4%)	0.2
Transfusion	3 (14.3%)	1 (3.4%)	0.2
Sexual promiscuity*	4 (19%)	5 (17.2%)	1.0
Unknown	8 (38.1%)	11 (38%)	1.0
Time** of infection (years) (median)	25	27	0.8

* Defined as sex relations with more than three partners during a period of 6 months.

** Estimated time of HBV infection for patients whose risk factors were blood transfusion and vertical transmission.

observed in the position of nucleotide 1858 in 6/19 (31.6%) cases and cytosine in 13/19 (68.4%). The only case of genotype F coarsed with thymine in the position of nucleotide 1858.

There was association between the presence of the G1896A mutation and the presence of the thymine nitrogen base in the position of nucleotide 1858 of the precore region ($p < 0.0001$). Sequencing of the precore region of all patients infected with the G1896A precore mutant variant revealed thymine in the position of nucleotide 1858. All patients infected with the G1896A precore mutant variant, genotype A, presented the association of a second mutation, i.e. an exchange of cytosine with thymine, in the position of nucleotide 1858, codon 15 (C1858T). In the wild type, cytosine was detected in 19/32 (59.4%) patients and thymine in 13/32 (40.6%) patients in the position of nucleotide 1858.

3.4. Serum ALT and HBV-DNA levels and G1896A precore mutation

Patients infected with the wild type and the G1896A precore mutant variant presented similar median ALT values (Fig. 1). Quantification of serum HBV-DNA was not possible in two patients (one from the group infected with the wild type and the other from the group infected with the G1896A mutant precore variant). There was no difference in median serum HBV-DNA levels between patients infected with the wild type and patients infected with the G1896A precore mutant variant (Fig. 2).

3.5. Liver histopathology and G1896A precore mutation

Patients infected with the G1896A mutant variant presented a higher frequency of moderate/severe HAI ($p: 0.03$) and of moderate/severe fibrosis/cirrhosis ($p: 0.03$) than patients infected with the wild type (Table 3). Immunohistochemistry for the detection of the HBe antigen was positive in 24/29 (82.7%) patients infected with the wild type and in 14/18 (77.8%) patients infected with the G1896A pre-

Fig. 2. Serum HBV-DNA levels in patients infected with the wild type and the mutant G1896A precore variant of HBV. Median: (—).

Table 3
Frequency of the different degrees of liver damage (HAI and fibrosis scores) and of intracellular HBe antigen expression in patients with chronic HBV infection with the wild type and the mutant G1896A precore variant of HBV

	Wild	G1896A precore mutant	p-value
Liver HAI score			0.03*
Minimal/mild	18/29 (62.1%)	6/18 (33.3%)	
Moderate/severe	11/29 (37.9)	12/18 (66.7)	
Liver fibrosis			0.03**
Absent/mild	18/29 (62.1%)	6/18 (33.3%)	
Moderate/severe	10/29 (34.5%)	11/18 (61.1%)	
Cirrhosis	1/29 (3.4%)	1/18 (5.6%)	
Positive HBeAg			1.0
Nucleus	13/24 (54.2%)	8/14 (57.1%)	
Cytoplasm	—	2/14 (14.3%)	
Nucleus/cytoplasm	11/24 (45.8%)	4/14 (28.6%)	

* Minimal/mild HAI vs. moderate/severe HAI.

** Absent/mild fibrosis vs. moderate/severe fibrosis and cirrhosis.

core mutant variant ($p: 0.7$). There was no difference in HBe expression predominantly in the nucleus or predominantly in the cytoplasm and nucleus/cytoplasm between patients infected with the wild type and patients infected with the G1896A precore mutant variant (Table 3).

4. Discussion

In the 1980 decade, infection with the precore mutant variant was believed to be limited to the Mediterranean area and to the Far East, with a frequency of 50% to 80% in Greece, Israel and Italy and of 40% to 55% in Hong Kong, Taiwan and Japan (Hadziyannis, 1995). However, different studies have demonstrated a wide geographic distribution of this variant, which has also been reported in the United Kingdom (Ballard and Boxall, 2000) and Germany (Knoll et al., 1999).

In the present study, the G1896A precore mutation occurred in 36% of Brazilian patients with chronic hepatitis B. In agreement with other authors (Grandjacques et al., 2000;

Fig. 1. Serum ALT levels in patients infected with the wild type and the mutant G1896A precore variant of HBV. Median: (—). Reference values: ALT: 10–44 U/L.

Lok et al., 1994; Mangia et al., 1996), the frequency of the G1896A mutation was significantly higher in the HBeAg-negative group, demonstrating a higher prevalence of this mutation during a later phase of infection, after HBeAg/anti-HBe seroconversion, thus permitting the persistence of viral replication and the consequent maintenance of the hepatic necro-inflammatory activity. The ϵ antigen is an important target for cell-mediated and antibody-mediated immune responses, so that the loss of ϵ antigen production by precore mutants may help the virus to evade the host immune response (Zuckerman and Zuckerman, 1999). Hence the occurrence of the precore mutation seems to be a strategy of viral selection secondary to the immunological pressure against HBV (Hadziyannis and Vassilopoulos, 2001).

Few data are available in Brazil about the prevalence of the HBV genotypes. In the present study, in agreement with previous observations about the genotypic distribution of HBV in HBV-infected Brazilian patients (Moraes et al., 1996; Teles et al., 1999), the most frequent genotypes were genotype D (56.5%) and genotype A (41.3%), whereas genotype F was rare (2.2%).

The G1896A precore mutation was more frequent in the group of patients with genotype D than in the patients with genotype A. However, possibly because of the small size of the sample studied, no difference was observed in the frequency of genotype distribution when the patients groups with wild type infection and precore mutant infection were compared. The relation between the G1896A precore mutation and the HBV genotypes is due to the base pairing of the stem loop structure of the encapsidation sequence of pregenome RNA. Nucleotide 1896 (codon 28) present in the descending part of the lower stem of pregenome RNA pairs with nucleotide 1858 (codon 15) located in the ascending part of this same stem. When the G1896A mutation is present, if nucleotide 1858 is composed of the thymine–nitrogen base, there is better stabilization of the lower stem, and the opposite occurs when cytosine is present at position 1858 (Li et al., 1993; Lindh et al., 1999; Lok et al., 1994). In the present study, all patients with the G1896A mutation presented thymine at position 1858 and all patients with HBV genotype A and with the G1896A mutation presented the C1858T mutation in association. These results support previous observations about the association between nucleotide 1858 consisting of the thymine–nitrogen base and the G1896A mutation precore mutation (Hunt et al., 2000; Lok et al., 1994; Lindh et al., 1995; Rodriguez-Frias et al., 1995).

There was no significant difference in ALT or HBV-DNA levels between patients infected with the G1896A precore mutant and patients infected with the wild type. However, 14 patients infected with the wild type and only three patients infected with the G1896A precore mutant presented serum levels of HBV-DNA higher than 4×10^7 copies/ml. We wish to point out that the upper limit of detection of serum HBV-DNA levels by the Amplicor HBV Monitor test is 4×10^7 copies/ml. It is possible that, if the real value of

HBV viremia had been available to us, we might have observed differences in serum HBV-DNA levels between the group of patients infected with the wild type and the group of patients infected with the G1896A precore mutant, with these levels being higher in the group of patients infected with the wild type. Grandjacques et al. (2000) studied 85 HBeAg-positive patients and 66 anti-HBe-positive patients and observed results similar to those obtained here with respect to ALT levels and the presence of the precore mutation. The same authors, however, observed lower HBV-DNA levels in patients infected with the mutant precore variant than in patients infected with the wild type. Based on *in vitro* studies, it has been suggested that precore mutant variant has better replicating ability than the wild type (Guidotti et al., 1996; Lamberts et al., 1993). A difference in viral replication between the mutant G1896A variant and the wild type has not been demonstrated using *in vivo* animal models (Chen et al., 1992).

The impact of the G1896A precore mutation on the natural history of hepatitis B, and its role in the pathogenesis of liver disease are still unknown. Acute infection with the precore mutant variant has been associated with a higher incidence of fulminant hepatitis (Bahn et al., 1995; Kosaka et al., 1991; Liang et al., 1991; Omata et al., 1991; Shafritz, 1991). In chronic infection, the emergence of the precore mutant variant during the immune clearance phase or after HBeAg/anti-HBe seroconversion, causing this to be the predominant variant in relation to the wild type, may be associated with episode of exacerbation of liver disease (Laskus et al., 1994) and with a lower response to antiviral treatment compared to the wild type (Rizzetto et al., 2000). However, the precore mutant variant has also been observed in inactive carriers (Nitsuma et al., 1995; Tur-Kaspa et al., 1992). Thus, variable results have been reported concerning the precore mutation and liver damage. In the present study, we observed an association between the presence of the precore mutation and severity of liver damage. Histopathological analysis of the liver revealed that patients infected with the G1896A precore mutant presented more severe liver damage, with a higher frequency of moderate/severe HAI scores (67%) and severe fibrosis (61%) compared to patients infected with the wild type. In the present study, positivity of the HBe antigen in the hepatocytes was similar for patients infected with the wild type and patients infected with the G1896A precore mutant. It has been reported that a predominantly cytoplasmic distribution of the core antigen (HBe) in the hepatocytes is related to greater liver aggression (Chu et al., 1995; Franca et al., 1989; Ramalho et al., 1988). An association between the presence of the G1896A precore mutation and the cytoplasmic expression of HBeAg has been recently reported (Grandjacques et al., 2000; Park et al., 1999). In contrast, in the present study no relation was observed between G1896A precore mutation and cytoplasmic expression of the core antigen, although the small number of cases evaluated should be considered. It is possible that that other, still no elucidated, mechanisms associated with the precore mutation which do

not depend on cytoplasmic expression of the HBc antigen participate in liver aggression.

We conclude that infection with the G1896A precore variant was frequent in the Brazilian population studied and that the presence of the G1896A precore mutation was associated with greater severity of liver damage.

Acknowledgements

The authors are grateful to Marie Secaf and Sandra S. Rodrigues for excellent technical assistance. This research was partly supported by FAPESP.

References

- Bahn A, Hilbert K, Martiné U, Westedt J, Weizsäcker FV, Wirt S. Selection of a precore mutant after vertical transmission of different hepatitis B virus variants is correlated with fulminant hepatitis in infants. *J Med Virol* 1995;47:336–41.
- Ballard AL, Bosall EH. Epidemiology of precore mutants of hepatitis B in the United Kingdom. *J Med Virol* 2000;62:463–70.
- Brunetto MR, Stemler M, Schodel F, Will H, Ottobrelli A, Rizzetto M, et al. Identification of HBV variants which cannot produce precore derived HBeAg and may be responsible for severe hepatitis. *Ital J Gastroenterol Hepatol* 1989;21:151–4.
- Brunetto MR, Stemler M, Bonino F, Schodel F, Oliveri F, Rizzetto M, et al. A new hepatitis B virus strain in patients with severe anti-HBe positive chronic hepatitis B. *J Hepatol* 1990;10:258–61.
- Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989;2:588–91.
- Chen HS, Kew MC, Hornbuckle WE, Tennant BC, Cote PJ, Gerin JL, et al. The precore gene of the woodchuck hepatitis virus genome is not essential for viral replication in the natural host. *J Virol* 1992;66:5682–4.
- Chu C-M, Yeh C-T, Sheen I-S, Liaw Y-F. Subcellular localization of hepatitis B core antigen in relation to hepatocyte regeneration in chronic hepatitis B. *Gastroenterology* 1995;109:1926–32.
- Desmet V, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;19:1513–20.
- Fattovich G, Brollo L, Giustina G, Noventa F, Pontisso P, Alberti A, et al. Natural history and prognostic factors for chronic hepatitis type B. *Gut* 1991;32:294–8.
- Franca ST, Kiyosawa K, Imai Y, Sodeyama T, Hayata T, Nakano Y, et al. Change of intrahepatic expression of hepatitis – B core antigen during the clinical course of type-B chronic hepatitis. *Scand J Gastroenterol* 1989;24:454–60.
- Grandjacques C, Pradat P, Stryver L, Chevallier M, Chevallier P, Pichoud C, et al. Rapid detection of genotypes and mutations in the pre-core promoter and the pre-core region of hepatitis B virus genome: correlation with viral persistence and disease severity. *J Hepatol* 2000;33:430–9.
- Guidotti LG, Matzke B, Pasquinelli C, Shoenberger JM, Rogler CE, Chisari FV. The hepatitis B virus (HBV) precore protein inhibits HBV replication in transgenic mice. *J Virol* 1996;70:7056–61.
- Hadziyannis SJ. Hepatitis B e antigen negative chronic hepatitis B: from clinical recognition to pathogenesis and treatment. *Viral Hepat Rev* 1995;1:7–36.
- Hadziyannis SJ, Vassilopoulos D. Hepatitis B e antigen-negative chronic hepatitis B. *Hepatology* 2001;34:617–24.
- Hunt CM, McGill JM, Allen MJ, Condreay LD. Clinical relevance of hepatitis B viral mutations. *Hepatology* 2000;31:1037–44.
- Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981;1:431–5.
- Knoll A, Rohrer A, Kochanowski B, Wurm EM, Jilg W. Prevalence of precore mutants in anti-HBe positive hepatitis B virus carriers in Germany. *J Med Virol* 1999;59:14–8.
- Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshida M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. *Gastroenterology* 1991;100:1087–94.
- Kranvis A, Kew MC, Bukofzer S. Hepatitis B virus precore mutants in serum and liver of southern african blacks with hepatocellular carcinoma. *J Hepatol* 1998;28:132–41.
- Lamberts C, Nassal M, Velhagen I, Zentgraf H, Schroder CH. Precore-mediated inhibition of hepatitis virus progeny DNA synthesis. *J Virol* 1993;67:3756–62.
- Laskus T, Rakela J, Tong MJ, Persing DH. Nucleotide sequence analysis of the precore region in patients with spontaneous reactivation of chronic hepatitis B. *Dig Dis Sci* 1994;39:2000–6.
- Li J-S, Tong S-P, Wen Y-M, Virvitski L, Zhang G, Trépo C. Hepatitis B virus genotype A rarely circulates as an HBe-minus mutant: possible contribution of a single nucleotide in the precore region. *J Virol* 1993;67:5402–10.
- Liang TJ, Hasegawa K, Rimmon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N Engl J Med* 1991;324:1705–9.
- Lindh M, Furuta Y, Vahlne A, Norkrans G, Horal P. Emergence of precore TAG mutation during hepatitis B e seroconversion and its dependence on pregenomic base pairing between nucleotides 1858 of 1896. *J Infect Dis* 1995;172:1343–7.
- Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. *J Infect Dis* 1999;179:775–82.
- Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histology activity in chronic hepatitis B. *J Viral Hepat* 2000;7:258–67.
- Lok ASF, Alcaraz U, Greene S. Mutation in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc Natl Acad Sci USA* 1994;91:4077–81.
- Mangia A, Chung YH, Hoofnagle JH, Birkenmeyer L, Mushahwar I, Di Bisceglie AM. Pathogenesis of chronic liver disease in patients with chronic hepatitis B virus infection without serum HBeAg. *Dig Dis Sci* 1996;41:2447–52.
- Moraes MT, Gomes AS, Niel C. Sequence analysis of pre-S/S gene of hepatitis B virus strains of genotypes A, D, and F isolated in Brazil. *Arch Virol* 1996;141:1767–73.
- Niitsuma H, Ishii M, Saito Y, Miura M, Kobayashi K, Hitoshi O, et al. Prevalence of precore-defective mutant of hepatitis B virus in HBV carriers. *J Med Virol* 1995;46:397–402.
- Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected host along with seroconversion to the antibody against e antigen. *J Virol* 1990;64:1298–303.
- Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991;324:1699–703.
- Park YN, Han KH, Kim KS, Chung JP, Kim S, Park C. Cytoplasmic expression of hepatitis B core antigen in chronic hepatitis B virus infection: role of precore stop mutants. *Liver* 1999;19:199–205.
- Pinho JRR, Santos CA, Gonzalez CLM, Bassit L, Barreto CC, Saez-Alquezar A, et al. Detection of hepatitis B virus DNA by the poly-

- merase chain reaction in anti-HBe positive chronic hepatitis B patients. *Rev Inst Med Trop São Paulo* 1993;35:515–20.
- Ramalho F, Bunetto MR, Rocca G, Piccari GG, Batista A, Chianberge E, et al. Serum markers of hepatitis B virus replication, liver histology and intrahepatic expression of hepatitis B core antigen. *J Hepatol* 1988;7:14–20.
- Rizzetto M, Volpes R, Smedile A. Response of pre-core mutant chronic hepatitis B infection to lamivudine. *J Med Virol* 2000;61:398–402.
- Rodriguez-Frias F, Buti M, Jardi R, Cotrina M, Viladomiu L, Esteban R, et al. Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. *Hepatology* 1995;22:1641–7.
- Shafiritz DA. Variants of hepatitis B virus associated with fulminant liver disease. *N Engl J Med* 1991;324:1737–9.
- Simik R, Pinho JRR, Da Silva LC, Fonseca LEP, Carrilho FJ, Bernardini AP. Hepatitis B virus genotypes and pre core mutants in chronic hepatitis B patients from São Paulo city Brazil. *Hepatology* 2000;32:587A abstract.
- Teles SA, Martins RMB, Vanderborgh B, Stuyver L, Gaspar AMC, Yoshida CFT. Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients. *Artif Organs* 1999;23:1074–8.
- Terazawa S, Kojima M, Yamanaka T, Yotsumoto S, Okamoto H, Tsuda F, et al. Hepatitis B virus mutants with precore-region defects in two babies with fulminant hepatitis and their mothers positive for antibody to hepatitis B e antigen. *Pediatr Res* 1991;29:5–9.
- Tur-Kaspa R, Klein A, Aharonson S. Hepatitis B virus precore mutants are identical in carriers from various ethnic origins and are associated with a range of liver disease severity. *Hepatology* 1992;16:1338–42.
- Zuckerman AJ, Zuckerman JN. Molecular epidemiology of hepatitis B virus mutants. *J Med Virol* 1999;58:193–5.

- 17- Bertolini DA, Ribeiro PC, Lemos MF, Saraceni CP, Pinho JR. Characterization of a Hepatitis B virus strain in southwestern Paraná, Brazil, presenting mutations previously associated with anti-HBs Resistance. Revista do Instituto de Medicina Tropical de São Paulo, 2010 Feb;52(1):25-30**

Rev. Inst. Med. trop. S. Paulo
52(1):25-29, January-February, 2010
doi: 10.1590/S0036-46652010000100005

CHARACTERIZATION OF A HEPATITIS B VIRUS STRAIN IN SOUTHWESTERN PARANÁ, BRAZIL, PRESENTING MUTATIONS PREVIOUSLY ASSOCIATED WITH ANTI-HBs RESISTANCE

Dennis Armando BERTOLINI(1), Patrícia Cristiane RIBEIRO(2), Marcílio Figueiredo LEMOS(3), Cláudia Patara SARACENI(3) & João Renato Rebello PINHO(4)

SUMMARY

The present study investigated if hepatitis B virus (HBV) mutants circulate in the southwestern region of the State of Paraná, Brazil, by analyzing samples from children who received immunoprophylaxis but were born to HBV carrier mothers. Samples from 25 children were screened for HBV serum markers and for HBV DNA by PCR. Only one sample was positive for HBsAg, anti-HBs and HBV DNA, although the child had been vaccinated. Analysis of the S gene sequence of this sample showed the presence of a proline at position 105, a serine at position 114, three threonines at positions 115, 116 and 140, and a glutamine at position 129. The presence of these amino acids, except for serine at position 114, has been related to monoclonal or polyclonal therapy with anti-HBs after liver transplantation, whereas the presence of threonine at position 116 has been described in immunized children from Singapore. This finding demonstrates the possible circulation of HBV strains resistant to hepatitis B immunoprophylaxis in southwestern Paraná, Brazil. The genotype of the sample was identified as genotype D, which is frequently found in the region studied. Since 36% of the children had received incomplete or no immunoprophylaxis, more extensive follow-up of children born to HBsAg-positive mothers is needed.

KEYWORDS: Hepatitis B; HBsAg; Vaccine; Hyperimmune gammaglobulin; Mutations.

INTRODUCTION

Hepatitis B virus (HBV) belongs to the genus *Orthohepadnavirus*, family *Hepadnaviridae*. HBV infection is one of the major global public health problems. Among the approximately two billion people infected worldwide, more than 350 million are chronic HBV carriers²⁰. Approximately 400,000 new cases are estimated to occur in Latin America each year²².

HBV is characterized by its wide genetic variability. These genetic differences have led to the classification of the HBV into eight genotypes (A-H), which are divided into subgenotypes with distinct virological and epidemiological properties²⁶. Few genotype data are available in Brazil, but it is known that A, D and F are the main genotypes circulating in the country. MELLO *et al.* (2007) identified genotypes A, D and F among 303 HBV isolates which were obtained from all geographic regions of Brazil, genotype D being the most prevalent in the south region (84.2%). SITNIK *et al.* (2004), studying 103 samples from patients with chronic hepatitis B originating from different regions of Brazil, found genotypes A (49.5%), B (2.9%), C (13.6%), D (24.3%), and F (9.7%). In Paraná, genotypes A (14.0%), C (1.3%), D (82.9%), F (1.3%) and H (0.4%) were identified in samples from HBsAg-positive blood donors (BERTOLINI *et al.*, unpublished data).

Hepatitis B is an important disease in the State of Paraná, Brazil^{8,10,28}.

An intermediate prevalence has been recently observed in Cascavel, Foz do Iguaçu and Francisco Beltrão³. Vaccination of risk groups started in 1991 but had no impact on infection control. Children under one year of age were vaccinated in 1995, when the hepatitis B vaccine was first introduced in the National Immunization Program throughout Brazil. Whereas in 1998 the vaccine was applied to children under 15 years of age only in the southwestern region of the State of Paraná, vaccination was extended to all states in 1999. In 2001, the age range comprised individuals under 21 years of age¹⁸. The measures mentioned above led to a rapid decline in the incidence of hepatitis B in the under-15-year-old population. Preliminary results show that vaccination strategies are effective in the control of hepatitis B in a certain age group¹⁸.

The exposure of the host's immune system to viral surface antigen (HBsAg) during HBV infection provides an important basis for the protective response against the virus. However, HBsAg exposure, together with the fact that HBV has a mutation rate ten times higher than that of other DNA viruses¹⁵, favors the emergence of mutations in the surface (S) gene. Data regarding the primary structure of HBsAg in different regions of the world are important for the understanding of failure of protection with current vaccines²⁷.

Point mutations in the S gene that result in amino acid substitutions in the common "a" antigenic determinant, which lies between amino acids 124 and 147, can alter B-cell epitopes of HBsAg, leading

Financial Support: CNPq

(1) Laboratory of Clinical Immunology, Department of Clinical Analyses, Universidade Estadual de Maringá, Maringá, PR, Brazil.

(2) Undergraduate Pharmacy Course, Universidade Estadual de Maringá, Maringá, PR, Brazil.

(3) Laboratory of Hepatitis, Instituto Adolfo Lutz, São Paulo, SP, Brazil.

(4) Laboratory of Tropical Gastroenterology, Institute of Tropical Medicine, Faculty of Medicine, Universidade de São Paulo, São Paulo, SP, Brazil.

Correspondence to: Dennis Armando Bertolini, Department of Clinical Analyses, Universidade Estadual de Maringá, Avenida Colombo 5790, 87020-900 Maringá, PR, Brazil. Phone.: +55 44 3261 4878; fax: +55 44 3261 4860. E-mail: dabertolini@uem.br

BERTOLINI, D.A.; RIBEIRO, P.C.; LEMOS, M.F.; SARACENI, C.P. & PINHO, J.R.R. - Characterization of a hepatitis B virus strain in southwestern Paraná, Brazil, presenting mutations previously associated with anti-HBs resistance. *Rev. Inst. Med. Trop. São Paulo*, 52(1): 25-29, 2010.

to variants that escape from the host immune response elicited by vaccination or previous infection¹⁶. WALLACE & CARMAN (1997) suggested increasing the area of investigation in the "S" region to include the entire major hydrophilic region (MHR) which comprises amino acids 99 to 169. The authors proposed the following system for the classification of mutations into five regions: HBs1, region upstream of amino acid 120; HBs2, region comprising amino acids 120 to 123; HBs3, region comprising amino acids 124 to 137; HBs4, region comprising amino acids 138 to 147, and HBs5, region comprising amino acids 148 to 169.

The most common HBsAg variant associated with resistance to vaccine-induced anti-HBs is a glycine-to-arginine substitution at amino acid 145 of the MHR of protein S^{4,24,31}. Vaccine-associated variants have been demonstrated in Italy⁴, the United Kingdom²⁰, the United States¹⁹, Singapore²³, Taiwan^{16,22}, Senegal¹¹, India²⁹, Indonesia⁵, and Japan²⁴. The following vaccine-associated HBsAg mutations were identified: I110V, T1126N, Q129H, M133L, K141E, P142S, D144A, G145R, and C149R^{6,7,32}. OON *et al.* (1999) detected various previously unknown mutations outside the region of the "a" determinant in immunized children from Singapore (N116T, V118A, A159V, F183C, and V184A). Recently, ARAÚJO *et al.* (2008) reported the presence of a single amino acid substitution, L215Q, in genotype F which may affect the conformation of HBsAg, possibly causing defective insertion in the endoplasmic reticulum membrane and intracellular retention.

Vaccine-induced escape mutants of the HBV S gene pose a high risk to society since the use of hepatitis B immunoglobulins (HBIG) and vaccines are not effective in preventing infection. Blood units containing HBV mutants are not detected by routine screening for HBsAg and may transmit HBV infection to recipients of blood transfusions. Since these mutants may actively replicate even in the presence of a powerful anti-HBc response in the host, the exclusion of blood units with high anti-HBc titers is expected to be effective in the prevention of their transmission by transfusion³¹.

Preliminary studies have detected mutations associated with vaccine resistance in HBV strains isolated from blood donors from the southeastern region of the State of Paraná²³. This fact emphasizes the urgent need to determine the presence of HBV mutants in children. Progression of the disease in children has serious consequences since it maintains infection within the population.

Therefore, the objective of the present study was to investigate if HBV mutants associated with resistance to vaccine and HBIG circulate in the southwestern region of the State of Paraná.

PATIENTS AND METHODS

Patients: A total of 123 children from the 8th Regional Health Division of the municipality of Francisco Beltrão were studied. These children were tested for HBIG between 1998 and 2004, but only 25 were located for the collection of biological material.

A blood sample (5-10 mL) was collected from each of the 25 children with disposable syringes under aseptic conditions. After serum separation the samples were divided into two tubes and stored in a freezer at -20 °C until the time of use.

The study was approved by the Ethics Committee on Human Research of the State University of Maringá, and written informed consent was obtained from all mothers.

Serology for HBV: Serum HBV markers (HBsAg, total anti-HBc, anti-HBc IgM and anti-HBs) were analyzed by microparticle immunoassay using commercial kits (Abbott Laboratories, Abbott Park, Illinois, USA) in an AXSYM device (Abbott Laboratories) according to manufacturer specifications.

Amplification of the HBV S gene region by the Polymerase Chain Reaction (PCR): All samples were submitted to PCR amplification of the HBV S gene region, i.e., the region of the viral genome containing the MHR, thus permitting the identification of mutations that confer resistance to vaccine and HBIG.

HBV DNA was extracted from 100 µL serum in a 300 µL guanidine isothiocyanate solution. Two primer sets were used for nested PCR: FHBS1 (5' GAG TCT AGA CTC GTG GTG GAC TTC 3') and RHBS1 (5' AAA TKG CAC TAG TAA ACT GAG CCA 3') for the first reaction, and FHBS2 (5' CGT GGT GGA CTT CTC TCA ATT TTC 3') and RHBS2 (5' GCC ARG AGA AAC GGR CTG AGG CCC 3') for the second reaction. The primers occupy the following positions in the HBV genome (strain HBVADW; GenBank accession number V00866): HBS1F (positions 244 to 267), HBS2F (positions 255 to 278), HBS2R (positions 648 to 671), and HBS1R (positions 668 to 691). The two reactions were carried out in a thermocycler (TPersonal Thermocycler, Biometra) programmed as follows: one cycle at 94 °C for 30 s for initial denaturation, 35 cycles at 94 °C for 20 s (denaturation), 56 °C for 20 s (annealing), and 72 °C for 30 s (extension), and one cycle at 72 °C for five min. The samples were stored at 4 °C for an indeterminate period of time²⁷.

After amplification in duplicate, the nested PCR product was identified by electrophoresis on 1.0% agar gel stained with ethidium bromide and photographed with a Polaroid camera. Samples were classified as positive when they presented a band with a molecular weight close to 450 bp.

Sequencing of the S gene region: The nested PCR products were sequenced in duplicate using dideoxynucleotide triphosphates (ddNTPs) labeled with fluorescent markers. Sequencing reactions were performed using the ABI Prism BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) in an automatic ABI Prism 377 sequencer (PE Applied Biosystems). Sequences were analyzed in both directions (forward and reverse) using primers FHBS2 and RHBS2.

HBV genotyping: The EditSeq and Megalign programs of the DNASTar package (Lasergene Inc., USA) were used for the analysis, correction and alignment of nucleotide and amino acid sequences. The accession number of the strain in GenBank is FJ613840.

The genotype was classified by alignment of the nucleotide sequence of the S gene with 53 already known sequences of 8 HBV genotypes (A-H), deposited at DDBJ/EMBL/GenBank (Table 1). Sequences were aligned using the CLUSTAL method³⁴ and the PAM250 weight residue table.

Identification of mutations in the S gene region causing resistance

deposited in GenBank. Genotype D is the most prevalent in the southern region of Brazil^{17,27} and in the State of Paraná².

The Technical Committee of the National Program of Immunization, National Health Foundation, Brazilian Ministry of Health³⁰, has suspended the supply of HBIG to children born to HBsAg-positive mothers between July 2000 and October 2002. It was reported that the vaccine itself would provide the same protection. However, the nine (36%) children who received incomplete or no immunoprophylaxis were not born during this period.

Positivity for HBsAg and anti-HBs serum markers might be observed when the patient is in the resolution phase of the infection. The child with this serum marker profile was born on May 31, 2004 and only received the hepatitis B vaccine on June 1, 2004, a fact that might have contributed to infection. The child may have been infected during the period of sample collection for the present study. When HBV positivity was confirmed by analysis of serum markers and PCR, the Epidemiological Service of the municipality of Salto do Lontra was notified to guarantee medical follow-up.

One child from the municipality of Cascavel, born on November 9, 1997, was not positive for any of the serum markers analyzed, although the child had been vaccinated on February 3, 1998. One child (5.3%) was positive for hepatitis B by analysis of serum markers and PCR. The presence of amino acids P, T, T, Q and T at positions 105, 115, 116, 129 and 140, respectively, may indicate the possibility of resistance to vaccine and HBIG. This fact shows that immunoprophylaxis-resistant HBV strains may circulate in the southwestern region of the State of Paraná, Brazil.

Most children (n = 12, 63.2%) received complete hepatitis B immunoprophylaxis, including vaccine and HBIG. However, nine (36%) children received incomplete or no immunoprophylaxis, although they were born during a period other than that from July 2000 to October 2002, a fact that may have exposed them to the HBV infection. Immunoprophylaxis of these children should be better controlled by health authorities to prevent the consequences of the disease and transmission of the infection.

RESUMO

Caracterização de uma cepa de hepatite por vírus B no sudoeste do Paraná, Brasil, apresentando mutações previamente associadas à resistência anti-HBs

O presente estudo investigou se mutantes do vírus da hepatite B (HBV) circulam na região Sudoeste do Estado do Paraná, Brasil, analisando amostras de crianças que receberam a imunoprofilaxia por terem nascido de mães portadoras do HBV. Amostras de 25 crianças foram analisadas para os marcadores sorológicos do HBV e para o DNA-HBV por PCR. Somente uma amostra foi positiva para AgHBs, anti-HBs e DNA-HBV, apesar da criança ter sido vacinada. Análises da sequência do gene S desta amostra mostrou a presença de uma prolina na posição 105, uma serina na posição 114, três treoninas nas posições 115, 116 e 140, e uma glutamina na posição 129. A presença destes aminoácidos, exceto para Serina na posição 114, tem sido relacionada a terapia monoclonal ou policlonal com anti-HBs após transplante de fígado, enquanto a presença da treonina na posição 116 tem sido descrita

em crianças imunizadas de Singapura. Este achado demonstra a possível circulação de cepas do HBV resistentes a imunoprofilaxia para hepatite B no Sudoeste do Paraná, Brasil. O genótipo da amostra foi identificado como genótipo D, o qual é frequentemente encontrado na região estudada. Desde que 36% das crianças tinham recebido incompleta ou nenhuma imunoprofilaxia, um seguimento mais intensivo das crianças nascidas de mães AgHBs positivo é necessário.

ACKNOWLEDGEMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazilian Ministry of Science and Technology. We thank the healthcare workers of the Departments of Epidemiological Surveillance of the municipalities participating in the study, and the 8th Regional Health Division of Francisco Beltrão.

REFERENCES

1. Araújo NM, Vianna COA, Soares CC, Gomes AS. A unique amino acid substitution, L215Q, in the hepatitis B virus small envelope protein of a genotype F isolate that inhibits secretion of hepatitis B virus subviral particles. *Intervirology*. 2008;51:81-6.
2. Bertolini DA. Estudo da prevalência do vírus da hepatite B e dos seus genótipos no Estado do Paraná, Brasil. (Tese). São Paulo: Universidade Federal de São Paulo; 2002.
3. Bertolini DA, Moreira RC, Saraceni CP, Granato CFH, Pinho JRR. Surface gene mutations of hepatitis B in blood donors samples from Paraná State - Brazil. In: 14th Encontro Nacional de Virologia, Florianópolis, 2003. (*Virus Res*. 2003;8:116-7).
4. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manziello G, Tanzi E, et al. Vaccine-induced escape mutant of hepatitis B virus. *Lancet*. 1990;336:325-9.
5. Carman WF, Korula J, Wallace L, Macpherson R, Mimms L, Decker R. Pulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. *Lancet*. 1995;345:1406-7.
6. Carman WF. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepatitis*. 1997;4(suppl.1):11-20.
7. Carman WF, Thomas HC, Zuckerman AJ, et al. Molecular variants of hepatitis B virus. In: Zuckerman AJ, Thomas HC. *Viral hepatitis*. 2. ed. London: Churchill Livingstone; 1998. p. 141-177.
8. Carman WF, Owsianka A, Wallace LA, Dow BC, Mutimer DJ. Antigenic characterization of pre- and post-liver transplant hepatitis B surface antigen sequences from patients treated with hepatitis B immune globulin. *J Hepatol*. 1999;31:195-201.
9. Carrilho FJ, Corrêa MCJM. Magnitude of hepatitis B and C in Latin America. In: Schinazi RF, Sommadossi JP, Thomas HC. *Therapies for viral hepatitis*. London: International Medical Press; 1998. p. 25-34.
10. Carvalho SP, Dias SLNG. Estado comparativo da taxa de incidência de sífilis, doença de Chagas, hepatites B e C, HIV e HTLV, em doadores de sangue por região do Paraná. *Rev Bras Anal Clin*. 1995;27:77-82.
11. Coursaget P, Yvonne B, Bourdill C, Mevelec MN, Adamowicz P, Barres JS, et al. HBsAg positive reactivity in man not due to hepatitis B virus. *Lancet*. 1987;2(8572):1354-58.
12. Fay OH, Fonseca JC, Marten A. The cooperative group. Hepatitis viral B vaccination in Latin America region. *Vaccine*. 1990;8(suppl.1):134-9.
13. Harrison TJ, Hopes EA, Oon CJ, Zanetti AR, Zuckerman AL. Independent emergence of a vaccine-induced escape mutant of hepatitis B virus. *J. Hepatol*. 1991;13(suppl.4):S105-7.

BERTOLINI, D.A.; RIBEIRO, P.C.; LEMOS, M.F.; SARACENI, C.P. & PINHO, J.R.R. - Characterization of a hepatitis B virus strain in southwestern Paraná, Brazil, presenting mutations previously associated with anti-HBs resistance. *Rev. Inst. Med. Trop. São Paulo*, 52(1): 25-29, 2010.

14. Higgins DG, Sharp PM. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene*. 1988;73:237-44.
15. Hunt CM, McGill JM, Allen MI, Condreay LD. Clinical relevance of hepatitis B viral mutations. *Hepatology*. 2000;31:1037-44.
16. Hsu HY, Chang MH, Ni YH, Lin HH, Wang SM, Chen DS. Surface gene mutants of hepatitis B virus in infants who develop acute or chronic infections despite immunoprophylaxis. *Hepatology*. 1997;26:786-91.
17. Mello FCA, Souto FJD, Nabuco LC, Villela-Nogueira CA, Coelho, HSM, Franz HCF *et al*. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiology*. 2007;7:103.
18. Moreira SDR, Thiel B, Wolski M, Entrés A. Primeiro resultado após a introdução da vacina contra a hepatite B na população menor de 15 anos, Paraná, 1993 a 2000. In: 5º Congresso Brasileiro de Epidemiologia, Curitiba; 2002. [resumo 553]. (*Braz J Epidemiol*. 2002;(supl. esp.):r.553).
19. Nainan OV, Khristova ML, Byun KS, Xia G, Taylor PE, Stevens CE, *et al*. Genetic variation of hepatitis B surface antigen coding region among infants with chronic hepatitis B virus infection. *J Med Virol*. 2002;68:319-27.
20. Ngui SL, O'Connell S, Eglin RP, Heptonstall J, Teo CG. Low detection rate and maternal provenance of hepatitis B virus S gene mutants in cases of failed postnatal immunoprophylaxis in England and Wales. *J Infect Dis*. 1997;176:1360-5.
21. Noerd H, Hammas B, Lee SD, Bile K, Couroucé AM, Mushahwar IK. Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol*. 1993;74:1341-8.
22. Oon CJ, Lim GK, Ye Z, Goh KT, Tan KL, Yo SL, *et al*. Molecular epidemiology of hepatitis B virus vaccine variants in Singapore. *Vaccine*. 1995;13:699-702.
23. Oon CJ, Ning CW, Shivan K, Keow LG. Identification of hepatitis B surface antigen variants with alterations outside the "a" determinant in immunized Singapore infants. *J Infect Dis*. 1999;179:259-63.
24. Okamoto H, Yano K, Nozaki Y, Mutsaers A, Miyazaki H, Yamamoto K. *et al*. Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. *Pediatr Res*. 1992;32:264-8.
25. Rodríguez-Frías F, Bull M, Jardi R, Vargas V, Quer J, Coltrina M, *et al*. Genetic alterations in the S gene of hepatitis B virus in patients with acute hepatitis B, chronic hepatitis B and hepatitis B liver cirrhosis before and after liver transplantation. *Liver*. 1999;19:177-82.
26. Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol*. 2007;13:14-21.
27. Siniuk R, Pinho JRR, Bertolini DA, Bernardini AP, Da Silva LC, Carrilho FJ. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol*. 2004;42:2455-60.
28. Souto FJD. Distribuição da hepatite B no Brasil: atualização do mapa epidemiológico e proposições para seu controle. *Gastroenterol Endosc Digest*. 1999;18:143-50.
29. Thakur V, Kazim S, Gupta R, Hasnain S, Bartholomeusz A, Malhotra V. *et al*. Transmission of G145 mutant of HBV to an unrelated contact. *J Med Virol*. 2005;76:40-6.
30. Universidade Estadual de Londrina. Hospital Universitário Regional Norte do Paraná. Centro de Referência em Imunobiológicos Especiais. Núcleo de Imunizações do Hospital de Clínicas. Ofício NIHC/CRIE nº 019, 2000.
31. Yamamoto K, Horikita M, Tsuda F, Itoh K, Akahane Y, Yotsumoto S. *et al*. Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J Virol*. 1994;68:2671-6.
32. Wallace LA, Carman WF. Surface gene variation of HBV: scientific and medical relevance. *Viral Hepatitis Rev*. 1997;3:5-16.
33. World Health Organization [homepage on the Internet]. Switzerland. 2000. Hepatitis. World Health Organization Fact Sheet 204 (Revised October 2000). WHO Web site. Available from: <http://www.who.int/mediacentre/factsheets/fs204/en/index.html>

Received: 7 August 2009

Accepted: 30 November 2009

- 18- Bertolini DA, Gomes-Gouvêa MS, Carvalho-Mello IM, Saraceni CP, Sitnik R, Grazziotin FG, Laurino JP, Fagundes NJ, Carrilho FJ, Pinho JR. Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil. Infection, Genetics and Evolution. 2012 Volume 12, Issue 6, August 2012, Pages 1295–1304 Apr 1**

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil

Dennis Armando Bertolini^{a,*}, Michele Soares Gomes-Gouvêa^b,
Isabel Maria Vicente Guedes de Carvalho-Mello^c, Cláudia Patara Saraceni^d, Roberta Sitnik^e,
Felipe Gobbi Graziotin^f, Jomar Pereira Laurindo^h, Nelson Jurandi Rosa Fagundes^g, Flair José Carrilho^b,
João Renato Rebello Pinho^{b,e}

^a Laboratório de Imunologia Clínica, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Maringá, Paraná, Brazil

^b Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil

^c Laboratório de Imunologia Viral, Instituto Butantan, São Paulo, São Paulo, Brazil

^d Serviço de Virologia, Instituto Adolfo Lutz, São Paulo, São Paulo, Brazil

^e Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, São Paulo, Brazil

^f Laboratório de Biologia Genética e Molecular, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

^g Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

^h Instituto de Biotecnologia, Universidade de Caxias do Sul, Rio Grande do Sul, Brazil

ARTICLE INFO

Article history:

Received 6 December 2011

Received in revised form 30 March 2012

Accepted 8 April 2012

Available online 17 April 2012

Keywords:

Hepatitis B virus

Genotypes

Genotype D

Migration

Brazil

Europe

ABSTRACT

Southern Brazil is considered an area of low Hepatitis B endemicity, but some areas of higher endemicity have been described in the Southwest of Paraná and Santa Catarina states. The aim of this study was to evaluate viral genotypes circulating throughout Paraná state. PCR amplification and partial sequencing of the S gene was carried out in 228 samples from HBsAg positive candidate blood donors. Samples have been collected in seven different counties (Cascavel, Curitiba, Foz do Iguaçu, Francisco Beltrão, Maringá, Londrina and Paranaguá). The most common HBV genotype in Paraná state was D (82.9%; 189/228), followed by A (14.1%; 32/228). Genotypes F (1.3%; 3/228), C (1.3%; 3/228) and H (0.4%; 1/228) were also found. Distribution of genotypes was different in the studied counties, but genotype D was the most frequent in all of them. In Francisco Beltrão, all studied samples belonged to genotype D. The high prevalence of HBV genotype D in South of Brazil is explained by the intense migration of settlers from European countries. Subgenotypes A1 and A2 were identified circulating in all cities where HBV/A was found. As observed in other areas of Brazil, HBV/A1 is more frequent than the HBV/A2 in Paraná state and its presence was significantly larger in black and mulatto individuals. Genotype C was found only in individuals with Asian ancestry from Londrina and Maringá. Most HBV/F sequences identified in this study were classified as subgenotype F2a that was previously described in Brazil. The sole case of subgenotype F4 was from Foz do Iguaçu city, near to Northern Argentina, where F4 is highly prevalent. The single genotype H sample was from Curitiba. This is the first case of this genotype described in Brazil. Further studies should be carried out to determine if more genotype H samples can be found in other populations from Brazil.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Hepatitis B virus (HBV) infection is still one of the major causes of chronic liver diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Lok and McMahon, 2009). HBV, as a member of the Hepadnaviridae family, is a circular, partially double-stranded DNA virus and is classified into eight genotypes, designated A to H (Kramvis et al., 2008; Norder et al.,

2004). Recently, two new additional, HBV genotypes, HBV/I and HBV/J, were proposed, which were isolated in Vietnam (Tran et al., 2008) and Japan (Tatematsu et al., 2009), respectively. However, because these strains are recombinants with other genotypes, their classification as new genotypes is under discussion (Kurbanov et al., 2010).

Currently, HBV genotypes are defined based on an intergroup divergence of 7.5% or more in the complete nucleotide sequence or 4% in the partial sequence of the open reading frame that code for the surface antigens (PreS1/PreS2/S) in the region covering the small protein (HBsAg) (Kramvis et al., 2008; Norder et al., 2004). Furthermore, based on a divergence of >4% and <8% in the complete nucleotide sequence within a genotype, subgenotypes

* Corresponding author. Address: Department of Clinical Analyses, Universidade Estadual de Maringá, Avenida Colombo 5790, 87020-900 Maringá, Paraná, Brazil. Tel.: +55 44 3011 4878; fax: +55 44 3011 4860. E-mail address: dabertolini@uem.br (D.A. Bertolini).

have been classified within genotypes A, B, C, D and F (Kramvis et al., 2008; Norder et al., 2004).

The importance of HBV genotypes/subgenotypes in the progression of liver disease and response to interferon therapy have been suggested by several studies, but results are still controversial (Kao and Lin, 2011; Lok and McMahon, 2009; Wiegand et al., 2008).

Most genotypes and some subgenotypes have distinct geographical distribution and are associated with different ethnicities (Kramvis et al., 2008; Kurbanov et al., 2010; Norder et al., 2004). Genotype A is highly prevalent in some regions of Africa, Northern and Middle Europe and North America; genotypes B and C are common in East Asia; genotype D prevails in the Mediterranean basin and in the Middle East; genotype E is indigenous to Africa and predominates throughout its western region; and genotype F is found in South America, especially in populations with Amerindian background (Kurbanov et al., 2010; Norder et al., 2004). The distribution of genotypes G and H is not well known: the first one was found in France, Germany, USA, and Mexico (Sanchez et al., 2007; Stuyver et al., 2000; Vieth et al., 2002); while the latter is frequent in Mexico and has also been found some case in other countries in Central and North America (Alvarado-Esquivel et al., 2006; Arauz-Ruiz et al., 2002; Sanchez et al., 2007). These two genotypes have recently been identified in some regions of South America: genotype G in Colombia (Alvarado Mora et al., 2011) and Brazil (Mendes-Correa et al., 2010); genotype H in Argentina (Flichman et al., 2009).

Brazil is a country with continental dimensions, consisting of five regions (North, Northeast, Central-West, South and Southeast) with distinct environmental and cultural characteristics, and population background. Furthermore, HBV epidemiology is different among these regions, with increasing prevalence from South to North (Souto, 1999). Nevertheless, there are some areas with elevated prevalence inside regions characterized with low or intermediate prevalence. The Southwest of Paraná state, in South of Brazil is an area where the prevalence of HBsAg reached 8.3% up to the 1990's (Carvalho and Dias, 1995; Souto, 1999). In another study carried out in 2006, after the introduction of universal vaccination we identified that HBV infection is still a serious health threat in this area with HBsAg prevalence of 3.8% (Bertolini et al., 2006).

Studies about the distribution of HBV genotypes in areas around the world have showed that it generally reflects the demographic history of this area (Alvarado Mora et al., 2011; Arankalle et al., 2003; Kramvis and Kew, 2007; Thedja et al., 2011). The differences in HBV prevalence among Brazilian regions may result, among other factors, from the origin of the people that live in these different areas nowadays. In this study, trying to elucidate the origins for the relevant prevalence of HBV infection in Southwest of Paraná state, we characterized the distribution of HBV genotypes in seven different regions of Paraná state and identified the demographic data that were associated with the different HBV genotypes identified in this state.

2. Materials and methods

2.1. Samples

Serum samples from 228 volunteers candidate blood donors (177 male and 51 female; age 31.8 ± 9.4 years) positive for HBV surface antigen (HBsAg) were collected in Blood Banks from seven geographically distinct localities at Paraná state, in South of Brazil: Cascavel ($n = 43$), Curitiba ($n = 35$), Foz do Iguaçu ($n = 53$), Francisco Beltrão ($n = 49$), Londrina ($n = 12$), Maringá ($n = 30$) and Paranaguá ($n = 6$).

Each enrolled in this study was submitted to a detailed interview about the following characteristics: sex, age, color (based

on self classification according to the Instituto Brasileiro de Geografia e Estatística – IBGE, 2007), education and income levels, ancestry (concerning family immigration from foreign countries as well as moving from other Brazilian regions or other states from southern Brazil – i.e., from Santa Catarina and Rio Grande do Sul states).

Blood samples were collected from May, 1998 to May, 2002 and sera were separated and stored at -20°C . This study was approved by the local institution ethics committee and all individuals provided written informed consent.

2.2. HBV DNA extraction, amplification and sequencing

HBV nucleic acid was extracted from 100 μL serum samples by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987) and eluted in 50 μL of sterile nuclease-free water.

A fragment of HBV DNA S gene was amplified by nested PCR as previously described (Sitnik et al., 2004). In order to prevent PCR carryover contamination, strict precautions and procedures were strictly followed.

PCR products were purified and submitted to sequencing, using second round primers with fluorescence-labeled dideoxynucleotide chain terminators using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequences were determined in an automated DNA sequencer (ABI Prism 377, Applied Biosystems, USA).

2.3. Sequence analysis

The quality of each electropherogram was evaluated using the Phred-Phrap software and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available in the web page Electropherogram quality analysis <(http://asparagin.cenargen.embrapa.br/phph/)>.

Sequences were submitted to EMBL/GenBank/DBJ under accession numbers EF172453–EF172680.

2.4. Phylogenetic analysis

HBV genotypes were determined by phylogenetic analysis of the amplified S gene fragment with sequences from different HBV genotypes and subgenotypes obtained from the GenBank public database <(http://www.ncbi.nlm.nih.gov/)>.

Sequences were aligned and compared using BioEdit (v. 7.0.8) and the integrated CLUSTAL W program (Hall, 1999). Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.6.1 (Drummond and Rambaut, 2007) under relaxed uncorrelated lognormal molecular clock using the model of nucleotide substitution (GTR + G + I) obtained previously by Modeltest v. 3.7 (Posada and Crandall, 1998). The maximum clade credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees and then 10% of burn-in was removed using Tree Annotator v.1.6.1 (Drummond and Rambaut, 2007).

2.5. Statistical analysis

Qualitative variables and genotypes were analyzed using the Chi square test and were described as percentages. Variance analysis was used to compare the mean age of the blood donors infected with different genotypes. Statistical significance level was defined as $P < .05$. SPSS software was utilized for all statistical analyses.

3. Results

3.1. HBV genotype distribution

HBV genotype was characterized in all 228 samples by phylogenetic analysis of a partial sequence of the S gene. The results showed that the most common HBV genotype in Paraná state was D (82.9%; 189/228), followed by genotype A (14.1%; 32/228). Genotypes F (1.3%; 3/228), C (1.3%; 3/228) and H (0.4%; 1/228) were also found.

The distribution of genotypes was different in the seven studied regions of Paraná state (Fig. 1), but genotype D was the most frequent in all of them. In Francisco Beltrão, all samples belonged to genotype D; genotype C was found only in Londrina and Maringá, while genotype F was found in Curitiba and Foz do Iguaçu. The single genotype H sample was from Curitiba.

3.2. Phylogenetic analysis

The strains of the most prevalent genotype (HBV/D) in Paraná state showed a nucleotide sequence identity in the analyzed genomic region ranging from 93.7% to 100%. Sequences with 100% similarity ($n = 64$) were removed from the data set used to construct the phylogenetic tree shown in Fig. 2. The phylogenetic tree with 125 sequences HBV/D from Paraná showed that these sequences grouped in different clades of the tree, even those samples from the same city. The same pattern was also observed with sequences isolated in other regions of Brazil and Europe.

Using the partial sequence of the S gene, we could not accurately verify HBV subgenotypes, but some reference sequences were obtained from complete genomes that have given their subgenotypes. Our genotype D sequences were mainly closely related with reference sequences classified as HBV subgenotypes D2 and D3, few of them grouped with D1 and one sequence grouped with D7. This last sequence obtained from Londrina was closely related with sequences isolated in Tunisia (Africa).

The phylogenetic tree with the other HBV genotypes is shown in Fig. 2. Analyses of genotype A sequences showed that the clades of subgenotypes A1 and A2 were well separated, but only A2 clade

had a high posterior probability value. Most (23/32) sequences of genotype A from Paraná grouped in A1 clade, and the other 9 were classified as A2. In all regions where genotype A was found, both subgenotypes (A1 and A2) were present and, as observed to genotype D, they did not group in the same clade according origin of the sample.

Sequences of genotype C grouped in a clade with reference sequences classified as C2 together with other sequences previously found in Brazil.

In the branch of genotype F, the different subgenotypes segregate with a proper posterior probability value, with the exception for HBV/F3. Our genotype F sequences clustered mainly with reference sequences classified as F2a, only one genotype F sequence from Foz do Iguaçu city grouped in F4 clade.

The sole genotype H sequence grouped close to a sequence isolated from a Mexican patient.

3.3. HBV genotypes and demographic data

Table 1 shows HBV genotypes distribution according to the demographic data: sex, age, color, education and income levels, social class, ancestry (concerning family immigration from foreign countries as well as moving from other Brazilian regions or other states from Southern Brazil – i.e., from Santa Catarina and Rio Grande do Sul states).

Genotype A1 was more frequent in males than others genotypes, but this difference was not statistically significant. The mean age of blood donors infected with genotype C was higher (42.7 years old) and those of genotypes F and H were lower (26.5 years old). This difference was neither statistically significant.

Blacks (61.5%) and mulattos (42.9%) were more frequently infected by subgenotype A1, while genotype D was the most frequent in whites (88.9%) – $p < 0.0001$. The three yellow individuals were Asian descendants and harbored all the genotype C strains found in this study.

The association of HBV genotypes with education level was statistically significant ($p = 0.019$). Genotypes A1 and A2 were more frequent in individuals with no education and fundamental

Fig. 1. Geographic location of Paraná state in Brazil and the distribution of HBV genotypes in the seven studied cities.

education level while genotype D was equally frequent in all education levels. Interestingly, genotype A1 frequency decreased with the education level.

On the other hand, in spite of a positive correlation found between education and income levels ($r = 0.466$; $P < 0.001$), no statistical significant difference between HBV genotype distribution and family income level was found ($p = 0.077$).

Among blood donors referring Brazilian and Paraguayan (only one individual) ancestry, a higher frequency of genotype A1 (38.5%) and lower frequency of genotype D (57.7%) was found when compared the others groups ($p < 0.001$). On the other hand, genotype D was more frequent in European descendants (89% – ($p < 0.001$)).

Genotype D was also the most prevalent in blood donors that reported a familial stemming from South (88.1%), Southeast (69.2%) and North (66.7%) regions, while genotype A1 was the most frequent in those from Center West (66.7%) and Northeast (60.0%) regions ($p < 0.0001$).

When the familial stemming was reported concerning only the three Brazilian southernmost states, genotype D was the most prevalent, i.e., in 95.8%, 94.3% and 82.1% of blood donors with origins in Santa Catarina, Rio Grande do Sul and Paraná states, respectively. No statistical significance was found ($p = 0.130$).

4. Discussion

In this study, we characterized HBV genotypes distribution among blood donor from different regions of Paraná state at South of Brazil. Using a methodology previously described by us (Sitnik et al., 2004), involving partial sequencing of the HBsAg coding gene, we were able to characterize the HBV genotypes circulating in Paraná state, as shown in the phylogenetic analysis (Figs. 2 and 3). This methodology was based in a concept that different HBV genotypes shows features at this gene that allow its identification with results consistent to those based in the sequencing of the complete genome (Norder et al., 1993).

Our results showed that genotype D was the most prevalent among the HBsAg seropositive candidate blood donors from different regions of Paraná state. Particularly in Francisco Beltrão, a region that shows the highest HBV prevalence in the state (Bertolini et al., 2006; Carvalho and Dias, 1995), genotype D was the sole one identified.

Previous studies in Brazil showed that HBV genotype A is the most prevalent one in different regions of the country, followed by genotypes D and F (Gomes-Gouveia et al., 2008; Mello et al., 2007; Ribeiro et al., 2006; Sitnik et al., 2004). On the other hand, when states located in South of Brazil (Rio Grande do Sul and Santa Catarina) were analyzed (Becker et al., 2010; Carrilho et al., 2004; Reis et al., 2011), this pattern changed and was most similar with the distribution here described for Paraná state, with a high prevalence of HBV/D.

Several published data have shown that HBV genotypes distribution in a specific region results from its demographic history. These studies showed that HBV genotypes found in some areas were probably introduced a long time ago through the migrations of the primeval populations or during the colonization period when the Europeans came to Africa and America; on the other hand, they may represent a more recent introduction by immigration from endemic areas (Alvarado Mora et al., 2011; Arankalle et al., 2003; Kramvis and Kew, 2007; Thedja et al., 2011).

The high prevalence of HBV genotype D in South of Brazil is explained by the intense migration of settlers from Europeans regions where HBV/D is prevalent. Genotype D is one of the most widespread genotypes worldwide, but it is predominant in the Mediterranean area, Middle East and Indian subcontinent (Chandra

Fig. 2. Phylogenetic tree of HBV/D strains isolated in seven different regions of Paraná state, in South of Brazil (highlighted in blue). The maximum clade credibility (MCC) tree was inferred from 378 partial sequences of HBsAg gene (378 nucleotides) identified in this study ($n = 125$) and sequences of HBV/D ($n = 114$) and other genotypes ($n = 139$) recovered in GenBank. Sequences retrieved from GenBank are denoted by their genotype, followed by accession numbers and the source country of the isolates. Brazilian sequences from GenBank (highlighted in red) are also identified by their state of origin in Brazil: MT (Mato Grosso), SP (São Paulo), SC (Santa Catarina), PR (Paraná), BA (Bahia), RO (Rondônia), RJ (Rio de Janeiro), AM (Amazonas), MG (Minas Gerais), MS (Mato Grosso do Sul). The collapsed clades correspond to the non-D HBV genotype. The posterior probabilities of the key nodes are depicted above the respective node. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2 (continued)

et al., 2009; Schaefer, 2007). In Europe, genotype D is found in the southern countries that border the Mediterranean Sea, summing up nearly 100% of the HBV cases found in Greece, Italy, Serbia and Albania (Schaefer, 2007).

During the years 1872 – 1975, 5435,735 million immigrants from Europe and Middle East came to Brazil encouraged by Brazilian government campaigns. These immigrants were Italians (34%), Portuguese (29%), Spanish (14%), Japanese (5%), Germans (4%), Lebanese and Syrians (2%) and others (12%) (IBGE, 2007). In Brazil, these people mainly came to São Paulo and Paraná states to work in coffee plantations or to sites named “core settlements” (i.e., regions with low population levels they were sending as settlers), mainly located in the South region of the country (IBGE, 2007). Paraná state specifically received large numbers of Italian, and German immigrants during this period and most of its population are descendant from people from these two nations (Lazier, 1997).

Human genomic analysis confirmed that in South of Brazil, most inhabitants have a high proportion of European genomic ancestry (Pena et al., 2011). In the present study, we showed an agreement with this fact concerning also the viral infection that they harbor: a significant association of HBV/D infection in white individuals was found, especially when they were European descendants (most of them Italian (63%) and German (26%) descendants).

The topology of phylogenetic tree showed that the sequences of HBV/D isolated in Paraná grouped in different clades of the tree, which also was observed concerning genotype D isolates in different countries of Europe. This suggests that different strains were introduced in Southern Brazil probably due the different European origin of the immigrants or because these different strains also circulated in the European regions from where they had come.

An intermediate to the high prevalence of HBV was identified in some areas, in Europe, especially in Italy, before the introduction of HBV vaccination and the introduction of other measures to avoid its spread (Stroffolini et al., 1988). Chronic HBV carriers probably were present among the European immigrants that came to Southern Brazil in the 19th and 20th centuries. This fact may explain the high prevalence of HBV/D infection observed in some areas of Paraná state, where most of the population has European ancestry.

Considering the Brazilian states origin of the studied blood donors, our results showed that genotype D was more frequent among those that reported a familial stemming from South and Southeast, but also from North region. Despite the fact that HBV genotype A has been found as the most prevalent genotype in all other Brazilians regions, some areas in these regions showed a different demographic history, reflecting in a different pattern of HBV genotypes prevalence. An example of this is Rondônia, a state located in the West of the North region of Brazil, where the most prevalent genotype was HBV/D (Santos et al., 2010). This genotype was introduced there mostly by people from the South region of Brazil that migrate during the years 70's to 80's to colonize some areas still non occupied in the Amazon region, including Rondônia, north region of Mato Grosso and the south region of Pará states. A previous study identified that these migrants were already infected when they arrived at these regions and continued to live in poor sanitary conditions, what favored HBV dissemination among themselves (Souto, 2004).

The results of the phylogenetic analysis of genotype D (Fig. 2) showed that the sequences obtained from Paraná state are present in several tree branches among other sequences from Rondônia and Mato Grosso states and other countries. Although some sequences clusters can be suggested, the sequence fragment was not long enough for deeper inferring their phylogenetical origins.

Genotype A was the second most prevalent genotype in Paraná state in a lower proportion (14.1%) of the HBV cases. Two HBV/A (A1 and A2) subgenotypes were identified circulating in all cities where HBV/A was found. HBV/A1 is mainly found in Eastern Africa and Asia (Kramvis and Kew, 2007) and is the most prevalent genotype in Brazil (Gomes-Gouveia et al., 2009; Mello et al., 2007; Santos et al., 2010). This subgenotype has been also identified in

Table 1
Characteristics of HBsAg positive candidate blood donors and their relation of HBV genotypes at Paraná state, 1998–2002.

Variable	N	HBV genotypes					P
		A1 n (%)	A2 n (%)	C n (%)	D n (%)	F/H n (%)	
Sex (n = 228)							
Female	51	2 (8.7)	2 (22.2)	1 (33.3)	44 (23.3)	2 (50)	P = .339
Male	177	21 (91.3)	7 (77.8)	2 (66.7)	145 (76.7)	2 (50)	
Age	210	33.0 ± 10.4	35.4 ± 10.8	42.7 ± 7.0	31.4 ± 9.1	26.5 ± 5.8	P = .213
Color (n = 222)							
White	199	10 (5.0)	9 (4.5)	0	177 (88.9)	3 (1.5)	P < .001
Black	13	8 (61.5)	0	0	4 (30.8)	1 (7.7)	
Yellow	3	0	0	3 (100.0)	0	0	
Mulatto	7	3 (42.9)	0	0	4 (57.1)	0	
Education level (n = 227)							
No education	8	2 (25.0)	1 (12.5)	0	5 (62.5)	0	P = .019
Fundamental	126	13 (10.3)	6 (4.8)	0	106 (84.1)	1 (0.8)	
Medium	75	6 (8.0)	1 (1.3)	1 (1.3)	64 (85.3)	3 (4.0)	
Superior	18	1 (5.6)	1 (5.6)	2 (11.1)	14 (77.8)	0	
Income level (n = 220)							
<2 MW ^a	88	12 (13.6)	4 (4.5)	1 (1.1)	71 (80.7)	0	P = .077
2 a 5 MW	101	8 (7.9)	4 (4.0)	0	85 (84.2)	4 (4.0)	
>5 MW	31	2 (6.5)	1 (3.2)	2 (6.5)	26 (83.9)	0	
Origin (n = 209)							
Brazil and Paraguay ^b	26	10 (38.5)	1 (3.8)	0	15 (57.7)	0	P < .001
Europe	164	7 (4.3)	8 (4.9)	0	146 (89.0)	3 (1.8)	
Others	19	1 (5.3)	0	3 (15.8)	15 (78.9)	0	
Region (n = 218)							
North	3	1 (33.3)	0	0	2 (66.7)	0	P < .001
Northeast	5	3 (60.0)	0	0	1 (20.0)	1 (20.0)	
Center-West	3	2 (66.7)	0	0	1 (33.3)	0	
Southeast	13	4 (30.8)	0	0	9 (69.2)	0	
South	194	11 (5.7)	7 (3.6)	2 (1.0)	171 (81.1)	3 (1.5)	
State (n = 189)							
Paraná	95	9 (9.5)	4 (4.2)	2 (2.1)	78 (82.1)	2 (2.1)	P = .130
S. Catarina	24	0	0	0	23 (95.8)	1 (4.2)	
Rio Grande do Sul	70	1 (1.4)	3 (4.3)	0	66 (94.3)	0	

^a Minimum Wage.

^b Only one individual related Paraguayan ancestry.

communities formed mostly by descendants of African slaves (Quilombos) reinforcing the theory that it was introduced by African slaves brought to Brazil during the slavery period (Alvarado-Mora et al., 2011; Motta-Castro et al., 2008). On the other hand, HBV/A2 has been characterized as a European genotype because of its high prevalence in some countries in this continent (Kurbanov et al., 2010; Schaefer, 2007).

As observed in other areas of Brazil, HBV/A1 is more frequent than HBV/A2 in Paraná state and its presence was significantly related with black and mulatto individuals. It was previously shown that black and mulatto people from Southern Brazil had higher African genomic ancestry than those from other regions of Brazil (Pena et al., 2011). Then, HBV/A1 currently circulating in Paraná was possibly introduced by slaves that lived in this area in the past or was introduced by internal migrants from other Brazilian region where this subgenotype is frequent (Mello et al., 2007). This last hypothesis is supported by the fact that familiar origins (concerning the regions of Brazil) of the most blood donors infected with HBV/A1 were from Northeast and Center-West regions.

Genotype distribution was also statistically different concerning the education level ($p = 0.019$) and a trend was observed concerning income level ($p = 0.077$). Subgenotype A1 was more frequent in "no education" and "fundamental education" levels. On the other hand, genotype D is equally distributed among the different education and income levels. The reasons for these differences are unclear. More studies on the evolution of the diseases caused by these different viral genotypes and the different ethnic groups should be

carried out to better understand this point. Careful attention should be deserved to respond if these differences are due to the genetical background concerning viral response or to socio-economical features. HBV/A2 is less frequent in other areas of Brazil; its presence in Paraná state is perhaps due the presence of European immigrants infected. HBV/A2 is common in Europe (Schaefer, 2007) and, in Germany, from where came the second major proportion of immigrants to South of Brazil, HBV/A is a most frequent genotype reaching 54% of the infected patients. In our study 44% of the individuals infected with HBV/A2 related German ancestry and 33% Italian.

Among the populations of blood donors from Paraná state, we also found three cases infected with genotype C that is a rare genotype in Brazil, but extremely common in regions of Asian continent (Huy and Abe, 2004; Kurbanov et al., 2010; Norder et al., 2004). In Brazil, this genotype is less prevalent and has been more frequently found among patients with Asian origin or their descendants (Clemente et al., 2009; Sitnik et al., 2004) and our results confirm these findings: the HBV/C infected individuals identified here were also Asian descendants. These patients were from the cities of Londrina and Maringá where there are the largest Japanese colonies in the Paraná state. A larger study on Japanese descendants living in Paraná state is needed to confirm if genotype C is the most frequent found in this population.

Another two genotypes were found in Paraná state: genotypes F and H, but as genotype C, they are not frequent. Genotype F represents the most prevailing genotype among Amerindians and

Fig. 3. Phylogenetic tree of HBV non D genotypes isolated in seven different regions of Paraná state, in South of Brazil (highlighted in blue) showing: (A) the whole tree with detailed subgenotypes A1 and A2 clusters; (B) genotype C branch; (C) genotypes F and H branches. The maximum clade credibility (MCC) tree was inferred from 260 partial sequences of HBsAg gene (378 nucleotides) identified in this study ($n = 39$) and sequences recovered in GenBank ($n = 221$). GenBank sequences were designated by their genotype, followed by accession numbers and the source country of the isolates. Brazilian sequences from GenBank (highlighted in red) are also identified by their state of origin in Brazil: MT (Mato Grosso), SP (São Paulo), SC (Santa Catarina), PR (Paraná), BA (Bahia), RO (Rondônia), RJ (Rio de Janeiro), AM (Amazonas), MG (Minas Gerais), MS (Mato Grosso do Sul). The posterior probabilities of the key nodes are depicted above the respective node. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3 (continued)

is found in South American countries with significant Amerindian genetic background such as Colombia, Venezuela and Chile (Alvarado Mora et al., 2011; Devesa et al., 2008; Di Lello et al., 2009; Mello et al., 2007). In Brazil, besides indigenous population, this genotype was also found in less proportion in non-indigenous population from all regions, but shows a higher prevalence in Amazon region at northern Brazil (Gomes-Gouveia et al., 2009; Mello et al., 2007; Sitnik et al., 2004). HBV/F from different geographic regions has been classified in four subgenotypes (F1–F4) (Norder et al., 2004). Most HBV/F sequences identified in this study were classified as F2a that is a common subgenotype that has been described in Brazil (Gomes-Gouveia et al., 2009; Mello et al., 2007). The sole case of subgenotype F4 isolated from a blood donor from Foz de Iguaçu city, close from Northern Argentina where F4 is higher prevalent (Pineiro et al., 2008). The grouping pattern in the phylogenetic tree suggests that our F4 sequence is closely related with a F4 sequence isolate in Argentina.

Genotype H case found in this study is a first case of this genotype described in Brazil. HBV/H epidemiology is not well known, but studies about molecular epidemiology around the world have been showed that it has a quite restricted global distribution. It is frequent in Mexico (Alvarado-Esquivel et al., 2006) and few cases have been reported in Nicaragua, USA (Arauz-Ruiz et al., 2002) and Japan (Ohnuma et al., 2005). Recently, HBV/H was also identified in two Argentine patients were closely related (sisters) and have not confirmed any contact with people from Central America (Flichman et al., 2009). Our HBV/H case was from Curitiba city, which is the capital of the Paraná state and where there is a great miscellany of immigrants from several regions of the world and

the country. We did not have more detailed information about the history of travels of this patient, we know that he was born in Paraná state, is an Italian descendant and is white, therefore, we cannot infer how he may be infected and the possible origin of this strain. More studies should be carried out at Curitiba to verify the presence of other cases infected with genotype H.

5. Conclusions

In conclusion, we reported the characterization of the HBV genotypes circulating in Paraná state and found that the genotype D is the most prevalent. This high prevalence of genotype D in some areas of the state of Paraná can be explained by the arrival of European immigrants in the South of Brazil in the 19th and 20th centuries, but also due to the migration of the South and Southeast. The subgenotype HBV/A1 was possibly introduced by slaves that lived in this area in the past or was introduced by internal migrants from other Brazilian region where this subgenotype is frequent, as the regions of the Midwest and Northeast. For the first time described the presence of H genotype in Brazil requiring further investigation to assess their origin. Thus, by identifying the genotype of the Hepatitis B virus can explain its origin, especially in areas of high endemicity, and understand their relationship with the local population. The use of larger fragments involving surface and polymerase HBV genes could provide further information about the origin of the virus in the region studied. Considering the territorial dimension of Brazil, further studies should be carried out to determine the genotype of the HBV.

Fig. 3 (continued)

Acknowledgements

This work had the financial support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 99/09551-0, FAPESP 2010/50081-9), Brazil. This study is dedicated to all patients and health professionals of the Paraná state, the City and state Health Secretaries and the Hemocentros (Blood Banks).

References

- Alvarado Mora, M.V., Romano, C.M., Gomes-Gouveia, M.S., Gutierrez, M.F., Botelho, L., Carrilho, F.J., Pinho, J.R., 2011. Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect. Genet. Evol.* 11, 103–108.
- Alvarado-Esquivel, C., Sablon, E., Conde-Gonzalez, C.J., Juarez-Figueroa, L., Ruiz-Maya, L., Aguilar-Benavides, S., 2006. Molecular analysis of hepatitis B virus isolates in Mexico: predominant circulation of hepatitis B virus genotype H. *World J. Gastroenterol.* 12, 6540–6545.

- Alvarado-Mora, M.V., Botelho, L., Gomes-Gouveia, M.S., de Souza, V.F., Nascimento, M.C., Pannuti, C.S., Carrilho, F.J., Pinho, J.R., 2011. Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão, Brazil. *Virol. J.* 8, 415.
- Arankalle, V.A., Murhekar, K.M., Gandhe, S.S., Murhekar, M.V., Ramdasi, A.Y., Padbidri, V.S., Sehgal, S.C., 2003. Hepatitis B virus: predominance of genotype D in primitive tribes of the Andaman and Nicobar islands, India (1989–1999). *J. Genet. Virol.* 84, 1915–1920.
- Arauz-Ruiz, P., Norder, H., Robertson, B.H., Magnus, L.O., 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J. Genet. Virol.* 83, 2059–2073.
- Becker, C.E., Mattos, A.A., Bogo, M.R., Branco, F., Sitnik, R., Kretzmann, N.A., 2010. Genotyping of hepatitis B virus in a cohort of patients evaluated in a hospital of Porto Alegre, South of Brazil. *Arq. Gastroenterol.* 47, 13–17.
- Bertolini, D.A., Pinho, J.R., Saraceni, C.P., Moreira, R.C., Granato, C.F., Carrilho, F.J., 2006. Prevalence of serological markers of hepatitis B virus in pregnant women from Paraná state, Brazil. *Braz. J. Med. Biol. Res.* 39, 1083–1090.
- Carrilho, F.J., Moraes, C.R., Pinho, J.R., Mello, L.M., Bertolini, D.A., Lemos, M.F., Moreira, R.C., Bassit, L.C., Cardoso, R.A., Ribeiro-dos-Santos, G., Da Silva, L.C., 2004. Hepatitis B virus infection in Haemodialysis Centres from Santa Catarina state, Southern Brazil. Predictive risk factors for infection and molecular epidemiology. *BMC Public Health* 4, 13.
- Carvalho, S.P., Dias, S.L.N.G., 1995. Estudo comparativo da taxa de incidência de sífilis, doença de Chagas, hepatites B e C, HIV e HTLV, em doadores de sangue por região do Paraná. *Revista Brasileira de análises clínicas* 27, 77–82.
- Chandra, P.K., Biswas, A., Datta, S., Banerjee, A., Panigrahi, R., Chakrabarti, S., De, B.K., Chakravarty, R., 2009. Subgenotypes of hepatitis B virus genotype D (D1, D2, D3 and D5) in India: differential pattern of mutations, liver injury and occult HBV infection. *J. Viral. Hepat.* 16, 749–756.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Clemente, C.M., Carrilho, F.J., Pinho, J.R., Ono-Nita, S.K., Da Silva, L.C., Moreira, R.C., Lemos, M.F., de Carvalho Mello, L.M., 2009. A phylogenetic study of hepatitis B virus in chronically infected Brazilian patients of Western and Asian descent. *J. Gastroenterol.* 44, 568–576.
- Devesa, M., Loureiro, C.L., Rivas, Y., Monsalve, F., Cardona, N., Duarte, M.C., Poblete, F., Gutierrez, M.F., Botto, C., Pujol, F.H., 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J. Med. Virol.* 80, 20–26.
- Di Lello, F.A., Leone, F.G.P.Y., Munoz, G., Campos, R.H., 2009. Diversity of Hepatitis B and C Viruses in Chile. *J. Med. Virol.* 81, 1887–1894.
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7, 214.
- Flichman, D., Galdame, O., Livellara, B., Vialat, M., Galdame, A., Campos, R., 2009. Full-length genome characterization of hepatitis B virus genotype H strain isolated from serum samples collected from two chronically infected patients in Argentina. *J. Clin. Microbiol.* 47, 4191–4193.
- Gomes-Gouveia, M.S., Pereira Soares Mdo, C., Guedes de Carvalho Mello, L.M., Brito, E.M., Pereira Moia Lde, J., Bensabath, G., Nunes, H.M., Carrilho, F.J., Pinho, J.R., 2008. Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin. *Acta Trop.* 106, 149–155.
- Gomes-Gouveia, M.S., Soares, M.C., Bensabath, G., de Carvalho-Mello, L.M., Brito, E.M., Souza, O.S., Queiroz, A.T., Carrilho, F.J., Pinho, J.R., 2009. Hepatitis B virus and hepatitis delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the western Brazilian Amazon region. *J. Gen. Virol.* 90, 2638–2643.
- Hall, T.A., 1998. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95–98.
- Huy, T.T., Abe, K., 2004. Molecular epidemiology of hepatitis B and C virus infections in Asia. *Pediatr. Int.* 46, 223–230.
- IBGE, 2007. Brasil: 500 anos de povoamento, second ed. Rio de Janeiro.
- Kao, J.H., Lin, C.L., 2011. The clinical implications of hepatitis B virus genotype: Recent advances. *J. Gastroenterol. Hepatol.* 26, 123–130.
- Kramvis, A., Arakawa, K., Yu, M.C., Nogueira, R., Stram, D.O., Kew, M.C., 2008. Relationship of serological subtype, basic core promoter and precore mutations to genotypes/subgenotypes of hepatitis B virus. *J. Med. Virol.* 80, 27–46.
- Kramvis, A., Kew, M.C., 2007. Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus. *Hepatol. Res.* 37, S27–S32.
- Kurbanov, F., Tanaka, Y., Mizokami, M., 2010. Geographical and genetic diversity of the human hepatitis B virus. *Hepatol. Res.* 40, 14–30.
- Lazier, H.G., 1997. Análise Histórica da Posse de Terra no Sudoeste Paranaense. *Gráfica e Editora Ltda, Francisco Beltrão, Paraná.*
- Lok, A.S., McMahon, B.J., 2009. Chronic hepatitis B: update 2009. *Hepatology* 50, 661–662.
- Mello, F.C., Souto, F.J., Nabuco, L.C., Villela-Nogueira, C.A., Coelho, H.S., Franz, H.C., Saraiva, J.C., Virgolino, H.A., Motta-Castro, A.R., Melo, M.M., Martins, R.M., Gomes, 2007. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol.* 7, 103.
- Mendes-Correa, M.C., Pinho, J.R., Locarnini, S., Yuen, L., Sitnik, R., Santana, R.A., Gomes-Gouveia, M.S., Leite, O.M., Martins, L.G., Silva, M.H., Gianini, R.J., Uip, D.E., 2010. High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. *J. Med. Virol.* 82, 1481–1488.
- Motta-Castro, A.R., Martins, R.M., Araujo, N.M., Niel, C., Facholi, G.B., Lago, B.V., Mello, F.C., Gomes, S.A., 2008. Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Arch. Virol.* 153, 2197–2205.
- Norder, H., Courouce, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., Magnus, L.O., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 47, 289–309.
- Norder, H., Hammes, B., Lee, S.D., Bile, K., Courouce, A.M., Mushahwar, I.K., Magnus, L.O., 1993. Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J. Gen. Virol.* 74 (Pt 7), 1341–1348.
- Pena, S.D., Di Pietro, G., Fuchshuber-Moraes, M., Genro, J.P., Hutz, M.H., Kehdy Fde, S., Kohlrausch, F., Magno, L.A., Montenegro, R.C., Moraes, M.O., de Moraes, M.E., de Moraes, M.R., Ojopi, E.B., Perini, J.A., Racciopi, C., Ribeiro-Dos-Santos, A.K., Rios-Santos, F., Romano-Silva, M.A., Sortica, V.A., Suarez-Kurtz, G., 2011. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. *PLoS ONE* 6, e17063.
- Pineiro, Y.L.F.G., Pezzano, S.C., Torres, C., Rodriguez, C.E., Eugenia Garay, M., Fainboim, H.A., Remondeguí, C., Sorrentino, A.P., Mbayed, V.A., Campos, R.H., 2008. Hepatitis B virus genetic diversity in Argentina: dissimilar genotype distribution in two different geographical regions; description of hepatitis B surface antigen variants. *J. Clin. Virol.* 42, 381–388.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Reis, L.M., Soares, M.A., Franca, P.H., Soares, E.A., Bonvicino, C.R., 2011. Clonal analysis of hepatitis B viruses among blood donors from Joinville, Brazil: evidence of dual infections, intragenotype recombination and markers of risk for hepatocellular carcinoma. *J. Med. Virol.* 83, 2103–2112.
- Ribeiro, N.R., Campos, G.S., Angelo, A.L., Braga, E.L., Santana, N., Gomes, M.M., Pinho, J.R., De Carvalho, W.A., Lyra, L.G., Lyra, A.C., 2006. Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver Int.* 26, 636–642.
- Sanchez, L.V., Tanaka, Y., Maldonado, M., Mizokami, M., Panduro, A., 2007. Difference of hepatitis B virus genotype distribution in two groups of Mexican patients with different risk factors. High prevalence of genotype H and G. *Intervirology* 50, 9–15.
- Santos, A.O., Alvarado-Mora, M.V., Botelho, L., Vieira, D.S., Pinho, J.R., Carrilho, F.J., Honda, E.R., Salcedo, J.M., 2010. Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virol. J.* 7, 315.
- Schaefer, S., 2007. Hepatitis B virus genotypes in Europe. *Hepatol. Res.* 37, S20–S26.
- Sitnik, R., Pinho, J.R., Bertolini, D.A., Bernardini, A.P., Da Silva, L.C., Carrilho, F.J., 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J. Clin. Microbiol.* 42, 2455–2460.
- Souto, F.J., 2004. Hepatitis B and the human migratory movements in the state of Mato Grosso, Brazil. *Rev. Soc. Bras. Med. Trop.* 37 (Suppl 2), 63–68.
- Souto, F.J.D., 1999. Distribution of hepatitis B in Brazil: update of the epidemiologic mapping and proposals for its control. *GED: gastroenterologia endoscopia digestiva* 18, 143–150.
- Stroffolini, T., Pasquini, P., Mele, A., 1988. HBsAg carriers among pregnant women in Italy: results from the screening during a vaccination campaign against hepatitis B. *Public Health* 102, 329–333.
- Struyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F., Rossau, R., 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.* 81, 67–74.
- Tatematsu, K., Tanaka, Y., Kurbanov, F., Sugauchi, F., Mano, S., Maeshiro, T., Nakayoshi, T., Wakuta, M., Miyakawa, Y., Mizokami, M., 2009. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype. *J. Virol.* 83, 10538–10547.
- Thedja, M.D., Muljono, D.H., Nuralini, N., Sukowati, C.H., Verhoef, J., Marzuki, S., 2011. Ethnogeographical structure of hepatitis B virus genotype distribution in Indonesia and discovery of a new subgenotype. *B9. Arch. Virol.* 156, 855–868.
- Tran, T.T., Trinh, T.N., Abe, K., 2008. New complex recombinant genotype of hepatitis B virus identified in Vietnam. *J. Virol.* 82, 5657–5663.
- Viet, S., Manegold, C., Drosten, C., Nippraschk, T., Gunther, S., 2002. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. *Virus Genes* 24, 153–156.
- Wiegand, J., Hasenclever, D., Tillmann, H.L., 2008. Should treatment of hepatitis B depend on hepatitis B virus genotypes? A hypothesis generated from an explorative analysis of published evidence. *Antivir. Ther.* 13, 211–220.

- 19- Flair J Carrilho, Cleusa R Moraes, JOÃO RR PINHO, Isabel MVGC Mello, Dennis A Bertolini, Marcílio F Lemos, Regina C Moreira, Leda C Bassit, Rita A Cardoso, Gabriela Ribeiro-dos-Santos & Luiz C Da Silva. Hepatitis B virus infection in Haemodialysis Centres from Santa Catarina State, Southern Brazil. Predictive risk factors for infection and molecular epidemiology. BMC Public Health 4 (1):13 (2004). doi: 10.1186/1471-2458-4-13**

Research article

Open Access

Hepatitis B virus infection in Haemodialysis Centres from Santa Catarina State, Southern Brazil. Predictive risk factors for infection and molecular epidemiology

Flair J Carrilho*¹, Cleusa R Moraes¹, João RR Pinho², Isabel MVGC Mello², Dennis A Bertolini², Marcílio F Lemos², Regina C Moreira², Leda C Bassit³, Rita A Cardoso¹, Gabriela Ribeiro-dos-Santos⁴ and Luiz C Da Silva¹

Address: ¹Hepatology Branch, Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, Brazil, ²Virology Branch, Adolfo Lutz Institute, São Paulo, Brazil, ³Blood Centre "Fundação Pró-Sangue/Hemocentro de São Paulo", Brazil and ⁴Biosciences Institute, University of São Paulo, Brazil

Email: Flair J Carrilho* - fjcarri@usp.br; Cleusa R Moraes - crem@zaz.com.br; João RR Pinho - jrrpinho@usp.br; Isabel MVGC Mello - imvgcmello@uol.com.br; Dennis A Bertolini - dabertolini@hotmail.com; Marcílio F Lemos - marcilio.lemos@uol.com.br; Regina C Moreira - rmoreira@ial.sp.gov.br; Leda C Bassit - zyv0@cdc.gov; Rita A Cardoso - rita@statistica.com.br; Gabriela Ribeiro-dos-Santos - gabrs@uol.com.br; Luiz C Da Silva - cepae@terra.com.br

* Corresponding author

Published: 27 April 2004

Received: 16 November 2003

BMC Public Health 2004, 4:13

Accepted: 27 April 2004

This article is available from: <http://www.biomedcentral.com/1471-2458/4/13>

© 2004 Carrilho et al; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

Abstract

Background: Patients under haemodialysis are considered at high risk to acquire hepatitis B virus (HBV) infection. Since few data are reported from Brazil, our aim was to assess the frequency and risk factors for HBV infection in haemodialysis patients from 22 Dialysis Centres from Santa Catarina State, south of Brazil.

Methods: This study includes 813 patients, 149 haemodialysis workers and 772 healthy controls matched by sex and age. Serum samples were assayed for HBV markers and viraemia was detected by nested PCR. HBV was genotyped by partial S gene sequencing. Univariate and multivariate statistical analyses with stepwise logistic regression analysis were carried out to analyse the relationship between HBV infection and the characteristics of patients and their Dialysis Units.

Results: Frequency of HBV infection was 10.0%, 2.7% and 2.7% among patients, haemodialysis workers and controls, respectively. Amongst patients, the most frequent HBV genotypes were A (30.6%), D (57.1%) and F (12.2%). Univariate analysis showed association between HBV infection and total time in haemodialysis, type of dialysis equipment, hygiene and sterilization of equipment, number of times reusing the dialysis lines and filters, number of patients per care-worker and current HCV infection. The logistic regression model showed that total time in haemodialysis, number of times of reusing the dialysis lines and filters, and number of patients per worker were significantly related to HBV infection.

Conclusions: Frequency of HBV infection among haemodialysis patients at Santa Catarina state is very high. The most frequent HBV genotypes were A, D and F. The risk for a patient to become HBV positive increase 1.47 times each month of haemodialysis; 1.96 times if the dialysis unit reuses the lines and filters ≥ 10 times compared with haemodialysis units which reuse < 10 times; 3.42 times if the number of patients per worker is more than five. Sequence similarity among the HBV S gene from isolates of different patients pointed out to nosocomial transmission.

Background

Hepatitis B Virus (HBV) can be detected in blood and derivatives as well as in saliva, semen, vaginal secretion and exudates from cutaneous ulcer. HBV contamination is mainly established during the first year of life in highly endemic areas (Asia, Africa and East Amazonia), while in low epidemic areas the most important infection is found among young adults belonging to risk groups (health workers, haemodialysis patients, haemophiliacs, homosexuals, prostitutes, drug abusers, Hansen's disease patients, immunosuppressed patients and contacts with HBV infected patients) [1].

During the 70's, HBV infection was recognized as a great risk to haemodialysis patients [2], as HBV infection prevalence overcame 50% in some centres [3]. Some of the factors associated with HBV propagation include blood and derivatives transfusion, duration and frequency of haemodialysis, equipment contamination and contact among patients as well among them and health-care workers [4]. Since that time, HBV incidence in haemodialysis patients has been dramatically decreased especially by selection of blood donors, HBsAg positive patients isolation during dialysis and routine vaccination of uraemic patients. In Brazil, HBV infection in haemodialysis centres varies from 7.5 to 28.0 % [1].

HBV is as ubiquitous as man and it is found in all inhabited region of the globe [5]. HBsAg heterogeneity is well established. Serological methods developed to distinguish HBsAg antigenic subtypes allowed its classification in nine different subtypes with variable frequencies in different countries [6].

Divergences of the complete genome sequence inside the same subtype is approximately 8%, similar to those found in different subtypes. Therefore, subtypes do not reflect a real genotypic variation and another classification was proposed based on the nucleotide sequence of the S gene [7]. Primarily, four genotypes were described – A, B, C and D – followed by two others, E and F, corresponding to subtypes *ayw4* and *adw4*, respectively [8,9]. Recently, two more genotypes were found: G, in North America and Europe [10] and H, in North and Central America [11].

Nucleotide sequence comparisons of HBV genome have been used to study the HBV routes of infection, like vertical or blood and derivatives accidental inoculation [12]. In our country, the viral genetic diversity of HBV has already been demonstrated [13], but the present study is the first to investigate the routes of transmission through HBV sequencing analysis.

The aims of this study were (i) to analyse the frequency of HBV serological markers (total anti-HBc, HBsAg and anti-

HBs) in haemodialysis patients, (ii) to analyse HBV DNA frequency in HBsAg positive patients submitted to haemodialysis, (iii) to characterize predictive factors to HBV infection in patients submitted to haemodialysis, and (iv) to study the epidemiology of HBV infection through molecular analysis of partial gene S sequence.

Methods

Data collection

From each patient, the following data were collected: name, age, gender, race, time in haemodialysis, change of dialysis units, number of haemodialysis sessions per week, number of times of equipment reuse, type of dialysis equipment and dialysis solution. Also, the dates of the first haemodialysis and the first haemodialysis session in the unit were recorded. A variable designated PSU (Patient always in the Same Unit) was created to distinguish patients coming from different dialysis units.

From each studied health care worker, data collected included current working unit, age, gender, professional assignment and labour time.

From each dialysis unit, the following data were collected: (i) type of dialysis equipment (proportional system, central system and tank), (ii) whether rooms were separated for patients with hepatitis B and/or C or temporal separation at the end of each day, (iii) whether separated types of reprocessing rooms for patients with hepatitis B and/or C were applied, (iv) frequency of disinfections of dialysis equipments with sodium hypochlorite (between shifts or by the end of the day), (v) and frequency of sterilization with formaldehyde or peracetic acid (daily, weekly or never).

Population

All the 813 patients and 149 (51.1%) out of 291 health-care workers from all the 22 dialysis units at Santa Catarina State, southern Brazil, were studied. Data and blood samples were prospectively collected between October 22, 1996 and December 03, 1997. As control group, 772 healthy adults – matched by sex and age (± 3 years) – were recruited from the same regions.

The studied population features were: age ranging from 14 to 86 years (47.1 ± 15.3); 349 (42.9%) females and 464 (57.1%) males; 764 (94.0%) whites, 25 (3.1%) mulattoes and 24 (3.0%) blacks. The 291 health-care workers studied included medical doctors (61), nurses (31), dialysis technicians and attendants (169) and cleaning professionals (30). All patients were submitted to four hours haemodialysis sessions three times a week using disposable needles to veno – arterial puncture and reuse of dialysis lines and equipments.

Some patients have been previously treated by peritoneal dialysis (30/813 – 3.7%), outpatient continuous peritoneal dialysis (14/813 – 1.7%) and renal transplantation (47/813 – 5.8%). Most (528/813 – 65.9%) of the patients have been previously submitted to blood transfusions: 342 (42.0%), 111 (13.7%) and 75 (9.2%) received one to five, six to ten and more than ten blood units, respectively. Previous serological data obtained from the dialysis units reported 58 (7.2%) and 246 (30.3%) out of 813 patients as HBsAg and anti-HCV positive, respectively.

The present study involved all the major dialysis units at Santa Catarina state. These units were geographically divided in: North state, units A, B, C and D; Itajaí River region, units E, H, I, R, O, P, Q, X; South state, units J, K, L, M and S; Mountain area, units F and G; and West state, units T, U and V.

Use of the different types of dialysis equipments

Among the 813 patients, proportional system, central system and tanks equipments corresponded to 161 (19.8%), 535 (65.8%) and 117 (14.4%), respectively, were used. In relation to the dialysis solution, 716 (88.1%) and 97 (11.9%) patients utilized bicarbonate and acetate, respectively.

Types of dialysis room

(i) W (white), for 268 (33%) HBsAg and anti-HCV negative patients; (ii) W/C: for 334 (41.0%) patients without HBsAg but occasionally anti-HCV positive; (iii) B: for HBsAg positive and anti-HCV negative 32 (3.9%) patients; (iv) C: for 117 (14.4%) HBsAg negative and anti-HCV positive patients; (v) B/C: for 62 (7.6%) HBsAg and/or anti-HCV positive patients.

Periodical disinfections

Frequency of disinfection of dialysis equipments was between each dialysis period for 267 (32.8%) patients and at the end of the day for 546 (67.2%) patients. No other sterilizing procedure was applied in equipments utilized by 303 (37.3%) patients, while 154 (18.9%) and 356 (43.8%) patients utilized equipments sterilized with 4% formaldehyde or peracetic acid daily or weekly, respectively.

Reuse of lines and dialysis equipments

(i) for HBsAg and anti-HCV negative patients (190 – 23.3%); (ii) for HBsAg positive patients (31 – 3.8%); (iii) for anti-HCV positive patients (102 – 12.5%); (iv) for HBsAg and/or anti-HCV positive patients (49 – 6.0%); and (v) for patients without HBsAg positive (441 – 54.2%).

Number of lines and dialysis equipments reuse

Less than 10 times for 369 (45.4%) patients, and between 10 and 20 times for 444 (54.5%) patients.

Samples

Blood samples were collected through venopuncture in dry tubes (9.5 ml) with vacuum. After clot retraction, samples were centrifuged at 1500 to 2000 rpm and stored in 1 mL aliquots at -20°C.

Serological reactions

Serological markers of hepatitis B virus were detected by ELISA using commercially available kits (Murex Biotech Ltd, United Kingdom): total anti-HBc (ICE* HBc Detection Pack), HBsAg (Murex HBsAg) and anti-HBs (Murex anti-HBs).

All samples were tested for anti-HBc and the positive ones were also tested for HBsAg. Finally, anti-HBs was tested in the HBsAg negative samples.

All samples were also investigated for contact with hepatitis C virus (HCV); samples repeatedly reactive to anti-HCV ELISA test (INNOTEST HCV Ab III* Innogenetics, Belgium) were further submitted to a confirmatory third generation immunoblot test (IB-III, INNO-LIA HCV Ab III* – Innogenetics, Belgium).

Polymerase chain reactions (PCR)

HBV DNA was detected by PCR using specific primers covering the core (C) and surface (S) genes of the HBV genome. In all HBsAg positive samples, extraction and amplification of HBV-DNA were carried out by nested PCR, as described by Kaneko *et al.* [14]. Samples with positive PCR result were further submitted to another PCR reaction covering a fragment of 417 bp of the S gene as developed by Sitnik *et al.*, 1999 [15]. HCV RNA was detected by a commercial RT-PCR kit following the manufacturer recommendations (INNO-LiPA HCVII PCR amplification, Innogenetics, Ghent, Belgium).

Sequencing reactions

PCR products covering the S gene were submitted to cycle sequencing reactions, using the second round primers and a method derived from Sanger *et al.* [16] with dideoxynucleotides (ddNTPs) labelled with fluorescent markers (ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit – Applied Biosystems, Foster City, CA, USA).

Genotyping

Genotype analysis was carried out through the comparison of the obtained sequences with sequences of the different HBV genotypes from the Genbank. For this

Table 1: Frequency of HBV serological and molecular markers in the 4 different studied subgroups.

Group	n	Anti-HBc +ve	HBsAg +ve	Anti-HBs +ve	PCR core +ve	PCR surface +ve
Haemodialysis Patients (HP)	813	262 (23.2%)	81 (10.0%)	126 (15.4%)	62 (7.6%)	49 (6.0%)
HP Controls	620*	150 (24.2%)	24 (3.8%)	89 (14.3%)	15 (2.4%)	8 (1.3%)
Health care Workers (HCW)	149	30 (20.1%)	4 (2.7%)	20 (13.4%)	4 (2.7%)	4 (2.7%)
HCW Controls	149	21 (14.1%)	4 (2.7%)	11 (7.4%)	3 (2.0%)	2 (1.3%)

*Three samples were not available for HBV markers detection. +ve = positive

Table 2: Frequency of anti-HCV ELISA reactivity and HCV infection in the 4 different studied subgroups.

Group	n	Anti-HCV +ve	HCV infection*
Haemodialysis Patients (HP)	813	276 (33.9%)	274 (33.4%)
HP Controls	623	7 (1.1%)	5 (0.8%)
Health care Workers (HCW)	149	7 (4.7%)	4 (2.7%)
HCW Controls	149	0	0

* anti-HCV positive by ELISA and immunoblot or PCR positive. +ve = positive

purpose, EditSeq and Megalign softwares from the DNAs-
tar package (Lasergene Inc., USA) were used.

Phylogenetical analysis

Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 2.1 program [17]. Sequences were aligned using CLUSTAL X version 1.81 [18] and then analyzed by the Neighbor-joining method using a distance matrix calculated according to the Kimura-2-parameter model [19] and gamma distribution.

Statistical analysis

For statistical analysis, HBsAg and anti-HBc positive cases were considered as HBV carriers, while concomitant anti-HBc and anti-HBs positive and HBsAg negative results were appointed as previous HBV infection.

All variables were descriptively analysed for patients, health-care workers and healthy controls using frequency and percentage for qualitative variables or, for quantitative variables, maximum, minimum, median, mean and standard deviation values.

For univariate analysis, Student's *t* and χ^2 tests were used to compare means and distributions among different groups. For multivariate analysis, a statistical logistic regression with stepwise method of variables selection was used to evaluate the significance of the variables obtained from the univariate analysis to predict HBV infection [20]. Through this procedure, it was possible to evaluate the contribution of each variable in diagnosis probability of HBV infection. Significance level was estab-

lished at 5%. All calculations were carried out using the Statistical Analysis System (SAS) [21].

Results

The results of HBV serological and molecular markers in haemodialysis patients and health-care workers and their respective controls are shown in Table 1.

Anti-HCV was detected in 276 (33.9%) out of 813 patients. Among these 276 patients, immunoblot confirmatory test was positive in 271 (98.2%) and inconclusive in five (1.8%). Among the latter, HCV RNA was detected by PCR in 171 (61.9%) patients. HCV infected cases were considered those with positive ELISA and at least another positive test (immunoblot and/or PCR); Table 2 shows the positive cases meeting these criteria.

Patient variables according to the presence (HBsAg and anti-HBc positive) or absence of HBV infection are shown in Table 3. Statistical significant differences were found for haemodialysis total time ($p = 0.0002$) and anti-HCV detection ($p < 0.0001$).

The relation between characteristics of haemodialysis units and HBV infection is shown in Table 4. Statistical significant differences were found for the type of dialysis equipment ($p = 0.0071$), frequency of disinfection with sodium hypochlorite ($p = 0.0002$), frequency of sterilization with 4% formaldehyde or peracetic acid ($p = 0.0043$), dialysis units rooms ($p < 0.0001$), type of reuse of lines and dialysis equipments ($p = 0.0009$) and patients/HCW ratio ($p < 0.0001$).

Table 3: Univariate analysis of patient features according to HBV infection

	HBV carriers	HBV non-carriers	P
Age			
mean	45.7	47.2	0.4026
standard deviation	13.6	15.4	
Gender			
Male	54 (11.6%)	410 (88.4%)	0.0660
Female	27 (7.7%)	322 (92.3%)	
Time (months) on haemodialysis			
mean	44.3	30.8	0.0002
standard deviation	39.1	32.7	
Previous renal transplantation			
Yes	5 (10.6%)	42 (89.4%)	0.8735
No	76 (9.9%)	690 (80.1%)	
Number of previous blood or derivatives transfused			
Zero	24 (8.4%)	261 (91.6%)	0.5017
1 – 5	34 (9.9%)	308 (90.1%)	
6 – 10	15 (13.5%)	96 (86.5%)	
> 10	8 (10.7%)	67 (89.3%)	
Patients always in the same unit (PSU)*			
Yes	64 (9.6%)	601 (90.4%)	0.1891
No	17 (13.5%)	109 (86.5%)	
Anti-HCV			
Reactive	44 (16.0%)	232 (84.0%)	<0.0001
Non-reactive	37 (6.9%)	500 (93.1%)	

HBV carrier = HBsAg and anti-HBc positive; HBV non-carrier = anti-HBc negative. * data not available for 22 patients

Stepwise logistic regression analysis

Seven independent variables were submitted to a multivariate analysis by a logistic regression model [20] with the purpose of determining the probability of HBV infection among Haemodialysis patients. Variables with statistical significant results are shown in Table 5.

It was observed that total time in haemodialysis, number of reuse of lines and dialysis equipments and the patient/employee relation adjusted a probabilistic model that allows predicting HBV infection among patients with 60.5% sensitivity, 71.2% specificity and 70.1 percentage of correctly classified patients.

Consequently, the probability of a haemodialysis patient to have a HBV infection is given by:

$$P = \frac{\exp(-4,5952 + 0,3963 \times T + 0,6731 \times R + 1,2297 \times P)}{1 + \exp(-4,5952 + 0,3963 \times T + 0,6731 \times R + 1,2297 \times P)}$$

– where, T = time on haemodialysis; R = Reuse number of lines and dialysis equipments; and P = Patients / Health-care workers proportion.

The following data could be drawn from the above equation: (i) the chance of HBV infection increases 1.47 times monthly whilst the patient is submitted to haemodialysis; (ii) the chance of HBV infection in a patient that is submitted to haemodialysis in a unit where the number of reuse of lines and dialysis equipments is > 10 times multiplies by 1.96 the chance of a patient that goes to a unit where the reuse is inferior to 10 times; (iii) the chance of HBV infection in a patient that is submitted to haemodialysis in units where the patient/employee ratio is higher than five is 3.42 times the chance of a patient that goes to a unit where this proportion is lower than five.

HBV genotypes

The frequency of HBV genotypes in haemodialysis patients and their controls is shown in Table 6. Genotype D was the most frequent in both groups, but genotypes A and F were found exclusively in haemodialysis patients.

HBV genotypes frequency in health-care workers and their controls at haemodialysis units is shown in Table 7. Genotype A was the most prevalent among health-care workers. All controls were infected with genotype D.

Table 4: Univariate analysis of haemodialysis-units variables and HBV infection

	HBV carriers	HBV non-carriers	P
Type of haemodialysis equipment			
proportional system	8 (4.9%)	153 (95.1%)	0.0071
central system	66 (12.3%)	469 (87.7%)	
double tank	7 (5.9%)	110 (94.1%)	
Hygiene frequency			
Between shifts	11 (4.1%)	256 (95.9%)	0.0002
End of the day	70 (12.8%)	476 (87.2%)	
Sterilization frequency			
daily	8 (5.2%)	146 (94.8%)	0.0043
weekly	30 (8.4%)	326 (91.6%)	
never	43 (14.2%)	260 (85.8%)	
Dialysis Unit Rooms			
B	26 (86.7%)	4 (13.3%)	<0.0001
B/C	15 (28.3%)	38 (71.7%)	
W	4 (1.5%)	265 (98.5%)	
C	13 (12.5%)	91 (87.5%)	
W/C	23 (6.4%)	334 (93.4%)	
Reuse of lines and dialysis equipments			
< 10 times	21 (5.7%)	348 (94.3%)	0.0009
≥ 10 times	60 (13.5%)	384 (86.5%)	
Patients/ HCW ratio			
1 - 4	19 (4.6%)	396 (95.4%)	<0.0001
5 - 8	60 (13.5%)	223 (86.5%)	
>9	17 (14.8%)	98 (85.2%)	

Where: B = for HBsAg positive and anti-HCV negative patients, B/C = for HBsAg and/or anti-HCV positive patients, W = for HBsAg and anti-HCV negative patients, C = for HBsAg negative and anti-HCV positive patients, W/C = for patients HBsAg negative but occasionally anti-HCV positive; HCW = health-care workers

Table 5: Data from the adjusted model of stepwise logistic regression

Variable	Estimative	Error	P	95% Confidence Interval		
				Odds ratio	Inferior	Superior
Intercept	-4.5952	0.4653	0.0001			
Time on haemodialysis (T)	0.3863	0.1138	0.0007	1.472	1.177	1.839
Reuse number of lines and dialysis equipments (R)	0.6731	0.2730	0.0137	1.960	1.148	3.347
Patients / Health-care workers Proportion (P)	1.2297	0.2784	0.0001	3.420	1.982	5.903

Table 6: HBV genotypes frequency in haemodialysis patients and controls

HBV Genotypes	Haemodialysis Patients		Controls	
	N (+) / Total (%)		N (+) / Total (%)	
A	15/49 (30.6)		-	
D	28/49 (57.1)		8/8 (100.0)	
F	6/49 (12.2)		-	

Table 7: Frequency of HBV genotypes in health-care workers and controls at haemodialysis units

HBV Genotypes	Health-care workers		Controls	
		N (+) / Total (%)		N (+) / Total (%)
A		2/4 (50.0)	-	
D		1/4 (25.0)	2/2 (100.0)	
F		1/4 (25.0)	-	

HBV phylogenetic tree

The phylogenetic tree drawn from the analysis with the HBV S-gene sequences (PCR-positive samples) shows that the branches of the different genotypes are clearly individualised (Figure 1).

Genotype A was found in haemodialysis groups from units H (Itajaí River region), K, M and S (South region). All cases were clustered in the same branch of the tree with a 100 bootstrap value. Genotype D was found in haemodialysis groups from units A, B, D, (North region), J, K (South region), O, R, (Itajaí River region), T, U and V (West region). Most of the cases were clustered in the same tree branch with a bootstrap value of 88. The two cases from unit J clustered in the same branch (bootstrap value = 98). Genotype F was found only in units F (Mountain region) and V (West state). All cases clustered in the same branch with a 79 bootstrap value. The sequences from the control population, being all genotype D, were scattered through different branches of the tree.

Discussion

Many studies on HBV infection in dialysis units have been published; nonetheless the landmark of the present investigation is the inclusion of all patients from all dialysis units of the Santa Catarina State.

All of the 813 patients of the 22 dialysis units have accepted to enrol on this study, allowing us to investigate 100% of the patients that received assistance at that time in the state of Santa Catarina. Besides, 51.1% of the health-care workers from the dialysis units and, as controls, 772 healthy workers matched by sex and age (± 3 years) from companies in the same area have volunteered to take part.

The 22 dialysis units were heterogeneous when comparing the use of disposable needle for artery-vein fistula, number of reuse of lines and dialysis equipments, type of dialysis machine, machine hygiene, machine sterilisation, type of dialysis rooms regarding infected patients, lines and dialysis equipments reprocessing rooms and the

patients/care workers ratio. In fact, after literature review, no related reference was found with such a large coverage.

The ideal human resources for dialysis units established by decree (number 2,042) from the Brazilian Ministry of Health's [23] define one technician or attendant per four patients. The reality disclosed by this study was quite different, though; the lack of qualified personnel and, sometimes, under qualified care workers was the main cause for the low human resource/patient ratio found at Santa Catarina State. Due to this distortion, this variable was adapted by taking into consideration the total number of technicians, auxiliaries and nursery attendants per attended patients.

As determined by a third-generation ELISA method in a previous work with hepatitis C virus [22], anti-HCV was found in 33.9% (276/813) of the present patient population, and confirmed by immunoblot in 98.2% (271 patients). Polymerase chain reaction for the 271 immunoblot-positives and five inconclusive results was positive in 171 (62%) patients. Anti-HCV serological marker was detected in 44 of the 813 patients infected by HBV, representing 5.4% of HBV + HCV co-infection.

In this study, the definition of present or past HBV infection was based on total anti-HBc reactivity. In our casuistic, anti-HBc was positive in 32.2% of the patients, 10.0% with HBsAg and 15.4% with anti-HBs. Such high frequency of HBsAg positive was observed in several investigations, not only in Brazil, but also in other countries. Hepatitis B serology was positive in 72.7% of the haemodialysis patients in Saudi Arabia – a hyperendemic area –, of which 10.9% was HBsAg positive [24]. On the other hand, HBsAg frequency was 1.6% and anti-HBc 36.7%, in non-hyperendemic areas in Japan [25]. In Brazil, the reported HBV-infection frequency varied from 14.1% in Porto Alegre city [26], 7.8 to 28.0% in São Paulo city [27-31], 12.0% in Goiania city [32] and up to 20.0% in Salvador city [33].

In a three-year prospective study carried out in São Paulo, the appearance of HBsAg in 17 patients submitted to

Figure 1
Neighbor-joining tree of a 348 nt fragment (including gaps) of the HBV S gene from isolates of the present studied population and Genbank sequences representing genotypes A to H. Tree was constructed with the distance matrix calculated with the Kimura 2-parameter method and γ distribution using MEGA version 2.1. Bootstrap test of phylogeny was performed with 100 replications and values equal or greater than 69 are indicated on the branches. Sequences from the present study are named as followed R_P_NNN_G where: R = region of haemodialysis unit (A to V); P (H = haemodialysis groups and C = controls); NNN = number of the sequence; and G = genotype (A, D or F). Genotype representative sequences from the Genbank (loci and accession numbers) are: A (HHVBA – X75666, HPBSAG – M32138); B (HPBADW2 – D00330, HPBADW3 – BD00331, S74815 – S74815); C (HHVBC – S75184, HHVCCHA – S75665; S81946 – S81946); D (RXXHEPA – V01460, HPVPS12SA – X77309); E (HHVBASS – X75657); F (HBVADW4A – X69798; HHVBF – S75663); G (IG9227 – AF160501) and H (U91827 – U91827). The Woolly Monkey Hepatitis B Virus (WM046996) was utilized as outgroup.

haemodialysis corresponded to 0.19 patients a year [34]. Seroconversion rates of 2% were similar to other dialysis unit in São Paulo during a six-month study [30].

HBV genotyping use as an epidemic marker allowed the demonstration that the infection was introduced by chronic patients and disseminated in a haemodialysis unit, probably due to equipment contamination and the unit environment [4].

In relation to the health-care workers and controls, HBsAg prevalence varied between 2.7% and 3.8%, lower than the one observed in haemodialysis patients. A similar HBsAg figure (2.9%) was observed in hospital workers in other Brazilian regions [35]. HBsAg frequency in health-care workers in the USA was 1.0%, thus lower than the observed in our country [36].

HBV-DNA was detected by PCR in 62 (7.6%) of the 813 patients and in 76.5% of the 81 positive HBsAg patients, confirming the high viral replication frequency in these patients. This figure was similar to the one observed by other authors in haemodialysis populations in Goiânia city, in which the PCR-positive frequency was between 67.6% and 88.2% of the HBsAg positive patients [32]. The observations above show that HBV infection continues to be an important problem in Brazilian haemodialysis units.

Univariate analysis of age and sex has not shown significant differences for presence or absence of HBV infection. Likewise, renal transplant background, number of blood and/or derivatives transfusions and patients always in the same unit (PSU) were not significantly associated with this infection.

On the other hand, significant differences among patients without HBV infection were observed with the following variables: total haemodialysis time in months and anti-HCV serological marker. Significant association between HBV and anti-HCV markers was also observed in São Paulo by other authors. It was reinforced the role of haemodialysis duration, previous background of haemodialysis, renal transplant background and blood transfusions background [27,34].

The presence of total anti-HBc in patients' serum has been associated to a higher prevalence of HCV infection, but this is still controversial. Several authors found such relation [35-46], while others denied [46,47].

All variables related to dialysis units showed, by univariate analysis, significant differences for HBV infection; those were (i) type of dialysis equipment, (ii) hygiene and sterilization frequency, (iii) patients distribution accord-

ing to the group of dialysis rooms, (iv) number of reuse of lines and dialysis equipments and (v) patients distribution according to the number of health-care workers (technicians, auxiliary and nursery attendants).

Reuse of dialysis equipments was initially proposed to reduce costs [48], as guidelines supporting this practice claimed no increase in HBV infection [49]. On the other hand, a previous case-control study had shown the importance of poor function of dialysis machines due primarily to rupture of dialysis membranes [50].

As observed by univariate analysis, HBV infection and the following variables were statistically significant: haemodialysis total time, machine hygiene frequency, machine sterilization frequency, number of reuse of lines and dialysis equipments, number of patients per number of health-care workers and HCV infection.

The multivariate analysis (stepwise-logistic regression) selected the variables haemodialysis total time, number of reuse of lines and dialysis equipments and the proportion patients per health-care workers. This analysis adjusted a probabilistic model that allows predicting HBV infection in the haemodialysis patients with 60.5% sensitivity, 71.2% specificity and 70.1% of correctly classified patients.

Other investigators, by means of multivariate analysis, observed an association of three variables only – haemodialysis duration, type of dialysis (haemodialysis or ACPD – ambulatory continuous peritoneal dialysis) and blood transfusion background – with HBV infection markers [27,34]. These results contradict the present work, as no relation between the number of blood/derivates transfusion and HBV infection was observed.

So far, the importance of the variables related to dialysis units and HBV infection has not been well emphasized in literature. The present investigation emphasizes some aspects of strong epidemiological importance: (i) the HBV chance of infection increases 1.47 times a month in haemodialysis patients; (ii) the chance of a haemodialysis patient to become HBV infected in a unit that reuses lines and dialysis equipments more than 10 times is 1.96 times the chance of a patient in a unit that reuses the same material less than 10 times; and (iii) the chance of HBV infection in a patient that does haemodialysis in a unit in which the patient/health-care worker ratio is higher than 5 is 3.42 times the chance of a patient that does so in units with lower ratio values.

With the purpose of a better understanding of the HBV spreading among haemodialysis patients, HBV genotyping and sequencing were applied. In 65 positive HBsAg,

the following frequency was found: genotype A – 28%, genotype D – 57% and genotype F – 15%. Therefore, it can be observed that genotypes B, C and E were not represented in the present study. As for genotypes B and C, frequently detected in patients from Southeast Asia, this result was expected due to the absence of Japanese in the studied patients. Likewise, genotype E – usually found in African populations – was not detected. In this study, 94% of the patients were white and only 3% black, but other studies did not show any evidence of genotype E in Brazil [15].

Due to the high miscegenation in Brazil and lack of data, the present study points out the need of similar epidemiological researches in other dialysis units with more heterogeneous populations for a comprehensive and significant genotype characterisation. Genotype F was found at a low frequency in 10/65 (15.4%) and 6/84 (7.1%), respectively to haemodialysis and chronic hepatitis patients [15].

It is worth noting that the HBV DNA-sequencing disclosed 100% similarity among isolates of various genotypes from 17 patients belonging to different dialysis units. This similarity found in a same unit strongly suggests contamination by the same viral strain and, consequently, nosocomial transmission. These findings represent important advances in HBV epidemiology and similar studies, from other regions of Brazil, are awaited.

Despite the knowledge of the risk factors related to haemodialysis, the high frequency of HBV infection observed in our dialysis environment shows that these units represent a closed system for this viral transmission.

Many governmental and institutional recommendations have been set to reduce HBV dissemination in haemodialysis patients. Unfortunately, those recommendations have not been followed by all units, as shown by HBV infection surveys [51] and the appearance of recent infection outbreaks [52].

The present investigation enabled us to suggest more rigorous measures to be adopted: (i) maintenance of nationwide debates on the subject; (ii) active immunoprophylaxis in uremic patients before their admission in dialysis units; (iii) reduction on reuse of lines and dialysis equipments; and (iv) reduction in the number of patients per health-care worker.

Conclusions

In face of the present results, it can be concluded that: (i) HBV serological markers were highly frequent in haemodialysis patients as in comparison to health-care workers and controls; (ii) HBV DNA was detected in most positive

HBsAg patients; (iii) predictive HBV infection factors included haemodialysis total time, number of reuse of lines and dialysis equipments and patients/health-care workers ratio; (iv) HBV genotypes found in these patients were A, D and F, with A and D predominance; (v) HBV DNA sequencing revealed 100% similarity in isolates from different patients, bounding them to a common and nosocomial source of infection; and (vi) the need for strict observance of the rules for correct technical functioning with qualified personnel and service surveillance.

Competing interests

None declared.

Authors' contributions

FJC: conceived and coordinated the study from its design to the manuscript confection; CRM: participated in the study design and collected clinical samples and data; JRRP: coordinated the molecular biology experiments and participated in the manuscript writing; IMVGC, DAB, MFL, RCM, LCB: carried out the laboratory determinations; RAC: participated in the study design and performed the statistical analysis; GRS: participated in the manuscript writing and revision; LCS: participated in the study design and in the manuscript writing. All authors read and approved the final manuscript.

Acknowledgements

This study was supported by research grants from Alkes de Queiroz Family Fund for Research.

References

1. Carrilho FJ, Corrêa MCJM: **Magnitude of hepatitis B and C in Latin America.** In *Therapy for Viral Hepatitis* Edited by: Schinazi RF, Sommadossi JP, Thomas HC. London: Int Med Press; 1998:25-34.
2. Garibaldi RA, Forrest JN, Bryan JA, Hanson BF, Dismukes WE: **Hemodialysis-associated hepatitis.** *J Amer Med Assoc* 1973, **225**:384-389.
3. Goffin E, Pirson Y, Strihou CVY: **Implications of chronic hepatitis B or hepatitis C infection for renal transplant candidates.** *Nephrol Dial Transplant* 1995, **10**(Suppl 61):88-92.
4. Castro-Figueiredo JF, Moysé Neto M, Gomes UA, Spalini-Ferraz A, Nardin-Batista MEP, Coimbra-Gaspar AM, Tachibana-Yoshida CF: **Hepatitis B virus infection in hemodialysis units: clinical features, epidemiological markers and general control measures.** *Brazilian J Med Biol Res* 1986, **19**:735-742.
5. Brancroft WH: **The geographical distribution of HBsAg subtypes.** *Biol Haematol* 1976, **42**:42-47.
6. Courouc-Pauty AM, Plançon A, Soulier JP: **Distribution of HBsAg subtypes in the World.** *Vox Sang* 1983, **44**:197-211.
7. Okamoto BH, Tsuda F, Sakugawa H, Sastrosowignjo RI, Imai M, Miyakawa Y, Mayumi M: **Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes.** *J Gen Virol* 1988, **69**:2575-2583.
8. Norder H, Hammas B, Lofdahl S, Courouc AM, Magnus LO: **Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains.** *J Gen Virol* 1992, **73**:1201-1208.
9. Norder H, Hammas B, Magnus LO: **Molecular basis of hepatitis B virus serotype variations within the four major subtypes.** *J Gen Virol* 1992, **73**:3141-3145.
10. Stuyver L, Gendt SD, Geyt CV, Zoulim F, Fried M, Schinazi RF, Rossau R: **A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness.** *J Gen Virol* 2000, **81**:67-74.

11. Arauz-Ruiz P, Norder H, Robertson BH, Magnus LO: **Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America.** *J Gen Virol* 2002, **83**:2059-2073.
12. Dumpis U, Holmes EC, Mendy M, Hill A, Thurst M, Hall A, Whittle H, Karayannis P: **Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis.** *J Hepatol* 2001, **35**:99-104.
13. Gaspar AM, Yoshida CF: **Geographic distribution of HBsAg subtypes in Brazil.** *Mem Inst Oswaldo Cruz* 1987, **82**:253-258.
14. Kaneko S, Feinstone SM, Miller RH: **Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the Polymerase Chain Reaction technique.** *J Clin Microbiol* 1989, **27**:1930-1933.
15. Sitnik R, Pinho JRR, Da Silva LC, Fonseca LEP, Carrilho FJ, Bernardini AP: **Hepatitis B Virus Genotypes and Pre Core Mutants in Chronic Hepatitis B Patients from São Paulo City, Brazil [abstract].** *Hepatology* 2000, **32**(4 Pt.2):587A.
16. Sanger F, Nicklen S, Coulson AR: **DNA sequencing with chain-terminating inhibitors.** *Proc Natl Acad Sci USA* 1977, **74**:5463.
17. Kumar S, Tamura K, Jakobsen IB, Nei M: **MEGA2: molecular evolutionary genetics analysis software.** *Bioinformatics* 2001, **17**:1244-1245.
18. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG: **The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools.** *Nucl Ac Res* 1997, **24**:4876-4882.
19. Kimura M: **A simple method for estimating evolutionary rates of bases substitutions through comparative studies of nucleotide sequences.** *J Mol Evol* 1980, **16**:111-120.
20. Cox DR: **The analysis of binary data.** London: Methuen 1970.
21. Cary NC: **SAS/STAT User's guide Volume 2.** 4th edition. New York: Statistical Analysis System Institute Inc; 1989.
22. Moraes CR: **Estudo epidemiológico caso - controle da infecção pelo vírus da hepatite C em pacientes e funcionários das Unidades de Diálise do Estado de Santa Catarina.** PhD thesis. University of São Paulo School of Medicine, Department of Gastroenterology 1998.
23. Ministry of Health - Brazil. Decree number 2,042.
24. Ashraf SJ, Arya SC, Parande CM: **Viral hepatitis markers in patients on haemodialysis in a hyperendemic area.** *J Med Virol* 1986, **19**:41-46.
25. Oguchi H, Miyazaki M, Tokunaga S, Hora K, Ichikawa S, Ochi T, Yamada K, Nagasawa M, Kanno Y, Aizawa T, Watanabe H, Yoshizawa S, Sato K, Terashima M, Yoshie T, Oguchi O, Tanaka E, Kiyosawa K, Furuta S: **Hepatitis virus infection (HBV and HCV) in eleven Japanese hemodialysis units.** *Clin Nephrol* 1992, **38**:36-43.
26. Mattos AA, Flores AL, Coral GP, Goldani JC, Loselmann A, Becker M, Asquidamini S, Fossati L: **Incidência dos marcadores virais para a hepatite B e resposta a vacinação em uma unidade de hemodiálise.** *Gastroenterol Endosc Digest* 1999, **18**:193-196.
27. Cendoroglo Neto M, Draibet SA, Silva AEB, Ferraz ML, Granato C, Pereira CAP, Sesso RC, Gaspar AMC, Ajzen H: **Incidence of and risk factors for hepatitis B virus and hepatitis C virus infection among haemodialysis and CAPD patients: evidence for environmental transmission.** *Nephrol Dial Transplant* 1995, **10**:240-246.
28. Ianhez LE, Cuelar MKS, Leaf C, Saldanha LB, Romão JE Jr, Sabbaga E: **Prognóstico hepático em pacientes transplantados com HBsAg positivo pré-transplante.** *Rev Hosp Clin Fac Med, S Paulo* 1985, **40**:53-57.
29. Kopstein J, Ugalde CB, Fiori AMC: **Hemodiálise e hepatite A vírus B.** *J Bras Nefrol* 1984, **6**:9-11.
30. Pinho JRR, Fares J, Oba IT, Siqueira ML, Moreira RC, Barreto CC, Albuquerque AS: **Acompanhamento dos marcadores das hepatites B e C em uma unidade de diálise [abstract].** *Rev Soc Bras Med Trop* 1994, **27**(Suppl):372.
31. Romão JE Jr: **Hepatite B em hemodiálise.** *J Bras Nefrol* 1994, **6**:2-3.
32. Teles SA, Martins RMB, Vanderborgh B, Stuyver L, Gaspar AMC, Yoshida CFT: **Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients.** *Artif Org* 1999, **23**:1074-1078.
33. Paraná R, Cotrim H, Mascarenhas R, Dutra M, Lyra L: **Incidence of markers of hepatitis B and C in patients undergoing hemodialysis [abstract].** *Acta Hepatol* 1991, **1**:23.
34. Cendoroglo Neto M, Manzano SIR, Carziani ME, Silva AEB, Cirenza MLF, Sesso RC, Ajzen H, Draibe SA: **Environmental transmission of hepatitis B and hepatitis C viruses within the hemodialysis unit.** *Artif Org* 1995, **19**:251-255.
35. Fernandes JV, Braz RFS, Neto FVA, Silva MA, Costa NF, Ferreira AM: **Prevalence of serologic markers of the hepatitis B virus in hospital personnel.** *Rev Saude Pùb* 1999, **33**:122-128.
36. Levy BS, Harris JC, Smith JL, Washburn JW, Mature J, Davis A, Crosson JT, Polesky H, Hanson M: **Hepatitis B in ward and clinical laboratory employees of a general hospital.** *Amer J Epidemiol* 1977, **106**:330-335.
37. Ayoola EA, Huraib S, Arif M, Al-Faleh FZ, Al-Rashed R, Ramia S, AL-Mofleh IA, Abu-Aisha H: **Prevalence and significance of antibodies to hepatitis C virus among Saudi haemodialysis patients.** *J Med Virol* 1991, **35**:155-159.
38. Beccari M, Sorgato G, Rizzolo L, Veneroni G: **Anti-HCV reactivity in dialysis patients: discrepancies between first and second generation tests [letter].** *Nephron* 1983, **63**:238.
39. Chan TM, Lok ASF, Cheng IKP: **Hepatitis C infection Among dialysis patients: a comparison between patients on maintenance haemodialysis and continuous ambulatory peritoneal dialysis.** *Nephrol Dial Transplant* 1991, **6**:944-947.
40. Dussol B, Chicheportiche C, Cantaloube J, Roubicek C, Biagini P, Berthézène P, Berland Y: **Detection of hepatitis C infection by polymerase chain reaction among hemodialysis patients.** *Amer J Kidney Dis* 1993, **22**:574-580.
41. Dussol B, Berthézène P, Brunet P, Roubicek C, Berland Y: **Hepatitis C virus infection among chronic dialysis patients in the South of France: a collaborative study.** *Amer J Kidney Dis* 1995, **25**:399-404.
42. Elsay M, Tsianos E, Mavridis A, Dardamanis M, Pappas M, Siamopoulos KC: **Antibodies against hepatitis C virus (anti-HCV) in haemodialysis patients: association with hepatitis B serological markers.** *Nephrol Dial Transplant* 1991, **6**:476-479.
43. Fabrizi F, Raffaele G, Bacchini G, Guarnieri I, Pontoriero G, Erba G: **Antibodies to hepatitis C virus and transaminase concentration in chronic haemodialysis patients: a study with second-generation assays.** *Nephrol Dial Transplant* 1993, **8**:744-747.
44. Kellerman S, Alter MJ: **Preventing hepatitis B and hepatitis C virus infections in end-stage renal disease patients: back to basics.** *Hepatology* 1999, **29**:291-293.
45. Lin D, Lin H, Huang C, Liaw Y: **High incidence of hepatitis C virus infection in hemodialysis patients in Taiwan.** *Amer J Kidney Dis* 1993, **21**:288-291.
46. Sturm JA, Gabriel GF, Pereira CFA, Suassuna JHR, Nogueira Neto JF, Lobão VI, Coelho CJP, Lauria C, Chong-Ghee T: **Reavaliação por testes de 2ª geração da prevalência de anticorpos da Hepatite C (soro e extrato sérico de papel de filtro) em uma unidade de transplante renal.** *Rev Bras Pat. Clin* 1993, **29**:14-19.
47. Machida J, Yamaguchi K, Ueda S, Yoshida M, Kusumoto Y, Nishimura Y, Futami G, Ishii T, Watanabe T, Takatsuki K: **High incidence of hepatitis virus antibodies in hemodialysis patients.** *Nephron* 1992, **60**:117-118.
48. Garcia VD: **Reuso dos dialisadores: formaldeído ou ácido peracético?** *J Bras Nefrol* 1994, **16**:55-57.
49. Centers for Disease Control and Prevention: **Guidelines for Dialysis Centers.** *Dial Transplant* 1989:543-55.
50. Snyderman DR, Bryan JA, London WT, Werner B, Bregm ND, Blumberg BS, Gregg MB: **Transmission of hepatitis B associated with hemodialysis: role of malfunction (blood leaks) in dialysis machines.** *J Infect Dis* 1976, **134**:562-570.
51. Lyster R, Kellerman S, Alter MJ: **Hepatitis B and hemodialysis: the impact of universal precautions in preventing the transmission of bloodborne viruses.** Letter to the editor. *Infect Control Hosp Epidemiol* 1999, **20**:156.
52. Favero MS, Alter MJ: **The reemergence of hepatitis B virus infection in hemodialysis centers.** *Sem Dialysis* 1996, **9**:373-374.

Pre-publication history

The pre-publication history for this paper can be accessed here:

<http://www.biomedcentral.com/1471-2458/4/13/prepub>

- 20- Andre C Lyra, João RR Pinho, Isabel MVGC Mello, Fernanda de M Malta, Michele MS Gomes, Adrian M Di Bisceglie, Luiz GC Lyra, Flair J Carrilho, Luiz C da Silva. Distribution of Hepatitis B Virus (HBV) genotypes among patients with acute viral hepatitis. Journal of Clinical Gastroenterology (2005), 39 (1): 81-82**

whose willingness to collaborate has been of crucial importance. The authors also thank the ABIS nurses Caroline Berggren, Christina Larsson, and Iris Franzén who have been so enthusiastic and encouraging.

Supported by JDF-Wallenberg Foundation (K98-99JD-12813-01A, No. 1997-73), the Swedish Medical Research Council (MFR) K99-72X-11242-05A, the Swedish Child Diabetes Foundation (Barndiabetesfonden), Söderbergs Foundation, Novo Nordisk Foundation, FORSS (2000-323), the Sven Jerring Foundation, and the Swedish Medical Association (2002-1101).

Jonas F. Ludvigsson, MD, PhD*†
Jeanette Wahlberg Topp, MD‡

Johnny Ludvigsson, MD, PhD‡
*Pediatric Department

Örebro University Hospital
Örebro, Sweden

†Clinical Epidemiology Unit
Department of Medicine

Karolinska University Hospital
Karolinska, Sweden

‡Division of Pediatrics
Department of Molecular and

Clinical Medicine
Faculty of Health Sciences

Linköping University
Linköping, Sweden

REFERENCES

1. Dieterich W, Laig E, Schopper H, et al. Autoantibodies to tissue transglutaminase as predictors of celiac disease [see comments]. *Gastroenterology*. 1998;115:1317-1321.
2. Salmela S, Haltanen T, Laurila K, et al. Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease [see comments]. *Gastroenterology*. 1998;115:1322-1328.
3. Troncone R, Maurano F, Rossi M, et al. IgA antibodies to tissue transglutaminase: an effective diagnostic test for celiac disease [see comments]. *J Pediatr*. 1999;134:166-171.
4. Lampasona V, Bazzigaluppi E, Batera G, et al. Tissue transglutaminase and combined screening for celiac disease and type 1 diabetes-associated autoantibodies [Letter]. *Lancet*. 1998;352:1192-1193.
5. Bilbao JR, Vitoria JC, Ortiz L, et al. Immunoglobulin G autoantibodies against tissue transglutaminase: a sensitive, cost-effective assay for the screening of celiac disease. *Autoimmunity*. 2002;35:255-259.
6. Ludvigsson JF, Ludvigsson J. Stressful life events, social support and confidence in the pregnant woman and risk of celiac disease in the offspring. *Scand J Gastroenterol*. 2003;38:516-521.
7. Ludvigsson JF, Falth-Magnusson K, Ludvigsson J. Tissue transglutaminase autoantibodies in cord blood from children to become celiacs. *Scand J Gastroenterol*. 2001;36:1279-1283.

8. Ivarsson A, Hernell O, Nyström L, et al. Children born in the summer have increased risk for celiac disease. *J Epidemiol Community Health*. 2003;57:36-39.
9. Afoke AO, Eeg-Olofsson O, Hed J, et al. Seasonal variation and sex differences of circulating macrophages, immunoglobulins and lymphocytes in healthy school children. *Scand J Immunol*. 1993;37:209-215.
10. Ciacci C, Cavallaro R, della Valle N, et al. The use of serum tTGab assay in patients on gluten-free diet as a measure of dietetic compliance. *Gastroenterology*. 2002;122:588.

Distribution of Hepatitis B Virus (HBV) Genotypes Among Patients With Acute Viral Hepatitis

To the Editor:

Hepatitis B virus (HBV) can be classified into at least seven genotypes, A to G.¹⁻³ Most studies that evaluated the molecular epidemiology of hepatitis B infection have included patients with chronic infection. We evaluated the distribution of HBV genotypes and subtypes among patients with acute viral hepatitis in the city of Salvador, Northeast Brazil.

We analyzed sera from 42 patients with acute hepatitis B who were referred by primary care institutions through a sentinel program for hepatitis between 1995 and 1999. All patients had signs and symptoms of acute hepatitis, as well as ALT and AST at least twofold the upper limit of normal (ULN). Serologic criteria consisted of seropositivity for anti-HBc IgM and for hepatitis B surface antigen (HBsAg), seronegativity for immunoglobulin M anti-hepatitis A antibody and for anti-hepatitis C antibody. HBV-DNA was detected in serum by partial amplification of the S gene using a nested-PCR assay as previously described.⁴ Both strands of HBV PCR products were sequenced and HBV isolates were genotyped and subtyped by comparison of amino acid mutations with sequences of previously published genomes, and by phylogenetic analysis using the Neighbor-joining method in MEGA program (version 2.1)⁵; the distance matrix was calculated with the Kimura 2-parameter model.⁶ HBV subtyping was done according to amino acid identity in the S protein.^{7,8}

Twenty-five patients were male and 17 were female. Their mean age was 32.2 ± 13.0 years. Mean ALT levels was 30.9 ± 20.8 ($\times$ ULN) and mean AST level was 26.3 ± 21.6 ($\times$ ULN). HBV genotype could be determined in 40 of 42 samples (GenBank accession nos. AY133252-AY133291). Thirty-five had HBV genotype A, three had genotype D, and two had genotype F. All 35 HBV genotype A sequences were subtype adw2 and both genotype F genomes were subtype adw4, while one HBV genotype D sequence was subtype ayw2 and two were subtype ayw3. Phylogenetic analysis of the sequences confirmed this genotype distribution.

This study showed that three distinct genotypes of HBV may be encountered among our population with community-acquired acute hepatitis B: genotypes A, D, and F. However, HBV genotype A was by far the most prevalent among these patients. The population of the city of Salvador, Brazil is composed mainly by African-Brazilians and by miscegenation of African-Brazilians and whites (Mulattoes), many of whom descend from European (Portugal and Spain) and African ethnic background. It is interesting to speculate this might explain the high frequency of HBV genotype A among these individuals. Of note, studies from cities of Southeast Brazil found a high frequency of genotype A and also genotype C among patients with chronic HBV infection.⁴ In this region of Brazil, there is an increased prevalence of individuals of Asian descent. This suggests that the HBV genotype distribution in Brazil may be related to the immigration pattern observed in different regions, as occurs in the United States.⁹ Further studies are necessary to evaluate the HBV genotype among patients with chronic hepatitis B infection in our population. This might provide a better understanding of the relevance of HBV genotypes on clinical outcome in this population.

Andre C. Lyra, MD*†
João RR Pinho, MD‡
Isabel M. V. G. C. Mello, MS†
Fernanda de M. Malta, BS†
Michele M. S. Gomes, BS†
Adrian M. Di Bisceglie, MD§
Luiz G. C. Lyra, MD‡

- 21- Nadia Regina Caldas Ribeiro, Gubio Soares Campos, Ana Luiza Dias Ângelo, Eduardo Lorens Braga, Nelma Santana, Michele Mesquita Soares Gomes, JOÃO RENATO REBELLO PINHO, Wilson Andrade de Carvalho, Luiz Guilherme Costa Lyra, André Castro Lyra. Distribution of Hepatitis B virus genotypes among patients with chronic infection. Liver International 26: 1 – 7 (2006).**

Clinical Studies

Liver International
DOI: 10.1111/j.1478-3231.2006.01280.x

Distribution of hepatitis B virus genotypes among patients with chronic infection

Ribeiro NRC, Campos GS, Angelo ALD, Braga EL, Santana N, Gomes MMS, Pinho JRR, De Carvalho WA, Lyra LGC and Lyra AC. Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver International* 2006; 26: 636–642.

© 2006 The Authors. Journal compilation © 2006 Blackwell Munksgaard

Abstract: Hepatitis B virus (HBV) can be classified into at least eight genotypes, A–H. We evaluated the distribution HBV genotypes among patients with chronic infection. **Methods:** We consecutively evaluated adult patients with chronic HBV infection from Salvador, Brazil. Patients were classified according to HBV infection chronic phases based on HBV-DNA levels and presence of serum HBV markers. HBV-DNA was qualitatively and quantitatively detected in serum by polymerised chain reaction (PCR). Isolates were genotyped by comparison of amino acid mutations and phylogenetic analysis. **Results:** One-hundred and fourteen patients were evaluated. HBV-DNA was positive in 96 samples. HBV genotype was done in 76. Mean age was 36 ± 11.3 . In 61 of 76 cases subjects were classified as inactive HBsAg carriers. Their mean HBV serum level was 1760 copies/ml and 53 of 61 were infected with HBV genotype A, seven with HBV genotype F and one with genotype B. Twelve of the 76 patients had detectable hepatitis B e-antigen (HBeAg) in serum. Ten were infected with HBV genotype A and two with genotype F; most had increased alanine aminotransferase and high HBV-DNA levels. Three patients were in the immunotolerant phase, two were infected with HBV genotype A and one with genotype F. HBV subtyping showed subtypes adw2 and adw4. **Conclusions:** HBV genotype A adw2 and genotype F adw4 were the most prevalent isolates found. We could not find differences in genotype distribution according to HBV clinical phases and DNA levels. We did not detect HBV genotype D in contrast to a previous study in our center with acute hepatitis B. All inactive HBsAg carriers had low HBV-DNA levels.

Nadia Regina Caldas Ribeiro¹, Gubio Soares Campos², Ana Luiza Dias Angelo², Eduardo Lorens Braga¹, Nelma Santana¹, Michele Mesquita Soares Gomes³, João Renato Rebello Pinho³, Wilson Andrade De Carvalho⁴, Luiz Guilherme Costa Lyra¹ and Andre Castro Lyra^{1,4}

¹Gastro-Hepatology Service, Federal University of Bahia and Hospital São Rafael, Bahia, Brazil, ²Virology Laboratory, Federal University of Bahia, Bahia, Brazil, ³Instituto Adolfo Lutz, São Paulo, Brazil, ⁴Molecular Biology Laboratory, Hospital São Rafael, Bahia, Brazil

Key words: chronic hepatitis B – HBV genotype – hepatitis B virus – inactive chronic carriers – orthohepadnavirus

Andre C. Lyra, MD, R. Sócrates Guanaes Gomes, 84/401, Salvador-Bahia, Brazil, CEP 40-280 630. Tel: +55 71 3351 0565
e-mail: adlyra@atale.com.br

Received 3 October 2005,
accepted 19 March 2006

It is estimated that 350 million people are infected with hepatitis B virus (HBV), approximately 5% of the world's population. Chronic HBV infection can result in cirrhosis and hepatocellular carcinoma. HBV can be classified into at least eight genotypes, A to H (1–4). Several studies have evaluated the molecular epidemiology and the relevance of HBV genotypes on clinical outcome. Thus, HBV genotype A appears to be the predominant genotype among patients with HBV infection in North America, Northwest Europe and Africa, while HBV genotype B and C are frequently found in China and Japan, and HBV genotype D is prevalent in South Europe, Middle East and India (5, 6). HBV genotype F has been reported in Central and South America (7–9). Most of these studies have evaluated patients

with chronic HBV infection and there are only a few studies that have analyzed the HBV genotype distribution in patients with acute hepatitis B. One study in Switzerland found that genotype A was more common among patients with chronic hepatitis B, whereas genotype D was more prevalent among patients with resolving acute hepatitis B (10). Brazil is a country geographically divided into several regions, which have been colonized by people of different ethnic background. Therefore, HBV genotype distribution may differ in these regions. We have previously demonstrated that HBV genotype A was the most prevalent among patients with acute HBV infection and HBV genotypes D and F was also detected among them (11). The aim of this study was to evaluate and describe the distribution of

Hepatitis B virus genotypes among patients with chronic infection

HBV genotypes and subtypes among patients with chronic HBV infection from a center in the city of Salvador, Northeast-Brazil.

Methods

Study population

We consecutively evaluated adult patients (18 years old or greater) with chronic HBV infection who attended the Hepatology outpatient service of Federal University of Bahia-Brazil during 1 year. The majority of these patients had been referred by the main blood bank of the city independent of the HBV replication status because of a partnership between the blood bank and the University Hospital. Between April 2003 and April 2004 all physicians of the hepatology outpatient service were requested to forward all patients with chronic HBV infection to be evaluated by one physician (N. R. C. R.). Patients were subjected to a clinical evaluation, which included physical exams and all charts were reviewed in order to register serum aminotransferases levels and HBV viral markers. All patients were requested to consent serum storage of their samples at -70°C . The institutional review boards approved the study.

All patients were HBsAg positive for at least 6 months. They were classified as follows:

- (a) Inactive HBsAg carrier state – characterized by detectable HBsAg and Anti-HBe in serum, low ($<100\,000$ copies/ml) or undetectable HBV-DNA, normal alanine aminotransferase (ALT). These patients were further characterized based on physical exams into patients with signs of HBV chronic liver disease or patients with no signs of HBV chronic liver disease.
- (b) Patients with chronic hepatitis and active HBV replication:
 - hepatitis B e-antigen (HBeAg)-positive chronic hepatitis – characterized by detectable HBsAg and HBeAg in serum, high HBV-DNA level ($>100\,000$ copies/ml) and increased ALT.
 - HBeAg-negative chronic hepatitis – characterized by detectable HBsAg and anti-HBe without HBeAg in serum; HBV-DNA level greater than $100\,000$ copies/ml and increased ALT.
- (c) Patients in the immunotolerant phase: characterized by detectable HBsAg and HBeAg in serum, high ($>100\,000$ copies/ml) HBV-DNA levels and normal or minimally elevated ALT level.

HBV-DNA detection

Qualitative detection of HBV-DNA in serum was done by partial amplification of the S gene using a nested-polymerised chain reaction (PCR) assay. The sense primers utilized were FHBS1 (5'-GAG TCT AGA CTC GTG GTG GAC TTC-3') and FHBS2 (5'-CGT GGT GGA CTT CTC TCA ATT TTC-3'), and the anti-sense primers were RHBS1 (5'-AAA TKG CAC TAG TAA ACT GAG CCA-3') and RHBS2 (5'-GCC ARG AGA AAC GGR CTG AGG CCC-3') (12). Nucleic acid was extracted from 200 μl of serum using QIAmp Kit (QIAGEN Valencia, CA) according to the manufacturer's instructions and resuspended in 50 μl of buffer. Twenty microliters of DNA dissolved in buffer were added to 80 μl of a PCR solution containing $1 \times$ PCR buffer, 2.0 mM/l MgCl_2 , 0.15 mM/l dNTPs, 1.0 μM /l of each primer (FHBS1 and RHBS1), and 1.5 U of Taq polymerase (Invitrogen, San Diego, CA). PCR cycles consisted of one cycle of 94°C for 30 s followed by 35 cycles (94°C , 20 s; 56°C , 20 s; 72°C , 30 s) plus an additional cycle of 72°C for 5 min. Identical conditions and primers FHBS2 and RHBS2 were used for the second round of amplification. After electrophoresis on 1.5% agarose gel, samples producing a single band of 417 bp were considered as positive. Stringent precautions to avoid contamination were taken as described previously (13).

Quantitative detection of HBV-DNA was done using the Amplicor HBV Monitor test (PCR assay) (Roche Molecular Systems, Branchburg, NJ) according to the manufacturer's instructions.

Sequencing and genotyping

Both strands of HBV-PCR products were sequenced utilizing ABI Prism BigDye Terminator Ready Reaction Kit version 2.0 (Applied Biosystems, Foster City, CA). Reactions were analyzed by automated DNA sequencer (ABI model 377). Sequences were validated after visual inspection and analysis using the Phred-Phrap-Consed software, and then aligned using CLUSTAL X version 1.81 (14–16). HBV isolates were genotyped by comparison of nucleotide or amino acid mutations with sequences of previously published genomes, and by phylogenetic analysis using the Neighbor-joining method in MEGA program (version 3.1) (17); the distance matrix was calculated with the Kimura 2-parameter model (18). HBV subtyping was done according to amino acid identity in the S protein (19, 20).

Results

During the study period, 114 patients with chronic HBV infection were evaluated. HBV-

Ribeiro et al.

Table 1. Clinical features of 76 patients subjected to HBV genotyping

Mean age $\pm$ SD (range)	36 $\pm$ 11.3 (18–67)
Gender (male/female)*	45/31
Inactive HBsAg carrier state*	61
Serum HBV DNA (copies/ml)†	1760 (<400–4000)
Patients in the immunotolerant phase*	3
Serum HBV DNA (copies/ml)‡	40 $\times$ 10 ⁶
Patients with HBeAg positive hepatitis	12
Seven subjects with high serum HBV DNA (copies/ml)†	28.6 $\times$ 10 ⁶ (8–40 $\times$ 10 ⁶)
Five subjects with low serum HBV DNA (copies/ml)†	2160 (<400–3200)
Total	76

*Absolute numbers. †Mean (range). ‡Mean and range. HBV, hepatitis B virus; HBeAg, hepatitis B e-antigen.

DNA was positive in 84.2% samples (96/114). HBV genotype was determined in 76 of 96 samples that were HBV-DNA positive. The GenBank accession numbers for these sequences are DQ121295 to DQ121370. In 20 samples HBV-DNA was weakly positive and we were unable to sequence them.

From the 76 patients subjected to HBV genotyping, 45 were male (59.2%), while 31 were female. Their age ranged from 18 to 67 years, mean 36 $\pm$ 11.3, median 35 (Table 1). In 80.3% (61/76) of cases, subjects were classified as inactive HBsAg carrier state. Their mean HBV serum level was 1760 copies/ml (range, <400–4000 copies/ml). In 10 cases HBV-DNA was undetectable by quantitative PCR. Most of the inactive carriers (53/61) were infected with HBV genotype A. Seven subjects were infected with HBV genotype F and one with genotype B. The latter was a female Chinese who had been recently residing in Brazil. Five of the inactive carriers had clinical or histological features of chronic liver disease.

Seven of the 76 patients had active HBV replication with detectable HBeAg in serum, increased ALT and high HBV-DNA levels (mean, 28.6 $\times$ 10⁶ copies/ml; range 8–40 $\times$ 10⁶ copies/ml). Five of them were infected with HBV genotype A and two with HBV genotype F. Four subjects had liver biopsies with chronic hepatitis and one with cirrhosis and were treated with α -interferon or lamivudine.

Five out of 76 were HBV genotype A infected patients with detectable HBeAg in serum and low HBV-DNA levels (mean, 2160 copies/ml; range <400–3200 copies/ml), three of them had normal or less than two times the upper limit of normal ALT level and two had increased ALT. One of these two patients had hepatocellular carcinoma and increased α -feto-protein levels of 882 ng/ml (0–10 ng/ml) and the other chronic hepatitis.

Three patients out of 76 had detectable HBeAg in serum, HBV-DNA levels of 40 $\times$ 10⁶ copies/ml and normal or minimally elevated ALT level (immunotolerant phase). Two were infected with HBV genotype A and one with genotype F.

Figures 1 and 2 show the HBV genotyping by comparison with the isolate HHVBAMAM (GenBank accession number X75669). Comparison with all sequences utilized for analysis is not shown. A phylogenetic tree was also created and confirmed the genotype distribution (Fig. 3).

HBV subtyping showed subtype adw2 in the 65 patients infected with HBV genotype A and in the only patient infected with HBV genotype B, and adw4 in the 10 patients infected with HBV genotype F. Clinical features and HBV-DNA levels according to HBV genotype are shown in Table 2.

Discussion

This study evaluated the distribution of HBV genotypes among individuals with chronic infection in the city of Salvador, Brazil. Three distinct HBV genotypes were detected among the studied population, genotypes A, B and F. Genotype A was the most prevalent, being detected in the large majority of the subjects. All were subtype adw2.

The geographic distribution of HBV genotypes A appears to be related to the ethnic background. In our study, the greater prevalence of HBV genotype A might reflect the ethnic background of the population of the city of Salvador, Brazil, where there is miscegenation of blacks and whites. The white population is descendant of Portuguese and there is a major Spanish colony in the city. Studies from Spain have showed HBV genotype A is frequent in that country. To our knowledge, there are no published investigations from Portugal regarding HBV genotype distribution. Other studies have also shown that genotype A is the most commonly found in North America, Africa and Northwestern Europe (5, 6, 21). It was also described in Philippines, possibly reflecting the closer relationship of this country with the United States in the last century. In South Africa, a variation of genotype A was also reported and denominated A' (22).

The clinical significance of HBV genotypes is still controversial. In a study from Mayerat et al. (10), it was suggested that the infection by HBV genotype A could be more frequently associated with chronic infection than genotype D. HBV genotype D has an universal distribution, however, it seems to be more prevalent in Southern Europe, Middle East, Northern Africa and India.

Hepatitis B virus genotypes among patients with chronic infection

	aa 110	aa114	aa 122	aa 127	aa131	aa 140	aa143	aa 156/159				
AA-HVYAMAM	VCLQKQKQSF	TNREPTSCF	PICQSYNNC	LAKPIPLFI	ILGLIFLIV	LADQGMFLV	CGLFPGSTT	STPGCTCTT	TAQSHMPFS	CCCEPFTAN	CTCEIPFSW	APAKYLNKA
HHV_0262												
HHV_0263												
HHV_0265												
HHV_0269												
HHV_0292												
HHV_0293												
HHV_0216												
HHV_027												
HHV_0229												
HHV_0214												
HHV_0238												
HHV_0246												
HHV_0231												
HHV_0254												
HHV_0247												
HHV_0259												
HHV_0256												
HHV_0240												
HHV_0245												
HHV_0266												
HHV_0268												
HHV_0254												
HHV_025												
HHV_0215												
HHV_0212												
HHV_0226												
HHV_0238												
HHV_0238												
HHV_0236												
HHV_0236												
HHV_024												
HHV_0239												
HHV_0230												
HHV_0251												
HHV_02155												
HHV_02199												
HHV_0243												
HHV_0244												
HHV_0228												
HHV_0252												
HHV_0258												
HHV_0277												
HHV_0248												
HHV_021												
HHV_0241												

Fig. 1. Partial amino acid hepatitis B virus sequences of 44 of 76 isolates from samples of patients with chronic hepatitis B of the study population. Genotype A isolate HHVBAMAM (GenBank accession number X75669) was used as a reference sequence for comparison. Subtyping was performed by comparison of amino acids at position 122, 127 and 160, of the S protein.

	aa 110	aa114	aa 122	aa 127	aa131	aa 140	aa143	aa 156/159				
AA-HVYAMAM	VCLQKQKQSF	TNREPTSCF	PICQSYNNC	LAKPIPLFI	ILGLIFLIV	LADQGMFLV	CGLFPGSTT	STPGCTCTT	TAQSHMPFS	CCCEPFTAN	CTCEIPFSW	APAKYLNKA
HHV_02150												
HHV_0206												
HHV_02144												
HHV_0257												
HHV_02187												
HHV_029												
HHV_0211												
HHV_0278												
HHV_0236												
HHV_0222												
HHV_0291												
HHV_0275												
HHV_0214												
HHV_0251												
HHV_0257												
HHV_0253												
HHV_0276												
HHV_02151												
HHV_02155												
HHV_0248												
HHV_02156												
AA-HVYAMAM	VCLQKQKQSF	TNREPTSCF	PICQSYNNC	LAKPIPLFI	ILGLIFLIV	LADQGMFLV	CGLFPGSTT	STPGCTCTT	TAQSHMPFS	CCCEPFTAN	CTCEIPFSW	APAKYLNKA
HHV_0249												
HHV_0255	G.P.....											
HHV_023	G.P.....											
HHV_029	G.P.....											
HHV_0224	G.P.....											
HHV_0256	G.P.....											
HHV_0292	G.P.....											
HHV_0245	G.P.....											
HHV_0253	G.P.....											
HHV_0219	G.P.....											
HHV_0279	H.P.....											

A

adw2

B

adw2

F

adw1

Fig. 2. Partial amino acid hepatitis B virus sequences of 32 of 76 isolates from samples of patients with chronic hepatitis B of the study population. Genotype A isolate HHVBAMAM (GenBank accession number X75669) was used as a reference sequence for comparison. Subtyping was performed by comparison of amino acids at position 122, 127 and 160, of the S protein.

This HBV genotype has also been described among intravenous illicit drug users (23). We have recently demonstrated that HBV genotype A was the most prevalent among patients with acute hepatitis B, however, HBV genotypes D and F were also found (11). In the current study,

HBV genotype D was not detected. Therefore, it would be interesting to speculate that HBV genotype A could indeed be more prone to chronic infection than genotype D. It has recently been suggested that infection with HBV genotype A in patients with acute hepatitis B in

Ribeiro et al.

Fig. 3. Neighbor-joining tree of 348–360 nt fragments of Hepatitis B virus (S gene) from previously published reference genomes and from the study population. Tree was constructed with the distance matrix calculated with the Kimura 2-parameter method using MEGA program (version 3.1). For reference strains, the GenBank accession numbers are indicated. Genotype designations are indicated and reflect designations of previous publications. Bootstrap test of phylogeny was performed with 1000 replications and values equal or greater than 70 are indicated on the branches.

Hepatitis B virus genotypes among patients with chronic infection

Table 2. Clinical and laboratorial features of patients according to HBV genotype

HBV genotype	A	B	F	Total
N (%)	65 (84.4%)	1 (1.3%)	10 (13%)	76
Age $\pm$ SD	36.0 $\pm$ 11.6	31.0	33.1 $\pm$ 10.0	36 $\pm$ 11.3
Males/female	39/26	0/1	6/4	45/31
Inactive HBsAg carriers	53	1	7	61
Immunotolerant phase	2	0	1	3
Patients with HBeAg-positive hepatitis				
High serum HBV DNA ($> 100\,000$ copies/ml)	5	0	2	7
Low serum HBV DNA ($< 100\,000$ copies/ml)	5	0	0	5
Serum HBV DNA level: median, range (copies/ml)	3000, < 400 – 40×10^6	800	3200, 1600– 40×10^6	3200, < 400 – 40×10^6

HBV, hepatitis B virus.

Japan where genotypes B and C are common tends to become chronic (24). However, prospective studies would be required to address this issue.

HBV genotypes B and C are more frequently found in Southeastern Asia, China and Japan, and have also been described among Asian descendants who live outside their native country (6, 25, 26). We detected one female patient infected with HBV genotype B. Interestingly she was originally from China and had been living in Brazil just recently.

We also detected 10 patients infected by HBV genotype F, which is found in South America, being highly prevalent in Argentina and Venezuela (7–9). In Brazil, genotype F has been described at prevalence as high as 77% in the Indian population from the Amazon (27).

As expected, most patients were chronic inactive HBsAg carriers no matter the HBV genotype was, A or F. Also, median serum HBV-DNA levels were similar in both genotype A and F infected subjects. Overall, the number of patients who were in the immunotolerant phase was too low to take any conclusion. We were also unable to identify subjects with laboratorial evidence of infection by a precore mutant strain (increased ALT levels, undetectable HBeAg and high serum HBV-DNA), probably because the majority of patients were infected by HBV genotype A.

Of note, serum HBV-DNA levels in the chronic inactive carrier state were lower than 10 000 copies/ml in all cases, reinforcing that the cut off of PCR assays during this phase of the chronic infection might be 10^4 copies/ml as has been debated worldwide (28, 29).

In summary, HBV genotype A was the most prevalent among patients with chronic HBV infection from the population of our center in the city of Salvador, Brazil, although HBV genotype F was also detected. HBV-DNA levels were similar in both HBV genotype A and F infected patients. As we did not detect HBV genotype D in

the current study and a previous one from our center found HBV genotype D among patients with acute HBV infection, this raises the hypothesis whether this genotype could be less prone to chronic infection. Nevertheless, it would require prospective follow-up of acutely infected patients for confirmation.

Acknowledgement

This study was supported by FAPESP Grant number 227/2003 and CNPq Grant number 303762/2003-9 (RD-2F).

References

- OKAMOTO H, TSUDA F, SAKUGAWA H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69(10): 2575–83.
- NORDER H, COUROUCE A M, MAGNIUS L O. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; 198: 489–503.
- STUYVER L, DE GENDT S, VAN GEYT C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; 81: 67–74.
- ARAÚZ-RUIZ P, NORDER H, ROBERTSON B H, MAGNIUS L O. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83: 2059–73.
- CHU C J, KEEFFE E B, HAN S H, et al. Hepatitis B virus genotypes in the united states: results of a nationwide study. *Gastroenterology* 2003; 125: 444–51.
- CHU C J, LOK A S. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002; 35: 1274–6.
- BLITZ L, PUJOL F H, SWENSON P D, et al. Antigenic diversity of hepatitis B virus strains of genotype F in Amerindians and other population groups from Venezuela. *J Clin Microbiol* 1998; 36: 648–51.
- MBAYED V A, LOPEZ J L, TELENTO P F, et al. Distribution of hepatitis B virus genotypes in two different pediatric populations from Argentina. *J Clin Microbiol* 1998; 36: 3362–5.
- TELENTO P F, POGGIO G P, LOPEZ J L, GONZALEZ J, LEMBERG A, CAMPOS R H. Increased prevalence of genotype F hepatitis B virus isolates in Buenos Aires, Argentina. *J Clin Microbiol* 1997; 35: 1873–5.
- MAYERAT C, MANTEGANI A, FREI P C. Does hepatitis B virus (HBV) genotype influence the clinical outcome of HBV infection? *J Viral Hepatol* 1999; 6: 299–304.

Ribeiro et al.

11. LYRA A C, PINHO J R, MELLO I M, et al. Distribution of hepatitis B virus (HBV) genotypes among patients with acute viral hepatitis. *J Clin Gastroenterol* 2005; 39: 81-2.
12. SITNIK R, PINHO J R, BERTOLINI D A, BERNARDINI A P, DA SILVA L C, CARRILHO F J. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 2004; 42: 2455-60.
13. KWOK S, HIGUCHI R. Avoiding false positives with PCR. *Nature* 1989; 339: 237-8.
14. EWING B, GREEN P. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 1998; 8: 186-94.
15. GORDON D, ABAJIAN C, GREEN P. Consed: a graphical tool for sequence finishing. *Genome Res* 1998; 8: 195-202.
16. THOMPSON J D, GIBSON T J, PLEWNIAK F, JEANMOUGIN F, HIGGINS D G. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; 25: 4876-82.
17. KUMAR S, TAMURA K, NEI M. MEGA3: integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinform* 2004; 5: 150-63.
18. KIMURA M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980; 16: 111-20.
19. MAGNIUS L O, NORDER H. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 1995; 38: 24-34.
20. NORDER H, COUROUCE A M, MAGNIUS L O. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol* 1992; 73(Part 12): 3141-5.
21. NORDER H, HAMMAS B, LEE S D, et al. Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J Gen Virol* 1993; 74(Part 7): 1341-8.
22. BOWYER S M, VAN STADEN L, KEW M C, SIM J G. A unique segment of the hepatitis B virus group A genotype identified in isolates from south africa. *J Gen Virol* 1997; 78(Part 7): 1719-29.
23. VAN STEENBERGEN J E, NIESTERS H G, OP DE COUL E L, et al. Molecular epidemiology of hepatitis B virus in Amsterdam 1992-1997. *J Med Virol* 2002; 66: 159-65.
24. SUZUKI Y, KOBAYASHI M, IKEDA K, et al. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J Med Virol* 2005; 76: 33-9.
25. KIDD-LJUNGREN K, MIYAKAWA Y, KIDD A H. Genetic variability in hepatitis B viruses. *J Gen Virol* 2002; 83: 1267-80.
26. SUMI H, YOKOSUKA O, SEKI N, et al. Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. *Hepatology* 2003; 37: 19-26.
27. BERTOLINI D A, MOREIRA R C, SOARES M C P, et al. Genotyping of hepatitis B virus in indigenous populations from Amazon region, Brazil [abstract]. In *Virus: Reviews and Research*, 2000; 5(Suppl 1): p.101.
28. DE FRANCHIS R, HADENGUE A, LAU G, et al. EASL International Consensus Conference on Hepatitis B. 13-14 September, 2002 Geneva, Switzerland. Consensus statement (long version). *J Hepatol* 2003; 39(Suppl 1): S3-25.
29. MARTINOT-PEIGNOUX M, BOYER N, COLOMBAT M, et al. Serum hepatitis B virus DNA levels and liver histology in inactive HBsAg carriers. *J Hepatol* 2002; 36: 543-6.

- 22- Moura IF, Lopes EP, Alvarado-Mora MV, Pinho JR, Carrilho FJ. Phylogenetic analysis and subgenotypic distribution of the hepatitis B virus in Recife, Brazil. Infection, Genetics and Evolution. 2013 Mar;14:195-9.**

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Phylogenetic analysis and subgenotypic distribution of the hepatitis B virus in Recife, Brazil

Izolda Fernandes Moura^a, Edmundo Pessoa Lopes^{b,*}, Mônica Viviana Alvarado-Mora^c, João Renato Pinho^c, Flair José Carrilho^c^a Central Laboratory of the Hospital das Clínicas, Federal University of Pernambuco, Brazil^b Division of Gastroenterology, Department of Internal Medicine, Federal University of Pernambuco, Brazil^c Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil

ARTICLE INFO

Article history:

Received 24 August 2012

Received in revised form 23 November 2012

Accepted 25 November 2012

Available online 23 December 2012

Keywords:

HBV genotype

HBV subgenotype

Phylogenetic analysis

Bayesian analysis

Hepatitis B virus

ABSTRACT

The analysis of the genomes of the hepatitis B virus in human hosts identifies phylogenetic variants called viral genotypes. Indeed, clinical and epidemiological observations suggest that differences in viral genotypes lead to distinct biological and clinical behaviors. The aim of this study was to evaluate the subgenotypic distribution and to conduct a phylogenetic analysis by Bayesian method of the hepatitis B virus (HBV) in patients from Recife, Brazil. From July 2009 to December 2010, 60 HBV infected patients were examined, 39 (65%) were males, whose mean age was 50 years old. 33 (55%) were genotyped by obtaining and amplifying a 1306 bp fragment comprising part of the DNA polymerase and the surface antigen of the HBV. The sequencing was performed on an ABI 3500 Automatic Sequencer and the consensus sequences were obtained by aligning both the sequenced strands (clockwise and anti-clockwise) using SEQUENCHER software. Phylogenetic analysis was conducted using the Markov Chain Monte Carlo simulation implemented by Bayesian evolutionary method by sampling trees. The following subgenotypic distribution was observed: A1 (79%), F2a (12%), A2 (6%) and F4 (3%) as was that those identified as subgenotype A1 were in the same cluster in the phylogenetic tree. In this study, the majority of patients presented the A1 subgenotype from the same viral strain. As per the distribution in the phylogenetic tree by Bayesian method, possibly this subgenotype was in the genetic make-up of Africans brought in centuries past to Brazil as slaves.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

The analysis of the genomes of hepatitis viruses in the human population identifies phylogenetic variants called viral genotypes. The genotype is characterized by nucleotide differences of 8% or more over the entire length of the genome of the hepatitis B virus (HBV) or 4% or more within the S gene of the viral surface (HBsAg) antigen (Kim et al., 2011; Tanwar and Dusheiko, 2012). In fact, biological and epidemiological observations suggest that differences in HBV genotypes induce distinct clinical and therapeutic behaviors (Raimondi et al., 2010). For example, patients infected with genotype A seem to respond better to interferon therapy than those infected with genotype D, while genotype B infected patients respond better than those infected with genotype C (Cao, 2009; Mello et al., 2012).

HBV is classified into nine genotypes from A through I, which are subdivided into subgenotypes that can be characterized by a

few amino acid substitutions in the determining "a" of gene S and named with digits A1 ... A5, F1 ... F4. These genotypes have distinct geographical distributions (Scheiblaue et al., 2010; Yu et al., 2010).

The genetic diversity of HBV and the geographical distribution of its subgenotypes are tools for reconstructing its evolutionary history, but this also can assist in coming to understand the evolution and migrations of humans in the past (Cao, 2009; Scheiblaue et al., 2010). It is worth mentioning that the flow of global migration can determine changes in the geographical distribution of HBV genotypes or subgenotypes, with the presence of some genotypes in areas where they had not previously circulated. Indeed, countries in South America have shown emigration flows to European countries such as Italy, which have led to the distribution of HBV genotypes, such as A and D (Palumbo et al., 2007).

In Brazil, studies on identifying HBV genotypes and subgenotypes show that variability occurs in the geographical distribution of the virus. As result of individuals coming from Europe and Africa to our country over many decades, the population became of mixed races and started to show a different genotypic circulation pattern to that found in other Latin American countries

* Corresponding author. Address: Rua Irmã Maria Davi, 154, Ap.3201, 52061-070 Recife, Pernambuco, Brazil. Tel./fax: +55 81 2126 8517.
E-mail address: epalopes@uol.com.br (E.P. Lopes).

(Alvarado-Mora et al., 2010). The presence of genotypes A and D suggest influences from African descendants as a result of the period of slavery and European colonization, respectively (Devesa et al., 2008; Alcalde et al., 2009; Tonetto et al., 2009). Nonetheless, in the native populations (indigenous) a higher prevalence of genotype F is observed (Mello et al., 2007; Dias et al., 2012).

Fig. 1 shows subgenotype distribution of HBV in some Brazilian states. It is noted that it has only been presented the articles that the sequencing of the subgenotype has been made (Andermach et al., 2009; Matos et al., 2009; Alvarado-Mora et al., 2011a; Santos et al., 2011; Bertolini et al., 2012).

Due to the scarcity of data on the distribution of subtypes of HBV in our region, this study set out to evaluate the subgenotypic distribution and to construct the phylogenetic tree and to conduct the Bayesian analysis of this virus.

2. Patients and methods

2.1. Patients

This was a cross sectional study to assess the genotypic and subgenotypic distribution of HBV in the outpatient's clinic for Hepatology at the Hospital das Clínicas of Federal University of Pernambuco between July 2009 and December 2010.

Patients were included consecutively provided they were above 18 years old, and had been HBsAg and anti-HBc positive for more than 6 months. The exclusion criteria were patients with positive serology for the hepatitis C virus (HCV) and immune-compromised patients.

The project was submitted to and approved by the Committee for Ethics in Research, Center for Health Sciences, UFPE and all patients signed an informed term of consent.

2.2. Methods

Blood samples were collected and taken to the Central Laboratory of the Hospital das Clínicas, where they were centrifuged and frozen at -20°C .

The frozen samples were then taken to the Laboratory of Gastroenterology and Hepatology of Institute of Tropical Medicine, University of São Paulo, where HBV genotyping and subgenotyping were conducted as was Bayesian and phylogenetic analysis.

HBV-DNA was extracted by withdrawing 100 μL of serum from each sample using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987; Kwok and Higuchi, 1989). A 1306 bp fragment comprising partially HBsAg and the DNA polymerase (S/Pol) coding regions was amplified by the nested PCR method using the primers PS3132F/2920R and PS3201F/P1285R (Alvarado-Mora et al., 2011b).

The amplified DNA was purified using the ChargeSwitch PCR Clean-Up Kit (Invitrogen). Sequencing was performed on an ABI 3500 Automatic Sequencer (Applied Biosystems, Foster City, CA) using dideoxynucleoside triphosphates (ddNTPs) containing fluorescent markers (Big Dye1 Terminator v3.1 Cycle Sequencing Ready Reaction Kit, Applied Biosystems).

The consensus sequences were obtained by aligning both sequenced strands (clockwise and anti-clockwise) using SEQUENCHER software (Gene Codes Corporation Ann Arbor, Michigan, United States of America).

The sequences were genotyped by means of phylogenetic reconstruction using the reference sequences of each genotype obtained from the GenBank ($n = 267$). The sequences were aligned using Muscle software and edited with SE-AL software. Phylogenetic analysis was conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST (Bayesian evolutionary analysis by sampling trees) v.1.5.3. Chains were run for 10 million

Fig. 1. Distribution of hepatitis virus B genotype and subgenotype in some Brazilian states.

generations which were sufficient to achieve the convergence of parameters. The maximum clade credibility (MCC) tree was obtained which summarized the 10,000 substitution trees and then 10% of the burn-in was removed using Tree Annotator v.1.5.3 (Drummond and Rambaut, 2007).

The sequences obtained were analyzed and grouped in the same "cluster". Then phylogenetics analysis was performed using BEAST v.1.5.4. A Bayesian skyline plot was drawn using the best model of nucleotide substitution (GTR+I+G), previously obtained in the Modeltest (Posada and Crandall, 1998). Ten million generations were sufficient to converge all parameters (effective sampling size > 200). The molecular clock with the best results was chosen by Bayes Factor comparison.

3. Results

We investigated 60 HBV infected patients, whose mean age was 50 years old, 39 (65%) were male and 21 (35%) were female. In 27 patients (45%) it was not possible to identify subgenotypes, and therefore, 33 HBV infected patients were analyzed. The samples from these patients revealed the following distribution: 28 A genotype patients (85%), there being 26 A1 subgenotype (79%) and 2 A2 (6%), and 5 F genotype patients (15%) of whom 4 were F2a subgenotype (12%) and 1 F4 (3%).

According to the Bayesian analysis, of the total number of sequences obtained, 24 of the A1 subgenotype were grouped in the same "cluster" with great support. The subgenotypes identified in this study are placed in the phylogenetic tree (Fig. 2).

4. Discussion

How the colonization of Brazil took place greatly influenced the distribution of HBV genotypes in each region of the country. In fact, a study involving 49 patients on hemodialysis in the state of Santa Catarina revealed that genotype D (57%) was the one that occurred most frequently, followed by the A (31%) and F (12%) genotypes (Carnilho et al., 2004). Another study of 67 patients from the state of Rio Grande do Sul also found that genotype D (60%) was the most frequent, followed by the A (34%) and F (5%) genotypes (Becker et al., 2010). Furthermore, a recently published study revealed that the most common genotype in state of Paraná was D (83%) followed by genotype A (14%) (Bertolini et al., 2012).

This finding should be related to the population of the South of Brazil being of predominantly European ancestry, mainly Italians and Germans, which would explain why genotype D is the one that has the highest occurrence.

On the other hand, in the Southeast region of Brazil was observed a predominance of HBV genotype A followed by D. In addition, the genotypes C and F were also found, suggesting that different HBV strain were introduced in this region (Alcalde et al., 2009; Tonetto et al., 2009).

In the North region of Brazil, in Western Brazilian Amazon at Lábrea District, a study evaluating 86 serum samples HBsAg reactive which whom 39 were found to be HBV-DNA, obtaining the following genotypic distribution A (60%), D (35%) and F (5%) (Dias et al., 2012). Nevertheless, an evaluation of 40 HBV infected patients in the Rondônia state found genotype D in 43%, A in 37% and F in 20% of the patients. The subgenotypic analysis showed the following distribution: A1 (37%), D3 (23%), F2a (20%), D4 (17%) and D2 (3%) (Santos et al., 2010). It is noteworthy that the HBV found in this study, according to phylogenetic analysis, did not come from a common strain, which suggests that different subgenotypic inputs occurred in this region of the Amazon. Probably, the genotype D was introduced there by people from South region

of Brazil that immigrates during the 1970s to 1980s decades. The F2a subgenotype was identified, even if its prevalence was low, as in our study, which is probably related to the influence of native South Americans. This genotype was found primarily in the indigenous populations of South America, especially Venezuela, Colombia and Brazil (Mello et al., 2007; Devesa et al., 2008; Alvarado-Mora et al., 2011b).

In the Midwest region, a study involving 26 hemodialysis patients from the state of Goiás verified the occurrence of genotype A in half the cases, followed by D (46%) and F (4%) (Teles et al., 1999). Two other studies conducted in this region on individuals from communities with African descendants (*quilombos*), as expected, showed a great predominance of the A genotype and a low percentage of genotype D (Motta-Castro et al., 2008; Matos et al., 2009).

In the Northeast, an evaluation conducted in a rural community of the semi-arid region in Bahia, involving 1476 individuals, found that 38 (2.6%) had chronic HBV infection and of these, 9 subjects (23.6%) presented positive HBV-DNA and were identified as A genotype (Almeida et al., 2006). Also in Bahia state, at Salvador city, were evaluated 61 HBV infected patients and revealed genotype distribution similar to our results. They found the following: genotype A (87%), D (11%) and F (2%), but the virus subgenotypes were not evaluated (Ribeiro et al., 2006). Another study analyzing subgenotyping and phylogenetic analysis in this region evaluated 72 individuals of African descent, residents of a *quilombo* in the state of Maranhão, and revealed that 4 (5.6%) were positive for HBsAg and all of them were identified as A1 subgenotype (Alvarado-Mora et al., 2011).

The present study which evaluated patients from a large city in the Northeast region of Brazil revealed that the A1 subgenotype was the most prevalent (79%). The subgenotypic placement in the phylogenetic tree indicated that most of them came from a common single strain from Africa (Fig. 2). It is noteworthy that no patients were found with genotype D, despite the influence of Portuguese and Dutch colonization in our region. The absence of genotype D could be justified because the patients included were from a public hospital and their socio-economic level was low. In other words, it may well be that the genetic make-up of these patients have minimal or no European elements in them. As the population evaluated had low incomes, the transmission could have occurred in the past by sexual or vertical pathway. Differently, a previous study in our city involving 24 blood donors revealed 4 individuals (17%) with genotype D, 54% and 29% with genotypes A and F, respectively (Mello et al., 2007). In our region, usually blood donors are of urban origin and have higher socio-economic level. Nor did our study find patients with genotypes B and C which have been found in Brazil (Tonetto et al., 2009; Bertolini et al., 2012), probably due to the immigration of people from the Far East to the Northeast being small.

All these data indicate that the HBV A genotype is widely prevalent in Brazil, except in the South region. As this genotype had an African origin, its introduction into Brazil could have occurred during the slave trade between the 16th and 19th centuries, mainly in the Northeast region where slaves were sent to work in the sugar cane fields. After the arrival in this region, some slaves ran away to other regions of the country especially North and Midwest where they raised the *quilombos*.

Similarly, in Haiti, where the population is predominantly of African origin, the A genotype was found to be the most prevalent, above all A1 and A5, followed by genotypes D and E (Kramvis and Kew, 2007; Andermach et al., 2009).

Current data unexpectedly show that the most common genotype in Angola is E and that the region which that country then covered was the main source of the slaves brought by the Portuguese to the Northeast of Brazil (Valente et al., 2010).

Fig. 2. Phylogenetic tree with the hepatitis B virus subgenotypes distribution of patients in Recife (2011).

As the A genotype has five subgenotypic differences (A1–A5), studies on evolution suggest that it has a long natural history in the African continent. As to the E genotype, it has less genetic diversity and must have arisen more recently in Africa (Kimbli et al., 2004; Kramvis and Kew, 2007). Thus, the high occurrence of the A genotype in the African-American descendants would be justified because of its having spread out in past centuries, when it was still endemic in Africa. As the emergence of the E genotype was more recent, it probably spread after the slave trade ended, namely from around the mid-to late 19th century.

5. Conclusion

In conclusion, this study demonstrated that most patients had the A genotype of HBV, the A1 subgenotype being the most common. The phylogenetic tree by Bayesian method indicates that its spread occurred from the same viral strain, suggesting that the introduction of this subgenotype in our region began when African slaves were brought here between the 16th and 19th centuries. Further studies in other cities of the Northeast and other regions of Brazil, however, are necessary to confirm these findings.

References

- Alcalde, R., Melo, F.L., Nishijima, A., Ferreira, S.C., Langhi Jr., M.D., Fernandes, S.S., Marcondes, L.A., Duarte, A.J.S., Casseb, J., 2009. Distribution of hepatitis B virus genotypes and viral load levels in Brazilian chronically infected patients in São Paulo city. *Rev. Inst. Med. Trop. S. Paulo* 51, 269–272.
- Almeida, D., Tavares-Neto, J., Vileitiski, L., Almeida, A., Mello, C., Santana, D., Tatch, F., Parani, R., 2006. Serological markers of hepatitis A, B and C viruses in rural communities of the Semiarid Brazilian Northeast. *B.J.I.D.* 10, 317–321.
- Alvarado-Mora, M.V., Botelho, L., Gomes-Gouveia, M.S., de Souza, V.F., Nascimento, M.C., Pannuti, C.S., Cardilho, F.J., Pinho, J.R., 2011a. Detection of hepatitis B virus subgenotype A1 in the Quilombo community from Maranhão, Brazil. *Virol. J.* 8, 415.
- Alvarado-Mora, M.V., Romano, C.M., Gomes-Gouveia, M.S., Gutierrez, M.F., Botelho, L., Camilho, F.J., Pinho, J.R., 2011b. Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect. Gen. Evol.* 11, 103–108.
- Alvarado-Mora, M.V., Romano, C.M., Gomes-Gouveia, M.S., Gutierrez, M.F., Carrilho, F.J., Pinho, J.R., 2010. Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J. Gen. Virol.* 91, 501–508.
- Andermach, L.E., Nolte, C., Pape, J.W., Müller, C.P., 2009. Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg. Infect. Dis.* 15, 1222–1228.
- Becker, C.E., Mattos, A.A., Bogo, M.R., Branco, F., Sitnik, R., Kretzmann, N.A., 2010. Genotyping of hepatitis B virus in a cohort of patients evaluated in a hospital of Porto Alegre, South of Brazil. *Arq. Gastroenterol.* 47, 13–17.
- Bertolini, D.A., Gomes-Gouveia, M.S., Carvalho-Mello, L.M.V.G., Saraceni, C.P., Sitnik, R., Gazziotin, F.G., Laurino, J.P., Fagundes, N.J.R., Carrilho, F.J., Pinho, J.R., 2012. Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil. *Infect. Genet. Evol.*
- Cao, G.W., 2009. Clinical relevance and public health significance of hepatitis B virus genomic variations. *World J. Gastroenterol.* 15, 5761–5769.
- Carrilho, F.J., Moraes, C.R., Pinho, J.R., Mello, L.M., Bertolini, D.A., Lemos, M.F., Moreira, R.C., Bassit, L.C., Cardoso, R.A., Ribeiro-dos-Santos, G., Da Silva, L.C., 2004. Hepatitis B virus infection in hemodialysis centers from Santa Catarina state, Southern Brazil. Predictive risk factors for infection and molecular epidemiology. *BMC Public Health* 4, 13.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Devesa, M., Loureiro, C.L., Rivas, Y., Monsalve, F., Cardona, N., Duarte, M.C., Poblete, E., Gutierrez, M.F., Botto, C., Pujol, F.H., 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and general population of Colombia. *J. Med. Virol.* 80, 20–26.
- Dias, A.L.B., Oliveira, C.M.C., Castilho, M.C., Silva, M.S.P., Braga, W.S.M., 2012. Molecular characterization of the hepatitis B virus in autochthonous and endogenous populations in the Western Brazilian Amazon. *Rev. Soc. Bras. Med. Trop.* 45, 9–12.
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sample trees. *BMC Evol. Biol.* 7, 214.
- Kim, B.K., Revill, P.A., Ahn, S.H., 2011. HBV genotypes: relevance to natural history, pathogenesis and treatment of chronic hepatitis B. *Antivir. Ther.* 16, 1169–1186.
- Kimbli, G.C., Kramvis, A., Kew, M.C., 2004. Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J. Gen. Virol.* 85, 1211–1220.
- Kramvis, A., Kew, M.C., 2007. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol. Res.* 37, S9–19.
- Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. *Nature* 339, 237–238.
- Matos, M.A.D., Reis, N.R.S., Kozłowski, A.G., Teles, A.S., Motta-Castro, A.R.C., Mello, F.C.A., Gomes, A.S., Martins, R.M.B., 2009. Epidemiological study of hepatitis A, B and C in the largest Afro-Brazilian isolated community. *Roy. Soc. Trop. Med. Hyg.* 103, 899–905.
- Mello, F.C.A., Fernandes, C.A., Gomes, S.A., 2012. Antiviral therapy against chronic hepatitis B in Brazil: high rates of lamivudine resistance mutations and correlation with HBV genotypes. *Mem. Inst. Oswaldo Cruz.* 107, 317–325.
- Mello, F.C.A., Souto, F.J.D., Nabuco, L.C., Villela-Nogueira, C.A., Coelho, H.S.M., Franz, H.C.F., Saniya, J.C.P., Virgilio, H.A., Motta-Castro, A.R.C., Melo, M.M.M., Martins, R.M.B., Gomes, A.S., 2007. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol.* 7, 103.
- Motta-Castro, A.R.C., Martins, R.M.B., Araújo, N.M., Niel, C., Facholi, G.B., Lago, B.V., Mello, F.C.A., Gomes, S.A., 2008. Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Arch. Virol.* 153, 2197–2205.
- Palumbo, E., Scotti, G., Falco, G., Cibelli, D.C., Angarano, G., 2007. Prevalence of HBV genotypes in South American immigrants affected by HBV-related chronic active hepatitis. *Braz. J. Infect. Dis.* 11, 311–313.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Raimondi, S., Maisonneuve, P., Bruno, S., Mondelli, M.U., 2010. Is response to antiviral treatment influenced by hepatitis B virus genotype? *J. Hepatol.* 52, 441–449.
- Ribeiro, N.R.C., Campos, G.S., Angelo, A.L.D., Braga, E.L., Santana, N., Gomes, M.M.S., Pinho, J.R., De Carvalho, W.A., Lyra, L.G.C., Lyra, A.C., 2006. Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver Int.* 26, 636–642.
- Santos, A.D., Alvarado-Mora, M.V., Botelho, L., Vieira, D.S., Pinho, J.R., Carrilho, F.J., Honda, E.R., Salcedo, J.M., 2010. Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virol. J.* 7, 315.
- Scheibauer, H., El-Nageh, M., Diaz, S., Nick, S., Zeichhardt, H., Gunert, H.-P., Pinne, A., 2010. Performance evaluation of 70 hepatitis B virus (HBV) surface antigen (HBsAg) assays from around the world by a geographically diverse panel with an array of HBV genotypes and HBsAg subtypes. *Vox Sang.* 98, 403–414.
- Tanwar, S., Dusheiko, G., 2012. Is there any value to hepatitis B virus genotype analysis? *Curr. Gastroenterol. Rep.* 14, 37–46.
- Teles, S.A., Martins, R.M.B., Vanderborght, B., Stuyver, L., Gaspar, A.M., Yoshida, C.E.T., 1999. Hepatitis B virus: genotypes and subtypes in Brazilian hemodialysis patients. *Artif. Organs* 23, 1074–1078.
- Tonetto, P.A., Gonçalves, N.S.L., Fais, V.C., Viganí, A.G., Gonçalves, E.S.L., Feltrin, A., Gonçalves Jr., F.I., 2009. Hepatitis B virus: molecular genotypes and HBeAg serological status among HBV-infected patients in the Southeast of Brazil. *BMC Infect. Dis.* 9, 149.
- Valente, F., Lago, B.V., Castro, C.A.V., Almeida, A.J., Gomes, A.S., Soares, C.C., 2010. Epidemiology and molecular characterization of hepatitis B virus in Luanda, Angola. *Mem. Inst. Oswaldo Cruz.* 105, 970–977.
- Yu, H., Yuan, Q., Ge, S.-X., Wang, H.-Y., Zhang, Y.-L., Chen, Q.-R., Zhang, J., Chen, P.-J., Xia, N.-S., 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype “T”. *PLoS ONE* 5, 1–9.

- 23- Alvarado-Mora, Monica V, Botelho, Livia, Gomes-Gouvea, Michele S, de Souza, Vanda F, Nascimento, Maria C, Pannuti, Claudio S, Carrilho, Flair J, Pinho, Joao RR**
Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão, Brazil. Virology journal, 8: 415, 25 August 2011

RESEARCH

Open Access

Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão, Brazil

Mônica V Alvarado-Mora^{1*}, Livia Botelho¹, Michele S Gomes-Gouvêa¹, Vanda F de Souza², Maria C Nascimento², Claudio S Pannuti², Flair J Carrilho¹ and João RR Pinho¹

Abstract

Background: The Brazilian population is mainly descendant from European colonizers, Africans and Native Americans. Some Afro-descendants lived in small isolated communities since the slavery period. The epidemiological status of HBV infection in Quilombos communities from northeast of Brazil remains unknown. The aim of this study was to characterize the HBV genotypes circulating inside a Quilombo isolated community from Maranhão State, Brazil.

Methods: Seventy-two samples from Fredchal Quilombo community at Maranhão were collected. All serum samples were screened by enzyme-linked immunosorbent assays for the presence of hepatitis B surface antigen (HBsAg). HBsAg positive samples were submitted to DNA extraction and a fragment of 1306 bp partially comprising HBsAg and polymerase coding regions (S/POL) was amplified by nested PCR and its nucleotide sequence was determined. Viral isolates were genotyped by phylogenetic analysis using reference sequences from each genotype obtained from GenBank (n = 320). Sequences were aligned using Muscle software and edited in the SE-AL software. Bayesian phylogenetic analyses were conducted using Markov Chain Monte Carlo (MCMC) method to obtain the MCC tree using BEAST v.1.5.3.

Results: Of the 72 individuals, 9 (12.5%) were HBsAg-positive and 4 of them were successfully sequenced for the 1306 bp fragment. All these samples were genotype A1 and grouped together with other sequences reported from Brazil.

Conclusions: The present study represents the first report on the HBV genotypes characterization of this community in the Maranhão state in Brazil where a high HBsAg frequency was found. In this study, we reported a high frequency of HBV infection and the exclusive presence of subgenotype A1 in an Afro-descendent community in the Maranhão State, Brazil.

Keywords: Hepatitis B virus, Genotype A1, Quilombo community, Maranhão state, Brazil, Bayesian Analysis

Introduction

It is estimated that two billion people have been infected with Hepatitis B virus (HBV) and that more than 350 million are chronic carriers of this virus [1]. HBV strains have distinct geographical distribution and are classified into nine genotypes, A to I, on basis of genome diversity [2]. Genotype A is found mainly in North America and Africa and has been found in six genetically distinct

subgenotypes (A1 to A6) [3]. Genotypes B and C are prevalent in Southeast Asia and the Far East. Genotype D has a worldwide distribution and is found predominantly in the Mediterranean region and Central Asia. Genotypes E and F are prevalent in West Africa and in the Native American population, respectively [4]. In addition, genotype G has been reported in the USA, France [5], Colombia [6] and Brasil [7] and genotype H has been found in North and Central America [8]. Recently, by using phylogenetic analyses, a new genotype was characterized in Vietnam and Laos and designated as genotype I [2,9,10].

* Correspondence: monica.viviana@usp.br

¹Laboratory of Tropical Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, Brazil

Full list of author information is available at the end of the article

© 2011 Alvarado-Mora et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In Brazil, a wide variation of HBV infection prevalence was reported, particularly dependent upon the geographical region of this country [11]. Genotype A is the most prevalent and genotypes B, C, D and F are also circulating in the population [12-15]. The presence of these genotypes reflects the mixture of cultures in the Brazil: Native American, European and African ancestral roots, showing this country as an important model for studies of population genetics [16].

During the slavery period in Brazil (from XVI to XIX centuries), some African people managed to escape to refuge areas, living with others in well hidden places in the woods. These places were known as Quilombos and they were regions of large concentration of runaway-slaves, far from urban centers and located in areas with difficult access. Their inhabitants generally stayed in culturally isolated communities without significant additional admixture since their establishment. Quilombos were located in different Brazilian states around the country: Pará (PA), Maranhão (MA), Alagoas (AL), Pernambuco (PE), Bahia (BA), Goiás (GO), Mato Grosso do Sul (MS), Minas Gerais (MG), Rio de Janeiro (RJ) and São Paulo (SP) [17].

The quilombo Frechal is located in the municipality of Mirinzal, at the region of lower western Maranhão State, Brazil (Figure 1). The Frechal community was founded in the late XVIII century and was devoted to sugar production till the XIX century. The Frechal community is one of the oldest and most important Quilombos located in Maranhão State (http://www.cpisp.org.br/comunidades/html/brasil/ma/ma_comunidades_frechal.html, accessed at 11/01/2011).

The aim of this study was to analyze the presence of current HBV infection by HBsAg and HBV DNA detection in the current inhabitants of Frechal community and to determine which HBV genotypes are found among these cases.

Materials and methods

Study Population

Seventy-two samples from inhabitants from Frechal, Maranhão were collected from 2000 to 2001 (Figure 1). Ethical Committee of the University of São Paulo, Medical School, São Paulo, Brazil, approved this protocol. All patients have signed an informed consent form before the samples were collected for research. The samples were screened for HBsAg using commercially available kits (DiaSorin Ltda, Saluggia, Italy). HBsAg positive samples were submitted to PCR amplification to detect HBV DNA.

HBV DNA extraction

Viral nucleic acids (HBV DNA and HDV RNA) extraction was carried out from 100 µl of sera for each sample

using the acid guanidinium thiocyanate/phenol/chloroform method [18]. To avoid false-positive results, strict procedures for nucleic acid amplification diagnostic techniques were followed [19].

HBV PCR Amplification

Samples were first amplified with the primers described by Sitnik et al. [13] in order to obtain a 416 base pairs (bp) fragment partially covering the HBsAg coding region (S) to confirm the presence of HBV-DNA in the sample.

To characterize HBV genotypes, a fragment of 1306 bp partially comprising HBsAg and the polymerase coding regions (S/POL) of HBV genome was amplified by nested PCR using the primers PS3132F (3132 nt - 3151 nt)/2920R (1417 nt - 1398 nt) and PS3201F (3201 nt - 3221 nt)/P1285R (1285 nt - 1266 nt) [6].

HBV Nucleotide Sequencing

Amplified DNA was purified using ChargeSwitch® PCR Clean-Up Kit (Invitrogen, São Paulo, Brazil). Sequencing was performed in an ABI Prism® 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA), based on the protocol described by Sanger et al. [20],

Figure 1 Geographical localization of the Afro-Brazilian community in Maranhão State - Brazil (modified of http://pt.wikipedia.org/wiki/Ficheiro:Maranhao_Municip_Mirinzal.svg).

using dideoxy nucleotide triphosphates (ddNTPs) containing fluorescent markers (*Big Dye[®] Terminator v3.1 Cycle Sequencing Ready Reaction kit* - Applied Biosystems, Foster City, CA, USA). The quality of each electropherogram was evaluated using the Phred-Phrap software [21] and consensus sequences were obtained by using an alignment constructed with CAP3 software available at the web page *Electropherogram quality analysis* (<http://asparagin.cenargen.embrapa.br/phph/>).

HBV Genotyping Analysis

Sequences were assigned to HBV genotypes after phylogenetic analysis using reference sequences from each HBV genotype obtained from the GenBank ($n = 320$). These sequences comprised partial HBsAg and polymerase coding regions (S/POL). They were aligned using Muscle Software [22] and edited with the SE-AL software (available at <http://tree.bio.ed.ac.uk/software/seal/>). To perform the phylogenetic analysis, the missing nucleotides were coded as "missing characters" in nexus block. Bayesian phylogenetic analyses were done applying Markov Chain Monte Carlo simulation using BEAST v.1.5.3 [23], and 10 million generations were sufficient to obtain the convergence of parameters. The relaxed uncorrelated lognormal was the best molecular clock for the dataset. The maximum clade credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees and then it was removed 10% of burn-in using Tree Annotator v.1.5.3 [23].

Results

Of the 72 samples, 9 (12.5%) were HBsAg-positive. Since we did not have epidemiological data about this population, it was not possible to compare HBsAg results with other demographic information. Of these nine samples, six were positive by nested PCR for the S fragment (416 bp) and among them, 4 also amplified the S/POL region (1306 bp).

To perform the phylogenetic analysis, the longest fragment available from each sample was sequenced and classified as subgenotype A1 (subtype *adw2*). Although the four samples from Frechal were grouped in the same cluster in the tree, it cannot be assumed that there was a founder effect in this community because this group is supported only with a low posterior probability (0.10) (Figure 2). Sequences were deposited in GenBank at accession numbers: HM772994 - HM772997.

Discussion

This is the first study that characterized HBV genotypes present in an isolated community from Maranhão state, Brazil. Since the genotype A1 has been reported as a common genotype in African and Brazilian populations [12,13,24-28], the four samples were compared with

others previously reported sequences from Quilombos in Brazil [28] and Venezuela [29] and with sequences from Rio de Janeiro, as in this place there was a constant slave traffic from Africa that was most intense between 1795 to 1811 [30]. The subgenotype A1 sequences from Quilombos and Rio de Janeiro did not assemble in a single group in the tree. These results suggest the presence of the different A1 strains within the Quilombos populations. These variants may have come from Africa before these groups were created and actually the HBV/A1 strains continued to evolve in the Afro-descendant population after they came to Brazil.

A lot of Afro-descendants live in Bahia in Northeast Brazil. The geographic distributions of HBV genotypes have showed that genotype A (subtype *adw2*) is the most frequent in according to the ethnic background of the population [31]. It was reported a high prevalence of genotype A in Bahia but short-length sequences available prevented performing phylogenetic analysis together with the other ones analyzed in this study. Moreover, it is possible that different A1 variants have different African origin, as during the slave trade time, slaves from different countries from Africa were mixed on the boats and then were sold and distributed in different Brazilian regions [32].

We found that the three sequences from Afro-Venezuelan population [29] were classified as subgenotype A2 (Figure 3), which suggest different origin of HBV strains circulating in this population compared with African descendants in Brazil. Moreover, a specific polymorphism was found in HBV S region that distinguish subgenotypes A1 and A2 and it was agreed with HBV genotype A classification previously reported [33]. The synonymous substitutions at nucleotides in the third-positions: 201 nt (TCC → TCA) Serine [S]; 222 nt (CCC → CCA) Proline [P]; 324 nt (TCA → TCG) Serine [S]; 327 nt (TCT → TCC) Serine [S] and 462 nt (TAC → TAT) Tryptofan [W] were identified and they confirm the results of subgenotype classification (Figure 3).

Finally, we found a high frequency of HBsAg in the Frechal population (12.5%). Kalunga population, the largest Afro-Brazilian isolated community located in Goiás state, showed lower frequency for HBsAg (1.8% - 16/878). HBV subgenotype (A1) was also found in all the positive samples from Goiás [34].

Another study, with 1058 individuals living in 12 different isolated Afro-descendant communities, was carried out in Mato Grosso do Sul state and showed that among 1058 individuals, 23 (2.2%) of them were HBsAg positive. The highest prevalence was detected in Furnas dos Dionisios community (42.4% to anti-HBc and 7.4% to HBsAg) and the overall prevalence for HBV infection was 19.8% [35]. Subgenotype A1 was the most frequent

Figure 2 The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 320 S/POL sequences with 1306 bp of Hepatitis B virus strains. The posterior probabilities of the key nodes (internal nodes) are depicted above the respective nodes. Samples HBV/A1 obtained from Frechal ($n = 4$, red taxa) were analyzed together with other worldwide strains by Maximum Likelihood (ML) method ($n = 134$). The clusters containing the strains of other Afro-Brazilian communities previously reported are highlighted (Blue taxa). Genotype B ($n = 19$), genotype C ($n = 20$), genotype D ($n = 24$), genotype E ($n = 7$), genotype F ($n = 25$), genotype G ($n = 4$), genotype H ($n = 6$) and genotype I ($n = 9$) branches were collapsed. Also, the subgenotypes A2 ($n = 52$), A3, A4, and A5 ($n = 10$), A6 ($n = 3$) and A7 ($n = 7$) branches were collapsed.

in this state, followed by subgenotype A2 and genotype D [28].

HBsAg frequency in Afro-Venezuelan communities (3.6%) and in rural populations from Venezuela were higher than those found in Brazilian studies involving Quilombo inhabitants (2.2%) [29,35].

In Frechal, seventy-two samples were collected and this sample size probably did not represent the population but our data suggest a high frequency of HBV in this community. However, further studies are needed to better evaluate the epidemiology of hepatitis B in this region.

In this study, we reported a high frequency of HBV infection and the exclusive presence of subgenotype A1 in an Afro-descendant community in the Maranhão State, Brazil. In conclusion, HBV subgenotype A1 was found in all African descendant population from South

America studied so far except in Venezuela, where subgenotype A2 was found. Also, this study shows the need to report more HBV sequences from Afro-descendant communities to complement the actual data and the establishment of an accurate substitution rate for this virus to understand the evolutionary origins. The study of HBV sequences from other Afro-descendant communities from American countries, as well as from other different African countries, will allow a better understanding on HBV spreading between these two continents.

Acknowledgements

We are deeply indebted to Maria Claudia Nascimento and Laura Sumita for provide the samples for this study. This work has been supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP 2007/53457-7 and 2008/0461-6 and CNPq.

Author details

¹Laboratory of Tropical Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, Brazil. ²Laboratory of Virology, São Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases, School of Medicine, University of São Paulo, Brazil.

Authors' contributions

MVAM participated in the design of the study, conducted the phylogenetic and evolutionary analysis, drafted the manuscript and in its design and coordination. LB participated in the PCR amplification and sequencing process. MSGG participated in the PCR amplification. VFS, MON, CSP and FJC participated in the design of the study. JRRP participated in the design of the study and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 15 May 2011 Accepted: 25 August 2011
Published: 25 August 2011

References

- World Health Organization: Hepatitis B. Fact Sheet 204 (Revised August 2011). World Health Organization [http://www.who.int/mediacentre/factsheets/fs204/en/index.html].
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ, Xia NS: Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "F". *PLoS One* 2010, **5**:e9297.
- Pourkarim MR, Amini-Bavil-Olyaei S, Lemey P, Maes P, Van Ranst M: HBV subgenotype misclassification expands quasi-subgenotype A3. *Clin Microbiol Infect* 2011, **12**:947-949.
- Magnius LO, Norder H: Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 1996, **38**:34-34.
- Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *The Journal of general virology* 2000, **81**:67-74.
- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Botelho L, Carilho FJ, Pinho JR: Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases* 2011, **11**:103-108.
- Botteccia M, Souto FJ, O KM, Amendola M, Brandao CE, Niel C, Gomes SA: Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *BMC microbiology* 2008, **8**:11.
- Araújo-Núñez P, Norder H, Robertson BH, Magnius LO: Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *The Journal of general virology* 2002, **83**:2059-2073.
- Hannou C, Norder H, Lindh M: An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. *J Gen Virol* 2000, **81**:2267-2272.
- Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000, **81**:67-74.
- Pereira LM, Martelli CM, Merchan-Hamann E, Montaneros UR, Braga MC, de Lima ML, Cardoso MR, Turchi MD, Costa MA, de Alencar LC, et al: Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am J Trop Med Hyg* 2009, **81**:340-347.
- Araújo NM, Mello FC, Yoshida CF, Nild C, Gomes SA: High proportion of subgroup A' (genotype A) among Brazilian isolates of Hepatitis B virus. *Archives of virology* 2004, **149**:1383-1395.
- Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Carilho FJ: Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *Journal of clinical microbiology* 2004, **42**:2455-2460.
- Viana S, Parana R, Moreira RC, Compi AP, Macedo V: High prevalence of hepatitis B virus and hepatitis D virus in the western Brazilian Amazon. *The American journal of tropical medicine and hygiene* 2005, **73**:808-814.
- Santos AO, Alvarado-Mora MV, Botelho L, Veira DS, Pinho JR, Carilho FJ, Honda ER, Salcedo JM: Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virology journal* 2010, **7**:315.
- Pena SD, Di Pietro G, Fuchshuber-Morales M, Genro JP, Hutz MH, Kehdy Fde S, Kohlrausch F, Magno LA, Montenegro RC, Moraes MD, et al: The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. *PLoS One* 2011, **6**:e17063.
- Rapoport Delegation on Afro-Brazilian Land Rights: Between the Law and Their Land. *Afro-Brazilian Quilombo Communities' Struggle for land rights*. 2008, 2-57.
- Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry* 1987, **162**:156-159.
- Kwok S, Higuchi R: Avoiding false positives with PCR. *Nature* 1989, **339**:237-238.
- Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 1977, **74**:5463-5467.
- Ewing B, Hillier L, Wendt MC, Green P: Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome research* 1998, **8**:175-185.
- Edgar RC: MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC bioinformatics* 2004, **5**:113.
- Drummond AJ, Rambaut A: BEAST: Bayesian evolutionary analysis by sampling trees. *BMC evolutionary biology* 2007, **7**:214.
- Bowyer SM, van Staden L, Kew MC, Sim JG: A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *The Journal of general virology* 1997, **78**(Pt 7):1719-1729.
- Kramvis A, Kew MC: Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus. *Hepatology research: the official journal of the Japan Society of Hepatology* 2007, **37**:S27-32.
- Kimbi GC, Kramvis A, Kew MC: Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *The Journal of general virology* 2004, **85**:1211-1220.
- Mello FC, Souto FJ, Nabuco LC, Vilela-Nogueira CA, Coelho HS, Franz HC, Saraiva JC, Virgolino HA, Motta-Castro AR, Mello MM, et al: Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC microbiology* 2007, **7**:103.
- Motta-Castro AR, Martins RM, Araújo NM, Niel C, Facholi GB, Lago BV, Mello FC, Gomes SA: Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Archives of virology* 2008, **153**:2197-2205.
- Quintero A, Martinez Q, Alarcon De Noya B, Castagliola A, Urbina L, Gonzalez N, Liprandi F, Castro De Guerra D, Pupol FH: Molecular epidemiology of hepatitis B virus in Afro-Venezuelan populations. *Archives of virology* 2002, **147**:1829-1836.
- Alencastro LF: O trato dos viventes: formação do Brasil no Atlântico Sul. São Paulo: Companhia das Letras; 2000, 9-523.
- Ribeiro NR, Campos GS, Angelo AL, Braga EL, Santana N, Gomes MM, Pinho JR, De Carvalho WA, Lyra LG, Lyra AC: Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver International: official journal of the International Association for the Study of the Liver* 2006, **26**:636-642.
- Ribeiro D: O Povo Brasileiro: a formação e o sentido do Brasil. Companhia das Letras São Paulo; 1995.
- Kimbi GC, Kramvis A, Kew MC: Distinctive sequence characteristics of subgenotype A1 isolates of hepatitis B virus from South Africa. *J Gen Virol* 2004, **85**:1211-1220.
- Matos MA, Reis NR, Kozłowski AG, Teles SA, Motta-Castro AR, Mello FC, Gomes SA, Martins RM: Epidemiological study of hepatitis A, B and C in the largest Afro-Brazilian isolated community. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 2009, **103**:899-905.
- Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Paniago AM, Lima KM, Gomes SA: Hepatitis B virus infection in isolated Afro-Brazilian communities. *Journal of medical virology* 2005, **77**:188-193.

doi:10.1186/1743-422X-8-415

Cite this article as: Alvarado-Mora et al.: Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão, Brazil. *Virology Journal* 2011 **8**:415.

24- Barros, Lena Maria Fonseca, Gomes-Gouvêa, Michele Soares, Kramvis, Anna, Mendes-Corrêa, Maria Cássia Jacintho, Santos, Alexsandro Dos, Souza, Letícia Alana Barros, Santos, Max Diego Cruz, Carrilho, Flair José, Domicini, Arnaldo De Jesus, Pinho, João Renato Rebello, Ferreira, Adalgisa De Souza Paiva. High prevalence of hepatitis B virus subgenotypes A1 and D4 in Maranhão State, Northeast Brazil. Infection, Genetics and Evolution 2014 Jun;24:68-75

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

High prevalence of hepatitis B virus subgenotypes A1 and D4 in Maranhão state, Northeast Brazil

Lena Maria Fonseca Barros^{a,1}, Michele Soares Gomes-Gouvêa^{b,*,1}, Anna Kramvis^c, Maria Cássia Jacintho Mendes-Corrêa^d, Alexandro dos Santos^a, Letícia Alana Barros Souza^a, Max Diego Cruz Santos^a, Flair José Carrilho^b, Arnaldo de Jesus Domicini^a, João Renato Rebello Pinho^{b,2}, Adalgisa de Souza Paiva Ferreira^{a,2}

^a Federal University of Maranhão, São Luís, Maranhão, Brazil^b Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil^c Hepatitis Virus Diversity Research Programme, Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa^d Department of Infectious Diseases, Medical School, University of São Paulo, São Paulo, Brazil

ARTICLE INFO

Article history:

Received 9 January 2014

Received in revised form 21 February 2014

Accepted 10 March 2014

Available online 16 March 2014

Keywords:

Phylogenetics
Subgenotype A1
Subgenotype D4
Slave trade
Maranhão
Brazil

ABSTRACT

In this study, we determined the prevalence of HBV subgenotypes in Maranhão state, located in north-eastern Brazil, where the population is heterogeneous, with a high proportion of African descendants. HBV was detected in 119 of 133 (89.5%) chronic hepatitis B patients, including 103 (86.5%) who were HBeAg-negative. Using phylogenetic analysis of the S/Polymerase region of HBV DNA, subgenotype A1 was found to be the most prevalent (67%), followed by genotype D (28%); subgenotype D4 was detected in 24%, D3 in 3%, and D2 in 1%. Genotype F, clustering with subgenotype F2a, was found in six (5%) patients. The topology of the phylogenetic tree showed that HBV/A1 sequences did not cluster together, suggesting that more than one strain was introduced into Maranhão. On the other hand, HBV/D4 sequences formed a monophyletic cluster, suggesting a single entry of this strain in this population. This study showed that HBV/A1 was the only subgenotype of HBV/A present in the population from Maranhão and indicated that in this region HBV/A1 was not restricted to an Afro-descendant community where it was previously reported, but is widely distributed among general population of HBV chronic carriers.

Unexpectedly, we found a high frequency of HBV subgenotype D4. Together with previously reported data on the distribution of HBV/D4 in the world, these findings suggest that this subgenotype was more prevalent in the African continent in the past and may have been introduced in Maranhão by means of the slave trade during the late XVIII century, when the largest number of African slaves arrived to this region.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Globally, the prevalence of chronic hepatitis B (CHB) varies considerably, with areas of high (>8%), intermediate (2–7%) and low (<2%) endemicity (WHO, 2002). A recent epidemiological study, carried out among a representative sample of the Brazilian population showed an HBsAg prevalence of less than 1% in

Northeast and Central-West regions (Pereira et al., 2009). However, in other regions, including some areas of the Amazon, south and southwest regions, hepatitis B virus (HBV) infection is hyperendemic and poses a serious health threat (Bertolini et al., 2006; Braga et al., 2012; Souto, 1999).

Brazil is a very large country and in several areas the epidemiological picture of HBV infection is unknown, especially in smaller cities or rural communities, where large reservoirs of HBV infection may persist. Maranhão state is located in the northeastern region of Brazil, is divided in 217 counties (Fig. 1), and has a population of approximately 6,794,301 inhabitants (<http://www.ibge.gov.br/estadosat/perfil.php?sigla=ma>). Population from Maranhão is one of the most heterogeneous in the country, with a high proportion of African descendants. In the few epidemiological studies carried

* Corresponding author. Address: Laboratório de Gastroenterologia e Hepatologia Tropical, Instituto de Medicina Tropical, FMUSP, Av. Dr. Enéas Carvalho de Aguiar, 500, 05403-000 São Paulo, SP, Brazil. Tel.: +55 1130618218.

E-mail address: gomesmic@yahoo.com.br (M.S. Gomes-Gouvêa).

¹ These authors contributed equally to this work.

² These authors also contributed equally to this work.

<http://dx.doi.org/10.1016/j.meegid.2014.03.007>
1567-1348/© 2014 Elsevier B.V. All rights reserved.

Fig. 1. Geographic location of Maranhão state in Brazil.

out to estimate HBV prevalence in this state, was observed that it was variable, depending on the studied group: a low HBV prevalence (0.5–0.9%) was found in two studies among the urban population from São Luís, the capital of Maranhão state (Pereira et al., 2009; Souza et al., 2012); whereas an intermediate prevalence (2.8%) was found in a rural area (Buriticupu) (El Khouri et al., 2010) and a surprisingly high prevalence (12.5%) reported in an African descendant population from another rural area (Frechal) (Alvarado-Mora et al., 2011).

HBV is currently classified into ten genotypes (A–J) based on a difference in genomic sequence of 7.5% or more and some of them have been further classified into subgenotypes (Kramvis et al., 2008; Norder et al., 2004). Most HBV genotypes and some of their subgenotypes have characteristic geographic distribution around

the world and a strong correlation with ethnicity (Kramvis et al., 2008; Kurbanov et al., 2010; Norder et al., 2004). Moreover, genotypes/subgenotypes can affect the natural course of infection and response to antiviral treatment (Cooksley, 2010; Kramvis and Kew, 2005; Lin and Kao, 2011).

Knowledge of HBV genotypes/subgenotypes prevalence in a geographical region is also important to understand the routes of HBV dissemination and to establish efficient strategies to prevent and control HBV infection. Genotypes B and C have been related to vertical transmission while subgenotype A1 and genotypes D, E, F and H are more related to horizontal transmission during early childhood. HBV/A2 has been frequently involved in sexual transmission and is more prone to establish chronic disease in adults. Like HBV/A2, genotype G infection is associated with sexual

transmission as a risk factor for infection and is frequently found in men who have sex with men (Araújo et al., 2011).

Genotypes A, D and F are the HBV genotypes more prevalent in Brazil (Bertolini et al., 2012; Gomes-Gouveia et al., 2009; Mello et al., 2007; Moura et al., 2013; Santos et al., 2010; Sitnik et al., 2004; Victoria et al., 2008). Although studies in particular regions of the country have shown the predominance of HBV subgenotypes A1, D3 and F2a (Bertolini et al., 2012; Gomes-Gouveia et al., 2009; Mello et al., 2007; Moura et al., 2013; Santos et al., 2010; Victoria et al., 2008), this information remains poorly understood in most of Brazilian regions. Specifically in Maranhão state, there is just one study that characterized the distribution of HBV subgenotypes in an Afro-descendant community where only HBV/A1 was identified (Alvarado-Mora et al., 2011), however the extent of its distribution in this state is unknown.

The aim of this study was to characterize the HBV genotypes and subgenotypes in chronic hepatitis B carriers from different regions of Maranhão state and to relate this data with demographic and clinical features of the patients.

2. Patients and methods

2.1. Patients

The study population included 133 patients with chronic hepatitis B from Maranhão state, in the northeast of Brazil. These patients were followed up at the Center for Liver Studies of the University Hospital, Federal University of Maranhão (HUUFMA) at São Luís, the capital city of Maranhão state. The local ethics committee approved this study and written informed consent was obtained from all patients included.

Demographic, clinical and laboratory data of the patients were retrieved from their medical files. All patients were HBsAg positive, for at least 6 months prior to inclusion in the study, and tested negative for hepatitis C virus or human immunodeficiency virus antibodies. The serum samples were collected during January 2008 to February 2010.

Chronic hepatitis B status was defined according to the following criteria:

- (1) Inactive carrier: positive HBsAg, negative HBeAg, positive anti-HBe, normal alanine aminotransferase (ALT) levels and HBV DNA below 2000 IU/mL;
- (2) Chronic hepatitis B: positive HBsAg, positive HBeAg, and high ALT levels; or positive HBsAg, negative HBeAg, positive anti-HBe and HBV DNA higher than 2000 IU/mL;
- (3) Liver cirrhosis: positive HBsAg with positive or negative HBeAg regardless of the viral load; signs or symptoms of advanced liver disease (ascites and/or presence of esophageal varices) or histological diagnosis of liver cirrhosis;
- (4) Immune tolerance: positive HBsAg and HBeAg with normal ALT levels and HBV DNA above 10^7 IU/mL.

2.2. Serological assays

Serum samples were tested for hepatitis B markers (HBsAg, total anti-HBc, anti-HBs, HBeAg, anti-HBe) by enzyme-linked immunosorbent assay (ELISA) using commercial kits (DiaSorin, Italy).

2.3. HBV DNA amplification and quantification

HBV DNA was extracted from 200 µL serum using the QIAamp DNA Blood Mini kit (Qiagen). Viral load was quantified by real-time PCR assay as described previously (Sitnik et al., 2010). Absolute

quantification of HBV DNA was performed with an ABI PRISM 7300 (Life Technologies) real-time analyzer using universal cycling conditions. The limit of detection of this assay was established in 50 IU/mL, but it can detect samples with viral load below this value (Sitnik et al., 2010).

For genotype identification, a fragment of 1306 base pairs (bp), comprising the whole HBsAg and part of the Polymerase coding genes, was amplified by nested PCR. For this, we used the enzyme Platinum Taq DNA polymerase (Invitrogen) and the primers PS3132F (5' CCT CCY GCH TCY ACC AAT CG 3'; nt 3132–3151 from the EcoRI site) and 2920RM (5' ACG TCC CKC GHA GRA TCC AG 3'; nt 1417–1398) for the first-round PCR, and PS3201F (5' CAY CCH CAG GCM ATG CAG TGG 3'; nt 3201–3221) and P1285R (5' CWA GGA GTT CCG CAG TAT GG 3'; nt 1285–1266) for the second round. PCR was carried out for 35 cycles of 94 °C for 30 s, 48 °C (first round) or 54 °C (second round) for 30 s, and 72 °C for 1 min and 40 s.

PCR products from the second-round reaction (nested PCR) were purified using Charge Switch® PCR Clean-Up Kit (Invitrogen) and were then sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Three inner and partially overlapping sequences were obtained in sense and antisense directions using three sets of primers: 1 – PS3201F and HBV477R (5' GGA CAV ACG GGC AAC ATA CCT T 3'; nt 477–456); 2 – L372 (5' – TCG YTG GAT GTR TCT GCG GCG TTT TAT – 3'; nt 370–396) and RADE2M (5' – TGR CAN ACY TTC CAR TCA ATN GG – 3'; nt 989–970); 3 – P781F (5' GAR TCC CTT TWT RCC KCT RTT ACC 3'; nt 781–804), and P1285R. Sequencing was carried out on an automated ABI 3500 DNA Sequencer (Applied Biosystems).

The quality of each electropherogram was evaluated using the Phred-Phrap software (Ewing and Green, 1998; Ewing et al., 1998) and consensus sequences were obtained from the alignment of these six sequences, for each single patient, using CAP3 software available at the web page <http://asparagin.cenargen.embrapa.br/phph/>.

2.4. HBV genotyping and subgenotyping

Sequences were aligned and edited using BioEdit (v. 7.0.8) and the integrated CLUSTAL W program. HBV genotypes and subgenotypes were classified by phylogenetic reconstructions using published reference sequences from the GenBank database (<http://www.ncbi.nlm.nih.gov/>).

Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.6.1 (Drummond and Rambaut, 2007) under uncorrelated lognormal relaxed molecular clock using the model of nucleotide substitution (GTR + G + I). The MCMC chains were run for 10 million generations, and sampled every 1000 steps. The maximum clade credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees and then 10% of burn-in was removed using Tree Annotator v.1.6.1 (Drummond and Rambaut, 2007).

HBV serotypes were deduced based on the presence of amino acids at positions 122, 127, 134 and 160 of S protein coding by S gene sequence: adw2 (Lys¹²², Pro¹²⁷ and Lys¹⁶⁰); ayw2 (Arg¹²², Pro¹²⁷, Tyr¹³⁴ and Lys¹⁶⁰); ayw3 (Arg¹²², Thr¹²⁷, Tyr¹³⁴ and Lys¹⁶⁰); adw4 (Lys¹²², Leu¹²⁷ and Lys¹⁶⁰) (Norder et al., 2004).

2.5. Nucleotide sequence accession numbers

The GenBank/EMBL/DBJ accession numbers for the sequences described in this study are JF298901, JF298903, JN983831–JN983947 for S/POL sequences and KJ470884–KJ470898 for complete genome sequences.

2.6. Statistical analysis

Statistical analyses to compare subgenotypes and different variables were performed. HBV/F2a, D2 and D3 subgenotypes were excluded from the statistical analysis because there were too few cases. Qualitative variables (gender and chronic HBV infection stage) were compared using Chi square test, t-test and Mann–Whitney were used to determine for significant differences in age and viral load values between subgenotype A1 and D4 groups, respectively. Differences were considered significant for p values less than 0.05. The statistical analysis software used was SPSS software, version 17.0.

3. Results

3.1. Clinical and demographic characteristics of HBV DNA positive patients

Out of the 133 patients included in this study, 119 (89%) were HBV DNA positive: 53% were male with mean age 40.5 (SD = 13) years. Considering the geographical origin inside Maranhão state, most of them (76%; 90/119) were from São Luís metropolitan area, which includes four cities: São Luís (capital of the state), São José de Ribamar, Raposa, and Paço do Lumiar. The remaining patients (24%; 28/119) were from 24 different small cities located in other areas of the state.

HBeAg-negative patients were predominant (86.5%; 103/119) and most of these (85%; 88/103) had viral loads varying from <2000 to 20,000 IU/mL (mean 2,565 IU/mL; SD 3857). On the other hand, higher viral loads (>20,000 IU/mL) were predominantly found in HBeAg-positive patients. The majority of the patients were classified as inactive carriers (46%; 55/119) or as chronic hepatitis (33%; 39/119).

3.2. HBV genotype and subgenotype distribution

Genotype A was the most prevalent (67%; 80/119), followed by genotypes D (28%; 33/119) and F (5%; 6/119). The phylogeny of genotype A sequences showed that all strains belonged to subgenotype A1, but grouped in different clusters of the HBV/A1 branch. HBV/A1 sequences from Maranhão mainly grouped together with sequences isolated from other regions of Brazil, but only some clusters were well supported (Fig. 2). All HBV/A1 strains were classified as serotype *adw2*.

HBV/D4 was the most frequent subgenotype of genotype D from Maranhão (24%; 29/119). Subgenotypes D2 (*ayw2*) and D3 (*ayw2/ayw3*) were found in a few cases with a frequency of 0.8% (1/119) and 2.4% (3/119), respectively (Fig. 3a and Supplementary Fig. S1). The HBV/D4 (*ayw2*) sequences isolated in Maranhão grouped in a monophyletic cluster with strong support (a posterior probability = 0.93) and clustered closer to HBV/D4 strains previously isolated in other Brazilian regions, but more distantly to other HBV/D4 strains from other countries. To confirm the subgenotype classification of the D4 isolates from Maranhão state, the complete genomes of fifteen isolates were sequenced and the phylogenetic analysis of the complete genomes showed similar grouping to that of S/POL genes (Fig. 3b). Moreover, the genetic diversity based on complete genome was less than 4.0% compared to previously characterized HBV/D4, confirming their subgenotype classification as D4.

HBV/F2a (*adw4*) was the unique subgenotype of genotype F found in Maranhão state. Most sequences clustered together with other F2a sequences previously isolated from two other Brazilian regions: Rondônia and Amazonas states (Supplementary Fig. S1).

3.3. HBV subgenotypes and demographic, clinical and laboratory characteristics of the patients

Table 1 shows HBV subgenotype distribution of 119 patients according gender, age, race (based in skin color), geographical origin, HBeAg profile, clinical status and HBV DNA levels. Overall, subgenotypes A1 and D4 comprised 92% of study population and a statistical comparison of demographic (gender and age), clinical and laboratory data of these patients was performed. Patients infected with HBV/D4 presented a higher frequency of HBeAg-positive status than those infected with HBV/A1 [8/29 (28%) vs. 8/80 (10%), $p = 0.02$] and were more frequently found in the immune tolerance stage of chronic HBV infection [7/29 (24%) vs. 3/80 (4%), $p = 0.003$]. There was no significant difference in gender, age and viral load median.

4. Discussion

This study determined the distribution of HBV genotypes and subgenotypes in Maranhão state. This state is the fourth most populous state of the Northeast region and its population is one of the most heterogeneous in Brazil, with a high proportion of African descendants, who live in isolated or semi-isolated rural communities.

The HBV genotype distribution in Maranhão state was similar to that reported in other Brazilian regions, showing a higher prevalence of genotype A, followed by genotypes D and F (Bertolini et al., 2012; Gomes-Gouveia et al., 2009; Mello et al., 2007; Moura et al., 2013; Santos et al., 2010; Sitnik et al., 2004; Victoria et al., 2008). However, molecular characterization at the subgenotype level revealed a relatively high prevalence of subgenotypes A1 and D4. It is noteworthy, that the population analyzed in this study was representative of HBV carriers from whole Maranhão state because the patients were recruited from a liver disease referral center that monitors the entire state.

To date only two subgenotypes of genotype A (A1 and A2) have been found in Brazil and in Brazilian regions, where data is available, HBV/A1 has been the single or most prevalent subgenotype of genotype A (Bertolini et al., 2012; Gomes-Gouveia et al., 2009; Mello et al., 2007; Moura et al., 2013; Santos et al., 2010; Victoria et al., 2008). The present study showed that under the period of study only HBV/A1 was circulating in Maranhão state and was the most prevalent (67%) subgenotype in this state. This result indicates that in Maranhão HBV/A1 was not restricted to an Afro-descendant community, where it was previously reported (Alvarado-Mora et al., 2011), but was widely distributed among the general population of HBV chronic carriers.

Phylogeographic studies of HBV sequences from different countries suggest that genotypes/subgenotypes can be used to trace human migrations (Kramvis and Paraskevis, 2013). Because of the presence of HBV/A1 in some regions of Africa and its distribution among Brazilian communities that are formed mostly by descendants of African slaves (Quilombos), it has been postulated that HBV/A1 was introduced in Brazil by African slaves, who arrived in Brazil between the 16th and 19th centuries (Alvarado-Mora et al., 2011; Araujo et al., 2004; Motta-Castro et al., 2008).

Brazil is a country with continental dimensions and different regions have diverse population histories, cultural habits and environmental characteristics. These features can influence the HBV genotype/subgenotype distribution and/or the spread of a specific HBV strain. Therefore it is important not to only know the genotype/subgenotype distribution in each region but also to study the phylogenetic relationship among the isolates identified.

The clustering pattern of HBV/A1 sequences from Maranhão state suggests different entries of this subgenotype into the state.

The phylogenetic analysis shows that HBV/A1 sequences isolated from other regions of Brazil also have this characteristic. This pattern may be a result of the introduction of different strains by slaves originating from diverse African regions or may be as a result of internal migration of infected slaves in Brazil (Klein, 1986). On the other hand, a recent study shows that HBV/A1 sequences, isolated in Pernambuco, another state in Northeastern Brazil, have a different phylogenetic grouping pattern, indicating that most of HBV/A1, introduced in this region, came from a common strain (Moura et al., 2013).

HBV genotype D, the second most common (28%) genotype found in Maranhão, is highly prevalent in southern Brazil and in some southeastern areas (Bertolini et al., 2012; Mello et al., 2007; Sitnik et al., 2004). This genotype has been observed throughout the world, but it predominates in northeastern Europe, the Mediterranean basin, northern Africa, and the Middle East (Schaefer, 2007; Yousif and Kramvis, 2013). The presence of genotype D in Brazil, especially in southern and in southeastern regions has been mainly attributed to the European migration that occurred during the years 1872–1975 (Bertolini et al., 2012; Mello et al., 2007).

In Maranhão state, three subgenotypes of genotype D were found, D2, D3 and D4. Unexpectedly subgenotype D4 was found in 88% of the cases harboring genotype D, whereas D1 and D2 were found in 3% and 9%, respectively. Subgenotype D4 has previously not been found frequently in Brazil (Mello et al., 2007). Subgenotype D4 has been identified in only a few cases in the Amazonas (Gomes-Gouveia et al., 2009) and Rondônia (Santos et al., 2010), states in the Amazon region of Brazil.

The occurrence of the few cases of HBV/D2 and D3 in Maranhão may be attributed to the internal migration of carriers from other Brazilian regions, where these subgenotypes are prevalent, as occurred in some regions of the Central West and North of the country (Mello et al., 2007; Santos et al., 2010; Souto, 2004). On the other hand, the presence of HBV/D4 in Maranhão is not as a result of European immigration because this subgenotype has not been found in Europe (Schaefer, 2007). Subgenotype D4 is not very common, with a few sequences identified from patients in Australia, Papua New Guinea (Norder et al., 2004), Rwanda (Hubschen et al., 2009) and Canada (Osiowy et al., 2011). In Haiti, HBV/D4 was found as the most frequent subgenotype of genotype D, corresponding to 80% (29/36) of samples classified as HBV/D (36/179) (Andernach et al., 2009) and to 57% (4/7) in another study (Brichler et al., 2013). The presence of HBV/D4 in two different regions in Latin America might reflect its dispersal as a result of the slave trade routes from Africa to Haiti and Brazil (Silva, 2008). The possible African origin of HBV/D4 circulating in Maranhão is supported by the fact that we found an African genotype of hepatitis delta virus (HDV-8) co-infecting two of the patients who were HBV/D4 carriers (Barros et al., 2011). Although currently HBV/D4 has not been reported frequently in Africa, having been described only in Rwanda (Hubschen et al., 2009), it is possible that this subgenotype was distributed in different regions of the African continent in the past. A recombinant of subgenotype D4 has been identified in the Sudan (Yousif et al., 2013). It is possible that D4 originated in Africa but has subsequently been replaced by other subgenotypes of D and the recombinant is a remnant of the original strain/s (Yousif et al., 2013). The fact that HBV/D4 is found in aboriginal populations in Papua New Guinea and Australia (Norder et al., 2004) and in the Canadian Inuit population (Osiowy et al., 2011) suggests that this is an early subgenotype dating from the time of the early human intercontinental migrations (Hudjashov et al., 2007). The HBV/D4 sequences from Maranhão grouped in a specific monophyletic cluster (with high posterior probability), indicating that a single virus entered this population, establishing a founder effect. It is possible that subgenotype D4 has a wider

global distribution and more research is required, especially in Africa, the Americas and Australasia. Moreover, the effect of subgenotype D4 on clinical manifestation has not been researched. In this study we observed that among HBV/D4 carriers the frequency of immune tolerant status was higher than among HBV/A1 carriers. Since there is not a statistically significant difference between the mean age of HBV/A1 and D4 carriers (42 ± 12 vs. 38 ± 13 , $p = 0.13$), HBsAg seroconversion to anti-HBe was a late event in cases of HBV/D4 infection and we may infer that perinatal transmission of the virus occurred in these cases. Studies with larger numbers of individuals infected with this subgenotype are required to confirm this observation.

As expected, the frequency of genotype F in Maranhão was low, corresponding to 5% of the cases. Accordingly, the prevalence of this genotype is low in most of the Brazilian regions and increases in the western Amazon region (Bertolini et al., 2012; Gomes-Gouveia et al., 2009; Mello et al., 2007; Santos et al., 2010; Sitnik et al., 2004; Victoria et al., 2008). Genotype F is considered indigenous to America because it has been found among Amerindians, mainly in South America, and is a most prevalent genotype in some countries of this region (Alvarado Mora et al., 2011; Devesa et al., 2008; Mello et al., 2007; Nakano et al., 2001; Pezzano et al., 2011; Venegas et al., 2008). The low prevalence of HBV genotype F in most of regions of Brazil may be related to a lower contribution of Amerindians to the formation of the Brazilian population, as demonstrated by genomic ancestry studies (Pena et al., 2011).

5. Conclusions

This study shows the distribution of HBV genotypes and subgenotypes in Maranhão state. In this region, the prevalence of HBV genotypes is similar to those reported in other Brazilian regions, where this was studied, with a predominance of genotypes A and D, and a lower prevalence of genotype F. However, the subgenotype distribution shows some specific characteristics, with a high frequency of HBV subgenotype D4 that is uncommon in other regions of Brazil. The great genetic variability of HBV strains circulating in Brazil needs to be taken into account when national guidelines, on how to treat patients with chronic hepatitis B, are formulated.

Acknowledgments

This work was supported by São Paulo Research Foundation (FAPESP) – grant 2010/50081-9 and 2010/51208-2; Fundação de Amparo à Pesquisa do Estado do Maranhão – FAPEMA (APP-2009/00950) and Secretaria de Saúde do Estado do Maranhão – Departamento de Vigilância Epidemiológica.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2014.03.007>.

References

- Alvarado Mora, M.V., Romano, C.M., Gomes-Gouveia, M.S., Gutierrez, M.F., Botelho, L., Carrilho, F.J., Pinho, J.R., 2011. Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect. Genet. Evol.* 11, 103–108.
- Alvarado-Mora, M.V., Botelho, L., Gomes-Gouveia, M.S., de Souza, V.F., Nascimento, M.C., Pannuti, C.S., Carrilho, F.J., Pinho, J.R., 2011. Detection of Hepatitis B virus subgenotype A1 in a Quilombo community from Maranhão. *Brazil. Virol. J.* 8, 415.
- Andernach, L.E., Nolte, C., Pape, J.W., Muller, C.P., 2009. Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg. Infect. Dis.* 15, 1222–1228.

- Araújo, N.M., Mello, F.C., Yoshida, C.F., Niel, C., Gomes, S.A., 2004. High proportion of subgroup A' (genotype A) among Brazilian isolates of Hepatitis B virus. *Arch. Virol.* 149, 1383–1395.
- Araújo, N.M., Waizbort, R., Kay, A., 2011. Hepatitis B virus infection from an evolutionary point of view: how viral, host, and environmental factors shape genotypes and subgenotypes. *Infect. Genet. Evol.* 11, 1199–1207.
- Barros, L.M., Gomes-Gouveia, M.S., Pinho, J.R., Alvarado-Mora, M.V., Dos Santos, A., Mendes-Correa, M.C., Caldas, A.J., Sousa, M.T., Santos, M.D., Ferreira, A.S., 2011. Hepatitis Delta virus genotype 8 infection in Northeast Brazil: inheritance from African slaves? *Virus Res.* 160, 333–339.
- Bertolini, D.A., Pinho, J.R., Saraceni, C.P., Moreira, R.C., Granato, C.F., Carrilho, F.J., 2006. Prevalence of serological markers of hepatitis B virus in pregnant women from Paraná State, Brazil. *Braz. J. Med. Biol. Res.* 39, 1083–1090.
- Bertolini, D.A., Gomes-Gouveia, M.S., Guedes de Carvalho-Mello, I.M., Saraceni, C.P., Sitnik, R., Grazzini, F.G., Laurino, J.P., Fagundes, N.J., Carrilho, F.J., Pinho, J.R., 2012. Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil. *Infect. Genet. Evol.* 12, 1295–1304.
- Braga, W.S., Castilho Mda, C., Borges, F.G., Martins, A.C., Rodrigues, L.S., Azevedo, E.P., Scazufca, M., Menezes, P.R., 2012. Prevalence of hepatitis B virus infection and carriage after nineteen years of vaccination program in the Western Brazilian Amazon. *Rev. Soc. Bras. Med. Trop.* 45, 13–17.
- Brichler, S., Lagathu, G., Chelkova, M.A., Le Gal, F., Edouard, A., Deny, P., Césaire, R., Gordien, E., 2013. African, Amerindian and European hepatitis B virus strains circulate on the Caribbean Island of Martinique. *J. Gen. Virol.* 94, 2318–2329.
- Cookley, W.G., 2010. Do we need to determine viral genotype in treating chronic hepatitis B? *J. Viral Hepat.* 17, 601–610.
- Devesa, M., Loureiro, C.L., Rivas, Y., Monsalve, F., Cardona, N., Duarte, M.C., Poblete, F., Gutierrez, M.F., Botto, C., Pujol, F.H., 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J. Med. Virol.* 80, 20–26.
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7, 214.
- El Khouri, M., Cordeiro, Q., Luz, D.A., Duarte, L.S., Gama, M.E., Corbett, C.E., 2010. Endemic hepatitis B and C virus infection in a Brazilian Eastern Amazon region. *Arq. Gastroenterol.* 47, 35–41.
- Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* 8, 186–194.
- Ewing, B., Hillier, L., Wendt, M.C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res.* 8, 175–185.
- Gomes-Gouveia, M.S., Soares, M.C.P., Bensabath, G., de Carvalho-Mello, I.M.V.G., Brito, E.M.F., Souza, O.S.C., Queiroz, A.T.J., Carrilho, F.J., Pinho, J.R., 2009. Hepatitis B virus and hepatitis delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the western Brazilian Amazon region. *J. Gen. Virol.* 90, 2638–2643.
- Hubschen, J.M., Mugabo, J., Peltier, C.A., Karasi, J.C., Sausy, A., Kirpach, P., Arendt, V., Müller, C.P., 2009. Exceptional genetic variability of hepatitis B virus indicates that Rwanda is east of an emerging African genotype E/A1 divide. *J. Med. Virol.* 81, 435–440.
- Hudjashov, G., Kivisild, T., Underhill, P.A., Endicott, P., Sanchez, J.J., Lin, A.A., Shen, P., Oefner, P., Renfrew, C., Villems, R., Forster, P., 2007. Revealing the prehistoric settlement of Australia by Y chromosome and mtDNA analysis. *Proc. Natl. Acad. Sci. USA* 104, 8726–8730.
- Klein, H.S., 1986. Slavery and the Plantation Economy in Brazil and the Guyanas in the 19th Century. African Slavery in Latin America and the Caribbean. Oxford University Press, New York, p. 123.
- Kramvis, A., Kew, M.C., 2005. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. *J. Viral Hepat.* 12, 456–464.
- Kramvis, A., Paraskevis, D., 2013. Subgenotype A1 of HBV – tracing human migrations in and out of Africa. *Antivir. Ther.* 18, 513–521.
- Kramvis, A., Arakawa, K., Yu, M.C., Nogueira, R., Stram, D.O., Kew, M.C., 2008. Relationship of serological subtype, basic core promoter and precore mutations to genotypes/subgenotypes of hepatitis B virus. *J. Med. Virol.* 80, 27–46.
- Kurbanov, F., Tanaka, Y., Mizokami, M., 2010. Geographical and genetic diversity of the human hepatitis B virus. *Hepatol. Res.* 40, 14–30.
- Lin, C.L., Kao, J.H., 2011. The clinical implications of hepatitis B virus genotype: recent advances. *J. Gastroenterol. Hepatol.* 26 (Suppl 1), 123–130.
- Mello, F.C., Souto, F.J., Nabuco, L.C., Villela-Nogueira, C.A., Coelho, H.S., Franz, H.C., Saraiva, J.C., Virgolino, H.A., Motta-Castro, A.R., Melo, M.M., Martins, R.M., Gomes, S.A., 2007. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol.* 7, 103.
- Motta-Castro, A.R., Martins, R.M., Araújo, N.M., Niel, C., Facholi, G.B., Lago, R.V., Mello, F.C., Gomes, S.A., 2008. Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Arch. Virol.* 153, 2197–2205.
- Moura, L.F., Lopes, E.P., Alvarado-Mora, M.V., Pinho, J.R., Carrilho, F.J., 2013. Phylogenetic analysis and subgenotypic distribution of the hepatitis B virus in Recife, Brazil. *Infect. Genet. Evol.* 14, 195–199.
- Nakano, T., Lu, L., Hu, X., Mizokami, M., Orito, E., Shapiro, C., Hadler, S., Robertson, B., 2001. Characterization of hepatitis B virus genotypes among Yucpa Indians in Venezuela. *J. Gen. Virol.* 82, 359–365.
- Norder, H., Courouce, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., Magnius, L.O., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 47, 289–309.
- Oslowy, C., Larke, B., Giles, E., 2011. Distinct geographical and demographic distribution of hepatitis B virus genotypes in the Canadian Arctic as revealed through an extensive molecular epidemiological survey. *J. Viral Hepat.* 18, e11–e19.
- Pena, S.D., Di Pietro, G., Fuchshuber-Moraes, M., Genro, J.P., Hutz, M.H., Kehdy Fde, S., Kohlrausch, F., Magno, L.A., Montenegro, R.C., Moraes, M.O., de Moraes, M.E., de Moraes, M.R., Ojopi, E.B., Perini, J.A., Racciopi, C., Ribeiro-Dos-Santos, A.K., Rios-Santos, F., Romano-Silva, M.A., Sortica, V.A., Suarez-Kurtz, G., 2011. The genomic ancestry of individuals from different geographical regions of Brazil is more uniform than expected. *PLoS One* 6, e17063.
- Pereira, L.M., Martelli, C.M., Merchan-Hamann, E., Montarroyos, U.R., Braga, M.C., de Lima, M.L., Cardoso, M.R., Turchi, M.D., Costa, M.A., de Alencar, L.C., Moreira, R.C., Figueiredo, G.M., Ximenes, R.A., 2009. Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am. J. Trop. Med. Hyg.* 81, 240–247.
- Pezzano, S.C., Torres, C., Fainboim, H.A., Bouzas, M.B., Schroder, T., Giuliano, S.F., Paz, S., Alvarez, E., Campos, R.H., Mbayed, V.A., 2011. Hepatitis B virus in Buenos Aires, Argentina: genotypes, virological characteristics and clinical outcomes. *Clin. Microbiol. Infect.* 17, 223–231.
- Santos, A.O., Alvarado-Mora, M.V., Botelho, L., Vieira, D.S., Pinho, J.R., Carrilho, F.J., Honda, E.R., Salcedo, J.M., 2010. Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virol. J.* 7, 315.
- Schaefer, S., 2007. Hepatitis B virus genotypes in Europe. *Hepatol. Res.* 37, S20–S26.
- Silva, D.B.D., 2008. The Atlantic Slave Trade to Maranhão, 1680–1846: Volume, Routes and Organisation. *Slavery Abolit.* 29, 477–501.
- Sitnik, R., Pinho, J.R., Bertolini, D.A., Bernardini, A.P., Da Silva, L.C., Carrilho, F.J., 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J. Clin. Microbiol.* 42, 2455–2460.
- Sitnik, R., Paes, A., Mangueira, C.P., Pinho, J.R., 2010. A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev. Inst. Med. Trop. São Paulo* 52, 119–124.
- Souto, F.J.D., 1999. Distribution of hepatitis B in Brazil: update of the epidemiologic mapping and proposals for its control. *GED: Gastroenterol. Endoscopia Dig.* 18, 143–150.
- Souto, F.J., 2004. Hepatitis B and the human migratory movements in the State of Mato Grosso, Brazil. *Rev. Soc. Bras. Med. Trop.* 37 (Suppl 2), 63–68.
- Souza, M.T., Pinho, T.L., Santos, M.D., Santos, A., Monteiro, V.I., Fonseca, L.M., Ferreira, P.A., Ferreira, A.S., 2012. Prevalence of hepatitis B among pregnant women assisted at the public maternity hospitals of São Luís, Maranhão, Brazil. *Braz. J. Infect. Dis.* 16, 517–520.
- Venegas, M., Munoz, G., Hurtado, C., Alvarez, L., Velasco, M., Villanueva, R.A., Brahm, J., 2008. Prevalence of hepatitis B virus genotypes in chronic carriers in Santiago, Chile. *Arch. Virol.* 153, 2129–2132.
- Victoria, F.d.S., Oliveira, C.M., Victoria, M.B., Victoria, C.B., Ferreira, L.C., 2008. Characterization of HBeAg-negative chronic hepatitis B in western Brazilian Amazonia. *Braz. J. Infect. Dis.* 12, 27–37.
- World Health Organization (WHO). 2002. Global Alert and Response.**
- Yousif, M., Kramvis, A., 2013. Genotype D of hepatitis B virus and its subgenotypes: An update. *Hepatol. Res.* 43, 355–364.
- Yousif, M., Mudawi, H., Bakht, S., Glebe, D., Kramvis, A., 2013. Molecular characterization of hepatitis B virus in liver disease patients and asymptomatic carriers of the virus in Sudan. *BMC Infect. Dis.* 13, 328.

- 25- Alcione O Santos, Monica V Alvarado Mora, Livia Botelho, Deusilene S Vieira, João R Rebello Pinho, Flair J Carrilho, Eduardo R Honda, Juan M Salcedo. Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. Virology Journal 2010, 7:315**

RESEARCH

Open Access

Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondônia, Brazil

Alcione O Santos¹, Mônica V Alvarado-Mora^{2*}, Livia Botelho², Deusilene S Vieira¹, João R Rebello Pinho², Flair J Carrilho², Eduardo R Honda¹, Juan M Salcedo¹

Abstract

Background: Hepatitis B virus (HBV) can be classified into nine genotypes (A-I) defined by sequence divergence of more than 8% based on the complete genome. This study aims to identify the genotypic distribution of HBV in 40 HBsAg-positive patients from Rondônia, Brazil. A fragment of 1306 bp partially comprising surface and polymerase overlapping genes was amplified by PCR. Amplified DNA was purified and sequenced. Amplified DNA was purified and sequenced on an ABI PRISM® 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were aligned with reference sequences obtained from the GenBank using Clustal X software and then edited with Se-AI software. Phylogenetic analyses were conducted by the Markov Chain Monte Carlo (MCMC) approach using BEAST v.1.5.3.

Results: The subgenotypes distribution was A1 (37.1%), D3 (22.8%), F2a (20.0%), D4 (17.1%) and D2 (2.8%).

Conclusions: These results for the first HBV genotypic characterization in Rondônia state are consistent with other studies in Brazil, showing the presence of several HBV genotypes that reflects the mixed origin of the population, involving descendants from Native Americans, Europeans, and Africans.

Background

Human hepatitis B virus (HBV), which is the prototype member of the family *Hepadnaviridae*, is a circular, partially double stranded DNA virus of approximately 3200 nt [1]. This highly compact genome contains four major open reading frames encoding the envelope (preS1, preS2 and surface antigen - HBsAg), polymerase (HBpPol) and X (HBx) proteins [2]. HBV infection is a relevant global health problem, with 2 billion people infected worldwide, including 350 million of them suffering from chronic HBV infection. HBV infection results in 500,000 to 1.2 million deaths per year caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma and is the 10th leading cause of death worldwide [3]. The mechanisms for persistent HBV infection are not fully understood, but they seem to involve several aspects, including genetic components [4]. The role of genetics components of the virus and the host in the natural history of hepatitis B

including HBV genotypes and subgenotypes; basal core promoter and pre core mutations; HBV DNA serum levels and co-infection with other viruses (particularly hepatitis C and human immunodeficiency viruses) have been recently reviewed [5].

HBV has been classified into nine different genotypes, designated from A to I [6], they that represent genetically stable viral populations that share a common, separate evolutionary history. They emerged in specific human populations and migrated with their hosts to other areas in the world, leading to their present geographical distribution [7]. Genotype A is distributed globally and is the main genotype found in Europe, North America, Africa and India. Genotypes B and C are predominant in East and Southeast Asia [8]. Genotype D is mainly found in the Middle East and Mediterranean countries but it has been reported globally, whereas genotype E seems to be predominant in western-sub-Saharan Africa [9,10]. HBV/E has not been found outside Africa, except for a few rare cases mostly in individuals with an African background. Nevertheless, it was recently reported the presence of this genotype in a specific community in Colombia [11] and in the north of India [12]. Genotype

* Correspondence: monica.viviana@usp.br

²Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo SP, Brazil
Full list of author information is available at the end of the article

© 2010 Santos et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

G has been characterized in samples from USA, Mexico and France and appears primarily to be present as a coinfection with another HBV genotypes, most commonly genotype A. Genotypes F and H are found almost exclusively in Central and South America [13,14]. Recently, HBV genotype I was described in northwestern China, Vietnam and Laos [6,15,16].

Most genotypes have been divided into subgenotypes with distinct virological and epidemiological properties. In addition, recombination among HBV genotypes increases the viral variability itself [17].

Genotype A is subdivided into seven subgenotypes (A1 to A7) [18,19]. Isolates belonging to subgroup A1 have been mostly identified in African populations and their descendants [20-22]. Subgenotype A2 is mainly found among Europeans, whereas subgenotype A3 has been identified in Central and West Africa [23,24]. Subgenotype A4 was reported in Gambia [18,22] and subgenotype A5 was reported in Nigeria and among African descendants in Haiti [25]. Subgenotype A6 includes strains from African-Belgian patients from Congo and Rwanda [26] and A7 was found in Rwanda and Cameroon [19].

Genotype D was previously divided in 4 subgenotypes (D1 - D4) [27] found in different continents, spreading particularly around the Mediterranean Basin to the Asian continent. New subgenotypes, D5 to D7 were later described in India [28], Indonesia [29], and in the Mediterranean Basin [30], respectively.

Genotypes E and G are not subdivided in subgenotypes [31,32]. Genotypes F and H are the "New World" genotypes found in indigenous populations from Alaska to Central and South America. Genotype F is divided into 4 subgenotypes: F1-F4. Subgenotypes F1 and F2 have been further divided in F1a, F1b, F2a and F2b [14,33-35]. Genotype H is very closely related to genotype F and was initially thought to be a clade of genotype F [13,36].

The state of Rondônia is located in the Southwest of Brazilian Amazon and borders with other Brazilian states (Mato Grosso - East, Amazonas - North, Acre - West) and Bolivia (West and South). Currently, it is not clear the general prevalence of HBV in Rondônia state. Katsuragawa et al., [37] found frequencies of 44.5% for anti-HBc and 6.7% for HBsAg studying the serologic markers of hepatitis B and C among the inhabitants of the upper Madeira river, between the localities of Santo Antonio and Abunã, in the Municipality of Porto Velho, Rondônia.

In the last Brazilian census, carried out in 2000, there were 1,380,952 inhabitants in Rondônia State, with the following ethnic background: European-descendants - 588,568 (42.62%); African-descendants - 63,452 (4.59%); Asian-descendants - 3,094 (0.22%); mixed - 698,309

(50.56%); Indigenous people - 10,683 (0.77%); not known - 16,846 (1.22%) [38].

The aims of the present study were to characterize the HBV genotypes circulating in Rondônia state, Brazil, and to infer about their origin using phylogenetical analyses approaches.

Methods

This study was carried out in the state of Rondônia, Brazil (Figure 1) and included 40 serum samples from patients chronically infected with HBV. Of these 40 patients, 26 (65%) were asymptomatic and 27 (67.5%) did not have liver cirrhosis. All samples are HBsAg positive for at least six months, but only 26 (65%) of them were also HBeAg positive, as previously determined by routine serological assays during patients follow up. Patients had between 18 and 60 years old (mean age: 30 years); sex distribution was 28 (69%) men and 12 (31%) women and all of them were under medical assistance at the Research Center for Tropical Medicine (CEPEM), in Rondônia. Indigenous people patients, pregnant women and patients with other associated chronic diseases were excluded from the present analysis.

For the viral DNA extraction from 200 µl serum, it was used QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's standards. The precipitated DNA was resuspended in 200 µl of elution buffer and stored at -20°C until use.

To avoid false-positive results, strict procedures proposed for nucleic acid amplification diagnostic techniques were followed [39]. Samples were first amplified with primers previously described [40] in order to get a 416 base pairs (bp) fragment partially covering the HBsAg coding region (S). A fragment of 1306 bp partially comprising HBsAg and Polymerase coding regions (S/POL) was then amplified from the samples that had been positive in the previous step [13].

Amplified DNA was purified using ChargeSwitch® PCR Clean-Up kit (Invitrogen, São Paulo, Brazil). Sequencing was performed in an ABI Prism® 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA) [41] using dideoxy nucleoside triphosphates (ddNTPs) containing fluorescent markers (Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction kit - Applied Biosystems, Foster City, CA, USA).

The quality of each electropherogram was evaluated using the Phred-Phrap software [42,43] and consensus sequences were obtained by alignment of both sequenced strands using CAP3 software available at the web page Electropherogram quality analysis <http://asparagin.cenargen.embrapa.br/phph/>.

Initially, sequences obtained in this study were genotyped by phylogenetic reconstructions using reference sequences from each HBV genotype obtained from the

Figure 1 Geographic location of Rondônia state, Brazil.

GenBank ($n = 383$) (data available upon request). These sequences comprising partial HBsAg and Polymerase coding regions (S/POL) were aligned using Clustal X software [44] and edited in the SE-AL software (available at <http://tree.bio.ed.ac.uk/software/seal/>). For the phylogenetic analysis, the missing nucleotides were coded as "missing characters" in nexus block. Bayesian phylogenetic analyses were through by Markov Chain Monte Carlo simulation implemented in BEAST v.1.5.3 [45] ten million generations were sufficient to obtain the convergence of parameters. The analyses were performed under relaxed uncorrelated lognormal molecular clock using the model of nucleotide substitution (GTR + G + I) obtained previously by Modeltest v3.7 [46]. The maximum clade credibility (MCC) tree was obtained

from summarizing the 10,000 substitution trees and then it was removed 10% of burn-in using Tree Annotator v.1.5.3 [45].

Results and Discussion

PCR for the S/POL region (1306 bp) was performed in all the 40 samples and 35 of them showed positive results (Figure 2). The HBV genotypes distribution found was: A (37.1%), D (42.8%), F (20.0%), while up to the subgenotype level we found A1 (37.1%), D3 (22.8%), F2a (20.0%), D4 (17.1%) and D2 (2.8%). Sequences were deposited in the GenBank at accession numbers: HM101096 - HM101130.

HBV/A1 samples from Rondônia state did not cluster together in a single group in the phylogenetic tree

and only few sequences remained close to previously reported Brazilian sequences. Subgenotypes D3 and F2a showed the same pattern. These results suggest that probably several different entries of these HBV subgenotypes occurred in this state. On the other hand, genotype D4 sequences clustered in a single group. Nevertheless, as there are few reported sequences from this subgenotype [25,47,48], it was not possible to robustly infer the entry pattern for this subgenotype.

This is the first study reporting the HBV genotypes in Rondônia state, Brazil. A molecular characterization of HBV sequences is important in establishing the evolutionary origins and patterns for viral dispersal. Several reports previously determined the preponderance of genotypes A, D and F in South America [40,49-53]. This finding agrees with the origins of Brazilian population, which is a mixture of European-descendants, Indigenous people and African-descendants.

Previous studies have shown that genotype A was the most frequent in different Brazilian populations [21,40,53-55]. Recently, this genotype was found in 75% patients from Rio de Janeiro [56]. Most of these cases belonged to subgenotype A1, which is the same that was detected in Rondônia. Genotype A was also found among HBV carriers in the state of Acre, which borders the state of Rondônia, in 25 (73.5%) of 34 HBV carriers [57].

Subgenotype A1 was related to the presence of isolated communities of African-descendants, as recently reported in Mato Grosso do Sul State, Central Brazil [58]. It is estimated that about 3.5 million Africans arrived in Brazil in the period between 1551 and 1850 [59]. Currently, there are over 1,000 communities officially identified as remnants of Quilombo, the Brazilian name for small isolated communities made from runaway-slaves where their descendants lived in communities since the slavery period [58,60]. Most of the African-descendants currently living in Rondônia came for the construction of the Madeira-Mamoré Railway, a hallmark in Rondônia state history, that was built by many African-descendants workers in the beginning of twentieth century. Most of them had come from the Caribbean Barbados in a different context from most of the slaves that came directly from Africa [61]. Studies analyzing HBV genotypes in Barbados should be carried to allow a better comparison among Rondônia and Barbados circulating virus. Based on the phylogenetic analysis, as the different sequences from Rondônia are interspersed in the tree and clustered together with other Brazilian sequences (that mostly come from Rio de Janeiro State), as well as with Haitians sequences in another branch, we suggest that subgenotype A1 had different entries in Rondônia, i.e., different viruses were the founders of this population.

Genotype D predominates in the Mediterranean area [62]. Subgenotype D1 occurs mostly in the Mediterranean basin and Middle East. D2 has been reported in India, Japan, Europe and the United States [63]. D3 was found in South Africa, Brazil, Rwanda, Costa Rica and the United States. Finally, D4 was reported in Australia, South Africa, Somalia, Rwanda and Oceania [27,47,48]. In this study, genotype D was prevalent (42.8%) and its subgenotypes were D2, D3 and D4. Since the number of sequences obtained for each HBV/D subgenotype found in Rondônia was small, it was not possible to infer about the origin for each one.

In all the three states located in Southern Brazil, HBV genotype D was previously detected: Paraná [64], Santa Catarina [65] and Rio Grande do Sul [66]. Genotype D is the most frequent in Southern Brazil, whereas genotype A is the most frequent in all other regions [67,68]. In Italy, genotype D is largely the most frequent and is found in 73 to 80% of the patients infected with HBV [69,70]. The Italian government claimed that there are 25 million Brazilians of Italian ancestry, which would comprise the largest population with Italian background outside Italy. During the last quarter of the nineteenth century, several Italians were stimulated to migrate to Brazil and other countries, such as Argentina and the United States. Italians migrants settled down mostly in Southeast (São Paulo state) and South Brazil (Paraná, Santa Catarina and Rio Grande do Sul states). South Region inhabitants latter migrate to Center West and Amazon states, including Rondônia. A deeper characterization of hepatitis B virus genotypes found in Southern Brazil is needed to better understand the migration of hepatitis B virus genotypes in Brazil, particularly for genotype D.

Genotype F is the most divergent and considered indigenous in the Americas. Mello *et al.* [55] showed that genotype F had a low prevalence in Brazil. All genotype F sequences here described belonged to subgenotype F2a, that it is the same that is found in other Brazilian and Venezuelan studies [14,55]. This probably is related to the important native American background in Rondônia population.

Conclusions

In conclusion, genotypes A, D and F found in Rondônia reflecting the ethnic background of its inhabitants, i.e., mainly descendants from European colonizers, African slaves, and indigenous people. Further studies should be carried out to investigate the clinical, virological and therapeutic response characteristics of HBV genotypes, using a large number of samples, including patients representing Rondônia State population with clinical data to characterize their HBV status (carrier, immunotolerance, acute and chronic hepatitis, cirrhosis and/or hepatocellular carcinoma).

Acknowledgements

This work was supported by IEPATRO, CEPEN, CNPq and Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (2007/03457-7 and 2008/04661-6), São Paulo, SP, Brazil.

Author details

¹Research Center for Tropical Medicine - CEPEN/Tropical Pathology Research Institute-IEPATRO, Porto Velho, RO, Brazil. ²Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, SP, Brazil.

Authors' contributions

ACS participated in the design of the study and drafted the manuscript. MMAM conducted the phylogenetic and evolutionary analysis, drafted the manuscript and in its design and coordination. LB participated in the PCR amplification and sequencing process. DSV participated in the design of the study. JRR participated in the elaboration of the manuscript. FJC, ERH and JMS participated in the design of the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 8 September 2010 Accepted: 12 November 2010

Published: 12 November 2010

References

- Magnius LO, Norder H: Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 1996, **38**:24-34.
- Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000, **81**:67-74.
- Rehermann B, Nascimben M: Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* 2005, **5**:215-229.
- Owada T, Mitsubayashi K, Sakata H, Ibara H, Sato S, Ikebuchi K, Kato T, Azuma H, Ikeda H: Interaction between desialylated hepatitis B virus and asialoglycoprotein receptor on hepatocytes may be indispensable for viral binding and entry. *J Virol* 2006, **80**:11-18.
- McMahon BJ: The natural history of chronic hepatitis B virus infection. *Hepatology* 2009, **49**:S45-S55.
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ, Xia NS: Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "F". *PLoS One* 2010, **5**:e1297.
- Echevarria JM, Audin A: Hepatitis B virus genetic diversity. *J Med Virol* 2006, **78**(Suppl 1):S36-42.
- Mahtab MA, Rahman S, Khan M, Karim F: Hepatitis B virus genotypes: an overview. *Hepatobiliary Pancreat Dis Int* 2008, **7**:457-464.
- Mulders MN, Venard V, Njaye M, Edouard AP, Bola Oyefolu AO, Kehinde MO, Muyembe-Tamfum JJ, Ntse YK, Maiga I, Ammerlaan W, Fack F, Omlahu SA, Le Faou A, Muller CP: Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 2004, **190**:400-408.
- Kramvis A, Restorp K, Norder H, Botha JF, Magnius LO, Kew MC: Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* 2005, **77**:47-52.
- Alvarado Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Camillo FJ, Pinho JR: Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol* 2010, **91**:501-508.
- Singh J, Didiens C, Pahal V, Kumar R, Chaudhary R, Kramvis A, Kew MC: First report of genotype e of hepatitis B virus in an Indian population. *Intervirology* 2009, **52**:235-238.
- Alvarado Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Botelho L, Camillo FJ, Pinho JR: Molecular characterization of the hepatitis B virus genotypes in Colombia: A Bayesian inferences on the genotype F. *Infection, genetics and Evolution*.
- Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, Duarte MC, Poblete F, Gutierrez MF, Botto C, Pujol FH: Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol* 2008, **80**:20-26.
- Jutavijittum P, Yousukh A, Samoury B, Samoury K, Ounavong A, Thammavong T, Keokhamphue J, Toriyama K: Seroprevalence of hepatitis B and C virus infections among Lao blood donors. *Southeast Asian J Trop Med Public Health* 2007, **38**:674-679.
- Olinger CM, Jutavijittum P, Hubschen JM, Yousukh A, Samoury B, Thammavong T, Toriyama K, Muller CP: Possible new hepatitis B virus genotype, southeast Asia. *Emerg Infect Dis* 2008, **14**:1777-1780.
- Schaefer S: Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol* 2007, **13**:14-21.
- Olinger CM, Venard V, Njaye M, Oyefolu AO, Maiga I, Kemp AJ, Omlahu SA, Le Faou A, Muller CP: Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 2004, **85**:1163-1173.
- Hubschen JM, Mbah PO, Forbi JC, Otegbayo JA, Olinger CM, Charpentier E, Muller CP: Detection of a new subgenotype of hepatitis B virus genotype A in Cameroon but not in neighbouring Nigeria. *Clin Microbiol Infect* 2010.
- Bowyer SM, van Staden L, Kew MC, Sim JG: A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997, **78**(Pt 2):1719-1729.
- Araujo NM, Mello FC, Yoshida CF, Nel C, Gomes SA: High proportion of subgroup A (genotype A) among Brazilian isolates of Hepatitis B virus. *Arch Virol* 2004, **149**:1383-1395.
- Hannoun C, Sodenstrom A, Norkans G, Lindh M: Phylogeny of African complete genomes reveals a West African genotype A subtype of hepatitis B virus and relatedness between Somali and Asian A1 sequences. *J Gen Virol* 2005, **86**:2163-2167.
- Kurbanov F, Tanaka Y, Fujiwara K, Suguchi F, Mbanya D, Zekeng L, Ndembu N, Ngansop C, Kaptue L, Miura T, Ido E, Hayami M, Ichimura H, Mizokami M: A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. *J Gen Virol* 2005, **86**:2047-2056.
- Makawa M, Souquiere S, Clifford SL, Mouling-Onemere A, Bawe-Johnson M, Wickings EJ, Latour S, Simon F, Roques P: Identification of hepatitis B virus genome in faecal sample from wild living chimpanzee (Pan troglodytes troglodytes) in Gabon. *J Clin Virol* 2005, **34**(Suppl 1):S83-88.
- Andemach IE, Nolte C, Pape JW, Muller CP: Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg Infect Dis* 2009, **15**:1222-1228.
- Pourkarim MR, Lemey P, Amini-Bavil-Olyaei S, Mies P, Van Ranst M: Novel hepatitis B virus subgenotype A6 in African-Belgian patients. *J Clin Virol* 2009, **47**:93-96.
- Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SQ, Mushahwar IK, Robertson BH, Locarnini S, Magnius LO: Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004, **47**:289-309.
- Chandra RK, Biswas A, Datta S, Banerjee A, Panigrahi R, Chakrabarti S, De BK, Chakravarty R: Subgenotypes of hepatitis B virus genotype D (D1, D2, D3 and D5) in India: differential pattern of mutations, liver injury and occult HBV infection. *J Virol* 2009, **83**:749-756.
- Utama A, Octavia TI, Dhenni R, Mirzad UA, Yusuf I, Tai S: Hepatitis B virus genotypes/subgenotypes in voluntary blood donors in Makassar, South Sulawesi, Indonesia. *Virol J* 2009, **6**:128.
- Meldal BH, Mousa NM, Barnes H, Boukief K, Allain JP: A novel hepatitis B virus subgenotype, D7, in Tunisian blood donors. *J Gen Virol* 2009, **90**:1622-1628.
- Kramvis A, Kew MC: Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res* 2007, **37**:S9-S19.
- Otiow C, Gordon D, Borlang J, Giles E, Wilenueve JP: Hepatitis B virus genotype G epidemiology and co-infection with genotype A in Canada. *J Gen Virol* 2008, **89**:3009-3015.
- Kato H, Fujiwara K, Gish RG, Sakagawa H, Yoshizawa H, Suguchi F, Oito E, Ueda R, Tanaka Y, Kato T, Miyakawa Y, Mizokami M: Classifying genotype F of hepatitis B virus into F1 and F2 subtypes. *World J Gastroenterol* 2005, **11**:6295-6304.

34. von Meltzer M, Vasquez S, Sun J, Wendt UC, May A, Gerlich WH, Radtke M, Schaefer S: A new clade of hepatitis B virus subgenotype F1 from Peru with unusual properties. *Virus Genes* 2008, 37:225-230.
35. Kurbanov F, Tanaka Y, Mizokami M: Geographical and genetic diversity of the human hepatitis B virus. *Hepatology* 2010, 40:14-30.
36. Chu CJ, Kieff EB, Han SH, Penito RP, Min AQ, Soldevilla-Pico C, Carey W, Brown RS Jr, Luketic VA, Terrault N, Lok AS: Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* 2003, 125:444-451.
37. Katsuragawa J, et al: High seroprevalence of hepatitis B and C markers in the upper Madeira river region, Porto Velho, Rondônia State, Brazil. *Rev Pan-Amaz Saude* 2010, 1:91-96.
38. IBGE: Características da População e dos Domicílios: Resultados do universo. *Censo Demográfico 2000* [http://ftp.ibge.gov.br/Censos/Censo_Demografico_2000/populacao/UFs/].
39. Kwok S, Higuchi R: Avoiding false positives with PCR. *Nature* 1989, 339:237-238.
40. Strik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Carliho FJ: Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 2004, 42:2455-2460.
41. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. 1977. *Biotechnology* 1992, 24:104-108.
42. Ewing B, Green P: Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 1998, 8:186-194.
43. Ewing B, Hillier L, Wendt MC, Green P: Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 1998, 8:175-185.
44. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG: The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997, 25:4876-4882.
45. Drummond AJ, Rambaut A: BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007, 7:214.
46. Posada D, Crandall KA: MODELTEST: testing the model of DNA substitution. *Bioinformatics* 1998, 14:817-818.
47. Okamoto H, Tsuda F, Sakagawa H, Saitohwagino R, Imai M, Miyakawa Y, Miyumi M: Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988, 69(Pt 10):2575-2583.
48. Norder H, Hammas B, Lofdah S, Courouce AM, Magnusson LO: Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992, 73(Pt 5):1201-1208.
49. Moraes MT, Gomes SA, Nél C: Sequence analysis of pre-S/S gene of hepatitis B virus strains of genotypes A, D, and F isolated in Brazil. *Arch Virol* 1996, 141:1767-1773.
50. Bliz L, Pujol RH, Swenson PD, Porto L, Attardo R, Araujo M, Costa L, Monsalve DC, Torres JR, Fields HA, Lambert S, Van Goyt C, Norder H, Magnusson L, Echeverria J, Stuyver L: Antigenic diversity of hepatitis B virus strains of genotype F in Amerindians and other population groups from Venezuela. *J Clin Microbiol* 1998, 36:648-651.
51. Mbayed VA, Lopez JL, Telenta PF, Palacios G, Badia I, Ferro A, Galoppo C, Campos R: Distribution of hepatitis B virus genotypes in two different pediatric populations from Argentina. *J Clin Microbiol* 1998, 36:3362-3365.
52. Quintero A, Martinez D, Alarcon De Noya B, Castagliola A, Urbina L, Gonzalez N, Liprandi F, Castro De Guerra D, Pujol FH: Molecular epidemiology of hepatitis B virus in Afro-Venezuelan populations. *Arch Virol* 2002, 147:1829-1836.
53. Teles SA, Martins RM, Gomes SA, Gaspar AM, Araujo NM, Souza KP, Carneiro MA, Yoshida CF: Hepatitis B virus transmission in Brazilian hemodialysis units: serological and molecular follow-up. *J Med Virol* 2002, 68:41-49.
54. Rezende RE, Fonseca BA, Ramalho LN, Zucoloto S, Pinho JR, Bertolini DA, Martindal AL: The precore mutation is associated with severity of liver damage in Brazilian patients with chronic hepatitis B. *J Clin Virol* 2005, 32:53-59.
55. Mello FC, Souto FJ, Nabuco LC, Vilela-Nogueira CA, Coelho HS, Franz HC, Saraiva JC, Virgolino HA, Motta-Castro AR, Melo MM, Martins RM, Gomes SA: Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol* 2007, 7:103.
56. Bottecchia M, Souto FJ, O KM, Amendola M, Brandao CE, Nél C, Gomes SA: Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *BMC Microbiol* 2008, 8:11.
57. Viana S, Parana R, Moreira RC, Compri AP, Macedo V: High prevalence of hepatitis B virus and hepatitis D virus in the western Brazilian Amazon. *Am J Trop Med Hyg* 2005, 73:808-814.
58. Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Paniago AM, Lima KM, Gomes SA: Hepatitis B virus infection in isolated Afro-Brazilian communities. *J Med Virol* 2005, 77:188-193.
59. Alves-Silva J, da Silva Santos M, Guimarães PE, Ferreira AC, Bandelt HJ, Pena SD, Prado VF: The ancestry of Brazilian mtDNA lineages. *Am J Hum Genet* 2000, 67:444-461.
60. Motta-Castro AR, Yoshida CF, Lemos ER, Oliveira JM, Cunha RV, Lewis-Ximenez LL, Cabello PH, Lima KM, Martins RM: Seroprevalence of Hepatitis B virus infection among an Afro-descendant community in Brazil. *Mem Inst Oswaldo Cruz* 2003, 98:13-17.
61. Marques RB: A dinâmica da escravidão no Brasil. *Resistência, tráfico negro e alforria, séculos XVII a XIX. Novos Estudos - CEBRAP, São Paulo* 2006, 74:107-123.
62. Gish RG, Gadano AC: Chronic hepatitis B: current epidemiology in the Americas and implications for management. *J Viral Hepat* 2006, 13:787-798.
63. Tallo T, Tefanova V, Primagi L, Schmidt J, Katargina O, Michailov M, Mikomolov S, Magnusson L, Norder H: D2: major subgenotype of hepatitis B virus in Russia and the Baltic region. *J Gen Virol* 2008, 89:1829-1839.
64. Bertolini DA, Ribeiro PC, Lemos MF, Saraceni CP, Pinho JR: Characterization of a Hepatitis B virus strain in southwestern Paraná, Brazil, presenting mutations previously associated with anti-HBs resistance. *Rev Inst Med Trop São Paulo* 2010, 52(1):25-30.
65. Carliho FJ, Moraes CR, Pinho JR, Mello IM, Bertolini DA, Lemos MF, Moreira RC, Bassi LC, Cardoso RA, Ribeiro-dos-Santos G, Da Silva LC: Hepatitis B virus infection in Haemodialysis Centres from Santa Catarina State, Southern Brazil. Predictive risk factors for infection and molecular epidemiology. *BMC Public Health* 2004, 4:13.
66. Bedier CE, Mattos AA, Bogo MR, Branco F, Strik R, Kretzmann NA: Genotyping of hepatitis B virus in a cohort of patients evaluated in a hospital of Porto Alegre, South of Brazil. *Arq Gastroenterol* 2007, 43:13-17.
67. Alcáide R, Melo FL, Nishiya A, Ferreira SC, Langhi Junior MQ, Fernandes SS, Marcondes LA, Duarte AJ, Casseb J: Distribution of hepatitis B virus genotypes and viral load levels in Brazilian chronically infected patients in São Paulo city. *Rev Inst Med Trop São Paulo* 2009, 51:269-272.
68. Mendes-Correia MC, Pinho JR, Locamini S, Yuen L, Strik R, Santana RA, Gomes-Gouveia MS, Leite OM, Martins LG, Silva MH, Gianini RJ, Uip DE: High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. *J Med Virol* 82:1483-1488.
69. Medici MC, Aioldi A, Martinelli M, Abelli LA, Casula F, Valcavi P, Dettori G, Chezzi C: HBV genotypes and antiviral-resistant variants in HBV infected subjects in Northern Italy. *New Microbiol* 2006, 29:63-67.
70. Dai Molin G, Poli A, Croce LS, D'Agaro P, Biagi C, Comar M, Tribelli C, Campello C: Hepatitis B virus genotypes, core promoter variants, and precore stop codon variants in patients infected chronically in North-Eastern Italy. *J Med Virol* 2006, 78:734-740.

doi:10.1186/1743-422X-7-315
Cite this article as: Santos et al: Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondônia, Brazil. *Virology Journal* 2010 7:315.

- 26- Conde SR, Moia L de J, Barbosa MS, Amaral I do S, Miranda EC, Soares M do C, Brito EM, Souza Odo S, de Araujo MT, Demachki S, REBELLO JR, Mesquita MG, Denis AB, Ishak R. Prevalence of Hepatitis B virus genotypes and the occurrence of precore mutation A-1896 and to correlate them with the clinical presentation of chronic hepatitis, in a population group of the Eastern Amazon region. Rev Soc Bras Med Trop. 2004; 37 (Supl 2):33-9 (2004).

Prevalência de genótipos e de mutantes pré-core A-1896 do vírus da hepatite B e suas implicações na hepatite crônica, em uma população da Amazônia oriental

Prevalence of hepatitis B virus genotypes and the occurrence of precore mutation A-1896 and to correlate them with the clinical presentation of chronic hepatitis, in a population group of the Eastern Amazon region

Simone Regina Souza da Silva Conde¹, Lizomar de Jesus Pereira Mória¹, Maria Silvia Brito Barbosa¹, Ivanete do Socorro Abarcado Amaral¹, Esther Castello Branco de Mello Miranda¹, Manoel do Carmo Pereira Soares², Elizabete Maria de Figueiredo Brito², Olgaíze do Socorro Costa Souza², Marialva Tereza de Araújo³, Sâmia Demachki³, João Renato Pinho Rebello⁴, Michele Gomes Soares Mesquita⁴, Denis Alberto Bertollini⁴ e Ricardo Ishak³

RESUMO

A infecção pelo vírus da hepatite B apresenta amplo espectro de manifestações clínicas. Objetivando conhecer os genótipos do HBV mais prevalentes e determinar a ocorrência da mutação pré-core A-1896, em uma população da Amazônia oriental, correlacionando com o diagnóstico clínico, foram selecionados 51 pacientes portadores crônicos de HBsAg e HBV-DNA positivos e divididos em três grupos: grupo A (n=14, pacientes assintomáticos); grupo B (n=20, sintomáticos HBeAg positivos) e grupo C (n=17, sintomáticos HBeAg negativos), sendo usado o sequenciador automático ABI modelo 377 para identificação de genótipos e mutantes pré-core. Os resultados evidenciaram o genótipo A como o mais prevalente, 81,8%, 89,5% e 93,7%, nos grupos A, B e C, respectivamente. A mutação pré-core A-1896 foi encontrada em 11,5% (3/26), sendo todos assintomáticos. Concluiu-se que na população estudada o genótipo A foi o mais prevalente e houve baixa ocorrência do mutante pré-core A-1896, ambos não se constituindo fatores agravantes da doença hepática.

Palavras-chaves: Hepatite B crônica. Genótipos. Mutação pré-core. Epidemiologia. Clínica.

ABSTRACT

Hepatitis B virus (HBV) infection presents itself with a variety of clinical manifestations. The present work aims to describe the prevalence of HBV genotypes and the occurrence of precore mutation A-1896 in a population group of the Eastern Amazon region of Brazil and to correlate them with the clinical presentation of chronic HBV infection. 51 HBsAg carriers (HBV-DNA positive) were selected and divided into three groups: A (14 asymptomatic subjects), B (20 HBeAg positive symptomatic patients) and C (17 HBeAg negative symptomatic patients). Using an automatic DNA sequencer ABI model 377 by sequencing for determined of genotypes and precore mutation. The results showed that the genotype A was the most commonly found (81,1%, 89,5% and 93,7% in groups A, B and C, respectively) and precore mutation A-1896 was described in 11,5% (3/26) of group A subjects. Genotype A of HBV was the most prevalent (89,1%) and low occurrence of precore mutation A-1896, both not associate with the worst outcome of the chronic infection of HBV.

Key-words: Chronic hepatitis B. Genotypes. Precore mutation. Epidemiology. Clinical.

1. Fundação Santa Casa de Misericórdia do Pará, Belém, PA, 2. Instituto Evandro Chagas, Belém, PA, 3. Universidade Federal do Pará, Belém, PA, 4. Instituto Adolfo Lutz, São Paulo, SP.

Endereço para correspondência: Dra. Simone Regina Souza da Silva Conde. Rua diogo Mória 197/901, Umarizal, 66055-170 Belém, PA.

Tel: 91 9112-8889

e-mail: conde@libnet.com.br

A infecção pelo vírus da hepatite B (HBV), apresenta distribuição universal, reconhecendo-se a existência de 350 milhões de portadores crônicos, sendo responsável por um milhão de óbitos por ano¹¹. O estudo da história natural da hepatite B evidenciou manifestações clínicas variáveis, na dependência de fatores relacionados ao hospedeiro e ao próprio vírus, sendo descritos quadros agudos assintomáticos ou oligossintomáticos ou ictericos, possibilidade de hepatite fulminante, cronificação e desenvolvimento de portadores inativo, cirrose hepática e hepatocarcinoma¹⁸.

O HBV é o protótipo da família *Hepadnaviridae* e em seu genoma composto por DNA de fita dupla incompleta se reconhece quatro principais regiões de transcrição (*ORF*): a) o gene *P* que codifica a polimerase viral; b) o gene pré-C/C (core) responsável pela codificação do HBcAg e de outra proteína não estrutural, o HBeAg; c) o gene pré-S/S2 e S (superfície) codificante do HBsAg e das proteínas conhecidas como L e M, e d) o gene *X* que através de sua transcrição produz a proteína *X*¹². O sequenciamento da região *S* do genoma do HBV revelou a presença de sete genótipos do HBV, de A a G^{33 34 41}, os quais possuem uma distribuição geográfica peculiar.

A presença do HBeAg no soro é epidemiologicamente relacionado com a replicabilidade e contagiosidade do vírus. Porém, em estudos pioneiros Carman *et al*¹ e Okamoto *et al*²⁵ identificaram mutantes que não sintetizavam o HBeAg, não interferindo, porém, no ciclo replicativo do mesmo, surgindo o termo mutação pré-core. A principal causa desta mutação deve-se à substituição da G (guanina) pela A (adenina) na posição do nucleotídeo (nt) 1896, e mais raramente no nt 1899, modificando o códon TGG específico para o aminoácido triptofano para um códon de parada precoce TAG, principalmente nos genótipos que possuem a timina (T) no nt 1854, portanto no B, C, D e E, e raro no A genótipo^{9 14}.

Vários estudos tentam correlacionar os diferentes genótipos com a maior ou menor gravidade dos quadros clínicos resultantes da infecção crônica pelo HBV^{26 43 44 45}, assim como a presença da mutação pré-core. Nesta última situação, os resultados ainda são mais controversos, visto que a prevalência destes mutantes em hepatopatias crônicas é elevada nas regiões do Mediterrâneo^{4 14 38}, no Oriente Médio²³ e no leste asiático^{13 24 28 32}, porém baixa ao norte europeu, EUA e Brasil^{18 29 39}; e ainda são identificados mutantes em portadores inativos do vírus^{15 32 36}.

Sendo a região Amazônica considerada uma área de alta prevalência do HBV e a falta de estudos sobre a prevalência de genótipos e mutantes pré-core nesta população, o presente trabalho objetiva conhecer os genótipos mais prevalentes do HBV e a descrição da prevalência de mutação pré-core A-1896 entre os pacientes portadores de hepatite crônica B, em uma população da Amazônia oriental, assim como correlacionar com as características epidemiológicas, clínicas e virológicas.

MATERIAL E MÉTODOS

População de estudo. Selecionou-se 51 pacientes portadores crônicos do HBsAg (HBV-DNA positivo) atendidos no Programa de Hepatopatias Crônicas do Hospital da

Fundação Santa Casa de Misericórdia do Pará, localizado na cidade de Belém. Excluíram-se do estudo pacientes com infecção simultânea pelo vírus da hepatite C (HCV), delta (HDV) ou vírus da imunodeficiência humana (HIV), ou os que utilizaram qualquer tipo de tratamento antiviral prévio. O total de pacientes selecionados foi dividido em três grupos: grupo A formado por pacientes assintomáticos HBeAg positivos e negativos (n: 14); grupo B, por aqueles pacientes sintomáticos HBeAg positivos (n: 20); e grupo C, por pacientes sintomáticos HBeAg negativos e anti-HBe positivos (n: 17). Estes foram submetidos a exames clínicos, laboratoriais, sorológicos, ultra-sonográficos, endoscópicos e, quando indicado, a biópsias hepáticas para estadiamento. Após a identificação destes grupos, procedeu-se a genotipagem de todos os espécimes e o sequenciamento da região pré-core nos portadores do HBV com HBeAg negativo, tanto assintomáticos quanto sintomáticos.

Testes sorológicos e de pesquisa do HBV-DNA. Para os exames sorológicos das hepatites B, C e D foram utilizados EIE, tipo ELISA, com kits comerciais. Todos os soros foram examinados para a presença de HBsAg (Organon Teknika®, Holanda), anti-HBc (Organon Teknika®, Holanda), anti-HBs (Abbott®, EUA) e anti-HCV (Ortho Clinical Diagnostics®, Alemanha). Nas amostras positivas para o HBsAg foi efetuada a pesquisa do HBeAg, anti-HBe, anti-HBc IgM e anti-HD (Organon Teknika®, Holanda).

O HBV-DNA foi pesquisado do modo qualitativo e quantitativo utilizando-se kits comerciais do laboratório Roche® (AMPLICOR HBV MONITOR™), que utiliza a PCR para a amplificação e hibridização do DNA.

Genotipagem do HBV. Para detecção da região *S* do HBV-DNA no soro por PCR, foi empregada a técnica de extração desenvolvida por Kaneko *et al*²⁹ e por Kwok & Higuchi²⁵ com poucas modificações. A extração do DNA viral se procedeu pelo NaOH e os primers utilizados nas PCR foram o FHBS1 (GAG TCT AGA CTC GTG GTG GAC TTC), o RHBS1, para a primeira reação e o FHBS2 (CGT GGT GGA CTT CTC TCA ATT TTC) e RHBS2 (GCC ARG AGA AAC GGR CTG AGG CCC) para a segunda.

A análise do produto de PCR se procedeu com a aplicação em gel de agarose, submetido à corrente elétrica de 150 V por 35 minutos e revelada a reação com a utilização de uma fonte de ultravioleta (UV). O produto foi fotografado com máquina tipo Polaroid. As amostras foram consideradas positivas quando apresentaram uma banda de 450pb, quando comparadas ao padrão. As amostras positivas foram sequenciadas por PCR, derivada da metodologia de Sanger *et al*³⁷ utilizando-se didesoxinucleotídeos (dNTPs) contendo marcadores fluorescentes, conforme o kit ABI Prism® BigDye™ Terminator (PE Applied Biosystems).

A análise dos genótipos foi realizada por meio da comparação das sequências obtidas com as já conhecidas dos diferentes genótipos de HBV. Para esta análise foram utilizados os programas EditSeq e Megalign do pacote DNASTAR (Lasergene Inc, USA).

Sequenciamento da região Pré-core do HBV. O sequenciamento da região pré-core do HBV foi efetuado nas

amostras HBeAg negativas dos grupos A e C. A extração do DNA viral se procedeu com a utilização da solução de isotiocianato de guanidina (solução GT) e os primers para a primeira e segunda PCR foram o EP1.1 (TCA TGG AGA CCA CCG TGA AC) e EP1.2 (GGA AAG AAG TCA GAA GGC AA) e o EP2.1 (CAT AAG AGG ACT CTT GGA CT) e EP2.2 (GGC AAA AAA GAG AGT AAC TC), respectivamente. Após a colocação do produto em gel de agarose a 1% e submetido a corrente elétrica, revelou-se a reação com a utilização de uma fonte de ultravioleta (UV), fotografando com máquina tipo Polaroid, sendo consideradas positivas quando apresentaram uma banda de 300pb.

Os produtos das reações de amplificação foram aplicados em gel de poliacrilamida e submetidos à eletroforese no sequenciador automático, modelo ABI Prism™ 377 DNA Sequencer (Perkin Elmer). As seqüências da região pré-core foram alinhadas a uma seqüência conhecida da mesma região, livre de mutações, utilizando-se para isto os programas EditSeq e Megalign do pacote DNASTAR (Lasergene Inc, USA). Posteriormente, analisou-se os pontos das seqüências que foram marcados por serem diferentes da seqüência consenso, visando o encontro de mutações A-1896 e A-1899, além de outras citadas em publicações científicas.

Para a análise estatística foram utilizados os programas EPIINFO 6.04 b e Biostat 2.0³. Utilizaram-se testes estatísticos paramétricos da ANOVA de um e de dois critérios e os não-paramétricos de Kruskal-Wallis e de Kolmogorov-Smirnov, nos casos de análise de variância de mais de duas amostras. Para duas amostras independentes, os testes não paramétricos empregados foram o qui-quadrado e o teste exato de Fisher. Estabeleceu-se em 0,05 (5%) o nível de rejeição da hipótese de nulidade (valor de $p \leq 0,05$).

O presente trabalho foi aprovada pelo Comitê de Ética Médica da Fundação Santa Casa de Misericórdia do Pará, e os pacientes selecionados foram conscientizados sobre os objetivos do estudo e assinaram um Termo de Consentimento Livre e Esclarecido, permitindo assim a realização da investigação.

RESULTADOS

Os três grupos formados se caracterizaram por um predomínio total do sexo masculino, cada um dos quais apresentando apenas um paciente do sexo feminino. A média das idades foram de $32,4 \pm 12,3$ anos (variação de 10-60 anos) para o grupo A, $41,6 \pm 26,3$ anos (variação de 1-84 anos) para o B e $52,3 \pm 15,3$ anos (variação de 6-72 anos) para o C. A diferença destas médias se mostrou estatisticamente significativa entre os grupos A e C, com valor de p igual a 0,028.

As complicações clínicas em decorrência da doença hepática, ocorridas em 7/20 (35%) pacientes do grupo B e em 9/17 (53%) do grupo C, e a letalidade de 15% (3/20) dos pacientes do grupo B e 35,2% (6/17) do C não apresentaram diferenças estatísticas entre si.

Nas dosagens de AST, ALT, bilirrubina total, FA, GGT e globulinas, o grupo A apresentou menores valores do que no grupo B ($p: 0,005$) e C ($p: 0,001$). As médias das dosagens

de albumina e atividade protrombínica foram menores nos grupos B e C em relação ao A (Tabela 1) e a diferença mostrou-se estatisticamente significativa ($p: 0,001$ e $p: 0,007$, respectivamente).

Tabela 1 - Exames laboratoriais complementares dos pacientes dos grupos A, B e C.

	Grupo A média $\pm$ DP	Grupo B média $\pm$ DP	Grupo C média $\pm$ DP	
ALT (08-54 IU/l)	33,2 $\pm 22,0$	135,5 $\pm 223,7$	115,1 $\pm 137,9$	$p: 0,002^*$
Bilirrubina total (0,4-1,4 mg/dl)	0,7 $\pm 0,3$	3,1 $\pm 3,8$	3,0 $\pm 3,0$	$p: 0,041^*$
GGT (08-63 IU/L)	34,3 $\pm 28,9$	122,0 $\pm 220,1$	98,6 $\pm 73,9$	$p: 0,020^*$
Fosfatase alcalina (36-55 g/dl)	61,1 $\pm 30,6$	181,0 $\pm 142,5$	155,3 $\pm 82,3$	$p: 0,001^*$
Albumina (3,5-5,5 g/dl)	4,7 $\pm 0,6$	3,2 $\pm 1,0$	3,6 $\pm 1,1$	$p: 0,001^*$
AP ($> 70\%$)	90,8 $\pm 14,6$	67,9 $\pm 29,5$	53,1 $\pm 24,8$	$p: 0,007^*$

DP: desvio padrão. (): valores normais e unidades de referência. ns: não significante ($p > 0,05$). * significância estatística entre os grupos A e B e entre A e C, porém não entre os grupos B e C.

Com relação às cargas virais, o grupo B apresentou níveis superiores às do A e C, com valor de p igual a 0,004 (Figura 1). Ressalta-se que o único indivíduo HBeAg positivo do grupo A possuía carga viral de 7,332 ($\log_{10}$), muito acima dos demais membros deste mesmo grupo.

O diagnóstico final dos pacientes do grupo B e C encontram-se expostos na Tabela 2. Não foi possível demonstrar diferença estatística entre eles.

Genotipagem do HBV. Das 51 amostras de soro de pacientes processadas para genotipagem, conseguiu-se êxito em 11 (79%) do grupo A, 19 (95%) do grupo B e 17 (94%) do grupo C. As amostras que não se conseguiu efetuar a

Figura 1 - Mediana e quartis das cargas virais (em $\log_{10}$) do HBV dos grupos A, B e C.

Tabela 2 - Diagnóstico final (clínico e/ou histopatológico) dos pacientes dos grupos B e C.

Diagnóstico	Grupo B (20)		Grupo C (17)		
	n°	%	n	%	
Hepatite crônica	8	40,0	5	29,5	ns
Cirrose hepática	9	45,0	7	41,0	ns
Hepatocarcinoma	3	15,0	5	29,5	ns

ns: não significante ($p > 0,05$)

extração do ácido nucleico apresentaram carga viral baixa, inferior a 2.500 cópias/mL (Figura 2).

A genotipagem das 46 amostras positivas revelou a presença do genótipo A na grande maioria dos pacientes, 89,1% (41/46), seguido dos genótipos F em 8,7% (4/46) e D em 2,2% (1/46). A distribuição dos genótipos encontrados nos grupos estudados está resumida na Tabela 3.

Figura 2 - Detecção por eletroforese em gel de agarose a 1% do produto de amplificação da região S do HBV (450 pb). Lanes a, b, c, f e g: amostras positivas; lane d: controle negativo; lane e: amostra negativa, lane h: padrão.

Tabela 3 - Distribuição dos genótipos do HBV nos grupos A, B e C.

Grupos	N°	Genótipos					
		A	%	D	%	F	%
A	11	9	81,8	1	9,1	1	9,1
B	19	17	89,5	0	-	2	10,5
C	16	15	93,7	0	-	1	6,3
Total		41	89,1	1	2,2	4	8,7

Quanto à procedência destes pacientes (Figura 3), observou-se que a maior parte dos pacientes com genótipo A, 68,3% (28/41), procediam da região metropolitana de Belém e da região sudeste do Pará. O paciente com genótipo D procedeu de Belém, assim como dois outros com genótipo F. Deste último genótipo, um terceiro paciente era oriundo de Cametá (Baixo Tocantins) e o quarto, de Vizeu, nordeste do Estado do Pará.

As características demográficas dos pacientes (Tabela 4) não mostraram diferenças estatísticas, quando distribuídas de acordo com o genótipo encontrado.

A presença do HBeAg entre os indivíduos portadores do genótipo A foi de 43,9% (18/41), taxa semelhante a encontrada nos do genótipo não A.

ESTADO DO PARÁ

Procedência	Genótipo A (41)	Genótipo não A (05)
Belém	20	3
Nordeste	3	1
Marajo	2	0
Baixo Tocantins	3	1
Sudeste	9	0
Sudoeste	3	0
Baixo Amazonas	1	0

Figura 3 - Procedência dos pacientes com genótipo A e não A do HBV.

Tabela 4 - Características demográficas dos pacientes com genótipos A e não A (D e F).

Características	Genótipo A (41)	Genótipo não A (D: 1 e F: 4)	
Sexo M/F	39 / 2	5 / 0	ns
Idade (anos) média $\pm$ DP	44 $\pm$ 22,6	33 $\pm$ 11,6	ns
Cor (%)			ns
branca	13,5	25,0	
negra	5,4	0	
parda	78,4	75,0	
amarela	2,7	0	

ns: não significante ($p > 0,05$)

A média da carga viral (em $\log_{10}$) das 41 amostras com genótipo A, 7.008 cópias/mL, e a dos cinco pacientes com genótipos não A, 6.716 cópias/mL não apresentaram diferença estatística significativa (Figura 4).

O caso de genótipo D encontrava-se na condição de assintomático. Nos quatro indivíduos com genótipo F, cada grupo de diagnóstico, assintomático, hepatite crônica, cirrose

Figura 4 - Distribuição da carga viral (em $\log_{10}$) das amostras de genótipos A, F e D.

hepática e hepatocarcinoma, recebeu um representante. Os representantes do genótipo A eram em 22% (9/41) assintomáticos, em 26,8% (11/41) portadores de hepatite crônica, 36,6% (15/41) portadores de cirrose hepática e, finalmente, 14,6% (6/41) portadores de CHC (Tabela 5).

Tabela 5 - Presença do HBeAg, dosagem da carga viral e o diagnóstico dos casos com genótipo A e dos genótipos não A (D e F).

	Genótipo A (41)	Genótipo diferente de A (D: 1 e F: 4)	
HBeAg positivo	18	2	ns
Média da carga viral (log ₁₀) ± DP (cópias/mL)	7,008 ± 7,156	6,716 ± 7,040	ns
Diagnóstico			ns
assintomático	9	2	
hepatite crônica	12	1	
cirrose hepática	14	1	
hepatocarcinoma	6	1	

ns: não significante ($p > 0,05$)

Mutação pré-core A-1896/ A-1899 do HBV. No total de amostras testadas, 11,5% (3/26) mostraram-se positivas para as mutações pesquisadas (Tabela 6). No grupo A (Figura 5), a presença de mutação pré-core foi encontrada em três (25%) amostras, sendo uma do tipo A-1896, uma do tipo A-1899 e uma do tipo combinado A-1896 com A-1899. No grupo C, não foi possível evidenciar tal mutação. Ainda assim, o resultado não mostrou diferença estatística entre os dois grupos analisados (valor de p igual a 1).

Tabela 6 - Prevalência de mutação pré-core A-1896 e A-1899 nos pacientes dos grupos A e C HBeAg negativos.

	Grupo A (12)	Grupo C (14)	Total
Positivo (n) %	(3) 25,0*	0	(3) 11,5
Negativo (n) %	(9) 75,0	(14) 100,0	(23) 88,5
Total	(12) 100,0	(14) 100,0	(26) 100,0

* Um caso de mutação A-1896, um de A-1899 e um caso de associação da A-1896 e A-1899.

Figura 5 - Sequências nucleotídicas da região pré-core do HBV de 12 amostras dos pacientes do grupo A. (-): nucleotídeos. (■): nt 1854. (■): nt 1896. nt(■): nt 1899. ■: cepa selvagem. ■: cepa com mutação pré-core A-1899/ A-1896 e 1899/ A-1896.

A presença do mutante pré-core foi observada em dois casos de genótipo A, nos quais também a mutação T-1858 estava presente, e em um caso de genótipo D. Contudo, outras mutações também foram encontradas, entre as quais destacam-se a T-1762 e a A-1764 (Tabela 7).

Os três indivíduos portadores do HBV mutante pré-core A-1896/ A-1899 eram do sexo masculino, procedentes da região metropolitana de Belém e com idades de 22, 34 e 29

Tabela 7 - Relação de tipos de mutações pré-core encontradas nos grupos A e C.

Tipo de mutação	Grupo A (12)		Grupo C (15)		Total (27)	
	n°	%	n°	%	n°	%
T-1858, A-1896	1	8,3	0	-	1	3,7
T-1762, A-1764, T-1858, A-1899	1	8,3	0	-	1	3,7
T-1762, A-1764, T-1858, A-1896, A-1899	1	8,3	0	-	1	3,7
T-1762, A-1764	5	41,8	8	53,2	13	48,2
C-1814	1	8,3	1	6,7	2	7,4
T-1762, A-1764, C-1814	2	16,7	2	13,3	4	14,8
T-1762	0	-	1	6,7	1	3,7
T-1762, A-1764, T-1858	0	-	1	6,7	1	3,7
Sem mutações	1	8,3	1	6,7	2	7,4

anos e assintomáticos. As médias das cargas virais (em log₁₀) dos três indivíduos 4,735 cópias/mL, e a do restante dos 23 sem mutação pré-core A-1896/A-1899, 4,922 cópias/mL, não apresentaram diferenças estatisticamente significantes entre si, com p : 0,399.

DISCUSSÃO

Na caracterização dos grupos estudados, observou-se um predomínio do sexo masculino e da faixa etária adulta nos três. Contudo, o grupo A possuía média de idade inferior aos demais, com significância estatística em relação ao grupo C. Este dado se assemelha com os trabalhos de Mangia *et al*⁹ e Niitsuma *et al*³², em que os pacientes portadores assintomáticos do HBV eram mais jovens em relação aos doentes crônicos HBeAg negativos. O grupo B, composto de pacientes HBeAg positivos, foi o que apresentou a maior média de carga viral (em log₁₀), com valores significativamente maiores do que os encontrados nos grupos A e C (p : 0,004). Chu *et al*⁹ ao analisarem 79 pacientes com hepatite crônica B, todos comprovados histologicamente, encontraram níveis séricos do HBV-DNA por dot-blot de 944 pg/mL nos HBeAg positivos, sendo maior do que os 58pg/mL encontrados nos anti-HBe positivos. A média de idade e a carga viral se mostraram inversamente proporcionais no grupo B e foi menor do que nos pacientes do grupo C.

A genotipagem das amostras do HBV mostrou que 89,1% pertenciam ao genótipo A, 8,7% ao F e 2,2% ao D. Os estudos de genotipagem descrevem uma ocorrência variada de genótipos de acordo com a situação geográfica examinada.

O genótipo A é o mais prevalente no norte europeu, na África (seguido em menor proporção pelo D e E), EUA e em descrições de prevalência de populações urbanas com alto fluxo migratório de países da América do Sul^{27 30 33 34 35}.

A genotipagem das amostras provenientes de cinco países da América Central (Nicarágua, Costa Rica, Guatemala, El Salvador e Honduras) detectou a presença do genótipo F em 79% das amostras, além de 14% do genótipo A, 6% do D e 1% do C 2, sugerindo ser o F o genótipo nativo da região³. Moraes *et al*³¹ demonstrando uma técnica de PCR específica para genotipar amostras de HBV, identificou em 54 espécimes provenientes de Manaus (AM) e Macapá (AP) 69% de genótipo

A, 31% de D e 7,4% de F, e sugeriu que em outros estudos houve uma superestimação do genótipo F. Em trabalho recente, Bertolini *et al*⁶ demonstraram em três comunidades indígenas da região Amazônica o predomínio total do genótipo F e em uma quarta 100% dos pesquisados pertenciam ao genótipo A, sendo que esta comunidade mantinha um alto grau de aculturação e contato com populações tri-híbridas.

O genótipo D é o predominante no sul da Europa e parte do Oriente Médio. Ao norte e sudoeste do continente asiático se destacam os genótipos B e C²⁷⁻³³. Trabalhos realizados com aborígenes australianos evidenciaram a presença do genótipo C¹.

Ao descreverem o genótipo G, em pacientes norte-americanos e franceses cronicamente infectados pelo HBV, Stuyver *et al*³⁴ observaram que o genótipo A também era o mais prevalente, com 54% do total de casos, seguido do D (19%), C (12%), G (11%), B (3%) e E (1%). Neste estudo, o genótipo F não foi encontrado.

Como se pode observar, a extensão geográfica do Brasil, as diferenças regionais de cultura, diferenças de poder aquisitivo, composição étnica, padrões de conforto, educação, dentre outros, conseguem influenciar na grande variabilidade não só da prevalência do HBV³⁰, como também de seus genótipos de acordo com a classificação atual no sequenciamento da região S³¹⁻³⁹.

Comunidades epidemiologicamente fechadas conseguem ter introduzido um genótipo e mantê-lo como o único circulante, enquanto que aqueles grupos em que acontece a circulação de vários genótipos, as frequências de ocorrência são variadas e não existe, aparentemente, algum fator biológico do vírus que favoreça o predomínio de um ou outro genótipo. A maior influência, provavelmente, se dá pelos fatores associados à transmissão do HBV.

No presente trabalho, não foi possível mostrar a influência de possíveis variações genotípicas com as manifestações clínicas e a gravidade da doença hepática crônica pelo HBV, como já proposto em outros estudos²⁹⁻³¹⁻⁴³⁻⁴⁵.

A procura da mutação pré-core A-1896 e/ou A-1899 nos pacientes HBeAg negativos, grupos A e C, mostrou que nos 12 sequenciamentos dos pacientes assintomáticos, três (25%) possuíam esta mutação, enquanto que em 14 pacientes do grupo sintomático HBeAg negativo, em nenhum foi possível mostrar a mutação. A baixa prevalência desta mutação, 11,5% (3/26) já era esperada devido a alta prevalência do genótipo A nos grupos estudados.

A mutação pré-core A-1896 em pacientes assintomáticos HBeAg negativos foi descrita por diversos autores¹⁵⁻²³⁻²⁹⁻³² em uma proporção extremamente variável, de 17,6% a 57,2%, de acordo com a região estudada. Nos locais onde predominam os genótipos B, D ou C, nos pacientes hepatopatas crônicos HBeAg negativos, a mutação pré-core é encontrada em 35% a 100% dos casos¹⁴⁻¹⁶⁻²³⁻³⁸⁻⁴⁶, enquanto nos países de maior prevalência do genótipo A, a mutação A-1896 se situa entre 28,5% a 45,2%, encontrada principalmente em pacientes com genótipos diferentes do A, nestas populações²⁹⁻³⁹⁻⁴⁵.

A presença da mutação mesmo em uma frequência baixa era esperada no grupo C. Por outro lado, a diferença entre

os grupos A e C não foi estatisticamente significativa, denotando não ser este um fator influenciador na evolução clínica dos pacientes em questão.

No presente trabalho, outras mutações nas regiões promotoras do core e pré-core também foram identificadas. Merecem destaque as mutações T-1762 e A-1764, as quais foram encontradas em 76,9% dos casos, incluindo os três grupos. A presença dessas mutações foi descrita por Takahashi *et al*⁴²⁻⁴³ e Karasawa *et al*⁴², os quais associaram a combinação das mesmas com a mutação A-1896 e descreveram a possibilidade das mutações influenciarem na evolução clínica dos pacientes infectados pelo HBV.

Estudos futuros com casuísticas maiores deverão ser efetuados para se buscar uma análise mais abrangente sobre a influência dos genótipos e mutação pré-core do HBV na evolução clínica dos pacientes cronicamente infectados, na Amazônia oriental, assim como o significado da presença das mutações promotora do core, T-1762 e A-1764.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Alestig E, Hannoun C, Horal P, Lindh M. Hepatitis B virus genotypes in Mongols and Australian Aborigines. *Archives of Virology* 146: 2321-2329, 2001.
2. Arauz-Ruiz P, Norder H, Visoná KA, Magnus LO. Molecular epidemiology of hepatitis B virus in Central America reflected in the genetic variability of small s gene. *The Journal of Infectious Diseases* 176: 851-858, 1997.
3. Ayres M, Ayres Júnior M, Ayres DL, Santos AS. BioEstat 2.0: aplicações estatísticas nas áreas de ciências biológicas e médicas. Belém: Sociedade Civil Mamirauá. Conselho Nacional de Desenvolvimento Científico e Tecnológico. Brasília, 2000.
4. Barbera C, Calvo F, Coscia A, Perugini L, Dastoli G, Randone A, Bonino E, Brunetto M. Precore mutant hepatitis B virus and outcome of chronic infection and hepatitis in hepatitis B e antigen-positive children. *Pediatric Research* 36: 347-350, 1994.
5. Beasley RP, Lin CC, Hwang LL, Chien CS. Hepatocellular carcinoma and hepatitis B. A prospective study of 22,707 men in Taiwan. *The Lancet* 21: 1129-1133, 1981.
6. Bertolini DA, Moreira RC, Soares M, Bensabath G, Lemos MF, Mello IMVGC, Pinho JRR. Genotyping of hepatitis B virus in indigenous populations from Amazon region, Brazil. *Reviews & Research* 5 (supl 1): 101, 2000.
7. Carman WF, Hadziyannis S, McGarvey MJ, Jacyna MR, Karayiannis P, Makris A, Thomas HC. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 2:588-591, 1989.
8. Castro LD, Niel C, Gomes SA. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. *BMC Microbiology* 1: 10, 2001.
9. Chan HLY, Hussain M, Lok ASF. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. *Hepatology* 29: 976-984, 1999.
10. Chu CM, Karayiannis P, Fowler MJE, Monjardino J, Liaw YF, Thomas HC. Natural history of chronic hepatitis B virus infection in Taiwan: studies on hepatitis B virus DNA in serum. *Hepatology* 5: 431-434, 1985.
11. Davey S. State of the world's vaccines and immunization. World Health Organization, Geneva. p.76-82, 1996.
12. Don G. *Hepadnaviridae and their replication*. In: Knipe DM, Howley PM (eds) *Fields Virology*. Lippincott-Raven, Philadelphia. p.2701-2738, 1996.
13. Ehata T, Chung WL, Omata M. Mutations of hepatitis B virus liver disease. *Gastroenterology Japonica* 28 (sup 4):7-11, 1993.
14. Frias FR, Buti M, Jardi R, Cotrina M, Viladomiu L, Esteban R, Guardia J. Hepatitis B virus infection: precore mutants and its relation to viral genotypes and core mutations. *Hepatology* 22: 1641-1647, 1995.

15. Fujiwara K, Yokosuka O, Ehata T, Chuang WL, Imezeki F, Saisho H, Omata M. The two different states of hepatitis B virus DNA in asymptomatic carriers HBe-antigen-positive versus anti-HBe-positive asymptomatic carriers. *Digestive Diseases and Sciences* 43: 368-376, 1998.
16. Hamasaki K, Nakata K, Nagayama Y, Ohtsuru A, Daikoku M, Taniguchi K, Tsutsumi T, Sato Y, Kato Y, Nagataki S. Changes in the prevalence of HBeAg-negative mutant hepatitis B virus during the course of chronic hepatitis B. *Hepatology* 20: 8-14, 1994.
17. Hasegawa K, Huang J, Wands JR, Ohata H, Liang TJ. Association of hepatitis B viral precore mutations with fulminant hepatitis B in Japan. *Virology* 185: 460-463, 1991.
18. Hoofnagle JH, Seeff LB. Natural history of chronic type B hepatitis. In: Popper H, Schaffner F (eds) *Progress in Liver Diseases*. Grune and Stratton, New York. p. 469-479, 1982.
19. Kaneko S, Feinstone SM, Miller RH. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using polymerase chain reaction technique. *Journal of Clinical Microbiology* 27: 1930-1933, 1989.
20. Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 118: 554-559, 2000.
21. Kao JH, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. *Journal of Clinical Microbiology* 40: 1207-1209, 2002.
22. Karasawa T, Shirasawa T, Okawa Y, Kuramoto A, Shimada N, Aizawa Y, Zeniya M, Toda G. Association between frequency of amino acid changes in core region of hepatitis B virus (HBV) and the presence of precore mutation in Japanese HBV carriers. *Journal of Gastroenterology* 32: 611-622, 1997.
23. Kaspar RT, Klein A, Aharonson S. Hepatitis B virus precore mutants are identical in carriers from various ethnic origins and are associated with a range of disease severity. *Hepatology* 16: 1338-1342, 1992.
24. Kim WH, Kim KH, Chung JP, Kang JK, Park IS. Mutations in the pre-core region of hepatitis B virus DNA in patients with chronic liver diseases. *Yonsei Medical Journal* 34: 158-165, 1993.
25. Kwok R, Higuchi R. Avoiding false positives with PCR. *Nature* 339: 237-238, 1989.
26. Liaw YF, Tai DI, Chu CM, Lin DY, Sheen IS, Chen TJ, Pao CC. Early detection of hepatocellular carcinoma in patients with chronic B hepatitis. A prospective study. *Gastroenterology* 90: 263-267, 1986.
27. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus - large-scale analysis using a new genotyping method. *Journal of Infectious Diseases* 175: 1285-1293, 1997.
28. Lindh M, Hannoun C, Dhillion AP, Norkrans G, Horal G. Core promoter mutations and genotypes in relation to viral replication and liver damage in east asian hepatitis B virus carriers. *Journal of Infectious Diseases* 179: 775-782, 1999.
29. Mangia A, Chung YH, Hoofnagle JH, Birkenmeyer L, Mushawar I, Di Bisceglie A. Pathogenesis of chronic liver disease in patients with chronic hepatitis B virus infection without serum HBeAg. *Digestive Diseases and Sciences* 41: 2447-2452, 1996.
30. Mbayed VA, Lopez JL, Telenta PFS, Palacios G, Badia I, Ferro A, Galoppo C, Campos R. Distribution of hepatitis B virus genotypes in two different pediatric populations from Argentina. *Journal of Clinical Microbiology* 36: 3362-3365, 1998.
31. Moraes MTB, Niel C, Gomes SA. A polymerase chain reaction-based assay to identify genotype F of hepatitis B virus. *Brazilian Journal of Medical and Biological Research* 32: 45-49, 1999.
32. Niitsuma H, Ishii M, Saito Y, Miura M, Kobayashi K, Ohori H, Toyoto T. Prevalence of precore defective mutant of hepatitis B virus in HBV carriers. *Journal of Medical Virology* 46: 397-402, 1995.
33. Norder H, Couroucé AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of hepatitis B virus, four of which represent two new genotypes. *Virology* 198: 489-503, 1994.
34. Okamoto H, Tsuda F, Sakugawa H, Sastrosowigno RI, Mitsunobu I, Miyakawa Y, Mayumi M. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *Journal General of Virology* 69: 2575-2583, 1988.
35. Okamoto H, Yotsumoto S, Akahane TY, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. Hepatitis B viruses with precore region defects prevail in persistently infect hosts along seroconversion to the antibody against e antigen. *Journal of Virology* 64: 1298-1303, 1990.
36. Raimondo G, Gianmichele M, Sardo MA, Rodino G, Salvatore C, Vecchi M, Pernice M, Rumi G, Tatarella M, Franchis R. Persistence of "wild-type" and "e-minus" hepatitis B virus infection in chronic healthy HBsAg/anti-HBe positive carriers. *Journal of Hepatology* 20: 148-151, 1994.
37. Sanger F, Nicklen S, Coulson AR. DNA sequencing chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* 74: 54-63, 1977.
38. Santantonio T, Jung MC, Miska S, Pastore G, Page GR, Will H. Prevalence and type of pre-C HBV mutants in anti-HBe positive carriers with chronic liver disease in highly endemic area. *Virology* 183: 840-844, 1991.
39. Sitrnik R, Pinho JRR, Silva LC, Fonseca LEP, Carrilho FJ, Bernardini AP. Genótipos do vírus da hepatite B e mutações na região pré-core (VHB) em pacientes brasileiros com hepatite crônica B. In: *Resumo na Gastroenterologia Endoscopia Digestiva* 18 (supl 1): S25, 1999.
40. Souto FJD. Distribuição da hepatite B no Brasil: atualização do mapa epidemiológico e proposições para seu controle. *Gastroenterologia Endoscopia Digestiva* 18: 143-150, 1999.
41. Stuyver L, Gendt S, Geys CV, Zoulim F, Fried M, Schinzel R, Rossau R. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *Journal of General Virology* 81: 67-74, 2000.
42. Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, Yoshizawa H, Mishihiro S. The precore/core promoter mutant (T1762/A1764) of hepatitis B virus: clinical significance and easy method for detection. *Journal of General Virology* 76: 3159-3164, 1995.
43. Takahashi K, Ohta Y, Kanai K, Akahane Y, Iwasa Y, Hino K, Ohno N, Yoshizawa H, Mishihiro S. Clinical implications of mutations C-to-T1653 and T-to-C/A/G1753 of hepatitis B virus genotype C in chronic liver disease. *Archives of Virology* 144: 1299-1308, 1999.
44. Thakur V, Gupta RC, Kazin SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver diseases patients in the Indian subcontinent. *Journal of Gastroenterology and Hepatology* 17: 165-170, 2002.
45. Tillmann H, Trautwein C, Walker D, Michitaka K, Böker K, Manns M. Clinical relevance of mutations in the precore genome of the hepatitis B virus. *Gut* 37: 568-573, 1995.
46. Tu H, Xiong SD, Trepo C, Wen YM. Frequency of hepatitis B virus e-minus mutants varies among patients from different areas of China. *Journal of Medical Virology* 51: 85-89, 1997.

27- Michele Soares Gomes-Gouvêa; Manoel do Carmo Pereira Soares; Isabel Maria Vicente Guedes C Mello; Elisabete Maria Figueiredo Brito; Lizomar de Jesus Pereira Moia; Gilberta Bensabath; Heloisa Marceliano Nunes; Flair José Carrilho; JOÃO RENATO REBELLO PINHO. Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin. Acta Tropica 106: 149 – 155 (2008)

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin

Michele Soares Gomes-Gouvêa^{a,*}, Manoel do Carmo Pereira Soares^b,
Isabel Maria Vicente Guedes de Carvalho Mello^{a,c}, Elisabete Maria Figueiredo Brito^b,
Lizomar de Jesus Maués Pereira Moia^d, Gilberta Bensabath^b, Heloisa Marceliano Nunes^b,
Flair José Carrilho^a, João Renato Rebello Pinho^a

^a Department of Gastroenterology, University of São Paulo School of Medicine, Av. Dr. Enéas Carvalho de Aguiar 500, 05403-000 São Paulo, SP, Brazil

^b Seção de Hepatologia, Instituto Evandro Chagas, Avenida Almirante Barroso 492, 66090-000 Belém, PA, Brazil

^c Laboratório de Imunologia Viral, Instituto Butantan, Avenida Vital Brasil 1500, 05503-900 São Paulo, SP, Brazil

^d Fundação Santa Casa de Misericórdia do Pará, Rua Oliveira Belo 395, 66050-380 Belém, PA, Brazil

ARTICLE INFO

Article history:

Received 1 August 2007

Received in revised form 26 February 2008

Accepted 29 February 2008

Available online 18 March 2008

Keywords:

HDV

HBV

Genotypes

Amazon

Brazil

ABSTRACT

Hepatitis D virus (HDV) is a defective hepatotropic virus whose infectivity is dependent on hepatitis B virus (HBV). HDV super- or co-infection leads to an increased risk of fulminant hepatitis or progression to severe chronic liver disease in HBV infected patients. The Brazilian Amazon Basin has been reported to be endemic for HBV and HDV, especially in the Western Amazon Basin. In this region, HDV infection is frequently associated with acute fulminant hepatitis with characteristic histologic features. HDV is classified into seven major clades (HDV-1 to HDV-7) and HBV is subdivided into eight genotypes (A–H). HDV and HBV genotypes have been shown to have a distinct geographic distribution. The aim of this study was to determine the HBV and HDV genotypes harbored by chronically infected patients from the Eastern Amazon Basin, Brazil. We studied 17 serum samples from HBV and HDV chronically infected patients admitted to a large public hospital (Santa Casa de Misericórdia) at Belém, state of Pará, Brazil, between 1994 and 2002. HDV-3 and HBV genotype A (subtype adw2) have been identified in all cases, in contrast to previous studies from other regions of the Amazon, where HBV genotype F has been found co-infecting patients that harbored HDV-3. The HDV-3/HBV-A co-infection suggests that there is not a specific interaction between HBV and HDV genotypes, and co-infection might merely reflect the most frequent genotypes found in a particular geographic area. The analysis of the carboxy-terminal region of the large hepatitis D antigen (L-HDAg), which interacts with the hepatitis B surface antigen (HBsAg) and is essential for HDV assembly, showed some diversity between the different isolates from the Eastern Amazon. This diversity is not observed among HDV-3 sequences from other South American regions.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Hepatitis D virus (HDV) is a defective hepatotropic virus whose infectivity is dependent on hepatitis B virus (HBV) for the provision of its envelope, which consists of hepatitis B surface antigen (HBsAg) (Karayiannis, 1998). HDV super- or co-infection is a major health risk in chronically or acutely HBV infected patients due to the increased chance of fulminant hepatitis or progression to severe chronic liver disease (Smedile et al., 1982).

HDV infection has a worldwide distribution but its frequency largely varies throughout different geographic regions. In Northern Europe and in North America, HDV infection is mainly confined to intravenous drug users. On the other hand, epidemics of HDV infections have been described in the Amazon Basin and Central Africa (Casey, 1996a). In some regions of South America, such as the Western Amazon Basin (including Brazil, Peru, Ecuador, Venezuela and Colombia), HDV infections have been associated with high morbidity and mortality outbreaks of hepatitis (Bensabath et al., 1987; Sjogren and Colichon, 1991; Casey et al., 1996b; Manock et al., 2000; Popper et al., 1983; Hadler et al., 1984; Buitrago et al., 1986; Ljunggren et al., 1985).

HDV used to be classified into three genotypes (I, II and III), but recent analyses of HDV sequences including strains isolated from African patients showed a greater genetic diversity than previously

* Corresponding author at: Laboratório de Gastroenterologia e Hepatologia Tropical, Instituto de Medicina Tropical, FMUSP, Av. Dr. Enéas Carvalho de Aguiar, 500, 05403-000 São Paulo, SP, Brazil. Tel.: +55 11 3061 8218; fax: +55 11 3064 5132.
E-mail address: gomesmic@yahoo.com.br (M.S. Gomes-Gouvêa).

described. A new classification was suggested proposing seven major clades (HDV-1 to HDV-7) individualized with strong phylogenetic support (Radjef et al., 2004). Different clades are related to their geographical origin: HDV-1 is found worldwide; HDV-2 (previously labeled HDV-IIa) is found in Japan, Taiwan, and Yakutia, Russia; HDV-4 (previously labeled HDV-IIb) in Taiwan and Japan; HDV-3 in the Amazon basin and HDV-5 to HDV-7 in Africa (Zhang et al., 1996; Shakil et al., 1997; Imazeki et al., 1990; Lee et al., 1996; Sakugawa et al., 1999; Wu et al., 1998; Ivaniushina et al., 2001; Casey et al., 1993, 1996b; Nakano et al., 2001; Radjef et al., 2004).

The severity of disease seems to be influenced by the HDV genotypes. Particularly, HDV-3 is closely associated with fulminant hepatitis in Northern South America (Casey et al., 1993, 1996b; Nakano et al., 2001). HDV-3 has been associated with the co-infecting HBV genotype F that is also indigenous to South America. It has been suggested that HDV-3 or the combination of HDV-3 with HBV genotype F may be responsible for the increased severity of disease reported in this region (Casey et al., 1996b; Nakano et al., 2001).

HBV genotypes from A to H have distinct geographical distributions and several studies highlighted that different HBV genotypes are related to different courses of the disease (Guettouche and Hnatyszyn, 2005), but few data have been published concerning HDV genotypes. The role of the association of different HBV and HDV genotypes with the clinical evolution of the disease has been even less studied.

In Brazil, especially in the Amazon Basin, there are some areas with high prevalence of both HBV and HDV and few reports concerning their genetic diversity. To our knowledge, no previous reports determining the HDV and HBV genotypes circulating in the Eastern Amazon Basin have been published until now. The aim of this study was to identify the HBV and HDV genotypes harbored by chronically infected patients from this region.

The determination of the prevalent genotypes in a particular area may contribute to a better understanding of the relationship between specific genotypes and the clinical course of the disease, providing that clinical data of the cases from that area are also available.

2. Methods

2.1. Patients

We studied 17 serum samples from HBV and HDV chronically infected patients from different counties within the Brazilian states of Pará and Amapá, in the Eastern Amazon Basin, which were admitted to the a large public hospital (Santa Casa de Misericórdia) in Belém, Pará, Brazil, between 1994 and 2002. Fig. 1 shows the studied patients' geographical origins in the Eastern Amazon.

This group included 16 males and one female ranging from 25 to 56 years old (mean, 37.5; standard deviation, 8.5). All patients had chronic liver disease and were positive both for HBsAg and for antibodies to the HDV (anti-HD). Human immunodeficiency or hepatitis C virus infections were ruled out in all of them through routine antibodies tests. Liver biopsies were available from 15 patients and in 14 of them cirrhosis was detected. Table 1 shows the characteristics of HBV and HDV-infected patients investigated.

2.2. Detection of viral nucleic acids

HDV RNA and HBV DNA were detected by polymerase chain reaction (PCR). For HDV, the partial delta antigen genomic region (403-nucleotide fragment) was amplified using primers described before (Casey et al., 1993; Zhang et al., 1996). For HBV, a 416-nucleotide fragment covering a conserved region of the surface

Fig. 1. Origin of the HDV-infected patients investigated. States of Pará and Amapá in the Eastern Amazon region of Brazil.

Table 1
Characteristics of HDV-infected patients investigated

Patient	Age (year)	Origin (city/state)	Sex ^a	Occupation	Liver disease (status)
1	37	Macapá/AP	M	Gold miner	Cirrhosis
2	27	Santarém/PA	M	Mason	Cirrhosis
3	25	Jacareacanga/PA	M	Student	Cirrhosis (died)
4	34	Jacareacanga/PA	M	Gold miner	Cirrhosis
5	49	Altamira/PA	M	Civil servant	NA ^b
6	35	Santarém/PA	M	Gold miner	Cirrhosis (died)
7	37	Altamira/PA	M	Gold miner	Cirrhosis
8	56	Altamira/PA	M	Peasant	Cirrhosis
9	34	Macapá/AP	M	Gold miner	Cirrhosis
10	37	Belém/PA	M	Guard	Cirrhosis (died)
11	39	Itaituba/PA	M	Gold miner	Cirrhosis
12	27	Jacareacanga/PA	F	Housewife	Cirrhosis
13	43	Santarém/PA	M	Mason	Cirrhosis (died)
14	42	Marabá/PA	M	Gold miner	Cirrhosis
15	38	Altamira/PA	M	Operator of Centrifugal machine	NA
16	28	Jacareacanga/PA	M	Gold miner	Cirrhosis
17	49	Barcarena/PA	M	NA	Chronic hepatitis (asymptomatic)

^a M, male; F female.

^b NA, nonavailable.

antigen coding gene was amplified, as previously described (Sitnik et al., 2004).

HDV RNA was extracted from 100 µl serum by the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987) and dissolved in 10 µl DEPC-treated water. RNA was previously denatured at 95 °C for 5 min and then it was added 20 µl of the reverse transcription (RT) mix [25 pmol of primer 1302OD, 0.2 mM each of the four dNTPs, 20 units RNaseOUT (Invitrogen™ Life Technologies, Carlsbad, CA, USA) and 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen™ Life Technologies, Carlsbad, CA, USA) in buffer supplied by the manufacturer]. The reaction mix was incubated at 37 °C for 60 min and stopped at 95 °C for 15 min. For the first polymerase chain reaction, 10 µl of the previous mix was added to 40 µl of a PCR mix [20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each of the four dNTPs, 25 pmol of primers 853IU and 1302OD, and 1.25 units of Taq DNA polymerase (Invitrogen™ Life Technologies, Carlsbad, CA, USA)]. PCR was carried out in a thermocycler under the following conditions: 2 min at 94 °C, followed by 40 cycles of 30 s at 94 °C, 50 s at 55 °C and 45 s at 72 °C with a final extension step of 5 min at 72 °C. For the second PCR, 5 µl of the product from the first round were added to another tube containing 45 µl of same PCR mix but with 25 pmol of inner primers HDV-E and HDV-A, using the same temperature cycling conditions for the first PCR.

HBV DNA was extracted with same methodology from HDV RNA and dissolved in 50 µl sterile water. HBV DNA (10 µL) was amplified by nested PCR as previously described by Sitnik et al. (2004). In order to confirm the HBV genotypes determined by the analysis of the S region, we also analyzed the pre-core/core region (501-nucleotide fragment) using a set of primers described by Kaneko et al. (1989) and Takahashi et al. (1995) with the following cycling temperatures: 1 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C with a final extension step of 5 min at 72 °C. Positive and negative controls were included in each set of reactions. The second PCR products were submitted to electrophoresis on a 2% agarose gel.

2.3. Precautions to avoid PCR contamination

To avoid false-positive results, strict procedures proposed for nucleic acid amplification diagnostic techniques were followed during pre- and post-amplification work (Kwok and Higuchi, 1989). The extraction of nucleic acids, the PCR setup and especially the addition of first round PCR reaction to second round PCR and PCR

product analysis were performed in completely separated areas. Each stage was performed with separate micropipettes and all PCR procedures were carried out using aerosol filter tips. Other precautions such as frequent changing of gloves, working with reagents divided in aliquot, control of daily flow of personnel only from pre- to post-amplification areas and the use of UV-light and sodium hypochlorite solution to decontaminate surfaces were strictly observed in order to prevent contamination.

2.4. Nucleotide sequencing of HDV and HBV

Both strands of the second PCR products were submitted to cycle sequencing with the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA) and the same primers utilized in the nested PCR, according to the manufacturer's instructions. Sequencing gels were run on an automated ABI Prism 377 DNA Sequencer (Applied Biosystems).

2.5. Sequence alignment and phylogenetic analyses

Sequences were validated using the Phred-Phrap software and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using Consed (Ewing et al., 1998; Ewing and Green, 1998; Gordon et al., 1998). Alignments of consensus sequences were carried out by using CLUSTAL X (version 1.81) (Thompson et al., 1997). MEGA version 2.1 (<http://www.megasoftware.net/>) was used to analyze amino acid sequences (Kumar et al., 2001).

The nucleotide sequences of HDV and HBV obtained in this study were compared with previously reported sequences retrieved from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA). The accession numbers of the reference sequences used were as follows:

HBV: AY738814, AF209400, AY344103, X75669, U55221, D00330, D00331, AF241411, D23680, X75656, AY090453, U95551, X97849, X75657, X75664, AB036911, AY090455, X69798, AB056513, AF160501, AY090457, AY090460.

HDV: Colombia (L22061), Peru-1 (L22063), Peru-2 (L22064), VnzD8624 (AB037949), VnzD8375 (AB037947), Somalia (U81988), Lebanon 1 (M84917), US-1 (D01075), Italy-A20 (X04451), CHINA (X77627), Japan-S (X60193), Taiwan 3 (U19598), Yakut-62 (AJ309880), Taiwan2476 (AF104264), Yakut26 (AJ309879), Taiwan-2b (AF018077), Miyako (AF309420), L215 (AB088679), Taiwan (AF209859), Tokyo (AB118847), dFr-910 (AX741159), dFr-47

(AX741149), dFr-73 (AX741154), dFr-69 (AJ583877), dFr-1953 (AJ583886), dFr-2020 (AJ583887), dFr-48 (AJ583871), dFr-1594 (AJ583884), dFr-45 (AJ583868), dFr-1843 (AJ583885), dFr-2066 (AJ583888).

Phylogenetic tree was constructed by maximum likelihood analyses using PAUP* software version 4.0b10 (Swofford, 2002), and the evolutionary model of DNA substitution and parameters used (HKY+G) were estimated by MODELTEST (Posada and Crandall, 1998). Robustness of the phylogenetic groups was evaluated using 1000 bootstrap replicates.

Nucleotide sequence dates have been deposited into the GenBank database under the following accession numbers: EF150915–EF150923 (HBV); EF150924–EF150937 (HDV).

3. Results

Among the 17 serum samples from patients from the Eastern Amazon that were positive for HBsAg and anti-HD, HDV RNA was detected in 15 (88.2%) and HBV DNA in 10 (58.8%) of them. HDV RNA and HBV DNA were both positive in nine (52.9%) samples. One sample was positive only for HBV DNA and another one was negative for both markers.

Fourteen HDV and nine HBV sequences were validated by Phred-Phrap-Consed analysis. One sequence from HDV and another from HBV did not achieve sufficient quality index and were not included in the phylogenetical analysis. These nine HBV sequences included the one from the patient that was HBV DNA positive only.

Fig. 2. (a) Phylogenetic tree of HDV partial delta antigen genomic region. (b) Phylogenetic tree of HBV S gene, partial region. ML analyses were performed with other sequences from different HDV or HBV genotypes described previously using PAUP* software. The Brazilian isolates are shown in bold letters and the HBV and HDV sequences obtained in GenBank are designated by their accession number or denominated according to the respective authors. Accession number of the all sequences from GenBank is described in Section 2. Bootstrap values from 1000 replicates are shown for the genotype branches (shown as a percentage).

Fig. 2. (Continued).

Phylogenetic analysis of HDV and HBV sequences were performed with other known sequences from different HDV and HBV genotypes described previously. The tree constructed showed that the all HDV sequences clustered with previously characterized sequences of HDV-3 from Peru, Venezuela and Colombia (Fig. 2a).

HDV isolates characterized in this study are closely related to one another and to other previously described HDV-3 sequences (nucleotide sequence identity in the analyzed region ranging from 93.4% to 99.3%), but different from other isolates (nucleotide sequence identity in the analyzed region was about 58.5–63%,

60.6–64%, 57.8–62.3%, 57.4–63.7%, 59.5–64.4%, 63.3–68.8% to genotypes 1, 2, 4, 5, 6 and 7, respectively).

The 19 carboxy-terminal amino acid sequence from the large hepatitis D antigen (L-HDAg) was not completely conserved among the different isolates from the Eastern Amazon Basin, having showed a greater diversity than other previously characterized HDV-3 sequences (Fig. 3).

Phylogenetic analysis of the S gene sequences showed that all HBV isolates clustered with HBV genotype A (Fig. 2b). Sequences of the pre-core/core region of HBV isolates from seven patients were also analyzed and the results confirmed the HBV genotype classi-

	119	129	139	149	159	169	179	189	199	209	
HDV-3 CO	GGRLSREER	ELERLMDDD	ERERRVAGPR	PGGVNHDGP	PHGAPGGQV	PSLQGVFSP	FSRYGEGIDI	RGNDHPP	YG	ETPPPPGQW	VPGCTDQ
HDV-3 PE	..M..Q...	..R...R..	Y...					..T....		X..	
HDV-3 VE	..M.....	..R...R..						..T....		X..	
Patient6	Q...	..R...R..	A...					..T....			
Patient7		..Q.....									A....
Patient13	..M.....	..R...R..	S...	V..		..N.....		..T....	..L....	..Y..F	
Patient12	..M..Q...	..R...R..				..N.....		..T....	..L....	..Y..	
Patient9	..M.....	..R...R..	Y...					..T....			
Patient8	..M.....	..R...R..	K...			..N.....		..L....	..A....	..Y..	
Patient1	Q...	..Q...R..	T...	A..				..T....	..S....	..Y..R	
Patient2	..M.....	..Q...R..					..L..V	..T....		..Y..R	
Patient3	..M..L..G	..Q...R..				..N.....		..T....			
Patient14	Q...	..Q...R..				..N.....		..T....			
Patient4	Q...	..Q...R..	Y...			..N.....		..T....			
Patient10	..M.....	..R...R..	S...								
Patient11	..M..Q...	..R...R..				..N.....					
Patient5	Q...		T...								
HDV-1	..M..K...	..R...TEE..E	..R...I...Q	..V...LE..G	..T...		..N.....	..T....	..S..G..	..DI..LF..	..PSSP..US..RP..
HDV-2	..I..K...	..R...TDE..E	..K...V..D...	..SR..G		..QNA.....		..T..G..	..VS..PS..	..QSLPL..LE..	..P..
HDV-4	..M.....	..R...TEE..E	..R...V..D...	..L..G		..HNDI.....	..T..R..D..L..V	..A..E..	..VS..PQ..	..RLPL..LE..	..P..
HDV-5	..S.....	..RK...TEE..E	..R...V..D...	..FE..		..QNEI.....	S..L..V		..HVD..PG..	..RLPL..LE..	..P..
HDV-6	..M.....	..G...TGE..E	..Q..K...T	..V..D...	..FG..D		..TNEH.....	L..V	..T...	..GN..TP..	..RLPL..LE..
HDV-7	..S..K...	..RK...TA...E	..Q..A...I	..V..D...	..FE..		..Q..L.....	L..	..DR..	..GP..SPT...	..RLPL..LE..

LHO Packaging signal
(prolin-rich domain)

LHD Packaging signal
(prolin-rich domain)

Fig. 3. Amino acid alignments of the sequences from the large hepatitis D antigen (L-HDAg) of the HDV isolates. Numbers indicate the amino-acid position in the HDV small and large antigens. The asterisks indicate the stop codon for S-HDAg, which is edited to render a tryptophan in the large HDV antigen. Genotype variation in the 19–20 carboxyl-terminal region of the large HDV antigen is showed in box.

fication as obtained by the analysis of the S gene sequences from these isolates (data not shown).

All HBV sequences encoded subtype *adw2*, defined by presence of Lys¹²², Lys¹⁶⁰ and Pro¹²⁷ in the S gene region.

4. Discussion

In this study, we describe HDV and HBV genotypes in chronically infected patients from the Eastern Amazon Basin. To our knowledge, this is the first study devoted to elucidate HDV genotypes circulating in this region. HDV genotype 3 was identified among these patients confirming data obtained from other authors which demonstrated that it is the most prevalent genotype in the North of South America (Casey et al., 1993, 1996b; Quintero et al., 2001; Nakano et al., 2001).

Despite the importance of HDV infection in some Brazilian regions, few studies have detailed the HDV genotypes circulating in Brazil. By means of RT-PCR and hybridization, Paraná et al. (2006) identified the HDV genotype 1 in 55% of the chronically infected patients from the Western Brazilian Amazon included in his study. This profile differed from what was encountered in previous studies, which demonstrated the prevalence of the genotype 3 in other regions of South America. These authors suggested that this result might be explained by the fact that they have studied few patients with Amerindian origin. However, in spite of the fact that our study did not include any Amerindian patient, only the genotype 3 was identified. Other studies should be carried out to better elucidate the discrepancy among HDV genotypes in patients from the Eastern and Western Amazon Basin, involving Amerindians and non-Amerindians individuals. The Amazon Basin is a region with continental dimensions and very diverse human populations, and this might be the cause of such discrepancies. This fact reinforces the need to analyze the differences in the distribution of HDV genotypes among its different regions and populations.

HDV genotype 3 is described as a more pathogenic strain, generally related to fulminant hepatitis. In this study, however, no case of fulminant hepatitis occurred. We have identified this genotype in 14 patients with cirrhosis and in only one patient with asymp-

tomatic infection. As suggested by other authors, HDV-3 infection was associated with severe liver disease (Casey et al., 1993, 1996b; Paraná et al., 2006) despite the absence of fulminant hepatitis cases.

Many previous works have reported that HDV infection suppress HBV replication. In our study, HBV was detected in only nine (60%) out of 15 HDV RNA positive samples.

All previous studies from South America reported HDV genotype 3 co-infecting HBV genotype F patients (Casey et al., 1996b; Quintero et al., 2001; Nakano et al., 2001). In contrast to them, in our study, HDV genotype 3 was found co-infecting HBV genotype A patients. To our knowledge, this is the first description of a co-infection of HDV genotype 3 with HBV genotype A (subtype *adw2*).

The finding of the co-infection HDV-3/HBV-A suggests that there is not a specific interaction between HBV and HDV genotypes, and co-infection may merely reflect the most frequent genotypes found in a particular geographic area. Genotype A has already been reported as the most prevalent in the Eastern Amazon (Conde et al., 2004).

On the other hand, it is possible that the association between HDV-3 and HBV-F leads to a more severe clinical course, as frequent fulminant hepatitis outbreaks have been reported. Bensabath et al. (1987) described the association of HDV infection and fulminant hepatitis at Boca do Acre, Western Amazon, which is the region where Viana et al. (2005) reported the finding of HBV genotypes A and F. The simultaneous description of HDV and HBV genotypes found in a particular area together with clinical data of cases from that area may contribute to elucidate this issue.

The analysis of the carboxy-terminal region of the L-HDAg, which interacts with the hepatitis B surface antigen and is essential for HDV assembly, showed some diversity between the different isolates from the Eastern Amazon. This diversity is not observed among HDV-3 sequences from other South American regions, as reported in some studies (Casey et al., 1993, 1996b; Quintero et al., 2001; Nakano et al., 2001). However, these studies have analyzed a limited quantity of samples and this may explain the smaller diversity. Therefore, the analysis of a higher number of sequences may be necessary to provide better information about the diversity of the carboxy-terminal region of the L-HDAg within the HDV genotype 3.

It is noteworthy that eight (47%) among 17 HDV positive patients were gold miners from Rio Tapajós valley where the Santarém, Itaituba and Jacareacanga counties are located. As this zone has a high prevalence of HBV infection, this particular group may potentially contribute to the dissemination of HDV infection among the population in this area (Santos et al., 1995).

Acknowledgments

This work was supported by FAPESP (No. 00/11457-1), Instituto Evandro Chagas/SVS/MS and Alves de Queiroz Family Fund for Research.

References

- Bensabath, G., Hadler, S.C., Soares, M.C., Fields, H., Dias, L.B., Popper, H., Maynard, J.E., 1987. Hepatitis delta virus infection and Labrea hepatitis. Prevalence and role in fulminant hepatitis in the Amazon Basin. *JAMA* 258, 479–483.
- Buitrago, B., Popper, H., Hadler, S.C., Thung, S.N., Gerber, M.A., Purcell, R.H., Maynard, J.E., 1986. Specific histologic features of Santa Marta hepatitis: a severe form of hepatitis delta-virus infection in northern South America. *Hepatology* 6, 1285–1291.
- Casey, J.L., Brown, T.L., Colan, E.J., Wignall, E.S., Gerin, J.L., 1993. A genotype of hepatitis D virus that occurs in northern South America. *Proc. Natl. Acad. Sci. U. S. A.* 90, 9016–9020.
- Casey, J.L., 1996a. Hepatitis Delta Virus: genetics and pathogenesis. *Clin. Lab. Med.* 16, 451–464.
- Casey, J.L., Niro, G.A., Engle, R.E., Vega, A., Gomez, H., McCarthy, M., Watts, D.M., Hyams, K.C., Gerin, J.L., 1996b. Hepatitis B virus (HBV)/hepatitis D virus (HDV) coinfection in outbreaks of acute hepatitis in the Peruvian Amazon basin: the roles of HDV genotype III and HBV genotype F. *J. Infect. Dis.* 174, 920–926.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Conde, S.R.S.S., Moia, L.J.P., Barbosa, M.S.B., Amaral, I.S.A., Miranda, E.C.B.M., Soares, M.C.P., Brito, E.M.F., Souza, O.S.C., Araújo, M.T., Demachki, S., Pinho, J.R.R., Gomes, M.M.S., Bertolini, D.A., Ishak, R., 2004. Prevalência de genótipos e de mutantes pré-core A-1896 do vírus da hepatite B e suas implicações na hepatite crônica, em uma população da Amazônia Oriental. *Rev. Soc. Bras. Med. Trop.* 37, 33–39.
- Ewing, B., Green, P., 1998. Base-Calling of automated sequencer traces using Phred II. Error Probabilities. *Genome Res.* 8, 186–194.
- Ewing, B., Hillier, L., Wendt, M.C., Green, P., 1998. Base-Calling of automated sequencer traces using Phred I. Accuracy Assessment. *Genome Res.* 8, 175–185.
- Gordon, D., Abajian, C., Green, P., 1998. Consed: a graphical tool for sequence finishing. *Genome Res.* 8, 195–202.
- Guettouche, T., Hnatyszyn, H.J., 2005. Chronic hepatitis B and viral genotype: the clinical significance of determining HBV genotypes. *Antivir. Ther.* 10, 593–604.
- Hadler, S.C., De Monzon, M., Ponzetto, A., Arzola, E., Rivera, D., Mondolfi, A., Bracho, A., Francis, D.P., Gerber, M.A., Thung, S., Gerin, J., Maynard, J.E., Popper, H., Purcell, R.H., 1984. Delta virus infection and severe hepatitis. An epidemic in the Yucpa Indians of Venezuela. *Ann. Intern. Med.* 100, 339–344.
- Imazeki, F., Omata, M., Ohto, M., 1990. Heterogeneity and evolution rates of delta virus RNA sequences. *J. Virol.* 64, 5594–5599.
- Ivanushina, V., Radjef, N., Alexeeva, M., Gault, E., Semenov, S., Salhi, M., Kiselev, O., Dery, P., 2001. Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia. *Russ. J. Gen. Virol.* 82, 2709–2718.
- Kaneko, S., Feinstone, S.M., Miller, R.H., 1989. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J. Clin. Microbiol.* 27, 1930–1933.
- Karayiannis, P., 1998. Hepatitis D Virus. *Rev. Med. Virol.* 8, 13–24.
- Kumar, S., Tamura, K., Jakobsen, I.B., Nei, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis Software. *Bioinformatics* 17, 1244–1245.
- Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. *Nature* 339, 237–238.
- Lee, C.M., Changchien, C.S., Chung, J.C., Liaw, Y.F., 1996. Characterization of a new genotype II hepatitis delta virus from Taiwan. *J. Med. Virol.* 49, 145–154.
- Ljunggren, K.E., Patarroyo, M.E., Engle, R., Purcell, R.H., Gerin, J.L., 1985. Viral hepatitis in Colombia: a study of the "hepatitis of the Sierra Nevada de Santa Marta". *Hepatology* 5, 299–304.
- Manock, S.R., Kelley, P.M., Hyams, K.C., Douce, R., Smalligan, R.D., Watts, D.M., Sharp, T.W., Casey, J.L., Gerin, J.L., Engle, R., Alava-Alprecht, A., Martinez, C.M., Bravo, N.B., Guevara, A.G., Russell, K.L., Mendoza, W., Vimos, C., 2000. An outbreak of fulminant hepatitis delta in the Waorani, an indigenous people of the Amazon basin of Ecuador. *Am. J. Trop. Med. Hyg.* 63, 209–213.
- Nakano, T., Shapiro, C.N., Hadler, S.C., Casey, J.L., Mizokami, M., Orito, E., Robertson, B.H., 2001. Characterization of hepatitis D virus genotype III among Yucpa Indians in Venezuela. *J. Gen. Virol.* 82, 2183–2189.
- Paraná, R., Kay, A., Molinet, F., Viana, S., Silva, L.K., Salcedo, J.M., Tavares-Neto, J., Lobato, C., Rios-Leite, M., Matteoni, L., D'Oliveira Jr., A., Tauli, P., Trepo, C., 2006. HDV genotypes in the Western Brazilian Amazon region: a preliminary report. *Am. J. Trop. Med. Hyg.* 75, 475–479.
- Popper, H., Thung, S.N., Gerber, M.A., Hadler, S.C., de Monzon, M., Ponzetto, A., Arzola, E., Rivera, D., Mondolfi, A., Bracho, A., 1983. Histologic studies of severe delta agent infection in Venezuelan Indians. *Hepatology* 3, 906–912.
- Posada, D., Crandall, K.A., 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Quintero, A., Uzcategui, N., Loureiro, C.L., Villegas, L., Illarramendi, X., Guevara, M.E., Ludert, J.E., Blitz, L., Liprandi, F., Pujol, F.H., 2001. Hepatitis delta virus genotypes I and III circulate associated with hepatitis B virus genotype F in Venezuela. *J. Med. Virol.* 64, 356–359.
- Radjef, N., Gordien, E., Ivanushina, V., Gault, E., Anais, P., Dragan, T., Trinchet, J.-C., Saito, D., Tamby, M., Milinkovitch, M.C., Dery, P., 2004. Molecular phylogenetic analyses indicate a wide and ancient radiation of African Hepatitis Delta Virus, suggesting a Deltavirus genus of at least seven major clades. *J. Virol.* 78, 2537–2544.
- Sakugawa, H., Nakasone, H., Nakayoshi, T., Kawakami, Y., Miyazato, S., Kinjo, F., Saito, A., Ma, S.P., Hotta, H., Kinoshita, M., 1999. Hepatitis delta virus genotype IIb predominates in an endemic area, Okinawa, Japan. *J. Med. Virol.* 58, 366–372.
- Santos, E.O., Loureiro, E.C.B., Jesus, I.M., Brabo, E., Da Silva, R.S.U., Soares, M.C.P., Câmara, V.M., Souza, M.R.S., Branches, F., 1995. Diagnóstico das condições de saúde de uma comunidade garimpeira na região do Rio Tapajós, Itaituba, Pará, Brasil 1992. *Cad. Saude Publica* 11, 212–225.
- Shakil, A.O., Hadziyannis, S., Hoofnagle, J.H., Di Bisceglie, A.M., Gerin, J.L., Casey, J.L., 1997. Geographic distribution and genetic variability of hepatitis delta virus genotype I. *Virology* 234, 160–167.
- Sitnik, R., Pinho, J.R.R., Bertolini, D.A., Bernardini, A.P., Da Silva, L.C., Carrilho, F.J., 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J. Clin. Microbiol.* 42, 2455–2460.
- Sjogren, M.H., Colichon, A., 1991. Hepatitis B and delta hepatitis infection in Peru. *Prog. Clin. Biol. Res.* 364, 97–104.
- Smedile, A., Farci, P., Verme, G., Caredda, F., Carniel, A., Caporaso, N., Dentico, P., Trepo, C., Opolon, P., Gimson, A., Vergani, D., Williams, R., Rizzetto, M., 1982. Influence of delta infection on severity of hepatitis B. *Lancet* 30, 945–947.
- Swofford, D.L., 2002. PAUP*, Phylogenetic Analysis Using Parsimony ("and Other Methods") Version 4. Sinauer Associates, Sunderland, Massachusetts.
- Takahashi, K., Aoyama, K., Ohno, N., Iwata, K., Akahane, Y., Baba, K., Yoshizawa, H., Mishi, S., 1995. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J. Gen. Virol.* 76, 3159–3164.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882.
- Viana, S., Paraná, R., Moreira, R.C., Compri, A.P., Macedo, V., 2005. High prevalence of hepatitis B virus and hepatitis D virus in the western Brazilian Amazon. *Am. J. Trop. Med. Hyg.* 73, 808–814.
- Wu, J.C., Chiang, T.Y., Sheen, I.J., 1998. Characterization and phylogenetic analysis of a novel hepatitis D virus strain discovered by restriction fragment length polymorphism analysis. *J. Gen. Virol.* 79, 1105–1113.
- Zhang, Y.Y., Tsega, E., Hansson, B.G., 1996. Phylogenetic analysis of hepatitis D viruses indicating a new genotype I subgroup among African isolates. *J. Clin. Microbiol.* 34, 3023–3030.

- 28- Gomes-Gouvêa MS, Soares MC, Bensabath G, de Carvalho-Mello IM, Brito EM, Souza OS, Queiroz AT, Carrilho FJ, PINHO JR. Hepatitis B and Delta virus genotypes in Outbreaks of fulminant Hepatitis (Labrea black fever) in the Western Brazilian Amazon region. Journal of General Virology. 2009 Nov;90 (Pt 11):2638-2643.**

**Short
Communication****Correspondence**

M. S. Gomes-Gouvêa
gomesmic@yahoo.com.br

Received 20 May 2009

Accepted 13 July 2009

Hepatitis B virus and hepatitis delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the western Brazilian Amazon region

M. S. Gomes-Gouvêa,^{1,2} M. C. P. Soares,² G. Bensabath,²
I. M. V. G. de Carvalho-Mello,^{1,3} E. M. F. Brito,² O. S. C. Souza,²
A. T. L. Queiroz,⁴ F. J. Carrilho¹ and J. R. R. Pinho¹

¹Laboratory of Tropical Hepatology and Gastroenterology, Tropical Medicine Institute, Department of Gastroenterology, University of São Paulo Medical School, Av. Dr Enéas Carvalho de Aguiar 500, São Paulo, SP 05403-000, Brazil

²Seção de Hepatologia, Instituto Evandro Chagas, Avenida Almirante Barroso 492, Belém, PA 66090-000, Brazil

³Laboratório de Imunologia Viral, Instituto Butantan, Avenida Vital Brasil 1500, São Paulo, SP 05503-900, Brazil

⁴Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, São Paulo, SP 05508-900, Brazil

The genotypes of hepatitis B (HBV) and delta (HDV) viruses circulating among fulminant hepatitis cases from the western Amazon Basin of Brazil were characterized in this study. HBV and HDV isolates were obtained from liver samples from 14 patients who developed fulminant hepatitis and died during 1978–1989. HBV DNA and HDV RNA were detected in all samples. Phylogenetic analyses of HDV sequences showed that they all clustered with previously characterized sequences of HDV genotype 3 (HDV-3). HBV genotypes F, A and D were found in 50.0, 28.6 and 21.4% of cases, respectively. These results confirm the predominance of HDV-3 in South America and its association with the severe form of hepatitis, and the finding of the co-infection of HDV-3 with different genotypes of HBV suggests that the association between HDV-3 and HBV-F is not necessarily causally related to a more severe clinical course of infection.

The Brazilian western Amazon Basin is considered a highly endemic area for hepatitis B (HBV) and delta (HDV) viruses (Bensabath & Dias, 1983; Bensabath *et al.*, 1987; Braga *et al.*, 2001; Fonseca *et al.*, 1988; Viana *et al.*, 2005). Severe hepatitis cases with peculiar clinical and histopathological features have been reported in this region. Cases of fulminant hepatitis with similar features have been described in other countries in northern South America (Colombia, Venezuela, Peru and Ecuador) and in the Central African Republic. This severe form of liver disease has been identified as HBV and HDV super- or co-infection (Bensabath *et al.*, 1987; Buñtrago *et al.*, 1986; Casey, 1996; Casey *et al.*, 1996; Hadler *et al.*, 1984; Lesbordes *et al.*, 1987; Ljunggren *et al.*, 1985; Manock *et al.*, 2000; Popper *et al.*, 1983; Sjogren & Colichon, 1991; Torres & Mondolfi, 1991).

The GenBank/EMBL/DBJ accession numbers for the sequences reported in this paper are FJ010634–FJ010647 (HDV) and FJ010648–FJ010661 (HBV).

A supplementary table and two supplementary figures are available with the online version of this paper.

The disease associated with HDV infection is typically more severe than that due to HBV infection alone, but its clinical spectrum ranges from asymptomatic carriage of the virus to very severe disease. A factor that may influence the course of disease is the genetical heterogeneity of HDV prevalent in different geographical areas (Casey, 1996; Rizzetto & Durazzo, 1991; Smedile *et al.*, 1982). Only HDV genotype 3 (HDV-3) was identified in cases of fulminant hepatitis from different countries of South America and has been associated with the co-infecting HBV genotype F (HBV-F), which is also indigenous to South America, suggesting that HDV-3 alone or in combination with HBV-F could be an important determinant of the particularly severe form of HDV-related disease in this region (Casey, 1996; Nakano *et al.*, 2001a, b).

Bensabath *et al.* (1987) carried out a 5 year protocol during 1979–1984 in the Brazilian western Amazon Basin to study the epidemiology of HDV infection and its role in the aetiology of fulminant hepatitis. In that study, the authors observed a high endemicity of HBV and HDV infection and confirmed the presence of HDV as a major factor for

fulminant hepatitis development in this area (Bensabath *et al.*, 1987). Nevertheless, as HDV infection could not be demonstrated in all cases of hepatitis fulminant, its exact role in these cases remains unclear. In the present study, we investigated the presence of HDV by molecular techniques and characterized the circulating HBV and HDV genotypes among fulminant hepatitis cases from the Brazilian western Amazon Basin detected during 1978–1989.

Liver samples from 14 patients who developed fulminant hepatitis and died were collected in three contiguous municipalities on the Purus River in the western Amazon Basin of Brazil (Boca do Acre and Pauini, in Amazonas state, and Sena Madureira, in Acre state) (Fig. 1). All patients were positive for HBsAg; two were also positive for anti-HBc IgM, a serological pattern consistent with acute HBV infection. Only eight (57%) patients had evidence of HDV infection in serum (HDsAg and anti-HD) and/or liver tissue (HDsAg) as shown by serological and immunoperoxidase assays, respectively. Anti-HAV IgM and anti-HCV were negative in all samples. The serological patterns found in these cases suggested HDV superinfection of HBV chronic carriers in 12 cases and HDV/HBV co-infection in two cases. Supplementary Table S1 (available in JGV Online) shows the demographic, clinical and epidemiological aspects of the studied cases. This study received institutional review board approval.

The results of the serological tests were confirmed by using bioMérieux kits for HBV and HDV serological markers. HBV DNA and HDV RNA were extracted from frozen liver samples stored at -70°C with QIAamp DNA and RNeasy Mini kits (Qiagen), respectively, according to the manu-

facturer's instructions. HDV RNA was amplified as described previously (Gomes-Gouveia *et al.*, 2008) and HBV DNA was amplified by using a set of primers that amplify a 734 bp fragment partially covering the overlapping DNA polymerase- and surface antigen-encoding genes. Primers used for HBV amplification were FHBS1 (Sitnik *et al.*, 2004) and RADE1M (5'-TGCRTCA-GCAAACACTTGGC-3'; nt 1175–1194) for the first round, and FHBS2 (Sitnik *et al.*, 2004) and RADE2M (5'-TGRCANACYTTCCARTCAATNGG-3'; nt 992–970) for the second round. The nucleotide position was based on the HBV-A sequence (GenBank accession no. M57663). The sequences of primers RADE1M and RADE2M were designed for this study. The PCR was carried out for 35 cycles of [94 $^{\circ}\text{C}$ for 30 s, 56 $^{\circ}\text{C}$ (first round) or 50 $^{\circ}\text{C}$ (second round) for 30 s, 72 $^{\circ}\text{C}$ for 1 min] after a step of 1 min at 94 $^{\circ}\text{C}$. The amplified products were separated in a 2% agarose gel stained with ethidium bromide to confirm the expected length of the amplicon. Strict procedures for nucleic acid amplification diagnostic techniques were followed to avoid false-positive results (Kwok & Higuchi, 1989). Moreover, positive and negative controls were included in each set of reactions.

HBV DNA and HDV RNA were positive in all analysed samples. The nested PCR products were purified by using a ChargeSwitch PCR Clean-Up kit (Invitrogen). Cycle-sequencing reactions of purified PCR products were performed by using a BigDye Terminator kit v3.1 (Applied Biosystems) and the same primers as were utilized in the second-round PCR for HDV and the primers FHBS2 (sense), RHBS2 (antisense), 5'IAM5 (sense) (Da Silva *et al.*, 2001; Sitnik *et al.*, 2004) and RADE2M (antisense) for HBV, according to the manufacturer's instructions. Each amplicon was analysed in both sense and antisense directions. The sequences were read in an automated ABI Prism 377 DNA Sequencer (Applied Biosystems).

Sequences obtained were aligned by using CLUSTAL_X (Thompson *et al.*, 1997) with previously reported sequences retrieved from GenBank, resulting in alignments of 627 nt for HBV and 319 nt for HDV. Phylogenetic analyses for genotyping were estimated by using distance and maximum-likelihood (ML) approaches implemented in the PAUP* v4.0b package (Swofford, 2002). The evolutionary model of DNA substitution and parameters used (HKY+I+G for HDV and GTR+I+G for HBV) were estimated by MODELTEST v3.06 (Posada & Crandall, 1998).

Phylogenetic analyses of HDV sequences from fulminant hepatitis cases showed that all of them clustered with previously characterized sequences of HDV-3 from South America (Fig. 2). Considering all sequenced isolates from the Brazilian Amazon (western and eastern), we observed that they did not group in one specific, closely related cluster. In the tree obtained by using the neighbour-joining method, three main clusters are clearly observed: clusters

Fig. 1. Geographical locations of the Boca do Acre, Pauini and Sena Madureira municipalities in the western Amazon Basin of Brazil.

Fig. 2. Unrooted phylogenetic tree constructed by the maximum-likelihood method based on HDV sequences from this study (marked ▲) and from GenBank (denoted by accession no. followed by the country of origin). Percentages at nodes represent bootstrap values obtained with 1000 replications. Only values >70% are shown. Genotypes are indicated.

with only Brazilian isolates, clusters with western Venezuelan and Colombian isolates and clusters with southern Venezuelan and Peruvian isolates (Supplementary Fig. S1a, b, available in JGV Online).

The phylogenetic trees show that some HDV sequences from patients of the same municipality of the western Amazon were grouped in different clusters. It is also observed in these trees that the fulminant cases occurring

in the same municipality in different years were caused by closely related viruses (Supplementary Fig. S1a, b, available in JGV Online).

The divergence in nucleotide sequence of HDV-3 from fulminant cases characterized in this study ranged from 0 to 7.4% ($4.7 \pm 1.6\%$, mean $\pm$ SD). Analyses of HDV-3 nucleotide sequences from the Brazilian Amazon (eastern and western) show a divergence of 0–8.4% ($4.4 \pm 1.4\%$), while between all HDV-3 sequences from other South American countries (Peru, Colombia and Venezuela), this divergence was 0–6.6% ($2.9 \pm 1.5\%$).

The 19 aa sequences of the C terminus of the large HDAG (the packaging-signal sequence) were not completely conserved between HDV-3 isolates characterized in this study, as only 14 (73.7%) of the 19 aa were conserved among them (Supplementary Fig. S2, available in JGV Online).

The tree constructed by phylogenetic analysis using the neighbour-joining (Fig. 3) and maximum-likelihood (data not shown) methods showed that HBV genotypes A, D and F were present in acute fulminant hepatitis cases that occurred in the western Brazilian Amazon. Genotype F was found in seven (50.0%) cases, whereas non-F genotypes were found in the other 50% of the cases: four (28.6%) genotype A and three (21.4%) genotype D. By using this dataset, it was also possible to assign subgenotypes within each genotype characterized in this study; subgenotypes A1, F2a, D3 and D4 were observed in Brazilian strains.

Our results confirmed that acute HBV and HDV co-infection and mainly acute HDV superinfection were the primary cause of severe hepatitis in the Brazilian western Amazon Basin, as has been observed in other countries of South America where outbreaks of severe hepatitis also occurred.

All HDV isolates from fulminant hepatitis cases that occurred in the western Amazon were classified as HDV-3. This result is consistent with other studies carried out in Peru, Colombia and Venezuela (Casey, 1996; Casey *et al.*, 1993; Nakano *et al.*, 2001b) and confirms the role of this genotype as the causal agent of outbreaks of fulminant hepatitis in South America.

The results of phylogenetic analysis showed that HDV-3 sequences from Brazil did not group as a specific, closely related cluster, as was observed with isolates from Venezuela (the country with the largest number of sequences characterized after Brazil), where HDV-3 grouped in specific clusters depending on the origin of the samples (northwest or south). These results and the higher nucleotide divergence observed between HDV-3 sequences from the Brazilian Amazon suggest that these isolates have a longer history within this region than HDV-3 characterized in other South American regions. This hypothesis needs to be confirmed by analysis of a larger number of HDV-3 sequences from other regions of South America where few HDV-3 sequences have been

Fig. 3. Unrooted phylogenetic tree constructed by the neighbour-joining method based on HBV sequences from this study (marked ▲) and from GenBank (denoted by accession no. followed by the country of origin). Percentages at nodes represent bootstrap values obtained with 1000 replications. Only values >70% are shown. Genotypes and subgenotypes are indicated.

characterized. More extensive sampling of HDV-3 sequences, mainly full genomes, would be helpful in understanding the evolutionary history of HDV-3 in South America.

In the tree topology of HDV sequences, it was observed that isolates from patients of the same municipality of the western Amazon were grouped in different clusters; this pattern may represent the introduction of different virus populations with distinct origins or indicate that they have diverged from a common ancestor and are probably evolving independently. We also observed that one specific strain was involved in fulminant cases in the same municipality in different years, suggesting the continual transmission of this HDV strain. This is likewise suggested by the fact that some cases showed evidence of having been exposed to the same source of infection, as their sequences showed 100% nucleotide similarity and grouped in the same branch in the phylogenetic tree with strong bootstrap support.

Contrary to previous studies involving outbreaks of fulminant hepatitis among Yucpa Indians in Venezuela (Nakano *et al.*, 2001a, b) and military troops in the Amazon Basin region of Peru (Casey *et al.*, 1996), where HDV-3 was always associated with HBV-F, we found HBV-F in 50.0% of the cases, HBV-A in 28.6% and HBV-D in 21.4%, suggesting that the association between HDV-3 and HBV-F is not necessarily causally related to a more severe clinical course of infection. These results confirm that there is not a specific interaction between particular HBV and HDV genotypes and that co-infections reflect the most frequent genotypes found in a particular geographical area. Previous studies have also described co-circulation of HBV-A, -D and -F in the western Brazilian Amazon (Viana *et al.*, 2005; Victoria Fda *et al.*, 2008). Our results also are in agreement with previous *in vitro* studies carried out by Shih *et al.* (2008), who did not observe any influence of the different HBV genotypes on assembly and secretion of different HDV genotypes.

In the western Amazon, HDV infections were described to be associated with different clinical manifestations (Bensabath *et al.*, 1987). This difference in the pattern of evolution may be caused by (i) factors related to the genetic characteristics of HDV or specifically of HDV-3; (ii) characteristics of the HBV helper, mainly the structure and level of HBsAg, or (iii) genetic background of the host or altered immune response due to other chronic infections and other cofactors, such as poor nutrition, alcohol, environmental toxins etc.

Different HDV genotypes may be related to the different outcomes observed between patients from this region. HDV-1 was reported previously in Venezuela (Quintero *et al.*, 2001) and Amazonas state, Brazil (Parana *et al.*, 2006). However, the fact that we previously found HDV-3 associated with chronic infection (Gomes-Gouveia *et al.*, 2008) and in this study with fulminant infection shows that there may be differences in the pathogenicity of the different HDV-3 isolates. Characterization of the HDV isolates associated with different clinical manifestations that occur in the Brazilian western Amazon may elucidate whether any genomic structure related to virus replication could explain this issue.

We found that the amino acid sequences of the C terminus of the large HDAg were not completely conserved between HDV-3 isolates from fulminant cases characterized in this study. We did not observe a particular pattern in this region associated with fulminant evolution, as some amino acid sequence patterns were similar to those of other HDV-3 isolates from Brazil (eastern Amazon) that are associated with chronic infection (Gomes-Gouveia *et al.*, 2008). Hsu *et al.* (2002) reported that the differences in amino acid sequence of this region were reflected in variations in packaging efficiencies between HDV-1 and HDV-2 and among isolates of the same genotype. The authors suggested that these differences could at least partially explain the differences observed in disease outcome between patients infected with HDV-1 and those infected with HDV-2. The identification of similar patterns of amino acid sequence at the C-terminus region between HDV-3 isolates associated with chronic and fulminant infection suggests that this factor is not related to disease outcome, at least among these cases. Neither did we observe any relationship between a specific pattern of amino acid sequence at the C-terminus region and a particular genotype of HBV. Further studies are needed to clarify the role of the variation in the C terminus of the large HDAg in HDV-3 packaging efficiencies and in the ability to interact with HBsAg from different HBV genotypes.

In conclusion, these results confirming the predominance of HDV-3 in South America and its association with the severe form of hepatitis, and the finding of co-infections of HDV-3 with different genotypes of HBV, suggest that the association between HDV-3 and HBV-F is not necessarily causally related to a more severe clinical course of infection.

Acknowledgements

This work was supported by FAPESP (no. 00/11457-1), Instituto Evandro Chagas/SVS/MS-INCT/CNPq, and Alves de Queiroz Family Fund for Research.

References

Bensabath, G. & Dias, L. B. (1983). Labrea hepatitis (Labrea black fever) and other fulminant forms of hepatitis in Sena Madureira, Acre and Boca do Acre, Amazonas, Brazil. *Rev Inst Med Trop São Paulo* 25, 182–194 (in Portuguese).

- Bensabath, G., Hadler, S. C., Soares, M. C., Fields, H., Dias, L. B., Popper, H. & Maynard, J. E. (1987). Hepatitis delta virus infection and Labrea hepatitis. Prevalence and role in fulminant hepatitis in the Amazon Basin. *JAMA* 258, 479–483.
- Braga, W. S., Brasil, L. M., de Souza, R. A., Castilho Mda, C. & da Fonseca, J. C. (2001). The occurrence of hepatitis B and delta virus infection within seven Amerindian ethnic groups in the Brazilian western Amazon. *Rev Soc Bras Med Trop* 34, 349–355 (in Portuguese).
- Buitrago, B., Popper, H., Hadler, S. C., Thung, S. N., Gerber, M. A., Purcell, R. H. & Maynard, J. E. (1986). Specific histologic features of Santa Marta hepatitis: a severe form of hepatitis delta virus infection in northern South America. *Hepatology* 6, 1285–1291.
- Casey, J. L. (1996). Hepatitis delta virus. Genetics and pathogenesis. *Clin Lab Med* 16, 451–464.
- Casey, J. L., Brown, T. L., Colan, E. J., Wignall, F. S. & Gerin, J. L. (1993). A genotype of hepatitis D virus that occurs in northern South America. *Proc Natl Acad Sci U S A* 90, 9016–9020.
- Casey, J. L., Niro, G. A., Engle, R. E., Vega, A., Gomez, H., McCarthy, M., Watts, D. M., Hyams, K. C. & Gerin, J. L. (1996). Hepatitis B virus (HBV)/hepatitis D virus (HDV) coinfection in outbreaks of acute hepatitis in the Peruvian Amazon basin: the roles of HDV genotype III and HBV genotype F. *J Infect Dis* 174, 920–926.
- Da Silva, L. C., Pinho, J. R., Sitnik, R., Da Fonseca, L. E. & Carrilho, F. J. (2001). Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *J Gastroenterol* 36, 476–485.
- Fonseca, J. C., Simonetti, S. R., Schatzmayr, H. G., Castejon, M. J., Cesario, A. L. & Simonetti, J. P. (1988). Prevalence of infection with hepatitis delta virus (HDV) among carriers of hepatitis B surface antigen in Amazonas State, Brazil. *Trans R Soc Trop Med Hyg* 82, 469–471.
- Gomes-Gouveia, M. S., Pereira Soares Mdo, C., Guedes de Carvalho Mello, I. M., Brito, E. M., Pereira Moia Lde, J., Bensabath, G., Nunes, H. M., Carrilho, F. J. & Pinho, J. R. (2008). Hepatitis D and B virus genotypes in chronically infected patients from the Eastern Amazon Basin. *Acta Trop* 106, 149–155.
- Hadler, S. C., De Monzon, M., Ponzetto, A., Anzola, E., Rivero, D., Mondolfi, A., Bracho, A., Francis, D. P., Gerber, M. A. & other authors (1984). Delta virus infection and severe hepatitis. An epidemic in the Yuca Indians of Venezuela. *Ann Intern Med* 100, 339–344.
- Hsu, S. C., Syu, W. J., Sheen, I. J., Liu, H. T., Jeng, K. S. & Wu, J. C. (2002). Varied assembly and RNA editing efficiencies between genotypes I and II hepatitis D virus and their implications. *Hepatology* 35, 665–672.
- Kwok, S. & Higuchi, R. (1989). Avoiding false positives with PCR. *Nature* 339, 237–238.
- Lesbordes, J. L., Ravisse, P., Georges, A. J., Chevallier, P., Pichoud, C., Vitvitski, L. & Trepo, C. (1987). Studies on the role of HDV in an outbreak of fulminant hepatitis in Bangui (Central African Republic). *Prog Clin Biol Res* 234, 451–459.
- Ljunggren, K. E., Patarroyo, M. E., Engle, R., Purcell, R. H. & Gerin, J. L. (1985). Viral hepatitis in Colombia: a study of the “hepatitis of the Sierra Nevada de Santa Marta”. *Hepatology* 5, 299–304.
- Manock, S. R., Kelley, P. M., Hyams, K. C., Douce, R., Smalligan, R. D., Watts, D. M., Sharp, T. W., Casey, J. L., Gerin, J. L. & other authors (2000). An outbreak of fulminant hepatitis delta in the Waorani, an indigenous people of the Amazon basin of Ecuador. *Am J Trop Med Hyg* 63, 209–213.
- Nakano, T., Lu, L., Hu, X., Mizokami, M., Orito, E., Shapiro, C., Hadler, S. & Robertson, B. (2001a). Characterization of hepatitis B virus genotypes among Yuca Indians in Venezuela. *J Gen Virol* 82, 359–365.

- Nakano, T., Shapiro, C. N., Hadler, S. C., Casey, J. L., Mizokami, M., Orito, E. & Robertson, B. H. (2001b). Characterization of hepatitis D virus genotype III among Yucpa Indians in Venezuela. *J Gen Virol* 82, 2183–2189.
- Parana, R., Kay, A., Molinet, F., Viana, S., Silva, L. K., Salcedo, J. M., Tavares-Neto, J., Lobato, C., Rios-Leite, M. & other authors (2006). HDV genotypes in the Western Brazilian Amazon region: a preliminary report. *Am J Trop Med Hyg* 75, 475–479.
- Popper, H., Thung, S. N., Gerber, M. A., Hadler, S. C., de Manzon, M., Ponzetto, A., Anzola, E., Rivera, D., Mondolfi, A. & other authors (1983). Histologic studies of severe delta agent infection in Venezuelan Indians. *Hepatology* 3, 906–912.
- Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Quintero, A., Uzategui, N., Loureiro, C. L., Villegas, L., Illarramendi, X., Guevara, M. E., Ludert, J. E., Bliz, L., Uprandi, F. & Pujol, F. H. (2001). Hepatitis delta virus genotypes I and III circulate associated with hepatitis B virus genotype F in Venezuela. *J Med Virol* 64, 356–359.
- Rizzetto, M. & Durazzo, M. (1991). Hepatitis delta virus (HDV) infections. Epidemiological and clinical heterogeneity. *J Hepatol* 13 (Suppl. 4), S116–S118.
- Shih, H. H., Jeng, K. S., Syu, W. J., Huang, Y. H., Su, C. W., Peng, W. L., Sheen, I. J. & Wu, J. C. (2008). Hepatitis B surface antigen levels and sequences of natural hepatitis B virus variants influence the assembly and secretion of hepatitis delta virus. *J Virol* 82, 2250–2264.
- Sitnik, R., Pinho, J. R., Berblini, D. A., Bernardini, A. P., Da Silva, L. C. & Carrilho, F. J. (2004). Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 42, 2455–2460.
- Sjogren, M. H. & Colichon, A. (1991). Hepatitis B and delta hepatitis infection in Peru. *Prog Clin Biol Res* 364, 97–104.
- Smedile, A., Farci, P., Verme, G., Caredda, F., Cargnel, A., Caporaso, N., Dentico, P., Trepo, C., Opolon, P. & other authors (1982). Influence of delta infection on severity of hepatitis B. *Lancet* 2, 945–947.
- Swofford, D. L. (2002). PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4. Sunderland, MA: Sinauer Associates.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876–4882.
- Torres, J. R. & Mondolfi, A. (1991). Protracted outbreak of severe delta hepatitis: experience in an isolated Amerindian population of the Upper Orinoco basin. *Rev Infect Dis* 13, 52–55.
- Viana, S., Parana, R., Moreira, R. C., Compri, A. P. & Macedo, V. (2006). High prevalence of hepatitis B virus and hepatitis D virus in the western Brazilian Amazon. *Am J Trop Med Hyg* 73, 808–814.
- Victoria Fda, S., Oliveira, C. M., Victoria, M. B., Victoria, C. B. & Ferreira, L. C. (2008). Characterization of HBeAg-negative chronic hepatitis B in western Brazilian Amazonia. *Braz J Infect Dis* 12, 27–37.

- 29- Carrilho FJ, Ono-Nita SK, Cardoso RA, Cançado ELR, PINHO JRR, Alves VAF, Da Silva LC. A prospective study of Hepatitis B virus markers in patients with chronic HBV infection from Brazilian families of Western and Asian origin. Brazilian Journal of Medical and Biological Research 38 (9): 1399-1408 (2005).**

A prospective study of hepatitis B virus markers in patients with chronic HBV infection from Brazilian families of Western and Asian origin

F.J. Carrilho¹,
S.K. Ono-Nita¹,
R.A. Cardoso¹,
E.L.R. Cancado¹,
J.R.R. Pinho¹,
V.A.F. Alves²
and L.C. Da Silva¹

¹Setor de Hepatologia, Departamento de Gastroenterologia,
²Departamento de Patologia, Faculdade de Medicina,
Universidade de São Paulo, São Paulo, SP, Brasil

Abstract

The purpose of the present study was to determine the frequency of hepatitis B virus (HBV) markers in families of HBsAg-positive patients with chronic liver disease. Serum anti-HBc, HBsAg and anti-HBs were determined by enzyme immunoassay and four subpopulations were considered: genetically related (consanguineous) and non-genetically related (non-consanguineous) Asian subjects and genetically related and non-genetically related Western subjects. A total of 165 and 186 relatives of Asian and Western origin were enrolled, respectively. The occurrence of HBsAg and anti-HBs antibodies was significantly higher ($P < 0.0001$) in family members of Asian origin (81.8%) than in family members of Western origin (36.5%). HBsAg was also more frequent among brothers (79.6 vs 8.5%; $P < 0.0001$), children (37.9 vs 3.3%; $P < 0.0001$) and other family members (33.9 vs 16.7%; $P < 0.0007$) of Asian than Western origin, respectively. No difference between groups was found for anti-HBs, which was more frequently observed in fathers, spouses and other non-genetic relatives. HBV infection was significantly higher in children of Asian than Western mothers ($P < 0.0004$). In both ethnic groups, the mothers contributed more to their children's infection than the fathers ($P < 0.0001$). Furthermore, HBsAg was more frequent among consanguineous members and anti-HBs among non-consanguineous members. These results suggest the occurrence of vertical transmission of HBV among consanguineous members and probably horizontal sexual transmission among non-consanguineous members of a family cluster. Thus, the high occurrence of dissemination of HBV infection characterizes family members as a high-risk group that calls for immunoprophylaxis. Finally, the study showed a high familial aggregation rate for both ethnic groups, 18/19 (94.7%) and 23/26 (88.5%) of the Asian and Western origin, respectively.

Key words

- Hepatitis B
- Familial clustering
- HBsAg
- Vertical transmission
- Anti-HBc
- Anti-HBs

Correspondence

F.J. Carrilho
R. Silva Correla, 153, Apto. 41
04537-040 São Paulo, SP
Brasil
Fax: +55-11-3063-1559
E-mail: fjcarri@usp.br

Research supported in part by FINEP
and Alves de Queiroz Family Fund
for Research.

Publication supported by FAPESP.

Received July 16, 2004
Accepted April 26, 2005

Introduction

Several sero-epidemiological studies on the general and blood-donor populations have identified asymptomatic carriers of the viral surface antigen of hepatitis B (HBsAg). Upon investigation, a significant proportion of those individuals turned out to have some liver injury ranging from minor alterations to chronic active hepatitis, liver cirrhosis and hepatocellular carcinoma (1). Human chronic hepatitis B virus (HBV) infection is frequent in certain regions of the world, where the prevalence of HBV carriers in the general population is high (2).

In Brazil, studies on HBsAg prevalence were carried out in the general population, but mainly in volunteer candidates for blood donation (1). The observed prevalence was 0.3% in the South of the country and 11.3% in the Amazon region (1). For the State of São Paulo, about 1.0% was found in the capital and 0.3 to 1.3% in the interior of the state (1).

The prevalence of asymptomatic carriers varies among countries, ranging from low (0.1-2.0%) to medium (2.0-7.0%) and high (8.0-20.0%) (2). As discussed below, this prevalence also varies within countries from region to region, depending on different factors, such as socioeconomic conditions, the extent of drug addiction, the extent of alcoholism, professional blood donation, homosexual behavior, and there is also a high familial incidence (3).

The familial occurrence of the HBV infection has been well established for some ethnic groups. Initially, Ohbayashi et al. (4) reported 3 Japanese families in which 36 of the 54 members were HBsAg positive. Of these, some were healthy carriers while others had liver cirrhosis and hepatocellular carcinoma. Similar observations have been reported for the American (5), European (6) and Asian continents (7,8). However, there are few comparisons among populations of different ethnic backgrounds living in the

same country (8,9).

Recently, we showed that family members and household contacts of chronic HBV carriers are at high risk to acquire hepatitis B in an area of low hepatitis B prevalence (10).

In view of the need to obtain more information about the familial occurrence of HBV in Brazil and to compare it among different ethnic groups, the purpose of this study was to prospectively determine the familial aggregation, HBV markers in the progeny of infected mothers and fathers, and the frequency and analysis of variance of HBV markers per ethnic group and degree of familial relation.

Material and Methods

Patients

Nineteen patients of Japanese background and 26 of Western origin were attended at the Hepatology Branch, Discipline of Clinical Gastroenterology, University of São Paulo School of Medicine. These patients were considered to be the probands (index cases) and after their first visit their relatives were prospectively studied as two groups in terms of familial relation, i.e., i) genetically related persons consisting of consanguineous individuals (parents, brothers, sisters, sons, daughters, grandparents, aunts, uncles, cousins, nieces, and nephews), and ii) non-genetically related persons consisting of non-consanguineous individuals (husbands, wives, brothers-in-law, sisters-in-law, and others that could directly or indirectly be in contact with the family). A total of 165 relatives of Asian (Japanese) background and 186 of Western origin were enrolled after being invited and giving informed consent to participate in the study.

Inclusion criteria

Probands were HBsAg-seropositive individuals with chronic liver disease such as

chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, diagnosed by liver biopsy. The parents of the probands belonged to the same ethnic group. Children of mixed marriages were not included in the study.

Methods

All study procedures were approved by the institutional review board of the Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, SP, Brazil.

Seroimmunologic markers for present (anti-HBc and HBsAg) and past (anti-HBc and anti-HBs) HBV infection were searched for in the relatives of the index cases.

Identification

For each index case and respective relatives, the parameters age, sex, ethnic origin, and degree of familial relation were evaluated.

HBsAg, anti-HBc and anti-HBs determination

The presence of anti-HBc, HBsAg and anti-HBs in serum was determined by the enzyme immunoassays provided by Abbott Laboratories (North Chicago, IL, USA), with a sensitivity, specificity and reproducibility of 100, 95 and 100% for Corzyme, of 100, 100 and 100% for Auszyme II, and of 100, 100 and 100% for Ausab, respectively (11).

Statistical analysis

The mean age of probands in both ethnic groups (Asian and Western) was compared by the *t*-test and categorical variables were compared by the chi-square or Fisher exact test. The influence of origin (Asian and Western) and degree of familial relation (genetic and non-genetic) on the serum immunologic markers was evaluated by linear model methods as reported by Grizzle et al. (12) and Koch et al. (13). Four subpopula-

tions were considered on the basis of ethnic group and degree of familial relation (Asian, genetically related; Asian, non-genetically related; Western, genetically related; Western non-genetically related). The results of the HBV serum markers were considered to be the response variable (HBsAg, anti-HBs and negative). The calculations were made using the SAS System according to the CATMOD procedure (14).

Results

Index cases

Nineteen patients of Asian origin and 26 of Western origin were the probands, who did not differ significantly in terms of gender (11 males/8 females and 21 males/5 females, respectively; $P = 0.0945$) or mean age (39.8 ± 11.3 (19-63) and 34.5 ± 17.3 (7-61) years old, respectively ($P > 0.05$)).

Familial HBV markers

Serum samples from relatives were first screened for hepatitis B infection and subjects positive for anti-HBc were selected.

HBsAg and anti-HBs

The overall frequency of both HBsAg and anti-HBs was 135/165 (81.8%) for relatives of Asian origin and 68/186 (36.5%) for relatives of Western origin ($P < 0.0001$). Significant differences in 106/134 (79.1%) and 38/143 (26.5%) were also observed between the brothers and sisters ($P < 0.0001$), sons and daughters ($P < 0.0001$) and other relatives ($P < 0.0007$) of the two groups (Asian and Western), as shown in Table 1. In contrast, among the parents of the probands the difference in 17/17 (100%) and 17/23 (73.9%), respectively, of Asian and Western origin, only tended to be significant ($P = 0.0578$). The small number of cases of fathers and mothers of the index cases was compensated for with the inclusion

of other parents belonging to the same kinship.

HBsAg

A significant difference ($P = 0.0001$) in the frequency of HBsAg was detected between Asian relatives, 79/165 (47.9%), and Western relatives, 10/186 (10.7%). Concerning the degree of familial relation, there was a significant difference between the two groups regarding the mothers, brothers and sisters and sons and daughters.

Anti-HBs

The anti-HBs frequency found for the Asian relatives was 56/165 (33.9%) and did not differ significantly ($P = 0.1209$) from that of the Western group, 48/186 (25.8%). Regarding the degree of familial relation, a significant difference was observed only for the parents and other family relatives.

Frequency of familial aggregation rate per ethnic group

The familial aggregation frequency, given

by the number of families having at least one HBsAg- or anti-HBs-positive member (except the index case) in relation to the total number of families studied, was 94.7 and 88.5% for the Asian and Western groups, respectively ($P = 0.4319$; Table 2).

HBV markers in the progeny of HBsAg-positive mothers

The progeny of 16 mothers of Asian origin and 7 of Western origin was studied (Table 3); of the 67 subjects of Asian origin, 56 (83.6%) presented HBsAg and 5 (7.5%) anti-HBs, whereas of the 20 subjects of Western origin, only 8 (40%) presented HBsAg and 4 (20.8%) anti-HBs ($P = 0.0004$).

HBV markers in the progeny of HBsAg-positive fathers

Ten fathers of Asian origin and 20 of Western origin were enrolled in the study (Table 4). Of the 30 subjects of the Asian progeny, 7 (23.3%) were HBsAg positive and 7 (23.3%) anti-HBs positive. For the 69 Western descendants, the markers were de-

Table 1. Frequency of HBsAg and anti-HBs antibodies in family members of probands with HBsAg-seropositive chronic liver disease.

Family members	Ethnic groups						χ^2	P
	Asian origin			Western origin				
	HBsAg N (%)	Anti-HBs N (%)	Negative N (%)	HBsAg N (%)	Anti-HBs N (%)	Negative N (%)		
Immediate	9 (52.9)	8 (47.1)	0	7 (30.4)	10 (43.5)	6 (26.1)	5.70	0.057
Mothers	9 (90.0)	1 (10.0)	0	4 (30.7)	6 (46.2)	3 (23.1)	-	-
Fathers	0	7 (100.0)	0	3 (30.0)	4 (40.0)	3 (30.0)	-	-
Brothers and sisters	39 (79.6)	10 (20.4)	0	4 (8.5)	10 (21.3)	33 (70.2)	61.47	<0.000
Sons and daughters	11 (37.9)	1 (3.5)	17 (58.6)	2 (3.3)	7 (11.7)	51 (85.0)	19.27	<0.000
Spouses	1 (7.1)	11 (78.6)	2 (14.3)	1 (5.0)	14 (70.0)	5 (25.0)	0.61	0.738
Other relatives	19 (33.9)	26 (46.4)	11 (19.7)	6 (16.7)	9 (25.0)	21 (58.3)	14.48	<0.000
Total	79 (47.9)	56 (33.9)	30 (18.2)	20 (10.7)	48 (25.8)	118 (63.5)	87.16	<0.000

The numbers within parentheses are percent, with the total being 100% for each ethnic group: Asian (N = 165) and Western (N = 186). The probands were 19 patients of Asian origin and 26 of Western origin. For detailed explanation of statistical analysis (chi-square test), see Results section.

tected in 8 (11.6%) and 6 (8.7%) members, respectively ($P = 0.0255$).

HBV markers in progeny from fathers or mothers (HBsAg) of Asian origin

Nine HBsAg-positive fathers and 15 HBsAg-positive mothers with a 26- and 63-member progeny, respectively, were enrolled in this investigation. HBsAg was detected in 3 (11.6%) and anti-HBs in 7 (26.9%) members of the first group, while for the second group, 52 (82.6%) were found to be HBsAg positive and 5 (7.9%) anti-HBs positive, with a significant difference between groups ($P < 0.0001$). The results for one family were excluded from the analysis because both father and mother were HBsAg positive.

HBV markers in progeny from fathers or mothers (HBsAg) of Western origin

Eighteen HBsAg-positive fathers with a progeny of 64 children and 5 mothers with a progeny of 15 children were studied. HBsAg was detected in 7 (10.9%) and anti-HBs in 6 (9.4%) of the 64 descendants of the first group, while for the 15 descendants of the second group, HBsAg was found in 7 (46.6%) and anti-HBs in 4 (26.7%) individuals ($P = 0.0002$). The results for two families were excluded from the analysis because both father and mother were HBsAg positive.

Frequency of HBV markers in the progeny of fathers or mothers (HBsAg) of Asian and Western origin

Progenies consisting of 90 and 78 individuals from 27 fathers and 20 HBsAg-positive mothers, respectively, were investigated. HBsAg was detected in 10 individuals (11.1%) and anti-HBs in 13 (14.4%) individuals in the first group, whereas in the second group the frequency found for these markers was 75.7% (59/78) and 11.5% (9/78), respectively ($P = 0.0001$).

Frequency of HBV markers per ethnic group and degree of familial relation

The frequency of HBV markers according to ethnic origin (Asian and Western) and the degree of familial relation (genetic and non-genetic) were compared and are presented in Table 5. Analysis of variance

Table 2. Frequency of familial aggregation rate with past or present hepatitis B virus infection as a function of ethnic group.

	Ethnic group	
	Asian origin	Western origin
No. of families studied	19	26
Familial aggregation rate - N (%)	18 (94.7)	23 (88.5)
P level (Fisher exact test)	0.4319	

Table 3. Frequency of hepatitis B virus (HBV) markers in the progeny of HBsAg-positive mothers.

	Ethnic group	
	Asian origin	Western origin
HBsAg (+) mothers (N)	16	7
Total number of children	67	20
HBsAg - N (%)	56 (83.6)	8 (40.0)
Anti-HBs - N (%)	5 (7.5)	4 (20.0)
HBV-negative markers - N (%)	6 (9.5)	8 (40.0)

$P = 0.0004$ and $\chi^2 = 15.54$ when HBsAg and anti-HBs frequency in 67 Asian and 20 Western origin children were compared.

Table 4. Frequency of hepatitis B virus (HBV) markers in the progeny of HBsAg-positive fathers.

	Ethnic group	
	Asian origin	Western origin
HBsAg (+) fathers (N)	10	20
Total number of children	30	69
HBsAg - N (%)	7 (23.3)	8 (11.6)
Anti-HBs - N (%)	7 (23.3)	6 (8.7)
HBV-negative markers - N (%)	16 (53.4)	55 (79.7)

$P = 0.0255$ and $\chi^2 = 7.34$ when HBsAg and anti-HBs frequency in 30 Asian and 69 Western origin children were compared.

Table 5. Frequency and analysis of variance of hepatitis B virus (HBV) markers per ethnic group and degree of familial relation.

Ethnic group	Degree of family relatedness	HBsAg (N)	Anti-HBs (N)	HBV-negative markers (N)	Total (N)
Asian origin	Genetically related (consanguineous)	76	28	22	126
	Non-genetically related (non-consanguineous)	3	28	8	39
Western origin	Genetically related (consanguineous)	19	30	105	154
	Non-genetically related (non-consanguineous)	1	20	10	31

Intercept: 2 degrees of freedom; $\chi^2 = 20.39$; $P = 0.0001$. Ethnic group: 2 degrees of freedom; $\chi^2 = 71.15$; $P = 0.0001$. Degree of familial relation: 2 degrees of freedom; $\chi^2 = 45.46$; $P = 0.0001$. Residual: 2 degrees of freedom; $\chi^2 = 2.71$; $P = 0.2580$.

Table 6. Frequency of hepatitis B virus (HBV) markers per ethnic group and degree of familial relation.

Ethnic group	Degree of family relatedness	HBV markers	Observed		Predicted	
			Probability	Standard error	Probability	Standard error
Asian origin	Genetically related (consanguineous)	HBsAg	0.60	0.04	0.60	0.04
		Anti-HBs	0.22	0.04	0.21	0.03
		Negative	0.17	0.03	0.19	0.03
	Non-genetically related (non-consanguineous)	HBsAg	0.08	0.04	0.10	0.04
		Anti-HBs	0.72	0.07	0.75	0.06
		Negative	0.20	0.06	0.15	0.04
Western origin	Genetically related (consanguineous)	HBsAg	0.12	0.03	0.13	0.03
		Anti-HBs	0.19	0.03	0.21	0.03
		Negative	0.68	0.04	0.67	0.04
	Non-genetically related (non-consanguineous)	HBsAg	0.03	0.03	0.02	0.01
		Anti-HBs	0.64	0.09	0.58	0.07
		Negative	0.32	0.08	0.40	0.07

Data are reported as observed and predicted probabilities.

showed a significant difference in both comparisons. Since the interaction between ethnic group and familial relation was not significant, the model was adjusted taking into consideration only the main effects. Finally, analysis of categorical data showed full agreement between the observed and expected probabilities (Table 6).

Discussion

Families represent an excellent model for the study of qualitative and quantitative

features concerning host response to certain etiologic agents and transmission mechanisms through people exposed to a variety of environmental characteristics. Family studies have proved to be important for obtaining information about viral hepatitis B, which shows few clinical symptoms but a clear trend to chronicity (5).

The mechanisms of hepatitis B transmission are not fully understood, but they seem to involve several aspects and the genetic component, in particular, is still being debated (15-17).

Despite all of these unclear features, few worldwide large-scale studies are available, including geographic areas of low and high incidence of HBV infection. Numerous reports of families with multiple cases of acute and chronic hepatitis B (5,18,19) have confirmed the possibility of familial clustering. However, they are of little value for estimating the frequency of these events in the population and for the understanding of certain aspects of HBV familial clustering (20).

On the basis of information from outside Brazil (6) and of preliminary Brazilian data (1) about relatives of HBV-positive patients with liver disease, two populations of distinct ethnic origins are described in the present report, i.e., the relatives of Brazilian probands of Asian and Western origin. The choice was based on the fact that immigrants of these two ethnic groups correspond to a relatively large proportion of the population of the State of São Paulo. In fact, São Paulo contains the largest Japanese descendant population after Japan.

Indeed, one of the main objectives of the present study was to determine whether our data are similar to those reported by others in the literature (4,5,20-22).

Concerning the inclusion criteria, it is worth mentioning work by other investigators. Bruguera et al. (21) studied 100 relatives of 29 patients with chronic HBV-related liver disease admitted to the Hospital of the University of Barcelona. HBsAg was detected in 12 members of the families studied and, of these, 8 were asymptomatic HBV carriers, 1 had cirrhosis and 3 progressed to acute hepatitis. There was no report of parenteral exposure among the familial contacts. The HBsAg frequency (12.0%) in the families was considerably higher than the prevalence of the antigen in patients (0.3%) without liver disease and in volunteer blood donors (0.4%).

The same group from Barcelona (20) published a study of familial contacts of probands with acute hepatitis and chronic hepatitis and healthy HBV carriers, and de-

termined the occurrence of HBsAg and anti-HBs compared to volunteer blood donors. The prevalence of these markers was significantly higher among the 178 relatives (39.2%) with domestic contact with the 54 HBsAg carriers than in the control group of 834 blood donors (12.1%; $P < 0.001$). The authors concluded that the risk of acquiring HBV infection for the relatives of patients with some HBV-related liver disease (acute = 33.0%; chronic = 46.3%) was higher than for individuals with no familial case of liver disorders (13.4%; $P < 0.01$). Similar data were reported by others (5,22).

Due to the relevance of these results and the ease to study patients with chronic liver diseases compared to patients with the acute forms, the families of the chronic cases were given priority in this study. The existence of a member with a chronic form of liver disease greatly motivates the family to join the study (Carrilho FJ and Da Silva LC, unpublished data).

Searching for HBsAg and anti-HBs has been generally adopted to detect present or past HBV infection (23). Anti-HBc is also another widely used marker of HBV infection (9).

The probands of Asian and Western origin were not significantly different regarding age and gender. The present study showed that the general occurrence of the HBsAg and anti-HBs markers, which indicate HBV contact, was 81.8% for the Asian families and 36.5% for the Western families (Table 1). This difference in the percent of members of a group who are HBsAg and anti-HBs positive was significant and our data are similar to those of Ohbayashi et al. (4) in Japan (79.9%) and of Tong et al. (8) in members of Chinese families living in the United States compared to members living in the Orient. However, our findings are also similar to those reported by various European (26.0-71.1%) (20,24) and American investigators (32.0-46.0%) (9,22) regarding Western people.

HBsAg was found in 90.0% of the mothers, 79.6% of the brothers and sisters and 37.9% of the progenies of Asian origin and, for the Western equivalents, in 30.7, 8.5 and 3.3%, respectively. These differences are significant and suggest, especially for the Asian subjects, that vertical or perinatal transmission from mothers is the most important way of acquiring HBV infection, as will be seen later. These results also agree with the literature for all groups (5,22).

The presence of anti-HBs, which in most cases indicates a host immune response to HBV and past infection (23), was detected in 33.9% of the members of the Asian families and in 25.8% of the members of families of Western origin. This difference was not significant, and the literature has reported the occurrence of this antibody in Asian (5,9) and Western subjects (9,20,25).

Anti-HBs antibodies were detected in 100% of the fathers and in 78.6% of the persons who are married to or are having an intimate (sexual) relationship with people of Asian origin. For those of Western origin, the occurrence was 40.0 and 70.0%, respectively, with a significant difference between the groups of Asian and Western fathers.

It is worth emphasizing that there is no similar concomitant study with these two ethnic groups (Japanese and Western) in the literature.

In general, 41 (91.1%) of the 45 families presented familial clustering, characterized by the existence of members (except the index case) with signs of present (HBsAg positive) or past (anti-HBs positive) infection. This clustering occurred in 94.7 and 88.5% of the Asian and Western families, respectively ($P = 0.4319$; Table 2). These values are higher than those reported by Bruguera et al. (20) (68.5%) in Spain and by Szmuness et al. (9) (8.4-21.0%) in the United States. This difference may be viewed, at least in part, as a consequence of the families submitted to different situations with the index cases, i.e., contact with chronic or

acute liver disorders or healthy HBsAg carriers (10,20,26).

The analysis of the HBsAg and anti-HBs markers in progenies of mothers or fathers positive for the antigen was carried out by considering all the familial possibilities; for instance, children from a genetically related relative of the index case were also included to increase the sampling for statistical analysis. The analysis of the HBV markers and of the degree of familial relation revealed that HBsAg was more frequent among genetically related individuals, whereas anti-HBs was more prevalent among non-genetically related individuals (Table 5). The Asian subjects showed an alarmingly high frequency of HBsAg among genetically related relatives such as parents, brothers, sisters, children (47.9%, Table 1), as well as other genetically related members of the family (60.3%, Table 1).

The present results show that: 1) there is a high frequency of intra-familial HBV spreading, especially in families of Asian origin, and 2) applying a certain statistical methodology (12-14) allows the prediction of the frequency of HBV markers per ethnic group and per degree of familial relation. In fact, the observed values agree with the expected ones (Table 6). This is a valuable contribution of practical usefulness and, as far as we know, there is no similar report in the literature. 3) Among the families of both ethnic groups, the infected mothers are the most likely to spread HBV, a fact strongly suggesting that vertical transmission is an important way of getting the infection. On the other hand, the non-genetically related relatives presented a higher tendency to develop anti-HBs upon contact with HBsAg-positive carriers. The reason is that the contact with HBV is in the adult phase, when the possibility to eliminate the virus is more likely.

The present results have shown fundamental differences in the occurrence of HBsAg between families of Asian and West-

ern origin, as also reported by others (9,27). It is worth noting, however, the lack of studies including different ethnic groups from a single country. In general, studies of this kind have shown that the spreading and persistence of HBV are more efficient among Asian people than other ethnic groups (9,28,29). To explain this phenomenon, hypotheses have been raised concerning environmental, nutritional and genetic factors, but they are unproved (17,30-37).

HBsAg carriers should be clinically investigated, monitored by laboratory tests and, if appropriate, submitted to a biopsy (3). The practice of these prophylactic measures including active immunization has significantly

decreased the incidence of HBV infection in both Western and Asian countries (38,39).

The search for other HBV markers (HBeAg, anti-HBe, HBsAg subtypes, and HBV genotyping) among the relatives of patients is currently under investigation by our group (40).

Acknowledgments

The authors thank Dr. Gabriela Ribeiro-dos-Santos, from Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, SP, Brazil, for critically reviewing the manuscript.

References

- Carrilho FJ & Corrêa MCJM (1998). Magnitude of hepatitis B and C in Latin America. In: Schinazi RF, Sommadossi J-P & Thomas HC (Editors). *Therapies for Viral Hepatitis*. 1st edn. International Medical Press, London, UK, 25-34.
- EASL International Consensus Conference on Hepatitis B (2003). 13-14 September, 2002: Geneva, Switzerland. Consensus statement. *Journal of Hepatology*, 38: 533-540.
- Lok AS & McMahon BJ (2001). Chronic hepatitis B. *Hepatology*, 34: 1225-1241.
- Ohbayashi A, Okochi K & Mayumi M (1972). Familial clustering of asymptomatic carriers of Australia antigen and patients with chronic liver disease or primary liver cancer. *Gastroenterology*, 62: 618-625.
- Tong MJ, Thursby MW, Lin JH et al. (1981). Studies on the maternal-infant transmission of the hepatitis B virus and HBV infection within families. *Progress in Medical Virology*, 27: 137-147.
- Bosch J, Bruguera M & Rodes J (1973). Familial spread of type-B hepatitis. *Lancet*, 2: 457.
- Beasley RP, Hwang LY, Stevens CE et al. (1983). Efficacy of hepatitis B immune globulin for prevention of perinatal transmission of the hepatitis B virus carrier state: final report of a randomized double-blind, placebo-controlled trial. *Hepatology*, 3: 135-141.
- Tong MJ, Weiner JM, Ashcovei MW et al. (1979). A comparative study of hepatitis B viral markers in the family members of Asian and non-Asian patients with hepatitis B surface antigen-positive hepatocellular carcinoma and with chronic hepatitis B infection. *Journal of Infectious Diseases*, 140: 506-512.
- Szmunes W, Harley EJ & Prince AM (1975). Intrafamilial spread of asymptomatic hepatitis B. *American Journal of Medical Sciences*, 270: 293-304.
- Ono-Nita SK, Carrilho FJ, Cardoso RA et al. (2004). Searching for chronic hepatitis B patients in a low prevalence area - role of racial origin. *BMC Family Practice*, 5: 7.
- Ratnam S & Tobin AM (1987). Comparative evaluation of commercial enzyme immunoassay kits for detection of hepatitis B seromarkers. *Journal of Clinical Microbiology*, 25: 432-433.
- Grizzle JE, Starnes CF & Koch GG (1969). Analysis of categorical data by linear models. *Biometrics*, 25: 489-504.
- Koch GG, Imrey PB, Singer JM et al. (1984). Lecture notes for analyses of categorical data. Postgraduate Course in the Department of Biostatistics. University of North Carolina Press, Chapel Hill, NC, USA.
- SAS Institute Inc. (1985). *SAS User's Guide: Statistics*. 5th edn. Cary: SAS Institute, Cary, NC, USA.
- Lok AS, Heathcote EJ & Hootnagle JH (2001). Management of hepatitis B: 2000 - summary of a workshop. *Gastroenterology*, 120: 1828-1853.
- Conjeevaram HS & Lok AS (2003). Management of chronic hepatitis B. *Journal of Hepatology*, 38 (Suppl 1): S90-S103.
- Liaw YF, Leung N, Guan R et al. (2003). Asian-Pacific consensus statement on the management of chronic hepatitis B: an update. *Journal of Gastroenterology and Hepatology*, 18: 239-245.
- Boss LP, Bender TR, Schreeder MT et al. (1981). Hepatitis B testing in the families and villages of five young Eskimos with primary hepatocellular carcinoma. *American Journal of Epidemiology*, 114: 95-101.
- Bruguera M, Bosch J & Rodes J (1973). Letter: Family outbreak of hepatitis B. *New England Journal of Medicine*, 289: 1144.
- Bruguera M, Caballeria J, Acero D et al. (1980). Transmisión intrafamiliar del virus de la hepatitis B. *Gastroenterology and Hepatology*, 3: 13-18.
- Bruguera M, Bosch J, Rodes J et al. (1974). Familial clustering of hepatitis B antigen: a study in relatives of patients with liver diseases and hepatitis B antigenaemia. *British Medical Journal*, 3: 495-497.
- Sampliner RE, Loevinger BL, Tabor E et al. (1981). Intrafamilial cluster of hepatitis B virus infection: study of a large family in the United States. *American Journal of Epidemiology*, 113: 50-54.
- Holland PV (1985). Hepatitis B surface antigen and antibody. In: Gerety RJ (Editor). *Hepatitis B*. Academic Press, Orlando, FL, USA, 5-25.

24. Hadziyannis SJ (1975). Nonparenteral transmission of viral hepatitis in Greece. *American Journal of Medical Sciences*, 270: 313-319.
25. Bemier RH, Sampliner R, Gerety R et al. (1982). Hepatitis B infection in households of chronic carriers of hepatitis B surface antigen: factors associated with prevalence of infection. *American Journal of Epidemiology*, 116: 199-211.
26. Pastore G, Dentico P, Angarano G et al. (1981). Infectivity markers in HBsAg chronic carriers and intrafamilial spread of hepatitis B virus infection. *Hepatology*, 28: 20-22.
27. Derso A, Boxall EH, Tarlow MJ et al. (1978). Transmission of HBsAg from mother to infant in four ethnic groups. *British Medical Journal*, 1: 949-952.
28. Pongpipat D, Suvatt V & Assateerawatts A (1980). Vertical transmission of the hepatitis B surface antigen in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, 11: 582-587.
29. Skinhoj P, Aldershvile J, Kjersem M et al. (1983). Hepatitis B infection in Vietnamese families. *Journal of Medical Virology*, 11: 125-129.
30. Chang MH, Hsu HC, Lee CY et al. (1984). Fraternal hepatocellular carcinoma in young children in two families. *Cancer*, 53: 1807-1810.
31. Follett EA, McMichael S, Goel KM et al. (1978). Hepatitis B in the school environment. *British Medical Journal*, 1: 1279-1280.
32. Helske T & Nevanlinna HR (1973). Familial accumulation of carriers of Au antigen. *Journal of Medical Genetics*, 10: 270-272.
33. Kashiwagi S, Naito S, Kaneoka H et al. (1978). A family study of HBsAg carriers and major histocompatibility antigens. *Fukuoka Igaku Zasshi*, 69: 156-161.
34. Nasrallah SM, Nassar VH & Shammaa MH (1978). Genetic and immunological aspects of familial chronic active hepatitis (type B). *Gastroenterology*, 75: 302-306.
35. Sampliner RE, Bias WB, Carney E et al. (1981). HLA antigens and HBV infection: evaluation in the chronic carrier state and in a large family. *Tissue Antigens*, 18: 247-251.
36. Tong MJ & Weiner JM (1979). Lack of supportive evidence for an autosomal recessive inheritance in chronic hepatitis B infections. *Digestive Diseases and Sciences*, 24: 286-288.
37. Vyas GN (1974). Evidence against recessive inheritance of susceptibility to the chronic carrier state for hepatitis B antigen. *Nature*, 248: 159-160.
38. Lee CL & Ko YC (1997). Hepatitis B vaccination and hepatocellular carcinoma in Taiwan. *Pediatrics*, 99: 351-353.
39. Beutels P, Edmunds WJ, Antonanzas F et al. (2002). Economic evaluation of vaccination programmes: a consensus statement focusing on viral hepatitis. *Pharmacoeconomics*, 20: 1-7.
40. Clemente CM (2003). Sequencing, genotyping and phylogenetic analysis of hepatitis B virus in Asian and Western background families of patients with chronic infection. Master's thesis, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brazil.

- 30- Cintia Mendes Clemente, Flair José Carrilho, João Renato Rebello Pinho, Suzane Kioko Ono-Nita, Luiz Caetano Da Silva, Regina Célia Moreira, Marcílio Figueiredo Lemos, Isabel Maria Vicente Guedes de Carvalho Mello. A phylogenetic study of Hepatitis B virus in chronically infected Brazilian patients of Western and Asian descent. Journal of Gastroenterology, 2009. 44 (6): 568-576**

A phylogenetic study of hepatitis B virus in chronically infected Brazilian patients of Western and Asian descent

Cintia Mendes Clemente · Flair José Carrilho · João Renato Rebello Pinho ·
Suzane Kioko Ono-Nita · Luiz Caetano Da Silva · Regina Célia Moreira ·
Marcilio Figueiredo Lemos · Isabel Maria Vicente Guedes de Carvalho Mello

Received: 22 May 2008 / Accepted: 2 December 2008 / Published online: 16 April 2009
© Springer 2009

Abstract

Background Hepatitis B virus (HBV) causes one of the most important chronic viral infections worldwide. HBV is classified into eight genotypes whose epidemiology varies geographically. In Brazil, genotypes A, D, and F are more frequent, while in East Asia, genotypes B and C predominate. Several studies showed that immigrants retain the HBV infection pattern of their ancestral country.

Purpose To identify HBV genotypes infecting chronic carriers in Brazilian families of Western and Asian descent by Hepatitis B surface antigen gene sequencing and analyze the route of viral transmission by phylogenetic analysis of viral sequences.

Methods Eighty-seven people chronically infected with HBV were separated into two groups: Western descent (27) and Asian descent (60). Surface and pre-core/core genes

were amplified from serum HBV-DNA and sequences were subjected to phylogenetic analysis.

Results HBV genotype A was found in 74% of Western subjects, while genotype C was found in 94% of Asian patients. Thirty-eight percent of Western families were infected with HBV with similar pre-core/core sequences, while only 25% of Asian families showed similarity in these sequences.

Conclusions Phylogenetic analysis of pre-core/core HBV gene suggested intra-familial transmission of HBV in 38% of Western families and 25% of Asian families. Analysis of HBsAg gene sequences helped to define the HBV genotype but did not allow inferring route of transmission as its sequences showed a smaller phylogenetic signal than pre-core/core sequences. Chronic HBV carriers of Asian descent born in or living in Brazil were infected with the same HBV genotype predominant in their ancestral country.

C. M. Clemente (✉) · F. J. Carrilho · J. R. R. Pinho ·
S. K. Ono-Nita · L. C. D. Silva · I. M. V. G. de Carvalho Mello
Departamento de Gastroenterologia,
Faculdade de Medicina,
Universidade de São Paulo,
Avenida Doutor Arnaldo 455,
São Paulo 01246-902, Brazil
e-mail: cintiamclemente@uol.com.br

F. J. Carrilho
e-mail: fjcarril@usp.br

J. R. R. Pinho
e-mail: jrpinho@usp.br

S. K. Ono-Nita
e-mail: skon@usp.br

L. C. D. Silva
e-mail: lucadasi@bol.com.br

I. M. V. G. de Carvalho Mello
e-mail: imvgcmello@butantan.gov.br

J. R. R. Pinho
Laboratório de Hepatologia e Gastroenterologia Tropical,
Instituto de Medicina Tropical da Universidade
de São Paulo, Avenida Doutor Enéas de Carvalho
Aguir 500, São Paulo 05403-000, Brazil

R. C. Moreira · M. F. Lemos
Serviço de Virologia, Instituto Adolfo Lutz,
Avenida Doutor Arnaldo 355,
São Paulo 01246-902, Brazil

R. C. Moreira
e-mail: rmoreira@ial.sp.gov.br

M. F. Lemos
e-mail: mlfemos@ial.sp.gov.br

I. M. V. G. de Carvalho Mello
Laboratório de Imunologia Viral, Instituto Butantan,
Avenida Doutor Vital Brasil, 1500,
São Paulo 05503-900, Brazil

Keywords Hepatitis B · Disease transmission · Genotype · Phylogeny · Family

Introduction

Hepatitis B virus (HBV), a partial double-stranded DNA virus, causes the most prevalent chronic viral infection worldwide. Currently, approximately 300 million people are infected with HBV, and at least 1 million die annually of HBV-related liver diseases. Although a highly effective vaccine is available, only a small percentage of individuals at risk receive it, due to various socio-economic reasons [1].

In evolutionary terms, HBV shows two opposing tendencies: its replication by error-prone reverse transcriptase leads to relatively elevated mutation rates, while its compact genome with overlapping reading frames is resistant to genetic variation [2].

HBV is classified into eight genotypes, and these genotypes show characteristic geographical distribution: in Brazil, HBV genotypes A, D, and F are the more frequent [3], while in East Asia, B and C predominate [4]. Prevalence of HBV infection in Brazil is variable, since this country has a large geographic extension and considerable ethnic and social-economic diversity. The majority of Brazilian states show intermediary endemicity: 1–2% of blood donor candidates test positive for the HBV surface antigen (HBsAg). However, the north region (particularly among Brazilian Indians) and the southwest of Paraná and Espírito Santo states show a higher HBsAg frequency [5].

Studies in other world regions, such as the USA, where a large number of inhabitants are immigrant descendants, have shown that immigrants retain the HBV infection pattern of their country of origin [6]. Nucleotide sequence comparisons of HBV genomes have been used to study the routes of HBV infection [7, 8]. In highly endemic areas, like East Asia, HBV infection is acquired at an early age and clustering analysis shows that intrafamilial transmission is an important transmission route [9].

The aim of this study was to identify the HBV genotypes infecting chronic carriers in familial Brazilian clusters of Western and Asian descent using HBsAg (S) coding gene sequencing. We also carried out phylogenetic analysis of S and pre-core/core HBV genes sequences in order to analyze the routes of viral transmission.

Methods

Study group

Immigrants from Japan, China, Korea, or Taiwan and their descendants were included in the Asian group, while

patients without this pattern were included in the Western group. This latter group included Brazilian patients without known foreign ancestry and patients with European ancestry (especially from Portugal, Spain, Italy, and Germany). Thirteen patients of Western descent and 20 patients of Asian (Japanese or Taiwanese) descent were identified as probands (index case), that is, the first person of the family diagnosed with HBV infection. Serum samples were collected from 1984 to 2000, and follow-up was carried out at the Hepatology Branch, Division of Clinical Gastroenterology, University of São Paulo School of Medicine, Brazil. Inclusion criteria for probands included testing serum HBsAg positive in two samples collected 6 months apart, having at least one other HBV-infected family member who was HBsAg serum positive, and positive HBV-DNA detection by PCR of HBV surface and/or pre-core/core coding genes from serum. Fourteen Western and 40 Asian descent relatives from the index cases were selected using the same criteria, and were excluded if they had been treated previously with interferon, lamivudine, or corticosteroids, had evidence of alcohol addiction, co-infection with hepatitis C virus (HCV), or human immunodeficiency viruses (HIV), or other hepatopathies (auto-immune hepatitis, Wilson's disease, iron overloaded hepatic disease, alpha-1-antitripsin deficiency, or toxic hepatic disease). Data concerning gender, age, HBV serologic markers, liver histology (when biopsy was clinically indicated), and family genealogical tree were obtained from each patient's medical records. We obtained informed consent from all subjects, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Serological tests

Blood samples were centrifuged and sera were stored in plastic tubes at -20°C within 2 h of collection. HBV

Table 1 Clinical and demographic data of the studied patients

	Western n (%)	Asian n (%)	P value
Number of patients	27 (100)	60 (100)	
Male	13 (48)	32 (53)	0.68
Female	14 (52)	28 (47)	
Age (years)	26.7 $\pm$ 13.7	31.9 $\pm$ 15.9	0.11
HBsAg+	17 (63)	39 (65)	1.0
ALT ($\geq 1.5 \times$ UNV)	11 (41)	16 (26)	0.12
Liver biopsies	14 (52)	43 (71)	
Normal liver	3 (22)	12 (29)	0.76
Chronic hepatitis	10 (72)	24 (55)	0.27
Cirrhosis	1 (6)	7 (16)	0.43

ALT Serum alanine aminotransferase, UNV upper normal value

serological markers (HBsAg, total anti-HBc, HBeAg, anti-HBe, anti-HBs) were detected by ELISA (Abbott Laboratories, North Chicago, IL, USA).

Detection and extraction of HBV-DNA by PCR

Extraction and amplification of HBV-DNA were carried out by nested PCR as previously described [10]. All reagents were obtained from Life Technologies of Brazil, São Paulo. For HBV genotyping, we amplified a partial fragment of the S gene, as previously described [3]. Pre-

core/core region amplification was carried out as described by Takahashi et al. [11].

Sequencing of PCR products

PCR products were sequenced as previously described [3] using second-round primers and ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA). After purification, samples were denatured and loaded in a 5% Long Ranger 6M urea gel (Long Ranger gel solution; FMC

Table 2 Familial relation of the index case to the other family members shown in this study

Index case	Sex	Family members					
Western families							
F1	F1-A	Male	F1-B (brother)				
F3	F3-A	Female	F3-B (son)				
F4	F4-A	Female	F4-B (son)				
F5	F5-A	Male	F5-B (brother)				
F6	F6-A	Male	F6-B (sister)				
F7	F7-A	Male	F7-B (father)				
F8	F8-A	Male	F8-B (brother)				
F9	F9-A	Male	F9-B (sister)				
F10	F10-A	Male	F10-B (brother)				
F12	F12-A	Female	F12-B (brother)				
F13	F13-A	Female	F13-B (son)	F13-C (daughter)			
F14	F14-A	Male	F14-B (mother)				
F15	F15-A	Female	F15-B (sister)	F15-C (mother)			
Asian descent families							
F16	F16-A	Male	F16-B (brother)				
F17	F17-A	Female	F17-B (son)	F17-C (son)	F17-D (son)		
F18	F18-A	Male	F18-B (brother)	F18-C (sister)			
F19	F19-A	Female	F19-B (brother)	F19-C (brother)	F19-D (nephew)	F19-E (niece)	
F20	F20-A	Female	F20-B (brother)	F20-C (brother)			
F21	F21-A	Female	F21-B (sister)	F21-C (son)	F21-D (niece)		
F22	F22-A	Female	F22-B (daughter)				
F23	F23-A	Male	F23-B (brother)	F23-C (sister)			
F24	F24-A	Male	F24-B (sister)				
F25	F25-A	Female	F25-B (mother)	F25-C (brother)	F25-D (sister)		
F26	F26-A	Female	F26-B (sister)				
F27	F27-A	Male	F27-B (son)	F27-C (daughter)			
F28	F28-A	Male	F28-B (brother)	F28-C (brother)			
F29	F29-A	Female	F29-B (son)				
F30	F30-A	Female	F30-B (mother)	F30-C (grandmother)			
F31	F31-A	Male	F31-B (niece)				
F32	F32-A	Female	F32-B (daughter)	F32-C (daughter)	F32-D (Fig. 1)	F32-E (Fig. 1)	F32-F (Fig. 1)
F33	F33-A	Male	F33-B (sister)				
F34	F34-A	Female	F34-B (sister)	F34-C (niece)			
F35	F35-A	Male	F35-B (sister)				

Family 32 heredogram is further detailed in Fig. 1

Bioproducts, Rockland, Maine, USA) in an automated ABI Prism 377 DNA sequencer (Applied Biosystems).

Analysis of the phylogenetic signal

Phylogenetic signal/noise in the S [290 nucleotides (nt)] and pre-core/core (425 nt) datasets was investigated by likelihood mapping analysis, investigating groups of four randomly chosen sequences called quartets. For a quartet, just three unrooted tree topologies are possible. The likelihood of each topology can be estimated with the maximum likelihood method, and the three likelihoods can be reported as a dot in an equilateral triangle. For N sequences, $(N/4)$ possible quartets exist, and the distribution of the dots in the triangle gives an overall impression of the tree-likeness of the data. When the N sequences are not clustered, the order of the sequences is not relevant, and the question as to which of the possible tree topologies is supported by any cluster is meaningless. Thus, we can distinguish two main areas in the equilateral triangle. The three corners represent fully resolved tree topologies and the presence of a tree-like phylogenetic signal in the data. The triangle center and the three areas on the sides represent a star-like and a net-like phylogeny, respectively, and indicate the presence of phylogenetic noise. The percentage of dots belonging to each area gives an idea about the significance of the dataset under investigation. Likelihood mapping analyses were performed with the program TREE-PUZZLE [12] using the HKY 85 substitution model.

Phylogenetic analysis

Multiple sequence alignments of the pre-core/core region with 425 nt and S region with 290 nt and the related sequences in the GenBank/EMBL database (accession numbers: AB036911, AY090453, D00330, D23680, M54923, M57663, U95551, X69798, X70185, X75656) were carried out with clustalX 1.81 program and further edited with the Bioedit 6.0.7. [13]. Neighbor-joining (NJ) phylogenetic analyses were performed with PAUP* version 4b10 [14] using K81 substitution model with the gamma distribution (G) variation among sites (for S region) and HKY 85 substitution model with the gamma distribution (G), which describes rates variation among sites and proportion of invariant sites (I) (for pre-core/core region). Evolutionary models were chosen by the hierarchical likelihood ratio test (hLRTs) using Modeltest 3.06 [15]. Phylogenetic groups were evaluated by using the bootstrap test with 1,000 replicates. Values ≥ 70 were considered significant.

Genotyping

Genotyping of HBV was done by phylogenetic analysis of the amplified fragments of the S gene, according to Sitnik et al. [3].

Statistical analysis

Statistical significance was determined using chi-square or Fisher exact tests.

Results

Clinical, serological, and histological analysis

Clinical and demographic data of the 87 individuals studied are presented in Table 1. The family relationship between the index case and the other cases shown in this study is shown in Table 2. The heredogram of one of these families is detailed in Fig. 1. Family 32 was chosen because this family had the largest numbers of relatives showing detectable serum HBV-DNA.

Surface gene sequence analysis

Phylogenetic analyses of the S gene sequences were used to classify the HBV genotypes, as shown in Fig. 2. HBV genotype A was detected in 19 (74.0%) of the people of Western descent followed by genotypes D-5 (18.5%) and

Fig. 1 Heredogram of Family 32

Fig. 2 Unrooted tree of partial S gene sequence from 87 HBV isolates, along with 2 sequences of each genotype from Genbank/EMBL (accession numbers: AY090453, D00330, D23680, M54923, M57663, U95551, X69798, X70185, X75656, AB036911). Bootstrap support values higher than 70 are shown in the appropriate tree branches. Nomenclature key example: F13-A, F family, 13 family number, A index case (B-E other family members)

F-2 (7.5%). Genotype C was found in 56 (94.0%) of people of Asian descent, and the remaining 4 (6.0%) were infected by HBV genotype B.

Analysis of the phylogenetic signal

S gene sequences showed a smaller phylogenetic signal than the pre-core/core gene sequences. Only 77% of the S

gene topologies studied sorted to the triangle regions, representing completely resolved trees versus 86% for the pre-core/core sequence analysis (Fig. 3).

Phylogenetic analysis of pre-core/core sequences

Phylogenetic tree analysis was carried out on the 87 pre-core/core gene sequences, 27 from the Western group and

Fig. 3 Phylogenetic signal obtained after the analysis of the 290 nt and 425 nt sequences from S gene (a) and from pre-core/core gene (b)

60 from the Asian group. The phylogenetic tree was constructed using all the sequences of both groups, including two sequences of each genotype from Genbank (A, B, C, D, and F) (Fig. 4).

In Family 14 (mother and son), pre-core/core gene from the mother's virus was not detectable by PCR, so only the son's viral pre-core/core sequence was submitted to phylogenetic analysis.

We observed that 5/13 (38%) of Western families and 5/20 (25%) of Asian families showed similarity in their HBV pre-core/core sequences, since they were in the same branch.

A higher number of relatives from Asian families than Western families were infected with HBV. In the Asian group, most members of large families were infected with highly similar HBVs (F-32), but other members were infected with non-similar viruses. In 8 Western families and 13 Asian families, family members were not infected with similar viruses, as illustrated in the phylogenetic tree. Sequences resided on the same branch, but there were also other viruses from different families on this branch. In some cases, the sequences from members of the same family resided on different branches, showing more similarity with the HBV from another family.

Discussion

Previous data from our group showed that Asian family members often become chronic HBV carriers since mother to child transmission is frequent in this group, and that infected Western family members are more often anti-HBc and anti-HBs positive, representing probable infection as adults via sexual transmission [16, 17]. Consequently, during patient accrual, we could identify fewer HBsAg

positive subjects in the Western group than in the Asian group (60 vs 27). In some Western cases, patients tested HBsAg-positive but HBV-DNA was not detectable in serum. In these cases, we hypothesize that an HBV or host genetic feature induces an immune response or influences HBV virulence, leading to a higher frequency of chronic inactive HBV infection in patients of Western descent [18].

HBV genotypes were determined by S gene sequencing, and genotypes A, D, and F were found in the Western group, while genotypes B and C were found in the Asian group. Chronic HBV carriers of Asian descent who were born in or have lived in Brazil tended to be infected with the same HBV genotype that predominates in their ancestral country. This phenomenon suggests that patients of Asian descent acquired their viruses in Brazil from infected Asian immigrants, and that transmission of the same HBV genotype strain was maintained by an intra-familial route of infection.

A previous study comparing HBV genotypes in Bolivia among Asian and Native residents in a closed society of Japanese immigrants and natives showed genotypes B, C, and F in patients with both origins [19]. These results are different from our results as we have not found genotypes A, D, and F among the Asian descendants patients studied by us. The differences in our results to those previously published from Bolivia are probably related to the lifestyles of these two different groups. Asian and Native populations in Bolivia probably have intense interactions in a more closed society. On the other hand, the patients analyzed in the present study live mostly in large urban areas in the São Paulo and Paraná states in southeast and south Brazil, where there are a large number of Japanese, Chinese, and Korean immigrants. These patients establish more interactions inside their own origin groups and this can explain

Fig. 4 Unrooted tree of partial pre-core/core gene sequence from 87 HBV isolates, along with 2 sequences of each genotype from Genbank/EMBL (accession numbers: AY090453, D00330, D23680, M54923, M57663, U95551, X69798, X70185, X75656, AB036911). Bootstrap support values higher than 70 are shown in the appropriate tree branches. Nomenclature key example: F13-A, *F* family, 13 family number, *A* index case (*B–E* other family members)

why HBV genotypes common in their native countries are almost restricted to them. In a previous study, we have already found that genotypes B and C are restricted to Asian descendants in Brazil [3]. Changes in the behavior of Asian and Western descendants in Brazil, leading to a higher admixture between these groups, will probably

change this figure, as HBV cases due to genotype A have already been found in Japanese descendants in São Paulo City [3].

Early studies of HBV transmission using PCR used the *S* gene sequencing to confirm the intra-familial route of HBV transmission [20–22], but this gene is too well

conserved to be appropriate for this kind of study [23]. Other approaches have used pre-S, pre-core, and core genes, as well as the complete genome, for these types of studies. Complete genome sequences would allow better resolution to evaluate intra-familial transmission [7, 24, 25], but we chose the pre-core/core region for phylogenetic analysis because it yields a better phylogenetic signal (86%), enough to clear the transmission in most cases without the need for exhaustive sequencing.

Two previous studies performed in Gambia and Italy demonstrated intra-familial HBV transmission by phylogenetic analysis of pre-core/core genes [7, 25], as this region of the HBV genome, because its sequence is relatively stable, is protected from mutations that could hide genetic relatedness, but not stable enough to possess identifying information about different HBV genotypes.

In HBV, the presence of overlapping open reading frames limits the extent of allowable sequence divergence; a silent mutation in one reading frame could be a lethal mutation in another. The S gene is well conserved and contains the 'a' antigenic determinant, which overlaps with the polymerase open reading frame. Therefore, this region of the genome is well conserved among all HBV genotype strains and does not provide sufficient information for reliable phylogenetic analysis. In our study, the pre-core/core gene sequences allowed a stronger phylogenetic signal than S gene, as previously reported [7].

The horizontal transmission of HBV occurred between siblings and between mothers and other non-offspring children. Children can become infected through close contact during playing, sharing the same bed during sleep, or other activities [24, 26].

Thirty-eight percent of Western families and 25% of Asian families demonstrated possible intra-familial HBV transmission, since their pre-core/core sequences sorted to the same tree branch in phylogenetic analysis. In the other families, it was not possible to confirm or discard the intra-familial HBV transmission route based on our sequence analysis of the 400-nt region of pre-core/core genome. Sequence analysis of the entire genome would allow a better phylogenetic comparison. It is likely that familial HBV transmission occurred in most of the cases studied, but host immune pressure led to mutations that altered the viral sequence.

In conclusion, according to phylogenetic analysis of the pre-core/core HBV gene, intra-familial HBV transmission probably occurred in 38% of Western families and 25% of Asian families; HBV S gene sequencing was important for defining the HBV genotypes of the family members. Chronic HBV carriers of Asian origin who were born in, or have lived in, Brazil tended to be infected by the same HBV genotype strain that predominates in their country of ancestry.

Acknowledgments FAPESP—Fundação de Amparo à Pesquisa do Estado de São Paulo (Grants 00/00998/1 and 00/11457/1 Viral Genetic Diversity Network) and Alves de Queiroz Family Fund for Research.

References

1. Lavanchy D. Epidemiology. In: Thomas HC, Lemon S, Zuckerman AJ, editors. *Viral Hepatitis*. 3rd ed. Malden: Blackwell; 2005. p. 181–92.
2. Kann M, Gerlich WH. Structure and molecular virology. In: Thomas HC, Lemon S, Zuckerman AJ, editors. *Viral Hepatitis*. 3rd ed. Malden: Blackwell; 2005. p. 149–80.
3. Simik R, Pinho JRR, Bertolini DA, Bernardini AP, da Silva LC, Carrilho FJ. Hepatitis B virus genotypes and precore and core mutations in Brazilian patients. *J Clin Microbiol*. 2004;42:2455–660.
4. Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. *J Gen Virol*. 2002;83:1267–80.
5. Carrilho FJ, Mendes-Correa MCJ. Magnitude of hepatitis B and C in Latin America. In: Schinazi RF, Sommadossi J-P, Thomas HC, editors. *Therapies for Viral Hepatitis*. London: International Medical Press; 1998. p. 25–34.
6. Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-Pico C, et al. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology*. 2003;125:444–51.
7. Dumps U, Holmes EC, Mendy M, Hill A, Thursz M, Hall A, et al. Transmission of hepatitis B infection in Gambian families revealed by phylogenetic analysis. *J Hepatol*. 2001;35:99–104.
8. Santantonio T, Jung MC, Pastore G, Angarano G, Günther S, Will H. Familial clustering of HBV pre-C and pre-S mutants. *J Hepatol*. 1997;26:221–7.
9. Lin CL, Kao JH, Chen BF, Chen PJ, Lai MY, Chen DS. Application of hepatitis B virus genotyping and phylogenetic analysis in intrafamilial transmission of hepatitis B virus. *Clin Infect Dis*. 2005;41:1576–81.
10. Kaneko S, Feinstone SM, Miller RH. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. *J Clin Microbiol*. 1989;27:1930–3.
11. Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, et al. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol*. 1995;76:3159–64.
12. Strimmer K, von Haeseler A. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. *Proc Natl Acad Sci USA*. 1997;94:6815–9.
13. Hall T. BioEdit, version 6.0.7. *Info therapeutics*. Carlshad, 2004.
14. Swofford DL. PAUP*. Phylogenetic analysis using Parsimony (*and Other Methods). Version 4b10. Sinauer, Sunderland, 2002.
15. Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. *Bioinformatics*. 1998;14:817–8.
16. Carrilho FJ, Ono-Nita SK, Cardoso RA, Cancado EL, Pinho JR, Alves VA, et al. A prospective study of hepatitis B virus markers in patients with chronic HBV infection from Brazilian families of Western and Asian origin. *Braz J Med Biol Res*. 2005;38:1399–408.
17. Ono-Nita SK, Carrilho FJ, Cardoso RA, Nita ME, da Silva LC. Searching for chronic hepatitis B patients in a low prevalence area—role of racial origin. *BMC Fam Pract*. 2004;5:7.
18. Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. *Immunity*. 1996;4:25–36.
19. Khan A, Tanaka Y, Saito H, Ebinuma H, Sekiguchi H, Iwama H, et al. Transmission of hepatitis B virus (HBV) genotypes among

- Japanese immigrants and natives in Bolivia. *Virus Res.* 2008; 132:174–80.
20. Zhong M, Hou J, Luo K. Identification of vertical transmission of hepatitis B virus from mother to children by direct sequencing a segment of surface gene of hepatitis B virus. *Zhonghua Yi Xue Za Zhi.* 1996;76:194–6.
 21. Martinson FE, Weigle KA, Royce RA, Weber DJ, Suchindran CM, Lemon SM. Risk factors for horizontal transmission of hepatitis B virus in a rural district in Ghana. *Am J Epidemiol.* 1998;147:478–87.
 22. Alaruk US, Greene S, Lok AS. Detection of precore hepatitis B virus mutants in asymptomatic HBsAg-positive family members. *Hepatology.* 1994;19:1366–70.
 23. Cacciola I, Cerenzia G, Pollicino T, Squadrito G, Castellaneta S, Zanetti AR. Genomic heterogeneity of hepatitis B virus (HBV) and outcome of perinatal HBV infection. *J Hepatol.* 2002;36:426–32.
 24. Thomas HC, Carman WF. Envelope and precore/core variants of hepatitis B virus. *Gastroenterol Clin North Am.* 1994;23:499–514.
 25. Zampino R, Lobello S, Chiaramonte M, Venturi-Pasini C, Dumpis U, Thursz M, et al. Intra-familial transmission of hepatitis B virus in Italy: phylogenetic sequence analysis and amino-acid variation of the core gene. *J Hepatol.* 2002;36:248–53.
 26. Sugauchi F, Mizokami M, Orito E, Sugauchi F, Mizokami M, Orito E, et al. Molecular evolutionary analysis of the complete nucleotide sequence of hepatitis B virus (HBV) in a case of HBV infection acquired through a needlestick accident. *Clin Infect Dis.* 2000;31:1195–201.

- 31- Sitnik R, Sette Jr H, Santana RAF, Menezes LC, Graça CHN, Dastoli GTF, Silbert S, PINHO JRR. Hepatitis B virus genotype E detected in Brazil in an African patient who is frequent traveler. Brazilian Journal of Medical and Biological Research (2007), 40 (12): 1689-92.**

Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler

R. Strik¹, H. Sette Jr.²,
R.A.F. Santana¹,
L.C. Menezes¹,
C.H.N. Craça¹,
C.T.F. Dastoli¹,
S. Silbert^{1,3} and
J.R.R. Pinho^{1,2}

¹Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil

²Departamento de Gastroenterologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil

³Departamento de Infectologia, Faculdade de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil

Abstract

Correspondence

R. Strik
Departamento de Patologia Clínica
Hospital Israelita Albert Einstein
Av. Albert Einstein, 627
05651-901 São Paulo, SP
Brasil
Fone: +55-11-3747-2122
E-mail: rstriki@hotmail.com

Publication supported by FAPESP.

Received February 21, 2007

Accepted July 10, 2007

Genotype E of hepatitis B virus (HBV) has not been described in Brazil and is found mainly in Africa. Genotype A is the most prevalent in Brazil, and genotypes B, C, D, and F have already been reported. We report here an HBV genotype E-infected patient and some characterization of surface (S) protein, DNA polymerase (P) and precore/core (preC/C) coding regions based on the viral genome. The patient is a 31-year-old black man with chronic hepatitis B who was born and raised in Angola. He has been followed by a hepatologist in São Paulo, Brazil, since November 2003, and he is a frequent traveler to Latin America, Africa, and Europe. In 2003, he was diagnosed with HBV infection and started treatment with lamivudine with the later addition of adefovir dipivoxil. No known risk factor was identified. Serologically, he is HBsAg and anti-HBe positive, but HBeAg and anti-HBs negative. DNA sequence analysis of the S/P region confirmed that this patient is infected with genotype E, subtype *ayw4*. The preC/C region showed G1896A and G1899A mutations but no mutations in the basal core promoter. Nucleotide substitutions common in genotype E were also observed (C1772, T1858 and A1757). Although this is not an autochthonous case and there is no evidence of further spread, the description of this case in Brazil highlights the current risk of viral genotypes spreading with unprecedented speed due to constant travel around the world.

Key words

- Hepatitis B
- HBV genotype E
- Precore mutation
- Brazil
- Africa
- African traveler

Hepatitis B virus (HBV) is a major cause of chronic liver disease worldwide. This virus is a small DNA virus with a partially double-stranded 3.2-kb genome (1) and has been classified into eight genotypes (A-H) based on sequence divergence over the entire genome. The genotypes show a distinct and well-defined geographic distribution (2).

Genotype E of HBV has not been described in Brazil and is found mainly in Africa. Genotype A is the most prevalent in Brazil, and genotypes B, C, D, and F have already been reported.

In the present study, we describe the clinical and epidemiological data of an African patient infected with HBV genotype E which

Figure 1. Phylogenetic tree. Phylogenetic analysis obtained with the MEGA3 software of a 593-bp fragment of hepatitis B virus (HBV) DNA from the index case (S gene - GenBank accession number EF151263), 29 genotype E African sequences and two sequences of each of the other HBV genotypes. The tree was constructed by the neighbor-joining method and only bootstrap values higher than 80 are shown. A woolly monkey HBV sequence was used as the outgroup. GenBank accession numbers as well as the original countries of all HBV isolates are indicated. The length of the horizontal bar indicates the number of nucleotide substitutions per site.

was detected in Brazil.

The patient provided informed written consent to perform all studies described here and sequences were deposited in Genbank (accession numbers EF151263 for the S/P region and EF153194 for the precore/core region).

A 31-year-old black man with a history of chronic hepatitis B infection has been followed by a hepatologist in São Paulo, Brazil, since November 2003, when he was diagnosed with HBV infection. This patient was born and raised in Angola and, as a lawyer, he frequently travels to Latin America, Africa, and Europe. Initial treatment consisted of lamivudine but, due to low compliance, adefovir dipivoxil was added. The patient suffers from morbid obesity and systemic arterial hypertension. No known risk factor was identified. Currently, he shows normal serum levels of hepatic enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and γ glutamyltransferase). Serologically, he is HBsAg and anti-HBe positive, but HBeAg and anti-HBs negative. Viral load determined by real time PCR was 291 IU/mL or 3,832.66 copies/mL in August 2006, when the consulting physician also asked for genotype and antiviral resistance tests.

HBV DNA was extracted from 200- μ L plasma samples using the QIAamp DNA mini-kit (QIAGEN, Hilden, Germany) according to manufacturer instructions. HBV DNA was detected by nested PCR amplifying the overlapping surface antigen (S) and DNA polymerase (P) genes, as previously described (3,4). For the detection of precore/core mutants, HBV DNA was amplified using the primers described by Takahashi et al. (5). HBV genotyping was carried out by direct sequencing of the amplified product using an ABI3130 DNA sequencer analyzer and the SeqScape Software V2.5 (Applied Biosystems, Foster City, CA, USA), which compares the sequence with a database of sequences from the different genotypes,

thereby generating a score of similarity. Sequences were submitted to BLAST in the GenBank database. A phylogenetic tree was constructed with the MEGA3 software (6) using a distance method (neighbor joining) and robustness of the tree was assessed by 1000 bootstrap replicates. The amino acid sequence obtained from the nucleotide sequence was also analyzed to assess subtype and possible drug resistance mutations in the HBV sequence.

Initial analysis of the nucleotide sequence of the S/P genomic region of HBV with the SeqScape Software V2.5 (Applied Biosystems) showed that it clustered with genotype E. Since this genotype has never been reported in Brazil, we performed a more extensive characterization of this virus. Phylogenetic analysis of the S/P region compared with reference isolates from GenBank representing all known HBV genotypes confirmed that it was classified into genotype E (Figure 1), and the HBeAg subtype was identified as *ayw4* based on the presence of Arg¹²², Lys¹⁴⁰, Leu¹²⁷, and Ser¹⁴⁰. Val to Leu mutation at position 209, present in all genotype E HBV sequences, was also found in this patient. No other amino acid mutations were found within the S protein and no drug resistance-related mutations were found in the HBV DNA polymerase protein. When submitted to BLAST in the GenBank, the sequence from the S/P region showed 99.07% similarity to several HBV genotype E sequences.

Mutation V209L is also found in genotype Aa, a discrete subtype within genotype A (7). Subgroup Aa has some amino acids differing from group Ae and was primarily found in sub-Saharan Africa. It was suggested that subgroup Aa may have evolved in indigenous populations of African countries. Later, this genotype was also found in South America (8,9), suggesting that this high proportion of subgroup Aa may be due to the intense slave trade from Africa to South America during the colonial period. In another study in a city with a large percent

of African descendants (Salvador, BA, Brazil), genotype A was also the most prevalent in the local population, followed by genotypes D and F, but no case of genotype E was identified (10). The Afro-Brazilian population has a higher prevalence of HBV markers compared to other ethnic groups (11) which might be related to a higher HBV infection rate among the first black inhabitants that were brought to Brazil and/or to the low socioeconomic level of that population. The lack of other genotype E cases in Brazil and South America is very intriguing, since this genotype has a high prevalence in Africa and may also have come with the African slaves, besides subgroup Aa.

The low sequence diversity of genotype E in isolates from African patients over a large geographical area suggests a short evolutionary history in humans (12,13). Indeed, genotype E has never been found within the Americas (14) or in the Afro-Brazilian population (8,10,11,15), a fact suggesting the emergence of genotype E as a human pathogen from the mid to the late 19th century.

Analysis of the precore/core region showed G1896A and G1899A precore mutations but no mutations in the basal core promoter (BCP). In another study with genotype E-infected patients, precore mutation was detected in 75% of the patients without HBeAg, in contrast to only 25% of the patients with HBeAg. All the strains described so far have T1858 (16), as also does the index case described here. This substitution allows the G1896A mutation and prevents the production of HBeAg. BCP mutation was detected in a few patients previously described and there was no relation to the presence of HBeAg (16,17). Other substitutions common to all genotype E sequences were also found (A1757 and C1772) in this patient. Mutation G1757A abolishes COUP TFI and HNF-1 nuclear factors binding to BCP in experiments *in vitro* (18) and may influence the clinical characteristics of genotype E, probably being related to the mild

evolution observed in this case.

We described here the major features of the first HBV genotype E case found in São Paulo State, Brazil. This genotype has never been reported in Brazil. It was recently found in 2 Argentinian sisters whose mother is from the Dominican Republic (19). In Brazil, genotypes A, B, C, D, and F have already been described and their distribution varies among Brazilian regions (4,10,20), suggesting an influence of the immigration pattern for each region. Further molecular epide-

miologic studies of HBV genotypes in the Afro-Brazilian population would be very important to determine if other cases can be found and to assess possible correlations of genotype E with the clinical course of infection and response to therapy. Furthermore, although this is not an autochthonous case and there is no evidence of further spread, the description of this case in Brazil highlights the current risk of viral genotypes spreading with unprecedented speed due to constant traveling around the world.

References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733-1745.
2. Liu CJ, Kao JH, Chen DS. Therapeutic implications of hepatitis B virus genotypes. *Liver Int* 2005; 25: 1097-1107.
3. da Silva LC, Pinho JR, Sitnik R, Da Fonseca LE, Carrilho FJ. Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *J Gastroenterol* 2001; 36: 476-485.
4. Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, da Silva LC, Carrilho FJ. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 2004; 42: 2455-2460.
5. Takahashi K, Aoyama K, Ohno N, Iwata K, Akahane Y, Baba K, et al. The precore/core promoter mutant (T1762A1764) of hepatitis B virus: clinical significance and an easy method for detection. *J Gen Virol* 1995; 76 (Part 12): 3159-3164.
6. Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 2004; 5: 150-163.
7. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 1997; 78 (Part 7): 1719-1729.
8. Araujo NM, Mello FC, Yoshida CF, Niel C, Gomes SA. High proportion of subgroup A' (genotype A) among Brazilian isolates of hepatitis B virus. *Arch Virol* 2004; 149: 1383-1395.
9. Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Paniago AM, Lima KM, et al. Hepatitis B virus infection in isolated Afro-Brazilian communities. *J Med Virol* 2005; 77: 188-193.
10. Lyra AC, Pinho JR, Mello IM, de M Malta F, Gomes MM, Di Bisceglie AM, et al. Distribution of hepatitis B virus (HBV) genotypes among patients with acute viral hepatitis. *J Clin Gastroenterol* 2005; 39: 81-82.
11. Bertolini DA, Pinho JR, Saraceni CP, Moreira RC, Granato CF, Carrilho FJ. Prevalence of serological markers of hepatitis B virus in pregnant women from Paraná State, Brazil. *Braz J Med Biol Res* 2006; 39: 1083-1090.
12. Kramvis A, Restorp K, Norder H, Botha JF, Magnus LO, Kew MC. Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* 2005; 77: 47-52.
13. Mulders MN, Venard V, Njaye M, Edorh AP, Bola Oyefolu AO, Kehinde MO, et al. Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 2004; 190: 400-408.
14. Quintero A, Martinez D, Alarcon De Noya B, Costagliola A, Urbina L, Gonzalez N, et al. Molecular epidemiology of hepatitis B virus in Afro-Venezuelan populations. *Arch Virol* 2002; 147: 1829-1836.
15. Ribeiro NR, Campos GS, Angelo AL, Braga EL, Santana N, Gomas MM, et al. Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver Int* 2006; 26: 636-642.
16. Suzuki S, Suguchi F, Orito E, Kato H, Usuda S, Siransy L, et al. Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in the Cote d'Ivoire: complete genome sequence and phylogenetic relatedness of HBV genotype E. *J Med Virol* 2003; 69: 459-465.
17. Fujiwara K, Tanaka Y, Orito E, Ohno T, Sugihara K, et al. Distribution of HBV genotypes among HBV carriers in Benin: phylogenetic analysis and virological characteristics of HBV genotype E. *World J Gastroenterol* 2005; 11: 6410-6415.
18. Li J, Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. *J Virol* 1999; 73: 1239-1244.
19. Mathet VL, Cuestas ML, Ruiz V, Minassian ML, Rivero C, Trinks J, et al. Detection of hepatitis B virus (HBV) genotype E carried - even in the presence of high titers of anti-HBs antibodies - by an Argentinean patient of African descent who had received vaccination against HBV. *J Clin Microbiol* 2006; 44: 3435-3439.
20. Sitnik R. Padronização de reações de seqüenciamento para caracterização do vírus da hepatite B: genótipos, subtipos, mutantes na região pré-core, promotor basal do core, epítipo "a" e resistência aos antivirais. [PhD thesis]. São Paulo: Faculdade de Medicina, USP; 2002.

32- Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvêa MS, de Azevedo Neto RS, Carrilho FJ, Pinho JR. Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population-how is the epidemiological situation? PLoS One. 2011 Apr 29;6(4):e18888

Hepatitis B (HBV), Hepatitis C (HCV) and Hepatitis Delta (HDV) Viruses in the Colombian Population—How Is the Epidemiological Situation?

Mónica Viviana Alvarado-Mora^{1*}, María Fernanda Gutiérrez Fernández², Michele Soares Gomes-Gouvêa¹, Raymundo Soares de Azevedo Neto³, Flair José Carrilho¹, João Renato Rebello Pinho¹

¹ Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, Brazil, ² Laboratory of Virology, Department of Microbiology, Pontifícia Javeriana University, Bogotá, Colombia, ³ Department of Pathology, School of Medicine, University of São Paulo, São Paulo, Brazil

Abstract

Background: Viral hepatitis B, C and delta still remain a serious problem worldwide. In Colombia, data from 1980s described that HBV and HDV infection are important causes of hepatitis, but little is known about HCV infection. The aim of this study was to determine the currently frequency of HBV, HCV and HDV in four different Colombian regions.

Methodology/Principal Findings: This study was conducted in 697 habitants from 4 Colombian departments: Amazonas, Chocó, Magdalena and San Andrés Islands. Epidemiological data were obtained from an interview applied to each individual aiming to evaluate risk factors related to HBV, HCV or HDV infections. All samples were tested for HBsAg, anti-HBc, anti-HBs and anti-HCV markers. Samples that were positive to HBsAg and/or anti-HBc were tested to anti-HDV. Concerning the geographical origin of the samples, the three HBV markers showed a statistically significant difference: HBsAg ($p=0.033$) and anti-HBc ($p<0.001$) were more frequent in Amazonas and Magdalena departments. Isolated anti-HBs (a marker of previous vaccination) frequencies were: Chocó (53.26%), Amazonas (32.88%), Magdalena (17.0%) and San Andrés (15.33%) - $p<0.001$. Prevalence of anti-HBc increased with age; HBsAg varied from 1.97 to 8.39% ($p=0.033$). Amazonas department showed the highest frequency for anti-HCV marker (5.68%), while the lowest frequency was found in San Andrés Island (0.66%). Anti-HDV was found in 9 (5.20%) out of 173 anti-HBc and/or HBsAg positive samples, 8 of them from the Amazonas region and 1 from them Magdalena department.

Conclusions/Significance: In conclusion, HBV, HCV and HDV infections are detected throughout Colombia in frequency levels that would place some areas as hyperendemic for HBV, especially those found in Amazonas and Magdalena departments. Novel strategies to increase HBV immunization in the rural population and to strengthen HCV surveillance are reinforced by these results.

Citation: Alvarado-Mora MV, Gutiérrez Fernández MF, Gomes-Gouvêa MS, de Azevedo Neto RS, Carrilho FJ, et al. (2011) Hepatitis B (HBV), Hepatitis C (HCV) and Hepatitis Delta (HDV) Viruses in the Colombian Population—How Is the Epidemiological Situation? PLoS ONE 6(4): e18888. doi:10.1371/journal.pone.0018888

Editors: Lise Lotte Gluud, Copenhagen University Hospital Gentofte, Denmark

Received: January 18, 2011; **Accepted:** March 23, 2011; **Published:** April 29, 2011

Copyright: © 2011 Alvarado-Mora et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work has been supported by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP 2007/53457-7 and 2008/50461-6 and CNPq, São Paulo, SP, Brazil and by Pontifícia Universidad Javeriana, Bogotá, Colombia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: monica.vi@usp.br

Introduction

Around two billion people worldwide have been infected by hepatitis B virus (HBV) and approximately 350 million people are chronic carriers. Every year about one million deaths are caused by chronic HBV infection [1]. Approximately 400,000 new cases are estimated to occur in Latin America each year and 10 to 70% of them may evolve to hepatocellular carcinoma [2]. Considering the HBV infection epidemiology throughout different geographical zones, the world is divided in high, intermediate and low endemicity areas, corresponding to infection rates higher than 8%, 2 to 8% and lower than 2%, respectively [3].

The routes of HBV transmission in South and Central America are highly variable. The highest prevalence was reported in 20 to 40 years old age group, and adult horizontal transmission is the

most common route of infection [4]. Furthermore, vertical and childhood horizontal transmission are also important in areas of high endemicity such as the Amazon Basin [5,6].

HBV strains have distinct geographical distribution that are classified in nine genotypes (A to I) based on genome diversity and some of them are further classified in subgenotypes [7]. In Colombia, subgenotype F3 is the most common subgenotype among the HBV infected patients, but it was also reported an important frequency of genotypes E, A2, G and F1b among HBV chronic patients [8,9].

Hepatitis D virus (HDV) is a subviral agent that requires a preexisting or concurrent infection with HBV [10]. HDV genome is a 1672 to 1697 nucleotides circular single-strand RNA of with extensive intramolecular complementarity [11,12]. Compared with infection with HBV alone, HDV coinfection with HBV is

associated with a higher rate of fulminant hepatitis in an acute infection and HDV superinfection in individuals with chronic HBV infection can lead to more severe progressive chronic liver disease [10].

HDV is classified in eight different genotypes: genotype 1 is the most frequent and found in Europe, Middle East, North America and North Africa [13–15]; genotype 2 is seen in the Far East; genotype 3 was reported in the Amazonian region of South America [16,17]; genotype 4 was isolated in Taiwan and Japan [18–20]; and genotypes 5 to 8 have been identified in Africans [21]. HDV is distributed worldwide with more than 24% of HBV carriers with HDV markers present in Africa, Southwest Asia and in the Mediterranean basin. In the United States, HDV prevalence is lower, ranging from 1 to 10% [22]. In Colombia, few reports are available on HDV prevalence, mainly showing its association to fulminant hepatitis outbreaks in departments with intermediate or high HBV endemicity, particularly in the Amazonas department where high HDV antibodies prevalence rates have been found among children younger than 4 years old [23].

Hepatitis C virus (HCV) infection is a public health problem throughout the world infecting around 3% of the world population. HCV is classified in six major genotypes and more than 70 subtypes [24,25]. It is an enveloped, single stranded positive sense RNA virus with a 50 nm diameter viral particle, classified in *Hepacivirus* genus of the *Flaviviridae* family [26]. Countries with the highest reported prevalence rates are located in Africa and Asia; while lower prevalence areas include North America, North and West Europe, and Australia [27]. In Latin America, HCV overall prevalence is 1.23% [4]. However, the HCV antibody in blood donors in Latin America varies from 0.2%–0.5% in Chile to 1.7%–3.4% in northeast of Brazil [4,28,29]. The main risk factors are unsafe injection techniques and blood transfusions [29]. Mandatory screening for HCV infection was gradually adopted in Latin America during the 1990s, leading to a currently decreased blood transmission in most South American countries [4,29]. One study carried out in Colombia during 2005 showed a prevalence of 9% of HCV among multi-transfused patients [30]. The distribution of HCV genotypes in Colombia showed that subtype 1b is the most prevalent and that HCV emerged in Bogotá around 60 years ago [31].

Since in Colombia there are few epidemiologic studies about the hepatitis virus, the aim of this study was to determine the frequency of serological markers for viral hepatitis B, C and D (delta) in four regions in Colombia and to analyze the risk factors associated with viral transmission in these populations.

Methods

Study population

This study was conducted in 697 habitants with ages varying from 12 to 72 years old from the rural and urban areas of four departments in Colombia: Amazonas (n = 184), Chocó (n = 138), Magdalena (n = 218) and San Andrés Islands (n = 156) (Figure 1). Epidemiological data were obtained from an interview applied to each individual aiming to evaluate risk factors related to HBV, HCV or HDV infections. The Ethical Committees of the Pontificia Universidad Javeriana, Bogotá, Colombia and the University of São Paulo Medical School, São Paulo, Brazil, approved this protocol. All patients have signed an informed consent form before joining the study.

Sampling was carried out for each department during the span of a week. The individuals were summoned in the public health laboratory from each region. Different risk groups were evaluated in this study. In the Amazonas department, sampling was

performed in the area of the Amazon River, involving Amerindian populations originating from four different ethnic groups (Ticuna, Huitoto, Huinane, and Yagua). In Chocó department and San Andrés Islands, sampling included female sex workers, doctors and nurses. In Magdalena department, it included medical workers and “desplazados” populations (i.e., people that were obliged to leave their homes due to the civil war).

Serological tests

All samples were tested for HBsAg, anti-HBc, anti-HBc and anti-HCV markers. Samples that were positive to for HBsAg and/or anti-HBc were tested to anti-HDV. These assays were performed using commercial available EIIISA kits (DiaSorin, Italy).

Statistical analyses

Statistical analyses were performed using Minitab Software v. 15. The χ^2 test for linear trend ($\alpha = 0.05$) was used to examine the distribution of HBV, HCV and HDV markers according to age group, sex, and geographical origin. Results were considered statistically significant when $p < 0.05$.

Results

The results for all serological markers tested in the samples and for the statistical analysis according to three analyzed variables (sex, age group and geographical origin of the samples) are shown in Table 1.

Hepatitis B virus markers (HBsAg, anti-HBc and anti-HBs)

In spite of the higher frequency of anti-HBc and anti-HBs positive cases when comparing males to females patients, these differences were not statistically significant ($p = 0.051$ and $p = 0.089$, respectively). Anti-HBc ($p < 0.001$) frequency increased with age, from the 11–15 years old group to the > 51 years old group. Anti-HBs ($p = 0.026$) frequency was also different in the several age groups and was higher in the 46–50 and > 51 years old groups. HBsAg frequency was not different according to sex and age ($p = 0.378$ and $p = 0.502$, respectively).

Amazonas and Magdalena departments showed the highest frequency for all HBV markers while San Andrés Islands and Chocó showed a low frequency for them. The differences for HBsAg ($p = 0.033$), anti-HBc ($p < 0.001$) and anti-HBs ($p < 0.001$) frequencies in the various geographical origins of the samples were statistically significant.

Concerning the geographical origin of the samples, the three HBV markers showed a statistically significant difference. HBsAg ($p = 0.033$) and anti-HBc ($p < 0.001$) were more frequent in Amazonas and Magdalena departments and less frequent in San Andrés Islands and Chocó. For anti-HBs ($p < 0.001$), the highest and lowest frequencies were also observed in Amazonas department and San Andrés Islands, respectively, but Chocó department showed the second highest frequency.

Another analysis was carried out to verify the frequency of isolated anti-HBs as a marker of previous vaccination. Its frequency by geographical origin of the samples was: Chocó (53.26%), Amazonas (32.88%), Magdalena (17.0%) and San Andrés (15.33%) - $p < 0.001$. Isolated anti-HBs distribution was not different according to sex and age ($p = 0.089$ and $p = 0.860$, respectively).

Hepatitis C marker (anti-HCV)

Amazonas department showed the highest frequency for anti-HCV marker (5.68%). The lowest frequency was found in San Andrés Island (0.66%) and intermediate levels were found in the

Figure 1. Geographic localization of the four places from where samples were collected in Colombia. The capital of each department is in red (●).
doi:10.1371/journal.pone.0018888.g001

two other departments, but these differences were not statistically significant ($p = 0.107$). Considering age groups, the higher frequency for anti-HCV was found from 26 to 30 (7.22%) years old, but it was not statistically significant ($p = 0.259$). Geographical origin of samples did not show statistically significant difference considering the presence of anti-HCV in the population ($p = 0.107$).

Hepatitis Delta marker (anti-HDV)

One-hundred seventy three anti-HBc positive and/or HBsAg-positive samples were screened for anti-HDV. Nine (5.20%) samples (from 3 males and 6 females) were positive for this marker. Eight of these samples were from the Amazonas region and one

sample came from Magdalena department. Anti-HDV positive patients ranged from 21 to 67 years old. No statistically significant differences were found for anti-HDV distribution and sex ($p = 0.949$). For age group and geographical origin of samples, it was not possible to carry out a statistical analysis due to the small number of positive samples.

Discussion

In Colombia, there are a few studies about hepatitis epidemiology. This is the first currently study reporting HBV, HCV and HDV serological profiles in these four departments (Amazonas, Chocó, Magdalena and San Andrés Islands).

Table 1. Results for each HBV, HDV and HCV serology marker evaluated in the Colombian population (>11 years old).

Variables	HBsAg Positives/n (%)	anti-HBc Positives/n (%)	anti-HBs Positives/n (%)	Isolated anti-HBs Positives/n (%)	anti-HDV Positives n (%)	anti-HCV Positives/n (%)
Sex						
Male group	7/163 (4.29%)	56/163 (34.36%)	73/149 (48.99%)	33/96 (34.38%)	3/56 (5.36%)	6/163 (3.68%)
Female group	28/455 (6.19%)	120/456 (26.32%)	175/427 (40.98%)	78/306 (25.49%)	6/117 (5.13%)	16/456 (3.51%)
<i>p</i>	0.378	0.051	0.089	0.089	0.949	0.919
Age group (years)						
11–15	1/32 (3.13%)	0/32 (0.00%)	6/30 (20.00%)	6/30 (20.00%)	no samples	0/32 (0.00%)
16–20	5/58 (8.62%)	7/58 (12.07%)	19/54 (35.19%)	11/45 (24.44%)	0/7 (0.00%)	1/58 (1.72%)
21–25	8/130 (6.15%)	25/130 (19.23%)	43/116 (37.07%)	25/94 (26.60%)	1/22 (4.55%)	6/130 (4.62%)
26–30	8/97 (8.25%)	26/97 (26.80%)	44/94 (46.81%)	23/66 (34.85%)	0/23 (0.00%)	7/97 (7.22%)
31–35	4/60 (6.67%)	20/69 (28.99%)	29/64 (45.31%)	12/45 (26.67%)	1/22 (4.55%)	2/69 (2.90%)
36–40	0/61 (0.00%)	20/62 (32.26%)	26/58 (44.83%)	11/38 (28.95%)	1/20 (5.00%)	0/62 (0.00%)
41–45	2/55 (3.64%)	19/55 (34.55%)	21/52 (40.38%)	7/33 (21.21%)	3/20 (15.00%)	2/55 (3.64%)
46–50	3/35 (8.57%)	17/35 (48.57%)	19/33 (57.58%)	5/16 (31.25%)	1/18 (5.56%)	0/35 (0.00%)
>51	4/81 (4.94%)	42/81 (51.85%)	41/75 (54.67%)	11/35 (31.43%)	2/41 (4.88%)	4/81 (4.94%)
<i>p</i>	0.502	<0.001	0.026	0.860	*	0.259
Geographical Origin						
Amazonas	14/176 (7.95%)	96/176 (54.55%)	105/168 (62.50%)	24/73 (32.88%)	8/97 (8.25%)	10/176 (5.68%)
Chocó	5/135 (3.70%)	25/136 (18.38%)	67/115 (58.26%)	49/92 (53.26%)	0/27 (0.00%)	5/136 (3.68%)
San Andrés Island	3/152 (1.97%)	6/152 (3.95%)	25/143 (17.48%)	21/137 (15.33%)	0/9 (0.00%)	1/152 (0.66%)
Magdalena	13/155 (8.39%)	40/155 (25.81%)	51/150 (34.00%)	17/100 (17.00%)	1/40 (2.50%)	6/155 (3.87%)
<i>p</i>	0.083	<0.001	<0.001	<0.001	*	0.107
Total	35/618 (5.66%)	176/619 (28.43%)	248/576 (43.06%)	111/402 (27.61%)	9/173 (5.20%)	22/619 (3.55%)

**p* value was not calculated due to the small number of positive samples.
doi:10.1371/journal.pone.0018888.t001

The overall anti-HBc and HBsAg frequencies found were 28.43% and 5.66%, respectively. These rates include Colombia as an intermediate endemicity region in at least these four regions that we have analyzed. HBsAg positivity rates ranged from 1.97% in San Andrés Islands to 8.39% in Magdalena department, slightly surpassing the intermediate endemicity rates (2 to 8%) in this last department.

Serological markers for HBV infection have been previously found to range from 25 to 83% among Amazonian peoples in Bolivia, Brazil, Peru and Venezuela [32,33]. In a study performed between 1977 and 1989, overall HBsAg seroprevalence rate was 4.7% in Colombia [34].

In a previous paper analyzing the different Colombian regions, HBsAg prevalence was 7.1%, 3.5% and 2.8% in the Central region, in the Pacific zone and in the Eastern region, respectively [35]. In the present paper, we have analyzed Chocó, in the Pacific region, and we have found a HBV intermediate prevalence of 3.7% of the studied population, very close to the previously reported.

In another study carried out in 1975 involving native Chocó people who lived in the Darien forest, it was reported a higher exposure to HBV: 42% anti-HBs positive but only 1.2% HBsAg positive [36]. The prevalence of HBV markers among these native Chocó people is lower than that found in the Chocó population studied in the present paper as well as that found in other native people who live in the Amazon department, as shown below. This difference might be related to the higher sensitivity of the methodology applied nowadays.

Western Amazonas basin is reported as one region with the highest rates of hepatitis B infection in the world as more than

80% of people living in some rural areas have been infected with HBV and more than 8% carry HBsAg [4,37,38]. In the present paper, we are reporting 7.95% HBsAg positivity among the studied population from Amazonas department, confirming that HBV infection remains a problem in this region.

Near the capital of Magdalena department, Santa Marta city, in northern Colombia, recurrent epidemics of severe hepatitis have been reported over many years and have acquired the name of "Santa Marta hepatitis". This hepatitis was described in 1930s [39] as a severe icteric illness with mortality as high as 10% [40]. HBsAg frequency found in Magdalena department in this study was 8.39%, the highest found among the 4 departments analyzed. Previous studies carried during the 80's found HBsAg levels higher in studies of the populations affected with "Santa Marta Hepatitis" [34]. The present study could not study the population of Sierra Nevada de Santa Marta where these epidemics have been described but the finding of higher levels of HBV endemicity in this area shows that it is remains a public health problem in this area.

To our knowledge, previous data on the frequency of hepatitis viruses markers at San Andrés Islands have not been published before. These islands showed the lowest prevalence for all HBV and HCV markers among the four studied regions. HDV infection was not detected.

Anti-HBc positivity increased in older age groups. This finding agrees with the known role of sexual transmission in HBV spreading. As we did not have studied any children with less than 12 years old in this study, we cannot evaluate the role of vertical transmission for hepatitis infection in this population. Anti-HBs

positivity also increased in older age groups, but surprisingly HBsAg positivity did not show a statistically significant difference with age as some age groups showed particularly lower levels of HBsAg detection (especially the 36 to 40 years old age group).

Cuba, Colombia and Brazil were the first countries in Latin America that introduced universal HBV vaccination in the early 1990s [41]. Isolated anti-HBs (i.e., immunized after vaccination) individuals constitute only 17.96% (n=111) of the studied population (n=618). Considering only the susceptible individuals (n=402), i.e., anti-HBc and HBsAg seronegatives but anti-HBs seropositives, only 27.61% of them were protected against HBV infection. Isolated anti-HBs was detected in more than half and in about one third of susceptible individuals in Chocó and Amazonas departments, respectively. In San Andrés Islands and Magdalena departments, isolated anti-HBs levels are only 15.33 and 17.00%, respectively. It is noteworthy that the highest HBsAg positive rate was detected in Magdalena department.

In 2002, it was reported a HBV vaccine coverage in Chocó about 26.2% [42]. In Chocó department, we found that 53.26% individuals with isolated anti-HBs, reflecting previous HBV vaccination, what would explain the HBsAg frequency found. At San Andrés Islands, we found a low frequency of isolated anti-HBs (15.3%) but also a lower frequency of HBV infection markers. Isolated anti-HBs frequency was 32.88% in Amazonas department.

These results show that HBV vaccination must be reinforced in Colombia as an effective health program for this population to prevent new hepatitis B cases. This is an important public health problem involving the costs for the implementation of widespread efficient vaccination programs in large regions inside scarcely inhabited areas where the populations are spread in small villages located in the middle of the equatorial rain forest. Furthermore, such program might face unexpected problems, as some also indigenous populations in the Amazonian region may not accept HBV vaccination for cultural reasons.

For hepatitis delta, areas of high prevalence include the Mediterranean Basin, the Middle East, Central Asia, Amazon Basin in South America and certain South Pacific islands [43]. Severe, often fatal, acute and chronic type D hepatitis occurs among indigenous people for Venezuela, Colombia, Brazil and Peru, all regions with high chronic HDV infection rates [44].

In Colombia, HDV infection is common in Amazonas and Magdalena departments [37,38,45,46]. These data were confirmed in this study as the only anti-HDV positive samples found were from these two departments: 8 (8.25%)/97 and 1 (2.5%)/40 among HBsAg and/or anti-HBc positive samples from Amazonas and Magdalena departments, respectively. These two departments have a huge jungle region and HDV genotype 3 predominates [10,17]. Buitrago et al., in 1986, suggested that HDV infection has been endemic in northern South America for more than 50 years. The high frequency of HBV, together with the presence of HDV markers, showed that these viruses represent the etiologic factors of the "hepatitis of the Sierra Nevada de Santa Marta" [40]. It is

important to reinforce that a continuous surveillance of this area allied to an intense HBV vaccination program is needed to control this severe hepatic disease, as cases co-infected with both viruses are still being detected.

The estimates of HCV prevalence in Colombia correspond to data collected from blood donors, since there is no study of prevalence in the general population [30]. A study reporting the potential risk for an infectious disease through tainted transfusion involving Colombian blood donors reported that 98.30% of them were submitted to anti-HCV screening and 0.90% of those were anti-HCV positive in 1993. This study also reported in Colombia a probability of 74.55 per 10,000 donors of receiving an infected transfusion and 67.09 per 10,000 donors of getting a transfusion-transmitted infection, which was the highest risk of receiving a blood unit infected with HCV and of contracting this infection in Latin America [47]. Colombia has made screening for HCV mandatory since 1993 [48] and the coverage for serology for that infection in the blood banks has increased since now. In another study, the screening coverage for anti-HCV in Colombia in 2002 increased to 99.70% and the risks of receiving an infected unit or developing an infection after receiving an infected unit of blood in decreased to 0.24 per 10,000 donors and 0.22 per 10,000 donors, respectively [49]. These results strongly agree with previous results from our group showing that HCV genotype 1b, the most frequent in Colombia, exponentially spread up to 1992, when its growth was controlled by HCV screening in Blood Banks [31].

In this study, anti-HCV frequency ranged from 0.66% in San Andrés Island up to 5.68% in Amazonas department. HCV is an infection that probably arrived recently in Colombia [31] when compared to HBV, which is found wide spread in Native Colombians people and some HBV genotypes are quite probably longer in South America [9]. Nevertheless, the widespread presence of this infection throughout the world, the known transmissions routes by blood supplies and other parenteral routes, led this infection to spread around the world and it is present in all the Colombian regions studied, in some of them in endemicity levels that deserve deeper attention of health policy authorities.

In conclusion, HBV, HCV and HDV infections are detected throughout Colombia in frequencies levels that would place some areas as hyperendemic for HBV, especially those found in Amazonas and Magdalena departments.

Acknowledgments

We thank to *Laboratorio de Salud Pública* in Quibdó, Santa Marta, San Andrés and Leticia, in Colombia, for kindly providing help in the sampling process for this study.

Author Contributions

Conceived and designed the experiments: MVAM MSGG EJC JRRP. Performed the experiments: MVAM MSGG MPGF. Analyzed the data: MVAM RSAN JRRP. Contributed reagents/materials/analysis tools: MVAM MSGG. Wrote the paper: MVAM JRRP.

References

- Lawachy D (2006) Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 13: 97–107.
- Fay OH (1990) Hepatitis B in Latin America: epidemiological patterns and eradication strategy. The Latin American Regional Study Group. *Vaccine* 8 Suppl: S100–106; discussion S134–109.
- Tanaka J (2000) Hepatitis B epidemiology in Latin America. *Vaccine* 18(Suppl 1): S17–19.
- Te HS, Jensen DM (2000) Epidemiology of hepatitis B and C viruses: a global overview. *Clin Liver Dis* 14: 1–21, vii.
- Torres JR (1996) Hepatitis B and hepatitis delta virus infection in South America. *Gut* 38(Suppl 2): S48–55.
- Gish RG, Gadeau AC (2006) Chronic hepatitis B: current epidemiology in the Americas and implications for management. *J Viral Hepat* 13: 787–798.
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, et al (2010) Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "T". *PLoS One* 5: e9297.
- Alvarado Mora MV, Romano GM, Gomez-Gouveia MS, Gutierrez MF, Garbino EJ, et al (2010) Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol* 91: 501–508.
- Alvarado Mora MV, Romano GM, Gomez-Gouveia MS, Gutierrez MF, Botelho L, et al (2011) Molecular characterization of the Hepatitis B virus

- genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol* 11: 103–108.
10. Casey JL, Brown TL, Cohen EJ, Wignall PS, Gerin JL (1993) A genotype of hepatitis D virus that occurs in northern South America. *Proc Natl Acad Sci U S A* 90: 9036–9040.
 11. Wang KS, Choo QJ, Weiner AJ, Ou JH, Najarian RG, et al. (1986) Structure, sequence and expression of the hepatitis delta (delta) viral genome. *Nature* 323: 568–574.
 12. Radjef N, Gordien E, Ivanishina V, Gault E, Anis P, et al. (2004) Molecular phylogenetic analyses indicate a wide and ancient radiation of African hepatitis delta virus, suggesting a *delavirus* genus of at least seven major clades. *J Virol* 78: 2537–2544.
 13. Makino S, Chang MF, Shieh CK, Kamahara T, Vannier DM, et al. (1987) Molecular cloning and sequencing of a human hepatitis delta (delta) virus RNA. *Nature* 329: 343–346.
 14. Chao YC, Chang MF, Gao I, Lai MM (1990) Sequence conservation and divergence of hepatitis delta virus RNA. *Virology* 178: 384–392.
 15. Shukil AO, Hadziyannis S, Hoodnagel JH, Di Biase AM, Gerin JL, et al. (1997) Geographic distribution and genetic variability of hepatitis delta virus genotype I. *Virology* 234: 160–167.
 16. Quintana A, Uzcategui N, Loureiro GL, Villegas I, Ilarrazendi X, et al. (2001) Hepatitis delta virus genotypes I and III circulate associated with hepatitis B virus genotype F in Venezuela. *J Med Virol* 64: 356–359.
 17. Gomes-Gouveia MS, Soares MC, Benabath G, de Carvalho-Mello IM, Brito EM, et al. (2009) Hepatitis B virus and hepatitis delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the western Brazilian Amazon region. *J Gen Virol* 90: 2638–2643.
 18. Wu JC, Chiang TY, Shern IJ (1998) Characterization and phylogenetic analysis of a novel hepatitis D virus strain discovered by restriction fragment length polymorphism analysis. *J Gen Virol* 79(Pt 5): 1105–1113.
 19. Sakaguchi H, Nakamura H, Nakayoshi T, Kawakami Y, Miyamoto S, et al. (1999) Hepatitis delta virus genotype IIb predominates in an endemic area, Okinawa, Japan. *J Med Virol* 58: 366–372.
 20. Watanabe H, Nagayama K, Iwamoto N, Ghisleri R, Yamashiro T, et al. (2003) Chronic hepatitis delta virus infection with genotype IIb variant is correlated with progressive liver disease. *J Gen Virol* 84: 3275–3289.
 21. Le Goff F, Gault E, Ripault MP, Serpagy J, Trinchet JC, et al. (2006) Eighth major clade for hepatitis delta virus. *Emerg Infect Dis* 12: 1447–1450.
 22. Gatta GR, Stroffolini T, Chiancone M, Accione T, Sornalaino G, et al. (2000) Chronic hepatitis D: a vanishing disease? An Italian multicenter study. *Hepatology* 32: 824–827.
 23. Espinal C (1998) Perfil Epidemiológico de la Hepatitis B y D en Colombia. *Biomedica* 18: 216–249.
 24. Paopier C, Njousm R, Ayoub A, Dabois M, Saire MT, et al. (2005) Distribution and heterogeneity of hepatitis C genotypes in hepatitis patients in Cameroon. *J Med Virol* 77: 390–398.
 25. Echeverría JM, Leon P (2003) Epidemiology of viruses causing chronic hepatitis among populations from the Amazon Basin and related ecosystems. *Cad Saude Publica* 19: 1583–91.
 26. Botani N, Mahmood T (2010) An overview about hepatitis C: A devastating virus. *Critical Reviews in Microbiology* 36(2): 91–133.
 27. Shepard CW, Finelli L, Alter MJ (2003) Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 3: 558–567.
 28. Caritho EJ, Corra MCJM (1998) Magnitude of hepatitis B and C in Latin America. In: Schinazi RF, Sommadossi JP, Thomas HG, eds. *Therapies for viral hepatitis*. London: International Medical Press. pp 25–34.
 29. Soza A, Riquelme A, Arrese M (2010) Routes of transmission of hepatitis C virus. *Ann Hepatol* 9 Suppl: 33.
 30. Beltrán M, Navas MC, De la Hoz F, Mercedes Muñoz M, Jaramillo S, et al. (2005) Hepatitis C virus seroprevalence in multi-transfused patients in Colombia. *J Clin Virol* 34(Suppl 2): S33–38.
 31. Mora MV, Romero CM, Gomez-Gouveia MS, Gutierrez MF, Caritho EJ, et al. (2010) Molecular characterization, distribution, and dynamics of hepatitis C virus genotypes in blood donors in Colombia. *J Med Virol* 82: 1889–1898.
 32. Benabath G, Hadler SC, Soares MC, Fields H, Maynard JE (1987) Epidemiologic and serologic studies of acute viral hepatitis in Brazil's Amazon Basin. *Bull Pan Am Health Organ* 21: 16–27.
 33. Hadler SC, De Monzon M, Ponzone A, Anzola E, Rivers D, et al. (1984) Delta virus infection and severe hepatitis: An epidemic in the Yupa Indians of Venezuela. *Ann Intern Med* 100: 339–344.
 34. Ljunggren KE, Patarroyo ME, Fagle R, Parrell RH, Gerin JL (1985) Viral hepatitis in Colombia: a study of the "hepatitis of the Sierra Nevada de Santa Marta". *Hepatology* 5: 299–304.
 35. Prieto F, Rojas D (2003) Situación serológica de la hepatitis B, Colombia. *Programa ITS/Aids, Instituto Nacional de Salud, Biomedica* 8: 2–11.
 36. Reeves WC, Peters CJ, Parrell RH (1975) The epidemiology of hepatitis B antigen and antibody among Panamanian Guana Indians. *Am J Trop Med Hyg* 24: 873–5.
 37. Gayotto LG (1991) Hepatitis delta in South America and especially in the Amazon region. *Prog Clin Biol Res* 364: 123–35.
 38. de la Hoz F, Martínez M, Iglesias A, Rojas MC (1992) Factores de riesgo en la transmisión de la hepatitis B en la Amazonia Colombiana. *Biomedica* 12: 5–9.
 39. Ramsey GH (1931) Fever with jaundice in the Province of Santa Marta, Colombia. Preliminary Report. Presented to the International Division of the Rockefeller Foundation.
 40. Buitrago B, Hadler SC, Popper H, Thung SN, Gerber MA, et al. (1986) Epidemiologic aspects of Santa Marta hepatitis over a 40-year period. *Hepatology* 6: 1292–1296.
 41. Shumway J (2000) Who needs vaccination against hepatitis viruses? *Vaccine* 18(Suppl 1): S4–5.
 42. Ministerio de Salud (2002) Boletín Epidemiológico Semanal: Situación de Hepatitis B en Colombia a la semana Epidemiológica. Available: www.col.ops-oms.org/sivigila/2002/BOLE40_02.pdf.
 43. Lai MM (1995) The molecular biology of hepatitis delta virus. *Annu Rev Biochem* 64: 259–286.
 44. World Health Organization (2001) Hepatitis Delta. Department of Communicable Disease Surveillance and Response. pp 18–20.
 45. Martínez M, De la Hoz F, Jaramillo IS, Rojas MC, Buitrago B, et al. (1991) Seroprevalencia de la infección por el virus de la hepatitis B en niños de la Amazonia Colombiana. *Biomedica* 11: 20–24.
 46. Buitrago B (1991) Historia natural de las hepatitis B y D en Colombia. *Biomedica* 11: 5–26.
 47. Schmunis GA, Zicker F, Pinheiro F, Brandling-Bennett D (1998) Risk for transfusion-transmitted infectious diseases in Central and South America. *Emerg Infect Dis* 4: 5–11.
 48. Ministerio de Salud (1996) Manual de normas técnicas, administrativas y de procedimientos. Capítulo 11. Garantía de calidad. Santa Fe de Bogotá, D.C., Colombia. Imprenta Nacional.
 49. Schmunis GA, Cruz JR (2005) Safety of the blood supply in Latin America. *Clin Microbiol Rev* 18: 12–29.

- 33- Alvarado-Mora MV, Romano CM, Gomes-Gouvêa MS, Gutierrez MF, Carrilho FJ, R Pinho JR. Phylogenetic analysis of complete genome sequences of hepatitis B virus from an Afro-Colombian community: presence of HBV F3/A1 recombinant strain. Virology Journal. 2012 Oct 24;9: 244**

RESEARCH

Open Access

Phylogenetic analysis of complete genome sequences of hepatitis B virus from an Afro-Colombian community: presence of HBV F3/A1 recombinant strain

Mónica V Alvarado-Mora^{1*}, Camila M Romano², Michele S Gomes-Gouvêa¹, Maria F Gutierrez³, Flair J Carrilho¹ and João R R Pinho¹

Abstract

Background: Hepatitis B virus (HBV) infection is one of the most prevalent viral infections in humans and represents a serious public health problem. In Colombia, our group reported recently the presence of subgenotypes F3, A2 and genotype G in Bogotá. The aim of this study was to characterize the HBV genotypes circulating in Quibdó, the largest Afro-descendant community in Colombia. Sixty HBsAg-positive samples were studied. A fragment of 1306 bp (S/POL) was amplified by nested PCR. Positive samples to S/POL fragment were submitted to PCR amplification of the HBV complete genome.

Findings: The distribution of HBV genotypes was: A1 (52.17%), E (39.13%), D3 (4.3%) and F3/A1 (4.3%). An HBV recombinant strain subgenotype F3/A1 was found for the first time.

Conclusions: This study is the first analysis of complete HBV genome sequences from Afro-Colombian population. It was found an important presence of HBV/A1 and HBV/E genotypes. A new recombinant strain of HBV genotype F3/A1 was reported in this population. This fact may be correlated with the introduction of these genotypes in the times of slavery.

Keywords: Hepatitis B virus, Subgenotype A1, Genotype E, Subgenotype D3, Bayesian analysis, Recombinant subgenotype F3/A1, Colombia

Introduction

Hepatitis B virus (HBV) infection is a relevant global health problem with 2 billion people that have been infected worldwide, including 350 million of them suffering from chronic HBV infection [1]. In Latin America, the estimated HBsAg seroprevalence ranges from 0.5% to 3.0%, with the total number of HBsAg carriers approaching 11 millions [2]. The highest prevalence surpassing 8.0 % is found among the native populations of the western Amazon basin, which includes Brazil [3], Colombia [4], Peru [5] and Venezuela [6].

HBV genome is a partially double-stranded circular DNA molecule of approximately 3,200 bp that encodes four overlapping open reading frames (ORFs) [7]. A genetic classification based on the comparison of complete HBV genomes has identified nine genotypes, A through I [8], that differ by at least 8% at nucleotide level from each other. Genotype A was initially identified in southern Africa [9]. Phylogenetic analysis of the complete genomes of subgenotype A1 isolates classified it in two clusters (African and Asian) [10]. The introduction of subgenotype A1 into Asia could be the result of movements along the East coast of Africa, from Somalia in the horn of Africa to the Arabic Peninsula in Asia [10]. Subgenotype A2, also denoted Ae, from "European" subgenotype, was isolated from South African carriers and is also found in Northern Europe and Greenland [11].

* Correspondence: monicaviviana@usp.br

¹Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, Brazil

Full list of author information is available at the end of the article

© 2012 Alvarado-Mora et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Subsequently, subgenotype A3 was characterized in Cameroon, Mali and Gambia [12]. Complete genomes of two HBV isolates from Mali (subgenotype A4) have been found to diverge by 6% from A3. Finally, four Nigerian genotype A isolates formed another group, tentatively designated subgenotype A5 [13]. More recently, subgenotypes A6 was detected in African-Belgian patients [14] and subgenotype A7 was reported in Cameroon [15].

Genotypes B and C are predominant in East and Southeast Asia. Subgenotype D1 occurs mostly in the Mediterranean basin and Middle East. D2 has been reported in India, Japan, Europe and the United States. D3 was found in South Africa, Brazil, Rwanda, India, Costa Rica, Iran, Serbia and the United States. Finally, D4 was reported in Australia, South Africa, Somalia, Rwanda and Oceania [11].

Genotype E was first described in West Africa in high prevalence but in a surprisingly low diversity, as the mean diversity over the whole genome is 1.75%. HBV/E is the most prevalent genotype in western and central Africa [10]. In Quibdó, Colombia we previously reported for the first time the presence of this genotype in nine cases [16]. Genotypes F and H are found in populations from Alaska to Central and South America [17,18].

The aim of the present study was to characterize the HBV genotypes circulating in Quibdó city, Colombia. We report for the first time the HBV complete genome sequences from Afro-Colombian infected people and inferred their origin using phylogenetic analyses approaches.

Materials and methods

Study population

To evaluate HBV genotypes distribution in Quibdó, the largest Afro-descendant community in Colombia, 60 positive samples for the Hepatitis B virus surface antigen (HBsAg) were obtained from sera stored at -20°C in a public health laboratory in Quibdó, Colombia in 2007. This protocol was approved by the Ethical Committees from Pontificia Universidad Javeriana, Bogotá Colombia and School of Medicine, University of São Paulo, São Paulo, Brazil. Before

HBV DNA extraction

HBV DNA extraction was carried out from 100 µL serum using the acid guanidinium thiocyanate/phenol/chloroform method [19]. Briefly, 300 µL GT solution was added to each sample. Ice-cold chloroform (50 µL) was added, followed by homogenization and centrifugation. The supernatant was transferred to a conical tube and precipitated with 300 µL cold ethanol. After discarding the ethanol, samples were dried at 94°C for 1 min, resuspended in 50 µL ultrapure MilliQ water and stored at -20°C.

HBV PCR amplification

To characterize HBV genotypes, a fragment of 1306 bp partially comprising HBsAg and DNA polymerase coding regions (S/POL) was amplified by nested PCR using the primers PS3132F/2920R and PS3201F/P1285R [18]. After purification of the PCR product through ChargeSwitch PCR Clean-Up Kit, Sanger sequencing was performed using dideoxynucleotide triphosphates (ddNTPs) in Big Dye Terminator v3.1 Cycle Sequencing Ready Reaction kit – Applied Biosystems, Foster City, CA, USA). The electrophoresis was done in an ABI Prism 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA). The quality of each electropherogram was evaluated using the Phred-Phrap software and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available at the web page Electropherogram quality analysis Phred (<http://asparagin.cenargen.embrapa.br/phph>).

Amplification of the whole HBV genome was performed with P1 and P2 primers described previously with slight modifications [20]. The quality of each electropherogram was evaluated as cited above.

HBV genotyping analysis

Sequences were genotyped by phylogenetic reconstructions using reference sequences from all HBV genotypes obtained from GenBank (n=412), comprising 1306 bp of partial HBsAg and DNA polymerase coding regions (S/POL). Complete genomes were also obtained from Genbank (n=192) and phylogenetic analyses were performed. All sequences were aligned using Muscle software [21] and edited with the SE-AL software (available at <http://tree.bio.ed.ac.uk/software/seal/>). The Bayesian Markov chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.4 [22] was done to obtain the best possible estimates under both relaxed uncorrelated lognormal and exponential molecular clock and using the model of nucleotide substitution (GTR+G+I). The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. After 10 million generations, the maximum credibility tree (MCC) was obtained by summarizing the 10,000 substitution trees after removing a burn-in of 10% using Tree Annotator v.1.5.3 [22]. Phylogenetic trees were visualized and midpoint rooted in FigTree v1.2.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

Results

Of the 60 HbsAg-positive samples, 29 (48.3%) were positive by nested PCR for S/POL region and among them, 23 were obtained with good quality sequenced for phylogenetic analysis (Figure 1). The distribution of HBV genotypes in these 23 samples was: A1 (52.17%), E (39.13%),

(See figure on previous page.)

Figure 1 The Maximum Clade Credibility (MCC) tree was estimated by a Bayesian analysis from a larger dataset comprising 413 sequences with 1306 nucleotides of S and Polymerase HBV region of the different subgenotypes. The posterior probabilities of the key nodes are shown above the respective clusters. The cluster containing the strains of other HBV subgenotypes were collapsed.

D3 (4.3%) and F3/A1 (4.3%). Nine genotype E sequences generated in this study from the same community were previously published [16] since they represented an exclusively African HBV genotype circulating in South America. Due to a small sample size, these results were considered inconclusive about the prevalence of these genotypes in the population.

This work shows for the first time globally a recombinant strain of HBV genotype F3/A1 with 1306bp. The break point was at position 941 of HBV genome (POL region). The analysis using the Simplot program and bootscanning analysis confirmed this complex recombination. The sequence was compared with a consensus sequence of each HBV genotype (A–H) in order to identify the breakpoints. The analysis was carried out using a window size of 200 bp, a step size of 20 bp, 100 bootstrap replicates, gapstrip on and neighbour-joining analysis. The break point at position 941 of HBV genome (POL region) was in the codon ATT (C) (HBV/F3) → ACT (C) (HBV/A1) (Figure 2). Furthermore, we obtained for the first time seven HBV Colombian complete genome sequences from A1, E and D3 genotypes (Figure 3). Complete genome HBV sequences were deposited at the GenBank under accession numbers: JQ023660–JQ023666.

Discussion

These results also showed that the distribution pattern of HBV genotypes and/or genotypes variation could be different in Colombia based on the region studied since unlike in Quibdó, a previous study in Bogotá found that

the most frequent subgenotype was the subgenotype F3 followed by A2 and G [18]. The presence of genotype E in more than two thirds of cases studied herein highlights the importance to carry out larger studies in Quibdó population to ascertain if this genotype is widespread in this region.

Such results could provide additional knowledge on the history of HBV/E around the world and further clarify molecular chronometer of such viral infections among humans. Based on our recent publication on genotype E in this afro-Colombian community [16], the TMRCA data suggested a recent infection; however such high frequency of infection in a population does not seem to match a recent infection. Further investigations are required to correlate these findings.

In Africa, viruses belonging to five genotypes, A (HBV/A) to E (HBV/E), have been found. Subgenotype A1 was identified in HBV isolates from South Africa using phylogenetic analysis of preS2/S sequences and confirmed by analysis of complete genomes from South Africa and Malawi [9]. This subgenotype has also been found in Somalia, Zimbabwe, Kenya, Rwanda, Philippines, India and Nepal and Yemen [10]. In South America, previous studies have shown that subgenotype A1 was the most frequent in Brazilian population [23]. While in Argentina there is a low prevalence of this subgenotype [24], in Haiti more than 90% of the population descended from African Slaves has the A1 as the most common subgenotype [25]. While this suggests that subgenotype A1 in South America is probably of similar origin, the subgenotype A1 in Colombia reported in this

(See figure on previous page)

Figure 3 The Maximum Clade Credibility (MCC) tree was estimated by a Bayesian analysis of 192 complete genome sequences of HBV strains. The posterior probabilities of the key nodes are shown above the respective nodes. The HBV Colombian complete genome sequences ($n = 7$) were analyzed together with other strains from around the world. The cluster containing the strains of other HBV subgenotypes were collapsed.

study though grouped in the same cluster, seems to suggest otherwise since the posterior probability was low (0.21), thus indicating a distant genetic relatedness.

An important result of this work was the presence of recombinant strain between subgenotype F3 and A1 found in a sixteen years old girl. Since this girl was pregnant when this sample was collected, it is probably that sexual contact was the way of HBV transmission. Several HBV recombinants inter genotypes have been reported around the world. In Bolivia, South America, several recombinants A/D, C/B, D/C and E/C have been detected [26]. Furthermore, recombination within and between genotypes created complex patterns and altered the cladistic structure of HBV genotypes [27]. For example, in previous study the B2 subgenotype proved to be a hybrid of genotypes B and C [28]. Unfortunately, we had no success on full-length genome amplification of our recombinant strain. Thus, although we know that the break point was in the polymerase region, we cannot exclude the possibility with of existence of other recombination points in this virus. In sum, this is the first HBV recombinant strain reported in Colombia.

Here, we present a study of the first seven complete HBV genomes of Colombian population. The results obtained from E and A1 genotypes support the theory that HBV may have been introduced into this Afro-descendent community in Colombia in the times of slavery.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MAM participated in the design of the study and drafted the manuscript, conducted the sequencing process and phylogenetic analysis. CMR conducted the recombination and evolutionary analysis. MSGG participated in the PCR amplification. MFG and FJC participated in the design of the study. JRRP participated in the elaboration of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This study was previously presented as a poster in 2011 in the 62nd annual meeting of the American association for the study of liver diseases in San Francisco, United States of America. This work has been supported by CNPq and by Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP 2007/53457-7/2011/50562-0 and 2008/50461-6, HCFMUSP and IFM.

Author details

¹Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, Brazil. ²São Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases (LIMHC), School of Medicine, University of São Paulo, São Paulo, Brazil. ³Laboratory of Virology, Department of Microbiology, Pontifícia Javeriana University, Bogotá, Colombia.

Received: 13 February 2012 Accepted: 22 October 2012
Published: 24 October 2012

References

- Lee WM: Hepatitis B virus infection. *N Engl J Med* 1997, **337**(24):1733-1745.
- Tanaka J: Hepatitis B epidemiology in Latin America. *Vaccine* 2000, **18**(Suppl 1):S17-S19.
- Abdoleda M, Castilho MC, Fonseca JC, Albuquerque BC, Saboia RC, Yoshida CP: Epidemiological aspects of hepatitis B and D virus infection in the northern region of Amazonas, Brazil. *Trans R Soc Trop Med Hyg* 1995, **89**:481-483.
- Alvarado-Mora MV, Fernandez MF, Gomes-Gouveia MS, de Azevedo Neto RS, Carilho FI, Pinho JR: Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population-how is the epidemiological situation? *PLoS One* 2011, **6**(4):e18888.
- Fay OH: Hepatitis B in Latin America: epidemiological patterns and eradication strategy. The Latin American Regional Study Group. *Vaccine* 1990, **8**:S100-S106.
- Duarte MC, Cardona N, Poblete F, González K, García M, Pacheco M, Botto C, Pujol FH, Williams JR: A comparative epidemiological study of hepatitis B and hepatitis D virus infections in Yanomami and Piara Amerindians of Amazonas State, Venezuela. *Trop Med Int Health* 2010, **15**(8):924-933.
- Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R: A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000, **81**(Pt 1):67-74.
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ, Xia NS: Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "T". *PLoS One* 2010, **5**(2):e9297.
- Kamris A, Weltmann L, Owiredu WK, Kew MC: Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. *J Gen Virol* 2002, **83**(Pt 4):835-839.
- Kamris A, Kew MC: Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus. *Hepatal Res* 2007, **37**(s1):S27-S32.
- Norder H, Courouce AM, Coudriet P, Echevarria JM, Lee SD, Mushahwar K, Robertson BH, Locamini S, Magnius LO: Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004, **47**(6):289-309.
- Hannoun C, Soderstrom A, Norkans G, Lindh M: Phylogeny of African complete genomes reveals a West African genotype A subtype of hepatitis B virus and relatedness between Somali and Asian A1 sequences. *J Gen Virol* 2005, **86**(Pt 8):2163-2167.
- Olinger CM, Venard V, Njayou M, Oyefolu AQ, Maiga I, Kemp AJ, Omilabu SA, le Faou A, Muller CP: Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 2006, **87**(Pt 5):1163-1173.
- Poulsen MR, Lemey P, Amiri-Bavil-Olyaei S, Maes P, Van Ranst M: Novel hepatitis B virus subgenotype A6 in African-Belgian patients. *J Clin Virol* 2010, **47**(1):93-96.
- Hubschen JM, Mbah PQ, Forbi JC, Oregbayo JA, Olinger CM, Chappentier E, Muller CP: Detection of a new subgenotype of hepatitis B virus genotype A in Cameroon but not in neighbouring Nigeria. *Clin Microbiol Infect* 2011, **17**(1):88-94.
- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Carilho FI, Pinho JR: Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol* 2010, **91**(Pt 2):501-508.
- Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, Duarte MC, Poblete F, Gutierrez MF, Botto C, Pujol FH: Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol* 2008, **80**(1):20-26.

18. Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Botelho I, Carilho FJ, Pinho JR: Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol* 2011, **11**(1):103-108.
19. Chomczynski P, Sacchi N: The single-step method of RNA isolation by add guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. *Nat Protoc* 2006, **1**(2):581-585.
20. Gomes-Gouveia MS: Characterization of complete genomes of the hepatitis B virus of different genotypes isolated in Brazil. São Paulo: Secretaria da Saúde/Coordenadoria de Controle de Doenças. Programa de Pós-Graduação em Ciências para obtenção do grau de Mestre; 2005:130.
21. Edgar RC: MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 2004, **5**:113.
22. Drummond AJ, Rambaut A: BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007, **7**:214.
23. Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Paniago AM, Lima KM, Gomes SA: Hepatitis B virus infection in isolated Afro-Brazilian communities. *J Med Virol* 2005, **77**(2):188-193.
24. Moayed VA, Pineiro y Leone FG, Pezzano SC, Campos RH: Molecular characterization of hepatitis B virus genotype A from Argentina and Brazil. *Arch Virol* 2009, **154**(3):525-529.
25. Adermach IE, Nolte C, Pape JW, Müller CP: Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg Infect Dis* 2009, **15**(8):1222-1228.
26. Simmonds P, Midgley S: Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol* 2005, **79**(4):15467-15476.
27. Purdy MA, Gonzales AC, Dimitrova Z, Khudyakov Y: Supragenotypic groups of the hepatitis B virus genome. *J Gen Virol* 2008, **89**(Pt 5):1179-1183.
28. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida, Chutaputti A, Lai CL, Ueda R, et al: Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J Virol* 2002, **76**(12):5985-5992.

doi:10.1186/1743-422X-9-244

Cite this article as: Alvarado-Mora et al: Phylogenetic analysis of complete genome sequences of hepatitis B virus from an Afro-Colombian community: presence of HBV F3/A1 recombinant strain. *Virology Journal* 2012 **9**:244.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

- 34- Mauricio Venegas, Mónica V. Alvarado-Mora, Rodrigo A. Villanueva, João R. Rebello Pinho, Flair J. Carrilho, Stephen Locarnini, Lilly Yuen, and Javier Brahm. Phylogenetic Analysis of Hepatitis B Virus Genotype F Complete Genome Sequences from Chilean Patients with Chronic Infection. Journal of Medical Virology 83:1530–1536 (2011). DOI: 10.1002/jmv.22129**

Phylogenetic Analysis of Hepatitis B Virus Genotype F Complete Genome Sequences From Chilean Patients With Chronic Infection

Mauricio Venegas,^{1*} Mónica V. Alvarado-Mora,² Rodrigo A. Villanueva,³ João R. Rebello Pinho,² Flair J. Carrilho,² Stephen Locarnini,⁴ Lilly Yuen,⁴ and Javier Brahm¹

¹Gastroenterology Section, Department of Medicine, University of Chile Clinical Hospital, Santiago, Chile

²Laboratory of Tropical Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo, SP, Brazil

³Laboratory of Hepatitis Viruses, Virology Program, Institute of Biomedical Sciences, Faculty of Medicine, Santiago, Chile

⁴Victorian Infectious Diseases Reference Laboratory, North Melbourne, Vic., Australia

Molecular epidemiological data concerning the hepatitis B virus (HBV) in Chile are not known completely. Since the HBV genotype F is the most prevalent in the country, the goal of this study was to obtain full HBV genome sequences from patients infected chronically in order to determine their subgenotypes and the occurrence of resistance-associated mutations. Twenty-one serum samples from antiviral drug-naïve patients with chronic hepatitis B were subjected to full-length PCR amplification, and both strands of the whole genomes were fully sequenced. Phylogenetic analyses were performed along with reference sequences available from GenBank ($n = 290$). The sequences were aligned using Clustal X and edited in the SE-AL software. Bayesian phylogenetic analyses were conducted by Markov Chain Monte Carlo simulations (MCMC) for 10 million generations in order to obtain the substitution tree using BEAST. The sequences were also analyzed for the presence of primary drug resistance mutations using CodonCode Aligner Software. The phylogenetic analyses indicated that all sequences were found to be the HBV subgenotype F1b, clustered into four different groups, suggesting that diverse lineages of this subgenotype may be circulating within this population of Chilean patients. *J. Med. Virol.* 83:1530–1536, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: HBV subgenotype F1b; resistance mutations; Santiago, Chile

INTRODUCTION

Hepatitis B virus (HBV) infection is a severe global health problem, with approximately 2 billion people infected worldwide, and with more than 350 million of them suffering from chronic hepatitis B (CHB) [Zuckerman and Zuckerman, 2000; Shepard et al., 2006]. HBV is a DNA virus of the *Hepadnaviridae* family, which contains a genome composed of approximately 3,200 nucleotides (nt), with four overlapping but frame-shifted open-reading frames for the *P*, *preC/C*, *preS1/preS2/S*, and *X* viral genes [Tiollais et al., 1981].

Molecular variation and sequence changes in the HBV genome over time have resulted in the emergence of at least nine genotypes. The HBV genotypes A to I are classified based on an intergroup divergence of 8% or more in their nucleotide sequence over the entire genome [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002; Yu et al., 2010]. Genotypes may influence the HBeAg

Grant sponsor: University of Chile Clinical Hospital, (to Dr Mauricio Venegas); Grant number: OAIC 362/09; Grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo FAPESP; Grant numbers: 07/53457-7; 08/50461-6; Grant sponsor: CNPq in Brazil.

The authors declare that they have no conflicts of interest.

*Correspondence to: Mauricio Venegas, Sección de Gastroenterología, Departamento de Medicina, Hospital Clínico Universidad de Chile, Santiago, Chile.

E-mail: mvenegas@redclinicauchile.cl

Accepted 12 May 2011

DOI 10.1002/jmv.22129

Published online in Wiley Online Library (wileyonlinelibrary.com).

seroconversion rate (related to mutational patterns in the pre-core and basal core promoter (BCP) regions), and the severity of liver disease. The differences encountered in the severity and progression of HBV-associated liver disease, as well as the response to anti-viral agents, in different regions of the world are probably attributed, at least in part, to the different HBV genotypes [Mahtab et al., 2008].

The natural history of CHB can be described by several distinct phases. These phases are characterized by particular serological, biochemical, and viral marker patterns, generally accompanied by the appearance of well-defined viral genomic mutations. Such mutations include the double A1762T/G1764A BCP mutation and the G1896A pre-C stop-codon mutation, often in combination with the G1899A mutation. Furthermore, additional mutations in the BCP region that may confer increased replication efficiency for the virus have also been found [Baumert et al., 1998; Parekh et al., 2003].

The HBV genotype F (HBV/F) has been identified as the most prevalent of the HBV genotypes in Central and South America, and it is mainly found among native indigenous people from South America [Devesa et al., 2008]. Genotype F can be further divided into four subgenotypes (F1–F4), with a genetic divergence of 4.3–6.1% [McMahon, 2009]. The subgenotype F1a has been found in Alaska, El Salvador, Guatemala, Costa Rica, and Nicaragua, whereas the F1b genotype has been reported in Peru and Argentina. The HBV subgenotype F2 was found in Venezuela and Brazil, where it was initially associated with fulminant hepatitis in patients co-infected with the hepatitis Delta virus. Subgenotype F3 has been identified in Venezuela, Colombia, and Panama, and, like the HBV subgenotype F2, it is also associated with fulminant hepatitis in these regions. Finally, subgenotype F4 was reported in Argentina and Bolivia [Blitz et al., 1998; Huy et al., 2006; Devesa et al., 2008; Santos et al., 2010; Alvarado-Mora et al., 2011].

In a previous study utilizing restriction fragment length polymorphism (RFLP), the HBV/F was found to be the most prevalent in Chile (84%), whereas genotypes A, B, C, and D were found at the frequencies of 3.8%, 3.8%, 6.1%, and 2.3%, respectively [Venegas et al., 2008]. In the current report, complete genome sequences of HBV isolates from 21 Chilean patients infected chronically with HBV/F were analyzed. The results shown herein identify HBV subgenotype- and antiviral resistance-associated substitutions, but no vaccine escape mutations, within the HBV genomes circulating in Chile.

MATERIALS AND METHODS

Study Population

Serum samples were collected between March 2005 and March 2010 from 21 patients in Chile attending the Gastroenterology Section, Clinical Hospital, University of Chile (Santiago, Chile), for routine HBV

DNA detection or quantitation. This laboratory is the national reference center in Chile for the molecular diagnostics of viral hepatitis, and it processes samples from medical centers throughout the country. In the current study, however, only samples collected from Santiago Metropolitan Area were included. All of the patients were anti-HBc and HBsAg positive (MEIA AcSym, Abbott, North Chicago, IL), and HBeAg+/anti-HBe negative, as determined by a commercially available kit from mimiVIDAS (Biomérieux, Craponne, France). The viral load was determined using a COBAS® TaqMan® Hepatitis B Virus test (Roche Molecular Systems, Branchburg, NJ). Viral genotyping was carried out by polymerase chain reaction (PCR) and RFLP as previously described [Venegas et al., 2008]. Chronic infection was defined by the detection of HBsAg in two serum samples collected at least 6 months apart. Three patients were co-infected with human immunodeficiency virus (HIV) (patients HCUCH3, HCUCH15, and HCUCH21), whereas none of the patients were co-infected with hepatitis C virus. All of the patients were male and their age ranged from 10 to 77 years old (mean age = 46 years). The ethics committee of the Clinical Hospital, University of Chile, approved this study and all participating patients signed an informed consent form. The HBV clinical data and GenBank accession numbers from the patients are shown in Table I.

HBV Complete Genome Amplification

Viral DNA was extracted from 500 µl of serum using a High Pure System Viral Nucleic Acid kit (Roche Molecular Systems). Amplification of the 21 complete HBV genome was carried out as previously described (P1 and P2 primers) [Günther et al., 1995].

HBV Nucleotide Sequencing

The 3.2 kb PCR products were gel-purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison WI). Complete genomes were sequenced from both strands of the viral DNA (Macrogen, Inc., Seoul, Korea) using the primers indicated in Table II. Consensus sequences were obtained by the alignment of both sequenced strands (sense and anti-sense) using MegAlign™ software from the DNASTar package (LaserGene, Inc., Madison, WI).

Phylogenetic Analyses

In order to analyze the distribution of the different HBV/F subgenotypes in the patients, the full sequences obtained from this study were genotyped by phylogenetic reconstructions using complete HBV genome reference sequences from each genotype retrieved from Genbank (n = 290). However, since there are only a few full HBV/F genome sequences, a larger dataset comprising 111 sequences with 1,278 nucleotides of the S/POL region of all HBV/F subgenotypes was also constructed with sequences obtained from

TABLE I. General Clinical Data of the Samples

Sample ID	Age (years)/gender (M/F)	Sample date	Viral load (cp/ml)	GenBank number
HCUCH1	55/M	June 1, 2007	>640,000,000	HM585198
HCUCH2	69/M	August 19, 2009	>640,000,000	HM585199
HCUCH3	20/M	October 7, 2009	22,989,000	HM590474
HCUCH4	10/M	July 1, 2008	25,957,200	HM585186
HCUCH5	30/M	May 28, 2008	>640,000,000	HM622135
HCUCH6	37/M	October 23, 2009	>640,000,000	HM585187
HCUCH7	61/M	December 20, 2006	137,352,000	HM585188
HCUCH8	48/M	March 15, 2005	>640,000,000	HM585189
HCUCH9	43/M	October 23, 2009	>640,000,000	HM585190
HCUCH10	77/M	November 2, 2009	>640,000,000	HM585191
HCUCH11	50/M	November 20, 2009	>640,000,000	HM585192
HCUCH12	45/M	October 7, 2009	>640,000,000	HM585193
HCUCH13	38/M	June 12, 2009	3,544,380	HM590471
HCUCH14	67/M	March 23, 2007	>640,000,000	HM590473
HCUCH15	48/M	December 9, 2009	>640,000,000	HM585200
HCUCH16	72/M	December 20, 2007	>640,000,000	HM585194
HCUCH17	45/M	March 15, 2010	>640,000,000	HM585195
HCUCH18	52/M	February 19, 2010	>640,000,000	HM585196
HCUCH19	14/M	February 19, 2010	21,243,000	HM590472
HCUCH20	33/M	September 26, 2007	>640,000,000	HM585197
HCUCH21	47/M	August 3, 2007	>640,000,000	HM627320

GenBank (datasets available from the authors upon request). The two datasets of the HBV sequences were aligned using Clustal X software [Thompson et al., 1997] and edited in the SE-AL software (available at <http://tree.bio.ed.ac.uk/software/seal/>). In order to perform the phylogenetic analysis, the missing nucleotides were coded as "missing characters" in the nexus block. Bayesian phylogenetic analyses were carried out using Bayesian Markov Chain Monte Carlo simulations implemented in BEAST v.1.5.3 [Drummond and Rambaut, 2007]. Analysis of the HBV dataset was performed under relaxed uncorrelated lognormal and relaxed uncorrelated exponential molecular clocks using the best model of nucleotide substitution (GTR + G + I) chosen in ModelTest [Posada and Crandall, 1998], and 10 million generations were sufficient to obtain the convergence of parameters. A Maximum Clade Credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees using Tree Annotator v.1.5.3 [Drummond and Rambaut, 2007].

Detection of Antiviral Resistance Substitutions

The presence of drug resistance substitutions was determined using CodonCode Aligner Software

v.3.5 (available at <http://www.codoncode.com/>). This program includes effective software for sequence assembly, contig editing, and mutation detection. The results were confirmed by analyzing the sequences with the SeqHepB program [Yuen et al., 2007]. A dataset with 290 complete HBV genomes was used to identify changes in the 21 patients. Firstly, the mutations associated with HBV anti-HBs monoclonal antibody and vaccination escape were screened using data reporting 39 relevant mutations in this region [Sitnik et al., 2004], which included sG145R. Primary antiviral drug resistance substitutions at the following positions were then screened: rtI169, rtL180, rtA181, rtT184, rtS202, rtM204, rtN236, and rtM250 [Zoulim and Locarnini, 2009]. Secondary (or compensatory) mutations were also included in the analysis, such as rtV173, as reported previously [Delaney et al., 2003; Zoulim and Locarnini, 2009]. In addition, any HBV subgenotypes were identified by the presence of specific substitutions at positions 122, 160, 127, and 140 in the S gene. Finally, BCP and pre-C mutations [Baumert et al., 1998; Parekh et al., 2003] were identified via sequence comparisons with other known sequences from different HBV genotypes.

TABLE II. Primers Used for Sequencing the HBV Genome

Primer name	Nucleotide position	Sequence (5'-3')
SB409	409-432	CAT CCT GCT GCT ATG CCT CAT CTT
SB1174	1174-1195	TGC CAA GTG TTT GCT GAC GCA A
SB1821	1821-1841	TTT TTC ACC TCT GCC TAA TCA
SB2373	2373-2392	GAA GAA CTC CCT CGC CTC GC
SB3010	3010-3031	GCA AAC AAG CTA GGA GTG GGA G
ASB432	432-408	AAG ATG AGG CAT AGC AGC AGG ATG
ASB1195	1195-1174	TTG CGT CAG CAA ACA CTT GGC A
AS1825	1825-1806	AAA AAG TTG CAT GGT GCT GG
ASB2392	2392-2373	GCG AGG CGA GGG AGT TCT TC
ASB3031	3031-3010	CTC CCA CTC CTA CCT TGT TTG C

RESULTS

Phylogenetic Analyses

The phylogenies showed that the 21 HBV/F samples were from subgenotype F1b (Figs. 1 and 2). The sequences of the 21 samples from the current study were grouped into four groups within the F1b cluster with a higher support. Figure 1 shows the first analysis of complete genomes from HBV/F derived from Chile, but, unfortunately, due to the insufficient number of complete genome sequences of HBV/F1b in GenBank, no further inferences about their distribution were possible. Figure 2 shows a phylogenetic tree that was constructed from 1,291 base-pair sequences, comprising all of the HBV/F subgenotypes. In this analysis, the 21 sequences from the patients were compared with other sequences previously reported from Chile, and from other countries. The phylogenetic analysis showed that these sequences were not

closely related to each other, suggesting that different lineages of subgenotype F1b are circulating in Chile. Additionally, because the HBV subgenotype F1b obtained in this study did not produce a single cluster, it can be argued that these sequences may have resulted from separate introductions into the community.

Detection of Antiviral Resistance Mutations

When the antiviral resistance mutations were analyzed, one naive patient (HCUCH15) was found to be infected with an HBV isolate that contained lamivudine (LMV), emtricitabine (FTC), and clevudine (INN) antiviral resistance-associated substitutions (rtV173L, rtL180M, and rtM204V). This male patient had the risk factor of having sex with men, and he was also co-infected with HIV. Both F and H genotypes have been described as having T237 and S238. Previously, P237H and N238T/D substitutions were associated

Fig. 1. The Maximum Clade Credibility (MCC) tree was estimated by a Bayesian analysis of 290 complete genome sequences of hepatitis B virus strains. The posterior probabilities of the key nodes are shown above the respective nodes. The HBV/F samples obtained from Chile ($n = 21$, HCUCH) were analyzed together with other strains from around the world. The clusters containing the strains of other HBV genotypes collapsed.

Fig. 2. The Maximum Clade Credibility (MCC) tree was estimated by a Bayesian analysis for a larger dataset comprising 111 sequences with 1,278 nucleotides of the S/POL region of all of the HIV/F subgenotypes. The posterior probabilities of the key nodes are shown above the respective nodes. The HIV/F samples obtained from Chile ($n = 21$, HCUCH) were analyzed together with other HIV/F strains from around the world.

with resistance to Adefovir [Shaw et al., 2006]. Our data reinforce the current view that these sites are highly polymorphic and that the mutations therein are not related to drug resistance.

HBsAg/Anti-HBs Monoclonal/Vaccine-Associated Changes

All of the samples from Chile were found to be from the subtype *adw4*. An analysis of the "a" determinant of HBsAg revealed that none of the isolates contained changes that would affect binding to HBsAg, the anti-HBs monoclonal antibody or the vaccination-associated anti-HBs. Furthermore, examination of the HCUC5 sequence showed that it contained a two amino acid deletion at the Pre-S1 region (codons 46 and 47). No significant changes were observed in any of the HBV DNA promoter regions. However, sequences from two patients (HCUC16 and HCUC21) presented BCP mutations: A1762T and G1764A. Besides this, the sequence from patient HCUC5 presented two core promoter mutations: C1768T and T1770A. Analyses of the nucleotide sequence at position 1858 showed the presence of thymine in all patients. Finally, none of the patients presented the T1753C mutation or the Pre-C mutations.

DISCUSSION

This is the first study to report a detailed analysis of complete HBV genomes circulating in a population in Chile. The phylogenetic analyses presented here revealed that all of the patients' sequences were of the HBV subgenotype F1b. In Chile, only data about HBV prevalence based on the detection of either surface antigens (HBsAg) or antibodies against the viral core protein (anti-HBc) have been published [Pereira et al., 2008, and references therein]. A recent report, based on RFLP profiles, showed that genotype F is the most prevalent genotype [Venegas et al., 2008]. Similar results were later published by others, based on partial sequencing of the HBV genome [DiLello et al., 2009]. The sequences obtained in the current study were compared to sequences previously reported from Chile, and it was possible to conclude that the HBV/F1b subgenotype distribution in this country is suggestive of a viral diversification process, since there are many viral lineages circulating within the population. Finally, since many strains are present in the country, they may have entered at different time points and/or from different origins. Unfortunately, it was not possible to estimate the time of the most recent common ancestor (TMRCA) for the subgenotype F1b in Chile. Since genotype F1b sequences are found in different and distant countries in the Americas, it is possible that this genotype was widely distributed over the continent after its introduction into different populations.

The two BCP mutations, C1768T and T1770A, both found in the HCUC5 isolate, are known to result in enhanced viral encapsidation and replication. The

effect of these mutations, leading to increased encapsidation, is mediated through enhanced core protein synthesis by the mutant virus [Baumert et al., 1998].

Variability of the HBV genome during the chronic phase of the disease determines the selection for viral-resistant strains [Zoulim and Locarnini, 2009]. Several studies have reported mutations in HBsAg that alter its antigenicity. In previous studies, it was observed that the LMV resistance mutations, rtV173L, rtL180M, and rtM204V, resulted in the reduced binding of antibodies to the neutralization domain ("a" determinant) of the HBsAg [Torres et al., 2002; Sloan et al., 2008]. Also, the rtV173L mutation, which accompanies rtL180M and rtM204V in about 10–20% of cases during LVD use, allows improved HBV replication fitness [Delaney et al., 2003; Poordad and Chee, 2010]. Moreover, genotypic resistance to TDF has been detected in several patients with HIV-HBV co-infection, and the substitution rtA194T (plus rtL180M and rtM204V) has been associated with TDF resistance [Sheldon et al., 2005; Zoulim and Locarnini, 2009]. However, the rtA194T mutation was not found in any of the samples from Chile in the current study. Reduced sensitivity to TDF has been described in patients infected with rtA181T/V and rtN236T [van Bömmel et al., 2010], but neither codon substitutions were also found. Other rt sequence changes have been implicated in Adefovir failure, including rtP237H and rtN238T/D [Shaw et al., 2006]. In this study, it was found that the genotype F presents T237 and S238 polymorphisms. These polymorphisms are not related to antiviral resistance. However, since one treatment-naïve patient with HBV antiviral resistance mutations was identified, it is important to elucidate the occurrence of potential genotypic resistance mutations in patients before they start antiviral treatment.

In conclusion, this study describes the complete genomic analysis of HBV/F1b from Chile. This subgenotype is also the most common in Argentina and the description of the different subgenotypes found in South American countries will help to understand the spread of this viral variant throughout this continent. Since so few complete HBV/F genomes have been reported to date, this analysis also provides a useful reference point for future molecular epidemiology studies of HBV in South America.

REFERENCES

- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Botelho L, Carrilho FJ, Pinho JR. 2011. Molecular characterization of the Hepatitis B virus genotypes in Colombia: A Bayesian inference on the genotype F. *Infect Genet Evol* 11:103–108.
- Arauz-Paiz P, Norder H, Robertson B, Magnus L. 2002. Genotype H: A new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83:2059–2073.
- Baumert T, Marrone A, Vergalla J, Liang T. 1998. Naturally occurring mutations define a novel function of the hepatitis B virus core promoter in core protein expression. *J Virol* 72:6785–6795.
- Blitz L, Pujol F, Swenson P, Porto L, Atencio R, Araujo M, Costa L, Monsalve D, Torres J, Fields H, Lambert S, Geyt CV, Norder H, Magnus L, Echevarria J, Stuyver L. 1998. Antigenic diversity of hepatitis B virus strains of genotype F in Amerindians and other population groups from Venezuela. *J Clin Microbiol* 36:648–651.

- Delaney W, Yang H, Westland C, Das K, Arnold E, Gibbs C, Miller M, Xiong S. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J Virol* 77:11833–11841.
- Devesa M, Loureiro C, Rivas Y, Monsalve F, Cardona N, Duarte M, Poblete F, Gutierrez M, Botto C, Pujol F. 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol* 80:20–26.
- Dilella F, Píñero Y, Leone F, Muñoz G, Campos R. 2009. Diversity of hepatitis B and C viruses in Chile. *J Med Virol* 81:1887–1894.
- Drummond A, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7:214.
- Günther S, Li B, Miska S, Krüger D, Meisel H, Will H. 1995. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 69:5437–5444.
- Huy T, Ishikawa K, Ampofo W, Izumi T, Nakajima A, Ansah J, Tetteh J, Nii-Trehi N, Aidoo S, Ofori-Adjei D, Sata T, Ushijima H, Abe K. 2006. Characteristics of hepatitis B virus in Ghana: Full length genome sequences indicate the endemicity of genotype E in West Africa. *J Med Virol* 78:178–184.
- Mahtab M, Rahman S, Khan M, Karim F. 2008. Hepatitis B virus genotypes: An overview. *Hepatobiliary Pancreat Dis Int* 7:457–464.
- McMahon B. 2009. The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatol Int* 3:334–342.
- Norder H, Courouce A, Magnus L. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 198:489–503.
- Okamoto H, Tsuda F, Sakugawa H, Sastroeswignjo R, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. *J Gen Virol* 69:2575–2583.
- Parekh S, Zoulim F, Ahn S, Tsai A, Li J, Kawai S, Khan N, Trépo C, Wands J, Tong S. 2003. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol* 77:6601–6612.
- Pereira S, Valenzuela B, Mora J, Vera L. 2008. Present situation of hepatitis B virus in Chile. *Rev Med Chil* 136:725–732.
- Poordad F, Chee G. 2010. Viral resistance in hepatitis B: Prevalence and management. *Curr Gastroenterol Rep* 12:62–69.
- Pozada D, Crandall KA. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics* 14:817–818.
- Santos AO, Alvarado-Mora MV, Botelho L, Vieira DS, Pinho JR, Carrilho FJ, Honda ER, Salcedo JM. 2010. Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virol J* 7:315.
- Shaw T, Bartholomeusz A, Locarnini S. 2006. HBV drug resistance: Mechanisms, detection and interpretation. *J Hepatol* 44:593–606.
- Sheldon J, Camino N, Rodés B, Bartholomeusz A, Kuiper M, Tacke F, Núñez M, Maus S, Lutz T, Klausen G, Locarnini S, Soriano V. 2005. Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. *Antivir Ther* 10:727–734.
- Shepard CW, Simard EP, Finelli L, Fiore AE, Bell BP. 2006. Hepatitis B virus infection: Epidemiology and vaccination. *Epidemiol Rev* 28:112–125.
- Sitnik R, Pinho J, Bertolini D, Bernardini A, Silva LD, Carrilho F. 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 42:2455–2460.
- Sloan R, Ijaz S, Moore P, Harrison T, Teo C, Tedder R. 2008. Antiviral resistance mutations potentiate hepatitis B virus immune evasion through disruption of its surface antigen a determinant. *Antivir Ther* 13:439–447.
- Stuyver L, Gendt SD, Geyt CV, Zoulim F, Fried M, Schinazi R, Rosau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. *J Gen Virol* 81:67–74.
- Thompson J, Gibson T, Plewniak F, Jeanmougin F, Higgins D. 1997. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882.
- Tollais P, Charnay P, Vyas G. 1981. Biology of hepatitis B virus. *Science* 213:406–411.
- Torres J, Earnest-Silveira L, Deliyannis G, Edgerton K, Zhuang H, Locarnini S, Fyfe J, Sozzi T, Jackson D. 2002. Reduced antigenicity of the hepatitis B virus HBsAg protein arising as a consequence of sequence changes in the overlapping polymerase gene that are selected by lamivudine therapy. *Virology* 293:305–313.
- vanBommel F, Man Rd, Wedemeyer H, Deterding K, Petemen J, Buggisch P, Erhardt A, Hüppe D, Stein K, Trojan J, Sarrazin C, Böcher W, Spengler U, Wasmuth H, Reinders J, Möller B, Rhode P, Feucht H, Wiedenmann B, Berg T. 2010. Long-term efficacy of tenofovir monotherapy for hepatitis B virus-monoinfected patients after failure of nucleoside/nucleotide analogues. *Hepatology* 51:73–80.
- Venegas M, Muñoz G, Hurtado C, Alvarez I, Velasco M, Villanueva R, Brahm J. 2008. Prevalence of hepatitis B virus genotypes in chronic carriers in Santiago, Chile. *Arch Virol* 153:2129–2132.
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen FJ, Xia NS. 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "T". *PLoS ONE* 5:e9297.
- Yuen L, Ayres A, Littlejohn M, Colledge D, Edgeley A, Maskill W, Locarnini S, Bartholomeusz A. 2007. SeqHepB: A sequence analysis program and relational database system for chronic hepatitis B. *Antiviral Res* 75:64–74.
- Zoulim F, Locarnini S. 2009. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 137:1593–1608.

- 35- Martinez AA, Zaldivar Y, Hong CCh, Alvarado-Mora MV, Smith R, Ortiz AY, Pinho JR, Cristina J, Pascale JM. Molecular characterisation of hepatitis B virus in the resident Chinese population in Panama City. Mem Inst Oswaldo Cruz. 2013 Aug;108(5):541-7**

Molecular characterisation of hepatitis B virus in the resident Chinese population in Panama City

Alexander Augusto Martinez^{1,2,3}, Yamitzel Zaldivar¹, Chen Ch Hong⁴,
Monica Viviana Alvarado-Mora⁵, Rebecca Smith¹, Alma Y Ortiz¹,
João Renato Rebello Pinho⁵, Juan Cristina⁶, Juan Miguel Pascale^{1,7,*}

¹Corgas Memorial Institute for Health Studies, Panama, Panama ²Department of Biotechnology, Acharya Nagarjuna University, Cuntur, India

³Instituto de Investigaciones Científicas y Servicios de Alta Tecnología-AIP, Ciudad del Saber, Clayton, Panama

⁴Internal Medicine, Massachusetts General Hospital, Boston, MA, USA ⁵Laboratório de Gastroenterologia e Hepatologia Tropical,

Departamento de Gastroenterologia, Instituto de Medicina Tropical, Faculdade de Medicina, Universidade de São Paulo,

São Paulo, SP, Brasil ⁶Laboratório de Virologia Molecular, Centro de Investigaciones Nucleares, Facultad de Ciencias,

Universidad de la República, Montevideo, Uruguay ⁷School of Medicine, University of Panama, Panama, Panama

Despite the effectiveness of current hepatitis B virus (HBV) vaccines, it is estimated that 350 million individuals suffer from chronic HBV infection and more than 50% of these affected individuals live on the Asian continent. Panama is a country with a great diversity of foreign groups; the Chinese community is a large example of this phenomenon. There is an urgent need to perform studies that evaluate the prevalence and the genetic diversity of HBV in this community. This study aimed to evaluate the prevalence of HBV and its genotypes and mutant variants in the Chinese population residing in Panama. In total, 320 subjects were enrolled in the study. Forty-two subjects (13.1%) were positive for HBsAg and HBV-DNA from 18 subjects revealed the presence of genotypes B2 and C1. Secondary mutations associated with drug resistance at positions rtV207L and rtN239T of the reverse transcriptase gene were identified. Additionally, the mutation pair A1762T/G1764A was found in three samples and the mutation G1896A was detected in an HBsAg-negative subject. In conclusion, to our knowledge, this is the first study to report high HBV prevalence rates in resident ethnic Chinese in Central America and the presence of genotypes B2 and C1 in this region.

Key words: hepatitis B - Panama - Bayesian analysis - genotype C1 - genotype B2

There are approximately 350 million individuals chronically infected with hepatitis B virus (HBV); more than 50% of these individuals live in Asian countries. HBV is classified into different genotypes based on 8% divergence over the complete genome or 4% divergence in the S gene (Okamoto et al. 1988, Kidd-Ljunggren et al. 2002, Kurbanov et al. 2010). According to this definition, 10 genotypes, A-I (Orto et al. 1989, Naumann et al. 1993, Blitz & Pujol 1998, Stuyver & Gendt 2000, Arauz-Ruiz & Norder 2002, Olinger et al. 2008, Yu et al. 2010), have been reported.

HBV genotypes have a variable geographical distribution: genotype A is the predominant genotype in Europe, India, Africa and North America, genotypes B and C are more prevalent in China, Japan and Southeast Asia, genotype D is found around the Mediterranean area, in the Middle-East and in Latin America (Kramvis et al. 2005), genotype E is found in West Africa and in the Af-

ro-Colombian population (Odemuyiwa et al. 2001, Alvarado Mora et al. 2010), genotype F is found in Central and South America (Pineiro y Leone et al. 2003, Torres et al. 2011), genotype G is found in France, Mexico and the United States of America (Stuyver & Gendt 2000), genotype H is found in Mexico (Torres et al. 2011) and Central America (Arauz-Ruiz & Norder 2002), genotype I is found in Vietnam and Laos (Olinger et al. 2008, Tran et al. 2008) and genotype J was recently found in a Japanese patient with hepatocellular carcinoma (HCC) (Tatematsu et al. 2009). Due to the world-wide distribution pattern of HBV genotypes, we can trace the spread of HBV infection in immigrants and within regions in a country (Campos et al. 2005). This also allows us to consider the possible frequency of recombination between genotypes within a region (Bowyer SM & Sim 2000, Khan et al. 2008, Shi et al. 2013) and to attempt to correlate genotypes with different clinical outcomes. Studies suggest that genotypes B-D are related to poor clinical outcomes (Echevarria & Avellon 2006).

Another factor affecting the prognosis in HBV infection is the presence of mutations in the C and D domains of the HBV reverse transcriptase (HBV-RT) gene, which produce changes in the primary structure of the HBV-RT and give rise to resistance to antiretroviral drugs (Michailidis et al. 2012). Mutations in the Enh II, pre-core (PC) and basal core promoter (BCP) regions of HBV may also cause changes in the binding of transcription factors in the BCP (Yin et al. 2011), affecting tran-

doi: 10.1590/0074-0276108052013002

Financial support: SENACYT-PANAMA (FID09-050)

AAM was supported by a fellowship from SENACYT-IFARHU.

* Corresponding author: jpascale@gorgas.gob.pa

Received 23 January 2013

Accepted 26 April 2013

online | memorias.ioc.fiocruz.br

scription of the PC mRNA and pre-genomic RNA and decreasing or even abolishing the secretion of HBeAg and enhancing viral replication rates, with subsequent hepatic damage (Buckwold et al. 1996).

Despite HBV vaccination policies around the world, there is still a high prevalence of chronic HBV (CHB) infection, especially among Asian populations (Merrill & Hunter 2011). Panama is a country with a high diversity of growing foreign communities; the Chinese population is a large example (INEC 2011). In the metropolitan areas of several developed cities (Lavanchy 2004, Pollack et al. 2006, Bini & Perumalswami 2010, Kallman et al. 2011), there is growing evidence of a high prevalence of HBV in Asian populations, but little is known regarding the molecular characteristics of the virus in these populations. To successfully implement a universal HBV vaccination program in Central American countries, it will be necessary to study the prevalence and molecular characteristics of the HBV genotypes that infect the risk groups in the region. The purpose of this study was to evaluate the prevalence of HBV infection and to describe the HBV genotypes and mutations in the resident Chinese population in Panama City.

PATIENTS, MATERIALS AND METHODS

Sample collection - The Institutional Review Board of the Gorgas Memorial Institute for Health Studies approved this study. All of the participants signed an informed consent form before inclusion in the study. This research was a cross-sectional study performed during 2003. Approximately 500 subjects of Chinese descent based on last name were randomly selected and contacted using the phone directory for the Chinese communities in the metropolitan area of Panama City. The subjects who answered the call were included in the study based on the completion of a consent form and their willingness to provide a blood sample at a designated sample collection site. The questionnaire was filled out with the assistance of a translator and blood samples were collected by venipuncture. Plasma was separated from the blood cells by centrifugation for 10 min at 2,500 rpm and was stored at -80°C until testing. The samples were evaluated for hepatitis B surface antigen [(HBsAg), AxSYM HBsAg (V2)] and hepatitis E antigen [(HBe), AxSYM HBe 2.0] according to the manufacturer's instructions.

Partial PreS gene amplification - DNA was extracted and amplified from the samples that were positive for HBsAg to detect and quantify the amount of HBV-DNA that was present using real-time polymerase chain reaction (PCR) according to previously described methods (Liu et al. 2007). Those samples with detectable viraemia were subjected to a nested PCR that yielded a 1,058-bp fragment that simultaneously covered amino acids 35-226 of the small surface gene and amino acids 42-280 of the HBV-RT gene, which corresponded to RT domains A-E. The following primers were used: HBV66 and HBV1121 for the first-round PCR and HBV095 (Takahashi et al. 2004b) and HBV1121 (Gunther et al. 1998) for the second-round PCR. The PCR conditions were similar in both reactions: 94°C for 6 min, 40 cycles

of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and a final extension for 7 min at 72°C.

The 1,058-bp product was sequenced using the following primers: HBV477, HB1842 (Takahashi et al. 2004b), HB066, HBV676 and HBV1121. The sequencing reaction was performed using Big Dye Terminator v.3.1 in an ABI 3130xl automatic DNA sequencer with an initial denaturation at 96°C followed by 30 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. The sequences obtained were inspected visually for quality and contigs were created using Sequencher v.4.5 software and submitted to GenBank with the accessions JX507200-JX507215 and JX86998-JX870001.

Full-length genome amplification - Samples that were positive for the short region (1,058 bp) were chosen for genome amplification. Primers P1 and P2 (Gunther et al. 1998) were used to amplify an approximately 3,220-bp region using Platinum® PCR Super Mix High Fidelity enzymes (Invitrogen) with the following PCR conditions: initial denaturation at 96°C for 15 min, 40 cycles of 94°C for 30 sec, 50°C for 90 sec and 68°C for 2 min, with increments of 90 sec every 10 cycles and a final extension step of 10 min at 68°C. DNA bands were visualised on a 1% agarose gel. The circular PCR amplicon obtained was sequenced directly as previously reported (Li et al. 2010).

Phylogenetic analysis - The partial HBV genome sequences obtained in this study were genotyped using phylogenetic reconstructions and reference sequences for each HBV genotype obtained from GenBank (n = 138) (data available upon request). These sequences were aligned using Muscle software (Edgar 2004) and edited in SE-AL software (tree.bio.ed.ac.uk/software/seal/). For the phylogenetic analysis, the missing nucleotides were coded as "missing characters" in the nexus block. Bayesian phylogenetic analyses were performed through Markov Chain Monte Carlo (MCMC) simulations implemented in BEAST v.1.5.3 (Drummond et al. 2007). Ten million generations were sufficient to obtain convergence of the parameters. MCMC simulations were performed to obtain the best possible estimates under both relaxed uncorrelated log_{normal} and exponential molecular clock conditions and when using the model of nucleotide substitution (GTR+G+I). The molecular clock that best fitted the data was chosen by Bayes factor comparison. The maximum clade credibility tree was obtained from summarising the 10,000 substitution trees and 10% of the burn-in was then removed using Tree Annotator v.1.5.3 (Drummond et al. 2007).

Mutation analysis - For the detection and analysis of mutations in the Enh II, PC and BCP regions, the nucleotide sequences were aligned with the reference sequence for genotype C X04615 using Muscle (Edgar 2004). For the analysis of the substitutions in the polymerase and small surface gene nucleotides, the sequences were translated and compared using MacClade v.4.08. The HBV-RT sequences were evaluated for the presence of residues previously reported as being involved in drug resistance (Michailidis et al. 2012). The major hydro-

philic region (MHR) of the small surface protein was evaluated for the presence of escape mutations (Coleman et al. 1999, Ma & Wang 2012).

RESULTS

Demographic and serological results - In total, 500 subjects answered the initial phone call and 180 individuals were excluded because they either did not sign the informed consent form or did not agree to give a blood sample for the study. A total of 320 samples were collected and included in the study: 135 men (42.2%) and 185 women (57.8%) with a mean age of 33 years old (95% confidence interval: 31.4-34.9 years old). The youngest participant was seven years old; the oldest participant was 79 years old. The birthplace for the majority of the participants was China (172, 53.8%); however, 115 participants (35.9%) were born in Panama (Table).

Forty-two participants (13.1%) were HBsAg-positive; of these, 10 subjects (23.8%) were also HBeAg-positive. Most of the positive subjects ($n = 27$) were in the age group of 19-45 years old, representing 64% of the study population. With respect to birthplace, 25 of the HBsAg-positive subjects (59.5%) were born in China, 12 (28.5%) in Panama and 5 (11.9%) did not answer the question.

Molecular biology results - DNA was extracted from all of the HBsAg-positive samples ($n = 42$) and the amount of HBV-DNA was quantified using real-time PCR. Eighteen samples had undetectable viral loads (< 50 copies/mL) and 24 were positive for HBV-DNA. The HBV-DNA from 18 of the 22 samples with higher copy numbers was sequenced. Phylogenetic analysis revealed that 11 samples (61.1%) were located in the genotype B2 cluster and that seven samples (38.9%) were located in the genotype C1 cluster (Fig. 1). Of the 24 positive subjects, 12 were born in China (6 B2, 6 C1), five were born in Panama (3 B2, 2 C1) and one did not answer the question (Fig. 2A). There was clustering among the Chinese-Panamanian samples, indicating possible common origins.

Considering the divergence found in this group of samples, evaluation of the mutations was performed to characterise other molecular risk factors such as resis-

tance to HBV-RT inhibitors and changes in several important regions, including the MHR of the surface antigen. Whole-genome amplification was performed on 10 samples to evaluate the PC and BCP regions and to explore the possible existence of recombinant strains.

No primary mutations associated with resistance to nucleos(t)ide HBV-RT inhibitors were identified upon analysis of the HBV polymerase gene. Two secondary mutations that were potentially associated with resistance, rtV207L and rtN238T, were identified in one (6%) and five (33%) of the analysed samples, respectively (Fig. 2A).

The analysis of the MHR mutations revealed a change in residue Q129H in two samples (11%). This change has been reported to affect the reactivity of at least one diagnostic assay. No other mutations related to escape from detection in HBsAg immunoassays, immunisation or hepatitis B immune globulin (HBIG) treatment were detected.

The analysis of mutations in the Enh II, PC and BCP regions of the 10 samples revealed the following results: the mutation G1613A was identified in two samples (20%), two samples (20%) had the mutation pair A1762T/G1764A and one sample carried both G1896A and the A1762T/G1764A double mutation. The patient from whom this sample was obtained had an HBeAg-negative phenotype (Fig. 2B).

Analysis to detect recombination among the samples using the RDP4 software gave no evidence of recombination.

DISCUSSION

The Republic of Panama had approximately 3,405,813 inhabitants in 2010 (INEC 2011), with approximately half of the population living in the capital city. The population of Chinese residents in Panama is estimated at 102,000 individuals, representing 2.9-3.1% of the total population. In this study, we found a high prevalence of HBV in the Chinese community in Panama City, approximately 13.1%, which is comparable to the prevalence in high endemic areas of China (Zeng et al. 2005) and is 20 times higher than the observed prevalence in female sex workers in Panama (Hakre et al. 2012). A similar

TABLE
Demographic, HBsAg, HBeAg and genotype data collected in 2003 for Panamanians of Chinese origin

Age range	Gender		Age range							Place of birth				Total n (%)
	Male	Female								Panama	China	Other	Unknown	
	n (%)	n (%)	0-10	11-18	19-30	31-45	46-60	> 61	n (%)	n (%)	n (%)	n (%)		
HBsAg positive	19 (5.9)	23 (7.2)	2	5	13	14	7	1	12 (3.8)	25 (7.8)	0 (0)	5 (1.6)	42 (13.1)	
HBeAg+	5 (1.6)	5 (1.6)	0	3	5	1	1	0	4 (1.3)	6 (1.9)	0 (0)	0 (0)	10 (3.1)	
HBeAg-	14 (4.4)	18 (5.6)	2	2	8	13	6	1	8 (2.5)	19 (5.9)	0 (0)	5 (1.6)	32 (10)	
HBsAg negative	116 (36.3)	162 (50.6)	20	30	86	71	57	14	103 (32.2)	147 (45.9)	6 (1.9)	22 (6.9)	278 (86.9)	
Total	135 (42.2)	185 (57.8)	22	35	99	85	64	15	115 (35.9)	172 (53.8)	6 (1.9)	27 (8.4)	320	

This study showed evidence that the HBV genotypes detected in the Chinese population of Panama are similar

The frequency of different HBV genotypes in a specific region and the fact that HBV has a higher evolution

Fig. 1: the maximum clade credibility tree was estimated by Bayesian analysis of 138 hepatitis B virus (HBV) sequences. The posterior probabilities of the key nodes are depicted above the respective nodes. Samples obtained from Panama ($n = 17$, red taxa) were analysed together with other worldwide strains. The clusters of other HBV genotypes were collapsed.

rate compared with other DNA viruses (Fares & Holmes 2002, Pineiro y Leone et al. 2003, Takahashi et al. 2004a) increase the possibilities of both recombination among the genotypes (Andernach et al. 2012, Shi et al. 2012, Zhou et al. 2012) and the development of greater variability, which has occurred in genotype I (Yu et al. 2010). Recombination analysis of 10 samples from this study did not show enough evidence for recombination in Panama, likely as a result of cultural and insular tendencies among Panamanian Chinese and immigrant communities in general.

In addition to the genotyping portion of this study, we implemented a PCR method that allows amplification of a region from amino acids 35-226 of the small surface gene and amino acids 42-280 of HBV-RT, which covers the A-E RT domains. This fragment was used to evaluate residue changes that cause antiretroviral drug resistance and allow escape from detection in HBsAg immunoassays, immunisation or HBIG treatment. Although primary drug resistance changes in the RT domains studied were completely absent, secondary drug resistance changes in domains C (rtV207L) and D (rtN238T) were identified

in one (6%) and five (31%) samples, respectively, which were higher than previous reports, although the study group was small ($n = 16$) (Zhong et al. 2012). However, we cannot discount the importance of these mutations when considering treatments for naïve populations.

With regard to the MHR region, in almost all of the samples, no residues were found with substitutions related to escape from detection in HBsAg immunoassays, immunisation or HBIG treatment. The substitution Q129H was observed in one sample; this residue has been reported as capable of reducing serological detection in some diagnostic assays (Coleman et al. 1999, Ma & Wang 2012).

Mutation analysis in the Enh II region found the G1613A mutation in two subjects; although both samples were HBsAg-positive, *in vitro* studies have linked this mutation with HBsAg suppression in CHB (Li et al. 2011).

In three samples, mutations were identified in the PC and BCP regions that are associated with either the suppression of HBsAg secretion and the development of liver cirrhosis or HCC. Mutations in the PC region affect the secretion of HBsAg (Tong et al. 2007, Tangkijvanich

Fig. 2A: amino acid alignments of hepatitis B virus reverse transcriptase (HBV-RT) gene isolated in this study from HBsAg positive subject naïve to treatment ($n = 18$). Four domains of RT protein are shown. Dots represent the same residue as in reference sequence. Substitutions of relevance are in box. Place of birth of the subjects is indicated (CN: China; NI: no information; PA: Panama); B: nucleotide alignments of the HBV, EnhII, precore and basal core promoter regions isolated in this study from HBsAg positive ($n = 10$). Dots represent the same nucleotide as in reference sequence. Mutations of relevance are in box. HBsAg status is shown.

et al. 2010) because this region controls the transcription of PC mRNA and pre-genomic RNA (Buckwold et al. 1996). The mutation pair A1762T/G1764A was found in three samples, with one sample concurrently showing the BCP mutation G1896A; this group of mutations abolished the secretion of HBeAg in this specimen, as evidenced by a negative result in the immunoassay used.

Collectively, these mutations are molecular indicators that patients may develop serious chronic illness, which could be a major public health problem in Panama that would not necessarily be restricted to the Chinese population.

Studies that determine the seroprevalence, genotype and mutation profile of HBV are useful from a public health point of view for identifying risk factors for the spread of different HBV genotypes and in applying epidemiological measures with the aim of decreasing the prevalence of HBV in particular risk groups. These measures are important in reducing the rate of evolution of HBV and in maintaining the efficacy of current vaccines.

In conclusion, to our knowledge, this is the first study that reports high HBV prevalence rates in resident ethnic Chinese in Central America and the presence of genotypes B2 and C1 in this region.

Further studies designed to evaluate the potential presence of the mutations found in Chinese populations in other countries from Central America are needed to achieve a better understanding of the regional impact of HBV infection.

ACKNOWLEDGEMENTS

To the Chinese-Panamanian Association, for their support with the study and help in translating the survey.

REFERENCES

- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Carrilho FJ, Pinho JR. 2010. Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol* 91: 501-508.
- Andernach IE, Jutavijittum P, Samouny B, Yousukh A, Thammavong T, Hubschen JM, Muller CP. 2012. A high variability of mixed infections and recent recombinations of hepatitis B virus in Laos. *PLoS ONE* 7: e30245.
- Arauz-Ruiz P, Norder H. 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83: 2059-2073.
- Bini EJ, Perumalswami PV. 2010. Hepatitis B virus infection among American patients with chronic hepatitis C virus infection: prevalence, racial/ethnic differences and viral interactions. *Hepatology* 51: 759-766.
- Blitz L, Pujol F. 1998. Antigenic diversity of hepatitis B virus strains of genotype F in Amerindians and other population groups from Venezuela. *J Clin Microbiol* 36: 648-651.
- Bowyer SM, Sim JG. 2000. Relationships within and between genotypes of hepatitis B virus at points across the genome: footprints of recombination in certain isolates. *J Gen Virol* 81: 379-392.
- Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. *J Virol* 70: 5845-5851.
- Campos RH, Mbayed VA, Pineiro YLFG. 2005. Molecular epidemiology of hepatitis B virus in Latin America. *J Clin Virol* 34 (Suppl. 2): S8-S13.
- Coleman PF, Chen YC, Mishahwar IK. 1999. Immunoassay detection of hepatitis B surface antigen mutants. *J Med Virol* 59: 19-24.
- Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7: 214.
- Echevarria JM, Avellon A. 2006. Hepatitis B virus genetic diversity. *J Med Virol* 78 (Suppl. 1): S36-S42.
- Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* 5: 113.
- Fares MA, Holmes EC. 2002. A revised evolutionary history of hepatitis B virus (HBV). *J Mol Evol* 54: 807-814.
- Günther S, Sommer G, Von Breunig F, Iwanska A, Kalinina T, Sterneck M, Will H. 1998. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: frequency and functional consequences of PCR-introduced mutations. *J Clin Microbiol* 36: 531-538.
- Hakre S, Arteaga G, Nunez AE, Bautista CT, Bolen A, Villarreal M, Peel SA, Paz-Bailey G, Scott PT, Pascale JM. 2012. Prevalence of HIV and other sexually transmitted infections and factors associated with syphilis among female sex workers in Panama. *Sex Transm Infect* 89: 156-164.
- INEC - Instituto Nacional de Estadística y Censo Panama. 2011. Censos Nacionales 2010. Available from: estadisticas.contraloria.gob.pa/Resultados2010/tabulados/Excel/Cuadro12.xls.
- Kallman JB, Tran S, Arsalla A, Haddad D, Stepanova M, Fang Y, Wrobel VI, Srishord M, Younossi ZM. 2011. Vietnamese community screening for hepatitis B virus and hepatitis C virus. *J Viral Hepat* 18: 70-76.
- Khan A, Tanaka Y, Saito H, Ebimura H, Sekiguchi H, Iwama H, Wakabayashi G, Kamiya T, Kurbanov F, Elkady A, Mizokami M. 2008. Transmission of hepatitis B virus (HBV) genotypes among Japanese immigrants and natives in Bolivia. *Virus Res* 132: 174-180.
- Kidd-Ljunggren K, Miyakawa Y, Kidd AH. 2002. Genetic variability in hepatitis B viruses. *J Gen Virol* 83: 1267-1280.
- Kramvis A, Kew M, Francois G. 2005. Hepatitis B virus genotypes. *Vaccine* 23: 2409-2423.
- Kurbanov F, Tanaka Y, Mizokami M. 2010. Geographical and genetic diversity of the human hepatitis B virus. *Hepatology Res* 40: 14-30.
- Lavanchy D. 2004. Hepatitis B virus epidemiology, disease burden, treatment and current and emerging prevention and control measures. *J Viral Hepat* 11: 97-107.
- Li MS, Lau TC, Chan SK, Wong CH, Ng PK, Sung JJ, Chan HL, Tsui SK. 2011. The G1613A mutation in the HBV genome affects HBeAg expression and viral replication through altered core promoter activity. *PLoS ONE* 6: e21856.
- Li Y, Zhu M, Guo Y, Chen W, Li G. 2010. Full-length hepatitis B virus sequences from naïve patients with fluctuation of viral load during ADV monotherapy. *Virus Genes* 40: 155-162.
- Liu Y, Hussain M, Wong S, Fung SK, Yim HJ, Lok ASF. 2007. A genotype-independent real-time PCR assay for quantification of hepatitis B virus DNA. *J Clin Microbiol* 45: 553-558.
- Ma Q, Wang Y. 2012. Comprehensive analysis of the prevalence of hepatitis B virus escape mutations in the major hydrophilic region of surface antigen. *J Med Virol* 84: 198-206.
- Merican I, Guan R, Amaraputika D, Alexander MJ, Chutaputti A, Chien RN, Hasnain SS, Leung N, Lesmana L, Phiet PH, Sjaifoellah Noer

- HM, Sollano J, Sun HS, Xu DZ 2000. Chronic hepatitis B virus infection in Asian countries. *J Gastroenterol Hepatol* 15: 1356-1361.
- Merrill RM, Hunter BD 2011. Seroprevalence of markers for hepatitis B viral infection. *Int J Infect Dis* 15: e78-e121.
- Michailidis E, Kirby KA, Hachiya A, Yoo W, Hong SP, Kim SO, Folk WR, Sarafianos SG 2012. Antiviral therapies: focus on hepatitis B reverse transcriptase. *Int J Biochem Cell Biol* 44: 1060-1071.
- Naumann H, Schaefer S, Yoshida CF, Gaspar AM, Repp R, Gerlich WH 1993. Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4. *J Gen Virol* 74: 1627-1632.
- Odeniyiwa SO, Mulders MN, Oyedele OI, Ola SO, Odaibo GN, Olaleye DO, Müller CP 2001. Phylogenetic analysis of new hepatitis B virus isolates from Nigeria supports endemicity of genotype E in West Africa. *J Med Virol* 65: 463-469.
- Okamoto H, Tsuda F, Sakugawa H, Sastroewignjo RI, Imai M, Miyakawa Y, Mayumi M 1988. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 69: 2575-2583.
- Olinger CM, Jutavijitum P, Hubschen JM, Yousukh A, Samountry B, Thammavong T, Toriyama K, Müller CP 2008. Possible new hepatitis B virus genotype, Southeast Asia. *Emerg Infect Dis* 14: 1777-1780.
- Orto E, Mizokami M, Ina Y, Moriyama EN, Kameshima N, Yamamoto M, Gojobori T 1989. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proc Natl Acad Sci* 86: 7059-7062.
- INEC - Instituto Nacional de Estadística y Censo Panama 2011. Censos Nacionales 2010. Available from: estadisticas.contraloria.gob.pa/Resultados2010/tabulados/Excel/Cuadro12.xls.
- Pineiro y Leone FG, Mbayed VA, Campos RH 2003. Evolutionary history of hepatitis B virus genotype F: an in-depth analysis of Argentine isolates. *Virus Genes* 27: 103-110.
- Pollack H, Wan K, Ramos R, Rey M, Sherman A, Tobias H, Tsang T, Tso A, Charles B, Korenblit P, Son S, Poon E, Bialek S, Bell B 2006. Screening for chronic hepatitis B among Asian/Pacific Islander populations New York City, 2005. *Morb Mortal Wkly Rep* 55: 505-509.
- Shi W, Carr MJ, Dunford L, Zhu C, Hall WW, Higgins DG 2012. Identification of novel inter-genotypic recombinants of human hepatitis B viruses by large-scale phylogenetic analysis. *Virology* 427: 51-59.
- Shi W, Zhang Z, Ling C, Zheng W, Zhu C, Carr MJ, Higgins DG 2013. Hepatitis B virus subgenotyping: history, effects of recombination, misclassifications and corrections. *Infect Genet Evol* 16C: 355-361.
- Stuyver L, Gendt S 2000. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 81: 67-74.
- Takahashi K, Toyota J, Karino Y, Kang JH, Maekubo H, Abe N, Mishi S 2004a. Estimation of the mutation rate of hepatitis E virus based on a set of closely related 7.5-year-apart isolates from Sapporo, Japan. *Hepatol Res* 29: 212-215.
- Takahashi M, Nishizawa T, Gotanda Y, Tsuda F, Komatsu F, Kawabata T, Hasegawa K, Altankhuu M, Chimedregzen U, Naranjaya L, Hoshino H, Hino K, Kagawa Y, Okamoto H 2004b. High prevalence of antibodies to hepatitis A and E viruses and viremia of hepatitis B, C, and D viruses among apparently healthy populations in Mongolia. *Clin Diagn Lab Immunol* 11: 392-398.
- Tangkijvanich P, Sa-Nguanmoo P, Mahachai V, Theamboonlers A, Poovorawan Y 2010. A case-control study on sequence variations in the enhancer II/core promoter/precore and X genes of hepatitis B virus in patients with hepatocellular carcinoma. *Hepatol Int* 4: 577-584.
- Tatematsu K, Tanaka Y, Kurbanov F, Suguchi F, Mano S, Maeshiro T, Nakayoshi T, Wakuta M, Miyakawa Y, Mizokami M 2009. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype I. *J Virol* 83: 10538-10547.
- Tong MJ, Blatt LM, Kao JH, Cheng JT, Corey WG 2007. Basal core promoter T1762/A1764 and precore A1896 gene mutations in hepatitis B surface antigen-positive hepatocellular carcinoma: a comparison with chronic carriers. *Liver Int* 27: 1356-1363.
- Torres C, Pineiro y Leone FG, Pezzano SC, Mbayed VA, Campos RH 2011. New perspectives on the evolutionary history of hepatitis B virus genotype F. *Mol Phylogenet Evol* 39: 114-122.
- Tran TT, Trinh TN, Abe K 2008. New complex recombinant genotype of hepatitis B virus identified in Vietnam. *J Virol* 82: 5657-5663.
- Yin J, Xie J, Liu S, Zhang H, Han L, Lu W, Shen Q, Xu G, Dong H, Shen J, Zhang J, Han J, Wang L, Liu Y, Wang F, Zhao J, Zhang Q, Ni W, Wang H, Cao G 2011. Association between the various mutations in viral core promoter region to different stages of hepatitis B, ranging of asymptomatic carrier state to hepatocellular carcinoma. *Am J Gastroenterol* 106: 81-92.
- Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ, Xia NS 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "T". *PLoS ONE* 5: e9297.
- Zeng G, Wang Z, Wen S, Jiang J, Wang L, Cheng J, Tan D, Xiao F, Ma S, Li W, Luo K, Naoumov NV, Hou J 2005. Geographic distribution, virologic and clinical characteristics of hepatitis B virus genotypes in China. *J Viral Hepat* 12: 609-617.
- Zhong Y, Lv J, Li J, Xing X, Zhu H, Su H, Chen L, Zhou X 2012. Prevalence, virology and antiviral drugs susceptibility of hepatitis B virus rtN238H polymerase mutation from 1,865 Chinese patients with chronic hepatitis B. *Antiviral Res* 93: 185-190.
- Zhou B, Wang Z, Yang J, Sun J, Li H, Tanaka Y, Mizokami M, Hou J 2012. Novel evidence of HBV recombination in family cluster infections in Western China. *PLoS ONE* 7: e38241.

- 36- Mónica Viviana Alvarado Mora, Camila Malta Romano, Michele Soares Gomes-Gouvêa, Maria Fernanda Gutierrez, Flair José Carrilho, João Renato Rebello Pinho. Molecular epidemiology and genetic diversity of Hepatitis B virus (HBV) genotype E in an Afro-Colombian isolated community. Journal of General Virology 2010 Feb; 91(Pt 2):501-8**

Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community

Mônica Viviana Alvarado Mora,¹ Camila Malta Romano,²
Michele Soares Gomes-Gouvêa,¹ Maria Fernanda Gutierrez,³
Flair José Carrilho¹ and João Renato Rebello Pinho¹

Correspondence:
Mônica Viviana Alvarado Mora
monica.viviana@usp.br

¹Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil

²Laboratory of Virology, São Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases, School of Medicine, University of São Paulo, Brazil

³Laboratory of Virology, Department of Microbiology, Pontificia Javeriana University Bogotá, Colombia

Hepatitis B virus (HBV) infection is a significant public health concern with 350 million chronic carriers worldwide. Eight HBV genotypes (A–H) have been described so far. Genotype E (HBV/E) is widely distributed in West Africa and has rarely been found in other continents, except for a few cases in individuals with an African background. In this study, we characterized HBV genotypes in Quidó, Colombia, by partial S/P gene sequencing, and found, for the first time, HBV/E circulating in nine Afro-Colombian patients who had no recent contact with Africa. The presence of HBV/E in this community as a monophyletic group suggests that it was a result of a recent introduction by some Afro-descendant contact or, alternatively, that the virus came with slaves brought to Colombia. By using sequences with sampling dates, we estimated the substitution rate to be about 3.2×10^{-4} substitutions per site per year, which resulted in a time to the most recent common ancestor (TMRCA) of 29 years. In parallel, we also estimated the TMRCA for HBV/E by using two previously estimated substitution rates (7.7×10^{-4} and 1.5×10^{-5} substitutions per site per year). The TMRCA was around 35 years under the higher rate and 1500 years under the slower rate. In sum, this work reports for the first time the presence of an exclusively African HBV genotype circulating in South America. We also discuss the time of the entry of this virus into America based on different substitution rates estimated for HBV.

Received 18 August 2009
Accepted 15 October 2009

INTRODUCTION

Hepatitis B virus (HBV) is estimated to cause chronic infection in more than 350 million people worldwide and death in 1 million per year. Nearly 20% of the chronic carriers live in Africa with excessively high prevalence rates reported, especially from the sub-Saharan region (Hübschen *et al.*, 2008). HBV strains have a distinct geographical distribution, and are traditionally classified into eight genotypes, A to H, on the basis of genome diversity (Stuyver *et al.*, 2000). Genotype A is found mainly in North and West Europe, North America and Africa. Genotypes B and C are prevalent in south-east Asia and the Far East. Genotype D has a worldwide distribution and is found predominantly in the Mediterranean region. Genotypes E and F are prevalent in West Africa and in

the Amerindian population, respectively (Magnius & Norder, 1995). Recently, genotype G has been reported in the USA and France (Stuyver *et al.*, 2000), and genotype H has been found in Central America (Arauz-Ruiz *et al.*, 2002).

In Africa, viruses belonging to five genotypes, A (HBV/A) to E (HBV/E), have been found. An extensive study of genotypes A, B, C and D in South Africa has been reported (Bowyer *et al.*, 1997). Genotype E was first described in 1992 (Norder *et al.*, 1992). Despite its wide geographical spread and high prevalence, genotype E viruses in Africa reveal a surprisingly low diversity. The mean diversity over the whole genome is 1.75%, compared with 4% diversity for African genotype A (Andernach *et al.*, 2009).

HBV/E is by far the most prevalent genotype in Mali, Burkina Faso, Nigeria, Togo, Benin, Central African Republic and the Democratic Republic of the Congo

The GenBank/EMBL/DBJ accession number for the Quidó HBV sequences identified in this study are GQ487690–GQ487698.

(Olinger *et al.*, 2006; Mulders *et al.*, 2004; Odemuyiwa *et al.*, 2001; Bekondi *et al.*, 2007). Also, this genotype has been reported from other West African countries like Senegal (Vray *et al.*, 2006), the Ivory Coast (Suzuki *et al.*, 2003), Ghana (Candotti *et al.*, 2006), Gambia (Dumppis *et al.*, 2001), Angola and Namibia (Kramvis & Kew, 2007). Finally, some strains were reported further East in Madagascar and Mozambique (Cunha *et al.*, 2007; Kramvis *et al.*, 2005).

Genotype E has not been found outside of Africa, except for a few rare cases mostly in individuals with an African background. In Europe, sporadic genotype E strains were reported from France (Halfon *et al.*, 2006), Italy (Palumbo *et al.*, 2007), Spain (Toro *et al.*, 2006), Belgium (Liu *et al.*, 2001) and The Netherlands (van Steenberg *et al.*, 2002). Others cases were reported in Argentina (Mathet *et al.*, 2006), Brazil (Sitnik *et al.*, 2007) and the USA (Kato *et al.*, 2004).

The analysis of DNA polymorphisms in the β -globin gene cluster mainly focuses on those linked to the HBB**S* gene, given that this mutation is absent among native American populations and was introduced into the American continent basically by the gene flow from Africa during the Atlantic slave trade from the 16th to the 19th century (Lemos Cardoso & Farias Guerreiro, 2006). Data described by Cuellar-Ambrosi *et al.* (2000) showed that the distribution of haplotypes from the western region of Colombia (which has the largest Afro-descendent population in the country) had typical African haplotypes: Bantu, 55.5%; Benin, 34.8%; Senegal, 4.3%; and Cameroon, 5.4%. These results closely agree with the historical data that indicate that most African slaves brought to Colombia originated from Angola (Bantu population) and São Tomé island in the Bight of Benin (Central West Africa).

Although these studies demonstrate the African origin of this population, they have not addressed the origins of HBV in this population. Also, there are no previous studies reporting the distribution of HBV genotypes in the Quibdó community. The aim of this study is to report the presence of genotype E of HBV in an Afro-descendent community from Quibdó, Colombia, and to determine the possible origin of its presence in this population using phylogenetic and Bayesian analysis.

RESULTS

Genotyping analysis

Nine samples were positive for the S region (416 bp) by nested PCR, but only five of them were also amplified in the S/POL region (734 bp) (GenBank accession nos GQ487690–GQ487698). To perform the phylogenetic analysis, the missing nucleotides were coded as 'missing characters' in nexus block. The longest fragment available from each sample was sequenced and classified into

genotype E by maximum-likelihood (ML) reconstruction (Fig. 1). Interestingly, the reconstruction of the nucleotides that change along the ML tree showed that all the Quibdó genotype E sequences have a synapomorphy of 2 nt, strongly suggesting that these sequences originated from a unique lineage introduced into this community in the past (Fig. 2). On the S ORF, these substitutions were non-synonymous and were found at position 133 ['a' determinant; Met (ATG) for Leu (TTG)] and at position 161 [Phe (TTC) for Tyr (TAC)]. On the Pol ORF, the first substitution at position 141 was also non-synonymous [Tyr (TAT) for Phe (TTT)], while the second substitution (162 aa) was synonymous [Ile (ATT) for Ile (ATA)]. Nevertheless, the two substitutions were localized out of all the known polymerase domains.

Bayesian analysis

Evolutionary dynamics of HBV/E. The mean rate of nucleotide substitution for HBV/E using 51 sequences for which sampling dates were known was estimated to be around 3.2×10^{-4} substitutions per site per year within highest probability densities (HPDs) (2.2×10^{-4} – 4.52×10^{-4}). Under this substitution rate, the estimated TMRCA for the origin of this genotype was around 29 years ago when only HBV S/Pol sequences were used; this increased to 90 years ago when non-recombinant regions of HBV genomes were utilized (Table 1).

As already expected, a huge variation of TMRCA was found for HBV/E by using the two different previously estimated substitution rates of 7.7×10^{-4} and 1.5×10^{-5} substitutions per site per year (Table 1). Under the higher rate, the TMRCA of genotype E estimated from the complete genomes was 30 years, but this value went up to 1536 years with the slower rate. The variation was also large when the TMRCA was evaluated using the non-recombinant region (the first 1799 nt of the HBV genome), ranging from 37.4 to 1907 years. Finally, the two datasets comprising the most conserved region of the virus (S/POL) revealed a TMRCA ranging between 24.4 and 1224 years or 9.7 and 469.5 years, when only genotype E sequences were utilized. The estimates in Table 1 correspond to the values obtained from the best-fit rate and molecular clock chosen by Bayes Factor comparison. The strict molecular clock best-fitted the dataset of the non-recombining region, and the relaxed uncorrelated lognormal was the best molecular clock for all the other HBV datasets. Therefore, there was little variation among estimates under distinct clock models (data available upon request).

DISCUSSION

HBV/E – what is the origin?

We showed, for the first time, the presence of HBV genotype E circulating inside a South American popu-

Fig. 1. ML phylogenetic tree of the S/Pol region of the HBV genome ($n=188$). The collapsed clades correspond to the non-E genotypes (A, B, C, D, F, G and H). The values of posterior probability (>0.9) are shown for key nodes. The nine HBV/E Quibdó sequences are indicated by a bracket. Cen Afr Rep, Central African Republic; SEN, Senegal; DRC, the Democratic Republic of the Congo; IVORY, the Ivory Coast.

Fig. 2. Multiple alignment of amino acid sequences of HBsAg of the nine Colombian sequences compared with others obtained from GenBank. The major hydrophilic region (100–160), including the 'a' determinant (124–147), is boxed (dotted line). The two synapomorphies are located at positions 133 and 161 of the HBV S gene (boxed, solid line).

lation. There have been many studies to characterize the HBV genotype prevalence in Afro-descendant populations in South America; in the Afro-Venezuelan population, genotypes A and F have already been found. These results might reflect the introduction of genotype A during the slavery period and the absence of the African genotype E supports

the hypothesis of a recent origin for this genotype, which is after the time of slavery (Quintero *et al.*, 2002). Also, in Afro-Brazilian communities, all HBV isolates belonged to genotype A, subtype AI (Motta-Castro *et al.*, 2005, 2008), which suggests that HBV was introduced from Africa to different South American countries during the slave trade.

Table 1. Substitution rates and TMRCA for human HBV/E sequences

TMRCA was calculated using three different substitution rates (substitutions per site per year).

Dataset	No. sequences	HBV/E TMRCA (years; 95 % HPD)		
		7.7×10^{-4}	3.2×10^{-4}	1.5×10^{-5}
Complete human HBV genomes	232	30 (25–34)	72 (60–85)	1536 (1293–1820)
HBV genomes, non-recombinant region	256	37.4 (30–47)	90 (72–115)	1907 (1525–2397)
HBV S/Pol region (all genotypes)	256	24.4 (14–37)	42 (29–57)	1224 (543–2094)
HBV S/Pol region (genotype E)	156	9.7 (6.3–14)	29.6 (16–52)	469.5 (257–738)

It is already known that genotype E is largely found only in Africa or African descendants and is very rare in any other continent. Currently, the few HBV/E carriers found outside Africa were recent travellers from this continent (Halfon *et al.*, 2006; Palumbo *et al.*, 2007; Toro *et al.*, 2006; Liu *et al.*, 2001; van Steenberg *et al.*, 2002; Mathet *et al.*, 2006; Sitnik *et al.*, 2007; Kato *et al.*, 2004). The fact that Quibdó (the Colombian city from where the samples in this work were isolated) inhabitants are descendent from the slaves that arrived in America up to the beginning of the 19th century raises the question of when this genotype was introduced in this population. Two hypotheses can explain the presence of this genotype in Quibdó: (i) the virus came with the slaves brought to Colombia and has been circulating in this population since that time; or (ii) the virus was introduced more recently, due to contact of its inhabitants with African people.

The slaves trafficked from Africa to America originated mostly from three main regions in West Central Africa: the Bight of Benin, the Bight of Nigeria and the Gold Coast in Ghana (Cuellar-Ambrosi *et al.*, 2000). Supporting the theory that the viruses circulating in Quibdó were introduced in the times of slavery, we found that the Colombian viruses constitute a closer cluster to sequences from Nigeria, Togo, Benin, the Democratic Republic of the Congo, the Ivory Coast and Cameroon, as seen in Fig. 1. Moreover, as far as we know, the Afro-Colombian population of Quibdó is very poor and there is no report of recent travel to Africa or visitors from African populations to this region.

Unfortunately, since there is no consensus about the evolutionary rate of HBV, it is difficult to estimate the TMRCA for this virus. For this reason, we decided to estimate the substitution rate of HBV/E. Furthermore, we also estimated TMRCA using the two previously published substitution rates. However, due to the large difference in these rates, our results on the evolutionary dynamics can support the two hypotheses about the origin of this genotype in Colombia. First, under our estimate of 3.2×10^{-4} substitutions per site per year, the TMRCA for genotype E sequences was around 30 years ago. The similar substitution rate estimated by Zhou & Holmes (2007) also suggested a recent entry of HBV/E into this community. In fact, this hypothesis is more consistent with the accepted

time estimated for the origin of genotype E in general. However, by considering a recent origin for HBV/E and consequently, a more recent introduction in America (less than 30 years ago), it is quite unexpected that the viruses found in Colombia are different from any other lineage found in Africa so far. Critically, this last hypothesis is also supported by the estimates of TMRCA using the slower rate and, by the monophyletic cluster (with high posterior probability) of nine sequences isolated from Quibdó (Fig. 2), which shows that a single virus entered the community, establishing a founder effect, and has remained there until now.

Variability in the substitution rate

The substitution rate estimated (3.2×10^{-4} substitutions per site per year) in this work for genotype E only was obtained using the same methodology used by Zhou & Holmes (2007) and the results were very similar to their findings and also to those of Zaaijer *et al.* (2008), even though they used a different approach. The first study estimating the substitution rate of HBV reported a rate between 1.4 and 3.2×10^{-5} substitutions per site per year (Okamoto *et al.*, 1987), obtained by dividing the amount of divergence accumulated in sequences obtained from a single patient by the total length of the sequence, assuming the common ancestor for the viruses at the patient's birth. By using a similar approach, other studies based on pair-wise sequence comparison sampled at different time points estimated a median substitution rate of 5.1×10^{-4} (Zaaijer *et al.*, 2008) and 7.9×10^{-5} (Osiowy *et al.*, 2006) substitutions per site per year. Similar evolutionary rates have also been estimated for other *Hepadnaviruses* such as duck hepatitis B virus: 0.8 and 4.5×10^{-5} substitutions per site per generation (Pult *et al.*, 2001).

In this work, we used a coalescent-based approach to estimate the substitution rate and the TMRCA of HBV/E. This method was also used previously to estimate the overall substitution rate for HBVs using different datasets (Zhou & Holmes, 2007). There are several advantages to using Bayesian approaches to calculate the rate of nucleotide substitution of pathogens. The first reason is that the estimates are based on thousands of phylogenetic trees instead of a unique distance value based on pair-wise

distances. Also, the method allows the use of matrices of substitution models that correct for distinct probabilities of substitution through the sequences (Huelsenbeck *et al.*, 2001). However, determining the rate of sequence change for HBV is difficult due to its complex organization. More than half of the HBV genome consists of overlapping frame-shifted ORFs. Moreover, overlapping genes and secondary structures of the HBV genome involved in the regulation of replication impose a constraint on the number and nature of substitutions occurring in the genome (Zaaijer *et al.*, 2008). Consequently, the best nucleotide substitution rate would be estimated from the third codon positions, with no overlapping regions. In fact, this has already been done and the values were around 9.63×10^{-4} substitutions per site per year. However, due to the large variation in the HPDs obtained from this dataset (from 0.4 to 19.2×10^{-4}), this specific rate may not be suitable (Zhou & Holmes, 2007). Another important factor influencing the evolution of HBV is the phase (chronic or acute) of natural infection. Zaaijer *et al.* (2008) noticed that the substitution rate in the HBV S gene was inversely related to the level of viraemia, with no substitutions occurring in the majority of highly viraemic HBV carriers during decades of cumulative follow-up.

In summary, this work reports, for the first time, the presence of HBV/E circulating in a small community in Colombia. Moreover, this virus appears to have experienced a unique entry into this population, since we found two particular nucleotide changes (synapomorphies) only in Quibdó sequences. We also attempt to estimate the evolutionary rate of HBV/E. The current available data and the substitution rate estimated in this work suggest a recent origin for HBV, but the great variability and the worldwide distribution of HBV genotypes argue against this theory. As an accurate substitution rate for this virus is crucial to understand its evolutionary origins, we argue that further analysis of HBV evolutionary patterns should be done, including relevant clinical and serological data.

METHODS

Genotyping analysis

Study population. Nine positive samples for the surface marker of HBV (HBsAg) were obtained from sera stored at -20°C in refrigerators in the Laboratorio de Salud Pública in Quibdó, Colombia. The sera were obtained from pregnant women living in Quibdó city, Chocó, Colombia, over 2 years (2006–2007). These samples came from nine Afro-descendant women aged from 16 to 23 years old. Specifically, the Laboratorio de Salud Pública received samples from different hospitals from this city and confirmed these using the HBsAg and anti-hepatitis B core antigen serological test.

HBV DNA extraction. HBV DNA extraction was carried out from 100 μl serum for each sample using the acid guanidinium isothiocyanate (GT)/phenol/chloroform method described by Chomczynski & Sacchi (1987). Briefly, 300 μl GT solution was added to each sample. Ice-cold chloroform (50 μl) was added, followed by homogenization and centrifugation. The supernatant was transferred

to a conical tube and precipitated with 300 μl cold ethanol. After discarding the ethanol, samples were dried at 94°C for 1 min, resuspended in 50 μl ultrapure MilliQ water and stored at -20°C .

HBV DNA amplification: S and S/Pol regions. To avoid false-positive results, strict procedures for nucleic acid amplification diagnostic techniques were followed (Kwok & Higuchi, 1989). Samples were first amplified with the primers described by Sitnik *et al.* (2004) in order to get a 416 bp fragment partially covering the HBsAg coding region (S). A fragment of 734 bp partially comprising HBsAg and Pol coding regions (S/Pol) was then amplified from the samples that were positive in the previous step, using the primers described by Gomes-Gouveia *et al.* (2009).

HBV sequencing. Amplified DNA was purified using ChargeSwitch PCR clean-up kit. Sequencing was performed in an ABI Prism 377 Automatic Sequencer (Applied Biosystems) based on the protocol described by Sanger *et al.* (1977), using dideoxy nucleoside triphosphates containing fluorescent markers (Big Dye terminator v3.1 cycle sequencing ready reaction kit; Applied Biosystems). The quality of each electropherogram was evaluated using the Phred-Phrap software (Ewing *et al.*, 1998; Ewing & Green, 1998) and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software, available at <http://asparagin.cenargen.embrapa.br/phph/>.

Evolutionary analysis

Phylogenetic analysis. Initially, the nine sequences obtained in this work were genotyped by phylogenetic reconstructions using reference sequences from each HBV genotype obtained from GenBank ($n=139$) (data available upon request). After genotyping, another dataset was constructed comprising a range of the most variable sequences of genotype E in order to obtain the best classification of the Colombian sequences within this group ($n=188$). These sequences comprising partial S/Pol coding regions were aligned using CLUSTAL_X software (Thompson *et al.*, 1997) and manually edited in the Se-Al software (available at <http://tree.bio.ed.ac.uk/software/seal/>). ML phylogenetic trees were inferred using the GARLI program (Zwickl, 2006), which employs an extensive branch-swapping protocol and optimizes the substitution model iteratively during the search. The evolutionary model of DNA substitution and initial parameters used in GARLI were estimated by MODELTEST v3.7 (Posada & Crandall 1998).

We also mapped the nucleotide changes along the ML tree using the MacClade v4.7 software (Maddison & Maddison, 2003).

Bayesian analysis

Data preparation. Initially, a large dataset comprising 256 complete genomes of all HBV genotypes was constructed with sequences obtained from GenBank (M. V. Alvarado Mora and others, unpublished data). Since there is evidence of recombination among HBV sequences, we undertook a detailed search for recombination using the RDP3 program (Marín, 2009) to exclude potential recombinants from the study. From this, additional datasets were obtained comprising different genome regions: (i) 232 sequences of the HBV complete genome without recombining sequences; (ii) 256 sequences with 1799 nt (without the recombining region of pre-core/C); (iii) 256 sequences with 834 nt of the S/Pol region from all genotypes; and (iv) 156 HBV/E sequences with 834 nt of the S/Pol region (datasets available from authors upon request). In order to estimate the substitution rate for genotype E, an additional dataset comprising 51 HBV/E sequences containing information about the sampling date was constructed.

Estimating evolutionary dynamics. The TMRCA in years was estimated by using the Bayesian Markov Chain Monte Carlo

approach (MCMC) implemented in BEAST v.1.4.8 (Drummond & Rambaut, 2007). We first estimated the nucleotide substitution rate and the TMRCA for HBV/E using the dataset with sampling date information. Yet, since there is no consensus on the HBV substitution rate, we also estimated the TMRCA using two other previously estimated evolutionary rates: (i) the recently estimated rate of 7.7×10^{-4} substitutions per site per year, also obtained from Bayesian methodology using the complete genome (Zhou & Holmes 2007), and (ii) a rate of 1.5×10^{-5} substitutions per site per year, which is the lowest estimated rate for HBV, and has been used in previous studies (Simmonds & Midgley, 2005; Hannoun *et al.*, 2005).

To estimate the TMRCA with as much accuracy as possible, we ran the Bayesian Skyline plot (BSL) under the strict and relaxed uncorrelated $\log_{normal}$ molecular clock using the best model of nucleotide substitution (GTR+G+I) obtained in MODELTEST. The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. Convergence of parameters during the MCMC was inspected with Tracer v.1.4 (Drummond & Rambaut, 2007), with uncertainties addressed as 95% HPD intervals. The number of generations run that were needed to achieve the convergence of all parameters (effective sampling size > 200) ranged between 10 and 60 million for distinct datasets.

Overall rates of evolutionary change and the TMRCA for HBV/E were estimated from the dataset with sampling dates. Uniform prior substitution rates and the relaxed uncorrelated $\log_{normal}$ molecular clock were used for estimates. The coalescent prior and the nucleotide substitution model were the same as used for the other HBV datasets. MCMC runs consisting of 10 million chains (with a 10% burning) were undertaken to obtain convergence of parameter estimates.

ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; 2007/53457-7 and 2008/50461-60), São Paulo, SP, Brazil, and by Pontificia Universidad Javeriana, Bogotá, Colombia. We thank the Laboratorio de Salud Pública, Quibdó, Colombia, for kindly providing the samples for this study.

REFERENCES

- Andernach, I. E., Hübschen, J. M. & Müller, C. P. (2009). Hepatitis B virus: the genotype E puzzle. *Rev Med Virol* 19, 231–240.
- Arauz-Ruiz, P., Norder, H., Robertson, B. H. & Magnus, L. O. (2002). Genotype HE a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 83, 2059–2073.
- Bekondi, C., Olinger, C. M., Bous, N., Talarmin, A., Müller, C. P., Le Faou, A. & Venard, V. (2007). Central African Republic is part of the West-African hepatitis B virus genotype E. *J Clin Virol* 40, 31–37.
- Bowyer, S. M., van Staden, L., Kew, M. C. & Sim, J. G. (1997). A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J Gen Virol* 78, 1719–1729.
- Candotti, D., Opere-Sem, O., Rezvan, H., Sarkodie, F. & Allain, J. P. (2006). Molecular and serological characterization of hepatitis B virus in deferred Ghanaian blood donors with and without elevated alanine aminotransferase. *J Viral Hepat* 13, 715–724.
- Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162, 156–159.
- Cuellar-Ambrosi, F., Mondragón, M. C., Figueroa, M., Prêhu, C., Galactéros, F. & Ruiz-Linares, A. (2000). Sickle cell anemia and

beta-globin gene cluster haplotypes in Colombia. *Hemoglobin* 24, 221–225.

- Cunha, L., Plouzeau, C., Ingrand, P., Gudo, J. P., Ingrand, L., Mondlane, J., Besuchant, M. & Agius, G. (2007). Use of replacement blood donors to study the epidemiology of major blood-borne viruses in the general population of Maputo, Mozambique. *J Med Virol* 79, 1832–1840.
- Drummond, A. J. & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 7, 214.
- Dumpis, U., Mendy, M., Hill, A., Thurs, M., Hall, A., Whittle, H. & Karayiannis, P. (2001). Prevalence of HBV core promoter/precore/core mutations in Gambian chronic carriers. *J Med Virol* 65, 664–670.
- Ewing, B. & Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8, 186–194.
- Ewing, B., Hillier, L., Wendt, M. C. & Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8, 175–185.
- Gomes-Gouveia, M. S., Soares, M. C., Bensabath, G., de Carvalho-Mello, I. M., Brito, E. M., Souza, O. S., Queiroz, A. T., Carrilho, F. J. & Pinho, J. R. (2009). Hepatitis B virus and hepatitis delta virus genotypes in outbreaks of fulminant hepatitis (Labrea black fever) in the western Brazilian Amazon region. *J Gen Virol* 90, 2638–2643.
- Halfon, P., Bourlière, M., Pol, S., Benhamou, Y., Ouzan, D., Roilly, M., Khiri, H., Renou, C., Pénaranda, G. & other authors (2006). Multicentre study of hepatitis B virus genotypes in France: correlation with liver fibrosis and hepatitis B e antigen status. *J Viral Hepat* 13, 329–335.
- Hannoun, C., Söderström, A., Norkrans, G. & Lindh, M. (2005). Phylogeny of African complete genomes reveals a West African genotype A subtype of hepatitis B virus and relatedness between Somali and Asian A1 sequences. *J Gen Virol* 86, 2163–2167.
- Hübschen, J. M., Andernach, I. E. & Müller, C. P. (2008). Hepatitis B virus genotype E variability in Africa. *J Clin Virol* 43, 376–380.
- Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. (2001). Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* 294, 2310–2314.
- Kato, H., Gish, R. G., Bzowej, N., Newsom, M., Suguchi, F., Tanaka, Y., Kato, T., Orito, E., Usuda, S. & other authors (2004). Eight genotypes (A–H) of hepatitis B virus infecting patients from San Francisco and their demographic, clinical, and virological characteristics. *J Med Virol* 73, 516–521.
- Kramvis, A. & Kew, M. C. (2007). Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res* 37, S9–S19.
- Kramvis, A., Restorp, K., Norder, H., Botha, J. F., Magnus, L. O. & Kew, M. C. (2005). Full genome analysis of hepatitis B virus genotype E strains from South-Western Africa and Madagascar reveals low genetic variability. *J Med Virol* 77, 47–52.
- Kwok, S. & Higuchi, R. (1989). Avoiding false positives with PCR. *Nature* 339, 237–238.
- Lemos Cardoso, G. & Farias Guerreiro, J. (2006). African gene flow to north Brazil as revealed by HBB*5 gene haplotype analysis. *Am J Hum Biol* 18, 93–98.
- Liu, H. F., Sokal, E. & Goubau, P. (2001). Wide variety of genotypes and geographic origins of hepatitis B virus in Belgian children. *J Pediatr Gastroenterol Nutr* 32, 274–277.
- Maddison, D. R. & Maddison, W. P. (2003). *MacClade 4: Analysis of Phylogeny and Character Evolution*, 4th edn. Sunderland, MA: Sinauer Associates.
- Magnus, L. O. & Norder, H. (1995). Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 38, 24–34.

- Martin, D. P. (2009). Recombination detection and analysis using RDP3. *Methods Mol Biol* 537, 185–205.
- Mathet, V. L., Cuestas, M. L., Ruiz, V., Minassian, M. L., Rivero, C., Trinks, J., Daleoso, G., León, L. M., Sala, A. & other authors (2006). Detection of hepatitis B virus (HBV) genotype E carried – even in the presence of high titers of anti-HBs antibodies – by an Argentinean patient of African descent who had received vaccination against HBV. *J Clin Microbiol* 44, 3435–3439.
- Motta-Castro, A. R., Martins, R. M., Yoshida, C. F., Teles, S. A., Paniago, A. M., Lima, K. M. & Gomes, S. A. (2006). Hepatitis B virus infection in isolated Afro-Brazilian communities. *J Med Virol* 77, 188–193.
- Motta-Castro, A. R., Martins, R. M., Araujo, N. M., Niel, C., Facholi, G. B., Lago, B. V., Mello, F. C. & Gomes, S. A. (2008). Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Arch Virol* 153, 2197–2205.
- Mulders, M. N., Venard, V., Njayou, M., Ederh, A. P., Bola Oyefolu, A. O., Kehinde, M. O., Muyembe Tamfum, J. J., Nebie, Y. K., Maiga, I. & other authors (2004). Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 190, 400–408.
- Norder, H., Hammas, B., Lofdhall, S., Courouze, A. M. & Magnus, L. O. (1992). Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 73, 1201–1208.
- Odemuyiwa, S. O., Mulders, M. N., Oyedele, O. I., Ola, S. O., Odaibo, G. N., Olaye, D. O. & Muller, C. P. (2001). Phylogenetic analysis of new hepatitis B virus isolates from Nigeria supports endemicity of genotype E in West Africa. *J Med Virol* 65, 463–469.
- Okamoto, H., Imai, M., Kametani, M., Nakamura, T. & Mayumi, M. (1987). Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through maternal-fetal transmission. *Jpn J Exp Med* 57, 231–236.
- Olinger, C. M., Venard, V., Njayou, M., Oyefolu, A. O., Maiga, I., Kemp, A. J., Omilabu, S. A., le Faou, A. & Muller, C. P. (2006). Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 87, 1163–1173.
- Osiowy, C., Giles, E., Tanaka, Y., Mizokami, M. & Minuk, G. Y. (2006). Molecular evolution of hepatitis B virus over 25 years. *J Virol* 80, 10307–10314.
- Palumbo, E., Scotto, G., Faleo, G., Cibelli, D. C., Saracino, A. & Angarano, G. (2007). Prevalence of HBV-genotypes in immigrants affected by HBV-related chronic active hepatitis. *Arg Gastroenterol* 44, 54–57.
- Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Pull, I., Abbott, N., Zhang, Y. Y. & Summers, J. (2001). Frequency of spontaneous mutations in an avian hepadnavirus infection. *J Virol* 75, 9623–9632.
- Quintero, A., Martínez, D., Alarcón De Noya, B., Costagliola, A., Urbina, L., González, N., Liprandi, F., Castro De Guerra, D. & Pujol, F. H. (2002). Molecular epidemiology of hepatitis B virus in Afro-Venezuelan populations. *Arch Virol* 147, 1829–1836.
- Rodas, C., Gelvez, N. & Keyeux, G. (2003). Mitochondrial DNA studies show asymmetrical Amerindian admixture in Afro-Colombian and Mestizo populations. *Hum Biol* 75, 13–30.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 74, 5463–5467.
- Simmonds, P. & Midgley, S. (2005). Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol* 79, 15467–15476.
- Sitnik, R., Pinho, J. R., Bertolini, D. A., Bernardini, A. P., Da Silva, L. C. & Carrilho, F. J. (2004). Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol* 42, 2455–2460.
- Sitnik, R., Sette, H., Jr, Santana, R. A., Menezes, L. C., Graca, C. H., Dastoli, G. T., Silbert, S. & Pinho, J. R. (2007). Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. *Braz J Med Biol Res* 40, 1689–1692.
- Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schirazi, R. F. & Rossau, R. (2000). A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 81, 67–74.
- Suzuki, S., Sugauchi, F., Orito, E., Kato, H., Usuda, S., Siranay, L., Arita, I., Sakamoto, Y., Yoshihara, N. & other authors (2003). Distribution of hepatitis B virus (HBV) genotypes among HBV carriers in the Cote d'Ivoire: complete genome sequence and phylogenetic relatedness of HBV genotype E. *J Med Virol* 69, 459–465.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876–4882.
- Toro, C., Jiménez, V., Rodríguez, C., Del Romero, J., Rodés, B., Holguin, A., Alvarez, P., García-Campello, M., Gómez-Hernando, C. & other authors (2006). Molecular and epidemiological characteristics of blood-borne virus infections among recent immigrants in Spain. *J Med Virol* 78, 1599–1608.
- van Steenberghe, J. E., Niesters, H. G., Op de Coul, E. L., van Doornum, G. J., Osterhaus, A. D., Leentvaar-Kuijpers, A., Coutinho, R. A. & van den Hoek, J. A. (2002). Molecular epidemiology of hepatitis B virus in Amsterdam 1992–1997. *J Med Virol* 66, 159–165.
- Vray, M., Debonne, J. M., Sire, J. M., Tran, N., Chevalier, B., Planter, J. C., Fall, F., Vernet, G., Simon, F. & Mb, P. S. (2006). Molecular epidemiology of hepatitis B virus in Dakar, Senegal. *J Med Virol* 78, 329–334.
- Zaaijer, H. L., Bouter, S. & Boot, H. J. (2008). Substitution rate of the hepatitis B virus surface gene. *J Viral Hepat* 15, 239–245.
- Zhou, Y. & Holmes, E. C. (2007). Bayesian estimates of the evolutionary rate and age of hepatitis B virus. *J Mol Evol* 65, 197–205.
- Zwickl, D. J. (2006). *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion*. PhD thesis, The University of Texas at Austin.

- 37- Alvarado, M.V., Romano, C. M., Gomes-Gouvea, M. S., Gutierrez, M. F., Carrilho, F. J., PINHO, J. R. R. 2010. Molecular Characterization of the Hepatitis B virus genotype in Colombia: A Bayesian Inference on the Genotype F. Infection, Genetics and Evolution 2011, 11 (1): 103-108**

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Molecular characterization of the Hepatitis B virus genotypes in Colombia: A Bayesian inference on the genotype F

Mónica Viviana Alvarado Mora^{a,*}, Camila Malta Romano^b, Michele Soares Gomes-Gouvêa^a, Maria Fernanda Gutierrez^c, Livia Botelho^a, Flair José Carrilho^a, João Renato Rebello Pinho^a

^aLaboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil

^bSão Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases (LIMHC), School of Medicine, University of São Paulo, Brazil

^cLaboratory of Virology, Department of Microbiology, Pontificia Javeriana University, Bogotá, Colombia

ARTICLE INFO

Article history:

Received 19 June 2010

Received in revised form 22 September 2010

Accepted 6 October 2010

Available online 15 October 2010

Keywords:

Hepatitis B virus

Genotype G

Genotype A2

Genotype F

Bayesian analysis

Colombia

ABSTRACT

Hepatitis B is a worldwide health problem affecting about 2 billion people and more than 350 million are chronic carriers of the virus. Nine HBV genotypes (A to I) have been described. The geographical distribution of HBV genotypes is not completely understood due to the limited number of samples from some parts of the world. One such example is Colombia, in which few studies have described the HBV genotypes. In this study, we characterized HBV genotypes in 143 HBsAg-positive volunteer blood donors from Colombia. A fragment of 1306 bp partially comprising HBsAg and the DNA polymerase coding regions (S/POL) was amplified and sequenced. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) approach to obtain the maximum clade credibility (MCC) tree using BEAST v.1.5.3. Of all samples, 68 were positive and 52 were successfully sequenced. Genotype F was the most prevalent in this population (77%) – subgenotypes F3 (75%) and F1b (2%). Genotype G (7.7%) and subgenotype A2 (15.3%) were also found. Genotype G sequence analysis suggests distinct introductions of this genotype in the country. Furthermore, we estimated the time of the most recent common ancestor (TMRCA) for each HBV/F subgenotype and also for Colombian F3 sequences using two different datasets: (i) 77 sequences comprising 1306 bp of S/POL region and (ii) 283 sequences comprising 681 bp of S/POL region. We also used two other previously estimated evolutionary rates: (i) 2.60×10^{-4} s/s/y and (ii) 1.5×10^{-5} s/s/y. Here we report the HBV genotypes circulating in Colombia and estimated the TMRCA for the four different subgenotypes of genotype F.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Hepatitis B virus (HBV) infection is a severe global health problem affecting 2 billion people worldwide and 350 million are suffering from chronic HBV infection (Chudy et al., 2006). A genetic classification based on the comparison of complete HBV genomes has identified eight genotypes, A through H (Mahtab et al., 2008). The distribution of HBV genotypes is geographically restricted. Genotype A is found mainly in northern and western Europe, North America, and Africa. Genotypes B and C are prevalent in South-east Asia and the Far East (Mahtab et al., 2008). Genotype D is predominant in the Mediterranean basin, the Middle East, and Central Asia. Genotype E is by far the most prevalent genotype in western and central Africa (Kramvis and Kew, 2007). HBV/E has not

been found outside Africa, except for a few rare cases mostly in individuals with an African background. However, recent reports have described the presence of this genotype in a specific community in Colombia (Alvarado Mora et al., 2010) and in the north of India (Singh et al., 2009). Genotypes F and H are prevalent in the Amerindian population and Central America, respectively (Arauz-Ruiz et al., 2002; Magnus and Norder, 1995). Recently, phylogenetic and distance analyses characterized a new genotype, designated as genotype I, in Vietnam and Laos (Yu et al., 2010).

Hepatitis B virus genotype G (HBV/G) is characterized by the presence of a unique 36 bp insertion located downstream of the core gene start codon and by mutations in the pre-C region (Kato et al., 2002). The origin of genotype G is still unknown (Lindh, 2005), and despite its apparent low prevalence in the world, it was reported in many countries in Europe (De Maddalena et al., 2007; Jardi et al., 2008; Lacombe et al., 2006; Vieth et al., 2002) and in the Americas (Alvarado-Esquivel et al., 2006; Bhat et al., 1990; Bottecchia et al., 2008; Chu et al., 2003; Osioy and Giles, 2003; Sanchez et al., 2007). Genotype G has also been reported in other places, namely Nigeria (Olinger et al., 2006), Vietnam (Toan et al.,

* Corresponding author at: Depto Gastroenterologia, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 500 segundo andar, Prédio IMT 2, São Paulo, SP, Brazil. Tel.: +55 11 30618218; fax: +55 11 30645932. E-mail address: monica.viviana@usp.br (M.V. Alvarado Mora).

2006), Japan (Shibayama et al., 2005), and Thailand (Suwannakarn et al., 2005). A high prevalence of genotype G has been reported in patients with the Acquired Immune Deficiency Syndrome (AIDS) (Alvarado-Esquivel et al., 2006; Perez-Olmeda et al., 2003), particularly in men who have sex with men (MSM) (Bottecchia et al., 2008; Chu et al., 2003). Finally, many studies have reported its transmission together with genotypes A (Kato et al., 2002) and H (Sanchez et al., 2007) through co-infected patients. Nevertheless, HBV/G mono-infection has also been reported (Alvarado-Esquivel et al., 2006; Pas et al., 2008).

Genotype A has been found in seven genetically distinct subgenotypes (A1 to A7) (Hubschen et al., 2010): A1 in Africa and Asia (Bowyer et al., 1997); A2 in northern Europe, the United States, and the Arctic, including Alaska and Greenland (Chu et al., 2003; Fung and Lok, 2004; Langer et al., 1997; Norder et al., 2004); A3 in Cameroon and Gabon (Hannoun et al., 2005); A4 in Gambia (Hannoun et al., 2005; Olinger et al., 2006); A5 was in Nigeria and African descendants in Haiti (Andemach et al., 2009); A6 subgenotype in African-Belgian patients from Congo and Rwanda (Pourkarim et al., 2010); and the recently identified A7 in Rwanda and Cameroon (Hubschen et al., 2010).

HBV genotype F was primarily found in indigenous populations from South America and is divided into four subgenotypes (F1 to F4), which present a genetic divergence of around 4.3–6.1% (Devesa et al., 2008). The subgenotype F1 was found in Alaska, Argentina, and Bolivia. F2 is prevalent in Venezuela and Brazil, where it is associated with fulminant hepatitis in patients coinfecting with HDV. Subgenotype F3 was found in Venezuela, Colombia, and Panama, and is also associated with fulminant hepatitis in co-infections with the hepatitis delta virus in these regions. F4 was reported in Argentina and Bolivia (McMahon, 2009).

Central and South America display marked differences in terms of Hepatitis B genotypes distribution and Colombia is the geographic connection between these two regions. Subgenotype F3 was reported in blood donors from Bogotá and Bucaramanga in a previous study (Devesa et al., 2008).

The aim of this study was to characterize HBV genotypes distribution in samples from 143 blood donors from five different regions in Colombia collected between 2003 and 2007. Furthermore, Bayesian analyses were performed to estimate the time of the most recent common ancestor (TMRCA) of the four HBV/F subgenotypes.

2. Materials and methods

2.1. Genotyping analysis

2.1.1. Study population

To evaluate HBV genotypes distribution in blood donors from Colombia, 143 positive samples for the Hepatitis B virus surface antigen (HBsAg) were obtained from sera stored at -20°C in the *Banco Nacional de Sangre de la Cruz Roja Colombiana*, Colombia, between 2003 and 2007. These samples were collected at five different places: Bogotá ($n=116$), Bucaramanga ($n=1$), Huila ($n=8$), Nariño ($n=11$), and Boyacá ($n=7$) (Fig. 1).

2.1.2. HBV DNA amplification: S and S/POL regions

HBV DNA extraction was carried out from 100 μl of sera for each sample using the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). To avoid false-positive results, strict procedures for nucleic acid amplification diagnostic techniques were followed (Kwok and Higuchi, 1989). Samples were first amplified with the primers described by Sitnik et al. (2004) in order to obtain a 416 base pairs (bp) fragment partially covering the HBsAg coding region (S) and to confirm the

Fig. 1. Geographic locations of cities where blood samples were collected.

presence of HBV-DNA in the sample. To characterize HBV genotypes, a fragment of 1306 bp partially comprising HBsAg and the DNA polymerase coding regions (S/POL) was amplified by nested PCR using the primers PS3132F/2920R and PS3201F/P1285R.

2.1.3. HBV sequencing

The amplified DNA was purified using ChargeSwitch[®] PCR Clean-Up Kit. Sequencing was performed in an ABI Prism[®] 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA), based on the protocol described by Sanger et al. (1977), using dideoxynucleotide triphosphates (ddNTPs) containing fluorescent markers (Big Dye[®] Terminator v3.1 Cycle Sequencing Ready Reaction kit – Applied Biosystems, Foster City, CA, USA). The quality of each electropherogram was evaluated using the Phred-Phrap software (Ewing and Green, 1998) and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available at the web page *Electropherogram quality analysis Phred* (<http://asparagin.cenargen.embrapa.br/phph/>).

2.2. Evolutionary analysis

2.2.1. Phylogenetic analysis

Sequences were genotyped by phylogenetic reconstructions using reference sequences from each HBV genotype obtained from GenBank ($n=301$). These sequences comprised partial HBsAg and DNA polymerase coding regions (S/POL). They were aligned using ClustalX software (Thompson et al., 1997) and edited with the SE-AL software (available at <http://tree.bio.ed.ac.uk/software/seal/>). To perform the phylogenetic analysis, the missing nucleotides were coded as “missing characters” in nexus format. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3 (Drummond and Rambaut, 2007) and 10 million generations were sufficient to obtain the convergence of parameters. The maximum clade credibility (MCC) tree was obtained by summarizing the 10,000 substitution trees and then it was removed 10% of burn-in using Tree Annotator v.1.5.3 (Drummond and Rambaut, 2007).

Fig. 2. The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 301 S/POL sequences with 1306 bp of Hepatitis B virus strains. The posterior probabilities of the key nodes are depicted above the respective nodes. Samples obtained from Colombian blood donors ($n = 34$) were analyzed together

2.2.2. Genotype F Bayesian analyses

Genotype F Bayesian analyses were performed to estimate the time of the most recent common ancestor (TMRCA) of the four HBV/F subgenotypes. This information led us to make inferences about the oldest and also the most recently emerged HBV/F subgenotype. To estimate the TMRCA of the four F subgenotypes, we constructed two datasets containing only genotype F sequences. To obtain the best possible estimates, we built the first dataset with 77 HBV/F sequences comprising 1306 bp of the S/POL region (the 31 Colombian sequences and other 46 from the GenBank). The second and larger dataset was built with 283 HBV/F sequences (the 31 Colombian sequences and other 252 from the GenBank) with the partial S/POL sequence (681 bp). Furthermore, since there is not a consensus on the HBV substitution rate, we estimated the TMRCA using two previously estimated evolutionary rates: (i) 2.60×10^{-4} s/s/y obtained from Bayesian approaches using the non-recombinant region of HBV (Zhou and Holmes, 2007) and (ii) the lower estimated rate for HBV 1.5×10^{-5} s/s/y that has been used in other studies (Alvarado Mora et al., 2010; Hannoun et al., 2005; Okamoto et al., 1987; Orito et al., 1989; Simmonds and Midgley, 2005).

TMRCA in years was estimated using MCMC implemented in BEAST v1.5.3 (Drummond and Rambaut, 2007). Bayesian Skyline plot (BSL) was applied under strict and relaxed uncorrelated lognormal molecular clock using the best model of nucleotide substitution (GTR + G + I) obtained in MODELTEST (Posada and Crandall, 1998). The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. Ten million MCMC runs were sufficient to achieve the convergence of all parameters (effective sampling size >200). The convergence was inspected in Tracer v1.4 (Drummond and Rambaut, 2007), with uncertainties addressed as 95% HPD intervals.

3. Results

3.1. Phylogenetic analysis

Of the 143 HBsAg-positive samples, 68 were positive by nested PCR for the S fragment (416 bp) and 31 among them were also positive for the S/POL region. Among the remaining 37 samples positive for the S fragment, good quality sequences were obtained in only 21 of them, allowing classifying them as subgenotype F3. Nevertheless, these shorter sequences were removed for further analysis because a good phylogenetic signal was not obtained with them.

A phylogenetic tree constructed with the 1306 bp sequences partially comprising HBsAg and DNA polymerase coding regions (S/POL) ($n = 301$) is shown in Fig. 2. The phylogeny showed that subgenotype F3 (*adw4*) was most prevalent in this population: 39 (75%) out of 52 sample sequences clustered together with F3 reference sequences. Genotypes G (*adw2*) (4–7.7%), A2 (*adw2*) (8–15.3%), and F1b (*adw4*) (1–2%) were also found in this population. The Colombian sequences were deposited in the GenBank database under accession numbers HM467737–HM467788.

3.2. Genotype F Bayesian analyses

Because substitution rates ranged among 10^{-4} to 10^{-5} , a huge variation of TMRCA was found for HBV/F and for each F subgenotype when using the two different previously estimated substitution rates 2.6×10^{-4} and 1.5×10^{-5} per site per year

with other worldwide strains. Black full rhombuses identify the sequences generated in this study. Genotype B ($n = 31$), genotype C ($n = 28$), genotype D ($n = 33$), genotype E ($n = 11$), genotype H ($n = 13$), and genotype I ($n = 6$) branches were collapsed. Also, the subgenotypes A1 ($n = 28$), A3, A4, and A5 ($n = 11$), A6 ($n = 2$), F2a ($n = 5$), F2b ($n = 6$), and F4 ($n = 6$) branches were collapsed.

Table 1

Substitution rates and TMRCAs for human HBV/F sequences. TMRCAs were calculated using two different substitution rates (2.6×10^{-4} and 1.5×10^{-5} substitutions per site per year).

Dataset genotype F	HBV TMRCAs (years; 95% HPD)	
	2.6×10^{-4}	1.5×10^{-5}
77 sequences of HBV S/POL region with 1306 bp		
Genotype F	150.9 (73.1–250.2)	2418.4 (113.68–3993.6)
F1	56.8 (28.3–93.4)	958.6 (490.9–1518.5)
F2	73.8 (31.6–131.1)	1136.3 (487.9–1922)
F3	118.6 (60.6–194.4)	1903.9 (1025–3003.6)
F3 Colombia	60 (35.7–89.2)	1005.1 (631.3–1450.5)
F4	39.3 (16.8–63.6)	677.3 (312.3–1060.5)
283 sequences of HBV S/POL region with 681 bp		
Genotype F	96 (28.9–361.6)	1713.94 (57.33–3325.29)
F1	37.1 (14.8–66.8)	759.75 (244–1684.34)
F2	39.8 (12.7–93.5)	659.42 (222.7–1406.4)
F3	73.1 (22.2–151.2)	1299.9 (416.2–2667.4)
F3 Colombia	22.9 (11.5–37.6)	412.56 (215.6–677.56)
F4	19.1 (9.9–31.5)	354 (171.4–622.5)

(Table 1). The estimates in Table 1 correspond to the values obtained from the best-fit molecular clock chosen by Bayes Factor comparison. The relaxed uncorrelated log_{normal} was the best molecular clock for the four HBV datasets. Therefore, little variation among estimates under distinct clock models was detected (Table 1 and Tables S1A/S1B in supplementary material).

4. Discussion

4.1. HBV genotypes in Colombia

Phylogenetic analysis showed that subgenotype F3 is the most common among Colombian blood donors (75%), followed by A2 (15.3%), G (7.7%) and F1b (2%).

Genotype F has been described as the indigenous representative of the virus in the Americas, since it is almost exclusively found in several Amerindian groups (Di Lello et al., 2009). HBV/F3 has mostly been found in Panamá (Norder et al., 2003) and in countries in the northern region of South America, i.e., Venezuela (Nakano et al., 2001) and Colombia (Devesa et al., 2008; Norder et al., 1994). In Colombia, subgenotype F3 was previously reported in Bogotá and Bucaramanga and in the Yupa population from Venezuela (Devesa et al., 2008). In Venezuela, subgenotypes F1, F2, and F3 circulate among Amerindian tribes located in the East and West regions of this country (Devesa et al., 2008).

The four samples of genotype G were obtained from men aged between 37 and 56 years. Despite the association of this genotype with MSM, we were not able to infer the route of infection, as information on sexual behavior was not available for these individuals. The phylogenies also showed that these sequences were not closely related to each other, which suggests that different lineages of genotype G are circulating in Colombia. Also, because Colombian genotype G did not produce a single cluster, we may argue that these sequences result from distinct introductions in the country (Fig. 2). Additionally, using MEGA 4.0 (Kumar et al., 2008) it was determined the mean distance between and within the different countries (groups) that reported the presence of the genotype G (Tables S2A/S2B of supplementary material). These results support this hypothesis, i.e., the mean distance between and within groups of HBV sequences genotype G in the maximum clade credibility (MCC) tree were similar from genotype G sequences from Colombia and from sequences obtained in other countries throughout the world (France, USA, Japan, Thailand, Germany and Italy). Curiously, the only country from where HBV genotype G showed higher mean distance values between and within groups was Brazil, where one of the sequences was more distinct than the other ones. The authors

that described this sequence hypothesized that it may represent a recombinant virus between genotypes A and G (Bottecchia et al., 2008). Another explanation is that it may represent a sequence composed of the sum of the sequences of these two genotypes co-infecting a particular patient.

A2 was the second most frequent subgenotype in the Colombian population studied herein. This subgenotype is found in Europe, the United States, and the Arctic, including Alaska and Greenland (Chu et al., 2003; Fung and Lok, 2004; Langer et al., 1997; Norder et al., 2004). In addition, in England, where this subgenotype was the most frequent in acute Hepatitis B cases, it was found that drug use and homosexual transmission were equally implicated as risks factors to infection by subgenotype A2 (Sloan et al., 2009). In the Netherlands, a cluster related to MSM in patients with acute Hepatitis B was also identified (van Steenbergen et al., 2002). Subgenotype A2 was also reported in Spanish patients (Echevarria et al., 2005) but the sequences are too short to be considered in our analysis.

Subgenotype A2 sequences from Colombian patients shared the same mutations found in other subgenotype A2 sequences described so far: (1) in all Colombian sequences, we confirmed the presence of Valine at position 209 in the S region while the other A subgenotypes (A1, A3, A4, A5, A6, and A7) had Leucine at this position; (2) Ser²⁰⁷ was confirmed in all Colombian sequences while the other subgenotypes had Asn²⁰⁷; and (3) Ala¹⁹⁴ was also found in all Colombian sequences.

Because only few longer sequences of HBV/A2 are available in the GenBank, we cannot make inferences about its origin in the country. Since in Colombia there was a huge European migration through the time of colonization, it is possible that subgenotype A2 entry was during this time. Coalescent studies comparing sequences from Colombian patients with sequences from other countries should be done to elucidate the origin of this subgenotype in Colombian patients.

4.2. HBV/F and its subgenotypes

By using the Bayesian analyses it was possible to make inferences about the time the different HBV/F subgenotypes emerged. We used all genotype F sequences available in the GenBank and applied a coalescent-based approach. Because there is no consensus about HBV evolutionary rate, it was difficult to estimate the TMRCAs for this virus assuming a single rate. Thus, we decided to use two previously published substitution rates commonly used in other studies (Simmonds and Midgley, 2005; Zhou and Holmes, 2007). This method was also used previously to estimate the overall substitution rate of HBVs using different datasets (Alvarado Mora et al., 2010; Hannoun et al., 2005; Simmonds and Midgley, 2005; Zhou and Holmes, 2007).

Genotype F has been divided into the four subgenotypes F1, F2, F3, and F4 (Devesa et al., 2004, 2008; Huy et al., 2006; Kato et al., 2005; Norder et al., 2003; von Meltzer et al., 2008) but the origin of its geographic distribution is still unclear. However, determination of the TMRCAs of each subgenotype is possible, as different strains from many countries (predominantly of Central and South America, where they are more prevalent) have been sequenced in previous studies (Arauz-Ruiz et al., 2002; Huy et al., 2006; Nakano et al., 2001; Naumann et al., 1993; Norder et al., 1994, 2003; Pineiro y Leone et al., 2003).

Although the median TMRCAs varied among the distinct HBV/F subgenotypes, the overlapping among all upper and lower 95% HPD did not allow us to make robust inferences about the order of their origins. The median TMRCAs of the subgenotypes F1 and F2 was very similar for both datasets, which may suggest that they appeared almost simultaneously (Table 1). Although different clades of these subgenotypes (F1a, F1b, F1c, F1d and F2a, F2b) have been described,

there are still a few numbers of reported sequences that do not allow determination of the TMRCA for each one with confidence.

The subgenotype F1a is prevalent in Central America, having been reported in Costa Rica and El Salvador (Arauz-Ruiz et al., 2002), whereas subgenotype F1b is prevalent in South America. The latter has been described in Argentina (Alestig et al., 2001), Chile (Di Lello et al., 2009), and now in Colombia. The subgenotype F1b was also found to be more prevalent among Alaska Native people presenting chronic HBV infection (Livingston et al., 2007). The presence of a unique F1b sequence in the samples is an indication that the sequence was introduced most likely by an immigrant from a place where this subgenotype was prevalent. Although we found only one F1b sequence in our study, it is possible that other circulating strains exist in the population but they may have a low prevalence.

We also analyzed the amino acid substitutions in the HBV genome that characterize subgenotypes F1 to F4. In a previous study, it was shown that genotypes F and H appear to have emerged from a common ancestral virus (Arauz-Ruiz et al., 2002), and that these two genotypes originated from Central and South American Indians (Campos et al., 2005). Devesa et al. (2008) also described specific amino acid motifs for each subgenotype and also showed that no specific motif could distinguish North and Central American clades of genotype F.

We observed that subgenotype F3 presents only three amino acid substitutions in the genome: S gene (Glu³) and POL gene (Glu³⁹⁰ and Thr⁶⁰⁷) that were different from other HBV/F subgenotypes. These three substitutions are found in the genotype H. Differently, the subgenotype F4 presents three other amino acid substitutions (I¹¹⁰, N⁴⁶⁶, and A⁶⁰⁷) that were only found in this subgenotype and were not similar to any substitutions found in genotype H. This finding suggests that the genotype H is more related to the subgenotype F3 than the F4. However, because only a few sequences of genotype H (Hichman et al., 2009) are available, it was not possible to estimate its TMRCA.

5. Conclusions

In conclusion, we reported the characterization of the HBV genotypes circulating in Colombia and found that the subgenotype F3 is the most prevalent. Subgenotypes G (7.7%), A2 (15.3%), and F1b (2%) were also found. Genotype G sequence analysis suggests distinct introductions of this genotype in the country. Finally, the amino acid pattern found among the distinct subgenotypes F and genotype H suggests that the HBV/F3 is more related to the genotype H.

Acknowledgements

This work has been supported by CNPq, Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP 2007/53457-7 and 2008/50461-6 and Pontificia Universidad Javeriana, Bogotá, Colombia. We thank Banco Nacional de Sangre de la Cruz Roja Colombiana for their kind provision of blood donor samples for this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2010.10.003.

References

- Alestig, E., Hannoun, C., Horal, P., Lindh, M., 2001. Hepatitis B virus genotypes in Mongols and Australian Aborigines. *Arch. Virol.* 146, 2321–2329.
- Alvarado Mora, M.V., Romano, C.M., Gomes-Gouveia, M.S., Gutierrez, M.F., Carlinho, F.J., Pinho, J.R., 2010. Molecular epidemiology and genetic diversity of hepatitis

- B virus genotype E in an isolated Afro-Colombian community. *J. Gen. Virol.* 91, 501–508.
- Alvarado-Esquivel, C., Sablon, E., Conde-Gonzalez, C.J., Juarez-Figueroa, I., Ruiz-Maya, L., Aguilar-Benavides, S., 2006. Molecular analysis of hepatitis B virus isolates in Mexico: predominant circulation of hepatitis B virus genotype H. *World J. Gastroenterol.* 12, 6540–6545.
- Andermach, L.E., Nolte, C., Pape, J.W., Muller, C.P., 2009. Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg. Infect. Dis.* 15, 1222–1228.
- Arauz-Ruiz, P., Norder, H., Robertson, B.H., Magnus, L.O., 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J. Gen. Virol.* 83, 2059–2073.
- Bhat, R.A., Ulrich, P.P., Vyas, G.N., 1990. Molecular characterization of a new variant of hepatitis B virus in a persistently infected homosexual man. *Hepatology* 11, 271–276.
- Botteccia, M., Souto, F.J., O, K.M., Amendola, M., Brandao, C.E., Nid, C., Gomes, S.A., 2008. Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *BMC Microbiol.* 8, 11.
- Bowyer, S.M., van Staden, L., Kew, M.C., Sim, J.C., 1997. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. *J. Gen. Virol.* 78 (Pt 7), 1719–1729.
- Campos, R.H., Mbayed, V.A., Pinheiro, Y.L.F.G., 2005. Molecular epidemiology of hepatitis B virus in Latin America. *J. Clin. Virol.* 34 (Suppl. 2), S8–S13.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Chu, C.J., Keeffe, E.B., Han, S.H., Perrillo, R.P., Min, A.D., Soldevila-Pico, C., Carey, W., Brown Jr., R.S., Luketic, V.A., Terrault, N., Lok, A.S., 2003. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology* 125, 444–451.
- Chudy, M., Schmidt, M., Czudal, V., Scheiblaue, H., Nick, S., Mosebach, M., Hourfar, M.K., Seifried, E., Roth, W.K., Grundel, E., Nubling, C.M., 2006. Hepatitis B virus genotype G monoinfection and its transmission by blood components. *Hepatology* 44, 99–107.
- De Maddalena, C., Giambelli, C., Tanzi, E., Colzani, D., Schiavini, M., Milazzo, L., Benini, F., Ebranati, E., Cargnel, A., Bruno, R., Galli, M., Zehender, G., 2007. High level of genetic heterogeneity in S and P genes of genotype D hepatitis B virus. *Virology* 365, 113–124.
- Devesa, M., Loureiro, C.I., Rivas, Y., Monsalve, F., Cardona, N., Duarte, M.C., Poblete, F., Gutierrez, M.F., Botto, C., Pujol, F.H., 2008. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J. Med. Virol.* 80, 20–26.
- Devesa, M., Rodriguez, C., Leon, G., Liprandi, F., Pujol, F.H., 2004. Clade analysis and surface antigen polymorphism of hepatitis B virus American genotypes. *J. Med. Virol.* 72, 377–384.
- Di Lello, F.A., Pinheiro, Y.L.F.G., Munoz, G., Campos, R.H., 2009. Diversity of hepatitis B and C viruses in Chile. *J. Med. Virol.* 81, 1887–1894.
- Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7, 214.
- Echevarria, J.M., Avellan, A., Magnus, L.O., 2005. Molecular epidemiology of hepatitis B virus in Spain: identification of viral genotypes and prediction of antigenic subtypes by limited sequencing. *J. Med. Virol.* 76, 176–184.
- Ewing, R., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Error probabilities. *Genome Res.* 8, 186–194.
- Hichman, D., Galdame, O., Livellara, B., Vlaut, M., Gadan, A., Campos, R., 2009. Full-length genome characterization of hepatitis B virus genotype H strain isolated from serum samples collected from two chronically infected patients in Argentina. *J. Clin. Microbiol.* 47, 4191–4193.
- Pung, S.K., Lok, A.S., 2004. Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? *Hepatology* 40, 790–792.
- Hannoun, C., Soderstrom, A., Nordmans, G., Lindh, M., 2005. Phylogeny of African complete genomes reveals a West African genotype A subtype of hepatitis B virus and relatedness between Somali and Asian A1 sequences. *J. Gen. Virol.* 86, 2163–2167.
- Hubschen, J.M., Mbah, P.O., Forbi, J.C., Otegbayo, J.A., Olinger, C.M., Charpentier, E., Muller, C.P., 2010. Detection of a new subgenotype of Hepatitis B virus genotype A in Cameroon but not in neighbouring Nigeria. *Clin. Microbiol. Infect.* <http://onlinelibrary.wiley.com/doi/10.1111/j.1469-0691.2010.03205.x/abstract>.
- Huy, T.T., Ishikawa, K., Ampo, W., Izumi, T., Nakajima, A., Ansah, J., Tetteh, J.O., Nii-Tsubi, N., Aido, S., Ofori-Adjei, D., Sato, T., Ushijima, H., Abe, K., 2006. Characteristics of hepatitis B virus in Ghana: full length genome sequences indicate the endemicity of genotype E in West Africa. *J. Med. Virol.* 78, 178–184.
- Jardi, R., Rodriguez-Frias, F., Schaper, M., Gigli, E., Tabernero, D., Homs, M., Esteban, R., Buti, M., 2008. Analysis of hepatitis B genotype changes in chronic hepatitis B infection: influence of antiviral therapy. *J. Hepatol.* 49, 695–701.
- Kato, H., Fujiwara, K., Gish, R.G., Sakugawa, H., Yoshiwara, H., Sugauchi, F., Orito, E., Ueda, R., Tanaka, Y., Kato, T., Miyakawa, Y., Mizokami, M., 2005. Classifying genotype F of hepatitis B virus into F1 and F2 subtypes. *World J. Gastroenterol.* 11, 6295–6304.
- Kato, H., Orito, E., Gish, R.G., Sugauchi, F., Suzuki, S., Ueda, R., Miyakawa, Y., Mizokami, M., 2002. Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes A through F. *J. Virol.* 76, 6131–6137.
- Kramvis, A., Kew, M.C., 2007. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol. Res.* 37, 59–519.

- Kumar, S., Nei, M., Dudley, J., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.* 9, 299–306.
- Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. *Nature* 339, 237–238.
- Lacombe, K., Massari, V., Girard, P.M., Serfaty, L., Gozlan, J., Pialoux, G., Mialhes, P., Molina, J.M., Lacombe, C., Wendum, D., Carra, F., Zoulim, F., 2006. Major role of hepatitis B genotypes in liver fibrosis during coinfection with HIV. *AIDS* 20, 419–427.
- Langer, B.C., Prosser, G.G., von Brunn, A., 1997. Epidemiological study of viral hepatitis types A, B, C, D and E among Inuits in West Greenland. *J. Viral Hepat.* 4, 339–349.
- Lindh, M., 2005. HBV genotype G—an odd genotype of unknown origin. *J. Clin. Virol.* 34, 315–316.
- Livingston, S.E., Simonetti, J.P., McMahon, B.J., Bulkow, L.R., Hurlburt, K.J., Homan, C.E., Snowball, M.M., Cagle, H.H., Williams, J.L., Chulanov, V.P., 2007. Hepatitis B virus genotypes in Alaska Native people with hepatocellular carcinoma: preponderance of genotype F. *J. Infect. Dis.* 195, 5–11.
- Magnius, L.O., Norder, H., 1995. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. *Intervirology* 38, 24–34.
- Mahtab, M.A., Rahman, S., Khan, M., Karim, F., 2008. Hepatitis B virus genotypes: an overview. *Hepatobiliary Pancreat. Dis. Int.* 7, 457–464.
- McMahon, B.J., 2009. The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatol. Int.* 3, 334–342.
- Nakano, T., Lu, L., Hu, X., Mizokami, M., Orito, E., Shapiro, C., Hadler, S., Robertson, B., 2001. Characterization of hepatitis B virus genotypes among Yuca Indians in Venezuela. *J. Gen. Virol.* 82, 359–365.
- Naumann, H., Schaefer, S., Yoshida, C.F., Caspar, A.M., Repp, R., Gerlich, W.H., 1993. Identification of a new hepatitis B virus HBV genotype from Brazil that expresses HBV surface antigen subtype adw4. *J. Gen. Virol.* 74 (Pt 8), 1627–1632.
- Norder, H., Arauz-Ruiz, P., Blitt, L., Pujol, F.H., Echevarria, J.M., Magnius, L.O., 2003. The T1858 variant: predisposing to the precore stop mutation correlates with one of two major genotype F hepatitis B virus clades. *J. Gen. Virol.* 84, 2083–2087.
- Norder, H., Courouge, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S., Magnius, L.O., 2004. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 47, 289–309.
- Norder, H., Courouge, A.M., Magnius, L.O., 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 198, 489–503.
- Okamoto, H., Imai, M., Kametani, M., Nakamura, T., Mayumi, M., 1987. Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through mother-to-fetus transmission. *Jpn. J. Exp. Med.* 57, 231–236.
- Ollinger, C.M., Venard, V., Njaye, M., Oyebolu, A.D., Maiga, I., Kemp, A.J., Omlahu, S.A., le Fau, A., Muller, C.P., 2006. Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J. Gen. Virol.* 87, 1163–1173.
- Orito, E., Mizokami, M., Ina, Y., Moriyama, E.N., Kameshima, N., Yamamoto, M., Gojobori, T., 1989. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proc. Natl. Acad. Sci. U.S.A.* 86, 7059–7062.
- Osley, C., Giles, E., 2003. Evaluation of the INNO-UPA HBV genotyping assay for determination of hepatitis B virus genotype. *J. Clin. Microbiol.* 41, 5473–5477.
- Pas, S.D., Tran, N., de Man, R.A., Burghoorn-Maas, C., Vermet, G., Niesters, H.G., 2008. Comparison of reverse hybridization, microarray, and sequence analysis for genotyping hepatitis B virus. *J. Clin. Microbiol.* 46, 1268–1273.
- Perez-Olmeda, M., Nunez, M., Garcia-Samaniego, J., Rios, P., Gonzalez-Lahoz, J., Soriano, V., 2003. Distribution of hepatitis B virus genotypes in HIV-infected patients with chronic hepatitis B: therapeutic implications. *AIDS Res. Hum. Retroviruses* 19, 657–659.
- Pinedo y Leone, F.G., Mbayed, V.A., Campos, R.H., 2003. Evolutionary history of Hepatitis B virus genotype F: an in-depth analysis of Argentine isolates. *Virus Genes* 27, 103–110.
- Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14, 817–818.
- Pourkarim, M.R., Lemey, P., Amini-Bavil-Olyaei, S., Maes, P., Van Ranst, M., 2010. Novel hepatitis B virus subgenotype A6 in African-Belgian patients. *J. Clin. Virol.* 47, 93–96.
- Sanchez, L.V., Tanaka, Y., Maldonado, M., Mizokami, M., Panduro, A., 2007. Difference of hepatitis B virus genotype distribution in two groups of Mexican patients with different risk factors. High prevalence of genotype H and G. *Intervirology* 50, 9–15.
- Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463–5467.
- Shibayama, T., Masuda, G., Ajiwaka, A., Hiruma, K., Tsuda, F., Nishizawa, T., Takahashi, M., Okamoto, H., 2005. Characterization of seven genotypes A to E, G and H of hepatitis B virus recovered from Japanese patients infected with human immunodeficiency virus type 1. *J. Med. Virol.* 76, 24–32.
- Simmonds, P., Midgley, S., 2005. Recombination in the genesis and evolution of hepatitis B virus genotypes. *J. Virol.* 79, 15467–15476.
- Singh, J., Dickens, C., Pahal, V., Kumar, R., Chaudhary, R., Kramvis, A., Kew, M.C., 2009. First report of genotype e of hepatitis B virus in an Indian population. *Intervirology* 52, 235–238.
- Sitnik, R., Pinho, J.R., Bertolini, D.A., Bernardini, A.P., Da Silva, L.C., Carrilho, F.J., 2004. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J. Clin. Microbiol.* 42, 2455–2460.
- Sloan, R.D., Strang, A.L., Ramsay, M.E., Teo, C.G., 2009. Genotyping of acute HBV isolates from England, 1997–2001. *J. Clin. Virol.* 44, 157–160.
- Suwanakarn, K., Tangkijvanich, P., Theamboonlers, A., Abe, K., Poovorawan, Y., 2005. A novel recombinant of Hepatitis B virus genotypes G and C isolated from a Thai patient with hepatocellular carcinoma. *J. Gen. Virol.* 86, 3027–3030.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876–4882.
- Toan, N.L., Song, L., Kremsner, P.G., Duy, D.N., Binh, V.Q., Koeberlein, B., Kaiser, S., Kandolf, R., Torresi, J., Bock, C.T., 2006. Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. *Hepatology* 43, 1375–1384.
- van Steenberghe, J.E., Nieters, H.G., Op de Coul, E.L., van Doornum, G.J., Osterhaus, A.D., Teunissen-Krijgers, A., Coutinho, R.A., van den Hoek, J.A., 2002. Molecular epidemiology of hepatitis B virus in Amsterdam 1992–1997. *J. Med. Virol.* 66, 159–165.
- Viehl, S., Manegold, C., Drosten, C., Nipprach, T., Gunther, S., 2002. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. *Virus Genes* 24, 153–156.
- von Melzer, M., Vasquez, S., Sun, J., Wendt, U.C., May, A., Gerlich, W.H., Radtke, M., Schaefer, S., 2008. A new clade of hepatitis B virus subgenotype F1 from Peru with unusual properties. *Virus Genes* 37, 225–230.
- Yu, H., Yuan, Q., Ge, S.X., Wang, H.Y., Zhang, Y.L., Chen, Q.R., Zhang, J., Chen, P.J., Xia, N.S., 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "T". *PLoS One* 5, e9297.
- Zhou, Y., Holmes, E.C., 2007. Bayesian estimates of the evolutionary rate and age of hepatitis B virus. *J. Mol. Evol.* 65, 197–205.

- 38- Godoy BA, Alvarado-Mora MV, Gomes-Gouvêa MS, Pinho JR, Fagundes NJ. Origin of HBV and its arrival in the Americas - the importance of natural selection on time estimates. Antivir Ther. 2013;18(3 Pt B):505-512.**

Original article

Origin of HBV and its arrival in the Americas – the importance of natural selection on time estimates

Bibiane A Godoy^{1,2}, Mônica V Alvarado-Mora³, Michele S Gomes-Gouvêa³, João R Rebello Pinho³, Nelson JR Fagundes^{1,2*}

¹Genetics Department, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

²National Institute of Medical Population Genetics, Porto Alegre, Brazil

³Laboratory of Tropical Gastroenterology and Hepatology 'João Alves de Queiroz and Castorina Bittencourt Alves', Institute of Tropical Medicine, Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, Brazil

*Corresponding author e-mail: nelson.fagundes@ufrgs.br

Background: The strong geographic structure shown by the global pattern of HBV lineages suggests an ancient origin for this virus; however, estimates based on the molecular clock suggest a very recent origin for the Native American genotypes F and H. In this study, we contribute to this debate by estimating the divergence times of genotypes F and H and by discussing how evolutionary rates estimated from recent samples may underestimate the divergence time of more ancient nodes in HBV phylogenies.

Methods: A total of 108 complete HBV genotype F and H genomes were compared to 44 reference genomes from other genotypes. Time estimates were based on a Bayesian method with evolutionary rates taken from the literature. To assess the pattern of substitutions in recent versus old branches we mapped the phylogenetic distribution of all mutations occurring in genotypes F and H using a maximum likelihood approach and compared the

number of synonymous and non-synonymous mutations in young and old branches of HBV genotype F and H phylogeny using a χ^2 test.

Results: Estimated divergence times between genotypes F and H depend heavily on the evolutionary rate. While fast rates suggest a recent separation of these genotypes (approximately 800 years ago), slow rates suggest an earlier divergence (up to approximately 13,000 years ago). There is a clear excess of non-synonymous substitutions in the most recent branches of HBV phylogeny ($P=4.87 \times 10^{-15}$), most likely suggesting the action of purifying selection.

Conclusions: These results suggest that rates estimated based on recent samples will overestimate the evolutionary rate and underestimate the coalescence times for ancient nodes in HBV phylogeny.

Introduction

HBV is an important human pathogen affecting millions of individuals worldwide [1]. HBV is a DNA virus from the *Hepadnaviridae*, and has a strong phylogeographic structure, with 10 known evolutionary lineages (from A to J), typically referred to as 'genotypes', segregating in human populations [2]. In addition to such strong phylogenetic structure, HBV also presents a remarkable geographic structure, with specific genotypes being typical from specific geographic regions [3]. For example, genotypes F and H are considered as typically 'American' and are usually associated with Native American populations or Native American ethnic backgrounds [4–6]. Although recent migration events may blur a clear-cut geographical association between HBV lineages and geographic origin, several studies associate the frequency of certain

HBV genotypes to more ancient migration events. For example, the finding of the (African) subgenotype A1 in Brazilian rural population isolates descending from African slaves brought to the country during colonial times is interpreted as being the result of the African slave trade during the 16th to 19th centuries [7,8]. Another example of a possible role of African slave trade for determining HBV distribution in the Americas is the finding of HBV genotype E in a poor rural Colombian community [9]. Likewise, the various prevalences of HBV genotypes in Argentina were interpreted as reflecting contributions of people from different ethnic backgrounds [10].

Despite such compelling historical accounts, which would suggest that HBV is a relatively old virus segregating within human populations for a long time, molecular

BA Godoy et al.

clock estimates for the origin of HBV and its genotypes provide a very different scenario for HBV evolution. Dating the most recent common ancestor for Colombian and African lineages of HBV genotype E resulted in a surprisingly recent date of 35 years ago [9]. Other studies estimated a very recent origin for the 'Native American' HBV genotype F, of approximately 284 years ago [11], and an even more extreme date of 229 years ago has been found for the origin of HBV itself [12]. In common, all these studies use extremely sophisticated methods and software to estimate HBV evolutionary times based on dated samples collected recently [11,12] or data from intra-familial transmissions [13]. Thus, while the geographic structure of HBV genotypes would be consistent with HBV being a relatively old virus with a moderate evolutionary rate, molecular clock studies suggest a very recent origin for HBV, with very fast evolutionary rates associated with it.

Recently, it has been suggested that time estimates based on molecular clocks may be highly dependent on the choice of the calibration of the evolutionary rate [14–18]. Evolutionary rates estimated from recent calibration points usually produce faster rates, while calibration with fossil data usually results in slower rates. Problems arise when evolutionary rates calibrated based on old nodes in the phylogeny are used to estimate times in recent nodes in the phylogeny and *vice versa*. One possible mechanism accounting for this phenomenon is purifying selection acting on deleterious substitutions that are still segregating in recent nodes of the phylogeny, but that would have been eliminated in more ancient nodes [15]. There is evidence that young human mitochondrial DNA haplogroups have an excess of non-synonymous substitutions as compared to old haplogroups [19], consistent with the purifying selection hypothesis if most non-synonymous mutations are deleterious or slightly deleterious. Therefore, if HBV evolutionary rates calibrated from recent samples are inflated, they would underestimate the deeper nodes in HBV phylogeny and may explain, at least partially, the discrepancy between 'molecular clock' and 'anthropological' accounts of HBV evolution.

In this study we compared the effect of different priors on the evolutionary rate to discuss how they impact divergence time estimates about the origin of genotypes F and H. We then compared the timing and distribution of HBV lineages in the Americas to discuss whether or not HBV is of pre-Columbian origin and if we could speculate its origins among the first American settlers who arrived in the Americas >15,000 years ago. Finally, we tested the hypothesis that purifying selection plays an important role in HBV evolution by comparing the number of synonymous and non-synonymous substitutions in lineages belonging to genotypes F and H and

discuss its impact on the estimates of an evolutionary rate for HBV.

Methods

We retrieved from GenBank sequences of 126 complete HBV genomes belonging to genotypes F and H. Because this dataset was also used in the analyses of natural selection, we excluded all sequences showing deletions, as they represent frame-shift mutations or long deletions of likely deleterious effects. Identical sequences were also excluded. The final dataset for HBV genotypes F and H consisted of 108 sequences (Additional file 1). For the phylogenetic analysis we also used a dataset of 44 complete genomes from HBV isolates belonging to other genotypes (A–E, G, I; Additional file 1).

To estimate the time of key events in HBV evolution we used the phylogenetic method implemented in BEAST version 1.6.2 [20]. More specifically, we estimated the time to the most recent common ancestor (TMRCA) of all F subgenotypes, the TMRCA of genotypes F and H, the TMRCA of the clades F and H and the root of the tree, which represents the divergence of genotypes F and H from the other Old World genotypes. As the full dataset included only different sequences, we used a Yule model as the tree prior. The Yule model is a birth-only process and it is an efficient way of describing a tree which is not based on population sampling, in which case a coalescent-based prior would be preferable. To evaluate the effect of the choice of the evolutionary rate and type of molecular clock (whether strict or relaxed [21]) different analyses were performed: a strict molecular clock using the fast evolutionary rate estimated by Torres *et al.* [11] using dated samples (1.67×10^{-4}); a strict clock using a slow rate of 1.0×10^{-5} , as discussed by Torres *et al.* [11]; a strict clock using a range of 'likely' rates suggested by Torres *et al.* [11], between 2.0×10^{-5} and 6.0×10^{-6} ; a log-normal relaxed clock using a fixed mean rate of 1.0×10^{-5} ; and a lognormal relaxed clock using a range of values between 2.0×10^{-5} and 6.0×10^{-6} as above. The prior for range of most 'likely' values was modelled using a truncated normal prior with a mean of 1.0×10^{-5} and a standard deviation of 5.0×10^{-6} , with minimum and maximum bounds set at 2.0×10^{-5} and 6.0×10^{-6} , respectively. For all analyses we ran 25,000,000 steps of the Markov Chain Monte Carlo sampling every 1,000 steps, discarding the first 5,000 samples as burn-in.

To evaluate the effect of natural selection on different parts of HBV genotype F and H phylogeny and the possible implications on inferred evolutionary rates, we estimated a maximum likelihood (ML) tree in PAUP 4.0 [22] using the TVM+I+G model with values obtained from Modeltest 3.01 [23]. We then ran PAUP 4.0 to map all substitutions occurring in the HBV genotype F and H dataset across all branches of the estimated ML tree. No

bootstrap was performed for this analysis as we wanted the most likely tree estimate (under the likelihood criterion) to map mutations directly in each branch under the same criterion. Nonetheless, the general robustness of HBV phylogeny estimated from this dataset can be evaluated using the clade posterior probabilities provided by the Bayesian tree estimate. Mutations were then assigned as non-synonymous or synonymous depending on their effect on the affected codon using DnaSP version 5.0 [24]. Complex codons (that is, codons affected by multiple substitutions that could be either synonymous or non-synonymous depending on their ancestral state) were checked manually. Because the HBV genome presents overlapping genes, we initially considered a mutation affecting two genes simultaneously as doubly non-synonymous, doubly synonymous, or as simultaneously synonymous and non-synonymous, depending on the exact effect it produces on the HBV genome. However, to be certain that this approach did not introduce any bias to our results we also performed alternative analyses using only genome positions occurring on non-overlapping regions or considering each gene separately.

'Young' branches were defined as those occurring within genotypes (or subgenotypes in the case of F1 and F2), whereas 'old' branches were defined as those connecting genotypes among each other. The branches connecting subgenotypes F1a and F1b to their common ancestors, and those connecting F2a and F2b to their common ancestors were considered 'intermediate' and were therefore excluded from the initial analyses (Figure 1). To test if our results were robust to this choice, we also re-analysed our data considering these branches as either 'young' or 'old'. This categorization of branches according to their age, despite being simple, is a robust way of dealing with phylogenetic uncertainty, because all genotypes and subgenotypes are monophyletic and one can therefore be certain that branches occurring within these categories are younger than those defining viral genotypes. The number of non-synonymous and synonymous mutations between 'young' and 'old' branches were then compared using a χ^2 test in the programme PEPI 4.0 [25]. Under the null hypothesis that the action of natural selection is similar between these groups of branches this test should result in statistically non-significant values.

Results

Time estimates for genotypes H and F, and subgenotypes of F

Not unexpectedly, the estimated date for the origin and divergence of HBV genotypes and subgenotypes is highly dependent on the evolutionary rate assumed. The root of the HBV tree can be dated as recently as approximately 800 years ago or as old as approximately 13,300

years ago, depending on the evolutionary rate assumed (Table 1), and represents the divergence between the clade formed by the Native American genotypes (F and H) and a clade formed by the remaining HBV genotypes. Importantly, depending on specific assumptions on the molecular clock model, the 95% CI included dates older than 12,000 years ago for all analyses except those based on the fast rate estimated by Torres *et al.* [11], and might be as old as approximately 19,000 years ago (Table 1). Concerning tree topology (Figure 2), the first divergence within the clade containing genotypes F and H separates genotype H from its sister genotype F. On its turn, the first divergence within genotype F separates subgenotype F1 from a sister clade containing all other F subgenotypes. These early divergences within genotypes F and H seem to be younger than 10,000 years ago. However, as previously mentioned, these estimates are highly sensitive to the choice of the evolutionary rate.

Effect of purifying selection on the estimated rates using recent samples

To test if the fast rates estimated from recent dated samples may be affected by the action of purifying selection on young branches, which would lead to an overestimation of the evolutionary rate (and a underestimation of divergence times), we compared the number of non-synonymous and synonymous substitutions in young versus old branches of HBV genotype F and H phylogeny (Figure 1). As shown in Table 2, there is a strong excess of non-synonymous substitutions in the young branches of HBV genotype F and H phylogeny ($P=4.87 \times 10^{-15}$ for the full genome comparison). This was also observed when the analysis considered only non-overlapping positions ($P=5.62 \times 10^{-15}$), or when individual genes were analysed separately, with the exception of gene X, which, despite an excess of non-synonymous to synonymous substitutions, did not show statistically significant values (Table 2). Importantly, considering 'intermediate' branches as either 'young' or 'old' did not change these results (Additional file 1).

Discussion

Estimates of HBV evolutionary rate: bias and solutions
The results presented here clearly indicate that there is an excess of non-synonymous substitutions in the young branches of HBV genotype F and H phylogeny. This provides a simple explanation for the discrepancy between 'historical' and 'molecular clock' estimates of HBV evolution, further suggesting that the latter is biased towards recent dates. These results contribute to the growing number of examples for which purifying selection acting on recent deleterious mutations will cause the evolutionary rate to be different

BA Godoy et al.

Figure 1. Schematic representation of the maximum likelihood HBV genotypes F and H tree based on complete genomes showing how mutations were counted along the phylogeny

Branches occurring within a given subgenotype were considered 'young' and are shown inside a light grey box. 'Intermediate' branches connect different lineages of the same subgenotype, as it is the case of F1a versus F1b, and F2a versus F2b. These are represented inside a white box. 'Old' branches are represented inside the dark grey box and connect different F subgenotypes and genotypes F and H. Non-synonymous and synonymous substitutions are represented scattered along the phylogeny as a white and black circles, respectively.

Table 1. Estimates of the time to the most recent common ancestor of specific nodes in HBV phylogeny according to the assumed evolutionary rate

Genotype	TMRCA in years (95% CI)				
	Strict clock, fixed fast rate of 1.67×10^{-4}	Strict clock, fixed slow rate of 1.0×10^{-5}	Strict clock, range of slow rates	Relaxed clock, fixed slow rate	Relaxed clock, range of slow rates
F1	85 (72, 100)	1,417 (1,185, 1,655)	1,140 (656, 2,053)	1,597 (1,107, 2,289)	1,300 (630, 2,451)
F2	113 (96, 132)	1,896 (1,602, 2,188)	1,522 (904, 2,770)	1,853 (1,255, 2,591)	1,488 (739, 2,765)
F3	116 (96, 137)	1,936 (1,610, 2,308)	1,568 (885, 2,816)	1,552 (999, 2,389)	1,164 (577, 2,173)
F4	86 (73, 100)	1,438 (1,226, 1,671)	1,158 (679, 2,095)	1,597 (952, 1,830)	1,072 (537, 1,994)
F	255 (229, 282)	4,254 (3,825, 4,712)	3,410 (2,033, 6,109)	4,134 (3,014, 5,457)	3,398 (630, 2,451)
H	103 (86, 120)	1,711 (1,443, 1,996)	1,374 (781, 2,474)	2,126 (1,418, 3,087)	1,773 (867, 3,351)
F and H	389 (350, 430)	6,498 (5,854, 7,170)	5,214 (3,093, 9,284)	5,769 (4,222, 7,917)	4,894 (2,587, 8,927)
Root	796 (732, 863)	13,295 (12,268, 14,413)	10,689 (6,438, 18,895)	8,657 (6,907, 10,510)	8,322 (4,631, 14,919)

Data are the time to the most recent common ancestor (TMRCA). 95% CI is the 95% credible interval for these data.

in young or old portions of the phylogeny [16,17]. Importantly, such excess would lead to an overestimation of the long-term evolutionary rate when calibrating HBV evolution with recent dated samples or data from intra-familial transmissions. For example, Zhou and Holmes [12] estimate evolutionary rates in the order of 10^{-4} , with corresponding TMRCA younger than 300 years ago. Our results suggest that this is an overestimation of the evolutionary rate, obtained

using recent calibrations, with corresponding underestimated TMRCA. However, even when only mutations occurring in 3rd codon positions and non-overlapping regions were considered, these authors found a global TMRCA for human HBV of approximately 477 years ago (with the 95% highest posterior density [HPD] estimate from 57 to 1,509 years ago) and an evolutionary rate of 9.63×10^{-4} , with a wide 95% HPD between 4.1×10^{-5} and 1.92×10^{-3} . If most mutations

Figure 2. Schematic representation of the Bayesian HBV phylogeny based on complete genomes

The scale bar represents a time span of 2,500 years. The arrow indicates the median estimate for the tree root assuming a relaxed clock with a range of 'slow rates', as explained in the text. The grey bars indicate the 95% CI for all time estimates. *All groups represented in this phylogeny had posterior probability values of at least 0.99, except the one indicated, which had a posterior probability of 0.74.

Table 2. Distribution of non-synonymous or synonymous nucleotide substitutions occurring in 'young' or 'old' branches according to the different partitions analysed

Partition	Complete HBV genome		Non-overlapping positions		Gene Pol		Gene PreS/S		Gene PreC/C		Gene X	
	NS	S	NS	S	NS	S	NS	S	NS	S	NS	S
Young branches	992	866	375	437	447	437	189	187	149	148	160	62
Old branches	170	334	44	198	104	167	26	69	2	60	24	15
P-value ^a	4.87×10^{-15}		5.62×10^{-15}		4.40×10^{-4}		6.25×10^{-5}		9.74×10^{-12}		0.183	

Data are number of non-synonymous (NS) or synonymous (S) substitutions in young and old branches. ^aP-value by χ^2 test.

in 3rd codon positions are synonymous, would this estimate be accurate? In highly linked systems, even synonymous (neutral) substitutions may be eliminated from the population if they are linked to deleterious non-synonymous mutations. Thus, if purifying natural selection is an important factor in HBV evolution, as our results suggest, in the long-term both deleterious non-synonymous and neutral synonymous substitutions linked to them will be eliminated from the population. The conclusion, therefore, is that using only synonymous substitutions is unlikely to alleviate the problems posed by purifying selection on evolutionary rate estimates. Moreover, a slower evolutionary rate in HBV is also consistent with the recent report of an HBV-C2 subgenotype found in a mummy from Korea dated to 330 years ago [26]. The excess of non-synonymous substitutions in young branches could also reflect positive selection in response to vaccine or immune escape. However, we think this is an unlikely explanation since implementation of vaccination programmes is very recent, and programmes still do not cover most parts of Latin American communities [27].

Another important implication of our findings is that this effect may be important for time estimates obtained from other viruses, as in these cases calibration points are usually given by intra-familial transmissions or recent dated samples, which are recent events that may overestimate the long-term evolutionary rate. We suggest that better estimates of HBV evolutionary rate will be obtained by including known historical events, such as African slave trade to Latin America, as calibration points, or by obtaining HBV isolates from old samples, as the case of the Korean mummy [26]. However, it should be noted that the difference between the short- and long-term evolutionary rates due to purifying selection is not the only problem for accurate estimates of HBV evolutionary time scale. It has been shown that strains expressing hepatitis B e antigen (HBeAg) have evolutionary rate orders of magnitude faster than those estimated for HBeAg-negative strains [28,29]. In the future it will be important to understand the interplay between these two factors in determining the bias on HBV evolutionary rate estimates.

Implications for the origin of HBV in the Americas
Results obtained with the fast HBV evolutionary rate would be consistent with a post-Columbian origin of HBV in the Americas. However, we think 'fast rate' estimates are flawed, as they are incompatible with convincing historical evidence about the distribution of HBV lineages and also do not take into account the effects of purifying selection, as discussed thus far. Thus, if HBV in the Americas is pre-Columbian, could we know when it arrived there? For genotypes other

than F and H the answer seems to be simple: they were carried here by African/European or Asian immigrants; but what about the 'indigenous' genotypes F and H?

Recent molecular studies about the peopling of the Americas suggest that the first New World settlers came from Asia through the Bering Strait around 18,000 years ago, following a fast expansion route through the Pacific [30,31]. This is corroborated by the fact that the oldest known archaeological site in the Americas (Monte Verde, dated at approximately 14,500 years ago) lies in Southern Chile [32]. After this initial entry, recurrent gene flow between Native Americans and Asians would have started around 9,750 years ago [33]. We can consider that these represent two alternative opportunities for HBV to spread from Asia to the Americas. The distribution of human HBV genotypes led Arauz-Ruiz *et al.* [34] to suggest that HBV New World genotypes F and H arrived from Asia with the initial settlers. As shown in Table 2, mutation rates in the order of 1.0×10^{-5} are easily compatible with divergence between genotypes F and H from the remaining genotypes older than 10,000 years ago, although they are hardly older than 18,000 years ago, for example. However, the uncertainty about the 'real' evolutionary rate of HBV makes it impossible to distinguish between different hypotheses or suggest that an alternative explanation should be invoked. Similarly, it is not possible to determine if these genotypes differentiated in America or if they arose elsewhere and were carried to the Americas.

The tree topology for HBV genotype F and H (Figure 2) is in agreement to those previously reported for HBV and HBV genotype F phylogeny [11,35]. Nonetheless, the fact that these two genotypes are sister clades in the phylogeny suggests that both differentiated in the Americas and that a 'pre-FH genotype' existed in the continent some time ago. Interestingly, the two most ancient divergences among the New World genotypes separate genotypes H and F1, respectively, from the remaining lineages. Genotype H is mostly distributed in Mexico and Central America [5,34], whereas subgenotype F1 is more common in Central America (F1a) [6,36] and in the Pacific coast of South America [26]. Therefore, it would be tempting to assume such divergences occurred as the first Native American human populations were moving southwards through the South American Pacific coast [30–32], but this would be highly speculative at this point, as these early divergences within genotypes F and H seem to be therefore more recent than the estimated times for the arrival of the first Native American populations in this region [32]. Better estimates of HBV evolutionary rate, taking into account purifying selection, will be crucial for an improved knowledge about the arrival of genotypes F and H in the Americas.

Acknowledgements

This study was funded by the National Council of Scientific and Technological Development (CNPq; grant number 573993/2008-4 to the National Institute of Medical Population Genetics), and supported by Fundação Faculdade de Medicina and Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. We would like to thank two anonymous reviewers for their helpful comments on an earlier version of the manuscript.

Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: Supplementary material including the GenBank accession numbers for the complete HBV genomes for genotypes A to I included in this study, and distributions of synonymous or non-synonymous nucleotide substitutions occurring in 'young' or 'old' branches according to the different partitions that were analysed, can be found at http://www.intmedpress.com/uploads/documents/AVT-12-SP-2655_Godoy_Add_file1.pdf

References

- Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet* 2003; 362:2089–2094.
- Yu H, Yuan Q, Ge SX, et al. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype T. *PLoS ONE* 2010; 5:e9297.
- Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol* 2007; 13:14–21.
- Devesa M, Pujol FH. Hepatitis B virus genetic diversity in Latin America. *Virus Res* 2007; 127:177–184.
- Roman S, Tanaka Y, Khan A, et al. Occult hepatitis B in the genotype H-infected Nahuas and Huichol native Mexican population. *J Med Virol* 2010; 82:1527–1536.
- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, et al. Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol* 2011; 11:103–108.
- Motta-Castro AR, Martins RM, Araújo NM, et al. Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Arch Virol* 2008; 153:2197–2205.
- Santos AO, Alvarado-Mora MV, Botelho L, et al. Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. *Virol J* 2010; 7:315.
- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MJ, Carrilho FJ, Pinho JRR. Molecular epidemiology and genetic diversity of hepatitis B virus genotype F in an isolated Afro-Colombian community. *J Gen Virol* 2010; 91:501–508.
- Piñeiro y Leone FG, Pezzano SC, Torres C, et al. Hepatitis B virus genetic diversity in Argentina: dissimilar genotype distribution in two different geographical regions; description of hepatitis B surface antigen variants. *J Clin Virol* 2008; 42:381–388.
- Torres C, Piñeiro y Leone FG, Pezzano SC, Mhayed VA, Campos RH. New perspectives on the evolutionary history of hepatitis B virus genotype F. *Mol Phylogenet Evol* 2011; 59:114–122.
- Zhou Y, Holmes EC. Bayesian estimates of the evolutionary rate and age of hepatitis B virus. *J Mol Evol* 2007; 65:197–205.
- Hannoun C, Horal P, Lindh M. Long-term mutation rates in the hepatitis B virus genome. *J Gen Virol* 2000; 81:75–83.
- Ho SYW, Larson G. Molecular clocks: when times are a-changin'. *Trends Genet* 2006; 22:79–83.
- Ho SYW, Phillips MJ, Cooper A, Drummond AJ. Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. *Mol Biol Evol* 2005; 22:1561–1568.
- Ho SYW, Saarma U, Barnett R, Haile J, Shapiro B. The effect of inappropriate calibration: three case studies in molecular ecology. *PLoS ONE* 2008; 3:e1615.
- Ho SYW, Lanfear R, Bromham L, et al. Time-dependent rates of molecular evolution. *Mol Ecol* 2011; 20:3087–3101.
- Crandall ED, Shrocco EJ, DeBoer TS, et al. Expansion dating: calibrating molecular clocks in marine species from expansions onto the Sunda Shelf following the last glacial maximum. *Mol Biol Evol* 2012; 29:707–719.
- Kivisild T, Shen P, Wall D, et al. The role of selection in the evolution of human mitochondrial genomes. *Genetics* 2006; 172:373–387.
- Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007; 7:214.
- Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. *PLoS Biol* 2006; 4:e88.
- Swofford DL. *PAUP*: Phylogenetic Analysis using Parsimony (*and other methods)*. Version 4.0b10. Sunderland, MA: Sinauer Associates 2003.
- Posada D, Crandall KA. Modeltest: testing the model of DNA substitution. *Bioinformatics* 1998; 14:817–818.
- Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009; 25:1451–1452.
- Abramson JH, Gahlinger PM. *Computer programs for epidemiologists: PEPI v 4.0*. Salt Lake City, UT: Sagebrush Press 2001.
- Bar-Gal GK, Kim MJ, Klein A, et al. Tracing hepatitis B virus to the 16th century in a Korean mummy. *Hepatology* 2012; 56:1671–1680.
- Ott JJ, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. *Vaccine* 2012; 30:2212–2219.
- Lim SG, Cheng Y, Guindon S, et al. Viral quasi-species during hepatitis B e antigen seroconversion. *Gastroenterology* 2007; 133:951–958.
- Harrison A, Lemey P, Hurler M, et al. Genomic analysis of hepatitis B virus reveals antigen state and genotype as sources of evolutionary rate variation. *Viruses* 2011; 3:83–101.
- Fagundes NJR, Kanitz R, Eckert R, et al. Mitochondrial population genomics supports a single pre-Clovis origin with a coastal route for the peopling of the Americas. *Am J Hum Genet* 2008; 82:583–592.
- Tamm E, Kivisild T, Reidla M, et al. Beringian standstill and spread of Native American founders. *PLoS ONE* 2007; 2:e829.
- Dillehay TD. *Monte Verde: a late Pleistocene settlement in Chile. The archaeological context and interpretation*. Vol. 2. Washington, DC: Smithsonian Institution Press 1997.
- Ray N, Wegmann D, Fagundes NJR, Wang S, Ruiz-Linares A, Excoffier L. A statistical evaluation of models for the initial settlement of the American continent emphasizes the importance of gene flow with Asia. *Mol Biol Evol* 2010; 27:337–345.
- Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83:2059–2073.

BA Godoy *et al.*

35. Venegas M, Alvarado-Mora MV, Villanueva RA, *et al.* Phylogenetic analysis of hepatitis B virus genotype F complete genome sequences from Chilean patients with chronic infection. *J Med Virol* 2011; 83:1530–1536.
36. Arauz-Ruiz P, Norder H, Visona KA, Magnus I.O. Genotype F prevails in HBV infected patients of hispanic origin in Central America and may carry the precore stop mutant. *J Med Virol* 1997; 51:305–312.

Accepted 13 January 2013; published online 21 June 2013

- 39- Bouckaert R, Alvarado-Mora MV, Pinho JR. Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods. Antivir Ther. 2013;18(3 Pt B):497-503**

Original article

Evolutionary rates and HBV: issues of rate estimation with Bayesian molecular methods

Remco Bouckaert¹*, Mónica V Alvarado-Mora², João R Rebello Pinho²¹University of Auckland, Auckland, New Zealand²Laboratory of Tropical Gastroenterology and Hepatology 'João Alves de Queiroz and Castorina Bittencourt Alves', Institute of Tropical Medicine, Department of Gastroenterology, University of São Paulo School of Medicine, São Paulo, Brazil

*Corresponding author email: remco@cs.auckland.ac.nz

Background: HBV infection is a public health problem affecting approximately 2 billion people and leading to >350 million chronic carriers of the virus worldwide. Phylogenetic analysis can give valuable insight to help in clarifying the history of viral infections around the world and in elucidating routes of transmission of the different viral strains present in the infected host population. These analyses rely on an accurate estimate of the rate of mutations.

Methods: In this study, we investigated the robustness of rate estimations based on Bayesian analysis obtained so far and examined, in particular, the choice of prior for the substitution rate.

Results: Most previous studies have concentrated on estimating the parameters of simple demographic models for HBV, such as exponential growth and constant population size. Here, we introduce a method that automatically

partitions the genome in components that show a different rate of mutation and fit different substitution models.

Conclusions: In conclusion, we find that, due to inaccuracy in the sampling dates from the samples where viral sequences were obtained, lack of a sufficiently large geographical and time spread of available and trustworthy sample dates, sensitivity to priors and model misspecification and rate estimation based on molecular methods, are not reliable. We suggest that rate estimates taking into account calibration points based on relevant historical events are more robust due to the lack of trustworthy sampling dates. For example, the known history of colonization of the Americas should be used to accurately study the current diversity of genotype F, which is the most frequent genotype in almost all Spanish speaking countries in South America.

Introduction

HBV belongs to the *Hepadnaviridae* family. Its members are divided into two genera; *Orthohepadnaviruses*, infecting mammals, and *Avihepadnaviruses*, affecting birds [1]. HBV infection in humans presents a global health problem. Worldwide, ≥ 2 billion people, or one-third of the world's population, have been infected with HBV [2]. The HBV genome is a partially double-stranded circular DNA molecule of approximately 3.2 kb in length. It contains four partly overlapping open reading frames: preS1/preS2/S (S), precore/core (C), polymerase (P) and X [3].

Molecular variation and sequence changes in the HBV genome over time have resulted in the emergence of ten genotypes, A to J, which HBV has been classified into, based on the sequence divergence over the entire genome [4–7]. Genotype A is found mainly in north and west Europe, North America and Africa. Genotypes B and C are prevalent in south-east Asia and the Far East. Genotype D has worldwide distribution and

is found predominantly in the Mediterranean region. Genotype E is predominant in the West/Central African crescent spanning from Senegal to Angola [8–10]. Despite the extensive spread of genotype E in this crescent, the virus is marked by a peculiar low overall sequence diversity of 1.75% over the whole genome [8]. Genotype F has been identified as the most prevalent in Central and South America and it is mainly found among native indigenous people from South America [11]. Genotype F can be further divided into four subgenotypes, F1 to F4, with a genetic divergence of 4.3–6.1% [12]. Genotype G has been reported in the United States and France [13]. Genotype H has been mainly described in Central America and Mexico [14]. Genotypes I and J have been identified in Vietnam and Borneo [7].

Two opposing forces can be discerned in terms of the diversity of HBV. First is the error-prone polymerase,

producing a high rate of nucleotide substitutions. Second, the extreme compactness of the genome prevents the occurrence of a large degree of genetic variability due to the overlapping open reading frames [15]. The true age of HBV is still the subject of much debate; however, a closer inspection of available genome sequence data indicates that the evidence for long-term codivergence is weaker than might be expected. The most divergent human genotypes are observed in populations either from the New World (genotypes F and H) or from Asia (genotype B). In some instances genotype F, predominant in the Americas, was the first to diverge, suggesting that the virus arose in the New World; by contrast, in other trees, sequences from genotype B, prevalent in East Asia, were the most divergent [16].

Sampling dates give information about the rate of evolution, and the difference between sampled sequences, together with the rate of evolution, give information about time of most recent common ancestors (MRCA) of a set of sequences. These MRCA times can be related to historic events. In this study, we investigate the factors that affect accuracy of substitution rate estimation based on molecular methods, from the sampling dates available to the diverse substitution rates that are presented in a virus with overlapping open reading frames that code for 1.5 proteins, which would be expected from its genome length. For this goal, we consider a number of previous molecular-method-based analyses and compare the sample dates. Due to the overlapping reading frames of HBV, it is expected that different parts of the genome follow a different evolutionary rate. We introduce a method for automatically partitioning the genome and determine a substitution model and mutation rate for each of the partitions.

Methods

Robustness of rate estimations

Since mutation rate estimates obtained from previous authors varied by ≥ 1 order of magnitude, we investigate robustness of the previously published rates by Zhou and Holmes [17], Alvarado-Mora *et al.* [18], Torres *et al.* [19] and Harrison *et al.* [20].

All experiments were performed using Markov Chain Monte Carlo (MCMC) methods, as implemented in BEAST 2.0 [21]. The MCMC chains were run long enough to ensure convergence, which was verified by inspecting the effective sample size. In all experiments, effective sample size was ≥ 100 , but in most cases was much larger than 200. The sequence dataset utilized by the authors is available upon request.

Robustness of sampling dates

Mutation rate estimation using molecular methods is based on the dates that sequences are sampled. To test

robustness of sampling dates, we used 19 full genome sequences from Harrison *et al.* [20] and applied the same model as in that study: the Hasegawa, Kishino and Yano (HKY) [22] substitution model with four gamma categories, a coalescent tree prior with a constant population size and an uncorrelated log normal distributed (UCLD) relaxed clock branch rate model. For the sample dates, we subtracted a value uniformly drawn from the interval 0 to 5.

Choice of priors

Another question that arises in any Bayesian analysis is the choice of priors. We examined, in particular, the choice of priors for the substitution rate. Three different priors were used to achieve more accurate estimated rates. By default, Bayesian evolutionary analysis utility, the graphical user interface used to specify BEAST analysis, suggests a uniform prior. Older versions, for example the one used in the analysis by Zhou and Holmes [17], do not even suggest there is another choice. We considered Jeffreys prior (also known as one-over-X prior) and an uninformative gamma prior (gamma 0.001, 0.001) as alternatives to the uniform prior. These were applied to the full genome alignments (among patients) from Harrison *et al.* [20] for the HBV A, C, D, E and F genotypes. We used the HKY substitution model with invariant sites, UCLD relaxed clock and coalescent tree prior with constant population.

Overlapping reading frames and estimation of the substitution model

Overlapping reading frames mean simple substitution models may not be appropriate. Different parts of the genome have different substitution rates. The presence of different coding genes along the HBV genome, using overlapping reading frames, suggest different substitution rates for different regions covering different genes. We propose a new model where we partition the genome in k consecutive parts. The boundaries of the partitions is randomly assigned and changed throughout the MCMC run. For each partition, a different substitution model is used. Since we do not know which substitution model is appropriate beforehand, we estimate the substitution model using a reversible-jump based method. The substitution model jumps between the following models: Felsenstein [23] (F81), HKY, Tamura and Nei [24] (TN93), transitional model (TIM), symmetrical model (SYM) and generalised time-reversible (GTR). The number of free parameters increases from 0 for F81 to 5 for GTR, and these parameters have a gamma (0.2, 5) prior each.

An experiment was performed on 68 full-genome HBV genotype F sequences using the above model with four gamma categories and invariant sites, since

this model gave the best fit. A UCLD relaxed clock and Bayesian skyline plot coalescent prior were applied.

Results

Sampling dates

To check the robustness of sampling dates, we repeated the experiment cited above 15 times. Results are shown in Figure 1. Another test to see whether there is any information regarding sampling dates is to randomly re-assign dates among sequences. This experiment was repeated 100 times and results are shown in Figure 2.

Choice of priors

Sensitivity of choice of priors was determined for the substitution rates considering uniform, Jeffreys and gamma priors for full-genome alignments for HBV genotypes A, C, D, E and F. The results are shown in Figure 3.

Estimation of the substitution model

Estimation of substitution model rates was carried out as described above. Results are shown in Figure 4. Notice that three different substitution models (HKY, GTR and SYM) and different mutations rates are

Figure 1. Robustness to sample dates

Rate estimates where sample dates had a random number from the interval 0 to 5 subtracted from the sample dates. HPD, highest probability density.

Figure 2. Information in sample dates

Sampling dates were randomly reassigned to taxa. HPD, highest probability density.

R Bouckaert et al.

Figure 3. Sensitivity of rate to prior

Rate estimates in substitution per site per year shown as 95% highest probability density (HPD) intervals and indication of the median for HBV genotypes A, C, D, E and F. The first data bar of each genotype displays a uniform prior (Uni), the second Jeffreys prior (1/X) and the third a gamma prior.

Figure 4. Varying rates in different part of the genome

Estimated mutation rates for different parts of the genome relative to the first partition are plotted against the site in the genome. The model picks up changes in overlapping reading frames. Parts that code for the polymerase (P) gene only have highest mutation rates, whereas the range where S and P overlap has the lowest. C, core; GTR, generalised time-reversible model; HKY, Hasegawa, Kishino and Yano model; Pre-C, precore; SYM, symmetrical model.

Table 1. Calibrated estimates

Prior	GTR		Partitioned	
	Median	95% HPD	Median	95% HPD
Uniform	2.07×10^{-04}	1.30×10^{-4} to 3.13×10^{-4}	1.79×10^{-04}	0.970×10^{-4} to 2.70×10^{-4}
1/X	2.06×10^{-04}	1.29×10^{-4} to 3.02×10^{-4}	1.64×10^{-04}	0.8991×10^{-4} to 2.43×10^{-4}
Gamma	2.09×10^{-04}	1.37×10^{-4} to 3.02×10^{-4}	1.62×10^{-04}	0.922×10^{-4} to 2.38×10^{-4}

Rate estimates for HBV genotype F assuming the time of most recent common ancestor is 300 years ago. Note that the sensitivity to the prior vanishes with this calibration. The generalised time-reversible (GTR) model gives slightly higher estimates than the partitioned model. Note that the partitioned model has a better fit to the data. HPD, highest probability density.

the most appropriate in different regions of the HBV genome that approximately correspond to the presence or absence of overlapping reading frames.

Time of most recent common ancestor

Although the real time for the introduction of HBV genotype is not known and previous estimates for the MRCA vary from 96 to 1,713.94 years, we have chosen to use a date corresponding to historical facts. We hypothesized that genotype F had a great increase in its spread after European colonization in Latin America, approximately 300 years ago. This date is included in the time interval cited above. This gives a calibration for the MRCA of HBV genotype F in Latin America. We estimated rates using GTR plus 4 gamma plus I, a UCLD relaxed clock and a Bayesian skyline plot tree prior, as well as the partitioned model with the same clock model and tree prior. Results are summarized in Table 1.

Discussion

The issue of sample dates

Consider the sequence with GenBank identification AB036905, which is a sample of HBV F3 from Venezuela. The GenBank submission year is 2009, whereas the journal submission year as recorded in GenBank is 2000. This already provides a choice of two sampling dates, although common sense says that the sampling date of the sequence should be 2000 or before. Anecdotal evidence suggests that these GenBank dates are being used in rate estimation studies. In the study by Harrison *et al.* [20] a sampling date of 1990 is used. The studies by Zhou and Holmes [17], who contacted all researchers for which there was no explicit sampling date in GenBank, and Torres *et al.* [19], who appear to have copied these dates, use a sampling date of 1982. We do want to point out that the discrepancy in dates could be traced, due to the excellent access to data provided by these studies. Therefore, the range of possible sampling dates for this particular sequence is 27 years. Note that the range of available HBV samples is just a few decades long. Fortunately, most HBV sequences

have a less extreme range. But it does raise the question of how sensitive the rate estimates are to the accuracy of the sampling dates.

Another issue is that sampling dates tend to be truncated to years [17,19]. For example, a sample taken on 31 December 1999 and another taken on 1 January 2000 are 1 year apart according to the sampling dates, whereas in reality they are just a day apart. Likewise, a sample from 31 December 2000 and another from 1 January 1999 are 1 year apart according to the sampling dates, whereas in reality they are almost 2 years apart. Figure 1 suggests that errors in the 0 to 5 range have a significant effect on substitution rate estimates. The effect is worse for errors in the 0 to 10 interval (unpublished observation). We conclude that accurate sampling dates do matter for estimating substitution rate.

Furthermore, rate estimates appear very sensitive to the choice of prior. Note that the y-axis of Figure 3 is in a log scale. The median systematically decreases when using a different prior from the uniform prior. Furthermore, the 95 highest probability density intervals become orders of magnitudes larger. Together, this indicates that rate estimates are very sensitive to the choice of prior. An alternative interpretation is that the prior dominates the rate estimate.

The reversible-jump-based substitution model has a posterior that is 100 log points larger than using GTR everywhere, which indicates that this model explains the data significantly better. Most of the genome is best explained using the HKY model, but the parts where S and P overlap and where X and P overlap are best explained by the GTR model.

Estimating HBV substitution rates

The notion that HBV has codiverged with animal populations over many millions of years is also incompatible with molecular clock estimates of the rate and timescale of viral evolution. The accurate estimation of nucleotide substitution rates in HBV is complicated by a number of factors, the most notable of which is the small size and overlapping nature of the HBV genome [25]. This results in strong selective constraints, so that substitution rates

measured in the short term may better reflect the intrinsic rate of mutation rather than the long-term rate of nucleotide substitution [25].

The first study estimating the substitution rate for HBV reported a rate between 1.4 and 3.2×10^{-5} substitutions per site per year [26], obtained by dividing the amount from a single patient by the total length of the sequence, assuming the common ancestor for the viruses at the patient's birth. By using a similar approach, other studies based on pairwise sequence comparison sampled at different time points estimated a median substitution rate of 5.1×10^{-4} [27] and 7.9×10^{-5} [28] substitutions per site per year. Similar evolutionary rates have also been estimated for other *Hepadnaviruses*, such as duck hepatitis B virus: 0.8 and 4.5×10^{-5} substitutions per site per year, respectively [29]. More recently, Alvarado-Mora *et al.* [18] used a coalescent-based approach to estimate the substitution rate for HBV genotype E of approximately 3.2×10^{-4} substitutions per site per year.

The common ancestor for the genotypes of human HBV would be >1 million years old if one assumes cospecies evolution. However, observed mutation rates and calculations based on genotype differences and human migration dates suggest that the HBV genome changes by a rate of approximately 2×10^{-5} to 10^{-6} substitutions per site per year. Based on such rates, the genotypes would have separated 2,000–40,000 years ago, while subgenotypes and the X/C recombinant would have evolved 1,000–20,000 years ago.

The origin of HBV is unclear. How and when the genotypes evolved and spread over the continents in humans and other species is not known. Several theories have been proposed, with both recent and distant emergence. The observation that HBV in apes and human genotypes A to E are more similar than are human genotypes F and H and genotypes A to E also points against cospecies evolution [16,30,31]. HBV genotypes may have evolved as a result of migration to separated geographical areas by humans carrying HBV (10,000–40,000 years ago). This idea is supported by the knowledge that an ice or land connection over the Bering Strait has joined present-day Alaska and eastern Siberia during several periods 10,000 to 25,000 years ago [32]. Finally, another theory suggests that the divergence of HBV has occurred by migration of infected persons into already populated areas. This model fits better with observed mutation rates [33]. The most extreme theory was the proposal that HBV originated from the Americas, and spread into the Old World as recently as 400 years ago [17].

The success of studies of viral evolution has been built on a combination of a growing database of gene and genome sequences coupled with advances in phylogenetic and coalescent methodology [25]. For all these results, an accurate estimate of mutation rates is crucial.

As pointed out in the *Results* section, there are a large number of difficulties in estimating rates using current phylogenetic methods. We demonstrated that rate estimates rely on accurate sampling dates. Unfortunately, accurate sampling dates are not readily available. Furthermore, the rate estimate is rather sensitive to the prior. The implication is that the information before the analysis has a larger effect on the rate estimate than the data. A problem specific to HBV rate estimation is that the genome has overlapping reading frames. Therefore, it can be expected that different parts of the genome have different mechanisms of evolution. This makes it unlikely that a full genome analysis with a single substitution model for the whole genome, as is generally used, leads to good results. In fact, we demonstrated that by automatically partitioning the data allowing each partition to have its own substitution model results in a much better fit to the data.

There are a number of other issues that make rate estimation cumbersome. For instance, it is known that hepatitis B e antigen (HBeAg)-positive patients have a slower rate than HBeAg-negative patients. If the HBeAg status is known, this phenomenon can be modelled by allowing extra mutation towards branches to these taxa [20]. Another issue is that different genotypes evolve at different rates, so genotypes cannot be simply grouped together. Currently, there is not enough information in the sequences due to the small sampling range of just a few decades. Another issue that is specific to HBV is that there is a rather small amount of data available. The genome is just 3.3 kb long, which makes it hard to distinguish between 10^{-5} substitutions per site per year and lower rates. In selecting sequences for alignments, care should be taken not to include sequences with recombination, since this would increase the estimate. Current phylogenetic analysis techniques do not readily deal with recombination. Finally, it is important that samples need to be from treatment-naïve patients. Otherwise rate estimate may be too high due to virus trying to mutate due to pressure by treatment.

One step towards getting more accurate rate estimates is by obtaining samples from a wider range in time, for example, by sequencing mummies, as for instance from the recently found Korean case [34]. These samples are difficult to obtain due to lack of sample candidates, and issues with preserving such samples intact through time. Another possibility is finding calibration from historic events. Most of the issues mentioned would be a lot less relevant if a phylogenetic reconstruction would be constrained by a calibration higher up in the tree. We demonstrated that rate estimates incorporating calibration points based on historic events are more robust to the choice of prior for HBV genotype E, and it is our hope that more such events can be found for other genotypes.

Acknowledgements

This work has been supported by Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP 2011/50562-0 and 2011/51597-1, CNPq, Alves de Queiroz Family Fund for Research, Fundação Faculdade de Medicina and HCFMUSP. Eddie Holmes and Philippe Lemey were very helpful in providing sequences in easily accessible file formats and answers to our questions.

Disclosure statement

The authors declare no competing interests.

References

- King AMQ, Lefkowitz E, Adams MJ, Carstens EB. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. Amsterdam: Elsevier 2011.
- Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337:1733–1745.
- Ganem D, Schneider RJ. Hepadnaviridae: the viruses and their replication. In Knipe DM, Howley PM (Editors). *Fields Virology*. 4th ed. Philadelphia: Lippincott Williams & Wilkins 2001; pp. 2923–2969.
- Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69:2575–2583.
- Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. *Virology* 1994; 198:489–503.
- Kramvis A, Arakawa K, Yu MC, Nogueira R, Stram DO, Kew MC. Relationship of serological subtype, basic core promoter and precore mutations to genotypes/subgenotypes of hepatitis B virus. *J Med Virol* 2008; 80:27–46.
- Yu H, Yuan Q, Ge SX, et al. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype “I”. *PLoS ONE* 2010; 5:e9297.
- Andernach IE, Hubschen JM, Muller CP. Hepatitis B virus: the genotype E puzzle. *Rev Med Virol* 2009; 19:231–240.
- Bekondi C, Olinger CM, Boua N, et al. Central African Republic is part of the West-African hepatitis B virus genotype E. *J Clin Virol* 2007; 40:31–37.
- Hubschen JM, Andernach IE, Muller CP. Hepatitis B virus genotype E variability in Africa. *J Clin Virol* 2008; 43:376–380.
- Devesa M, Loureiro CL, Rivas Y, et al. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol* 2008; 80:20–26.
- McMahon BJ. The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatol Int* 2009; 3:334–342.
- Stuyver L, De Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; 81:67–74.
- Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83:2059–2073.
- Mizokami M, Orito E, Ohba K, Ikey K, Lau JY, Gojobori T. Constrained evolution with respect to gene overlap of hepatitis B virus. *J Mol Evol* 1997; 44 Suppl 1:S83–S90.
- Bollyky PL, Holmes EC. Reconstructing the complex evolutionary history of hepatitis B virus. *J Mol Evol* 1999; 49:130–141.
- Zhou Y, Holmes EC. Bayesian estimations of the evolutionary rate and age of hepatitis B virus. *J Mol Evol* 2007; 65:197–205.
- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, Gutierrez MF, Carrilho FJ, Pinho JR. Molecular epidemiology and genetic diversity of hepatitis B virus genotype F in an isolated Afro-Colombian community. *J Gen Virol* 2010; 91:501–508.
- Torres C, Guadalupe Piñero y Leone F, Pezzano SC, Mbayed VA, Campos RH. New perspectives on the evolutionary history of hepatitis B virus genotype F. *Mol Phylogenet Evol* 2011; 59:114–122.
- Harrison A, Lemey P, Hurler M, et al. Genomic analysis of hepatitis B virus reveals antigen state and genotype as sources of evolutionary rate variation. *Viruses* 2011; 3:83–101.
- Drummond A, Bouckaert R. Bayesian evolutionary analysis with BEAST 2.0. Cambridge: Cambridge University Press 2013.
- Hasegawa M, Kishino H, Yano T. Dating of human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* 1985; 22:160–174.
- Felsenstein J. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* 1981; 17:368–376.
- Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* 1993; 10:512–526.
- Alvarado-Mora MV, Romano CM, Gomes-Gouveia MS, et al. Molecular characterization of the hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol* 2011; 11:103–108.
- Holmes EC. Evolutionary history and phylogeography of human viruses. *Annu Rev Microbiol* 2008; 62:307–328.
- Okamoto H, Imai M, Kametani M, Nakamura T, Mayumi M. Genomic heterogeneity of hepatitis B virus in a 54-year-old woman who contracted the infection through maternal-fetal transmission. *Jpn J Exp Med* 1987; 57:231–236.
- Zaaijer HL, Bouter S, Boot HJ. Substitution rate of the hepatitis B virus surface gene. *J Viral Hepat* 2008; 15:239–245.
- Osiowy C, Giles F, Tanaka Y, Mizokami M, Minuk GY. Molecular evolution of hepatitis B virus over 25 years. *J Virol* 2006; 80:10307–10314.
- Pult I, Abbott N, Zhang YY, Summers J. Frequency of spontaneous mutations in an avian hepadnavirus infection. *J Virol* 2001; 75:9623–9632.
- Fares MA, Holmes EC. A revised evolutionary history of hepatitis B virus (HBV). *J Mol Evol* 2002; 54:807–814.
- MacDonald DM, Holmes EC, Lewis JC, Simmonds P. Detection of hepatitis B virus infection in wild-born chimpanzees (*Pan troglodytes verus*): phylogenetic relationships with human and other primate genotypes. *J Virol* 2000; 74:4253–4257.
- O'Rourke DH, Raff JA. The human genetic history of the Americas: the final frontier. *Curr Biol* 2010; 20:R202–R207.
- Jazayeri SM, Alavian SM, Carman WF. Hepatitis B virus: origin and evolution. *J Viral Hepat* 2010; 17:229–235.

Accepted 13 September 2012; published online 21 June 2013

CONCLUSÕES

4-CONCLUSÕES

Neste texto sistematizado, estão apresentados 39 trabalhos científicos selecionados sobre hepatite B envolvendo os desenvolvimentos nas técnicas de biologia molecular adotadas para seu estudo bem como as suas diferentes aplicações em estudos clínicos e epidemiológicos. As principais conclusões destes trabalhos estão colocadas abaixo

✓ **Desenvolver metodologias moleculares aplicadas para a detecção do vírus da hepatite B (trabalhos 1 a 4):**

Saímos das aplicações das primeiras metodologias desenvolvidas, como os testes de detecção da atividade de DNA polimerase viral no soro, passamos para a técnica de hibridação molecular e logo entramos na reação em cadeia da polimerase (PCR), passando pela primeira metodologia qualitativa e finalizamos com a técnica de PCR em tempo real, que foi durante muito tempo a única metodologia disponível em nosso meio para a quantificação do HBV DNA, metodologia esta que foi com o passar do tempo sendo incorporada na rotina do tratamento dos pacientes com hepatite B, indicando o tipo e a duração do tratamento e tendo importância fundamental para a tomada de decisões clínicas sobre a duração, continuidade ou alterações no esquema terapêutico.

✓ **Avaliar a resposta ao tratamento da hepatite B utilizando marcadores moleculares**

Em paralelo, desenvolvíamos metodologias para a caracterização das linhagens virais circulantes, utilizando principalmente a técnica do sequenciamento automatizado de ácidos nucleicos. Aliando as técnicas de detecção e/ou quantificação de genomas virais pela PCR com a análise do aparecimento de mutações de resistência a drogas pela técnica do sequenciamento de ácidos nucleicos com os marcadores sorológicos e bioquímicos mais convencionais, em conjunto com o acompanhamento clínico destes pacientes, pudemos:

5) descrever em detalhes o processo de clareamento viral dos marcadores sorológicos e moleculares da infecção pelo HBV, fenômeno na época ainda controverso na literatura, pois os tratamentos para a hepatite B crônica começavam a apresentar resultados positivos nesta época;

6) descrever a importância da carga viral e a detecção do HBeAg na previsão da resposta ao tratamento com a lamivudina; bem como descrever pela primeira vez em nosso país a importância de mutações no gene da DNA polimerase do HBV associadas com a resistência ao tratamento com a lamivudina;

7) descrever a dinâmica do aparecimento de cepas com resistência a lamivudina em pacientes sob tratamento prolongado e a volta de cepas selvagens em 6 a 18 meses após a interrupção do tratamento;

8) descrever diferentes perfis de quantificação do DNA viral e do HBeAg em função da resposta aos antivirais. Os pacientes que tinham o melhor prognóstico de

resposta ao tratamento eram aqueles que apresentavam queda conjunta destes dois marcadores eram os que apresentavam o melhor prognóstico.

9) na população coinfectada com HIV, avaliar que o uso do Tenofovir para o tratamento conjunto das duas doenças estava associado com o sucesso do tratamento para HBV

- ✓ **Avaliar os diferentes genótipos e/ou subgenótipos virais e a presença de mutações com impacto clínico em diferentes grupos populacionais do Brasil e de outros países da América Latina (Chile, Colômbia e Panamá).**

a) Brasil

Em dois trabalhos publicados com 11 anos de diferença, encontramos em trabalhos publicados em 2004 e 2015, as seguintes distribuição de genótipos - A (49,5 para 67 %), B – (2,9 para 0,2 %), C (13,6 para 0,9 %), D (24,3 para 25,8 %), E (0 para 0,6 %), F (9,7 para 5,0%) e G (0 para 0,4%). As amostras do primeiro trabalho foram colhidas entre 1998 e 2001 em um laboratório privado que recebia amostras de pacientes com hepatite crônica B em acompanhamento médico. As amostras do segundo trabalho foram colhidas entre 2006 e 2011 de pacientes que não haviam recebido tratamento e eram provenientes de diferentes localidades, em geral de serviços públicos. As diferentes origens podem explicar algumas diferenças obtidas, especialmente em relação aos genótipos B e C que foram mais frequentes no primeiro estudo pois todos os pacientes portadores destes genótipos tinham origem oriental e estes pacientes costumam ser mais frequentemente infectados por estes genótipos. Como a casuística também era maior no segundo trabalho (103 vs.702), foi possível se

encontrar genótipos E e G neste estudo, se bem que os casos de genótipo E eram todos de indivíduos com contato com a África. Os subgenótipos só foram avaliados no segundo trabalho, e foram encontrados os subgenotipos A1, A2, B1,B2, C2, D1 a D4, F2a e F4.

b) São Paulo (capital)

Entre pacientes co-infectados com o HIV na Grande São Paulo, foram encontrados os genótipos A, D, F e G. Mutações de resistência a lamivudina foram encontradas em metade dos pacientes, mas em nenhum deles foram encontradas as mutações de resistência ao tenofovir. Também foram encontradas mutações associadas com resistência ao anti-HBs. Um caso de hepatite B infectado com o subgenótipo C2 foi encontrado também em uma paciente brasileira que tinha morado no Japão.

c) São Paulo – Ribeirão Preto

Em Ribeirão Preto, foram encontrados os genótipos A, D e F. O genótipo D apresenta uma alta frequência de mutantes pré-core, que foi verificada nesta população, assim como esta mutação foi associada com maior gravidade da doença.

d) Paraná

Neste estado, onde encontramos uma elevada prevalência da Hepatite B na região sudoeste, encontramos uma alta frequência de infecção pelo genótipo D bem como uma cepa com mutações associadas com resistência ao anti-HBs, que pode estar associada com hepatite B mesmo após imunoprofilaxia.

e) Santa Catarina

Em um estudo em unidades de diálise de diferentes localidades deste estado, encontramos os genótipos A, D e F e a análise das sequencias obtidas apontou para transmissão nosocomial.

f) Bahia

Encontramos os genótipos A, D e F em casos de hepatite aguda, sendo o genótipo A encontrado em 87,5% dos casos, o que é esperado em um estado com importante frequência de população de origem étnica africana. Nos casos de hepatite crônica, também foi mais frequente o genótipo A (86,93%).

g) Pernambuco

Em Pernambuco, também foi mais frequente o genótipo A (85% - quase todos pertencentes ao subgenótipo A1), sendo também só encontrado o genótipo F (15% - subgenótipos F2a e F4).

h) Maranhão

No Maranhão, em uma comunidade quilombola encontramos exclusivamente o subgenótipo A1. Em outro estudo mais amplo neste estado, encontramos o subgenótipo A1 como o preponderante (67%), mas encontramos também o subgenótipo D4 em 24% dos casos. Este subgenótipo só foi encontrado neste estado, o que sugere que esta infecção tenha sido trazida da África durante o período da escravidão, pois este genótipo só foi encontrado na África e em outros países com população de origem africana.

i) Rondônia

Foram encontrados os subgenótipos A1, D3, F2a, D4 e D2, coerentes com as origens populacionais da população deste estado (ameríndios, europeus e africanos).

j) Amazônia

Os genótipos A, F e D foram os mais frequentes no estado do Pará. Nesta região, encontramos a presença de co-infecção com o vírus da hepatite delta (HDV) com HBV de genótipos A e F, o que não tinha sido descrito anteriormente. Esta associação com HDV com diferentes genótipos do HBV foi confirmada também na região da febre negra de Lábrea, onde foi encontrada a co-infecção também com o genótipo D.

k) Imigrantes Orientais

Constatada a alta frequência da infecção pelo HBV nesta população, foi também constatada a elevada frequência de infecção pelo genótipo C, característico do oriente da Ásia e por métodos filogenéticos se comprovou transmissão intrafamiliar em muitas famílias.

l) Imigrantes Africanos

Em um viajante africano que vinha com frequência ao Brasil se encontrou o genótipo E nunca antes encontrado por aqui, chamando atenção para o risco de sua introdução na população brasileira com o aumento de contato com estas nações.

m) Colômbia

Confirmamos a elevada presença de infecção pelo HBV em algumas regiões da Colômbia, nas quais esta infecção se mostra associada com a infecção pelo vírus delta. Em uma comunidade afro-colombiana, encontramos os subgenótipos A1 e E, característicos da África, bem como um novo recombinante A1/F3.

n) Chile

Estudando casos de hepatite B crônica do Chile, encontramos apenas o subgenótipo F1b, típico da população ameríndia.

o) Panamá

Estudando uma população de chineses que migrou para o Panamá, contatou-se elevada prevalência de infecção pelo HBV causada apenas pelos subgenótipos B2 e C1, característicos da China.

✓ **Avaliar os tempos de infecção pelo vírus da hepatite B em diferentes grupos populacionais utilizando métodos de análise filogenéticos**

Como encontramos casos de HBV genótipo E na população afro-colombiana da África e não havia história de contato recente com a África, tentamos estabelecer o tempo de infecção por este genótipo na Colômbia. O cálculo deste tempo por métodos bayesianos nos forneceu resultados extremamente variáveis, entre 35 e 1500, de acordo com as diferentes estimativas disponíveis sobre a taxa de mutação viral.

Para o genótipo F, um vírus de possível origem no continente americano, encontramos este tempo variando entre 96 a 2418, conforme a taxa de mutação utilizada. Dentre os subgenótipos, nossos dados sugerem que o subgenótipo F3 seja o mais antigo e pode compartilhar a origem comum com o genótipo H.

Como nossos resultados tinham uma ampla faixa de variação, começamos a trabalhar com diferentes métodos para avaliar as taxas evolucionárias deste vírus. Em um trabalho, concluímos que as taxas estimadas apenas em amostras recentes tendem a subestimar os tempos de coalescência. Em outro, sugerimos que datas de eventos históricos importantes devem ser levados em consideração para tentar solucionar este problema.

REFERÊNCIAS

5-REFERÊNCIAS

1. Beadle GW, Tatum EL. Genetic Control of Biochemical Reactions in *Neurospora*. *Proc Natl Acad Sci U S A*. 1941 Nov 15;27(11):499-506.
2. Avery OT, Macleod CM, McCarty M. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from *Pneumococcus* Type Iii. *J Exp Med*. 1944 Feb 1;79(2):137-58.
3. Griffith F. The Significance of Pneumococcal Types. *J Hyg (Lond)*. 1928 Jan;27(2):113-59.
4. Hershey AD, Chase M. Independent functions of viral protein and nucleic acid in growth of bacteriophage. *J Gen Physiol*. 1952 May;36(1):39-56.
5. Chargaff E, Zamenhof S, Green C. Composition of human desoxypentose nucleic acid. *Nature*. 1950 May 13;165(4202):756-7.
6. Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature*. 1953 Apr 25;171(4356):737-8.
7. Meselson M, Stahl FW. The Replication of DNA in *Escherichia Coli*. *Proc Natl Acad Sci U S A*. 1958 Jul 15;44(7):671-82.
8. Jacob F, Monod J. Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol*. 1961 Jun;3:318-56.
9. Crick FH, Barnett L, Brenner S, Watts-Tobin RJ. General nature of the genetic code for proteins. *Nature*. 1961 Dec 30;192:1227-32.
10. Smith HO, Wilcox KW. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. *J Mol Biol*. 1970 Jul 28;51(2):379-91.
11. Roberts RJ. How restriction enzymes became the workhorses of molecular biology. *Proc Natl Acad Sci U S A*. 2005 Apr 26;102(17):5905-8.
12. Weiss B, Richardson CC. Enzymatic breakage and joining of deoxyribonucleic acid, I. Repair of single-strand breaks in DNA by an enzyme system from *Escherichia coli* infected with T4 bacteriophage. *Proc Natl Acad Sci U S A*. 1967 Apr;57(4):1021-8.
13. Lederberg J. Cell genetics and hereditary symbiosis. *Physiol Rev*. 1952 Oct;32(4):403-30.
14. Maxam AM, Gilbert W. A new method for sequencing DNA. *Proc Natl Acad Sci U S A*. 1977 Feb;74(2):560-4.
15. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A*. 1977 Dec;74(12):5463-7.
16. Jackson DA, Symons RH, Berg P. Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1972 Oct;69(10):2904-9.
17. Cohen SN, Chang AC. Recircularization and autonomous replication of a sheared R-factor DNA segment in *Escherichia coli* transformants. *Proc Natl Acad Sci U S A*. 1973 May;70(5):1293-7.
18. Rutgers T CT, De Wilde M. Production of viral antigens in prokaryotic and eukaryotic cells Immunohistochemistry of viruses II: the basis for serodiagnosis and vaccines Amsterdam: Elsevier; 1990. p. 103-23.
19. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature*. 1982 Jul 22;298(5872):347-50.
20. Kim KS, Park SA, Ko KN, Yi S, Cho YJ. Current status of human papillomavirus vaccines. *Clin Exp Vaccine Res*. 2014 Jul;3(2):168-75.

21. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.* 1975 Nov 5;98(3):503-17.
22. Mullis KB. The unusual origin of the polymerase chain reaction. *Sci Am.* 1990 Apr;262(4):56-61, 4-5.
23. Ansorge W, Voss H, Wirkner U, Schwager C, Stegemann J, Pepperkok R, et al. Automated Sanger DNA sequencing with one label in less than four lanes on gel. *J Biochem Biophys Methods.* 1989;20(1):47-52.
24. Morey M, Fernandez-Marmiesse A, Castineiras D, Fraga JM, Couce ML, Cocho JA. A glimpse into past, present, and future DNA sequencing. *Mol Genet Metab.* 2013 Sep-Oct;110(1-2):3-24.
25. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR, et al. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics.* 2012;13:341.
26. Lauck M, Alvarado-Mora MV, Becker EA, Bhattacharya D, Striker R, Hughes AL, et al. Analysis of hepatitis C virus intrahost diversity across the coding region by ultradeep pyrosequencing. *J Virol.* 2012 Apr;86(7):3952-60.
27. McCallum F. Homologous serum jaundice. *Lancet.* 1947(2):691-2.
28. Hollinger F. Hepatitis B virus. In: Knipe BNFaDM, editor. *Virology.* 2^a ed. New York: Raven Press Ltd; 1990. p. 2171-236.
29. Blumberg BS, Alter HJ, Visnich S. A "New" Antigen in Leukemia Sera. *Jama.* 1965 Feb 15;191:541-6.
30. Prince AM. An antigen detected in the blood during the incubation period of serum hepatitis. *Proc Natl Acad Sci U S A.* 1968 Jul;60(3):814-21.
31. Okochi K, Murakami S. Observations on Australia antigen in Japanese. *Vox Sang.* 1968;15(5):374-85.
32. Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet.* 1970 Apr 4;1(7649):695-8.
33. WHO. Hepatitis B; 2008.
34. Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: a global overview. *Clin Liver Dis.* 2010 Feb;14(1):1-21, vii.
35. Merrill RM, Hunter BD. Seroprevalence of markers for hepatitis B viral infection. *Int J Infect Dis.* 2011 Feb;15(2):e78-121.
36. Souto FJ, Fontes CJ, Gaspar AM. Prevalence of hepatitis B and C virus markers among malaria-exposed gold miners in Brazilian Amazon. *Mem Inst Oswaldo Cruz.* 2001 Aug;96(6):751-5.
37. Braga WS, Silva EB, Souza RA, Tosta CE. [Seroprevalence of hepatitis B and malaria infection in Labrea, Brazilian western Amazon: estimates of coinfection rates]. *Rev Soc Bras Med Trop.* 2005 May-Jun;38(3):218-23.
38. Estudo de Prevalência de Base Populacional das Infecções pelos vírus das Hepatites A, B e C nas Capitais do Brasil. 2010 [cited 2015 16/02/2015]; Available from: http://www.aids.gov.br/publicacao/2010/estudo_de_prevalencia_de_base_populacional_das_infeccoes_pelos_virus_das_hepatites_b
39. Keating GM, Noble S. Recombinant hepatitis B vaccine (Engerix-B): a review of its immunogenicity and protective efficacy against hepatitis B. *Drugs.* 2003;63(10):1021-51.
40. Venters C, Graham W, Cassidy W. Recombivax-HB: perspectives past, present and future. *Expert Rev Vaccines.* 2004 Apr;3(2):119-29.
41. Orenstein WA, Pickering, L.K., Mawle, A., Hinman, A.R., Wharton, M. Immunization. In: Mandell GL, Bennett, J.E., Dolin, R., editor. *Principles and Practice of Infectious Diseases.* 7^a ed: Churchill Livingstone; 2010. p. 1577-92.
42. Gust ID, Burrell CJ, Coulepis AG, Robinson WS, Zuckerman AJ. Taxonomic classification of human hepatitis B virus. *Intervirology.* 1986;25(1):14-29.

43. Summers J, Mason WS. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell*. 1982 Jun;29(2):403-15.
44. Marion PL, Oshiro LS, Regnery DC, Scullard GH, Robinson WS. A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. *Proc Natl Acad Sci U S A*. 1980 May;77(5):2941-5.
45. Testut P, Renard CA, Terradillos O, Vitvitski-Trepo L, Tekaiia F, Degott C, et al. A new hepadnavirus endemic in arctic ground squirrels in Alaska. *J Virol*. 1996 Jul;70(7):4210-9.
46. Lanford RE, Chavez D, Brasky KM, Burns RB, 3rd, Rico-Hesse R. Isolation of a hepadnavirus from the woolly monkey, a New World primate. *Proc Natl Acad Sci U S A*. 1998 May 12;95(10):5757-61.
47. He B, Li Z, Yang F, Zheng J, Feng Y, Guo H, et al. Virome profiling of bats from Myanmar by metagenomic analysis of tissue samples reveals more novel Mammalian viruses. *PLoS One*. 2013;8(4):e61950.
48. Mimms LT, Solomon LR, Ebert JW, Fields H. Unique preS sequence in a gibbon-derived hepatitis B virus variant. *Biochem Biophys Res Commun*. 1993 Aug 31;195(1):186-91.
49. MacDonald DM, Holmes EC, Lewis JC, Simmonds P. Detection of hepatitis B virus infection in wild-born chimpanzees (*Pan troglodytes verus*): phylogenetic relationships with human and other primate genotypes. *J Virol*. 2000 May;74(9):4253-7.
50. Warren KS, Heeney JL, Swan RA, Heriyanto, Verschoor EJ. A new group of hepadnaviruses naturally infecting orangutans (*Pongo pygmaeus*). *J Virol*. 1999 Sep;73(9):7860-5.
51. Grethe S, Heckel JO, Rietschel W, Hufert FT. Molecular epidemiology of hepatitis B virus variants in nonhuman primates. *J Virol*. 2000 Jun;74(11):5377-81.
52. Mason WS, Seal G, Summers J. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J Virol*. 1980 Dec;36(3):829-36.
53. Sprengel R, Kaleta EF, Will H. Isolation and characterization of a hepatitis B virus endemic in herons. *J Virol*. 1988 Oct;62(10):3832-9.
54. Prassolov AV. Detection of a new avian hepadnavirus with an unexpectedly broad host range. *Dokl Biol Sci*. 2002 Nov-Dec;387:593-6.
55. Pult I, Netter HJ, Bruns M, Prassolov A, Sirma H, Hohenberg H, et al. Identification and analysis of a new hepadnavirus in white storks. *Virology*. 2001 Oct 10;289(1):114-28.
56. Piasecki T, Kurenbach B, Chrzastek K, Bednarek K, Krabberger S, Martin DP, et al. Molecular characterisation of an avihepadnavirus isolated from *Psittacula krameri* (ring-necked parrot). *Arch Virol*. 2012 Mar;157(3):585-90.
57. Piasecki T, Harkins GW, Chrzastek K, Julian L, Martin DP, Varsani A. Avihepadnavirus diversity in parrots is comparable to that found amongst all other avian species. *Virology*. 2013 Apr 10;438(2):98-105.
58. Kann M GWH. Structure and Molecular Virology. In: Zuckerman AJ TH, editor. *Viral Hepatitis 2ª ed*. Londres: Churchill-Livingstone; 1998. p. 77-105.
59. Glebe D, Bremer CM. The molecular virology of hepatitis B virus. *Semin Liver Dis*. 2013 May;33(2):103-12.
60. Gerlich WH, Deepen R, Heermann KH, Krone B, Lu XY, Seifer M, et al. Protective potential of hepatitis B virus antigens other than the S gene protein. *Vaccine*. 1990 Mar;8 Suppl:S63-8; discussion S79-80.
61. Gerlich WH. Medical Virology of Hepatitis B: how it began and where we are now. *Virology Journal*. 2013 Jul 20;10.
62. Bruss V, Gerhardt E, Vieluf K, Wunderlich G. Functions of the large hepatitis B virus surface protein in viral particle morphogenesis. *Intervirology*. 1996;39(1-2):23-31.
63. Bruss V. Hepatitis B virus morphogenesis. *World J Gastroenterol*. 2007 Jan 7;13(1):65-73.

64. Meier A, Mehrle S, Weiss TS, Mier W, Urban S. Myristoylated PreS1-domain of the hepatitis B virus L-protein mediates specific binding to differentiated hepatocytes. *Hepatology*. 2013 Jul;58(1):31-42.
65. Baumert TF, Meredith L, Ni Y, Felmlee DJ, McKeating JA, Urban S. Entry of hepatitis B and C viruses - recent progress and future impact. *Curr Opin Virol*. 2014 Feb;4:58-65.
66. Hallen S, Mareninova O, Branden M, Sachs G. Organization of the membrane domain of the human liver sodium/bile acid cotransporter. *Biochemistry*. 2002 Jun 11;41(23):7253-66.
67. Salisse J, Sureau C. A function essential to viral entry underlies the hepatitis B virus "a" determinant. *J Virol*. 2009 Sep;83(18):9321-8.
68. Abou-Jaoude G, Sureau C. Entry of hepatitis delta virus requires the conserved cysteine residues of the hepatitis B virus envelope protein antigenic loop and is blocked by inhibitors of thiol-disulfide exchange. *J Virol*. 2007 Dec;81(23):13057-66.
69. Lepere-Douard C, Trotard M, Le Seyec J, Gripon P. The first transmembrane domain of the hepatitis B virus large envelope protein is crucial for infectivity. *J Virol*. 2009 Nov;83(22):11819-29.
70. Gripon P, Le Seyec J, Rumin S, Guguen-Guillouzo C. Myristylation of the hepatitis B virus large surface protein is essential for viral infectivity. *Virology*. 1995 Nov 10;213(2):292-9.
71. Bruss V, Hagelstein J, Gerhardt E, Galle PR. Myristylation of the large surface protein is required for hepatitis B virus in vitro infectivity. *Virology*. 1996 Apr 15;218(2):396-9.
72. Le Seyec J, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P. Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. *J Virol*. 1999 Mar;73(3):2052-7.
73. Blanchet M, Sureau C. Infectivity determinants of the hepatitis B virus pre-S domain are confined to the N-terminal 75 amino acid residues. *J Virol*. 2007 Jun;81(11):5841-9.
74. Ni Y, Sonnabend J, Seitz S, Urban S. The pre-s2 domain of the hepatitis B virus is dispensable for infectivity but serves a spacer function for L-protein-connected virus assembly. *J Virol*. 2010 Apr;84(8):3879-88.
75. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A*. 2002 Nov 26;99(24):15655-60.
76. Gripon P, Cannie I, Urban S. Efficient inhibition of hepatitis B virus infection by acylated peptides derived from the large viral surface protein. *J Virol*. 2005 Feb;79(3):1613-22.
77. Petersen J, Dandri M, Mier W, Lutgehetmann M, Volz T, von Weizsacker F, et al. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nat Biotechnol*. 2008 Mar;26(3):335-41.
78. Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, et al. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife*. 2012;1:e00049.
79. Meyer zum Buschenfelde KH, Gerken G, Hess G, Manns M. The significance of the pre-S region of the hepatitis B virus. *J Hepatol*. 1986;3(2):273-9.
80. Gerlich WH. Prophylactic vaccination against hepatitis B: achievements, challenges and perspectives. *Med Microbiol Immunol*. 2015 Feb;204(1):39-55.
81. Hildt E, Urban S, Lauer U, Hofschneider PH, Kekule AS. ER-localization and functional expression of the HBV transactivator MHBst. *Oncogene*. 1993 Dec;8(12):3359-67.
82. Hildt E, Saher G, Bruss V, Hofschneider PH. The hepatitis B virus large surface protein (LHBs) is a transcriptional activator. *Virology*. 1996 Nov 1;225(1):235-9.
83. Karai K IS, Kokie K. et al Pre-S(2) antigen in chronic hepatitis B virus infection: a new marker for HBV replication. In: Zuckermann A, editor. *Viral Hepatitis and Liver Disease*: Alan R. Liss; 1988. p. 284-5.
84. Sun L, Zhang Y, Zhao B, Deng M, Liu J, Li X, et al. A new unconventional HLA-A2-restricted epitope from HBV core protein elicits antiviral cytotoxic T lymphocytes. *Protein Cell*. 2014 Apr;5(4):317-27.

85. Uy A, Bruss V, Gerlich WH, Kochel HG, Thomssen R. Precore sequence of hepatitis B virus inducing e antigen and membrane association of the viral core protein. *Virology*. 1986 Nov;155(1):89-96.
86. Nassal M, Galle PR, Schaller H. Proteaselike sequence in hepatitis B virus core antigen is not required for e antigen generation and may not be part of an aspartic acid-type protease. *J Virol*. 1989 Jun;63(6):2598-604.
87. Miller RH. Proteolytic self-cleavage of hepatitis B virus core protein may generate serum e antigen. *Science*. 1987 May 8;236(4802):722-5.
88. Nassal M. The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J Virol*. 1992 Jul;66(7):4107-16.
89. Chen MT, Billaud JN, Sallberg M, Guidotti LG, Chisari FV, Jones J, et al. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci U S A*. 2004 Oct 12;101(41):14913-8.
90. Liang TJ. Hepatitis B: the virus and disease. *Hepatology*. 2009 May;49(5 Suppl):S13-21.
91. Zhang XD, Wang Y, Ye LH. Hepatitis B virus X protein accelerates the development of hepatoma. *Cancer Biol Med*. 2014 Sep;11(3):182-90.
92. Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. *J Hepatol*. 2011 Nov;55(5):996-1003.
93. Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, et al. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. *Proc Natl Acad Sci U S A*. 2009 Nov 24;106(47):19975-9.
94. van Breugel PC, Robert EI, Mueller H, Decorsiere A, Zoulim F, Hantz O, et al. Hepatitis B virus X protein stimulates gene expression selectively from extrachromosomal DNA templates. *Hepatology*. 2012 Dec;56(6):2116-24.
95. Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, et al. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology*. 2006 Mar;130(3):823-37.
96. Ezzikouri S, Ozawa M, Kohara M, Elmdaghri N, Benjelloun S, Tsukiyama-Kohara K. Recent insights into hepatitis B virus-host interactions. *J Med Virol*. 2014 Jun;86(6):925-32.
97. Koshy R, Hofschneider PH. Transactivation by hepatitis B virus may contribute to hepatocarcinogenesis. *Curr Top Microbiol Immunol*. 1989;144:265-81.
98. Capovilla A, Carmona S, Arbuthnot P. Hepatitis B virus X-protein binds damaged DNA and sensitizes liver cells to ultraviolet irradiation. *Biochem Biophys Res Commun*. 1997 Mar 6;232(1):255-60.
99. Kaplan PM, Greenman RL, Gerin JL, Purcell RH, Robinson WS. DNA polymerase associated with human hepatitis B antigen. *J Virol*. 1973 Nov;12(5):995-1005.
100. Tavis JE, Ganem D. Expression of functional hepatitis B virus polymerase in yeast reveals it to be the sole viral protein required for correct initiation of reverse transcription. *Proc Natl Acad Sci U S A*. 1993 May 1;90(9):4107-11.
101. Schlicht HJ, Schaller H. Analysis of hepatitis B virus gene functions in tissue culture and in vivo. *Curr Top Microbiol Immunol*. 1989;144:253-63.
102. Bartenschlager R, Schaller H. The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. *Embo J*. 1988 Dec 20;7(13):4185-92.
103. Radziwill G, Tucker W, Schaller H. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. *J Virol*. 1990 Feb;64(2):613-20.
104. Dallmeier K, Nassal M. Hepadnaviruses have a narrow host range - do they? In: Weber O, Protzer-Knolle U., editor. *Comparative Hepatitis - Birkhäuser Advances in Infectious Diseases*. Birkhäuser, Basel; 2008. p. 303-40.

105. Steitz TA. Visualizing polynucleotide polymerase machines at work. *Embo J*. 2006 Aug 9;25(15):3458-68.
106. Chen Y, Robinson WS, Marion PL. Selected mutations of the duck hepatitis B virus P gene RNase H domain affect both RNA packaging and priming of minus-strand DNA synthesis. *J Virol*. 1994 Aug;68(8):5232-8.
107. Nassal M. Hepatitis B viruses: reverse transcription a different way. *Virus Res*. 2008 Jun;134(1-2):235-49.
108. Siddiqui A, Jameel S, Mapoles J. Transcriptional control elements of hepatitis B surface antigen gene. *Proc Natl Acad Sci U S A*. 1986 Feb;83(3):566-70.
109. Raney AK, Easton AJ, Milich DR, McLachlan A. Promoter-specific transactivation of hepatitis B virus transcription by a glutamine- and proline-rich domain of hepatocyte nuclear factor 1. *J Virol*. 1991 Nov;65(11):5774-81.
110. Raney AK, Zhang P, McLachlan A. Regulation of transcription from the hepatitis B virus large surface antigen promoter by hepatocyte nuclear factor 3. *J Virol*. 1995 Jun;69(6):3265-72.
111. Cattaneo R, Will H, Hernandez N, Schaller H. Signals regulating hepatitis B surface antigen transcription. *Nature*. 1983 Sep 22-28;305(5932):336-8.
112. Malpierce Y, Michel ML, Carloni G, Revel M, Tiollais P, Weissenbach J. The gene S promoter of hepatitis B virus confers constitutive gene expression. *Nucleic Acids Res*. 1983 Jul 11;11(13):4645-54.
113. Faktor O, De-Medina T, Shaul Y. Regulation of hepatitis B virus S gene promoter in transfected cell lines. *Virology*. 1988 Feb;162(2):362-8.
114. Masuda M, Lee G, Yuasa T, Yoshikura H. Upstream region of hepatitis B virus S gene responsible for transcriptional stimulation by dexamethasone. *Microbiol Immunol*. 1988;32(7):741-7.
115. Tseng YP, Kuo YH, Hu CP, Jeng KS, Janmanchi D, Lin CH, et al. The role of helioxanthin in inhibiting human hepatitis B viral replication and gene expression by interfering with the host transcriptional machinery of viral promoters. *Antiviral Res*. 2008 Mar;77(3):206-14.
116. Yuh CH, Chang YL, Ting LP. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. *J Virol*. 1992 Jul;66(7):4073-84.
117. Treinin M, Laub O. Identification of a promoter element located upstream from the hepatitis B virus X gene. *Mol Cell Biol*. 1987 Jan;7(1):545-8.
118. Yaginuma K, Koike K. Identification of a promoter region for 3.6-kilobase mRNA of hepatitis B virus and specific cellular binding protein. *J Virol*. 1989 Jul;63(7):2914-20.
119. Chen IH, Huang CJ, Ting LP. Overlapping initiator and TATA box functions in the basal core promoter of hepatitis B virus. *J Virol*. 1995 Jun;69(6):3647-57.
120. Yu X, Mertz JE. Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. *J Virol*. 1996 Dec;70(12):8719-26.
121. Yu X, Mertz JE. Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. *J Virol*. 1997 Dec;71(12):9366-74.
122. Buckwold VE, Chen M, Ou JH. Interaction of transcription factors RFX1 and MIBP1 with the gamma motif of the negative regulatory element of the hepatitis B virus core promoter. *Virology*. 1997 Jan 20;227(2):515-8.
123. Uchida T, Takahashi K, Tatsuno K, Dhingra U, Eliason JF. Inhibition of hepatitis-B-virus core promoter by p53: implications for carcinogenesis in hepatocytes. *Int J Cancer*. 1996 Sep 17;67(6):892-7.
124. Guo WT, Wang J, Tam G, Yen TS, Ou JS. Leaky transcription termination produces larger and smaller than genome size hepatitis B virus X gene transcripts. *Virology*. 1991 Apr;181(2):630-6.

125. Zheng YW, Riegler J, Wu J, Yen TS. Novel short transcripts of hepatitis B virus X gene derived from intragenic promoter. *J Biol Chem*. 1994 Sep 9;269(36):22593-8.
126. Cherrington J, Russnak R, Ganem D. Upstream sequences and cap proximity in the regulation of polyadenylation in ground squirrel hepatitis virus. *J Virol*. 1992 Dec;66(12):7589-96.
127. Rieger A, Nassal M. Specific hepatitis B virus minus-strand DNA synthesis requires only the 5' encapsidation signal and the 3'-proximal direct repeat DR1. *J Virol*. 1996 Jan;70(1):585-9.
128. Quade K, Saldanha J, Thomas H, Monjardino J. Integration of hepatitis B virus DNA through a mutational hot spot within the cohesive region in a case of hepatocellular carcinoma. *J Gen Virol*. 1992 Jan;73 (Pt 1):179-82.
129. Kidd AH, Kidd-Ljunggren K. A revised secondary structure model for the 3'-end of hepatitis B virus pregenomic RNA. *Nucleic Acids Res*. 1996 Sep 1;24(17):3295-301.
130. Knaus T, Nassal M. The encapsidation signal on the hepatitis B virus RNA pregenome forms a stem-loop structure that is critical for its function. *Nucleic Acids Res*. 1993 Aug 25;21(17):3967-75.
131. Bartenschlager R, Schaller H. Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. *Embo J*. 1992 Sep;11(9):3413-20.
132. Lok AS, Akarca U, Greene S. Mutations in the pre-core region of hepatitis B virus serve to enhance the stability of the secondary structure of the pre-genome encapsidation signal. *Proc Natl Acad Sci U S A*. 1994 Apr 26;91(9):4077-81.
133. Su H, Yee JK. Regulation of hepatitis B virus gene expression by its two enhancers. *Proc Natl Acad Sci U S A*. 1992 Apr 1;89(7):2708-12.
134. Quasdorff M, Protzer U. Control of hepatitis B virus at the level of transcription. *J Viral Hepat*. 2010 Aug;17(8):527-36.
135. Shaul Y, Rutter WJ, Laub O. A human hepatitis B viral enhancer element. *Embo J*. 1985 Feb;4(2):427-30.
136. Antonucci TK, Rutter WJ. Hepatitis B virus (HBV) promoters are regulated by the HBV enhancer in a tissue-specific manner. *J Virol*. 1989 Feb;63(2):579-83.
137. Elfassi E. Broad specificity of the hepatitis B enhancer function. *Virology*. 1987 Sep;160(1):259-62.
138. Chou HW, Harrell D, Forough R, Watabe K. Binding of tissue-specific factors to the enhancer sequence of hepatitis B virus. *FEBS Lett*. 1988 Mar 14;229(2):349-54.
139. Fukai K, Takada S, Yokosuka O, Saisho H, Omata M, Koike K. Characterization of a specific region in the hepatitis B virus enhancer I for the efficient expression of X gene in the hepatic cell. *Virology*. 1997 Sep 29;236(2):279-87.
140. Yee JK. A liver-specific enhancer in the core promoter region of human hepatitis B virus. *Science*. 1989 Nov 3;246(4930):658-61.
141. Ori A, Zauberman A, Doitsh G, Paran N, Oren M, Shaul Y. p53 binds and represses the HBV enhancer: an adjacent enhancer element can reverse the transcription effect of p53. *Embo J*. 1998 Jan 15;17(2):544-53.
142. Wang SH, Yeh SH, Lin WH, Wang HY, Chen DS, Chen PJ. Identification of androgen response elements in the enhancer I of hepatitis B virus: a mechanism for sex disparity in chronic hepatitis B. *Hepatology*. 2009 Nov;50(5):1392-402.
143. Wang Y, Chen P, Wu X, Sun AL, Wang H, Zhu YA, et al. A new enhancer element, ENII, identified in the X gene of hepatitis B virus. *J Virol*. 1990 Aug;64(8):3977-81.
144. Yuh CH, Ting LP. The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. *J Virol*. 1990 Sep;64(9):4281-7.
145. Tur-Kaspa R, Burk RD, Shaul Y, Shafritz DA. Hepatitis B virus DNA contains a glucocorticoid-responsive element. *Proc Natl Acad Sci U S A*. 1986 Mar;83(6):1627-31.

146. Tur-Kaspa R, Shaul Y, Moore DD, Burk RD, Okret S, Poellinger L, et al. The glucocorticoid receptor recognizes a specific nucleotide sequence in hepatitis B virus DNA causing increased activity of the HBV enhancer. *Virology*. 1988 Dec;167(2):630-3.
147. Lopez-Alcorocho JM, Cabrerizo M, Bartolome J, Cotonat T, Carreno V. Analysis of hepatitis B virus precore variants in hepatitis B e antibody-positive patients treated with prednisone plus interferon. *J Viral Hepat*. 1995;2(6):279-84.
148. Pollack JR, Ganem D. Site-specific RNA binding by a hepatitis B virus reverse transcriptase initiates two distinct reactions: RNA packaging and DNA synthesis. *J Virol*. 1994 Sep;68(9):5579-87.
149. Zoulim F, Seeger C. Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. *J Virol*. 1994 Jan;68(1):6-13.
150. Liu N, Tian R, Loeb DD. Base pairing among three cis-acting sequences contributes to template switching during hepadnavirus reverse transcription. *Proc Natl Acad Sci U S A*. 2003 Feb 18;100(4):1984-9.
151. Le Pogam S, Chua PK, Newman M, Shih C. Exposure of RNA templates and encapsidation of spliced viral RNA are influenced by the arginine-rich domain of human hepatitis B virus core antigen (HBcAg 165-173). *J Virol*. 2005 Feb;79(3):1871-87.
152. Koniger C, Wingert I, Marsmann M, Rosler C, Beck J, Nassal M. Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. *Proc Natl Acad Sci U S A*. 2014 Oct 7;111(40):E4244-53.
153. Norder H, Courouce AM, Magnius LO. Molecular basis of hepatitis B virus serotype variations within the four major subtypes. *J Gen Virol*. 1992 Dec;73 (Pt 12):3141-5.
154. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol*. 1988 Oct;69 (Pt 10):2575-83.
155. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol*. 1992 May;73 (Pt 5):1201-8.
156. Kramvis A, Kew M, Francois G. Hepatitis B virus genotypes. *Vaccine*. 2005 Mar 31;23(19):2409-23.
157. Kurbanov F, Tanaka Y, Kramvis A, Simmonds P, Mizokami M. When should "I" consider a new hepatitis B virus genotype? *J Virol*. 2008 Aug;82(16):8241-2.
158. Locarnini S, Littlejohn M, Aziz MN, Yuen L. Possible origins and evolution of the hepatitis B virus (HBV). *Semin Cancer Biol*. 2008 Dec;23(6 Pt B):561-75.
159. Shi W, Zhang Z, Ling C, Zheng W, Zhu C, Carr MJ, et al. Hepatitis B virus subgenotyping: history, effects of recombination, misclassifications, and corrections. *Infect Genet Evol*. 2013 Jun;16:355-61.
160. Andernach IE, Nolte C, Pape JW, Muller CP. Slave trade and hepatitis B virus genotypes and subgenotypes in Haiti and Africa. *Emerg Infect Dis*. 2009 Aug;15(8):1222-8.
161. Yousif M, Kramvis A. Genotype D of hepatitis B virus and its subgenotypes: An update. *Hepatol Res*. 2013 Apr;43(4):355-64.
162. Andernach IE, Hubschen JM, Muller CP. Hepatitis B virus: the genotype E puzzle. *Rev Med Virol*. 2009 Jul;19(4):231-40.
163. Kramvis A, Kew MC. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res*. 2007 Jul;37(s1):S9-S19.
164. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, Pinho JR. Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community. *J Gen Virol*. 2010 Feb;91(Pt 2):501-8.

165. Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, Duarte MC, et al. Subgenotype diversity of hepatitis B virus American genotype F in Amerindians from Venezuela and the general population of Colombia. *J Med Virol.* 2008 Jan;80(1):20-6.
166. Mello FC, Souto FJ, Nabuco LC, Villela-Nogueira CA, Coelho HS, Franz HC, et al. Hepatitis B virus genotypes circulating in Brazil: molecular characterization of genotype F isolates. *BMC Microbiol.* 2007;7:103.
167. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Botelho L, Carrilho FJ, et al. Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. *Infect Genet Evol.* 2011 Jan;11(1):103-8.
168. Di Lello FA, Pineiro YLFG, Munoz G, Campos RH. Diversity of hepatitis B and C viruses in Chile. *J Med Virol.* 2009 Nov;81(11):1887-94.
169. Torres C, Pineiro y Leone FG, Pezzano SC, Mbayed VA, Campos RH. New perspectives on the evolutionary history of hepatitis B virus genotype F. *Mol Phylogenet Evol.* 2011 Apr;59(1):114-22.
170. Locarnini S, Littlejohn M, Aziz MN, Yuen L. Possible origins and evolution of the hepatitis B virus (HBV). *Semin Cancer Biol.* 2013 Dec;23(6 Pt B):561-75.
171. Panduro A, Maldonado-Gonzalez M, Fierro NA, Roman S. Distribution of HBV genotypes F and H in Mexico and Central America. *Antivir Ther.* 2013;18(3 Pt B):475-84.
172. Mata Marin JA, Arroyo Anduiza CI, Calderon GM, Cazares Rodriguez S, Fuentes Allen JL, Arias Flores R, et al. Prevalence and resistance pattern of genotype G and H in chronic hepatitis B and HIV co-infected patients in Mexico. *Ann Hepatol.* 2012 Jan-Feb;11(1):47-51.
173. van der Kuyl AC, Zorgdrager F, Hogema B, Bakker M, Jurriaans S, Back NK, et al. High prevalence of hepatitis B virus dual infection with genotypes A and G in HIV-1 infected men in Amsterdam, the Netherlands, during 2000-2011. *BMC Infect Dis.* 2013;13:540.
174. Bertolini DA, Gomes-Gouvea MS, Guedes de Carvalho-Mello IM, Saraceni CP, Sitnik R, Grazziotin FG, et al. Hepatitis B virus genotypes from European origin explains the high endemicity found in some areas from southern Brazil. *Infect Genet Evol.* 2012 Aug;12(6):1295-304.
175. Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, Carrilho FJ. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol.* 2004 Jun;42(6):2455-60.
176. Barros LM, Gomes-Gouvea MS, Kramvis A, Mendes-Correa MC, dos Santos A, Souza LA, et al. High prevalence of hepatitis B virus subgenotypes A1 and D4 in Maranhao state, Northeast Brazil. *Infect Genet Evol.* 2014 Jun;24:68-75.
177. Ribeiro NR, Campos GS, Angelo AL, Braga EL, Santana N, Gomes MM, et al. Distribution of hepatitis B virus genotypes among patients with chronic infection. *Liver Int.* 2006 Aug;26(6):636-42.
178. Sitnik R, Sette H, Jr., Santana RA, Menezes LC, Graca CH, Dastoli GT, et al. Hepatitis B virus genotype E detected in Brazil in an African patient who is a frequent traveler. *Braz J Med Biol Res.* 2007 Dec;40(12):1689-92.
179. Gomes-Gouvea MS, Ferreira AC, Teixeira R, Andrade JR, Ferreira AS, Barros LM, et al. HBV carrying drug-resistance mutations in chronically infected treatment-naive patients. *Antivir Ther.* 2015 Jan 27.
180. Bottecchia M, Souto FJ, O KM, Amendola M, Brandao CE, Niel C, et al. Hepatitis B virus genotypes and resistance mutations in patients under long term lamivudine therapy: characterization of genotype G in Brazil. *BMC Microbiol.* 2008;8:11.
181. Mendes-Correa MC, Pinho JR, Locarnini S, Yuen L, Sitnik R, Santana RA, et al. High frequency of lamivudine resistance mutations in Brazilian patients co-infected with HIV and hepatitis B. *J Med Virol.* 2010 Sep;82(9):1481-8.
182. Silva AC, Spina AM, Lemos MF, Oba IT, Guastini Cde F, Gomes-Gouvea MS, et al. Hepatitis B genotype G and high frequency of lamivudine-resistance mutations among human

- immunodeficiency virus/hepatitis B virus co-infected patients in Brazil. *Mem Inst Oswaldo Cruz*. 2010 Sep;105(6):770-8.
183. Lobato C, Tavares-Neto J, Rios-Leite M, Trepo C, Vitvitski L, Parvaz P, et al. Intrafamilial prevalence of hepatitis B virus in Western Brazilian Amazon region: epidemiologic and biomolecular study. *J Gastroenterol Hepatol*. 2006 May;21(5):863-8.
 184. Nebbia G, Peppia D, Maini MK. Hepatitis B infection: current concepts and future challenges. *Qjm*. 2012 Feb;105(2):109-13.
 185. Taylor BC, Yuan JM, Shamliyan TA, Shaukat A, Kane RL, Wilt TJ. Clinical outcomes in adults with chronic hepatitis B in association with patient and viral characteristics: A systematic review of evidence. *Hepatology*. 2009 May;49(5 Suppl):S85-95.
 186. Kwon H, Lok AS. Hepatitis B therapy. *Nat Rev Gastroenterol Hepatol*. 2011 May;8(5):275-84.
 187. Da Silva LC, Madruga CL, Carrilho FJ, Pinho JR, Saez-Alquezar A, Santos C, et al. Spontaneous hepatitis B surface antigen clearance in a long-term follow-up study of patients with chronic type B hepatitis. Lack of correlation with hepatitis C and D virus superinfection. *J Gastroenterol*. 1996 Oct;31(5):696-701.
 188. Bowden S. Serological and molecular diagnosis. *Semin Liver Dis*. 2006 May;26(2):97-103.
 189. Hadziyannis E, Hadziyannis SJ. Hepatitis B surface antigen quantification in chronic hepatitis B and its clinical utility. *Expert Rev Gastroenterol Hepatol*. 2014 Feb;8(2):185-95.
 190. Maylin S, Boyd A, Martinot-Peignoux M, Delaugerre C, Bagnard G, Lapalus M, et al. Quantification of hepatitis B e antigen between Elecsys HBeAg and Architect HBeAg assays among patients infected with hepatitis B virus. *J Clin Virol*. 2013 Apr;56(4):306-11.
 191. Da Silva LC, Nova ML, Ono-Nita SK, Pinho JR, Sitnik R, Santos VA, et al. Simultaneous quantitation of serum HBV DNA and HBeAg can distinguish between slow and fast viral responses to antiviral therapy in patients with chronic hepatitis B. *Rev Inst Med Trop Sao Paulo*. 2009 Sep-Oct;51(5):261-8.
 192. Lin HJ, Wu PC, Lai CL, Chak W. Micromethod for phosphonoformate inhibition assay of hepatitis B viral DNA polymerase. *Clin Chem*. 1984 Apr;30(4):549-52.
 193. Miyamoto YP, JRR. Análise de alguns parâmetros envolvidos com o ensaio da DNA polimerase do vírus da Hepatite B. *Revista do Instituto Adolfo Lutz* 1990;50(1):297 – 9
 194. Scotto J, Hadchouel M, Hery C, Yvart J, Tiollais P, Brechot C. Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique: comparison with results for other viral markers. *Hepatology*. 1983 May-Jun;3(3):279-84.
 195. Sitnik R, Paes A, Manguiera CP, Pinho JR. A real-time quantitative assay for hepatitis B DNA virus (HBV) developed to detect all HBV genotypes. *Rev Inst Med Trop Sao Paulo*. 2010 May-Jun;52(3):119-24.
 196. Thibault V, Pichoud C, Mullen C, Rhoads J, Smith JB, Bitbol A, et al. Characterization of a new sensitive PCR assay for quantification of viral DNA isolated from patients with hepatitis B virus infections. *J Clin Microbiol*. 2007 Dec;45(12):3948-53.
 197. Bowden S, Locarnini S. Molecular Diagnostics in Hepatitis B. In: Marschall S, Runge MD P, Cam Patterson MD, editor. *Principles of Molecular Medicine*: Humana Press; 2006. p. 554-66.
 198. Haleboua-De Marzio D, Hann HW. Then and now: the progress in hepatitis B treatment over the past 20 years. *World J Gastroenterol*. 2014 Jan 14;20(2):401-13.
 199. Da Silva LC, Pinho JR, Sitnik R, Da Fonseca LE, Carrilho FJ. Efficacy and tolerability of long-term therapy using high lamivudine doses for the treatment of chronic hepatitis B. *J Gastroenterol*. 2001 Jul;36(7):476-85.
 200. EASL. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol*. 2012 Jul;57(1):167-85.

201. Araujo NM, Waizbort R, Kay A. Hepatitis B virus infection from an evolutionary point of view: how viral, host, and environmental factors shape genotypes and subgenotypes. *Infect Genet Evol.* 2011 Aug;11(6):1199-207.
202. Guirgis BS, Abbas RO, Azzazy HM. Hepatitis B virus genotyping: current methods and clinical implications. *Int J Infect Dis.* 2010 Nov;14(11):e941-53.
203. Lin CL, Kao JH. The clinical implications of hepatitis B virus genotype: Recent advances. *J Gastroenterol Hepatol.* 2011 Jan;26 Suppl 1:123-30.
204. McMahon BJ. The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. *Hepatol Int.* 2009 Jun;3(2):334-42.
205. Tanwar S, Dusheiko G. Is there any value to hepatitis B virus genotype analysis? *Curr Gastroenterol Rep.* 2012 Feb;14(1):37-46.
206. McMahon G, Ehrlich PH, Moustafa ZA, McCarthy LA, Dottavio D, Tolpin MD, et al. Genetic alterations in the gene encoding the major HBsAg: DNA and immunological analysis of recurrent HBsAg derived from monoclonal antibody-treated liver transplant patients. *Hepatology.* 1992 May;15(5):757-66.
207. Cooksley WG. Do we need to determine viral genotype in treating chronic hepatitis B? *J Viral Hepat.* 2010 Sep;17(9):601-10.
208. Alavian SM, Carman WF, Jazayeri SM. HBsAg variants: diagnostic-escape and diagnostic dilemma. *J Clin Virol.* 2013 Jul;57(3):201-8.
209. Rodriguez-Frias F, Buti M, Tabernero D, Homs M. Quasispecies structure, cornerstone of hepatitis B virus infection: mass sequencing approach. *World J Gastroenterol.* 2013 Nov 7;19(41):6995-7023.
210. Ponde RA. The underlying mechanisms for the "simultaneous HBsAg and anti-HBs serological profile". *Eur J Clin Microbiol Infect Dis.* 2011 Nov;30(11):1325-40.
211. Huang CH, Yuan Q, Chen PJ, Zhang YL, Chen CR, Zheng QB, et al. Influence of mutations in hepatitis B virus surface protein on viral antigenicity and phenotype in occult HBV strains from blood donors. *J Hepatol.* 2012 Oct;57(4):720-9.
212. Zanetti AR, Tanzi E, Manzillo G, Maio G, Sbreglia C, Caporaso N, et al. Hepatitis B variant in Europe. *Lancet.* 1988 Nov 12;2(8620):1132-3.
213. Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, et al. Vaccine-induced escape mutant of hepatitis B virus. *Lancet.* 1990 Aug 11;336(8711):325-9.
214. Okamoto H, Yano K, Nozaki Y, Matsui A, Miyazaki H, Yamamoto K, et al. Mutations within the S gene of hepatitis B virus transmitted from mothers to babies immunized with hepatitis B immune globulin and vaccine. *Pediatr Res.* 1992 Sep;32(3):264-8.
215. Bertolini DA, Ribeiro PC, Lemos MF, Saraceni CP, Pinho JR. Characterization of a Hepatitis B virus strain in southwestern Parana, Brazil, presenting mutations previously associated with anti-HBs Resistance. *Rev Inst Med Trop Sao Paulo.* 2010 Jan-Feb;52(1):25-30.
216. Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, Decker R. Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. *Lancet.* 1995 Jun 3;345(8962):1406-7.
217. Rasenack JW, Schlayer HJ, Hettler F, Peters T, Preisler-Adams S, Gerok W. Hepatitis B virus infection without immunological markers after open-heart surgery. *Lancet.* 1995 Feb 11;345(8946):355-7.
218. Sallie R, Rayner A, Naoumov N, Portmann B, Williams R. Occult HBV in NANB fulminant hepatitis. *Lancet.* 1993 Jan 9;341(8837):123.
219. Wright TL, Mamish D, Combs C, Kim M, Donegan E, Ferrell L, et al. Hepatitis B virus and apparent fulminant non-A, non-B hepatitis. *Lancet.* 1992 Apr 18;339(8799):952-5.
220. Chazouilleres O, Mamish D, Kim M, Carey K, Ferrell L, Roberts JP, et al. "Occult" hepatitis B virus as source of infection in liver transplant recipients. *Lancet.* 1994 Jan 15;343(8890):142-6.

221. Gomes-Gouvêa M. Prevalência de resistência primária aos antivirais utilizados no tratamento da hepatite B entre pacientes com infecção crônica pelo vírus da hepatite B não submetidos a tratamento São Paulo: Universidade de São Paulo; 2014.
222. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology*. 2009 Nov;137(5):1593-608 e1-2.
223. Stuyver LJ, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, et al. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. *Hepatology*. 2001 Mar;33(3):751-7.
224. Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, Pawlotsky JM, et al. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology*. 2007 Jul;46(1):254-65.
225. Locarnini SA, Yuen L. Molecular genesis of drug-resistant and vaccine-escape HBV mutants. *Antivir Ther*. 2010;15(3 Pt B):451-61.
226. Karatayli E, Karatayli SC, Cinar K, Gokahmetoglu S, Guven K, Idilman R, et al. Molecular characterization of a novel entecavir mutation pattern isolated from a multi-drug refractory patient with chronic hepatitis B infection. *J Clin Virol*. 2012 Feb;53(2):130-4.
227. Shaw T, Bartholomeusz A, Locarnini S. HBV drug resistance: mechanisms, detection and interpretation. *J Hepatol*. 2006 Mar;44(3):593-606.
228. Sheldon J, Rodes B, Zoulim F, Bartholomeusz A, Soriano V. Mutations affecting the replication capacity of the hepatitis B virus. *J Viral Hepat*. 2006 Jul;13(7):427-34.
229. Fu L, Cheng YC. Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(-)SddC (3TC) resistance. *Biochem Pharmacol*. 1998 May 15;55(10):1567-72.
230. Bartholomeusz A, Locarnini S. Hepatitis B virus mutations associated with antiviral therapy. *J Med Virol*. 2006;78 Suppl 1:S52-5.
231. Ji D, Liu Y, Si LL, Li L, Chen GF, Xin SJ, et al. Variable influence of mutational patterns in reverse-transcriptase domain on replication capacity of hepatitis B virus isolates from antiviral-experienced patients. *Clin Chim Acta*. 2011 Jan 30;412(3-4):305-13.
232. Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J Hepatol*. 2006 Feb;44(2):422-31.
233. Lai MW, Huang SF, Hsu CW, Chang MH, Liaw YF, Yeh CT. Identification of nonsense mutations in hepatitis B virus S gene in patients with hepatocellular carcinoma developed after lamivudine therapy. *Antivir Ther*. 2009;14(2):249-61.
234. Lee YS, Chung YH, Kim JA, Jin YJ, Park WH, Kim SE, et al. rtL180M mutation of hepatitis B virus is closely associated with frequent virological resistance to adefovir dipivoxil therapy. *J Gastroenterol Hepatol*. 2012 Feb;27(2):300-5.
235. Karatayli E, Karayalcin S, Karaaslan H, Kayhan H, Turkyilmaz AR, Sahin F, et al. A novel mutation pattern emerging during lamivudine treatment shows cross-resistance to adefovir dipivoxil treatment. *Antivir Ther*. 2007;12(5):761-8.
236. Liu LJ, Wang JH, Du SC, Tian JH, Yang RF, Wei L. rtE218G, a novel hepatitis B virus mutation with resistance to adefovir dipivoxil in patients with chronic hepatitis B. *J Viral Hepat*. 2010 Mar;17 Suppl 1:66-72.
237. Qin Bo PR, HE TingTing, Huang ZhaoHui, PAN GuoShao, TU ChunYu, LU MengJi, CHEN XinWen Polymerase mutations rtN238R, rtT240Y and rtN248H of hepatitis B virus decrease susceptibility to adefovir. *Chinese Science Bulletin*. 2013;58(15):1760-6.
238. Locarnini S. Molecular virology and the development of resistant mutants: implications for therapy. *Semin Liver Dis*. 2005;25 Suppl 1:9-19.
239. Amini-Bavil-Olyaei S, Herbers U, Mohebbi SR, Sabahi F, Zali MR, Luedde T, et al. Prevalence, viral replication efficiency and antiviral drug susceptibility of rtQ215 polymerase mutations within the hepatitis B virus genome. *J Hepatol*. 2009 Oct;51(4):647-54.

240. Baldick CJ, Tenney DJ, Mazzucco CE, Eggers BJ, Rose RE, Pokornowski KA, et al. Comprehensive evaluation of hepatitis B virus reverse transcriptase substitutions associated with entecavir resistance. *Hepatology*. 2008 May;47(5):1473-82.
241. Sheldon J, Camino N, Rodes B, Bartholomeusz A, Kuiper M, Tacke F, et al. Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. *Antivir Ther*. 2005;10(6):727-34.
242. Delaney WEt, Ray AS, Yang H, Qi X, Xiong S, Zhu Y, et al. Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. *Antimicrob Agents Chemother*. 2006 Jul;50(7):2471-7.
243. Qin B, Budeus B, Cao L, Wu C, Wang Y, Zhang X, et al. The amino acid substitutions rtP177G and rtF249A in the reverse transcriptase domain of hepatitis B virus polymerase reduce the susceptibility to tenofovir. *Antiviral Res*. 2013 Feb;97(2):93-100.
244. Zhu Y, Curtis M, Borroto-Esoda K. The YMDD and rtA194T mutations result in decreased replication capacity in wild-type HBV as well as in HBV with precore and basal core promoter mutations. *Antivir Chem Chemother*. 2011;22(1):13-22.
245. Huang SF, Chen YT, Lee WC, Chang IC, Chiu YT, Chang Y, et al. Identification of transforming hepatitis B virus S gene nonsense mutations derived from freely replicative viruses in hepatocellular carcinoma. *PLoS One*. 2014;9(2):e89753.
246. Chotiayaputta W, Lok AS. Hepatitis B virus variants. *Nat Rev Gastroenterol Hepatol*. 2009 Aug;6(8):453-62.
247. Lee SA, Kim K, Kim H, Kim BJ. Nucleotide change of codon 182 in the surface gene of hepatitis B virus genotype C leading to truncated surface protein is associated with progression of liver diseases. *J Hepatol*. 2012 Jan;56(1):63-9.
248. Quarleri J. Core promoter: a critical region where the hepatitis B virus makes decisions. *World J Gastroenterol*. 2014 Jan 14;20(2):425-35.
249. Barros LM, Gomes-Gouvea MS, Pinho JR, Alvarado-Mora MV, Dos Santos A, Mendes-Correa MC, et al. Hepatitis Delta virus genotype 8 infection in Northeast Brazil: inheritance from African slaves? *Virus Res*. 2011 Sep;160(1-2):333-9.
250. Schaefer S. Hepatitis B virus genotypes in Europe. *Hepatol Res*. 2007 Jul;37(s1):S20-6.
251. Ito K, Tanaka Y, Orito E, Sugiyama M, Fujiwara K, Sugauchi F, et al. T1653 mutation in the box alpha increases the risk of hepatocellular carcinoma in patients with chronic hepatitis B virus genotype C infection. *Clin Infect Dis*. 2006 Jan 1;42(1):1-7.
252. Brichtler S, Lagathu G, Chekaraou MA, Le Gal F, Edouard A, Deny P, et al. African, Amerindian and European hepatitis B virus strains circulate on the Caribbean Island of Martinique. *J Gen Virol*. 2013 Oct;94(Pt 10):2318-29.
253. Liu S, Zhang H, Gu C, Yin J, He Y, Xie J, et al. Associations between hepatitis B virus mutations and the risk of hepatocellular carcinoma: a meta-analysis. *J Natl Cancer Inst*. 2009 Aug 5;101(15):1066-82.
254. Margeridon-Thermet S, Svarovskaia ES, Babrzadeh F, Martin R, Liu TF, Pacold M, et al. Low-level persistence of drug resistance mutations in hepatitis B virus-infected subjects with a past history of Lamivudine treatment. *Antimicrob Agents Chemother*. 2013 Jan;57(1):343-9.