MÓNICA VIVIANA ALVARADO MORA

Estudos sobre infecções pelos vírus da Hepatite B (HBV), Hepatite C (HCV), Hepatite Delta (HDV) e vírus GB-C (GBV-C) em diferentes regiões da América do Sul

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências.

Programa de Gastroenterologia Clinica

Orientador: Dr. João Renato Rebello Pinho

São Paulo

2011

Dados Internacionais de Catalogação na Publicação (CIP)

Preparada pela Biblioteca da
Faculdade de Medicina da Universidade de São Paulo

Alvarado-Mora, Mónica Viviana
Estudos sobre infecções pelos vírus da Hepatite B (HBV), Hepatite C (HCV), Hepatite Delta (HDV) e vírus GB-C (GBV-C) em diferentes regiões da América do Sul / Mónica Viviana Alvarado Mora. -- São Paulo, 2011.
Tese(doutorado)--Faculdade de Medicina da Universidade de São Paulo. Programa de Gastroenterologia Clínica.
Orientador: João Renato Rebello Pinho.

Descritores: 1.Hepatite B 2.Hepatite C 3.Vírus delta da hepatite 4.Vírus GB-C 5.Epidemiologia 6.Doadores de sangue 7.População indígena 8.Colômbia 9.Brasil 10.Chile

USP/FM/DBD-287/11

DEDICATÓRIA

A minha mãe, Edilma.

A meu pai, Jorge.

A meu irmão, Alejandro.

"All our dreams can come true, if we have the courage to pursue them"

Walt Disney

AGRADECIMENTOS

A Deus pela sua companhia, cuidado, amor e confiança durante todo esse período de novos conhecimentos e crescimento pessoal;

A minha família: mãe, pai e irmão pelo acolher incondicional, confiança e estímulo em cada um dos dias que passei longe de casa. Agradeço por suportarmos juntos a saudade da distância, pois sabemos que apesar de ser difícil conseguimos vence-la e atingir os objetivos propostos;

Ao meu orientador, Dr. João Renato Rebello Pinho, por ser meu amigo e guia em todo esse processo, por confiar e acreditar em cada objetivo proposto, pela sua dedicação e apoio incondicional que enriqueceram a cada dia minha formação profissional e pessoal;

A minha co-orientadora, Dra. Maria Fernanda Gutierrez Fernandez, amiga e cúmplice durante a realização do meu trabalho, pelo incentivo e carinho que sempre me proporciona;

Ao Prof. Dr. Flair José Carrilho pela oportunidade de fazer parte do departamento de Gastroenterologia da FMUSP, pelo reconhecimento e aprovação para cada trabalho proposto;

A Dra. Camila Malta Romano, amiga e mestre nesse processo, pela sua dedicação e interesse em cada um dos trabalhos realizados, pela vontade de me ensinar e compreender cada uma das fases do processo;

A Michele Soares Gomes-Gouvêa, amiga e companheira, agradeço pela dedicação, interesse no trabalho e companhia dia a dia no processo assim como pelo incentivo de continuar acreditando em cada uma das minhas ideias;

A Lívia de Souza Botelho agradeço pela contribuição incondicional e dedicação que sempre demostrou na realização dos nossos objetivos, por me permitir orienta-lhe e ensinar-lhe com carinho e confiança os processos na bancada do laboratório;

Ao Dr. Raymundo de Azevedo Neto pela contribuição com as analises estatísticas em varias fases do estudo;

A Anna Nishiya pela sua disposição e dedicação no trabalho com o vírus GB-C;

A todas as pessoas que participaram do estudo aportando uma amostra de sangue para os estudos, agradeço pela confiança depositada em mim e em toda a equipe que dia a dia se dedicou a contribuir com dados epidemiológicos e moleculares importantes na área das hepatites virais;

Aos enfermeiros, médicos e pessoal administrativo dos laboratórios de saúde publica de Leticia, Quibdó, Santa Marta e San Andrés, por ter apoiado nosso projeto permitindo a utilização da sua infraestrutura no processamento das amostras;

Ao *Banco Nacional de Sangre de la Cruz Roja Colombiana* pelo apoio e contribuição com as amostras dos doadores de sangue;

Aos nosso colaboradores no Brasil, estado de Rondônia: Dr. Juan Miguel Villalobos Salcedo, Alcione Santos, Deusilene Vieira, assim como aos nosso colaboradores de Pernambuco; Izolda Maria Moura e Dr. Edmundo Lopes, pela confiança e oportunidade de trabalharmos juntos;

Aos nosso colaboradores Chilenos, Dr. Mauricio Venegas, Dr. Rodrigo Villanueva e Dr. Javier Brahms do *Clinical Hospital*, *University of Chile* em Santiago do Chile, pela amizade e oportunidade de trabalharmos juntos;

Ao Dr. Stephen Locarnini e Dra. Lilly Yuen do *Victorian Infectious Diseases Reference Laboratory,* Austrália, pela oportunidade de trabalhamos juntos no estudo do Chile;

A Tania Tozetto, Dra. Vanda Akiko Ueda de Souza e Dr. Claudio Sergio Panutti por permitir-me trabalhar em colaboração com as amostras do estado do Maranhão;

Aos meus amigos e colegas do *Laboratório de Gastroenterologia e Hepatologia Tropical - FMUSP* pela sua companhia, incentivo e apoio nesse processo;

As minhas amigas e amigos do *Instituto de Medicina Tropical - FMUSP* pelo incentivo e reconhecimento constante ao meu trabalho;

A minha banca de qualificação pela avaliação detalhada de cada um dos trabalhos assim como pelas sugestões propostas e reconhecimento a minha dedicação;

A cada uma das pessoas que de uma ou outra forma sempre estiveram perto de mim com sua alegria e disposição de me ajudar muitas vezes para

aprender melhor a língua portuguesa assim como para viver na cidade de São Paulo.

RESUMO

Alvarado-Mora, MV. Estudos sobre infecções pelos vírus das Hepatite B (HBV), Hepatite C (HCV), Hepatite Delta (HDV) e vírus GB-C (GBV-C) em diferentes regiões da América do Sul. [Tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2011.

Descritores: Hepatite B, Hepatite C, Vírus Delta da hepatite (HDV), vírus GB-C, Epidemiologia, Doadores de Sangue, população indígena, Colômbia, Brasil, Chile.

As hepatites virais estão entre as mais importantes pandemias mundiais da atualidade. Existem várias causas de hepatite, entre elas, o vírus da hepatite B (HBV), o vírus da hepatite C (HCV) e o vírus da Hepatite Delta (HDV). Da mesma forma, o vírus GB-C (GBV-C) é importante na co-infecção com outros vírus, como o HIV. Nesse estudo, várias regiões da América do Sul foram analisadas. Na Colômbia, os estados do Amazonas e Magdalena foram encontradas como regiões hiperendêmicas para HBV. O genótipo F3 (75%) foi o mais prevalente. Determinou-se que o subgenótipo F3 é o mais antigo dos subgenótipos F. No estado de Chocó, encontrou-se o subgenótipo A1 (52,1%) como o mais prevalente. Surpreendentemente, nesse mesmo estado foram encontrados nove casos autóctones de infecção pelo genótipo E (39,1%). Para o HCV, em Bogotá, encontrou-se o subtipo 1b (82,8%) como o mais prevalente. Da mesma forma, estimou-se que esse subtipo foi introduzido por volta de 1950 e se propagou exponencialmente entre 1970 a 1990. O HDV foi identificado em casos de hepatite fulminante do estado de Amazonas, todos classificados como genótipo 3. Se determinou que o HDV/3 se espalhou exponencialmente a partir de 1950 a 1970 na América do Sul e depois desta época, esta infecção deixou de aumentar, provavelmente devido a introdução de vacinação contra o HBV. GBV-C foi procurado em doadores de sangue colombianos infectados com HCV e/ou HBV de Bogotá e em povos indígenas com infecção pelo HBV no Amazonas. A análise filogenética revelou a presença do genótipo 2a como o mais prevalente entre os doadores de sangue e o 3 nos povos indígenas estudados. A presença do genótipo 3 na população indígena foi previamente relatada na região de Santa Marta, na Colômbia e nos povos indígenas da Venezuela e da Bolívia. No Chile, foi realizado um estudo com 21 pacientes cronicamente infectados pelo HBV sem tratamento antiviral prévio. Todas as sequências obtidas eram do subgenótipo F1b e se agrupavam em quatro diferentes grupos, sugerindo que diferentes linhagens desse subgenótipo estão circulando no Chile. No Brasil, no estado de Rondônia, para o HCV, encontramos o subtipo 1b (50,0%) como o mais frequente. Esse foi o primeiro relato sobre os genótipos do HCV neste estado. Para o HBV, o subgenótipo A1 (37,0%) foi o mais frequente. Os resultados do estado de Rondônia são consistentes com outros estudos no Brasil, mostrando a presença de vários genótipos do HBV, refletindo a origem mista da população Brasileira. Estudando o estado do Maranhão, avaliamos a frequência da infecção pelo HBV e seus genótipos. Foram

encontradas 4 sequencias genótipo A1 que agruparam com outras sequências reportadas do Brasil. Em outro estudo, caracterizamos os subgenótipos do HBV em 68 pacientes com hepatite crônica B em Pernambuco, encontrando 78,7% de presença do subgenótipo A1. Finalmente, em um estudo realizado com amostras da cidade de São Paulo, encontramos um caso de HBV genótipo C em um brasileiro nativo, sendo essa a primeira sequência completa do genoma de HBV/C2 notificados no Brasil.

ABSTRACT

Alvarado-Mora, MV. Studies on viral infections by Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis Delta virus (HDV) and GB virus C (GBV-C) in different regions of South America. [Thesis]. São Paulo: School of Medicine, University of São Paulo, 2011.

Keywords: Hepatitis B, Hepatitis C, Hepatitis Delta Virus (HDV), GB virus C, Epidemiology, Blood Donors, indigenous, Colombia, Brazil, Chile.

Viral hepatitis are among the major pandemics in the world nowadays. There are many causes of hepatitis, including hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis delta virus (HDV). Similarly, GB virus C (GBV-C) is a relevant agent in co-infection with HIV. In this study, several regions of South America were studied. In Colombia, the states of Amazonas and Magdalena were identified as highly endemic areas for HBV. Genotype F3 (75%) was the most prevalent. It was determined that subgenotype F3 is the oldest among all F subgenotypes. In the state of Chocó, subgenotype A1 (52.1%) was the most prevalent. Surprisingly, nine indigenous cases of infection by genotype E (39.1%) were found in this state. For HCV, in Bogotá, subtype 1b (82.8%) was the most frequent. Likewise, it was estimated that this subtype was introduced around 1950 and spread exponentially from 1970 to 1990. HDV has been identified in cases of fulminant hepatitis in the state of Amazonas, all of them classified as genotype 3. It was determined that the HDV/3 spread exponentially from 1950 to 1970 in South America and after this time, this infection stopped to increase, probably due to introduction of vaccination against HBV. GBV-C was sought in Colombian blood donors infected with HCV and/or HBV in Bogotá and indigenous peoples with HBV infection in the Amazon. The phylogenetic analysis revealed the presence of genotype 3 as the most prevalent among blood donors and in three studied indigenous people. The presence of genotype 3 in the indigenous population has been previously reported in the region of Santa Marta, Colombia, and in the indigenous peoples of Venezuela and Bolivia. In Chile, a study was carried out with 21 patients chronically infected with HBV without any prior antiviral treatment. All sequences obtained belonged to subgenotype F1b and clustered into four different groups, suggesting that different strains that are circulating in Chile. In Brazil, the state of Rondônia, we found HCV subtype 1b (50.0%) as the most frequent. This was the first report on HCV genotypes in this state. For HBV, subgenotype A1 (37.0%) was the most frequent. The results of the state of Rondônia are consistent with other studies carried out in Brazil, showing the presence of several HBV genotypes, reflecting the mixed origin of the Brazilian population. Studying the state of Maranhão, we evaluated the frequency of HBV infection and its genotypes and we found 4 genotype A1 sequences that grouped with other sequences reported in Brazil. In another study, we characterized HBV subgenotypes in 68 patients with chronic hepatitis B in Pernambuco and we found subgenotype A1 in 78.7%

cases. Finally, in a study of samples from São Paulo, we found a case of HBV genotype C in a native Brazilian patient and this is the first complete genome sequence of HBV/C2 reported in Brazil.

SUMÁRIO

1. INTRODUÇÃO

As interações entre os vírus e os seres humanos levam a diferentes patologias, com variados graus de gravidade, que se apresentam na forma de infecções agudas ou crônicas. Aqueles patógenos que provavelmente interagem com o ser humano há muito tempo, tendem a originar infecções crônicas de evolução lenta, que podem evoluir ou não após muitos anos de infecção para formas mais graves da doença, associadas a debilitações importantes de funções orgânicas ou mesmo com carcinogênese.

O estudo de agentes virais com estas características, através da detecção de sua presença e caracterização de seus variantes genéticos em diferentes grupos populacionais, permite que se possa adotar medidas efetivas para o controle destas infecções, não só pelo conhecimento dos mecanismos de transmissão, como também pela indicação do tratamento antiviral mais adequado para os pacientes infectados. Além disso, estudos detalhados das características das populações virais em diferentes regiões do mundo, através da avaliação da diversidade genética viral pela análise das diferentes sequências nucleotídicas obtidas, tornam possível inferir a dinâmica da expansão da infecção viral nos diferentes grupos populacionais.

As hepatites virais estão entre as mais importantes infecções mundiais na atualidade. Existem várias causas de hepatite, sendo as mais frequentes

aquelas causadas pelos vírus da Hepatite A (HAV), Hepatite B (HBV), Hepatite C (HCV), Hepatite Delta (HDV) e Hepatite E (HEV). O vírus da Hepatite G ou vírus GB-C (GBV-C) foi descrito inicialmente como um agente envolvido com hepatites virais, mas esta relação etiológica não se sustentou. Entre os cinco agentes infecciosos causadores de hepatites; HBV, HCV e HDV, podem persistir após a infecção aguda e causar infecções crônicas, que podem levar a doença grave do fígado, como hepatite crônica, cirrose hepática e carcinoma hepatocelular.

Um outro aspecto importante do comportamento epidemiológico dos vírus causadores de doença hepática crônica é a tendência de alguns desses agentes se estabelecerem em alguns grupos populacionais devido a fatores geográficos, sociais ou culturais.

Nossos estudos envolveram principalmente a Colômbia, país do qual foram obtidas amostras de diferentes localidades. Este foi o foco principal desse trabalho, pois haviam poucos dados publicados sobre estes vírus neste país e que desperta também grande interesse por este estar localizado na extremidade norte do continente sul americano, por onde, obrigatoriamente, as primeiras populações humanas tiveram que passar para o povoamento da América do Sul a partir da América do Norte há pelo menos 10.000 anos atrás.

A Colômbia, oficialmente República da Colômbia, é uma república constitucional do noroeste da América do Sul. Este país faz fronteira à leste com Venezuela e Brasil; ao sul, com o Equador e Peru; à norte, com o Mar do

Caribe; ao noroeste, com o Panamá; e a oeste, com o Oceano Pacífico. Com uma população de mais de 45 milhões de habitantes, tem a 29ª maior população do mundo e a 2ª maior da América do Sul, após o Brasil. Da mesma forma, tem a 4ª maior população de língua espanhola no mundo, depois do México, EUA e Espanha.

A Colômbia é etnicamente diversa e a interação entre os descendentes da população nativa indígenas com colonos espanhóis, com africanos trazidos como escravos entre os séculos XVI e XIX, e com imigrantes da Europa e do Oriente Médio durante o século XX, produziu uma população particular com características próprias. As condições de saúde na Colômbia têm melhorado muito desde a década de 1980. Uma reforma em 1993 transformou a estrutura do financiamento da saúde pública. Como resultado, os empregados pagam planos de saúde aos quais os empregadores também contribuem. Embora este sistema tenha ampliado a cobertura da população pelo sistema de segurança social e de saúde de 21% (pré-1993) para 56% em 2004 e 66% em 2005, persistem disparidades de saúde e a população mais pobre apresenta taxas de morbidade e mortalidade mais elevadas.

Quanto às pesquisas sobre os vírus das hepatites, até a presente data, existem poucos estudos realizados. Alguns estudos demonstram que o HBV é endêmico nas regiões de Santa Marta e Amazonas, causando vários casos graves de hepatite fulminante, com elevada mortalidade. Da mesma forma, a cobertura dos doadores de sangue com anti-HCV nos bancos de sangue vem

aumentando desde o começo dos anos 90, mas poucos estudos têm avaliado a prevalência destes agentes virais causadores de hepatites na população colombiana.

Os estudos realizados no presente compêndio abrangeram também outros países da América do Sul, no qual nos concentramos apenas nas hepatites virais B e C. No Brasil, foram realizados em três estados: 1) Rondônia, na região Norte do país, complementando outros estudos de nosso grupo realizados no estado do Amazonas, por ser um estado com fronteiras com países de colonização espanhola; 2) Maranhão, na região Nordeste, que foi estudado por ser um estado com uma importante população de origem africana, em semelhança ao estado de Quibdó, na Colômbia; 3) Pernambuco, no extremo leste do continente, onde foi possível verificar a presença dos genótipos do HBV e comparar com os descritos nas outras regiões.

Ademais, tivemos a oportunidade de estudar o Chile, outro país de colonização espanhola, no extremo sul de nosso continente, mas que guarda com a Colômbia muitas semelhanças, não só em termos de sua história, como também pelo fato de ser outro país onde já se descrevia o predomínio do genótipo F da hepatite B, que é o genótipo mais frequentemente encontrado em outros países latino-americanos de língua espanhola já estudados, desde o México até a Argentina.

Finalmente, foi realizado um estudo em uma população de pacientes com hepatite B acompanhados na cidade de São Paulo, no qual encontramos

pela primeira vez no Brasil um caso de genótipo C em uma paciente de origem ocidental que havia morado no Japão.

OBJETIVOS

O objetivo deste estudo é relatar as frequências das infecções por HBV, HCV e HDV em várias regiões da Colômbia, assim como caracterizar os genótipos circulantes no país e avaliar a dinâmica das infecções virais nas populações onde são encontrados.

Além disso, considerando o trabalho dispendido durante a coleta das amostras, realizamos vários estudos em paralelo com o vírus GB-C nas amostras previamente positivas para HBV e HCV.

Através de outras colaborações com grupos do Brasil e Chile, foi possível aplicar as mesmas metodologias desenvolvidas para o trabalho com a Colômbia em outros trabalhos de caracterização dos vírus das hepatites de transmissão parenteral em outras regiões. Estes estudos foram importantes para a determinação do perfil epidemiológico destas infecções nestas regiões, em especial quanto aos genótipos virais encontrados e para estudos da dinâmica do espalhamento destas infecções virais pelo continente sul-americano.

Sendo assim, com este projeto foram publicados 11 trabalhos em revistas de impacto, e mais de 25 resumos já foram apresentados em vários eventos nacionais e internacionais. Dessa forma, quatro capítulos estão sendo apresentados nesse texto evidenciando as principais conclusões dos trabalhos realizados e em cada capitulo estão anexados os respectivos trabalhos publicados. Finalmente, foi obtida a aprovação pela Pós-graduação da

Faculdade de Medicina da USP da apresentação desta tese como um compêndio dos trabalhos publicados (Anexo 1).

CAPÍTULO 1 : O VÍRUS DA HEPATITE B NA AMÉRICA DO SUL

1.1. Características gerais do vírus da hepatite B

O vírus da hepatite B (HBV) está classificado na família *Hepadnaviridae,* gênero *Orthohepadnavírus*. Apresenta tropismo preferencial pelo fígado e se caracteriza por ser um vírus de DNA circular de dupla fita parcial, que se replica através de um RNA intermediário mediante transcrição reversa. A partícula viral apresenta 42 nm de diâmetro e possui um envelope constituído por proteínas de superfície: grande (L), média (M) e pequena (HBsAg) (Schaefer *et al.,* 2007).

O HBV é transmitido através do contato com secreções corporais infectadas. Embora o sangue seja o veículo mais importante para a transmissão, outros fluidos também possuem relação com a infecção, como o sêmen e saliva. Atualmente, três modos de transmissão de HBV estão reconhecidos: (i) vertical, (ii) sexual e (iii) parenteral/percutânea (Hou *et al.,* 2005). Acredita-se que outras formas de transmissão, como a horizontal, causada por contatos próximos, mas não sexuais, devem ter seu papel na elevada frequência desta infecção.

A prevalência do HBV e seus padrões de transmissão variam nas diferentes partes do mundo, já que dependem da taxa de infecção crônica, da proporção de pessoas com o vírus em replicação ativa e da via de transmissão predominante em cada região (Mahoney, 1999). Em função dessas

características, as populações no mundo foram classificadas em três grupos de acordo com a prevalência do antígeno de superfície (HBsAg) do HBV: alta (>8%), intermediária (2-8%) e baixa (<2%).

O genoma do HBV é constituído por um DNA com cerca de 3200 pb (pares de bases), circular, com uma fita parcialmente dupla apresentando uma região de fita simples com tamanho variável (Sánchez *et al.,* 2007). O genoma apresenta 4 fases de leitura aberta que se sobrepõem entre si: Pré-S / S, Pré-Core / Core, Polimerase e X. A região Pré -S / S codifica as proteínas do envelope viral, sendo que a tradução das regiões Pré-S1, Pré-S2 e S formam a proteína grande ("L"); a proteína média ("M") deriva da tradução das regiões Pré-S2 e S; e a proteína pequena, conhecida também como HBsAg, forma-se a partir da tradução da região "S". Esta é a principal proteína do envelope, que possui o epitopo "a", contra o qual são dirigidos os anticorpos neutralizantes anti-HBs (Sánchez *et al.,* 2007).

O gene C codifica a proteína do nucleocapsídeo viral, de aproximadamente 21 kDa, o HBcAg. Esta proteína é formada por 183 aminoácidos, e várias subunidades desta proteína agregam-se formando o capsídeo, que tem a função de empacotar o RNA pré-genômico (RNApg) e a polimerase viral (Tiollais *et al.,* 1981; Crowther *et al.,* 1994; Sanchez *et al.,* 2007). O antígeno "e" (HBeAg) é uma forma modificada desta proteína, com peso molecular de 17 kDa, formado quando a tradução se inicia na região Pré – Core. Sua sequência corresponde à proteína do capsídeo com a adição de 29

aminoácidos provenientes da região Pré - C na extremidade amino, que sofre posterior hidrólise nas extremidades amina e carboxila, resultando na secreção do HBeAg, com um peso molecular de 15 a 18 kDa. O HBeAg não é essencial para o vírus, pois existem mutantes viáveis que não produzem esta proteína (Brunetto *et al.,* 1999).

A terceira fase de leitura aberta é a maior região do genoma viral, contém 2.436 nucleotídeos e codifica a polimerase viral. Esta proteína possui 4 domínios: proteína terminal, que se liga à extremidade 5´ do genoma viral durante a replicação; espaçador; DNA polimerase; e RNAse H. A determinação da sequência nucleotídica dos diferentes domínios da DNA polimerase permite avaliar a resistência do HBV às drogas antivirais.

Por último, a quarta fase de leitura aberta é o gene X, que possui 1386 pb. Este gene codifica a proteína X, que pode se ligar ao DNA e ativar promotores celulares (Koike *et al.,* 1995; Sanchez *et al.,* 2007).

O HBV, apesar de possuir DNA como ácido nucléico, utiliza a transcrição reversa para propagar seu genoma, mas a integração do ácido nucléico viral dentro do genoma da célula hospedeira não é obrigatória. A maneira exata pela qual os vírus entram na célula é desconhecida. Após a ligação com receptores celulares, ocorre a fusão da partícula viral com a membrana celular e penetração do nucleocapsídeo na célula, que transporta o DNA viral até o núcleo. No núcleo, ocorre a síntese completa da fita positiva e o DNA viral é

convertido em uma molécula de DNA circular covalentemente fechada (cccDNA - do inglês “covalently closed circular DNA”).

Muitas cópias deste cccDNA são sintetizadas através de um ciclo viral intracelular para servir como molde de RNA mensageiro pré-genômico e subgenômico. No núcleo da célula, ocorre a síntese de RNAs mensageiros destinados à tradução, os quais sintetizam as proteínas estruturais e não estruturais, entre as quais se encontra a polimerase com função de transcriptase reversa, que desencadeia a formação do DNA viral a partir das moléculas de RNA pré - genômico. Estes mRNAs saem pelos poros nucleares e, no citoplasma, ocorre a tradução de proteínas por parte de ribossomos celulares que sintetizam proteínas centrais dos vírus e a polimerase do HBV (Liang T, 2009).

O RNA pré - genômico e a polimerase viral são seletivamente englobados no citoplasma por nucleocapsídeos nascentes formados pelas proteínas do core. Os nucleocapsídeos virais montados são envelopados e as partículas virais completas são exportadas por vesículas transportadoras para fora da célula. Como as proteínas do envelope são produzidas em grande quantidade, ocorre a formação de partículas virais vazias (sem ácido nucléico), constituídas apenas por essas proteínas.

Para a manutenção da infecção, alguns nucleocapsídeos não são envelopados e exportados, mas reiniciam o ciclo de replicação. O DNA genômico replicado é redirecionado para o núcleo com o objetivo de aumentar e

manter o reservatório de moléculas de cccDNA na célula, sem haver necessidade de re-infecção. Algumas moléculas de HBV DNA podem ser integradas ao DNA dos hepatócitos, porém essa integração não é um passo essencial no ciclo de vida do vírus (Nassal, 1999).

1.2 Epidemiologia do HBV na Colômbia

Em torno de dois bilhões de pessoas estão infectadas com o vírus da Hepatite B (HBV) e aproximadamente 350 milhões de pessoas são portadoras crônicas dessa doença no mundo. Cerca de 15 - 40% do pacientes infectados pelo HBV no mundo irão desenvolver cirrose, insuficiência hepática, ou carcinoma hepatocelular (CHC) (Lok *et al.,* 2002). A infecção pelo HBV apresenta de 500.000 a 1,2 milhões de mortes a cada ano, sendo a décima principal causa de morte no mundo (Mahoney*,* 1999). A incidência do carcinoma hepatocelular vem aumentando e causando a morte de 300.000 a 500.000 pessoas a cada ano.

Na América Latina, existem em torno de 400.000 mil novos casos por ano sendo que destes, 10 a 70% desenvolvem carcinoma hepatocelular (CHC) (Lavanchy, 2004; Fay, 1990). Dados epidemiológicos sugerem que 7 a 12 milhões de latino-americanos estão infectados com o HBV. As vias de transmissão na América do Sul e Central são altamente variáveis. A maior prevalência foi relatada para grupos de pessoas com 20 a 40 anos de idade,

suportando a transmissão horizontal entre adultos como a rota mais comum de infecção. Além disso, algumas práticas culturais, tais como a tatuagem, também contribuem para o risco de infecção (Te & Jensen, 2010). Na América Latina, os países que reportam baixa prevalência incluem Bahamas, Barbados, Cuba, Jamaica, Trinidad e Tobago, Costa Rica, El Salvador, Nicarágua, Panamá, México, Argentina, Bolívia, Brasil, Chile, Equador, Paraguai e Uruguai. Entre os países com prevalência intermediária (2 a 8%) encontram-se Haiti, República Dominicana, Guatemala, Honduras, Brasil, Colômbia, Venezuela. As regiões com alta prevalência (>8%) são encontradas na Bacia Amazônica, em partes do norte do Brasil, Colômbia, Peru e Venezuela, onde se estima que mais de 30% dos portadores na América do Sul, estejam localizados (Tanaka, 2000).

Na Colômbia, existem poucos estudos epidemiológicos sobre os vírus das hepatites. Existem cinco áreas na Colômbia nas quais mais de 70% da população apresenta sinais de infecção pelo HBV: costa do Caribe, Golfo Urabá, costa do Pacífico, Bacia Amazônica e na fronteira com a Venezuela. Um primeiro estudo epidemiológico realizado em 1980, relatou prevalência de HBsAg de 3% a 8% em diferentes grupos etários. A partir desses achados estimou-se que existam 600.000 portadores do HBV e quatro milhões pessoas que tiveram contato com o HBV na Colômbia (Buitrago *et al.,* 1986a; Buitrago *et al.,* 1986b; Martinez *et al.,*1991), o qual classificou a Colômbia inicialmente dentro do grupo de países com prevalência elevada para o HBV.

Em 2007, foi realizada uma avaliação dos marcadores sorológicos do HBV (HBsAg, anti-HBc e anti-HBs) numa população de 696 habitantes provenientes de quatro localidades da Colômbia: Amazonas (n=184); Chocó (n=138); Magdalena (n=218) e San Andrés (n=156) **(Alvarado-Mora *et al.,* 2011a)**. Selecionando a população de pessoas maiores de 11 anos, encontrou-se prevalência de HBsAg de 5,66% e de anti-HBc de 28,43%, o qual classifica atualmente a Colômbia dentro dos países com prevalência intermediária para o HBV. Entretanto, foram encontradas diferenças entre as populações avaliadas: os estados de Amazonas e Magdalena apresentaram maiores frequências para todos os marcadores do HBV em comparação com San Andrés e Chocó. Encontrou-se significância estatística quanto a distribuição das frequências para HBsAg ($p=0,033$), anti-HBc ($p<0,001$) e anti-HBs ($p<0,001$) em relação à origem das amostras. Da mesma forma, foi encontrado que a frequência do anti-HBc aumenta de forma significante com a idade, com menor frequência no grupo entre 11 e 15 anos e maior frequência no grupo com mais de 51 anos de idade ($p<0,001$). Para o anti-HBs isolado (marcador de vacinação prévia), foi encontrada uma frequência de 27,61% na população em geral. O estado de Chocó apresentou a maior porcentagem de vacinação na população (53,26%) e o estado do Amazonas apresentou a menor porcentagem de vacinação (17,00%).

Esse estudo mostrou que a Colômbia apresenta regiões de prevalência elevada (>8%), intermediária (2-8%) e baixa (<2%). Os resultados mostram

também que a vacinação contra hepatite B deve ser reforçada na Colômbia como um programa de saúde pública eficaz para evitar novos casos de hepatite B na população. Este é um importante problema de saúde pública, cuja resolução envolve custos para a implementação generalizada de programas de vacinação eficientes em grandes regiões pouco habitadas, como o estado de Amazonas, onde as populações estão espalhadas em pequenas aldeias situadas no meio da floresta equatorial. Além disso, esse programa pode enfrentar problemas inesperados, como acontece nos estado de Amazonas e Magdalena, já que algumas etnias indígenas não aceitam a vacinação por razões culturais e terminam por se contaminar com o HBV.

Com os trabalhos citados previamente, pode-se concluir que a Colômbia se classifica como um pais de prevalência intermediária para o vírus da hepatite B, e se espera uma melhora no programa de vacinação obrigatória que vem sendo aplicado no país desde 1992, especialmente em algumas regiões onde a prevalência do HBV continua sendo alta.

1.3 Caracterização dos genótipos do HBV em algumas regiões da América Latina

O HBV é classificado atualmente em nove genótipos com distribuição geograficamente restrita (Yu *et al.,* 2010). O genótipo A (HBV/A) é encontrado principalmente no norte e oeste da Europa, na América do Norte e na África

(Bowyer *et al.,* 1997; Sugauchi *et al.,* 2004a; Kramvis & Kew, 2007; Gulube *et al.,* 2011). Genótipos HBV/B e HBV/C são prevalentes no sudeste da Ásia e no Extremo Oriente (Suguachi *et al.,* 2004b; Chan *et al.,* 2005; Tanaka *et al.,* 2005; Mahtab *et al.,* 2008). No Brasil, especificamente em São Paulo e no norte do Paraná, houve grande migração de descendentes do leste da Ásia, sendo, portanto, encontrados os genótipos C (subgenótipo C2) e B (Sitnik *et al.,* 2004; **Alvarado-Mora *et al.,* 2011b**). HBV/D é predominante na bacia do Mediterrâneo, no Oriente Médio e na Ásia Central (Norder *et al.,* 2004; Elkady *et al.,* 2008). O genótipo HBV/E é o genótipo mais prevalente na África Ocidental e África Central (Kramvis e Kew, 2007). HBV/E não é encontrado fora da África, com exceção de alguns casos de indivíduos com origem africana. No entanto, em um dos trabalhos realizados, pela primeira vez na América do Sul foi descrita a presença deste genótipo em uma comunidade de afro-descendentes na Colômbia (**Alvarado-Mora *et al.,* 2010c**). A origem do genótipo G é atualmente desconhecida, mas, apesar de ser pouco frequente, este genótipo tem sido relatado em vários países da Europa (Vieth *et al.,* 2002; Jardi *et al.,* 2008;) e nas Américas (Alvarado-Esquivel *et al.,* 2006; Bottecchia *et al.,* 2008), muitas vezes em co-infecção com o HIV (Silva *et al.,* 2010). Os genótipos HBV/F e HBV/H são encontrados nas Américas, sendo o genótipo F descrito desde o Alaska até a Argentina, enquanto o genótipo H foi descrito principalmente na América Central 4e México (Arauz-Ruiz *et al.,* 2002; Devesa *et al.,* 2004; 2008). Recentemente, foi caracterizado um novo genótipo,

designado como o genótipo I, no Vietnã e Laos (Yu *et al.,* 2010). Na Figura 1, está mostrada a distribuição dos genótipos do HBV na América Latina.

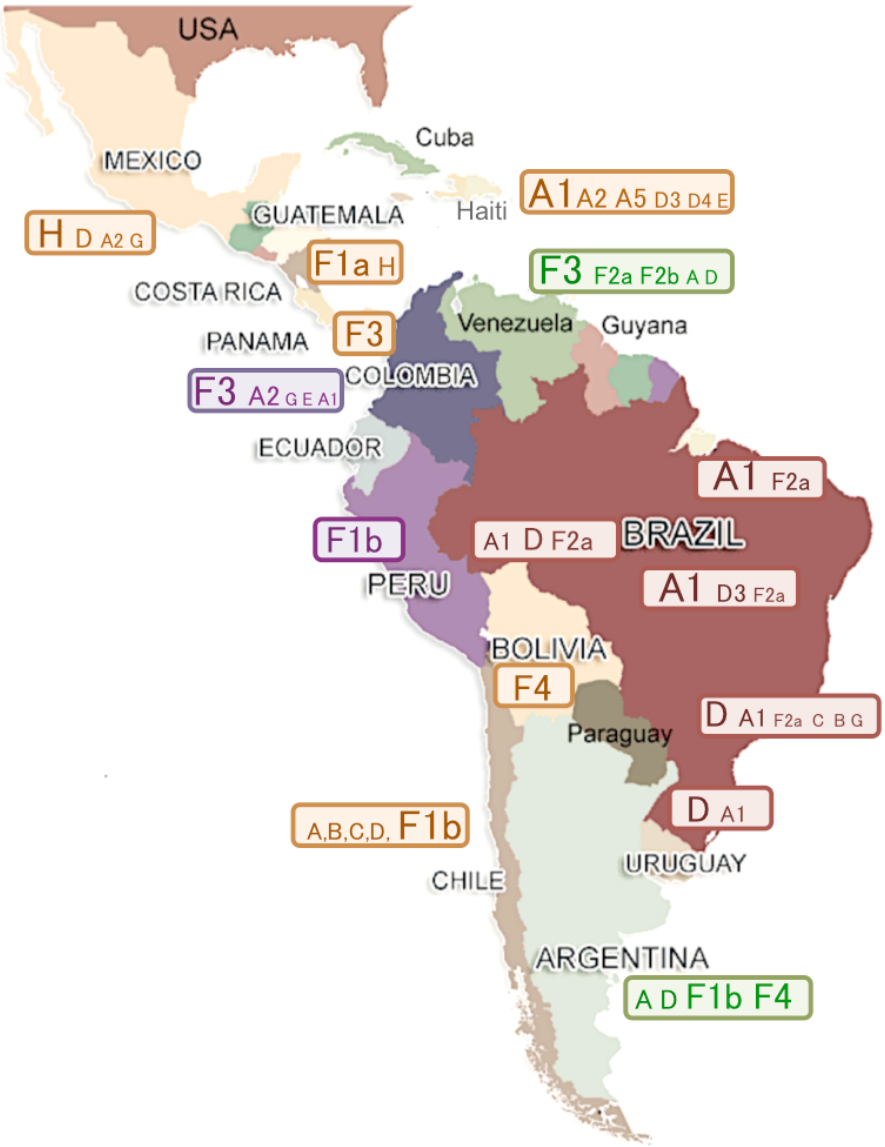


Figura 1 – Distribuição dos genótipos do vírus da Hepatite B na América Latina.

No estudo realizado na população de doadores de sangue na Colômbia (n=143), o subgenótipo do HBV mais prevalente foi o F3 (75%), mas também se encontraram os subgenótipos F1b (2%), A2 (15,3%) e G (7,7%) (**Alvarado-Mora *et al.,* 2011d**). Os resultados das análises filogenéticas realizadas nessa população sugerem que o genótipo G na Colômbia teve varias introduções, pois as sequências encontradas não agruparam no mesmo clado da árvore filogenética. Da mesma forma, a presença de somente uma sequência do subgenótipo F1b sugere a entrada de alguma cepa migrante nessa população proveniente de uma região aonde este subgenótipo é prevalente, como o Chile (**Venegas *et al.,* 2011**) ou Argentina (Campos *et al.,* 2005).

O segundo subgenótipo mais prevalente encontrado na Colômbia foi o subgenótipo A2 (15,3%), encontrado com frequência na Europa, nos Estados Unidos e na região do Ártico (Langer *et al.,* 1997; Chu *et al.,* 2003; Norder *et al.,* 2004; Osiowy *et al.,* 2011). Existem atualmente poucas sequências longas deste genótipo nos bancos de dados, portanto não podemos fazer inferências específicas sobre sua origem na Colômbia. De qualquer forma, na Colômbia, houve uma importante migração europeia no tempo da colonização espanhola, o que faz acreditar que possivelmente o subgenótipo A2 foi introduzido nessa época **(Alvarado-Mora *et al.,* 2011d)**.

O genótipo F tem sido descrito como representante da população indígena nas Américas, já que é encontrado em diversos grupos ameríndios de forma quase exclusiva (Di Lello *et al.,* 2009). O subgenótipo F3 tem sido

relatado no Panamá (Norder *et al.,* 2004), Venezuela (Nakano *et al.,* 2001) e na Colômbia (Norder *et al.,* 2004; Devesa *et al.,* 2008). Em nosso trabalho, encontrou-se o subgenótipo F3 como o mais frequente na população da Colômbia e também determinamos o ancestral comum mais recente (TMRCA) para cada um dos subgenótipos do genótipo F (F1, F2, F3 e F4) circulantes nas Américas. Como resultado, foi obtido que o genótipo F3 é provavelmente o genótipo mais antigo e o subgenótipo F4 é o mais recente **(Alvarado-Mora *et al.,* 2011d)**. Da mesma forma, observamos que o subgenótipo F3 aparentemente circula há mais tempo na Venezuela do que na Colômbia. Realizando uma análise das substituições nucleotídicas características de cada um dos quatro subgenótipos do genótipo F, foi encontrado que o subgenótipo F3 apresenta três substituições não sinônimas no genoma que não estão presentes nos outros subgenótipos, mas estão presentes no genótipo H. Dessa forma, podemos sugerir que o subgenótipo F3 está mais relacionado ao genótipo H do que qualquer outro subgenótipo do genótipo F.

Na população de Chocó, estado do país que apresenta mais de 65% de população afro-descendente (http://es.wikipedia.org/wiki/Chocó), encontrou-se os subgenótipos A1 e E como os mais prevalentes **(Alvarado-Mora *et al.,* 2011e)**. O genótipo E foi descrito de forma autóctone pela primeira vez na América do Sul nessa população **(Alvarado-Mora *et al.,* 2010c)**. A partir desse resultado, foram realizadas análises evolutivas para determinar a possível origem desse genótipo nessa comunidade. A população de Chocó é

descendente na sua maior parte dos escravos africanos que foram trazidos para as Américas no começo do século XIX. Dessa forma, foram testadas duas hipótese da presença do genótipo E: (i) HBV/E foi trazido para as Américas com os escravos ou (ii) HBV/E foi introduzido recentemente por algum contato dos seus habitantes com a África. Dessa forma, foi determinada uma taxa de substituição específica para o HBV/E de 3,2 x 10^{-4} substituições / sítio / ano (s/s/y) e foram testadas mais duas taxas previamente relatadas para o HBV (Simmonds & Midgley, 2005; Hannoun *et al.,* 2005; Zhou & Holmes, 2007). Os resultados das análises das sequências demonstraram que possivelmente o HBV/E pode estar circulando há algum tempo nessa região, sendo que foram encontradas duas sinapomorfias nas sequências de Chocó. Entretanto, os resultados do ancestral comum mais recente (TMRCA) obtidos pela taxa calculada nesse estudo sugerem que o genótipo E é um genótipo recente. ,Os valores obtidos com a taxa mais lenta (1.5x10^{-5} s/s/y) indicam um TMRCA de em torno 500 anos, o qual favorece a hipótese de que a entrada deste genótipo na Colômbia pode ter sido através do trafico de escravos. De qualquer forma, a presença do subgenótipo A1, que também tem uma origem africana (e não foi encontrado nas outras populações estudadas da Colômbia) pode favorecer a hipótese da possível entrada do genótipo E e do próprio subgenótipo A1 na época do tráfico de escravos. Interessantemente, uma sequência recombinante foi encontrada entre os subgenótipos F3 e A1 na posição 941 da região da polimerase (POL) **(Alvarado-Mora *et al.,* 2011e)**. Embora tenha sido uma única sequência que foi encontrada nesta região, já foram relatadas várias

cepas recombinantes deste vírus no mundo (Chen *et al.,* 2004; Suwannakarn *et al.,* 2005; Abdou-Chekaraou *et al.,* 2010; Mahgoub *et al.,* 2011). Como o evento de recombinação é raro, sugerimos que a presença desta cepa recombinante deve estar relacionada à alta prevalência do genótipo F3 assim como do genótipo A1, que podem estar circulando há muito tempo nessa região, mas também existe a hipótese desse recombinante ter sido introduzido a partir de outra região geográfica.

Comparando com outros países da América do Sul, o genótipo E só foi relatado em casos de africanos nativos que vieram recentemente para o Brasil (Sitnik *et al.,* 2007) ou Argentina (Mathet *et al.,* 2006). Entretanto, o subgenótipo A1 tem sido relatado como um genótipo prevalente em comunidades de quilombos no estado de Mato Grosso, no Brasil (Motta-Castro *et al.,* 2005, 2008). Da mesma forma, o subgenótipo A1 foi caracterizado em quatro casos de uma comunidade de Quilombos (Frechal) no estado de Maranhão (**Alvarado-Mora *et al.,* 2011f**) e em mais de 70% de prevalência em pacientes com hepatite B crônica do estado de Pernambuco (**Moura *et al.,* 2011**). Já no estado de Rondônia, no Brasil, este subgenótipo foi encontrado em 37,1% dos casos, enquanto os subgenótipos D3 e F2a foram encontrados em 22,8% e 20,0% dos casos, respectivamente (**Santos *et al.,* 2010**).

1.4 Referências

1. Abdou-Chekaraou M, Brichler S, Mansour W, Le Gal F, Garba A, et al. A novel hepatitis B virus (HBV) subgenotype D (D8) strain, resulting from recombination between genotypes D and E, is circulating in Niger along with HBV/E strains. *J Gen Virol.* 2010; 91: 1609-1620.

2. Alvarado-Esquivel C, Sablon E, Conde-Gonzalez CJ, Juarez-Figueroa L, Ruiz-Maya L, et al. Molecular analysis of hepatitis B virus isolates in Mexico: predominant circulation of hepatitis B virus genotype H. *World J Gastroenterol.* 2006; 12: 6540-6545.

3. **Alvarado-Mora MV**, **Gutierrez Fernandez MF, Gomes-Gouvea MS, de Azevedo Neto RS, Carrilho FJ, et al.** Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population - how is the epidemiological situation? **2011a; *PLoS One* 6: e18888.**

4. **Alvarado-Mora MV, Santana RA, Sitnik R., Abrão Ferreira P, Mangueira CL, Carrilho F, Pinho JR.** Full-length genomic sequence of hepatitis B vírus genotype C2 isolated in a Native Brazilian patient. ***Mem Inst Osw Cruz.*** **2011b; 106(4): 495-498**

5. **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*. 2010c; 91: 501-508**.

6. **Alvarado-Mora MV**, **Romano CM, Gomes-Gouvea MS, Gutierrez MF, Botelho L, et al.** Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F. ***Infect Genet Evol*. 2011d; 11: 103-108.**

7. **Alvarado-Mora MV**, **Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, Pinho, JR .** Phylogenetic Analysis of Complete Genome Sequences of Hepatitis B Virus From Afro-Colombian Community: Presence of HBV F3/A1 Recombinant Strain. ***AASLD The Liver Meeting 2011. Aceito.***

8. **Alvarado-Mora MV, Botelho L, Gomes-Gouvêa MS, de Souza VA, Nascimento MC, Pannuti C, Carrilho F, Pinho JR.** Detection of Hepatitis B vírus subgenotype A1 in a Quilombo community from Maranhão, Brazil. ***Virology Journal. 2011f; in print***.

9. 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

10. Bottecchia M, Souto FJ, O KM, Amendola M, Brandao CE, 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.

11. 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 (7): 1719-1729.

12. Brunetto MR, Rodriguez UA, Bonino F. Hepatitis B virus mutants. *Intervirology.* 1999: 42; 69-80.

13. Buitrago B, Popper H, Hadler SC, Thung SN, Gerber MA, et al. Specific histologic features of Santa Marta hepatitis: a severe form of hepatitis delta-virus infection in northern South America. *Hepatology*.1986a; 6: 1285-1291.

14. Buitrago B, Hadler SC, Popper H, Thung SN, Gerber MA, et al. Epidemiologic aspects of Santa Marta hepatitis over a 40-year period. *Hepatology*.1986b; 6: 1292-1296.

15. Campos RH, Mbayed VA, Pineiro YLFG. Molecular epidemiology of hepatitis B virus in Latin America. *J Clin Virol*. 2005; 34(2): S8-S13.

16. Chan HL, Tsui SK, Tse CH, Ng EY, Au TC, et al. Epidemiological and virological characteristics of 2 subgroups of hepatitis B virus genotype C. *J Infect Dis.* 2005; 191: 2022-2032.

17. Chen BF, Kao JH, Liu CJ, Chen DS, Chen PJ. Genotypic dominance and novel recombinations in HBV genotype B and C co-infected intravenous drug users. *J Med Virol.* 2004; 73: 13-22.

18. Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, et al. Hepatitis B virus genotypes in the United States: results of a nationwide study. *Gastroenterology.* 2003; 125: 444-451.

19. Crowther RA, Kiselev NA, Böttcher B, Berriman JA, Borisova GP, Ose V, Pumpens P. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell*. 1994; 77: 943–950.

20. Devesa M, Rodriguez C, Leon G, Liprandi F, Pujol FH. Clade analysis and surface antigen polymorphism of hepatitis B virus American genotypes. *J Med Virol*. 2004; 72: 377-384.

21. Devesa M, Loureiro CL, Rivas Y, Monsalve F, Cardona N, 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.

22. Di Lello FA, Pineiro YLFG, Munoz G, Campos RH. Diversity of hepatitis B and C viruses in Chile. *J Med Virol.* 2009; 81: 1887-1894.

23. Fay OH. Hepatitis B in Latin America: epidemiological patterns and eradication strategy. The Latin American Regional Study Group. 1990. *Vaccine* 8: S100-106.

24. Elkady A, Tanaka Y, Kurbanov F, Oynsuren T, Mizokami M. Virological and clinical implication of core promoter C1752/V1753 and T1764/G1766 mutations in hepatitis B virus genotype D infection in Mongolia. *J Gastroenterol Hepatol.* 2008; 23: 474-481.

25. Gulube Z, Chirara M, Kew M, Tanaka Y, Mizokami M, et al. Molecular characterization of hepatitis B virus isolates from Zimbabwean blood donors. *J Med Virol*. 2011; 83: 235-244.

26. Hannoun C, Soderstrom A, Norkrans 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.

27. Hou J, Liu Z, Gu F. Epidemiologic and prevention of Hepatitis B virus Infection. *IJMMS.* 2005; 2: 50-57.

28. Jardi R, Rodriguez-Frias F, Schaper M, Giggi E, Tabernero D, et al. Analysis of hepatitis B genotype changes in chronic hepatitis B infection: Influence of antiviral therapy. *J Hepatol.* 2008; 49: 695-701.

29. Kramvis A, Kew MC. Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes. *Hepatol Res.* 2007; 37: S9-S19.

30. Koike K, Takada S. Biochemistry and Functions of Hepatitis B virus X protein. *Intervirology.* 1995; 38(1-2): 89-99.

31. Langer BC, Frosner GG, von Brunn A. Epidemiological study of viral hepatitis types A, B, C, D and E among Inuits in West Greenland. *J Viral Hepat.* 1997; 4: 339-349.

32. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat.* 2004; 11: 97-107.

33. Lok AS. Chronic hepatitis B. *N Engl J Med.* 2002; 346(22): 1682–1683.

34. Mahgoub S, Candotti D, El Ekiaby M, Allain JP. Hepatitis B virus (HBV) infection and recombination between HBV genotypes D and E in asymptomatic blood donors from Khartoum, Sudan. *J Clin Microbiol*. 2011; 49: 298-306.

35. Mahoney FJ. Update on diagnosis, management, and prevention of hepatitis B virus infection. *Clin Microbiol Rev*. 1999; 12(2): 351–366.

36. Mahtab MA, Rahman S, Khan M, Karim F. Hepatitis B virus genotypes: an overview. *Hepatobiliary Pancreat Dis Int*. 2008; 7: 457-464.

37. Martinez M, De la Hoz F, Jaramillo LS, Rojas MC, Buitrago B, Boshell J, et al. Seroepidemiologia de la infección por el virus de la hepatitis B en niños de la Amazonia Colombiana. *Biomédica*. 1991; 11:20-24.

38. Mathet VL, Cuestas ML, Ruiz V, Minassian ML, Rivero C, 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.

39. Motta-Castro AR, Martins RM, Yoshida CF, Teles SA, Paniago AM, et al. Hepatitis B virus infection in isolated Afro-Brazilian communities. *J Med Virol.* 2005; 77: 188-193.

40. Motta-Castro AR, Martins RM, Araujo NM, Niel C, Facholi GB, et al. Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community. *Arch Virol.* 2008;153: 2197-2205.

41. **Moura M, Alvarado-Mora MV, Pinho JR, Carrilho F, Lopes E.** High prevalence of hepatitis B virus genotype A1 in patients with chronic infection from Pernambuco state, Brazil. ***AASLD The Liver Meeting 2011. Submetido.***

42. Nassal M. Hepatitis B virus replication: novel roles for virus-host interactions. *Intervirology.* 1999; 42: 100-116.

43. Nakano T, Lu L, Hu X, Mizokami M, Orito E, et al. Characterization of hepatitis B virus genotypes among Yucpa Indians in Venezuela. *J Gen Virol.* 2001; 82: 359-365.

44. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology.* 2004; 47: 289-309.

45. Osiowy C, Larke B, Giles E. 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*. 2011; 18: e11-19.

46. Sánchez 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. *Intervirology.* 2007; 50: 9–15.

47. **Santos AO, Alvarado-Mora MV, Botelho L, Vieira DS, Pinho JR, et al.** Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil. ***Virol J.*** **2010; 7: 315**.

48. Schaefer S. Hepatitis B virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol.* 2007; 13: 14-21.

49. Silva AC, Spina AM, Lemos MF, Oba IT, Guastini Cde F, 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 Osw Cruz.* 2010; 105: 770-778.

50. Simmonds P, Midgley S. Recombination in the genesis and evolution of hepatitis B virus genotypes. *J Virol.* 2005; 79: 15467-15476.

51. Sitnik R, Pinho JR, Bertolini DA, Bernardini AP, Da Silva LC, et al. Hepatitis B virus genotypes and precore and core mutants in Brazilian patients. *J Clin Microbiol*. 2004; 42: 2455-2460.

52. Sitnik R, Sette H, Jr., Santana RA, Menezes LC, Graça CH, 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: 1689-1692.

53. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol*. 2004a; 85: 811-820.

54. Sugauchi F, Kumada H, Sakugawa H, Komatsu M, Niitsuma H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. *Clin Infect Dis.* 2004b; 38:1222-1228.

55. Suwannakarn K, Tangkijvanich P, Theamboonlers A, Abe K, Poovorawan Y. A novel recombinant of Hepatitis B virus genotypes G and C isolated from a Thai patient with hepatocellular carcinoma. *J Gen Virol.* 2005; 86: 3027-3030.

56. Tanaka J. Hepatitis B epidemiology in Latin America. *Vaccine.* 2000; 18(1): 17–19.

57. Tanaka Y, Orito E, Yuen MF, Mukaide M, Sugauchi F, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. *Hepatol Res.* 2005; 33: 216-224.

58. Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: a global overview. *Clin Liver Dis* 2010; 14: 1-21.

59. Tiollais P, Chárnay P, Vyas GN. Biology of the Hepatitis B virus. S*cience.* 1981; 21(4506): 406 – 411.

60. **Venegas M, Alvarado-Mora MV, Villanueva R, Pinho JRR, Carrilho F, Locarnini S, Yuen L, Brahm J.** Phylogenetic analysis of Hepatiti B virus genotype F complete genome sequences from patients with chronic infection from Chile. ***J Med Virol. 2011;*** **83(9):1530-6.**

61. Vieth S, Manegold C, Drosten C, Nippraschk T, Gunther S. Sequence and phylogenetic analysis of hepatitis B virus genotype G isolated in Germany. *Virus Genes.* 2002; 24: 153-156.

62. Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, et al. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". *PLoS One*. 2010; 5: e9297.

63. Zhou Y, Holmes EC. Bayesian estimates of the evolutionary rate and age of hepatitis B virus. *J Mol Evol.* 2007; 65: 197-205.

OPEN ACCESS Freely available online

PLoS one

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 Gutierrez Fernandez[2], Michele Soares Gomes-Gouvêa[1], Raymundo Soares de Azevedo Neto[3], Flair José Carrilho[1], João Renato Rebello Pinho[1]

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, Pontificia 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 Andres 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, Gutierrez Fernandez 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

Editor: Lise Lotte Gluud, Copenhagen University Hospital Gentofte, Denmark

Received January 18, 2011; **Accepted** March 23, 2011; **Published** April 29, 2011

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 Pontificia Universidad Javeriana, Bogóta, 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.viviana@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 Andres 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 Andres 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-HBs 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 ELISA 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 Andres 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 (6.15%)	120/456 (26.32%)	175/427 (40.98%)	78/306 (25.49%)	6/117 (5.13%)	16/456 (3.51%)
p	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	4/69(5.80%)	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 (15.0%)	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%)
p	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%)	49/155 (31.61%)	51/150 (34.00%)	17/100 (17.00%)	1/40 (2.50%)	6/155 (3.87%)
p	0.033	<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 showed 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 Andres 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 Colombians 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 thanks to *Laboratorio de Salud Publica*, in Quibdó, Santa Marta, San Andres and Leticia, in Colombia, for kindly providing help in the sampling process for this study.

Author Contributions

Conceived and designed the experiments: MVAM MSGG FJC JRRP. Performed the experiments: MVAM MSGG MFGF. Analyzed the data: MVAM RSAN JRRP. Contributed reagents/materials/analysis tools: MVAM MSGG. Wrote the paper: MVAM JRRP.

References

1. Lavanchy D (2004) Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 11: 97–107.
2. 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.
3. Tanaka J (2000) Hepatitis B epidemiology in Latin America. Vaccine 18(Suppl 1): S17–19.
4. Te HS, Jensen DM (2010) Epidemiology of hepatitis B and C viruses: a global overview. Clin Liver Dis 14: 1–21, vii.
5. Torres JR (1996) Hepatitis B and hepatitis delta virus infection in South America. Gut 38(Suppl 2): S48–55.
6. Gish RG, Gadano AC (2006) Chronic hepatitis B: current epidemiology in the Americas and implications for management. J Viral Hepat 13: 787–798.
7. Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, et al. (2010) Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". PLoS One 5: e9297.
8. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, 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.
9. Alvarado Mora MV, Romano CM, Gomes-Gouvea 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, Colan EJ, Wignall FS, Gerin JL (1993) A genotype of hepatitis D virus that occurs in northern South America. Proc Natl Acad Sci U S A 90: 9016–9020.
11. Wang KS, Choo QL, Weiner AJ, Ou JH, Najarian RC, et al. (1986) Structure, sequence and expression of the hepatitis delta (delta) viral genome. Nature 323: 508–514.
12. Radjef N, Gordien E, Ivaniushina V, Gault E, Anais P, et al. (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.
13. Makino S, Chang MF, Shieh CK, Kamahora 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, Gust I, Lai MM (1990) Sequence conservation and divergence of hepatitis delta virus RNA. Virology 178: 384–392.
15. Shakil AO, Hadziyannis S, Hoofnagle JH, Di Bisceglie AM, Gerin JL, et al. (1997) Geographic distribution and genetic variability of hepatitis delta virus genotype I. Virology 234: 160–167.
16. Quintero A, Uzcategui N, Loureiro CL, Villegas L, Illarramendi 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-Gouvea MS, Soares MC, Bensabath 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, Sheen 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. Sakugawa H, Nakasone H, Nakayoshi T, Kawakami Y, Miyazato 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, Enomoto N, Chinzei 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 Gal F, Gault E, Ripault MP, Serpaggi J, Trinchet JC, et al. (2006) Eighth major clade for hepatitis delta virus. Emerg Infect Dis 12: 1447–1450.
22. Gaeta GB, Stroffolini T, Chiaramonte M, Ascione T, Stornaiuolo 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. Biomédica 18: 216–249.
24. Pasquier C, Njouom R, Ayouba A, Dubois M, Sartre MT, et al. (2005) Distribution and heterogeneity of hepatitis C genotypes in hepatitis patients in Cameroon. J Med Virol 77: 390–398.
25. Echevarria 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. Bostan 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 (2005) Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 5: 558–567.
28. Carrilho FJ, Corrêa MCJM (1998) Magnitude of hepatitis B and C in Latin America. In: Schinazi RF, Somadossi JP, Thomas HC, 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. Beltran M, Navas MC, De la Hoz F, Mercedes Munoz 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, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, 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. Bensabath 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, Ponzetto A, Anzola E, Rivero D, et al. (1984) Delta virus infection and severe hepatitis. An epidemic in the Yucpa Indians of Venezuela. Ann Intern Med 100: 339–344.
34. Ljunggren KE, Patarroyo ME, Engle R, Purcell 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 semestral de la hepatitis B, Colombia. Programa ITS/sida, Instituto Nacional de Salud. Biomédica 8: 2–11.
36. Reeves WC, Peters CJ, Purcell RH (1975) The epidemiology of hepatitis B antigen and antibody among Panamanian Cuna Indians. Am J Trop Med Hyg 24: 873–5.
37. Gayotto LC (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 Amazonía 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. Slusarczyk J (2000) Who needs vaccination against hepatitis viruses? Vaccine 18(Suppl 1): S4–5.
42. Ministerio de Salud (2002) Boletin Epidemiologico Semanal: Situacion de Hepatitis B en Colombia a la semana Epidemiologica. Available: www.col.ops-oms.org/sivigila/2002/BOLE49_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. Martinez M, De la Hoz F, Jaramillo LS, Rojas MC, Buitrago B, et al. (1991) Seroepidemiologia de la infeccion por el virus de la hepatitis B en ninos 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. Capitulo 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.

Full-length genomic sequence of hepatitis B virus genotype C2 isolated from a native Brazilian patient

Mónica Viviana Alvarado-Mora[1]/+, Rúbia Anita Ferraz Santana[2], Roberta Sitnik[2], Paulo Roberto Abrão Ferreira[3], Cristovão Luís Pitangueira Mangueira[2], Flair José Carrilho[1], João Renato Rebello Pinho[1,2]

[1]Laboratório 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 [2]Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, SP, Brasil [3]Universidade 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).

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

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

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-Gouvea 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, LAM1F, 2817R, P184, P192, P194, P197, P1193R, P970F, 2029R, X1577F (Gomes-Gouvea 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/).

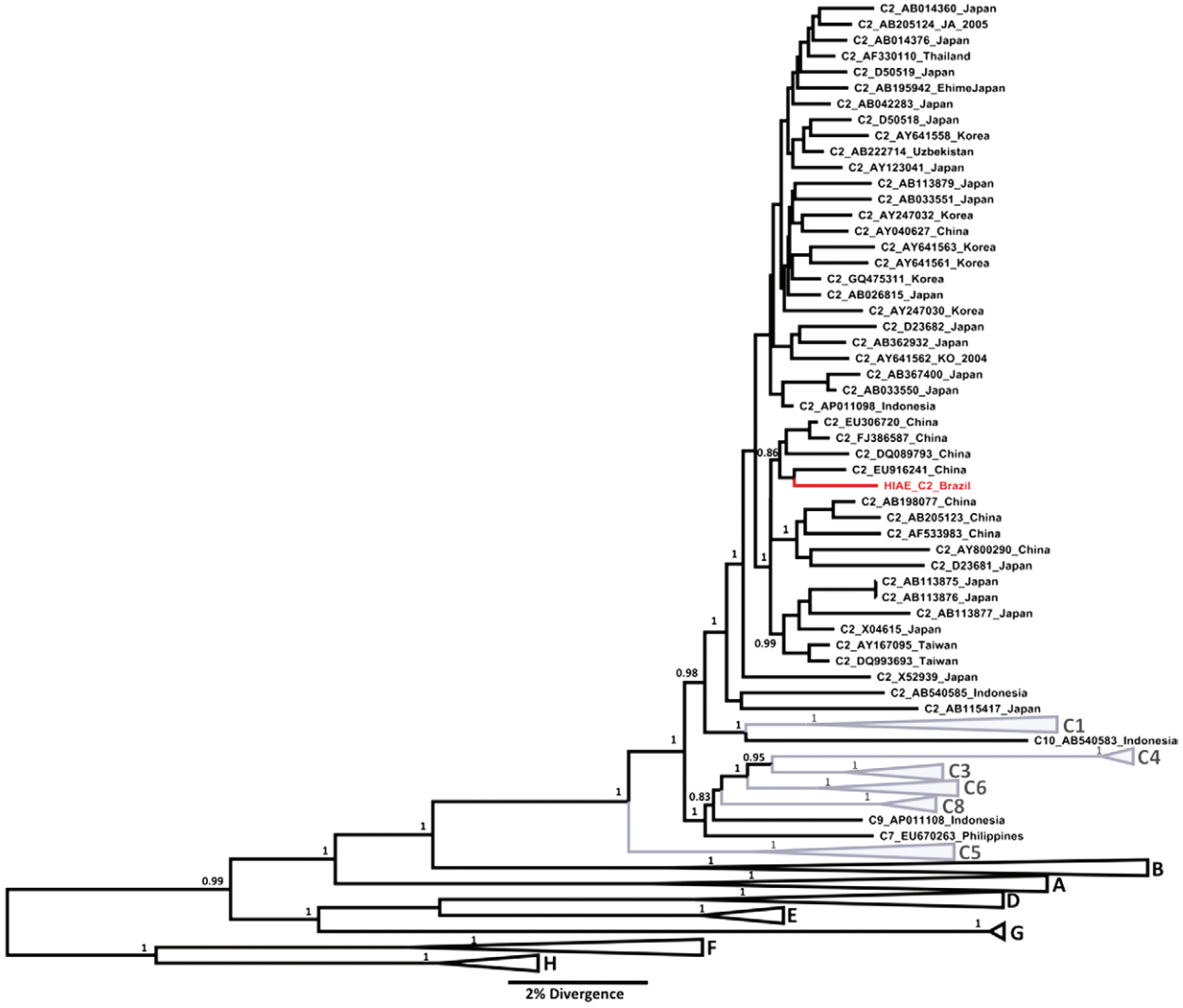
The isolated complete genome was aligned with other previously reported complete genome sequences (n = 192) using ClustalX software (Thompson et al. 1997) and was edited with the SE-AL software (available from: tree.bio.ed.ac.uk/software/seal/). The Bayesian Markov chain Monte Carlo simulation implemented in BEAST v.1.4.8 (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 v.1.5.3 (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 descendents 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 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 county 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 hypothesise 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, Romano CM, Gomes-Gouvea 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, Magnius 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, Bartholomeusz 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 Sao 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, Wendl MC, Green P 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res 8*: 175-185.

Gomes-Gouvea MS 2005. *Characterization of complete genomes of the hepatitis B virus of different genotypes isolated in Brazil*, Secretaria da Saúde/Coordenadoria 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, Tjahyono 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, Magnius 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.

Stuyver 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, 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.

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, Goncales NS, Fais VC, Vigani AG, Goncales ES, Feltrin A, Goncales 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 "I". *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.

Journal of General Virology (2010), **91**, 501–508 DOI 10.1099/vir.0.015958-0

Molecular epidemiology and genetic diversity of hepatitis B virus genotype E in an isolated Afro-Colombian community

Mónica Viviana Alvarado Mora,[1] Camila Malta Romano,[2] Michele Soares Gomes-Gouvêa,[1] Maria Fernanda Gutierrez,[3] Flair José Carrilho[1] and João Renato Rebello Pinho[1]

Correspondence
Mónica Viviana Alvarado Mora
monica.viviana@usp.br

[1]Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil

[2]Laboratory of Virology, São Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases, School of Medicine, University of São Paulo, Brazil

[3]Laboratory 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 Quibdó, 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-descendent 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/DDBJ accession number for the Quibdó 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 (Dumpis *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 Steenbergen *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 TMRCAs 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 $\log_{normal}$ 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.

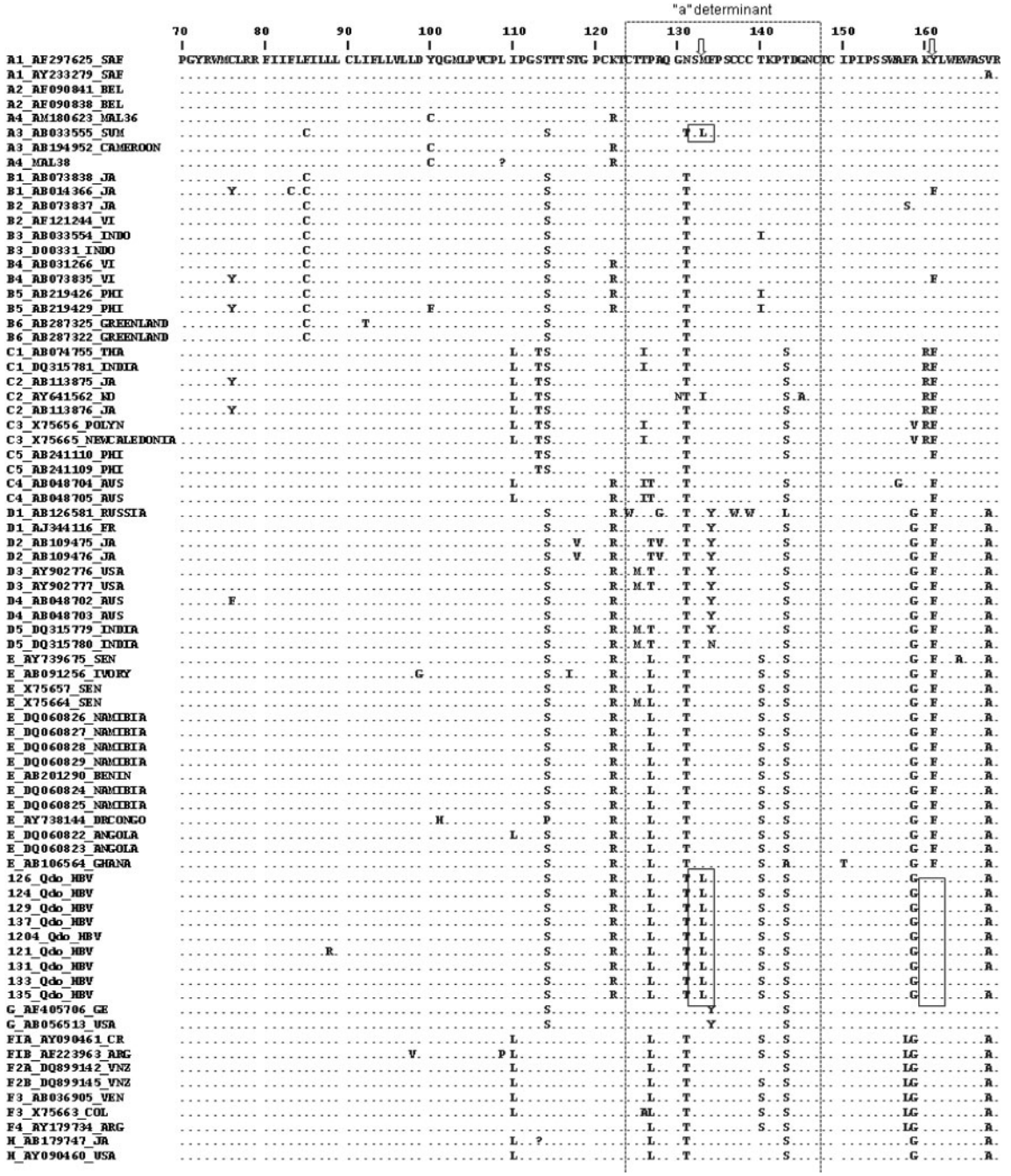


Fig. 2. Multiple alignment of amino acid sequences of HBsAg of the nine Colombian sequences compared with others obtained from GenBank. The major hydrophilic region (100–160), including the 'a' determinant (124–147), is boxed (dotted line). The two synapomorphies are located at positions 133 and 161 of the HBV S gene (boxed, solid line).

lation. There have been many studies to characterize the HBV genotype prevalence in Afro-descendent 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 A1 (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 descendents 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 Steenbergen *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 Publica 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-descendent women aged from 16 to 23 years old. Specifically, the Laboratorio de Salud Publica 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-Gouvêa *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 v.3.7 (Posada & Crandall 1998).

We also mapped the nucleotide changes along the ML tree using the MacClade v.4.7 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 (Martin, 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, Bogóta, Colombia. We thank the Laboratorio de Salud Publica, Quibdó, Colombia, for kindly providing the samples for this study.

REFERENCES

Andernach, I. E., Hübschen, J. M. & Muller, C. P. (2009). Hepatitis B virus: the genotype E puzzle. *Rev Med Virol* **19**, 231–240.

Arauz-Ruiz, P., Norder, H., Robertson, B. H. & Magnius, L. O. (2002). Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* **83**, 2059–2073.

Bekondi, C., Olinger, C. M., Boua, N., Talarmin, A., Muller, C. P., Le Faou, A. & Venard, V. (2007). Central African Republic is part of the West-African hepatitis B virus genotype E crescent. *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., Opare-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.

Cuéllar-Ambrosi, F., Mondragón, M. C., Figueroa, M., Préhu, 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, I., Mondlane, J., Beauchant, 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., Thursz, 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., Wendl, M. C. & Green, P. (1998). Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* **8**, 175–185.

Gomes-Gouvêa, 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., Rotily, 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. & Muller, 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., Sugauchi, 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., Magnius, 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*S 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.

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.

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. (2005). 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., Edorh, 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., Lofdahl, S., Courouce, A. M. & Magnius, 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., Olaleye, 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 materno-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. *Arq Gastroenterol* **44**, 54–57.

Posada, D. & Crandall, K. A. (1998). MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817–818.

Pult, 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., Schinazi, 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., Siransy, 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., Holguín, 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 Steenbergen, 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., Plantier, 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.

Infection, Genetics and Evolution 11 (2011) 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]

[a] Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil
[b] São Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases (LIMHC), School of Medicine, University of São Paulo, Brazil
[c] Laboratory 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.

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; Magnius 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).

1567-1348/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2010.10.003

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 monoinfection 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 descendents in Haiti (Andernach 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 coinfected 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 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.

Fig. 1. Geographic locations of cities where blood samples were collected.

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 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 (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 Clustal X 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 block. 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).

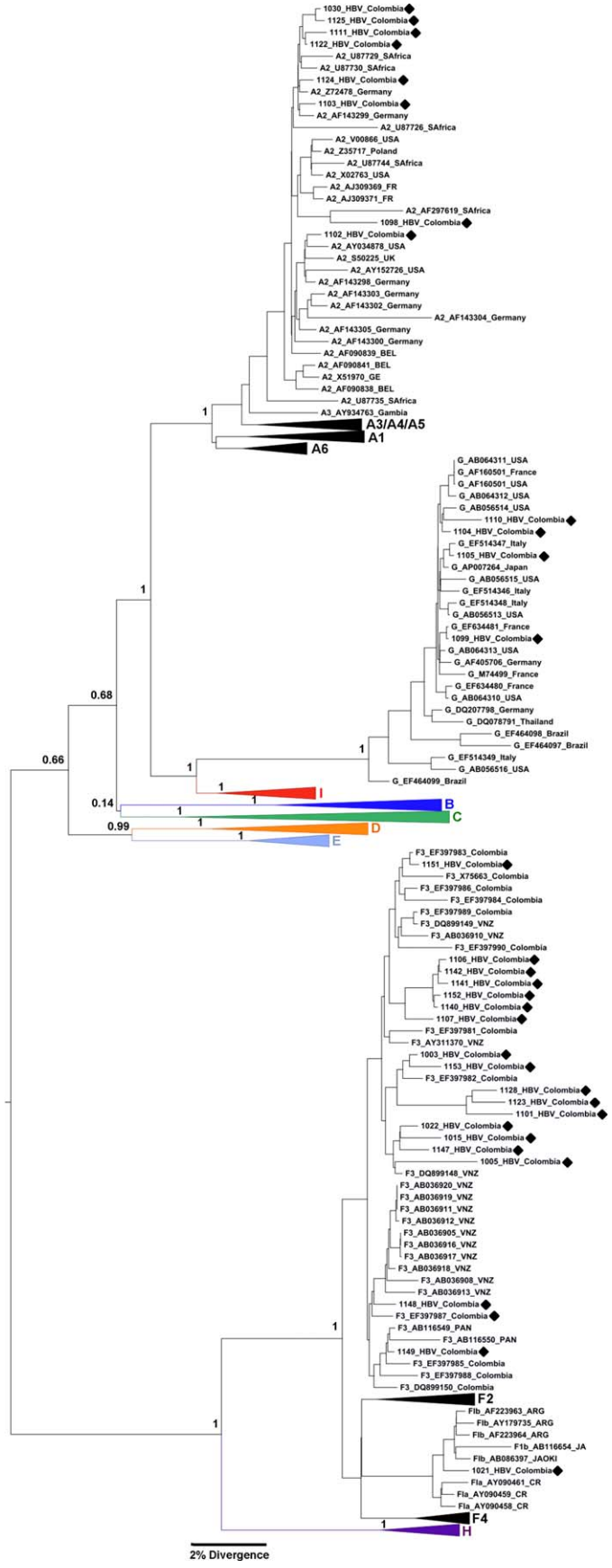


Fig. 2. The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 301 S/POL sequences with 1306 bp of Hepatitis B virus strains. The posterior probabilities of the key nodes are depicted above the respective nodes. Samples obtained from Colombian blood donors ($n = 34$) were analyzed together 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.

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 v.1.5.3 (Drummond and Rambaut, 2007). Bayesian Skyline plot (BSL) was applied under strict and relaxed uncorrelated $\log_{normal}$ 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 TMRCAs 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

Table 1
Substitution rates and TMRCA for human HBV/F sequences. TMRCA was calculated using two different substitution rates (2.6×10^{-4} and 1.5×10^{-5} substitutions per site per year).

Dataset genotype F	HBV TMRCA (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 (1136.8–3993.6)
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 (573.3–3325.29)
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 S1.A/S1.B 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 Yucpa 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 S2.A/S2.B 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) Ser^{207} was confirmed in all Colombian sequences while the other subgenotypes had Asn^{207}; and (3) Ala^{194} 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 TMRCA 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 TMRCA 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 TMRCA 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 TMRCA 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 (Glu^{2}) and POL gene (Glu^{359} and Thr^{607}) 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 (I^{110}, N^{466}, and A^{607}) 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 (Flichman 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-Gouvea, 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.

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.

Andernach, I.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., Magnius, 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.

Bottecchia, M., Souto, F.J., O, K.M., Amendola, M., Brandao, C.E., Niel, 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.G., 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., Pineiro, 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., Czudai, V., Scheiblauer, H., Nick, S., Mosebach, M., Hourfar, M.K., Seifried, E., Roth, W.K., Grunelt, 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., Bernini, 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.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.

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., Pineiro, 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., Avellon, A., Magnius, 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, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8, 186–194.

Flichman, D., Galdame, O., Livellara, B., Viaut, M., Gadano, 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.

Fung, 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., 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.

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., Ampofo, W., Izumi, T., Nakajima, A., Ansah, J., Tetteh, J.O., Nii-Trebi, 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.

Jardi, R., Rodriguez-Frias, F., Schaper, M., Giggi, 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., Yoshizawa, 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, S9–S19.

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., Lascoux-Combe, C., Wendum, D., Carrat, F., Zoulim, F., 2006. Major role of hepatitis B genotypes in liver fibrosis during coinfection with HIV. AIDS 20, 419–427.
Langer, B.C., Frosner, 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 Yucpa Indians in Venezuela. J. Gen. Virol. 82, 359–365.
Naumann, H., Schaefer, S., Yoshida, C.F., Gaspar, 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., Blitz, 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., 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.
Norder, H., Courouce, 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 materno-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.
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.
Osioy, C., Giles, E., 2003. Evaluation of the INNO-LiPA 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., Vernet, 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.
Pineiro 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-Olyaee, 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 chain18 terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.
Shibayama, T., Masuda, G., Ajisawa, 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.
Suwannakarn, 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 le, H., 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 Steenbergen, 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.
Vieth, 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.
von Meltzer, 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 "I". 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.

Alvarado-Mora et al., 2011e

PRESENTATION TYPE: Oral or Poster

CURRENT CATEGORY: Hepatitis B

CURRENT DESCRIPTORS: SO5. Epidemiology/Prevention/Control

TITLE: Phylogenetic Analysis of Complete Genome Sequences of Hepatitis B Virus From Afro-Colombian Community: Presence of HBV F3/A1 Recombinant Strain

AUTHORS (FIRST NAME, LAST NAME): Monica V. Alvarado-Mora[1], Camila M. Romano[2], Michele Gomes-Gouvêa[1], Maria F. Gutiérrez[3], Flair J. Carrilho[1], João R. Pinho[1]

Institutional Author(s):

INSTITUTIONS (ALL): 1. Gastroenterology, University of São Paulo , São Paulo , São Paulo, Brazil.
2. Infectious and Parasitic Diseases (LIMHC), University of São Paulo , São Paulo , São Paulo, Brazil.
3. Microbiology, Pontificia Javeriana University, Bogotá, Bogotá, Colombia.

ABSTRACT BODY: Hepatitis B virus (HBV) infection is one the most prevalent viral infections in humans and it represents a serious public health problem in many countries. HBV strains have a distinct geographical distribution and are traditionally classified into nine genotypes (A to I) on the basis of genome diversity. In Colombia, there are few epidemiologic studies about the hepatitis B virus. Recently our group reported the presence of genotypes F3, A2 and G in blood donors from Bogotá. The aim of this study was to characterize the HBV genotypes circulating in Quibdó, a largest Afro-descendent community in Colombia. In order to identify the HBV genotypes in this community, sixty HBsAg-positive samples were obtained from the public health laboratory in Quibdó, Colombia. A fragment of 1306 bp partially comprising HBsAg and the DNA polymerase coding regions (S/POL) was amplified by nested PCR. Positive samples to S/POL fragment were submitted to PCR amplification of the HBV complete genome. Amplified DNA was purified and sequenced. Sequences were genotyped by phylogenetic reconstructions using reference sequences from each HBV genotype obtained from GenBank (n = 412). They were aligned using Clustal X software (and edited with the SE-AL software. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3. The maximum clade credibility (MCC) tree was obtained by summarizing the 20,000 substitution trees. Putative HBV recombinant sequences were identified with the use of SimPlot program. Out of 60 HBsAg positive samples, 48.3% (n=29) were positive for HBV DNA. Of these HBV DNA positive samples, 23 sequences were obtained from S/POL region with good quality for phylogenetic analysis. Additionally, seven HBV complete genome sequences were obtained from A1, E and D3 genotypes. The phylogeny showed that subgenotype A1 was the most prevalent in this population. Also, a HBV recombinant strain genotype F3/A1 was found for the first time in the world. The recombination point was at position 941 of HBV genome (POL region). The distribution of HBV genotypes was: A1 (52.17%), E (39.13%), D3 (4.3%) and F3/A1 (4.3%). In conclusion, this study is the first analysis of complete HBV genome sequences from Afro-Colombian population. The complete genome of genotype D3 clustered in the same group with other two sequences from the United States of America. We found that the Colombian genotype E constitutes a closer cluster to sequences from Benin and Namibia. The results from E and A1 genotypes support the theory that HBV was introduced into this Afro-descendent community in Colombia in the times of slavery.

Virology journal, submetido

DETECTION OF HEPATITIS B VIRUS SUBGENOTYPE A1 IN A QUILOMBO COMMUNITY FROM MARANHÃO, BRAZIL

Mónica V Alvarado-Mora[1], Lívia Botelho[1], Michele S Gomes-Gouvêa[1], Vanda F de Souza[2], Maria C Nascimento[2], Claudio S Pannuti[2], Flair J Carrilho[1], João RR Pinho[1]

[1]Laboratory 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. [2]Laboratory of Virology, São Paulo Institute of Tropical Medicine, Department of Infectious and Parasitic Diseases, School of Medicine, University of São Paulo, Brazil. Email: monica.viviana@usp.br

*Corresponding author:

Monica Viviana Alvarado-Mora: 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. Phone: +55-11-30618218. Fax: +55-11-30645932. E-mail: monica.viviana@usp.br

Running title: Hepatitis B genotype A1 in a Quilombo Community.

ABSTRACT

Background: The Brazilian population is mainly descendant from European colonizers, Africans and Amerindians. 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 Frechal Quilombo community 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 1306bp partially comprising HBsAg and polymerase coding regions (S/POL) of HBV genome was amplified and sequenced. Viral isolates were genotyped by phylogenetic analysis using reference sequences from each genotype using BEAST v.1.5.3 (n=320). **Results:** Of the 72 individuals, 9 (12.5%) were HBsAg-positive and 4 of them were successfully sequenced for the 1306bp 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 it was found. We reported a high frequency of HBV infection and the exclusive presence of subgenotype A1 in an Afro-descendent community in Maranhão, Brazil.

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. HBV strains have distinct geographical distribution and are traditionally classified into nine genotypes, A to I, on basis of genome diversity [1]. Genotype A is found mainly in North America and Africa and has been found in seven genetically distinct subgenotypes (A1 to A7) [2]. 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 Amerindian population, respectively [3]. In addition, genotype G has been reported prevalent in the USA and France [4] and genotype H has been found in North and Central America [5]. Recently, by using phylogenetic analyses, a new genotype was characterized in Vietnam and Laos and designated as genotype I [1].

In Brazil, a wide variation of HBV infection prevalence was reported, particularly dependent upon the geographical region of this country [6]. Genotype A is the most prevalent and genotypes B, C, D and F are also circulating in the population [7-10]. The presence of these genotypes reflects the mixture of cultures in the country since Brazilian population is descendant mainly from African, European and native Amerindians [10]. More recently, migrations from other world regions, such as Asian countries contributed to increase Brazilian miscegenation [11].

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) (http://pt.wikipedia.org/wiki/Quilombo, accessed at 11/01/2011).

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.

MATERIAL AND METHODS

Study Population

Seventy-two samples were collected from inhabitants from Frechal, Maranhão State, Brazil (Figure 1). 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) extraction was carried out from 100µl of sera for each sample using the acid guanidinium thiocyanate / phenol / chloroform method [12]. To avoid false-positive results, strict procedures for nucleic acid amplification diagnostic techniques were followed [13].

HBV PCR Amplification

Samples were first amplified with the primers described by Sitnik et al. [8] 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/2920R and PS3201F/P1285R [14].

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. [15], 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 [16] 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

To perform evolutionary inferences, sequences from Frechal were genotyped by phylogenetic reconstructions using reference sequences from each HBV genotype obtained from the GenBank (n=320). These sequences comprised partial HBsAg and polymerase coding regions (S/POL) of HBV genome. They were aligned using Muscle Software [17] and edited with the SE-AL software (available at http://tree.bio.ed.ac.uk/software/seal/).

Bayesian phylogenetic analyses were done applying Markov Chain Monte Carlo simulation using BEAST v.1.5.3 [18], and 10 million generations were sufficient to obtain the convergence of parameters. 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 [18].

RESULTS

Of the 72 samples, 9 (12.5%) were HBsAg-positive. Since we did not have access to more 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 the four samples were classified as subgenotype A1 (subtype *adw2*). Interestingly, the four samples clustering in a single group but with a low posterior probability about 0.10 (Figure 2). Sequences were deposed in the 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 [7,8,19-23] the four samples were compared with others previously reported sequences from Quilombos in Brazil [23] and Venezuela [24] and with sequences from Rio de Janeiro, as in this place there was constant slave traffic from Africa that was more intense between 1795 to 1811 [25]. The genotype 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. Since the Quilombos communities present few contact with other urban communities, these HBV/A1 variants may have come from Africa before these groups were created and actually the HBV/A1 strains continued to evolve in the Afro-descendent population after they came to Brazil. However, most reported sequences with a long fragment of the HBV genome are needed to infer the specific dates on the presence of this genotype in Quilombos people in Brazil.

A lot of Afro-descendants live in Bahia in Northeast Brazil. The geographic distributions of HBV genotypes there are showed that genotype A (subtype *adw2*) is the most frequent in according to the ethnic background of the population[26]. It was reported a high prevalence of genotype A in Bahia but the sequences are shorter to perform the 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 [27].

We found that the three sequences from Afro-Venezuelan population [24] 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 confirm the HBV classification reported in this study. The synonymous substitutions at nucleotides in the third-positions: 201nt (TC**C** → TC**A**) Serine [S]; 222nt (CC**C** → CC**A**) Proline [P]; 324nt (TC**A** → TC**G**) Serine [S]; 327nt (TC**T** → TC**C)** Serine [S] and 462nt (TA**C** → TA**T)** Tryptofan [W] were identified and they confirm the results of subgenotype classification (Figure 3).

Finally, we found a higher frequency of HBsAg in the Frechal population (12.5%). Kalunga population, a largest Afro-Brazilian isolated community located in Goiás state, showed less frequency for HBsAg (1.8% -

16/878). The same HBV subgenotype (A1) was also found in all the positive samples from Goiás [28].

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% [11]. Subgenotype A1 was the most frequent in this state, followed by subgenotype A2 and genotype D [23].

HBsAg frequencies in Afro-Venezuelan communities (3.6%) and in rural populations from Venezuela were lower than those found in Brazilian studies involving Quilombo inhabitants (2.2%) [24].

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 conclusion, HBV subgenotype A1 is found prevalent in all African descendant population from South America studied so far but interestingly in Venezuela, where subgenotype A2 was found.

AUTHORS' CONTRIBUTIONS

MVAM participated in the design of the study, conducted the phylogenetic and evolutionary analysis and drafted the manuscript. LB and MSGG participated in the PCR amplification and sequencing process. VAUFS, MCN and CSP participated in the sample collection. FJC and JRRP participated in the elaboration of the manuscript.

COMPETING INTEREST

The authors declare that they have no financial or competing interest with this article.

ACKNOWLEDGEMENTS

We are deeply indebted to Dra. Maria Claudia Nascimento and Dra. 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 2008/50461-6 and CNPq.

REFERENCES

1. 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 "I". *PLoS One*, **5:**e9297.

2. 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*, **17:**88-94.

3. Magnius LO, 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.

4. 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.

5. 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.

6. Naumann H, Schaefer S, Yoshida CF, Gaspar AM, Repp R, Gerlich WH: **Identification of a new hepatitis B virus (HBV) genotype from Brazil that expresses HBV surface antigen subtype adw4.** *J Gen Virol* 1993, **74 (Pt 8):**1627-1632.

7. 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.

8.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.

9. 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.

10. Santos AO, Alvarado-Mora MV, Botelho L, Vieira DS, Pinho JR, Carrilho FJ, Honda ER, Salcedo JM: **Characterization of hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil.** *Virol J*, **7:**315.

11. 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.

12. Chomczynski P, Sacchi N: **Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.** *Anal Biochem* 1987, **162:**156-159.

13. Kwok S, Higuchi R: **Avoiding false positives with PCR.** *Nature* 1989, **339:**237-238.

14. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Botelho L, Carrilho FJ, Pinho JR: **Molecular characterization of the Hepatitis B virus genotypes in Colombia: a Bayesian inference on the genotype F.** *Infect Genet Evol*, **11:**103-108.

15. Sanger F, Nicklen S, Coulson AR: **DNA sequencing with chain-terminating inhibitors.** *Proc Natl Acad Sci U S A* 1977, **74:**5463-5467.

16. Ewing B, Green P: **Base-calling of automated sequencer traces using phred. II. Error probabilities.** *Genome Res* 1998, **8:**186-194.

17. Edgar RC: **MUSCLE: a multiple sequence alignment method with reduced time and space complexity.** *BMC Bioinformatics* 2004, **5:**113.

18. Drummond AJ, Rambaut A: **BEAST: Bayesian evolutionary analysis by sampling trees.** *BMC Evol Biol* 2007, **7:**214.

19. 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 7):**1719-1729.

20. Kramvis A, Kew MC: **Molecular characterization of subgenotype A1 (subgroup Aa) of hepatitis B virus.** *Hepatol Res* 2007, **37:**S27-32.

21. 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.

22. Mello FC, Souto FJ, Nabuco LC, Villela-Nogueira CA, Coelho HS, Franz HC, Saraiva 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. Motta-Castro AR, Martins RM, Araujo NM, Niel C, Facholi GB, Lago BV, Mello FC, Gomes SA: **Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community.** *Arch Virol* 2008, **153:**2197-2205.

24. Quintero A, Martinez D, Alarcon De Noya B, Costagliola 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.

25. Alencastro LF: **O trato dos viventes: formação do Brasil no Atlântico Sul.**

São Paulo: *Companhia das Letras.* 2000; 9-523.

26. 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 Int* 2006, **26:**636-642.

27. Ribeiro D: **O Povo Brasileiro: a formação e o sentido do Brasil.** *Companhia das Letras*, São Paulo. 1995.

28. Matos MA, Reis NR, Kozlowski 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.** *Trans R Soc Trop Med Hyg* 2009, **103:**899-905.

FIGURES LEGENDS

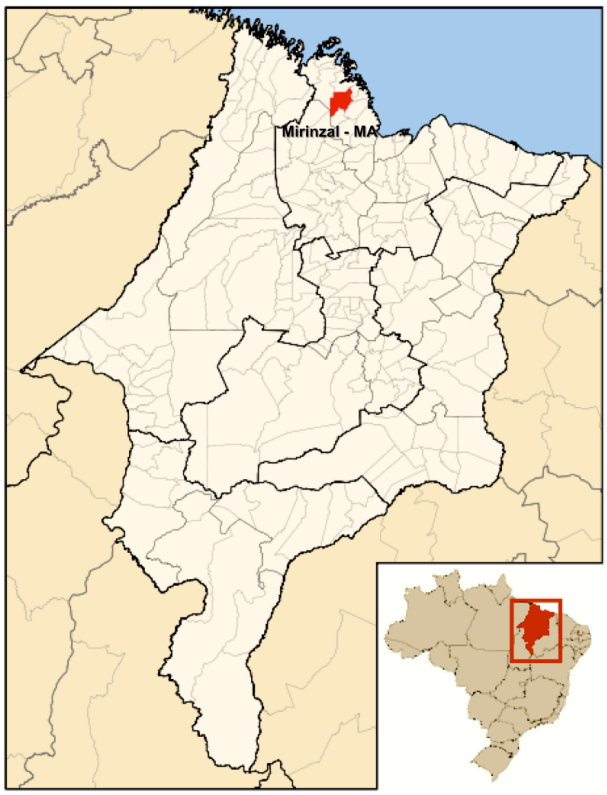


Figure 1 - Geographical localization of the Afro-Brazilian community in Maranhão State – Brazil.

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 are depicted above the respective nodes. Samples HBV/A1 obtained from Frechal (n=4, red taxa) were analyzed together with other worldwide strains**.** The clusters containing the strains of others Afro-Brazilian communities previously reported are highlighted (Blue taxes). 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.

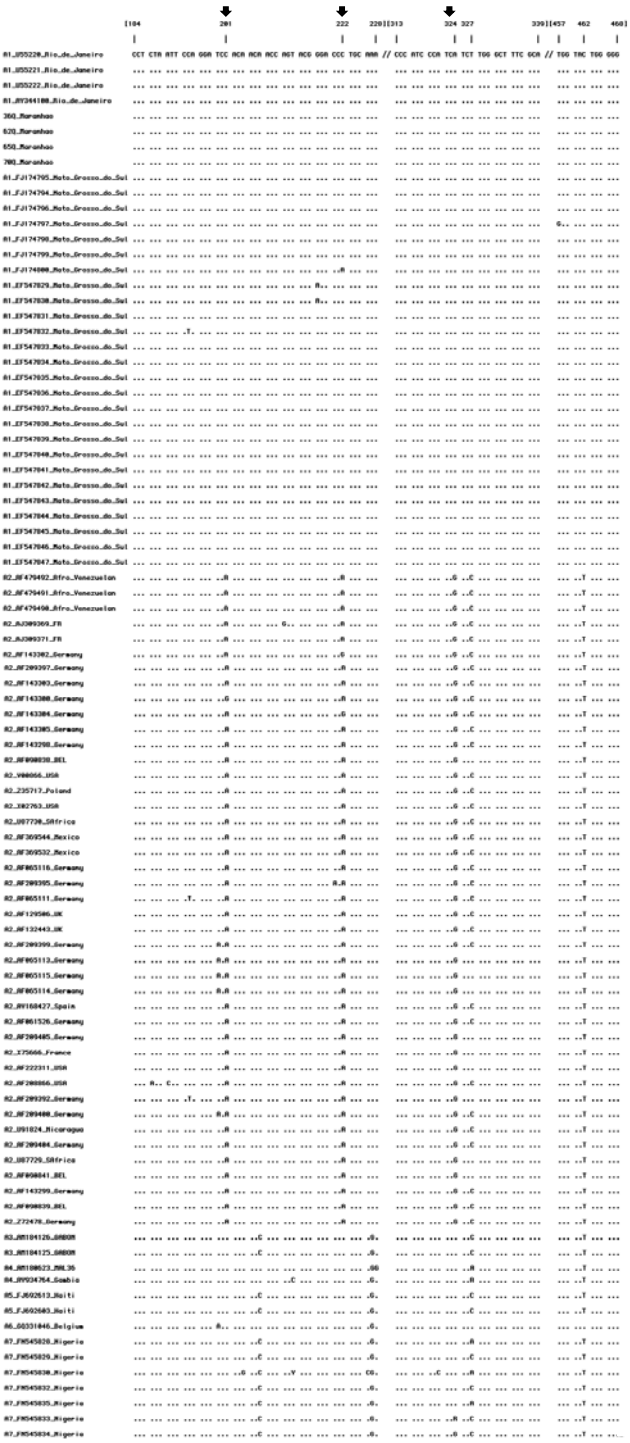


Figure 3 - Multiple alignment of partial HBV/S gene (184 to 468 nt) comprising 34 HBV/A1 sequences from Brazil, which are comparing with other sequences of subgenotypes HBV/A2 to HBV/A7 previously reported. Five positions are indicated (➔) in the figure.

The Liver Meeting®
THE 62ND ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR THE STUDY OF LIVER DISEASES 2011
San Francisco, CA • Moscone West • November 4 -8, 2011

PRESENTATION TYPE: Oral or Poster

CURRENT CATEGORY: Hepatitis B

CURRENT DESCRIPTORS: SO5. Epidemiology/Prevention/Control

TITLE: High prevalence of hepatitis B virus genotype A1 in patients with chronic infection from Pernambuco state, Brazil

AUTHORS (FIRST NAME, LAST NAME): Izolda M. Moura[2], Monica V. Alvarado-Mora[1], João R. Pinho[1], Flair J. Carrilho[1], Edmundo P. Lopes[2]

Institutional Author(s):

INSTITUTIONS (ALL): 1. Gastroenterology, Sao Paulo University, São Paulo , São Paulo, Brazil.
2. Gastroenterology, Federal University of Pernambuco, Recife, Pernambuco, Brazil.

ABSTRACT BODY: Infection with hepatitis B virus (HBV) is a worldwide health problem, infecting about two billion people, with more than 350 million chronic carriers. HBV has been classified into nine different genotypes, designated from A to I, that represent genetically stable viral populations that share a common ancestor but show diverse evolutionary history. They emerged in specific human populations and migrated with their hosts to other areas in the world, leading to their current geographical distribution. In Brazil, the prevalence of HBV varies throughout the country and is especially high in the North and Northeast regions. Some studies showed that genotypes A, D and F are the most frequent around the country. The aim of this study was to characterize for the first time the HBV subgenotypes circulating in patients with chronic hepatitis B from Pernambuco, a Brazil state located in the Northeast region of the country. We included 68 patients with chronic infection. A fragment of 1306 bp comprising part of the DNA polymerase and the HBsAg (S/POL) was amplified and sequenced. The sequences obtained were genotyped by phylogenetic analysis using reference sequences from each genotype obtained from GenBank (n=267). They were aligned using Clustal X software and edited with the SE-AL software. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3. 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. The frequency of subgenotype found was A1 (78.7%), F2a (12.1%), A2 (6.2%) and F4 (3.0%). Subgenotype A1 was the most prevalent in this study and most of subgenotype A1 sequences grouped within the same cluster with high posterior probability in the phylogenetic tree. Isolates belonging to subgroup A1 have been mostly identified in African populations and their descendants. This genotype has been reported in several studies related to the presence of Afro-descendants in Brazil. The high prevalence of subgenotype A1 and the results of the phylogenetic analysis strongly suggest that these sequences originated from a unique lineage. This lineage was introduced into this community possibly between XVI and XVII centuries when the slaves came from West Africa. This finding agrees with the origins of Brazilian population, which is a mixture of European-descendants, Indigenous people and African-descendants.

VIROLOGY JOURNAL

RESEARCH Open Access

Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondônia, Brazil

Alcione O Santos[1], Mónica V Alvarado-Mora[2*], Lívia Botelho[2], Deusilene S Vieira[1], João R Rebello Pinho[2], Flair J Carrilho[2], Eduardo R Honda[1], Juan M Salcedo[1]

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-Al 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 (HBPol) 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 immunodefficiency 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
[2]Laboratory 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

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 Eletropherogram 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

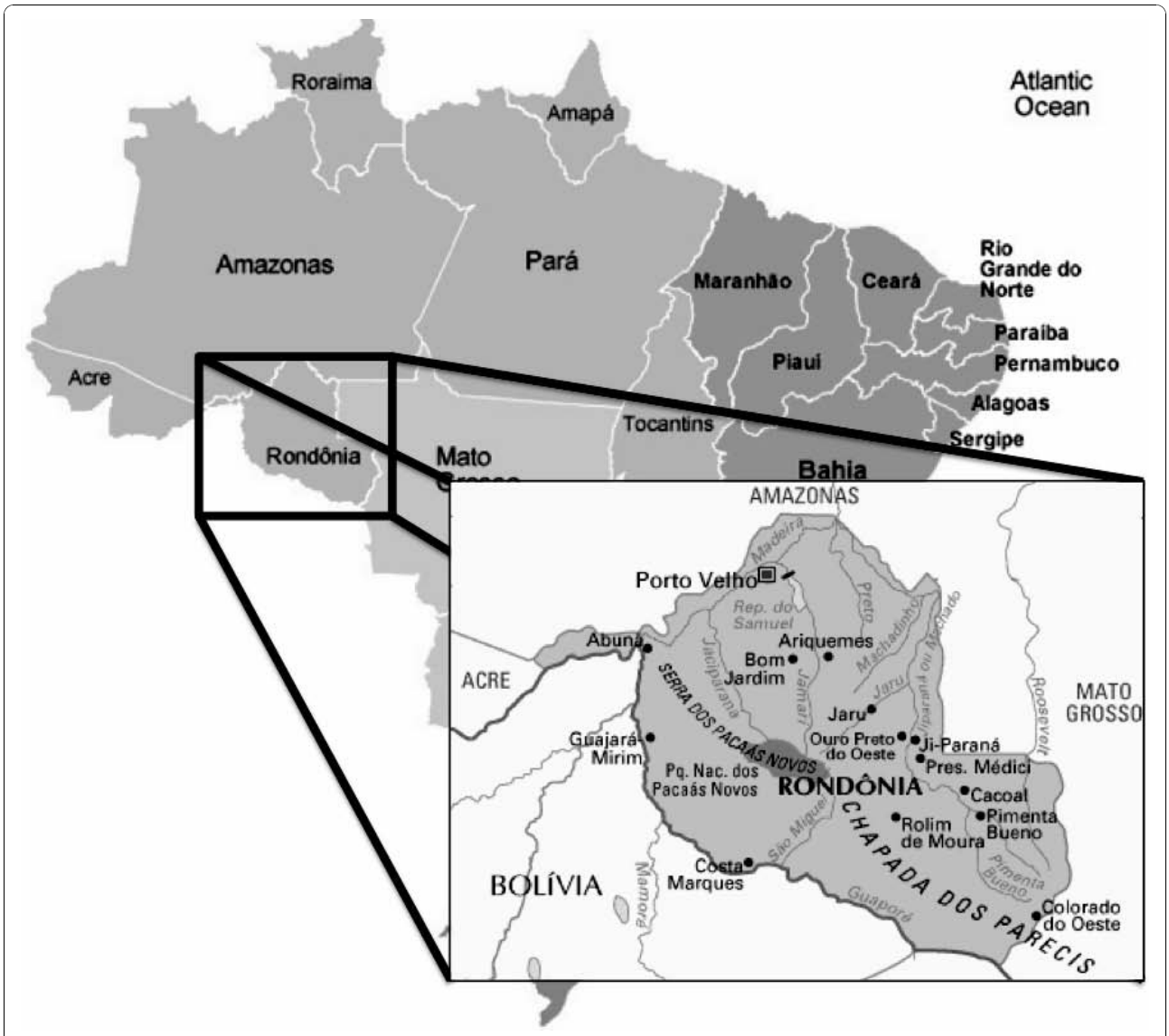


Figure 1 Geographic location of Rondônia state, Brazil.

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 deposed 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

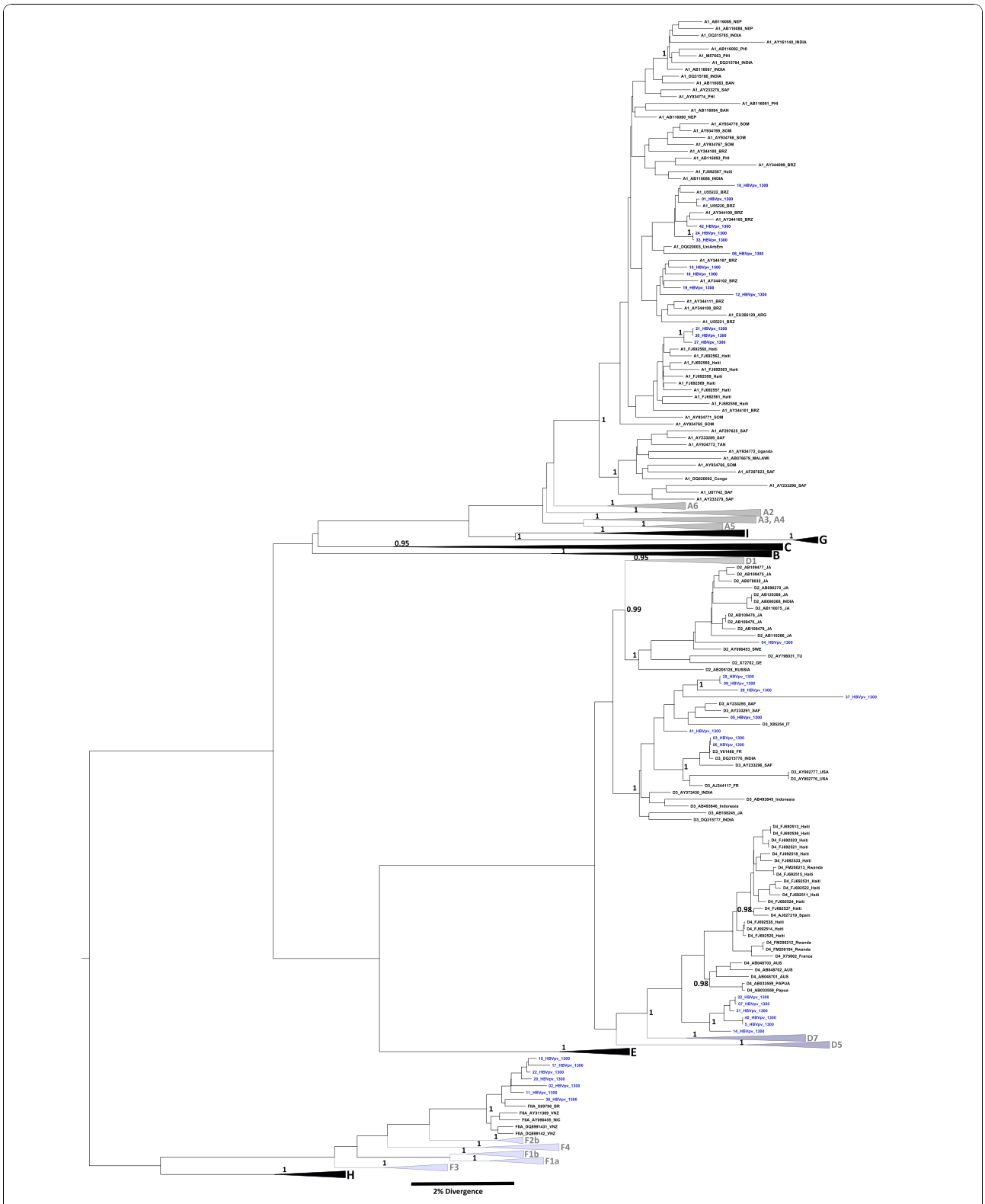


Figure 2 The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 383 S/POL sequences with 1306 bp of Hepatitis B virus strains. The posterior probabilities (> 0.95) of the key nodes are depicted above the respective nodes. The HBV isolates from Rondônia state are represented in blue and were analyzed together other worldwide strains. The collapsed clades correspond to the other genotypes of HBV.

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 therapeutical 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, immunotolerancy, acute and chronic hepatitis, cirrhosis and/or hepatocellular carcinoma).

Acknowledgements
This work was supported by IPEPATRO, CEPEM, CNPq and Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (2007/53457-7 and 2008/50461-60), São Paulo, SP, Brazil.

Author details
[1]Research Center for Tropical Medicine - CEPEM/Tropical Pathology Research Institute-IPEPATRO. Porto Velho, RO, Brazil. [2]Laboratory 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
AOS participated in the design of the study and drafted the manuscript. MVAM 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. JRRP 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

1. Magnius LO, 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.
2. 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.
3. Rehermann B, Nascimbeni M: **Immunology of hepatitis B virus and hepatitis C virus infection.** *Nat Rev Immunol* 2005, **5**:215-229.
4. Owada T, Matsubayashi K, Sakata H, Ihara 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 Viral Hepat* 2006, **13**:11-18.
5. McMahon BJ: **The natural history of chronic hepatitis B virus infection.** *Hepatology* 2009, **49**:S45-55.
6. 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 "I".** *PLoS One* 2010, **5**:e9297.
7. Echevarria JM, Avellon A: **Hepatitis B virus genetic diversity.** *J Med Virol* 2006, **78(Suppl 1)**:S36-42.
8. Mahtab MA, Rahman S, Khan M, Karim F: **Hepatitis B virus genotypes: an overview.** *Hepatobiliary Pancreat Dis Int* 2008, **7**:457-464.
9. Mulders MN, Venard V, Njayou M, Edorh AP, Bola Oyefolu AO, Kehinde MO, Muyembe Tamfum JJ, Nebie YK, Maiga I, Ammerlaan W, Fack F, Omilabu 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.
10. 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.
11. 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, **91**:501-508.
12. 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.
13. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Botelho L, Carrilho FJ, Pinho JRR: **Molecular characterization of the Hepatitis B virus genotypes in Colombia: A Bayesian inferences on the genotype F.** *Infection, genetics and Evolution* .
14. 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.
15. Jutavijittum P, Yousukh A, Samountry B, Samountry 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.
16. Olinger CM, Jutavijittum P, Hubschen JM, Yousukh A, Samountry B, Thammavong T, Toriyama K, Muller CP: **Possible new hepatitis B virus genotype, southeast Asia.** *Emerg Infect Dis* 2008, **14**:1777-1780.
17. Schaefer S: **Hepatitis B virus taxonomy and hepatitis B virus genotypes.** *World J Gastroenterol* 2007, **13**:14-21.
18. Olinger CM, Venard V, Njayou M, Oyefolu AO, 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**:1163-1173.
19. 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.
20. 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 7)**:1719-1729.
21. 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.
22. Hannoun C, Soderstrom A, Norkrans 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.
23. Kurbanov F, Tanaka Y, Fujiwara K, Sugauchi F, Mbanya D, Zekeng L, Ndembi 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.
24. Makuwa M, Souquiere S, Clifford SL, Mouinga-Ondeme 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.
25. 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, **15**:1222-1228.
26. Pourkarim MR, Lemey P, Amini-Bavil-Olyaee S, Maes P, Van Ranst M: **Novel hepatitis B virus subgenotype A6 in African-Belgian patients.** *J Clin Virol* 2009, **47**:93-96.
27. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, 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.
28. Chandra PK, 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 Viral Hepat* 2009, **16**:749-756.
29. Utama A, Octavia TI, Dhenni R, Miskad UA, Yusuf I, Tai S: **Hepatitis B virus genotypes/subgenotypes in voluntary blood donors in Makassar, South Sulawesi, Indonesia.** *Virol J* 2009, **6**:128.
30. Meldal BH, Moula NM, Barnes IH, Boukef K, Allain JP: **A novel hepatitis B virus subgenotype, D7, in Tunisian blood donors.** *J Gen Virol* 2009, **90**:1622-1628.
31. Kramvis A, Kew MC: **Epidemiology of hepatitis B virus in Africa, its genotypes and clinical associations of genotypes.** *Hepatol Res* 2007, **37**: S9-S19.
32. Osiowy C, Gordon D, Borlang J, Giles E, Villeneuve JP: **Hepatitis B virus genotype G epidemiology and co-infection with genotype A in Canada.** *J Gen Virol* 2008, **89**:3009-3015.
33. Kato H, Fujiwara K, Gish RG, Sakugawa H, Yoshizawa H, Sugauchi F, Orito 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.** *Hepatol Res* 2010, **40**:14-30.
36. Chu CJ, Keeffe EB, Han SH, Perrillo RP, Min AD, Soldevila-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 , *et al*: **High soroprevalence 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 [ftp://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. 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.
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, Wendl 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, Sakugawa H, Sastrosoewignjo 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(Pt 10)**:2575-2583.
48. 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, **73(Pt 5)**:1201-1208.
49. Moraes MT, Gomes SA, 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**:1767-1773.
50. Blitz L, Pujol FH, Swenson PD, Porto L, Atencio R, Araujo M, Costa L, Monsalve DC, Torres JR, Fields HA, Lambert S, Van Geyt C, Norder H, Magnius 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, Costagliola 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, Martinelli 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, Villela-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, 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 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, Guimaraes 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. Marquese RB: **A dinâmica da escravidão no Brasil. Resistência, tráfico negreiro e alforrias, 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, Priimagi L, Schmidt J, Katargina O, Michailov M, Mukomolov S, Magnius 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 Sao Paulo* 2010, **52(1)**:25-30.
65. Carrilho FJ, Moraes CR, Pinho JR, Mello IM, Bertolini DA, Lemos MF, Moreira RC, Bassit 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. Becker CE, Mattos AA, Bogo MR, Branco F, Sitnik 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* **47**:13-17.
67. Alcalde R, Melo FL, Nishiya A, Ferreira SC, Langhi Junior MD, Fernandes SS, Marcondes LA, Duarte AJ, Casseb J: **Distribution of hepatitis B virus genotypes and viral load levels in Brazilian chronically infected patients in Sao Paulo city.** *Rev Inst Med Trop Sao Paulo* 2009, **51**:269-272.
68. Mendes-Correa MC, Pinho JR, Locarnini S, Yuen L, Sitnik R, Santana RA, Gomes-Gouvea 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**:1481-1488.
69. Medici MC, Aloisi 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. Dal Molin G, Poli A, Croce LS, D'Agaro P, Biagi C, Comar M, Tiribelli 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.

Journal of Medical Virology 83:1530–1536 (2011)

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 Brahm[1]

[1]*Gastroenterology Section, Department of Medicine, University of Chile Clinical Hospital, Santiago, Chile*
[2]*Laboratory 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*
[3]*Laboratory of Hepatitis Viruses, Virology Program. Institute of Biomedical Sciences , Faculty of Medicine, Santiago, Chile*
[4]*Victorian 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-naive 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 AxSym, Abbott, North Chicago, IL), and HBeAg+/anti-HBe negative, as determined by a commercially available kit from mimiVIDAS (Biomèrieux, 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 HBIg, 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 GTA 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

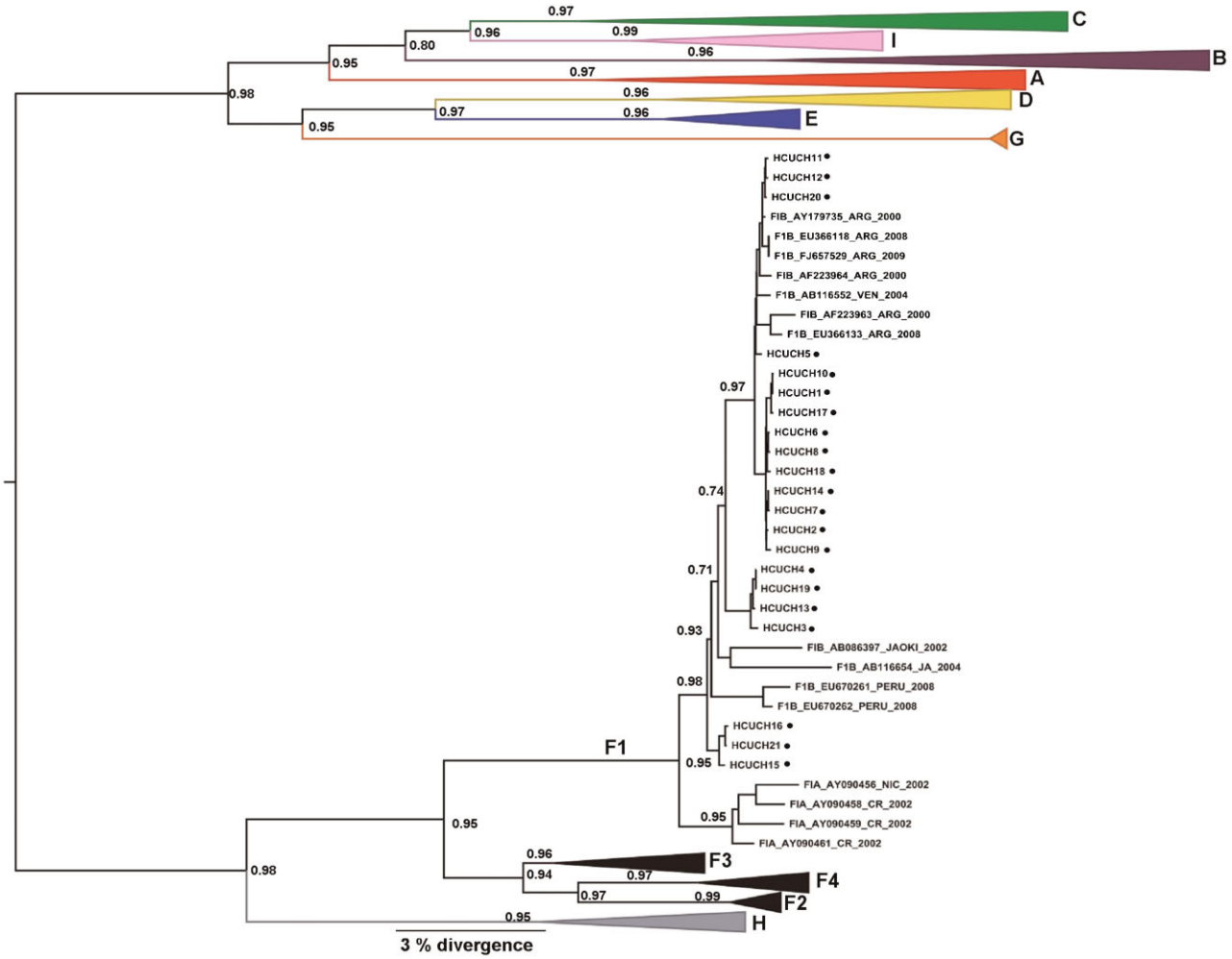


Fig. 1. The Maximum Clade Credibility (MCC) tree was estimated by a Bayesian analysis of 290 complete genome sequences of hepatitis B virus strains. The posterior probabilities of the key nodes are shown above the respective nodes. The HBV/F samples obtained from Chile (n = 21, HCUCH) were analyzed together 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 HBV/F subgenotypes. 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 HBV/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.

HBIg/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 HBIg, the anti-HBs monoclonal antibody or the vaccination-associated anti-HBs. Furthermore, examination of the HCUCH5 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 (HCUCH16 and HCUCH21) presented BCP mutations: A1762T and G1764A. Besides this, the sequence from patient HCUCH5 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 HCUCH5 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 [Torresi 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 tN236T [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-naive 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-Gouvea 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 B, Magnius 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, Magnius L, Echevarría 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.

DiLello F, Piñeiro 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-Trebi 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, Couroucé A, Magnius 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, Sastrosoewignjo 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.

Posada 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, Mauss 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, Rossau 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.

Tiollais P, Charnay P, Vyas G. 1981. Biology of hepatitis B virus. Science 213:406–411.

Torresi J, Earnest-Silveira L, Deliyannis G, Edgtton 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.

vanBömmel F, Man Rd, Wedemeyer H, Deterding K, Petersen 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 L, 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 PJ, Xia NS. 2010. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". PLoS ONE 5:e9297.

Yuen L, Ayres A, Littlejohn M, Colledge D, Edgely 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.

CAPÍTULO 2 : O VÍRUS DA HEPATITE C NA AMÉRICA DO SUL

2. 1 Características gerais do vírus da hepatite C

O vírus da hepatite C (HCV) é classificado dentro da família *Flaviviridae,* embora apresente algumas diferenças importantes em relação a outros vírus da mesma família, sobretudo no que diz respeito à organização de seu genoma, portanto está classificado no gênero *Hepacivirus*. Conhecem-se seis genótipos filogeneticamente distintos, denominados de HCV-1 até HCV-6, os quais chegam a diferir em 30 a 35% de seu genoma. Dentro dos genótipos, foram relatados mais de 100 subtipos, os quais se diferenciam entre si de 20 até 25% no seu genoma (Cristina & Colina, 2006; Katsoulidou *et al.*, 2006).

Genótipos 1, 2 e 3 possuem distribuição mundial, enquanto que os genótipos 4 a 6 são restritos a áreas distintas (Pasquier *et al.*, 2005). Os genótipos são ainda subdivididos em subtipos identificado pelas letras minúsculas. Os subtipos 1a, 1b, 2c e 3a são os de maior prevalência mundial e se encontram por todas as regiões do mundo. O genótipo 4 é o predominante no Egito, Norte e Centro da África e no Oriente Médio; o genótipo 5, na África e o genótipo 6, na Indochina (Pasquier *et al.*, 2005).

Atualmente, é bem estabelecido que a infecção pelo HCV é de importância global, constituindo um grave problema de saúde que requer amplas e ativas intervenções para a sua prevenção e controle. Estudos

prospectivos têm demonstrado que 80% dos casos de hepatite C aguda progridem para infecção crônica e 10 a 20% destes desenvolverão complicações da doença hepática crônica, como cirrose hepática e/ou carcinoma hepatocelular. Se estima que 6,8 a 8,9 milhões de adultos sejam anti-HCV positivos na América Latina e uma prevalência de 1,5% nas Américas em 2010 (Lavanchy, 2010; Kershenobich *et al.,* 2011).O HCV é um vírus envelopado, genoma RNA fita simples, de polaridade positiva, com aproximadamente 9.600 nucleotídeos, organizados numa fase de leitura aberta (ORF) que codifica para as proteínas estruturais (core, E1 e E2) e para sete proteínas não estruturais (p7, NS2 a NS5b). Nos extremos dessa ORF, encontram-se regiões não traduzidas (5`UTR e 3`UTR) que têm função importante na regulação da ativação e replicação viral (Cristina & Colina, 2006).

Cada indivíduo apresenta clara variabilidade genética que está associada à baixa fidelidade da enzima RNA polimerase RNA dependente e à alta taxa de replicação viral, o que faz com que o vírus possa se adaptar ao hospedeiro. Esta variabilidade genética ocorre devido a uma taxa de mutação espontânea de 1,5 a 2 x 10^{-3} substituições de nucleotídeos por sítio por ano (Katsoulidou *et al.,* 2006; Pasquier *et al.,* 2005), que é mais evidente nos segmentos genéticos que codificam as proteínas E1 e E2, em contraste com o alto nível de conservação da região 5`UTR (Lole *et al.*, 2003).

As proteínas não estruturais do HCV e o RNA viral têm sido detectados no fígado de pacientes ou de chimpanzés inoculados experimentalmente,

confirmando que o fígado é o lugar de replicação do vírus. Infelizmente, a concentração de proteínas e de RNA em tecidos infectados é baixa, gerando assim a necessidade de métodos de detecção mais sensíveis (Bartenschlager & Lohmann, 2000).

A análise cuidadosa da dinâmica da replicação viral durante o tratamento antiviral com IFN-α revelou a produção de 10^{12} partículas por dia, sendo a meia vida do vírion de 3 a 5 horas (Neumann *et al.*, 1998). Apesar deste número elevado, poucos vírus são gerados quando se faz a relação com o número total de hepatócitos, i.e., assume-se que aproximadamente 10% dos cerca de $2x10^{11}$ hepatócitos são infectados, o que nos leva a uma taxa de produção de 50 partículas de vírus por hepatócito por dia (Neumann *et al.,* 1998).

A infecção na célula hospedeira se inicia com a liberação da fita de RNA (+) dentro do citoplasma e tradução da mesma. A poliproteína é processada e as proteínas virais permanecem associadas firmemente às membranas do reticulo endoplasmático (RE). Uma fita de RNA (-) é sintetizada por uma replicase composta por NS3-5B e serve como molde para a produção excessiva da fita positiva de RNA. A interação do RNA (+) com as proteínas estruturais forma a partícula viral. Estas partículas são envelopadas dento do lúmen do retículo endoplasmático e são levadas para fora através do complexo de Golgi (Bartenschlager & Lohmann, 2000).

2.2 Epidemiologia do HCV na Colômbia

A infecção pelo HCV é um problema de saúde pública no mundo, sendo que ao redor de 3% da população mundial está infectada com este vírus. Os indivíduos infectados cronicamente apresentam um risco aumentado de desenvolver cirrose e carcinoma hepatocellular (Alter & Seelf, 2000; McHutchison & Bacon, 2004).

As regiões do mundo com as maiores taxas de prevalência estão localizadas na África e na Ásia, enquanto as áreas de baixa prevalência incluem América do Norte, Europa e Austrália. Na China, já foram relatadas prevalências para o HCV de até 9,6% (Zhou *et al.,* 2010). Na América Latina, a prevalência do HCV em toda a região é de 1,23% (Te & Jensen, 2010). No entanto, esta varia de região para região, de 0,2% - 0,5% no Chile até 1,7% - 3,4% no Nordeste do Brasil (Carrilho & Mendes-Correa, 1998; Soza *et al.,* 2010; Te & Jensen, 2010).

As estimativas da prevalência do HCV na Colômbia correspondem aos dados coletados na população de doadores de sangue, uma vez que não existem estudos publicados da prevalência na população em geral. Dessa forma, este estudo é o primeiro trabalho que estuda a prevalência para o HCV na população da Colômbia **(Alvarado-Mora *et al.,* 2011a)**.

Na Colômbia, a triagem para anti-HCV é realizada desde 1991, sendo obrigatória desde 1993 (Ministério de la Salud, 1996), o que tem incrementado

a cobertura deste teste na maior parte dos bancos de sangue. Em um estudo realizado em 2002, encontrou-se que o risco de receber uma transfusão infectada pelo HCV na Colômbia era de 0,24 em cada 10.000 doações (Schmunis & Cruz, 2005).

Mais recentemente, outro estudo realizado na Colômbia em 2005 teve como objetivo determinar a prevalência de marcadores sorológicos do HCV e avaliar os fatores de risco para a infecção pelo HCV nas cidades de Bogotá e Medellín. O estudo constatou que 9% (n=45) entre 500 pacientes multi-transfundidos estavam infectados com este vírus. Na Colômbia, conhecer a distribuição dos genótipos do HCV em pacientes politransfundidos pode fornecer indicações sobre a epidemia do HCV no país, sendo que a transfusão de sangue ainda é o principal fator de risco (Beltran *et al.,* 2005).

No estudo realizado na população da Colômbia em 2007 **(Alvarado-Mora *et al.,* 2011a)**, encontrou-se uma prevalência total de 3,55% na população estudada, sendo que o estado do Amazonas apresentou as maiores taxas de anti-HCV positivo (5,68%). A menor frequência foi encontrada na Ilha de San Andrés (0,66%) e níveis intermediários foram encontrados em dois outros estados (Magdalena e Amazonas), mas essas diferenças não foram estatisticamente significativas (p= 0,107). Considerando os grupos etários, a maior frequência de anti-HCV foi encontrada na população entre 26 a 30 anos de idade (7,22%), mas esta frequência não foi estatisticamente maior quando comparada com os outros intervalos de idade (p = 0,259).

Surpreendentemente, apesar de não ter sido encontrada significância estatística, a maior prevalência foi encontrada na região do Amazonas da Colômbia, sendo que os relatos prévios não tem relatado altas prevalências na região amazônica de outros países como Venezuela (Blitz-Dorfman *et al.,* 1994) e Brasil (De Paula *et al.,* 2001). Estes resultados mostram que a infecção pelo HCV é também importante nesta região do país, devendo ser reforçado o acompanhamento desta infecção nas diferentes regiões da Colômbia, pois em algumas localidades, em especial no estado do Amazonas, o acesso ao diagnóstico desta infecção é ainda muito difícil. Na América Central, tem-se relatado valores até de 6,3% de prevalência, como é o caso de San Juan de Porto Rico em 2001 (Perez *et al.,* 2005). No México, relatou-se prevalência de 1,2% (Uribe & Mendes-Sanchez, 2002). Na América Latina, o genótipo 1 é o mais prevalente (Pujol *et al.,* 1997; Colina *et al.,* 1999, Vega *et al.,* 2001; **Viera *et al.,* 2011**). Entretanto, diferenças na distribuição dos genótipos do HCV têm sido observadas dentro do nosso continente, particularmente na região do Caribe, onde os genótipos 2 e 4 tem uma frequência importante, diferindo do resto da América Latina (Quarleri *et al.,* 2000; San Roman *et al.,* 2002). Na figura 2, encontra-se a distribuição atual dos genótipos e subtipos do HCV na América Latina.

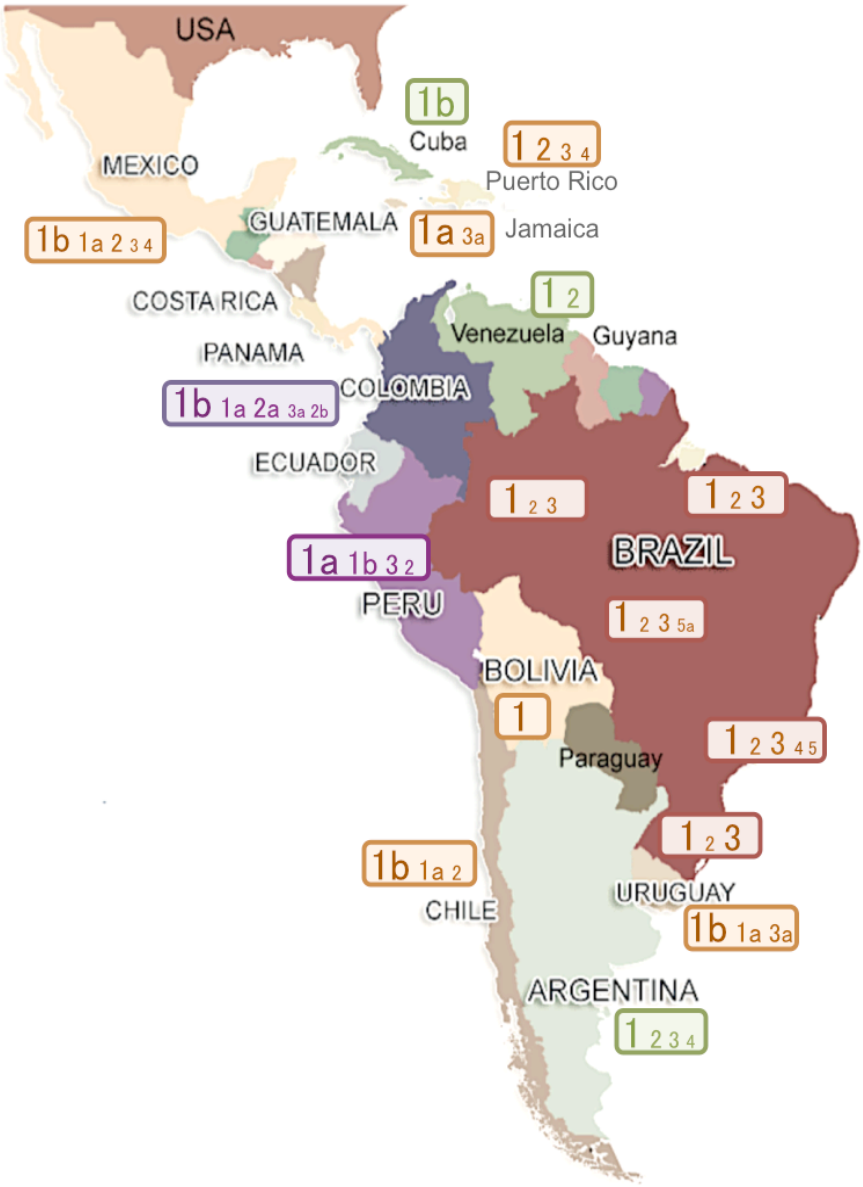


Figura 2 – Distribuição dos genótipos e subtipos do vírus da Hepatite C na América Latina.

Na população de 184 doadores de sangue de Bogotá, foi realizada pelo nosso grupo a caracterização dos subtipos do HCV, encontrando o subtipo 1b

como o mais frequente (82,8%). Da mesma forma, foram encontrados em menor frequência os subtipos 1a (5,7%), 2a (5,7%), 2b (2,8%) e 3a (2,8%) **(Alvarado-Mora *et al.,* 2010g)**. Embora altas frequências do subtipo 1b entre doadores de sangue tenham sido relatadas em outros países da América do Sul, como Brasil (Amorim *et al.,* 2004), México (Garcia Montalvo & Macosay-Castillo, 2007), Chile (Munos *et al.,* 1998) e Peru (Sanchez *et al.,* 2000), o valor encontrado na Colômbia (82.8%) foi o mais alto encontrado até o presente momento.

Sendo o subtipo 1b o mais prevalente, foi realizada uma análise que procurou determinar a origem e o crescimento deste subtipo na população, encontrando que o mesmo está circulando possivelmente na população da Colômbia desde 1950. Da mesma forma, foi determinado que esse subtipo apresentou sinais de crescimento exponencial desde o final da década de 70 até começo da década de 90, quando começou uma queda no crescimento da infecção viral na população. Os resultados encontrados demonstram que possivelmente o fator mais importante da infecção pelo HCV foi a transfusão de sangue antes de 1995 quando a prática de doação de sangue voluntaria era comum na Colômbia e eram realizadas doações pagas de forma clandestina perto dos hospitais públicos. Dessa forma, um estudo mostrou que nessa época a população apresentava um alto risco de contaminação com bolsas de sangue infectadas pelo HCV (Schmunis & Cruz, 2005). Esses fatores foram envolvidos na explosão do subtipo 1b que descrevemos. Finalmente, em 1991,

iniciou-se o teste para anti-HCV nos bancos de sangue e, em 1993, este foi implementado de forma obrigatória. Dessa forma, observou-se que a partir da introdução da triagem de anticorpos do HCV até a presente data a infecção pelo HCV, em especial pelo subtipo 1b, está sendo controlada na população da Colômbia.

Finalmente, podemos concluir que a infecção pelo HCV provavelmente chegou mais recentemente na capital da Colômbia **(Alvarado-Mora et al., 2010g)**, quando comparado ao HBV, que se encontra nos povos indígenas colombianos. O subtipo 1b é o mais frequente em doadores de sangue, mas nossos dados indicam que a triagem nos bancos de sangue sendo eficaz no controle desta infecção. Mas ainda persiste a necessidade de melhorar a triagem para esta infecção viral em comunidades isoladas, como é o caso daquelas que habitam o estado de Amazonas.

2.4 Referencias

1. Alter HJ, Seeff LB. Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. *Semin Liver Dis*. 2000; 20: 17-35.

2. **Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvea MS, de Azevedo Neto RS, Carrilho FJ, et al.** Hepatitis B (HBV), hepatitis C

(HCV) and hepatitis delta (HDV) viruses in the Colombian population-how is the epidemiological situation? ***PLoS One*. 2011a; 6: e18888**

3. **Alvarado-Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, et al**. Molecular characterization, distribution, and dynamics of hepatitis C virus genotypes in blood donors in Colombia. ***J Med Virol*. 2010g; 82: 1889-1898.**

4. Amorim RM, Oliveira CP, Wyant PS, Cerqueira DM, Camara GN, et al. Hepatitis C virus genotypes in blood donors from the Federal District, Central Brazil. *Mem Inst Osw Cruz.* 2004; 99: 895-897.

5. Bartenschlager R, Lohmann V. Replication of hepatitis C vírus. *J Gen Virol*. 2000; 81, 1631–1648.

6. Beltran M, Navas MC, De la Hoz F, Mercedes Munoz M, Jaramillo S, et al. Hepatitis C virus seroprevalence in multi-transfused patients in Colombia. *J Clin Virol.* 2005; 34 Suppl 2: S33-38.

7. Blitz-Dorfman L, Monsalve F, Porto L, Weir J, Arteaga M, et al. Epidemiology of hepatitis C virus in western Venezuela: lack of specific antibody in Indian communities. *J Med Virol.* 1994; 43: 287-290.

8. Carrilho FJ, Corrêa MCJM. Magnitude of hepatitis B and C in Latin America. In: Schinazi RF, Somadossi JP, Thomas HC, editors. Therapies for viral hepatitis. London: International Medical Press. 1998; 25–34.

9. Colina R, Azambuja C, Uriarte R, Mogdasy C, Cristina J. Evidence of increasing diversification of hepatitis C viruses. *J Gen Virol.* 1999; 80 (6): 1377-1382.

10. Cristina J, Colina R. Evidence of structural genomic region recombination in Hepatitis C virus. *Virol J.* 2006; 3: 53.

11. de Paula VS, Arruda ME, Vitral CL, Gaspar AM. Seroprevalence of viral hepatitis in riverine communities from the Western Region of the Brazilian Amazon Basin. *Mem Inst Osw Cruz.* 2001; 96: 1123-1128.

12. Garcia-Montalvo BM, Macossay-Castillo M. Preliminary data for genotype distribution and epidemiological aspects of hepatitis C virus infection in blood donors from Yucatan, Mexico. *Transfus Med*. 2007; 17: 488-490.

13. Katsoulidou A, Sypsa V, Tassopoulos NC, Boletis J, Karafoulidou A, et al. Molecular epidemiology of hepatitis C virus (HCV) in Greece: temporal trends in HCV genotype-specific incidence and molecular chárácterization of genotype 4 isolates. J Viral Hepat. 2006; 13: 19-27.

14. Kershenobich D, Razavi HA, Sanchez-Avila JF, Bessone F, Coelho HS, Dagher L, et al. Trends and projections of hepatitis C virus epidemiology in Latin America. *Liver Int.* 2011; 31(2): 18-29.

15. Lavanchy D. Evolving epidemiology of hepatitis C virus. *Clin Microbiol Infect.* 2010; 17: 107-115.

16. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol*. 1999; 73(1):152-60.

17. McHutchison JG, Bacon BR. Hepatitis C: a 20-year debt comes due. *Am J Manag Care*. 2004; 10: S20.

18. Ministerio de Salud. Manual de normas técnicas, administrativas y de procedimientos. Capítulo 11. Garantía de calidad. Santa Fe de Bogotá, D.C., Colombia. Imprenta Nacional. 1996.

19. Munoz G, Velasco M, Thiers V, Hurtado C, Brahm J, et al. Prevalencia y genotipos del virus de la hepatitis C en donantes de sangre y en pacientes con enfermedad hepática crónica y hepatocarcinoma en población chilena. Rev Med Chil.1998; 126: 1035-1042.

20. Neumann AU, Lam N P, Dahári H, Gretch DR, Wiley TE, Layden TJ, Perelson AS. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alphá therapy. *Science.* 1998*;* 282:103 -107.

21. Pasquier C, Njouom R, Ayouba A, Dubois M, Sartre M, Vessiere A, Timba I, Thonnon J, Izopet J, Nerrienet E. Distribution and heterogeneity

of hepatitis C genotypes in Hepatitis Patients in Cameroon. *J Med Virol.* 2005; 77: 390-398.

22. Perez CM, Suarez E, Torres EA, Roman K, Colon V. Seroprevalence of hepatitis C virus and associated risk behaviours: a population-based study in San Juan, Puerto Rico. *Int J Epidemiol.* 2005; 34: 593-599.

23. Pujol FH, Loureiro CL, Devesa M, Blitz L, Parra K, et al. Determination of genotypes of hepatitis C virus in Venezuela by restriction fragment length polymorphism. *J Clin Microbiol.* 1997; 35: 1870-1872.

24. Quarleri JF, Robertson BH, Mathet VL, Feld M, Espinola L, et al. Genomic and phylogenetic analysis of hepatitis C virus isolates from argentine patients: a six-year retrospective study. *J Clin Microbiol.* 2000; 38: 4560-4568.

25. San Roman M, Lezama L, Rojas E, Colina R, Garcia L, et al. Analysis of genetic heterogeneity of hepatitis C viruses in Central America reveals a novel genetic lineage. Brief report. *Arch Virol.* 2002; 147: 2239-2246.

26. Sanchez JL, Sjogren MH, Callahan JD, Watts DM, Lucas C, et al. Hepatitis C in Peru: risk factors for infection, potential iatrogenic transmission, and genotype distribution. *Am J Trop Med Hyg.* 2000; 63: 242-248.

27. Schmunis GA, Cruz JR. Safety of the blood supply in Latin America. *Clin Microbiol Rev.* 2005; 18: 12–29

28. Soza A, Riquelme A, Arrese M. Routes of transmission of hepatitis C virus. *Ann Hepatol.* 2010; (9)1: 30-33.

29. Te HS, Jensen DM. Epidemiology of hepatitis B and C viruses: a global overview. *Clin Liver Dis.* 2010; 14: 1-21.

30. Uribe M, Mendez-Sanchez N . Hepatitis C in Mexico. *Rev Gastroenterol Mex*. 2002; 67(2): 7-8.

31. Vega I, Colina R, Garcia L, Uriarte R, Mogdasy C, et al. Diversification of hepatitis C viruses in South America reveals a novel genetic lineage. *Arch Virol*. 2001; 146: 1623-1629.

32. **Vieira DS, Alvarado-Mora MV, Botelho L, Carrilho FJ, Pinho JR, et al.** Distribution of hepatitis c virus (hcv) genotypes in patients with chronic infection from Rondonia, Brazil. **Virol J. 2011; 8: 165.**

33. Zhou S, Zhao Y, He Y, Li H, Bulterys M, et al. Hepatitis B and hepatitis C seroprevalence in children receiving antiretroviral therapy for human immunodeficiency virus-1 infection in China, 2005-2009. *J Acquir Immune Defic Syndr.* 2010; 54: 191-196.

Journal of Medical Virology 82:1889–1898 (2010)

Molecular Characterization, Distribution, and Dynamics of Hepatitis C Virus Genotypes in Blood Donors in Colombia

Mónica Viviana Alvarado Mora,[1]* Camila Malta Romano,[2] Michele Soares Gomes-Gouvêa,[1] Maria Fernanda Gutiérrez,[3] Flair José Carrilho,[1] and João Renato Rebello Pinho[1]

[1]*Laboratory of Gastroenterology and Hepatology, Department of Gastroenterology, School of Medicine, São Paulo Institute of Tropical Medicine, University of São Paulo, São Paulo, Brazil*
[2]*Laboratory of Virology, Department of Infectious and Parasitic Diseases, School of Medicine, São Paulo Institute of Tropical Medicine, University of São Paulo, São Paulo, Brazil*
[3]*Laboratory of Virology, Department of Microbiology, Pontificia Javeriana University, Bogotá, Colombia*

Hepatitis C virus (HCV) is a frequent cause of acute and chronic hepatitis and a leading cause for cirrhosis of the liver and hepatocellular carcinoma. HCV is classified in six major genotypes and more than 70 subtypes. In Colombian blood banks, serum samples were tested for anti-HCV antibodies using a third-generation ELISA. The aim of this study was to characterize the viral sequences in plasma of 184 volunteer blood donors who attended the ''Banco Nacional de Sangre de la Cruz Roja Colombiana,'' Bogotá, Colombia. Three different HCV genomic regions were amplified by nested PCR. The first of these was a segment of 180 bp of the 5′UTR region to confirm the previous diagnosis by ELISA. From those that were positive to the 5′UTR region, two further segments were amplified for genotyping and subtyping by phylogenetic analysis: a segment of 380 bp from the NS5B region; and a segment of 391 bp from the E1 region. The distribution of HCV subtypes was: 1b (82.8%), 1a (5.7%), 2a (5.7%), 2b (2.8%), and 3a (2.8%). By applying Bayesian Markov chain Monte Carlo simulation, it was estimated that HCV-1b was introduced into Bogotá around 1950. Also, this subtype spread at an exponential rate between about 1970 to about 1990, after which transmission of HCV was reduced by anti-HCV testing of this population. Among Colombian blood donors, HCV genotype 1b is the most frequent genotype, especially in large urban conglomerates such as Bogotá, as is the case in other South American countries. ***J. Med. Virol. 82:1889–1898, 2010.***

KEY WORDS: hepatitis C virus; genotype 1b; blood donors; Colombia; Bayesian analysis

INTRODUCTION

Infection by hepatitis C virus (HCV) is a public health problem throughout the world and, ~3% of the world population is infected with this virus. This means that 3–4 million individuals are being infected every year. Individuals infected chronically are at an increased risk of developing cirrhosis of the liver and hepatocellular carcinoma [Echevarria and Leon, 2003; Pasquier et al., 2005; Katsoulidou et al., 2006].

HCV is a positive sense, single-stranded RNA virus with a genome size of ~9,400 bases. It contains a large open reading frame that encodes a precursor polyprotein of about 3,000 amino acids. The virus represents the genus *Hepacivirus* of the family *Flaviviridae* [Ishii et al., 1999]. The polyprotein precursor is cleaved into at least 10 different proteins: the structural core (C) and envelope (E1, E2, and p7) proteins, and the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [Suzuki et al., 2007].

Many tests have been evaluated for viral subtyping by analysis of different regions of the viral genome, such as 5′UTR, C, E1, and NS5B. Genotyping methods used commonly include 5′UTR amplification followed by restriction fragment length polymorphism [Furione et al., 1999]; hybridization with genotype-specific

Disclosures: Authors have no conflict of interest.

Grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Grant numbers: 2007/53457-7, 2008/50461-6; Grant sponsor: Pontificia Universidad Javeriana, Bogotá, Colombia.

*Correspondence to: Mónica Viviana Alvarado Mora, Faculdade de Medicina, Departamento Gastroenterologia, Universidade de São Paulo, Av Dr. Enéas de Carvalho Aguiar, 500 2º andar, Prédio IMT 2, São Paulo, SP, Brazil.
E-mail: monica.viviana@usp.br

Accepted 22 June 2010

DOI 10.1002/jmv.21908

Published online in Wiley Online Library (wileyonlinelibrary.com)

primers [Spada et al., 1998]; or direct nucleotide sequencing. Nevertheless, these methods cannot establish HCV subtypes with certainty [Halfon et al., 1997; Sandres-Saune et al., 2003]. For this purpose, the sequencing of the NS5B has been standardized and used for identification of HCV subtypes, as the region contains subtype-specific motifs, being more appropriate for epidemiological applications [Maertens and Stuyver, 1997]. Consequently, partial genome sequences, particularly those from the E1 and NS5B genes, are used commonly in HCV genotyping, especially when evolutionary analysis is of interest [Simmonds et al., 1993; Smith et al., 1997].

The sequencing of HCV isolates has identified 6 genotypes and more than 70 subtypes [Pawlotsky, 2003]. These genotypes differ by 31–34% in their nucleotide sequence and by 30% in their amino acid sequence. Accurate HCV genotyping can be used for predicting response to anti-viral therapy, as genotypes 1 and 4 are less likely to respond to interferon than genotypes 2 and 3 [Pawlotsky, 2003]. Furthermore, genotyping is an essential tool for epidemiological studies, as HCV genotypes vary among geographical regions [Martell et al., 2004; Cantaloube et al., 2006].

A study done in Colombia in 2005 aimed to determine the prevalence of HCV serological markers and investigate the risk factors for this infection in the cities of Bogotá and Medellín. The study found that 45 (9%) among 500 multi-transfused patients were infected with this virus. In this study, we aimed to report the current distribution of HCV genotypes in Bogotá, Colombia. Knowing the distribution of HCV genotypes in multi-transfused patients may provide indications on the course of HCV epidemics, as blood transfusion is still the main risk factor for HCV infection in Colombia and other countries [Beltran et al., 2005]. The possible time of appearance of the most prevalent subtype of HCV in Colombia was also investigated.

MATERIALS AND METHODS

Genotyping Analysis

Study population. A total of 184 serum samples considered to be reactive for the presence of anti-HCV antibodies were obtained from the blood bank of Cruz Roja Colombiana in Bogotá city, Colombia and stored at −20°C. These samples were tested using a third-generation ELISA and were collected between 2003 and 2007. Of the 184 volunteer blood donors, 90 were women (mean age was 40 years) and 94 were men (mean age was 37; standard deviation 4.45).

HCV RNA extraction. To avoid false-positive results, rigorous procedures used for nucleic acid amplification diagnostic techniques were followed [Kwok and Higuchi, 1989]. HCV-RNA extraction was carried out from 140 μl of serum using QIAamp® Viral RNA Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. The synthesis of the complementary DNA (cDNA) was done immediately after RNA extraction.

Synthesis of the complementary DNA (cDNA). Reverse transcriptase reaction was performed using the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and random primers. The final volume of the reaction was 60 μl in the following concentrations: 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 450 ng random primers, 30 U RNAse enzyme inhibitor (RNase OUT™), and 300 U MMLV-RT.

Samples were submitted to the following temperature cycles: 70°C for 10 min, 25°C for 15 min, 37°C for 60 min, and 95°C for 15 min in a thermocycler (Eppendorf Mastercycler ®, Eppendorf, Hamburg, Germany).

Polymerase chain reaction (PCR). Polymerase chain reactions (PCR) were done in two stages to increase sensitivity.

Three segments of the HCV genome were amplified: 5′UTR (180 bp), NS5B (380 bp), and E1 (391 bp). The 5′UTR was amplified to detect current viral infection in anti-HCV-positive samples. NS5B and E1 regions were amplified for genotyping and evolutionary analysis. Specific primers used for amplification and amplification cycles were as described [Enomoto et al., 1990; Garson et al., 1990, 1991; Bukh et al., 1993; Sandres-Saune et al., 2003]. Reactions were done in a final volume of 50 μl. The cDNA (5 μl) was added to 20 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 pmol/μl of each primer, and 2.5 U *Platinum* Taq DNA polymerase. All the reagents used were from Invitrogen™ Life Technologies (Carlsbad, CA).

HCV sequencing. Amplified cDNA was purified using the ChargeSwitch® PCR Clean-Up Kit. Sequencing was done in an ABI Prism® 377 Automatic Sequencer (Applied Biosystems, Foster City, CA) using dideoxy nucleoside triphosphates (ddNTPs) 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 and Green, 1998] and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using the CAP3 software available at the web page for electropherogram quality analysis (http://asparagin.cenargen.embrapa.br/phph/).

Evolutionary Analysis

Phylogenetic analysis. For genotyping, sequences obtained from NS5B and E1 regions were aligned with other reference sequences available in the GenBank using Clustal X software [Thompson et al., 1997] and manually edited using the SE-AL software (available at: http://tree.bio.ed.ac.uk/software/seal/). Maximum likelihood (ML) phylogenetic trees of the 212 NS5B sequences and 187 E1 sequences were inferred using the GARLi program (Genetic Algorithm for Rapid Likelihood Inference) [Zwickl, 2006], which uses an extensive branch-swapping protocol that optimizes the substitution model parameters iteratively during the

search. The parameters of the evolutionary model of DNA substitution were estimated by MODELTEST v. 3.7 [Posada and Crandall, 1998] and were used on GARLi, which optimized the parameters during searches. Phylogenetic trees were visualized and midpoint rooted in FigTree v1.2.2 (http://tree.bio.ed.ac.uk/software/figtree/). The robustness of the phylogenetic groups was evaluated by 1,000 bootstrap replicates.

Bayesian analysis. To investigate the demographic history of hepatitis C in Bogotá, the Bayesian Markov chain Monte Carlo simulation implemented in BEAST v.1.4.8 [Drummond and Rambaut, 2007] was used. The time of the most recent common ancestor (TMRCA) was estimated using two data sets. The first data set comprised the 25 Colombian sequences that clustered together in the maximum a posteriori (MAP) tree reconstructed with worldwide sequences. As the coalescent analysis based on a small data set might be inaccurate, the TMRCA of Colombian viruses was also estimate using a larger data set (n = 88) containing only sequences from subtype 1b, to ensure that the most accurate possible parameters were obtained.

Four Colombian sequences clustered together viruses from other countries in both MAP tree and ML tree, which means that they do not represent the viruses that are largely circulating in Colombia. Therefore, since these sequences do not share the same common ancestor with the 25 other Colombian viruses, they were excluded from demographic analysis. The Bayesian Skyline plot (BSL) was performed under strict and relaxed uncorrelated lognormal molecular clock using the best model of nucleotide substitution (GTR + G + I) obtained in MODELTEST. The previously estimated substitution rate (9.2×10^{-4} s/s/y) obtained from the NS5B region of HCV-1b circulating in Brazil [Romano et al., 2010] was used for Bayesian inference. The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. In order to describe which demographic model better explain the data, we performed the analysis under the Constant, Exponential, Expansion, and Logistic demographic models. The substitution rate of 9.2×10^{-4} s/s/y was used under the strict molecular clock. Convergence of parameters during both the Bayesian Markov chain Monte Carlo simulation (MCMC) and the distinct demographic models analysis was inspected with Tracer v.1.4 [Drummond and Rambaut, 2007], with uncertainties addressed as 95% highest probability density (HPD) intervals. Twenty million and 50 million generations run were enough to achieve the convergence of all parameters (effective sample size (ESS) >200) for the BSL analysis and for the distinct demographic analysis, respectively.

RESULTS

HCV Amplification

PCR for the 5′UTR was positive in 53 out of the 184 anti-HCV-positive samples. Among them, a 380 bp fragment for the NS5B region was amplified successfully in 35 samples. The E1 region from 20 of these samples was also amplified to confirm the genotype and the cluster of these samples.

Genotyping Analysis

To analyze the distribution of the different HCV genotypes in this population, phylogenetic trees were reconstructed with both NS5B and E1 HCV regions. Once all sequences were genotyped, the presence of exclusive mutations in these samples was evaluated and compared to sequences obtained from the GenBank. Other sequences were further added to each alignment of the two regions in order to increase the search for variants from different HCV genotypes. Finally, 32 5′UTR sequences were sequenced to discard the unlikely possibility of recombination (GenBank accession numbers: HCV 5′UTR [GQ379738–GQ379769], HCV NS5B [GQ379770–GQ379804], and HCV E1 [GU220398–GU220416]).

Phylogenetic analysis: NS5B and E1 regions. The NS5B phylogenetic analysis was performed with 35 Colombian sequences obtained in this work and also with reference sequences from GenBank (n = 212). The subtype 1b was the most prevalent in this population (29–82.8%), followed by 1a (2–5.7%) and 2a (2–5.7%). Subtypes 2b and 3a were represented by a single patient each (1–2.8%). Of the 29 HCV-1b sequences, 25 (86%) clustered as a single monophyletic group, suggesting that these viruses represent the main strain circulating in the population of Bogotá (Fig. 1).

An ML phylogeny for the E1 region using the 19 sequences obtained from Colombian samples and reference sequences from the GenBank (n = 187) was also reconstructed. All the six samples from subtypes 1a, 2a, 2b, and 3a, but only 13 out of 29 1b samples, were amplified successfully and sequenced for E1 region (Fig. 2). Genotyping was consistent when analyzed by either NS5B or E1 regions. The E1 topology of the 13 sequences of subtype 1b confirmed the cluster sequences obtained with the NS5B phylogenetic analysis.

Aminoacid variability. A specific variation was found in the NS5B region of the Colombian sequences from subtype 2a (Fig. 3). A substitution of Glutamine (G)–Alanine (A) to Leucine (L)–Serine (S), is due to the substitution at nucleotides position 241 (C**A**G → C**T**G) and 242 (**G**CC → **T**CC). This LS motif is characteristic of HCV subtype 2c. However, the other three amino acid substitutions common for subtype 2c (Threonine [T] at position 267, Leucine [L] at position 293 and an Asparagine [N] at position 304) were not found in the samples here analyzed.

In this study, another amino acid substitution was found in subtype 2a of our strains. The residue at position 289 changed from Leucine [L] to Methionine [M] (Fig. 3). Among all genotype 2 sequences obtained from GenBank, this mutation was only found in the subtype 2e.

Additionally, the sequence identity matrix was estimated using BioEdit version 7.0.0 for all NS5B sequences for genotype 2, and the percentage of similarity

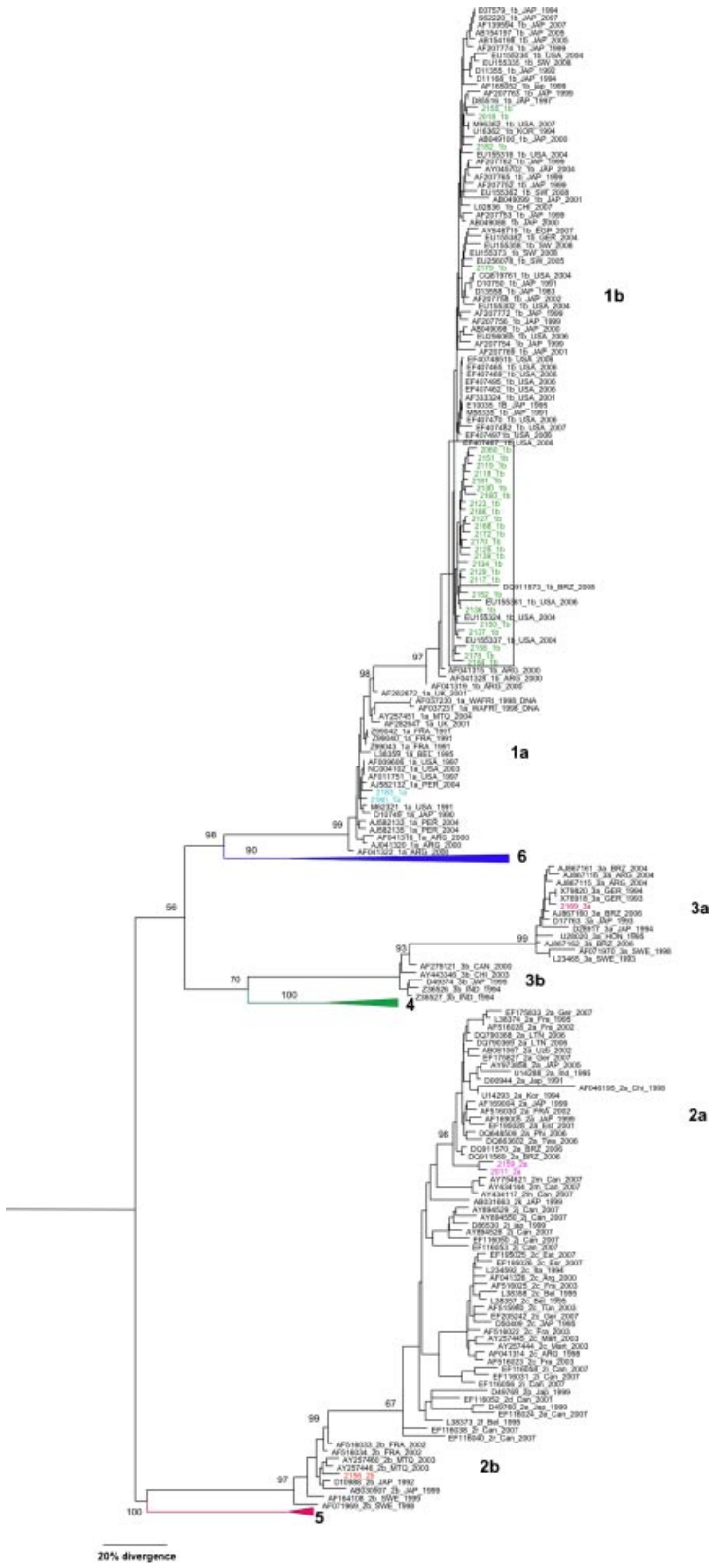


Fig. 1. Phylogenetic tree of 212 NS5B sequences with 380 bp of hepatitis C virus strains. Samples obtained from Colombian blood donors (n = 35, colored taxa) were analyzed together with other worldwide strains by maximum likelihood (ML) method. The cluster containing the strains that are mostly circulating in Colombia is highlighted. Genotype 4 (n = 6), genotype 5 (n = 2), and genotype 6 (n = 8) branches were collapsed. Bootstrap values (1,000 replicates) are depicted in the main nodes of the tree.

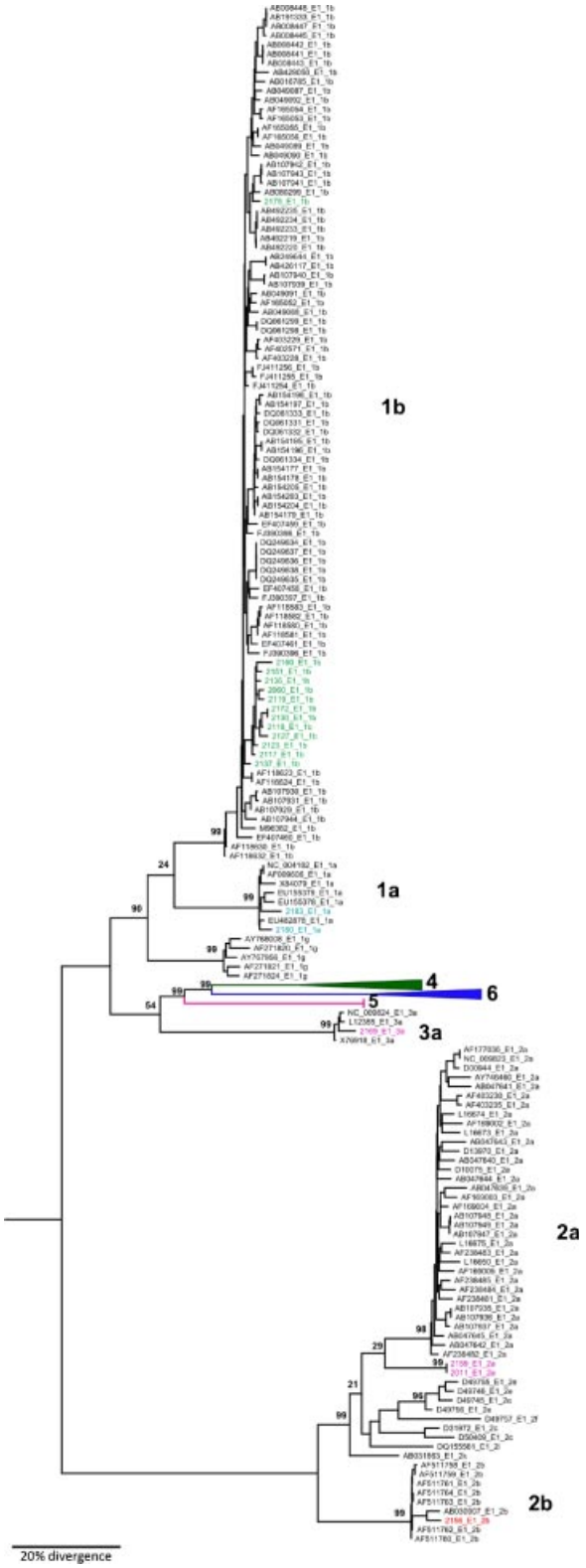


Fig. 2. Phylogenetic tree analysis of 187 E1 sequences with 391 bp of hepatitis C virus strains. Samples obtained from Colombian blood donors (n = 19, colored taxa) were analyzed together with other worldwide strains by maximum likelihood (ML) method. Genotype 4 (n = 6), genotype 5 (n = 2), and genotype 6 (n = 8) branches were collapsed. Bootstrap values (1,000 replicates) are depicted in the main nodes of the tree.

between the two Colombian samples of subtype 2a and the reported sequences of subtype 2e was 84%. Furthermore, the same value of similarity was found when the sequences were compared to other genotype 2a sequences from GenBank. Finally, there was no evidence of recombination among the sequences reported in this study when compared to other genotype 2 sequences (data available upon request).

Bayesian analysis. The sequences of genotype 1b here described grouped in a monophyletic cluster in the MAP tree (Fig. 1, Supplementary Material), corresponding to the viruses largely circulating in Bogotá, and they coalesce around 1950. The evolutionary analysis performed on samples from this study also suggested that HCV-1b has been circulating in Bogotá since the 1950s, with lower and upper values occurring in 1910 and 1974 (Table I). After that, the virus presented signals of exponential growth from the end of the 70s until the beginning of the 90s (Fig. 4), when a reduction in the curve slope was observed. These estimates correspond to the values obtained under the best-fitted molecular clock chosen by BF comparison (Table IA and B, Supplementary Material). The strict molecular clock best fitted the data set of 25 Colombian viruses, and the relaxed uncorrelated lognormal was the best molecular clock for the HCV-1b 88 sequences. Therefore, there was a slight variation among the parameters estimated under distinct clock models (Table I). We also did run the distinct demographic models (Constant, Exponential, Expansion, and Logistic growth) for the Colombian data set. Although the BF analysis revealed that any demographic model could be rejected, the TMRCA estimated by all models covered essentially the same range as the obtained by the Bayesian skyline analysis (see Supplementary Table IC for the detailed statistical analysis), suggesting that our results are robust.

DISCUSSION

HCV Genotypes Distribution

Similarly to findings in other countries in South America, HCV-1b prevalence in blood donors from Colombia found in this study (82.8%) is among the highest so far described (Table II).

Additionally, subtype 1b is mostly found among people who have a previous history of blood transfusion. The high prevalence of this subtype in this population, even 18 years after implementation of HCV tests in blood banks, can be explained by the fact that HCV carrier patients present a long asymptomatic phase and survive for long periods after infection. Because the prevalence of other genotypes in Bogotá is far lower than HCV-1b, it is suggested that this genotype has been maintained in the population through other transmission routes (intravenous and nasal drug use, sexual intercourse, vertical transmission, and alternative routes such as tattoos, body piercing, shared razors, and acupuncture) [Booth et al., 2001].

Although the NS5B region provides sufficient phylogenetic signals to perform the genotyping and subtyping

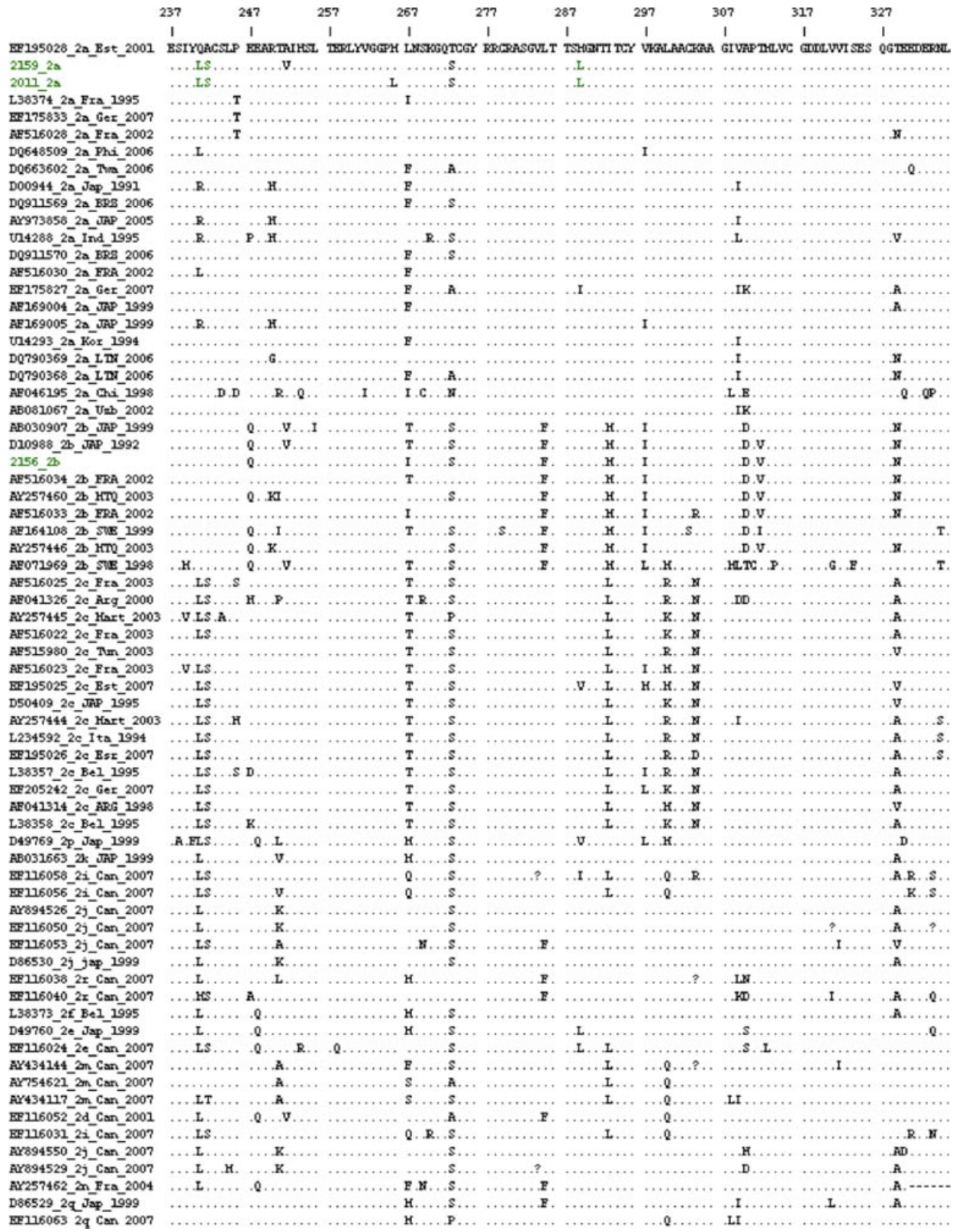


Fig. 3. Multiple alignment of partial NS5B region (237–336 aa) comprising 69 strains of all subtypes of the HCV genotype 2. Aminoacid substitutions exclusive of Colombian viruses from blood donors were compared to other reported viruses from GenBank. Colombian sequences are in green.

TABLE I. Estimated Evolutionary Parameters for Each Data Set Analyzed

NS5B data set	Molecular clock (best Bayes factor)	TMRCA[a] (95% HPD)	ESS for TMRCA
Colombian (n = 25)	Strict clock	1950 (1910–1974)[b]	352
Worldwide (n = 88)	Relaxed uncorrelated lognormal	1944 (1923–1962)[b]	315

[a]TMRCA (time of the most common ancestor is expressed in years).
[b]The parameters were obtained by previously estimated substitution rate of 9.2E−4 [Romano et al., 2010].

of HCV, the sequenced segment of the E1 region also showed a strong phylogenetical signal, as observed in previous work [Salemi and Vandamme, 2002]. The E1 phylogeny confirmed the genotyping analysis and the results for recombination analysis while the similarity matrix also agreed with those obtained for NS5B. Although insufficient sequences were obtained to evaluate the dynamics of other genotypes in the country, the low frequency of genotypes other than HCV-1b indicates that this subtype still prevails, at least in Bogotá.

Genotype 1b: Evolutionary Analysis

To analyze the origin and spread of the HCV genotype 1b, several studies have been developed recently. In Chile [Di Lello et al., 2009], the TMCRA of HCV-1b from Chilean patients is around 1893. In a small Sicilian town of Italy, it was also reported that the HCV-1b was introduced in the late 1940s [Ferraro et al., 2008]. In Argentina, the TMCRA estimated for HCV-1b ranged between 1964 and 1979 and revealed that this genotype arrived recently in that country [Di Lello et al., 2008]. Finally, in Brazil and in the United States the MCRA of HCV-1b is reported to be between 1880 and 1920 [Nakano et al., 2004; Romano et al., 2010]. Accordingly, the results from Colombia are similar to those reported previously for other countries and indicate that the genotype 1b is slowly spreading throughout South America. It is important to remember that the viruses described in this study were obtained from patients living in Bogotá, which is the capital and largest city of Colombia. Nevertheless, the estimates obtained in the present work may not represent the oldest entry of this virus in Colombia. The evolutionary analysis of Colombian viruses also revealed a growth rate around 0.2, which is quite similar to that obtained for HCV-1b that circulates in São Paulo, the largest Brazilian State [Romano et al., 2010]. Together, the data support the notion that HCV-1b growth pattern in Bogota may be similar to those found in other large metropolis.

It is noteworthy that the BSL description recovered signals of decreasing growth rate at the time of the implementation of the HCV blood screening tests in Colombia, in 1991 [WHO, 1991]. In fact, some works pinpointed the importance of the HCV screening tests in blood banks to control the spread of the genotype 1b. Since HCV screening of blood donors was established, the incidence of post-transfusion hepatitis C has continued to decline in many countries [Aach et al., 1991; Schmunis et al., 2001; Schmunis and Cruz, 2005]. In Colombia, it has been shown that after the introduction of HCV screening tests in blood banks the risk of infection decreased three times between 1993 and 1995, before reaching 99% coverage [Beltran et al., 2005],

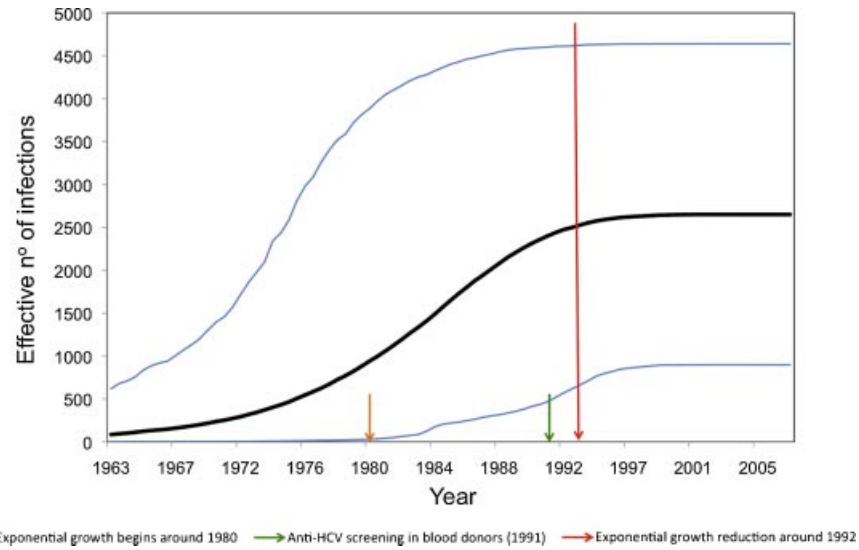


Fig. 4. Bayesian skyline (BSL) plot obtained from Colombia NS5B region sequences genotype 1b (n = 25). The figure shows the superimposed median values of Ne.g through time. Time is presented in years, and the size of the effective population time per generation time (Ne.g) is given in a linear scale with the 95% HPD values shown in blue.

TABLE II. Prevalence of HCV Genotypes Among Blood Donors and Other Risk Groups, Reported for Many Countries of the World

Country	Group	Genotype	Prevalence (%)	Refs.
Uzbekistan	Blood donors	3a	25.0	Kurbanov et al. [2003]
		1b	**55.0**	
		2a	10.0	
Italy	Blood transfusion	**1b**	**66.6**	Dal Molin et al. [2002]
		1a	13.8	
		2	13.8	
	IDUs	**1a**	**42.85**	
		3a	37.6	
		1b	14.2	
	Blood donors	**1b**	**50.7**	Guadagnino et al. [1997]
		2c	44.6	
Germany	Blood transfusion	**1a**	**51.5**	Ross et al. [2000]
		1b	42.7	
		3a	1.9	
		2a	1.0	
	IDUs	**1a**	**51.3**	
		1b	11.5	
		3a	30.8	
		2a	1.2	
China	Blood donors	**2a**	78.6	Liu et al. [2008]
		1b	21.4	
French	Blood donors	1a	20.0	Elghouzzi et al. [2000]
		1b	**34.3**	
		2	11.4	
		3a	24.1	
	Blood donors	**1b**	**61.8**	Nousbaum et al. [1995]
Brazil	Hemodialysis	**1a**	**65.7**	Espirito-Santo et al. [2007]
		1b	26.7	
		3a	7.6	
	Blood donors	**1**	**60.9**	Amorim et al. [2004]
		3	39.1	
	Blood donors	**1a**	**34.3**	Martins et al. [1998]
		1b	30.0	
		3a	25.7	
México	Blood donors	**1**	**43.4**	Garcia-Montalvo and Macossay-Castillo [2007]
		2	34.7	
		3	8.6	
Chile	Blood donors	**1b**	**46.6**	Munoz et al. [1998]
	Blood transfusion	**1b**	**82.0**	Soza et al. [2004]
Peru	Blood donors, hemodialysis	**1a**	**74.0**	Sanchez et al. [2000]
		1b	12.0	
		3	10.0	
Colombia	Blood donors	1a	5.7	This work
		1b	**82.8**	
		2a	5.7	
		2b	2.8	
		3a	2.8	

The HCV genotype prevalent in each study is in bold and underlined.

while after 1995 this reduction was greater than 90%. These results are most likely due to an increased sensitivity of the anti-HCV ELISA, with a reduced window period, leading to a decrease in the transmission through blood transfusion [Andrade et al., 2006]. Since 1993 the detection of HCV infection in blood banks increased from >10% to 150% in many Latin American countries, such as Colombia [Schmunis and Cruz, 2005].

The evolutionary analysis suggested that HCV-1b entered Colombia around 50 years ago, but started to grow in the late 1970s, possibly carried by blood transfusions that became a common practice in Colombia at the end of that decade. Before this period, the transmission of HCV-1b was possibly maintained by minor and sporadic practices. Our results agreed with the notion that the most important risk factor for HCV infection was the transfusion of blood components before 1995, when voluntary blood donation was practically nonexistent in Colombia [Beltran et al., 2005] and the donors were scaled only to repository donation. Interestingly, before 1995, the population presented the highest risk of receiving a blood unit infected with HCV (74 per 10,000 donors) and also of contracting that infection (66 per 10,000 donors), as compared to other countries in Latin America [Schmunis and Cruz, 2005]. Since 1980, other practices have become common in Colombia, such as replacement donors (~80%) or at a low level, paid donors [Pan American Health Organization, 1996]. The practice of paid donors may have turned into blood donors some individuals who belonged

originally to different HCV risk groups. This behavior may have led to an explosion of genotype 1b in the 1980s in this population (Fig. 4), which could be controlled only after the introduction of the ELISA test. However, further control measures might be needed to control the spread of other HCV genotypes present in the Colombian population and for which transmission is not associated with blood transfusions.

This study is the first report of the distribution of HCV genotypes among blood donors in Colombia. Subtype 1b was the most prevalent to date, even after HCV transmission control measures have been implemented in blood banks. Also, it is estimated that HCV emerged in Bogotá around 59 years ago, which is similar to the time of HCV-1b entry into many other countries in Latin America.

ACKNOWLEDGMENTS

We thank Banco Nacional de Sangre de la Cruz Roja Colombiana for their kind provision of blood donor samples for this study.

REFERENCES

Aach RD, Stevens CE, Hollinger FB, Mosley JW, Peterson DA, Taylor PE, Johnson RG, Barbosa LH, Nemo GJ. 1991. Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. N Engl J Med 325:1325–1329.

Amorim RM, Oliveira CP, Wyant PS, Cerqueira DM, Camara GN, Flores LS, Martins RM, Martins CR. 2004. Hepatitis C virus genotypes in blood donors from the Federal District, Central Brazil. Mem Inst Oswaldo Cruz 99:895–897.

Andrade AF, Oliveira-Silva M, Silva SG, Motta IJ, Bonvicino CR. 2006. Seroprevalence of hepatitis B and C virus markers among blood donors in Rio de Janeiro, Brazil, 1998–2005. Mem Inst Oswaldo Cruz 101:673–676.

Beltran M, Navas MC, De la Hoz F, Mercedes Munoz M, Jaramillo S, Estrada C, Del Pilar Cortes L, Arbelaez MP, Donado J, Barco G, Luna M, Uribe GA, de Maldonado A, Restrepo JC, Correa G, Borda P, Rey G, de Neira M, Estrada A, Yepes S, Beltran O, Pacheco J, Villegas I, Boshell J. 2005. Hepatitis C virus seroprevalence in multi-transfused patients in Colombia. J Clin Virol 34:S33–S38.

Blood bank situation in Latin America. 1996. Serological markers for communicable diseases in blood donors. Epidemiol Bull 19:12–14.

Booth JC, O'Grady J, Neuberger J. 2001. Clinical guidelines on the management of hepatitis C. Gut 49:I1–I21.

Bukh J, Purcell RH, Miller RH. 1993. At least 12 genotypes of hepatitis C virus predicted by sequence analysis of the putative E1 gene of isolates collected worldwide. Proc Natl Acad Sci USA 90:8234–8238.

Cantaloube JF, Laperche S, Gallian P, Bouchardeau F, de Lamballerie X, de Micco P. 2006. Analysis of the 5' noncoding region versus the NS5b region in genotyping hepatitis C virus isolates from blood donors in France. J Clin Microbiol 44:2051–2056.

Dal Molin G, Ansaldi F, Biagi C, D'Agaro P, Comar M, Croce L, Tiribelli C, Campello C. 2002. Changing molecular epidemiology of hepatitis C virus infection in Northeast Italy. J Med Virol 68:352–356.

Di Lello F, Garcia G, Kott V, Sookoian S, Campos R. 2008. Diversity of hepatitis C virus genotype 1b in Buenos Aires, Argentina: Description of a new cluster associated with response to treatment. J Med Virol 80:619–627.

Di Lello FA, Pineiro YLFG, Munoz G, Campos RH. 2009. Diversity of hepatitis B and C viruses in Chile. J Med Virol 81:1887–1894.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol 7:214.

Echevarria JM, Leon P. 2003. Epidemiology of viruses causing chronic hepatitis among populations from the Amazon Basin and related ecosystems. Cad Saude Publica 19:1583–1591.

Elghouzzi MH, Bouchardeau F, Pillonel J, Boiret E, Tirtaine C, Barlet V, Moncharmont P, Maisoneuve P, du Puy-Montbrun MC, Lyon-Caen D, Courouce AM. 2000. Hepatitis C virus: Routes of infection and genotypes in a cohort of anti-HCV-positive French blood donors. Vox Sang 79:138–144.

Enomoto N, Takada A, Nakao T, Date T. 1990. There are two major types of hepatitis C virus in Japan. Biochem Biophys Res Commun 170:1021–1025.

Espirito-Santo MP, Carneiro MA, Reis NR, Kozlowski AG, Teles SA, Lampe E, Yoshida CF, Martins RM. 2007. Genotyping hepatitis C virus from hemodialysis patients in Central Brazil by line probe assay and sequence analysis. Braz J Med Biol Res 40:545–550.

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, Wendl MC, Green P. 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 8:175–185.

Ferraro D, Genovese D, Argentini C, Giordano V, Pizzillo P, Stroffolini T, Craxi A, Rapicetta M, Di Stefano R. 2008. Phylogenetic reconstruction of HCV genotype 1b dissemination in a small city centre: The Camporeale model. J Med Virol 80:1723–1731.

Furione M, Simoncini L, Gatti M, Baldanti F, Grazia Revello M, Gerna G. 1999. HCV genotyping by three methods: Analysis of discordant results based on sequencing. J Clin Virol 13:121–130.

Garcia-Montalvo BM, Macossay-Castillo M. 2007. Preliminary data for genotype distribution and epidemiological aspects of hepatitis C virus infection in blood donors from Yucatan. Mexico Transfus Med 17:488–490.

Garson JA, Tedder RS, Briggs M, Tuke P, Glazebrook JA, Trute A, Parker D, Barbara JA, Contreras M, Aloysius S. 1990. Detection of hepatitis C viral sequences in blood donations by "nested" polymerase chain reaction and prediction of infectivity. Lancet 335:1419–1422.

Garson JA, Ring CJ, Tuke PW. 1991. Improvement of HCV genome detection with "short" PCR products. Lancet 338:1466–1467.

Guadagnino V, Stroffolini T, Rapicetta M, Costantino A, Kondili LA, Menniti-Ippolito F, Caroleo B, Costa C, Griffo G, Loiacono L, Pisani V, Foca A, Piazza M. 1997. Prevalence, risk factors, and genotype distribution of hepatitis C virus infection in the general population: A community-based survey in southern Italy. Hepatology 26:1006–1011.

Halfon P, Ouzan D, Khiri H, Feryn JM. 1997. Serotyping and genotyping of hepatitis C virus (HCV) strains in chronic HCV infection. Commission Hepatologie du CREGG. Club de Reflexion des Cabinets de Groupes en GastroEnterologie. J Med Virol 52:391–395.

Ishii K, Tanaka Y, Yap CC, Aizaki H, Matsuura Y, Miyamura T. 1999. Expression of hepatitis C virus NS5B protein: Characterization of its RNA polymerase activity and RNA binding. Hepatology 29:1227–1235.

Katsoulidou A, Sypsa V, Tassopoulos NC, Boletis J, Karafoulidou A, Ketikoglou I, Tsantoulas D, Vafiadi I, Hatzis G, Skoutelis A, Akriviadis E, Vasiliadis T, Kitis G, Magiorkinis G, Hatzakis A. 2006. Molecular epidemiology of hepatitis C virus (HCV) in Greece: Temporal trends in HCV genotype-specific incidence and molecular characterization of genotype 4 isolates. J Viral Hepat 13:19–27.

Kurbanov F, Tanaka Y, Sugauchi F, Kato H, Ruzibakiev R, Zalyalieva M, Yunusova Z, Mizokami M. 2003. Hepatitis C virus molecular epidemiology in Uzbekistan. J Med Virol 69:367–375.

Kwok S, Higuchi R. 1989. Avoiding false positives with PCR. Nature 339:237–238.

Liu P, Xiang K, Tang H, Zhang W, Wang X, Tong X, Takebe Y, Yang R. 2008. Molecular epidemiology of human immunodeficiency virus type 1 and hepatitis C virus in former blood donors in central China. AIDS Res Hum Retroviruses 24:1–6.

Maertens G, Stuyver L. 1997. Genotypes and genetic variation of Hepatitis C virus In The molecular medicine of viral Hepatitis. England: John Wiley & Sons. pp. 182–223.

Martell M, Briones C, de Vicente A, Piron M, Esteban JI, Esteban R, Guardia J, Gomez J. 2004. Structural analysis of hepatitis C RNA genome using DNA microarrays. Nucleic Acids Res 32:e90.

Martins RM, Vanderborght BO, Yoshida CF. 1998. Hepatitis C virus genotypes among blood donors from different regions of Brazil. Mem Inst Oswaldo Cruz 93:299–300.

Munoz G, Velasco M, Thiers V, Hurtado C, Brahm J, Larrondo-Lillo M, Guglielmetti A, Smok G, Brechot C, Lamas E. 1998. Prevalence and genotypes of hepatitis C virus in blood donors and in patients with chronic liver disease and hepatocarcinoma in a Chilean population. Rev Med Chil 126:1035–1042.

Nakano T, Lu L, Liu P, Pybus OG. 2004. Viral gene sequences reveal the variable history of hepatitis C virus infection among countries. J Infect Dis 190:1098–1108.

Nousbaum JB, Pol S, Nalpas B, Landais P, Berthelot P, Brechot C. 1995. Hepatitis C virus type 1b (II) infection in France and Italy. Collaborative Study Group. Ann Intern Med 122:161–168.

Pasquier C, Njouom R, Ayouba A, Dubois M, Sartre MT, Vessiere A, Timba I, Thonnon J, Izopet J, Nerrienet E. 2005. Distribution and heterogeneity of hepatitis C genotypes in hepatitis patients in Cameroon. J Med Virol 77:390–398.

Pawlotsky JM. 2003. Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C. Antiviral Res 59:1–11.

Posada D, Crandall KA. 1998. MODELTEST: Testing the model of DNA substitution. Bioinformatics 14:817–818.

Romano CM, de Carvalho-Mello IMVG, Jamal LF, Melo FL, Iamarino A, Motoki M, Pinho JRR, Holmes EC, Zanotto PMA. VGDN consortium, 2010.Social networks shape the transmission dynamics of hepatitis C virus. PLoS One (in press).

Ross RS, Viazov S, Renzing-Kohler K, Roggendorf M. 2000. Changes in the epidemiology of hepatitis C infection in Germany: Shift in the predominance of hepatitis C subtypes. J Med Virol 60:122–125.

Salemi M, Vandamme AM. 2002. Hepatitis C virus evolutionary patterns studied through analysis of full-genome sequences. J Mol Evol 54:62–70.

Sanchez JL, Sjogren MH, Callahan JD, Watts DM, Lucas C, Abdel-Hamid M, Constantine NT, Hyams KC, Hinostroza S, Figueroa-Barrios R, Cuthie JC. 2000. Hepatitis C in Peru: Risk factors for infection, potential iatrogenic transmission, and genotype distribution. Am J Trop Med Hyg 63:242–248.

Sandres-Saune K, Deny P, Pasquier C, Thibaut V, Duverlie G, Izopet J. 2003. Determining hepatitis C genotype by analyzing the sequence of the NS5b region. J Virol Methods 109:187–193.

Schmunis GA, Cruz JR. 2005. Safety of the blood supply in Latin America. Clin Microbiol Rev 18:12–29.

Schmunis GA, Zicker F, Cruz JR, Cuchi P. 2001. Safety of blood supply for infectious diseases in Latin American countries, 1994–1997. Am J Trop Med Hyg 65:924–930.

Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PL, Kolbeg J, Urdea MS. 1993. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 74:2391–2399.

Smith DB, Pathirana S, Davidson F, Lawlor E, Power J, Yap PL, Simmonds P. 1997. The origin f hepatitis C virus genotypes. J Gen Virol 78:321–328.

Soza A, Arrese M, Gonzalez R, Alvarez M, Perez RM, Cortes P, Patillo A, Riquelme A, Riqueme A. 2004. Clinical and epidemiological features of 147 Chilean patients with chronic hepatitis C. Ann Hepatol 3:146–151.

Spada E, Ciccaglione AR, Dettori S, Chionne P, Kondili LA, Amoroso P, Guadagnino V, Greco M, Rapicetta M. 1998. Genotyping HCV isolates from Italy by type-specific PCR assay in the core region. Res Virol 149:209–218.

Suzuki T, Aizaki H, Murakami K, Shoji I, Wakita T. 2007. Molecular biology of hepatitis C virus. J Gastroenterol 42:411–423.

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 analyss tools. Nucleic Acids Res 25:4876–4882.

WHO. 1991. Consensus statement on screening of blood donations for infectious agents transmissible through blood transfusion. Geneva: World Health Organization Global Programme on AIDS/League of Red Cross and Red Crescent Societies. p 13.

Zwickl DJ. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion: The University of Texas at Austin.

VIROLOGY JOURNAL

RESEARCH Open Access

Distribution of hepatitis c virus (hcv) genotypes in patients with chronic infection from Rondônia, Brazil

Deusilene S Vieira[2], Mónica V Alvarado-Mora[1*], Lívia Botelho[1], Flair J Carrilho[1], João RR Pinho[1] and Juan M Salcedo[2]

Abstract

Background: Hepatitis C virus (HCV) is an important human pathogen affecting around 3% of the human population. In Brazil, it is estimated that there are approximately 2 to 3 million HCV chronic carriers. There are few reports of HCV prevalence in Rondônia State (RO), but it was estimated in 9.7% from 1999 to 2005. The aim of this study was to characterize HCV genotypes in 58 chronic HCV infected patients from Porto Velho, Rondônia (RO), Brazil.

Methods: A fragment of 380 bp of NS5B region was amplified by nested PCR for genotyping analysis. Viral sequences were characterized by phylogenetic analysis using reference sequences obtained from the GenBank (n = 173). Sequences were aligned using Muscle software and edited in the SE-AL software. Phylogenetic analyses were conducted using Bayesian Markov chain Monte Carlo simulation (MCMC) to obtain the MCC tree using BEAST v.1.5.3.

Results: From 58 anti-HCV positive samples, 22 were positive to the NS5B fragment and successfully sequenced. Genotype 1b was the most prevalent in this population (50%), followed by 1a (27.2%), 2b (13.6%) and 3a (9.0%).

Conclusions: This study is the first report of HCV genotypes from Rondônia State and subtype 1b was found to be the most prevalent. This subtype is mostly found among people who have a previous history of blood transfusion but more detailed studies with a larger number of patients are necessary to understand the HCV dynamics in the population of Rondônia State, Brazil.

Background

Hepatitis C virus (HCV) infects around 170 million people, 3% of the world population, and is considered a worldwide public health problem [1,2]. The HCV genome is a 9.4 Kb single stranded RNA sequence with two untranslated regions at both ends (5 'UTR and 3' UTR) [3]. This coding region contains a single open reading frame encoding a polyprotein of approximately 3,000 amino acids that originate at least 10 viral gene products (C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) [4,5].

The virus represents the genus *Hepacivirus* of the family *Flaviviridae* [6] and it is classified into six major genotypes (1 to 6) and more than 80 subtypes. HCV genotypes may show extensive subtype diversity in some regions of the world, representing the spreading of this epidemic [7,8]. These genotypes differ by 31 to 34% in their nucleotide sequence and by around 30% in their amino acid sequence. Accurate HCV genotyping can be used for predicting response to anti-viral therapy, as genotypes 1 and 4 are less likely than genotypes 2 and 3 to respond to interferon [9].

Molecular tests are essential for confirmation of persistent HCV infection and to monitor and verify the success or failure of therapy. The advantages of these tests include the possibility of early diagnosis in acute viral infection, diagnosis of infection in patients unable

* Correspondence: monica.viviana@usp.br
[1]Laboratory 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
Full list of author information is available at the end of the article

to mount antibody response, and confirmation of active infection [10]. Besides its use for determining the type or duration of HCV, HCV genotyping is useful epidemiological studies, as genotypes vary among geographical regions and among different risk groups [11,12]. Sequencing of the NS5B has been standardized and used for identification of HCV subtypes, as the region contains subtype-specific motifs, and it is also appropriate for epidemiological applications [13].

In Brazil, it has been estimated that around 1.5% of the Brazilian population (> 2.5 million people) is anti-HCV positive [14,15]. Distribution of cases of chronic hepatitis C by transmission routes from 1998 to 2006 showed 21% of cases associated with intravenous drug use and 16% with blood transfusion, but in 40% of cases there was not any known risk factor [16]. An extensive review on HCV infection data in Brazil showed the following prevalence in healthy adults and/or blood donors in the different Brazilian regions: 0.9 to 2.4% (North), 1.7 to 3.4% (Northeast), 1.0 to 1.4% (Middle West), 0.8 to 2.8% (Southeast) and 1.1 to 2.1% (South) [17].

Rondônia (RO) State, in North-Western Brazil, has characteristics of a highly endemic region for viral hepatitis. A study carried out with the local population in the Madeira River at Porto Velho-RO showed the prevalence of hepatitis C reached 7.4% [18]. Ferrari et al. [19] investigated HCV seroprevalence among Karitiana Indians living in Rondônia and found that the HCV prevalence was 1.7%. According to the Brazilian Ministry of Health, during the period from 1999 to 2005, 1831 cases of hepatitis C were confirmed in the North Region and 400 (21.8%) of these cases were from Rondônia [18].

Recently, we reported the genotype distribution of HBV in Rondônia [20] and the present study is the first report on HCV distribution in this state. The aim of this study was to characterize the HCV genotypes circulating in Rondônia State (RO), Brazil.

Methods

Study Population

This study was carried out in the state of Rondônia, Brazil (Figure 1) and included 58 anti-HCV positive patients (29 males, 29 females, age ranging from 27 to 85 years old). All patients signed a consent form for this study. The samples were collected between January 2007 and October 2009 in the Oswaldo Cruz Foundation, Fiocruz Noroeste, Porto Velho-RO, Brazil. Data on previous blood transfusion and presence of liver cirrhosis or fibrosis were obtained from the review of each patient medical data.

HCV RNA extraction

HCV-RNA extraction was carried out from 140 μL of serum using QIAamp® Viral RNA Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's instructions. The synthesis of the complementary DNA (cDNA) was made immediately after the RNA extraction. To avoid false-positive results, rigorous procedures proposed for nucleic acid amplification diagnostic techniques were followed [21].

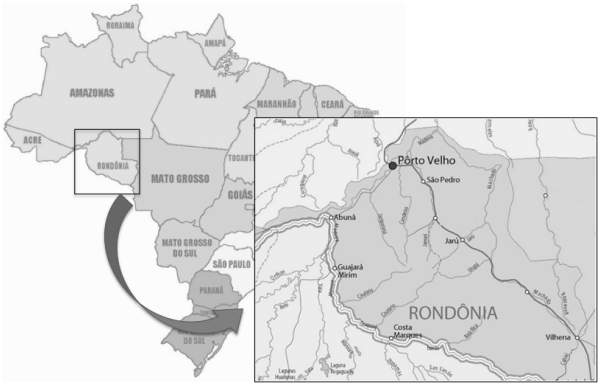


Figure 1 Geographical localization of Rondônia state, Brazil.

Synthesis of the complementary DNA (cDNA)

The reverse transcriptase reaction was performed using the enzyme Reverse Transcriptase of *Moloney Murine Leukemia Virus* and random primers. The final volume of the reaction was 60 μL with the following concentrations: 50 mM Tris-HCl (pH = 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dNTP (10 mM), 450 ng random primers, 30 units RNAse enzyme inhibitor (RNase OUT™) and 300 units M-MLV. Samples were submitted to the following temperature cycles: 70°C for 10 minutes, 25°C for 15 minutes, 37°C for 60 minutes and 95°C for 15 minutes in a thermocycler (Eppendorf Mastercycler ®, Eppendorf, Hamburg, Germany).

Polymerase Chain Reaction (PCR)

Polymerase Chain Reactions (PCR) were carried out in two stages, first and second PCR, aiming to increase the reaction sensitivity. The NS5B (380 bp) region was amplified for genotyping analysis. Amplification primers and cycling conditions are previously described [22,23]. Reactions were carried out in a final volume of 50 μL. The cDNA (5 μl) was added to 20 mM Tris-HCl (pH = 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (10 mM), 0.4 pmol/μL of each primer, and 2.5 units *Platinum* Taq DNA polymerase. All the reagents used were from Invitrogen ™ Life Technologies, Carlsbad, CA, USA.

HCV sequencing

Amplified cDNA was purified using ChargeSwitch® PCR Clean-Up Kit. Sequencing was performed in an ABI

Prism® 377 Automatic Sequencer (Applied Biosystems, Foster City, CA, USA) 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 [24,25] and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available at the web page *Eletropherogram quality analysis* http://asparagin.cenargen.embrapa.br/phph/.

Phylogenetic Analysis

The HCV sequences were genotyped by phylogenetic reconstructions using reference NS5B sequences from each HCV subtype obtained from GenBank (n = 173). The sequences were aligned using Muscle software [26] and edited in the SE-AL program (available at http://tree.bio.ed.ac.uk/software/seal/). Phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3 [27] by both relaxed uncorrelated $\log_{normal}$ and relaxed uncorrelated exponential molecular clock using the best model of nucleotide substitution (GTR+G+I) obtained by MODELTEST [28]. Twenty million generations were run to obtain the convergence of parameters. The maximum clade credibility (MCC) tree was obtained from summarizing the substitution trees and then it was removed 10% of burn-in using Tree Annotator v.1.5.3 [27].

Results and Discussion

Among the 58 positive anti-HCV samples, only 22 were positive by nested PCR for NS5B region.

After sequencing, the phylogenetic analyses in this study showed that subtype 1b was the most prevalent in this population (50%). Also, the subtypes 1a (27.3%), 2b (13.6%) and 3a (9,0%) were detected in Rondônia State (Figure 2). Sequences were deposed in GenBank at accession numbers: HQ630386 - HQ630407.

Several studies were carried out to investigate the HCV distribution in the different states and different risk groups in Brazil [29-32]. Campiotto et al., [33] reported that genotype 1 was the most frequently genotype found in all regions of Brazil. Specifically, in the North region, genotype 1 was the most prevalent in Amazonas and Acre states (78.0 and 64.3%, respectively), followed by genotype 3. Genotype 2 was only observed in Amazonas state (7.1%). Also, genotype 1 predominates in the Southeastern Region of Brazil where the prevalence is around 70% in the states of Rio de Janeiro and São Paulo [34-36], and 84% in Minas Gerais [37]. In Salvador, Bahia state, genotype 3 was the most common genotype (53.3%), followed by genotypes 1 (40%) and 2 (6.7%) but, as the number of positive cases was small, other studies are necessary to confirm the high frequency of genotype 3 in Salvador [15]. Another study in Piauí state, Brazil, found 49.0% of subtype 3a, 26.0% of subtype 1a, 24.0% of subtype 1b and 1.0% of subtype 2b [38]. Furthermore, subtypes 1a (34.3%), 1b (30.0%) and 3a (25.7%) were frequently found in Brazilian blood donors, while 2b was rare, and 2a was not detected [39].

HCV subtypes found in Brazil represent several independent lineages probably originated from multiple introductions of each subtype into Brazil; analysis of Brazilian-specific clades provides a more accurate representation of the population dynamics of HCV in the country [40]. Furthermore, as Brazil is a large country with many different population backgrounds, a large variation in the frequencies of HCV genotypes is expected throughout its territory. In this study, as shown in Figure 2, phylogenetic analyses showed that subtypes 1a and 2b sequences from Rondônia clustered together but subtypes 1b and 3a have a more heterogeneous distribution along the tree. Since there are only 22 sequences reported in this study we cannot make any further inferences about HCV genetic variability.

Subtype 1b is mostly found among older members of the population who have a previous history of blood transfusion [41-43] but in this study the patients did not report previous blood transfusion. Also, subtype 1b is associated with a higher rate of chronic active hepatitis or cirrhosis, and with a poorer response to treatment with α-interferon than genotypes 2 or 3 [16]. In this study, all patients were naïve and two subtype 1a patients, as well as one subtype 1b patient had cirrhosis.

Genotype 3 has been associated with transmission through intravenous drug users what could have contributed to a large dissemination of this genotype [44]. The two subtype 3a female patients did not report previous drug injection and it is possible that this association is not valid in this region. However, the present study was not intended to analyze the association between HCV subtypes and routes of transmission.

In conclusion, this study is the first report on HCV genotypes from Rondônia state where subtype 1b was found to be the most prevalent. There was not any data from this region previously published. Rondônia state is one region where the population is growing faster in

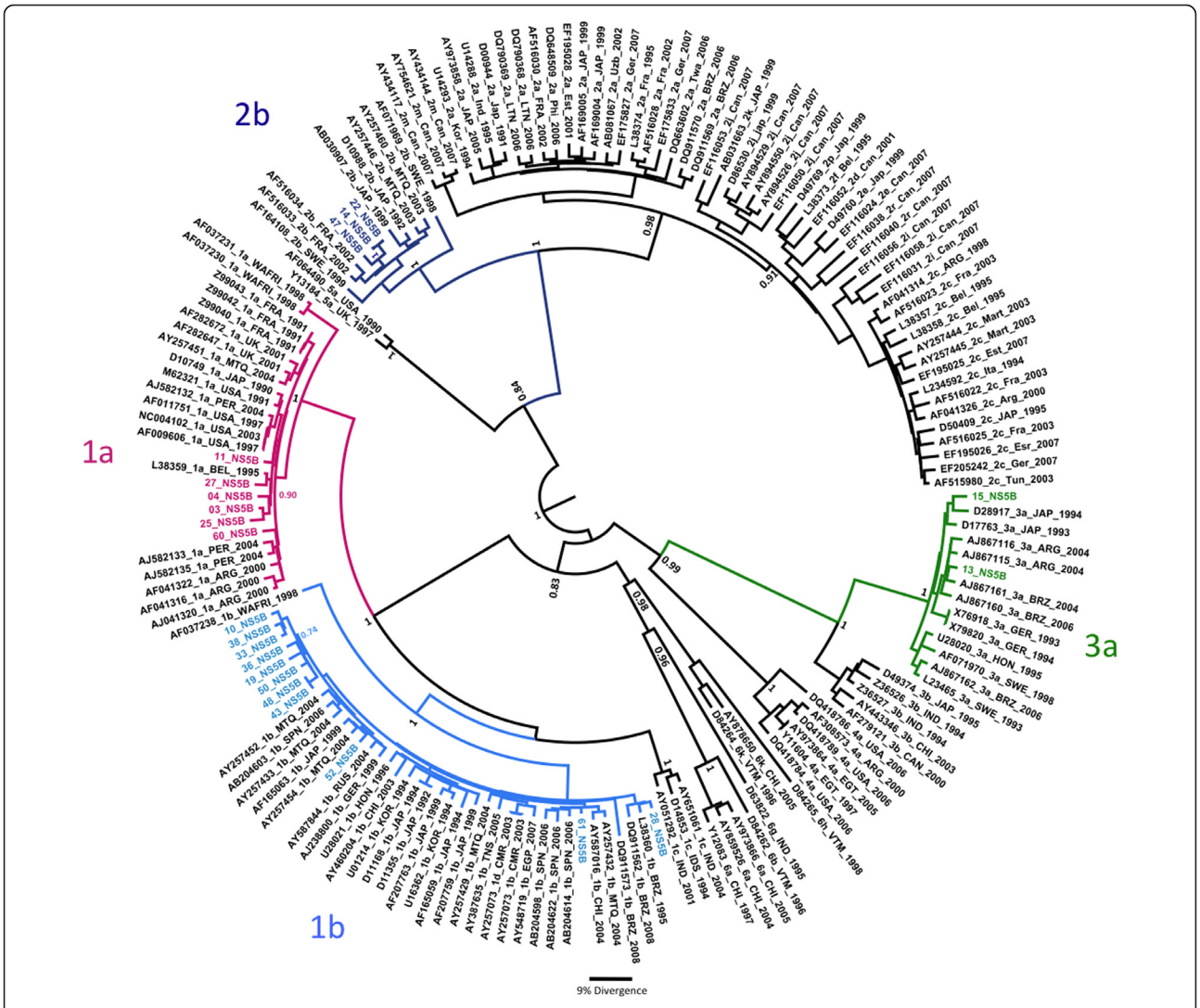


Figure 2 The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 170 NS5B sequences with 313 bp of Hepatitis C virus strains. The posterior probabilities of the key nodes are depicted above the respective nodes. The twenty-two sequences from Rondônia were highlighted: Six sequences subtype 1a (red), eleven sequences subtype 1b (blue), three sequenced subtype 2b (dark blue) and two sequences subtype 3a (green).

Brazil and it is relevant to analyze its health issues to allow the early implementation of health policies.

Acknowledgements
Oswaldo Cruz Foundation, Fiocruz Noroeste, CNPq and Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (2007/53457-7 and 2008/50461-6), São Paulo, SP, Brazil supported this work.

Author details
[1]Laboratory 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. [2]Oswaldo Cruz Foundation, Fiocruz Noroeste. Porto Velho, RO, Brazil.

Authors' contributions
DVS participated in the design of the study. MVAM conducted the phylogenetic and evolutionary analysis, drafted the manuscript and participated in its design and coordination. LB participated in the PCR amplification and sequencing process. FJC and JMS participated in the design of the study. JRRP participated in the elaboration of the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 22 February 2011 Accepted: 12 April 2011
Published: 12 April 2011

References

1. Alter HJ, Seeff LB: **Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome.** *Semin Liver Dis* 2000, **20**: 17-35.
2. McHutchison JG, Bacon BR: **Hepatitis C: a 20-year debt comes due.** *Am J Manag Care* 2004, **10**: S20.
3. Simmonds P: **Genetic diversity and evolution of hepatitis C virus–15 years on.** *J Gen Virol* 2004, **85**: 3173-3188.

4. Penin F, Brass V, Appel N, Ramboarina S, Montserret R, Ficheux D, Blum HE, Bartenschlager R, Moradpour D: **Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A.** *J Biol Chem* 2004, **279**: 40835-40843.
5. Drexler JF, Kupfer B, Petersen N, Grotto RM, Rodrigues SM, Grywna K, Panning M, Annan A, Silva GF, Douglas J, *et al*: **A novel diagnostic target in the hepatitis C virus genome.** *PLoS Med* 2009, **6**: e31.
6. Ishii K, Tanaka Y, Yap CC, Aizaki H, Matsuura Y, Miyamura T: **Expression of hepatitis C virus NS5B protein: characterization of its RNA polymerase activity and RNA binding.** *Hepatology* 1999, **29**: 1227-1235.
7. WHO: **Global surveillance and control of hepatitis C.** In *Journal of Viral Hepatitis. Volume 6.* Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium; 1999: 35-47.
8. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, Halfon P, Inchauspe G, Kuiken C, Maertens G, *et al*: **Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes.** *Hepatology* 2005, **42**: 962-973.
9. Pawlotsky JM: **Mechanisms of antiviral treatment efficacy and failure in chronic hepatitis C.** *Antiviral Res* 2003, **59**: 1-11.
10. Germer JJ, Zein NN: **Advances in the molecular diagnosis of hepatitis C and their clinical implications.** *Mayo Clin Proc* 2001, **76**: 911-920.
11. Cavlek TV, Margan IG, Lepej SZ, Kolaric B, Vince A: **Seroprevalence, risk factors, and hepatitis C virus genotypes in groups with high-risk sexual behavior in Croatia.** *J Med Virol* 2009, **81**: 1348-1353.
12. Cantaloube JF, Laperche S, Gallian P, Bouchardeau F, de Lamballerie X, de Micco P: **Analysis of the 5' noncoding region versus the NS5b region in genotyping hepatitis C virus isolates from blood donors in France.** *J Clin Microbiol* 2006, **44**: 2051-2056.
13. Maertens G, Stuyver L: **Genotypes and genetic variation of Hepatitis C virus In: The molecular medicine of viral Hepatitis.** England: John Wiley & Sons; 1997, 182-223.
14. Focaccia R, da Conceicao OJ, Sette H Jr, Sabino E, Bassit L, Nitrini DR, Lomar AV, Lorenco R, Vieira De Souza F, Kiffer CR, *et al*: **Estimated Prevalence of Viral Hepatitis in the General Population of the Municipality of Sao Paulo, Measured by a Serologic Survey of a Stratified, Randomized and Residence-Based Population.** *Braz J Infect Dis* 1998, **2**: 269-284.
15. Zarife MA, Silva LK, Silva MB, Lopes GB, Barreto ML, Teixeira M, da G, Dourado I, Reis MG: **Prevalence of hepatitis C virus infection in north-eastern Brazil: a population-based study.** *Trans R Soc Trop Med Hyg* 2006, **100**: 663-668.
16. Diament D: **Epidemiological Aspects of Hepatitis C in Brazil.** *Braz J Infect Dis* 2007, **11(5)**: 6-7.
17. Carrilho FJ, Corrêa MCJM: **Magnitude of hepatitis B and C in Latin America.** In *Therapies for Viral Hepatitis.* Edited by: Schinazi RF, Sommadossi JP, Thomas HC. International Medical Press, Atlanta, GA, USA; 1998:.
18. Katsuragawa , *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.
19. Ferrari JO, Ferreira MU, Tanaka A, Mizokami M: **The seroprevalence of hepatitis B and C in an Amerindian population in the southwestern Brazilian Amazon.** *Rev Soc Bras Med Trop* 1999, **32**: 299-302.
20. Santos AO, Alvarado Mora MV, Botelho L, Vieira D, Rebello Pinho JR, Carrilho FJ, Honda E, Salcedo JM: **Characterization of Hepatitis B virus (HBV) genotypes in patients from Rondonia, Brazil.** *Virology Journal* 2010, **7**: 315.
21. Kwok S, Higuchi R: **Avoiding false positives with PCR.** *Nature* 1989, **339**: 237-238.
22. Enomoto N, Takada A, Nakao T, Date T: **There are two major types of hepatitis C virus in Japan.** *Biochem Biophys Res Commun* 1990, **170**: 1021-1025.
23. Sandres-Saune K, Deny P, Pasquier C, Thibaut V, Duverlie G, Izopet J: **Determining hepatitis C genotype by analyzing the sequence of the NS5b region.** *J Virol Methods* 2003, **109(2)**: 187-93.
24. Ewing B, Green P: **Base-calling of automated sequencer traces using phred. II. Error probabilities.** *Genome Res* 1998, **8**: 186-194.
25. Ewing B, Hillier L, Wendl MC, Green P: **Base-calling of automated sequencer traces using phred. I. Accuracy assessment.** *Genome Res* 1998, **8**: 175-185, 44.
26. Edgar RC: **MUSCLE: a multiple sequence alignment method with reduced time and space complexity.** *BMC Bioinformatics* 2004, **5**: 113.
27. Drummond AJ, Rambaut A: **BEAST: Bayesian evolutionary analysis by sampling trees.** *BMC Evol Biol* 2007, **7**: 214.
28. Posada D, Crandall KA: **MODELTEST: testing the model of DNA substitution.** *Bioinformatics* 1998, **14**: 817-818.
29. Lopes CL, Teles SA, Espirito-Santo MP, Lampe E, Rodrigues FP, Motta-Castro AR, Marinho TA, Reis NR, Silva AM, Martins RM: **Prevalence, risk factors and genotypes of hepatitis C virus infection among drug users, Central-Western Brazil.** *Rev Saude Publica* 2009, **43(Suppl 1)**: 43-50.
30. Silva MB, Andrade TM, Silva LK, Rodart IF, Lopes GB, Carmo TM, Zarife MA, Dourado I, Reis MG: **Prevalence and genotypes of hepatitis C virus among injecting drug users from Salvador-BA, Brazil.** *Mem Inst Oswaldo Cruz* **105**: 299-303.
31. Germano FN, dos Santos CA, Honscha G, Strasburg A, Gabbi B, Mendoza-Sassi RA, Soares EA, Seuanez HN, Soares MA, Martinez AM: **Prevalence of hepatitis C virus among users attending a voluntary testing centre in Rio Grande, southern Brazil: predictive factors and hepatitis C virus genotypes.** *Int J STD AIDS* **21**: 466-471.
32. Freitas NR, Teles SA, Matos MA, Lopes CL, Reis NR, Espirito-Santo MP, Lampe E, Martins RM: **Hepatitis C virus infection in Brazilian long-distance truck drivers.** *Virol J* **7**: 205.
33. Campiotto S, Pinho JR, Carrilho FJ, Da Silva LC, Souto FJ, Spinelli V, Pereira LM, Coelho HS, Silva AO, Fonseca JC, *et al*: **Geographic distribution of hepatitis C virus genotypes in Brazil.** *Braz J Med Biol Res* 2005, **38**: 41-49.
34. Oliveira ML, Bastos FI, Sabino RR, Paetzold U, Schreier E, Pauli G, Yoshida CF: **Distribution of HCV genotypes among different exposure categories in Brazil.** *Braz J Med Biol Res* 1999, **32**: 279-282.
35. Bassit L, Da Silva LC, Ribeiro-Dos-Santos G, Maertens G, Carrilho FJ, Fonseca LE, Alves VA, Gayotto LC, Pereira AN, Takei K, *et al*: **Chronic hepatitis C virus infections in brazilian patients: association with genotypes, clinical parameters and response to long term alpha interferon therapy.** *Rev Inst Med Trop Sao Paulo* 1999, **41**: 183-189.
36. Focaccia R, Baraldo DC, Ferraz ML, Martinelli AL, Carrilho FJ, Goncales FL Jr, Pedroso ML, Coelho HS, Lacerda MA, Brandao CE, *et al*: **Demographic and anthropometrical analysis and genotype distribution of chronic hepatitis C patients treated in public and private reference centers in Brazil.** *Braz J Infect Dis* 2004, **8**: 348-355.
37. Oliveira GC, Carmo RA, Rocha MO, Silva MO, Lima AT, Guimaraes MD, Correa-Oliveira R: **Hepatitis C virus genotypes in hemophiliacs in the state of Minas Gerais, Brazil.** *Transfusion* 1999, **39**: 1194-1199.
38. Veras KN, Jacobina KS, Soares VY, Avelino MA, Vasconcelos Cde M, Parente JM: **Chronic hepatitis C virus in the state of Piaui, northeastern Brazil.** *Braz J Infect Dis* 2009, **13**: 125-129.
39. Martins RM, Vanderborght BO, Yoshida CF: **Hepatitis C virus genotypes among blood donors from different regions of Brazil.** *Mem Inst Oswaldo Cruz* 1998, **93**: 299-300.
40. Lampe E, Espirito-Santo MP, Martins RM, Bello G: **Epidemic history of Hepatitis C virus in Brazil.** *Infect Genet Evol* 2010, **10**: 886-895.
41. Pawlotsky JM, Tsakiris L, Roudot-Thoraval F, Pellet C, Stuyver L, Duval J, Dhumeaux D: **Relationship between hepatitis C virus genotypes and sources of infection in patients with chronic hepatitis C.** *J Infect Dis* 1995, **171**: 1607-1610.
42. Kurbanov F, Tanaka Y, Sugauchi F, Kato H, Ruzibakiev R, Zalyalieva M, Yunusova Z, Mizokami M: **Hepatitis C virus molecular epidemiology in Uzbekistan.** *J Med Virol* 2003, **69**: 367-375.
43. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, Pinho JR: **Molecular characterization, distribution, and dynamics of hepatitis C virus genotypes in blood donors in Colombia.** *J Med Virol* **82**: 1889-1898.
44. Silva GF, Nishimura NF, Coelho KI, Soares EC: **Grading and staging chronic hepatitis C and its relation to genotypes and epidemiological factors in Brazilian blood donors.** *Braz J Infect Dis* 2005, **9**: 142-149.

doi:10.1186/1743-422X-8-165
Cite this article as: Vieira *et al.*: Distribution of hepatitis c virus (hcv) genotypes in patients with chronic infection from Rondônia, Brazil. *Virology Journal* 2011 **8**:165.

CAPÍTULO 3: O VÍRUS DA HEPATITE DELTA NA REGIÃO AMAZÔNICA – AMÉRICA DO SUL

3.1 Características gerais do vírus da Hepatite Delta

O vírus da Hepatite Delta (HDV) foi descrito em 1977 e está associado à co-infecção ou super-infecção com portadores do HBV (Rizzetto *et al.,* 1977). A prevalência do HDV varia amplamente dependendo da região geográfica. Em algumas regiões do Mediterrâneo, África e Oriente Médio, mais de 24% dos portadores do HBV apresentam marcadores para HDV. No entanto, essa infecção é pouco comum em países como Estados Unidos da América, onde basicamente essa infecção é restrita a grupos de risco como usuários de drogas e hemofílicos (Gaeta *et al.,* 2000). No Brasil, o HDV tem sido reportado na região da Amazônia Ocidental, onde um grande número de casos de infecções agudas e crônicas por este vírus foram descritos (Bensabath *et al.,* 1983; Bensabath *et al.,* 1987). Nesta região, a porcentagem de portadores de HBsAg com anticorpos anti-HDV é 32% (Fonseca *et al.,* 1988; Fonseca, 2002).

Estudos genéticos e de sequenciamento do genoma do HDV revelaram uma alta heterogeneidade do vírus, o qual gerou uma classificação nos genótipos 1, 2 e 3 (Chao *et al.,* 1990; Casey *et al.,* 1993). O genótipo 2 tem sido dividido em subtipos (2a, 2b). Num estudo recente, usando analises filogenéticas incluindo um grande numero de sequências da África sugeriu que

existem mais de três genótipos do HDV (Radjef *et al.,* 2004). Cada genótipo do HDV tem uma distribuição geográfica diferente e associação com diferentes graus de doença do fígado. O genótipo 3 e subtipo 2a são considerados os mais patogênicos (Casey *et al.,* 1996).

3.2 Epidemiologia do HDV na América do Sul

A infecção pelo HDV está sob controle em países desenvolvidos, embora, em países em desenvolvimento, ainda é um grave problemas de saúde pública (Braga *et al.,* 2001; de Paula *et al.,* 2001). Especificamente, na região da Amazônia Ocidental do Brasil, embora vários centros de referência em medicina tropical relatem frequentemente surtos de hepatite aguda e crônica pelos HDV/HBV, há uma escassez de estudos clínicos detalhados e biomoleculares (dados dos relatórios de vigilância epidemiológica da Secretaria de Saúde Pública do Estado do Acre). Em outras regiões amazônicas de outros países, os genótipos 1 e 3 prevalecem, mas o genótipo 3 é mais frequente em casos de hepatite agudas e crônicas graves. Estes resultados justificam a importância do estudo de genotipagem do HDV porque contribuirão para um melhor entendimento da epidemiologia molecular e aspectos clínicos da infecção pelo HDV no Brasil.

Estima-se que dois bilhões de pessoas estão infectadas com o HBV no mundo e mais de 350 milhões deles são portadores crônicos deste vírus (Lavanchy *et al.,* 2005) e 15 milhões estão co-infectados ou superinfectados

pelo vírus da hepatite Delta (HDV) (Hadziyannis *et al.,* 1997). O HDV geralmente induz a uma doença grave, mas suas manifestações clínicas são muito amplas, que vão desde casos assintomáticos até casos de hepatite fulminante (Bonino *et al.,* 1987, Hadziyannis *et al.,* 1991).

Os genótipos do HDV são divididos em oito grupos distintos: O genótipo HDV/1 é o mais comum e é prevalente na Europa, Oriente Médio, América do Norte e África do Norte (Makino *et al.,* 1987; Chao *et al.,* 1990; Shakil *et al.,* 1997); HDV/2 prevalece no Japão (Imazeki *et al.,* 1990), Taiwan (Wu *et al.,* 1995) e Rússia (Ivaniushina *et al.,* 2001). HDV/3 prevalece na região amazônica da América do Sul (Casey *et al.,* 1993); HDV/4 é prevalente no Japão (Sakugawa *et al.,* 1999; Watanabe *et al.,* 2003) e Taiwan (Wu *et al.,*1998) e os genótipos HDV/5 ao HDV/8 são encontrados no continente africano (Le Gal *et al.,* 2006).

Na Colômbia, há poucos estudos disponíveis sobre a prevalência do HDV, principalmente, mostrando sua associação com os focos hepatite fulminante em regiões com endemicidade alta ou intermediária do HBV. No estado do Amazonas, altas taxas de prevalência para o anti-HDV foram encontrados entre as crianças menores de 4 anos (Espinal *et al.,* 1998). Além disso, HDV tem sido descrito há mais de 50 anos na região de Santa Marta (estado de Magdalena), no norte da Colômbia (Ljunggren *et al.,* 1985; Buitrago *et al.,* 1986).

No estudo realizado em 2007 **(Alvarado-Mora *et al.,* 2011a)**, cento e setenta e três amostras HBsAg ou/e anti-HBc positivas foram testadas para o

anti-HDV, sendo encontrados 5,20% (n=9) casos positivos para anti-HDV. Oito destas amostras foram provenientes da região do Amazonas e uma amostra veio do estado de Magdalena. Da mesma forma, foi caracterizado o genótipo do HDV em cinco de sete casos de hepatite fulminante provenientes do Amazonas, sendo encontrada a presença do HDV/3 **(Alvarado-Mora *et al.,* 2011h**). Nesse mesmo trabalho, foi estimada a taxa de substituição do HDV/3 em torno de $1,07x10^{-3}$ s/s/y, determinando um ancestral comum mais recente de 85 anos para este genótipo. Além disso, foi observado que o HDV/3 apresentou um crescimento exponencial entre a década de 50 até os anos 70 no norte da América do Sul. Esses resultados estão de acordo com os relatos que demonstram a presença do HDV na América do Sul desde 1930 (Ljunggren *et al.,* 1985; Buitrago *et al.,* 1986).

Diferentes razões estão associadas possivelmente com a diminuição no crescimento do HDV/3 depois de 1970. Primeiro, é possível associar a alta mortalidade causada por hepatite fulminante relatada na década de 80 (Popper *et al.,* 1983; Buitrago *et al.,* 1986; Hadler *et al.,* 1992). Em segundo lugar, entre 1976 e 1979, o Brasil implementou um programa epidemiológico em Sena Madureira (Acre) e Boca do Acre (Amazonas) para controlar as infecções pelo HBV e HDV (Bensabath *et al.,* 1983). Mas o fato possivelmente mais importante associado com a diminuição encontrada na infecção pelo HDV/3 foi a introdução dos programas de vacinação contra o HBV. Colômbia e Brasil foram os primeiros países a introduzir a vacinação universal contra o HBV na América Latina (de la Hoz *et al.,* 2005). No Brasil, a vacinação começou em

setembro de 1989 na cidade de Lábrea (AM), onde a infecção era predominante e uma redução significativa na frequência do anti-HDV foi relatada após a introdução da vacina contra o HBV (Braga et al., 1998; Braga, 2004). Na Colômbia, no estado de Amazonas, iniciou-se o programa de vacinação contra o HBV em 1992. Na Venezuela, especificamente na população de Yucpas e Bari na Sierra de Perijea, fronteira com a Colômbia, desde metade da década dos 80 se realiza a vacinação entre crianças. Dessa forma, os resultados aqui obtidos mostram a importância dos estudos sobre a dinâmica do vírus da Hepatite Delta na América do Sul e no resto do mundo, nas regiões onde estão circulando os outros genótipos, dos quais ainda não se conhece a sua dinâmica nas diferentes populações.

3.3 Referências

1. **Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvea MS, de Azevedo Neto RS, Carrilho FJ, et al.** Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population - how is the epidemiological situation? **PLoS One; 2011a; 6: e18888.**

2. **Alvarado-Mora MV., Romano CM., Gomes-Gouvêa M, Gutierrez MF, Carrilho F, Pinho, JR.** Dynamics of Hepatitis D (Delta) Vírus genotype 3 in the Amazon region of South America. ***Infect Genet Evol.*** **2011h; 11(6):1462-8.**

3. Bensabath G, Dias LB. Hepatite de Lábrea (febre negra de Lábrea) e outras hepatites fulminantes em Sena Madureira, Acre e Boca do Acre, Amazonas, Brasil. *Rev Inst Med Trop Sao Paulo.* 1983; 25: 182-194.

4. Bensabath G, Hadler SC, Soares MC, Fields H, Dias LB, Popper H, Maynard JE. Hepatitis delta virus infection and Labrea hepatitis. Prevalence and role in fulminant hepatitis in the Amazon Basin. J*AMA;* 1987; 258(4): 479-483.

5. Bonino F, Negro F, Baldi M, Brunetto MR, Chiaberge E, et al. The natural history of chronic delta hepatitis. *Prog Clin Biol Res.* 1987; 234: 145-152.

6. Braga WS, Melo H, Cossate MDB, Castilho MC, Souza RAB, Brasil LM, Fonseca JCF. Prevalência de marcadores sorológicos dos vírus da hepatite B e Delta em população assintomática: estudo do impacto do uso da vacina contra hepatite B em áreas hiperendêmicas, Itamarati-Amazonas, Vale do rio Juruá. *Rev Soc Bras Trop.* 1998; 31, 31

7. Braga WS, Brasil LM, de Souza RA, Castilho Mda C, da Fonseca JC. The occurrence of hepatitis B and delta virus infection within seven Amerindian ethnic groups in the Brazilian western Amazon. *Rev Soc Bras Med Trop*. 2001; 34(4): 349-355.

8. Braga WS. Hepatitis B and D virus infection within Amerindians ethnic groups in the Brazilian Amazon: epidemiological aspects. *Rev Soc Bras Med Trop.* 2004; 37, 2: 9-13.

9. Buitrago B, Popper H, Hadler SC, Thung SN, Gerber MA, Purcell RH, Maynard JE. Specific histologic features of Santa Marta hepatitis: a severe form of hepatitis delta-virus infection in northern South America. *Hepatology.* 1986; 6, 1285-1291.

10. Casey JL, Brown TL, Colan EJ, Wignall FS, Gerin JL. A genotype of hepatitis D virus that occurs in northern South America. *Proc Natl Acad Sci U S A.* 1993; 90: 9016-9020.

11. Casey JL, Niro GA, Engle RE, Vega A, Gomez H, McCarthy M, Watts DM, Hyams KC, Gerin JL. 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.* 1996; 174(5): 920-926.

12. Chao YC, Chang MF, Gust I, Lai MM. Sequence conservation and divergence of hepatitis delta virus RNA. Virology. 1990; 178: 384-392.

13. de la Hoz F, Perez L, Wheeler JG, de Neira M, Hall AJ. Vaccine coverage with hepatitis B and other vaccines in the Colombian Amazon: do health worker knowledge and perception influence coverage? *Trop Med Int Health.* 2005; 10: 322-329.

14. de Paula VS, Arruda ME, Vitral CL, Gaspar AM. Seroprevalence of viral hepatitis in riverine communities from the Western Region of the Brazilian Amazon Basin. *Mem Inst Oswaldo Cruz.* 2001; 96(8):1123-112.

15. Espinal C. Perfil Epidemiológico de la Hepatitis B y D en Colombia. *Biomédica*. 1998; 18: 216-249.

16. Fonseca JC, Simonetti SR, Schatzmayr HG, Castejon MJ, Cesario AL, Simonetti JP. 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.* 1988; 82(3):469-471.

17. Fonseca J. C. Hepatitis D. *Rev Soc Bras Med Trop*. 2002; 35(2): 181-190.

18. Gaeta GB, Stroffolini T, Chiaramonte M, Ascione T, Stornaiuolo G, Lobello S, Sagnelli E, Brunetto MR, Rizzetto M. Chronic hepatitis D: a vanishing Disease? An Italian multicenter study. *Hepatology* 2000; 32(4): 824-827.

19. Hadler SC, Alcala de Monzon M, Rivero D, Perez M, Bracho A, et al. Epidemiology and long-term consequences of hepatitis delta virus infection in the Yucpa Indians of Venezuela. *Am J Epidemiol.* 1992; 136: 1507-1516.

20. Hadziyannis SJ, Papaioannou C, Alexopoulou A. The role of the hepatitis delta virus in acute hepatitis and in chronic liver disease in Greece. *Prog Clin Biol Res.* 1991; 364: 51-62.

21. Hadziyannis SJ. Review: hepatitis delta. *J Gastroenterol Hepatol.* 1997; 12: 289-298.

22. Imazeki F, Omata M, Ohto M. Heterogeneity and evolution rates of delta virus RNA sequences. *J Virol.* 1990; 64: 5594-5599.

23. Ivaniushina V, Radjef N, Alexeeva M, Gault E, Semenov S, et al. Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia, Russia. *J Gen Virol.* 2001; 82: 2709-2718.

24. Lavanchy D. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. *J Clin Virol.* 2005; 34(1): 1-3.

25. Le Gal F, Gault E, Ripault MP, Serpaggi J, Trinchet JC, et al. Eighth major clade for hepatitis delta virus. *Emerg Infect Dis.* 2006; 12: 1447-1450.

26. Ljunggren KE, Patarroyo ME, Engle R, Purcell RH, Gerin JL. Viral hepatitis in Colombia: a study of the "hepatitis of the Sierra Nevada de Santa Marta". *Hepatology.* 1985; 5: 299-304.

27. Makino S, Chang MF, Shieh CK, Kamahora T, Vannier DM, et al. Molecular cloning and sequencing of a human hepatitis delta (delta) virus RNA. *Nature.* 1987; 329: 343-346.

28. Popper H. Concerning particularly delta agent infection, chronic hepatitis, and relation of hepatitis B infection to hepato-cellular carcinoma. *Prog Clin Biol Res.* 1983; 143: 397-410.

29. Radjef N, Gordien E, Ivaniushina V, Gault E, Anais P, Drugan T, Trinchet JC, Roulot D, Tamby M, Milinkovitch MC, Deny P. 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* 2004; 78(5): 2537-2544.

30. Rizzetto M, Canese M, Aroci S, Crivelli O, Trépo C, Bonino F, Verme G. Immunofluorescence detection of a new antigen-antibody system (Delta/anti-Delta) associated with hepatitis B virus in liver and serum of HBsAg carriers. *Gut* 1977; 18:997-1003.

31. Sakugawa H, Nakasone H, Nakayoshi T, Kawakami Y, Miyazato S, et al. Hepatitis delta virus genotype IIb predominates in an endemic area, Okinawa, Japan. *J Med Virol.* 1999; 58: 366-372.

32. Shakil AO, Hadziyannis S, Hoofnagle JH, Di Bisceglie AM, Gerin JL, et al. Geographic distribution and genetic variability of hepatitis delta virus genotype I. *Virology*. 1997; 234: 160-167.

33. Watanabe H, Nagayama K, Enomoto N, Chinzei R, Yamashiro T, et al. Chronic hepatitis delta virus infection with genotype IIb variant is

correlated with progressive liver disease. *J Gen Virol.* 2003; 84: 3275-3289.

34. Wu JC, Chen CM, Sheen IJ, Lee SD, Tzeng HM, et al. Evidence of transmission of hepatitis D virus to spouses from sequence analysis of the viral genome. *Hepatology.* 1995; 22: 1656-1660

35. Wu JC, Chiang TY, Sheen IJ (1998) Characterization and phylogenetic analysis of a novel hepatitis D virus strain discovered by restriction fragment length polymorphism analysis. *J Gen Virol.* 1998; 79 (5): 1105-1113.

Infection, Genetics and Evolution 11 (2011) 1462–1468

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Dynamics of Hepatitis D (delta) virus genotype 3 in the Amazon region of South America

Mónica Viviana Alvarado-Mora [a,*], Camila Malta Romano [b], Michele Soares Gomes-Gouvêa [a], Maria Fernanda Gutierrez [c], Flair José Carrilho [a], João Renato Rebello Pinho [a]

[a] Laboratory of Gastroenterology and Hepatology, São Paulo Institute of Tropical Medicine and Department of Gastroenterology, School of Medicine, University of São Paulo, Brazil
[b] Institute of Tropical Medicine of São Paulo, Department of Infectious and Parasitic Diseases (LIMHC), School of Medicine, University of São Paulo, Brazil
[c] Laboratory of Virology, Department of Microbiology, Pontificia Javeriana University, Bogotá, Colombia

ARTICLE INFO

Article history:
Received 18 April 2011
Received in revised form 20 May 2011
Accepted 22 May 2011
Available online 27 May 2011

Keywords:
Hepatitis Delta
Genotype 3
South America
Bayesian Inference
Dynamics

ABSTRACT

Hepatitis delta virus (HDV) is widely distributed and associated with fulminant hepatitis epidemics in areas with high prevalence of HBV. Several studies performed in the 1980s showed data on HDV infection in South America, but there are no studies on the viral dynamics of this virus. The aim of this study was to conduct an evolutionary analysis of hepatitis delta genotype 3 (HDV/3) prevalent in South America: estimate its nucleotide substitution rate, determine the time of most recent ancestor (TMRCA) and characterize the epidemic history and evolutionary dynamics. Furthermore, we characterized the presence of HBV/HDV infection in seven samples collected from patients who died due to fulminant hepatitis from Amazon region in Colombia and included them in the evolutionary analysis. This is the first study reporting HBV and HDV sequences from the Amazon region of Colombia. Of the seven Colombian patients, five were positive for HBV-DNA and HDV-RNA. Of them, two samples were successfully sequenced for HBV (subgenotypes F3 and F1b) and the five samples HDV positive were classified as HDV/3. By using all HDV/3 available reference sequences with sampling dates ($n = 36$), we estimated the HDV/3 substitution rate in 1.07×10^{-3} substitutions per site per year (s/s/y), which resulted in a time to the most recent common ancestor (TMRCA) of 85 years. Also, it was determined that HDV/3 spread exponentially from early 1950s to the 1970s in South America. This work discusses for the first time the viral dynamics for the HDV/3 circulating in South America. We suggest that the measures implemented to control HBV transmission resulted in the control of HDV/3 spreading in South America, especially after the important raise in this infection associated with a huge mortality during the 1950s up to the 1970s. The differences found among HDV/3 and the other HDV genotypes concerning its diversity raises the hypothesis of a different origin and/or a different transmission route.

1. Introduction

Hepatitis B virus (HBV) infection is one of the leading causes of acute and chronic hepatitis disease in the world and is also associated with liver cirrhosis and hepatocellular carcinoma (Fan et al., 2010). It is estimated that 2 billion people are infected with HBV, more than 350 millions of them are chronic carriers of this virus (Lavanchy, 2005) and 15 millions of them are coinfected or superinfected with HDV (Hadziyannis, 1997). The natural history of chronic HBV infection appears to exhibit significant geographical different behaviors what may be linked to differences in viral, host and environmental factors (Hadziyannis, 2011).

HBV is classified into nine genotypes (HBV/A to HBV/I) where each one differs from each other by 7.5–13% at the nucleotide level (Kramvis et al., 2008; Yu et al., 2010). Specifically, HBV/F and HBV/H genotypes are the most divergent viral genotypes in relation to the others and HBV/F is characteristic of indigenous populations from South America. HBV/F presents high frequency in several American countries, especially those with Spanish colonization (Alvarado Mora et al., 2011; Arauz-Ruiz et al., 2002; Devesa et al., 2004, 2008; Santos et al., 2010), but it is also found in other sites with Native American population, such as Inuits from Alaska (Livingston et al., 2007) to the Brazilian Amazon rainforest (Gomes-Gouvêa et al., 2009). The presence of these genotypes in the American countries appears to be very antique and much is speculated about their origin (Alvarado Mora et al., 2011; Blitz et al., 1998; Livingston et al., 2007).

* Corresponding author at: Depto. Gastroenterologia, Faculdade de Medicina da 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).

1567-1348/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.meegid.2011.05.020

HDV is associated with HBV, as a primary co-infection with HBV or as a superinfection in an HBV carrier. HDV has a negative-sense circular RNA genome with about 1700 nucleotides that only expresses delta antigen (Imazeki et al., 1990). HDV usually induces a severe disease but its clinical manifestations are very broad, ranging from asymptomatic cases to fulminant hepatitis (Bonino et al., 1987; Hadziyannis et al., 1991). The virus is found worldwide but is not uniformly distributed, as determined by seroprevalence studies of anti-HD in HBsAg-positive patients (Rizzetto, 2000). HDV genotypes are classified in eight different groups: genotype 1 is the most common genotype and is prevalent in Europe, Middle East, North America and North Africa (Chao et al., 1990; Makino et al., 1987; Shakil et al., 1997); genotype 2 prevails in Japan (Imazeki et al., 1990), Taiwan (Wu et al., 1995) and Russia (Ivaniushina et al., 2001); genotype 3 in the Amazon region in South America (Casey et al., 1993); genotype 4 in Japan (Sakugawa et al., 1999; Watanabe et al., 2003) and Taiwan (Wu et al., 1998) and HDV genotypes 5 to 8 prevails in Africa (Le Gal et al., 2006).

In Colombia, few reports are available on HDV prevalence, mainly showing its association to fulminant hepatitis outbreaks in regions with intermediate or high HBV endemicity, particularly in the Amazonas department, where high prevalence rates for anti-HDV have been found among children younger than 4 years old (Espinal, 1998). Furthermore, HDV has been described for more than 50 years in Santa Marta region, in the north of Colombia, where HBsAg seroprevalence was 4.7% (Ljunggren et al., 1985; Buitrago et al., 1986). In another paper analyzing 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 (Prieto and Rojas, 2003). Moreover, in Colombia, HBV genotype F3 is the most prevalent but genotypes A2, G, E and F1b were also reported (Alvarado Mora et al., 2010, 2011).

The present study was conducted to obtain insight into the molecular epidemiology and dynamics of HDV/3 genotype. We also intended to estimate its nucleotide substitution rate and the time of most recent ancestor (TMRCA). Also, we verify the presence of HDV infection among 7 fulminant hepatitis cases of hepatitis B identified in the Amazon Public Health Laboratory in Leticia, Amazonas Department, Colombia. This is the first study reporting HBV and HDV sequences from the Amazon region of Colombia.

2. Materials and methods

2.1. Study population

Seven HBsAg positive serum samples were collected between January and December of 2007 in the Amazon Public Health Laboratory in Leticia city, Amazonas, Colombia and stored at −70 °C. The age of the patients (five women and two men) ranged from 17 to 39 years old. These samples came from inhabitants with fulminant hepatitis from different small villages at the Amazonas department, in Colombia. This protocol was approved from Ethical Committees from the Pontificia Universidad Javeriana, Bogotá, Colombia and University of São Paulo Medical School, São Paulo, Brazil.

2.2. HBV DNA and HDV RNA amplification

To avoid false-positive results, rigorous procedures used for nucleic acid amplification techniques were followed (Kwok and Higuchi, 1989). HBV DNA extraction was carried out from 100 μL of each serum sample using the acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). HDV RNA was extracted with same methodology from HBV DNA and dissolved in 10 μL Milli Q water.

Amplification was performed by nested PCR covering a 737 bp region corresponding to the S gene and a partial fragment of the polymerase gene. The amplification took place with the primers described by Sitnik et al. (2004) and Gomes-Gouvêa et al. (2009), known as FBHS1 (5′-GAGTCTAGACTCGTGGTGGACTTC-3′; nt 244–267) and FBHS2 (5′-CGTGGTGGACTTCTCTCAATTTTC-3′; nt 255–278), and primers RADE1 (5′-TGCRTCAGCAAACACTTGGC-3′; nt 1175–1194) and RADE2 (5′-TGRCANACYTTCCARTCAATNGG-3′; nt 989–970).

For HDV, the partial delta antigen genomic region (403-nucleotide fragment) it was amplified. For the detection of HDV RNA, extracted RNA was previously denatured at 95 °C for 5 min and then 20 μL of the reverse transcriptase was added with the reverse primer 1302OD. Reaction mix was incubated at 37 °C for 60 min and stopped at 95 °C for 15 min. The amplification of a region of 403 bp was performed using the protocols described previously by Casey et al. (1993) and Zhang et al. (1996). The primers used were: 8531U (5′-CGGATGCCCAGGTCGGACC-3′; nt 853–871); 1302D (5′-GGATTC ACCGACAAGGAGAG-3′; nt 1322–1303); HDV-E (5′-GAAGGAAGGCCCTCGAGAACAAGA-3′; nt 887–910) and HDV-A (5′-GAGATGCCATGCCGACCCGAAGAG-3′; nt 1290–1267).

2.3. HDV and HBV sequencing

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) using dideoxy nucleotide triphosphates (ddNTPs) containing fluorescent markers (*Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction kit* – Applied Biosystems, Foster City, CA, USA).

Quality of each electropherogram was evaluated using the Phred-Phrap software (Ewing and Green, 1998; Ewing et al., 1998) and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software on the web page eletropherogram quality analysis (http://asparagin.cenargen.embrapa.br/phph/).

2.4. Phylogenetic analyses

HBV and HDV sequences were genotyped by phylogenetic reconstructions using reference sequences from HBV ($n = 276$) and HDV genotype ($n = 242$) obtained from GenBank. Sequences were aligned and edited using Clustal X (Thompson et al., 1997) and Se-AL (available at: http://tree.bio.ed.ac.uk/software/seal/) software, respectively. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3 (Drummond and Rambaut, 2007). HBV and HDV datasets were analyzed under relaxed uncorrelated lognormal and relaxed uncorrelated exponential molecular clock 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. The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. The maximum clade credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees after excluding 10% of burn-in using Tree Annotator v.1.5.3 Drummond and Rambaut, 2007). Phylogenetic trees were visualized in FigTree v.1.2.2 (available at: http://tree.bio.ed.ac.uk/software/figtree/).

2.5. Evolutionary analysis for HDV/3

All HDV/3 sequences reported in Genbank and five additional sequences reported in this study ($n = 41$) (25 from Brazil, 8 from Venezuela, 6 from Colombia and 2 from Peru) were analyzed in this

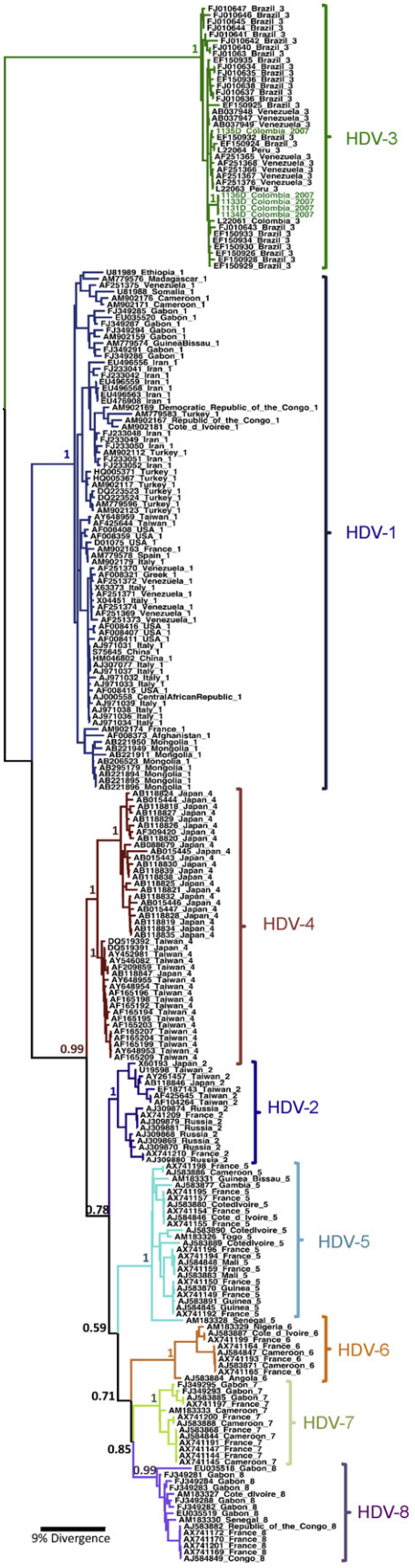


Fig. 1. The(MCC) tree was estimated by Bayesian analysis of 242 hepatitis delta virus strains. The posterior probabilities of the key nodes are depicted above the respective nodes. Samples obtained from Colombia Amazon region ($n = 5$; marked in green) were analyzed together with other worldwide strains. The Genbank accession number, HDV genotype and geographic origin of each sequence are indicated in the tree. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

paper. The BSL analysis was carried out using all from them, except 5 from Venezuela, as the dates of sample collection were not available from them. Bayesian Skyline Plot (BSL) was performed 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).

3. Results

3.1. HBV and HDV genotyping

HBV and HDV PCR assays were carried out on the seven samples that had been previously reported as positive for the presence of HBsAg by an ELISA test (DiaSorin, Italy). In five of them, it was possible to amplify the 737 bp fragment of HBV genome and the 403 bp fragment of HDV genome. Sequencing of all the amplified fragments was attempted but sequences with good resolution were obtained for five HDV and two HBV sequences. The two HBV sequences were typed by phylogenetic analysis and they belong to genotype F (F1b and F3) (Fig. 1 supplementary material). All HDV sequences were classified as genotype 3 (Fig. 1). HBV and HDV sequences were deposited at the GenBank under accession numbers: EU287873–EU287874 and EU287868–EU287872.

3.2. Evolutionary analysis for HDV/3

We obtained a substitution rate about 1.07×10^{-3} substitution/site/year (s/s/y) for HDV/3 by running the BSL analysis with the HDV/3 dataset with sampling dates. The evolutionary analysis performed only on South American Amazon HDV/3 sequences ($n = 36$) suggested that this genotype was circulating in South America since the 1920s (95% high probability density (HPD) from 1821 to 1974). Furthermore, this genotype presented signals of exponential growth from the end of the 50s until the end of the 70s (Fig. 2), where after a reduction in infections was found. These estimates correspond to the values obtained under the best-fitted molecular clock chosen by Bayes Factor comparison where the relaxed uncorrelated exponential was the best molecular clock for these analyses.

4. Discussion

4.1. HBV/F and HDV/3 co-infection

The two HBV sequences found in this study were classified as subgenotypes F1b and F3. HBV/F3 was also reported as the most prevalent in Bogotá, Colombia, followed by genotypes F1b, A2 and G (Alvarado Mora et al., 2011). In Venezuela, subgenotypes F2 and F3 were reported in Yucpa indigenous groups (Devesa et al., 2004; Devesa and Pujol, 2007; Nakano et al., 2001).

All the 5 HDV isolates from Amazonas department, in southern Colombia were classified as genotype 3. These results agree with previous data describing that HDV/3 is the only genotype found in the Peruvian Amazon Basin (Casey et al., 1993) and Western Brazilian Amazon Basin (Gomes-Gouvêa et al., 2009). There was just one sequence from Colombia reported formerly (L22061): this sequence was also HDV genotype 3 and was obtained from one case from Santa Marta region (Ljunggren et al., 1985). It should be stressed that the five positive strains for HDV were collected within the Colombian Amazonas department but not in the same location.

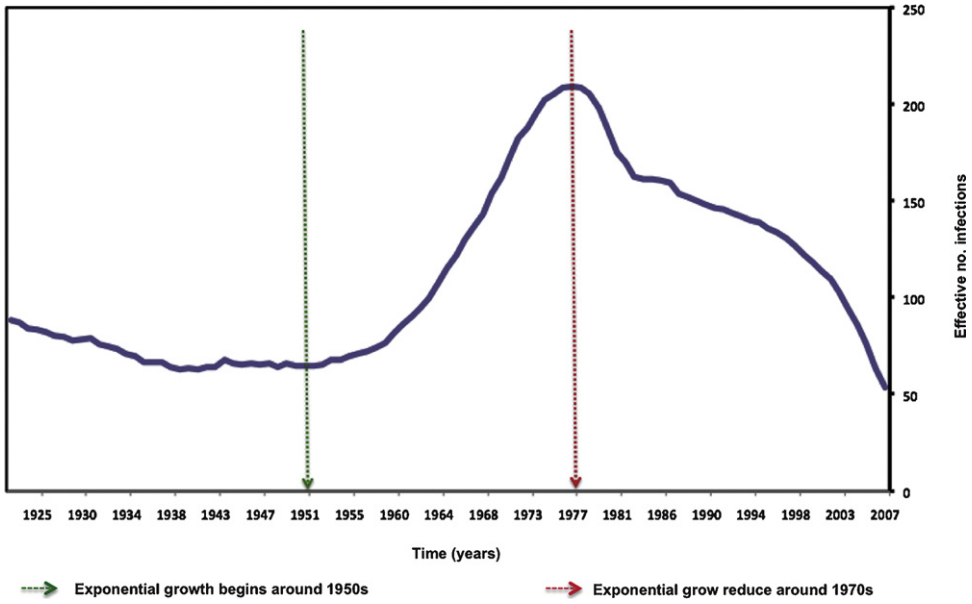


Fig. 2. Bayesian skyline (BSL) plot obtained from South American HDV/3 sequences (n = 36). The figure shows the superimposed median values of Ne.g through time. Time is presented in years.

Four out of these five sequences presented a 100% similarity and they probably represent the commonest strain circulating in this Colombian region (Fig. 1). A similarity of 100% is not completely unexpected since it was already reported in Brazil a 100% of similarity among viruses of patients infected with HDV/3, all of them probably exposed to the same source of infection (Gomes-Gouvêa et al., 2009).

Two HDV/3 cases were co-infected with HBV/F3 and HBV/F1b, respectively. Co-infection of HDV/3 with HBV/F is the most frequent in the Amazon basin However, co-infection of HDV/3 with HBV/A and HBV/D was previously reported in the same region in Brazil (Gomes-Gouvêa et al., 2009). In Venezuela, HDV genotypes 1 and 3 were reported and HDV/1 was associated with HBV/D while HDV/3 was associated with HBV/F. The founding of HDV/1 in a Yucpa Indian, that live in Sierra de Perijá, Zulia state, Venezuela, probably reflects an transmission from European immigrants, since, in the last century, these Indians have been the ones most in contact with Amerindians communities (Quintero et al., 2001).

4.2. Substitution rate for HDV/3

The substitution rate estimated (1.07×10^{-3} substitutions per site per year) in this work for HDV/3 was obtained using Bayesian approach previously used for other viruses such as HBV, HCV and GBV-C (Alvarado Mora et al., 2010, 2011; Romano et al., 2008, 2010; Zhou and Holmes, 2007). Other studies previously estimated similar substitution rate for HDV, even though using very small number of samples and distinct methods based on analysis of similarity. The previously estimated evolutionary rates ranged from 0.59×10^{-3} s/s/y to 3.0×10^{-2}, which agrees with the data obtained in this work (Chao et al., 1990, 1994; Imazeki et al., 1990; Lee et al., 1992).

Using the sequences obtained from small populations living in the Amazon region (n = 36), we estimated a TMRCA of about 85 years for the HDV genotype 3 strains circulating in these populations. Since there are considerable variations on evolutionary rates previously reported and different substitution rates can occur among distinct genotypes of the same virus (Alvarado Mora et al., 2010, 2011; Romano et al., 2010; Zehender et al., 2008; Zhou and Holmes, 2007), we did not evaluate the TMRCA for the delta genotypes other than HDV/3 using the substitution rate estimated in this study.

4.3. Dynamics of Hepatitis Delta genotype 3 in South America

Our evolutionary results agree with the previous data supporting that hepatitis delta has been circulating in South America since early 1930s, involved in many different outbreaks. The first report probably associated with this agent is known as "hepatitis de la Sierra Nevada de Santa Marta" (Ljunggren et al., 1985; Buitrago et al., 1986). Another severe hepatitis outbreak caused by this virus was also observed in the Labrea region on Purus River, Acre, Brazil and was subsequently named Labrea hepatitis or black fever (Bensabath and Dias, 1983). Such cases have been reported since 1934 throughout the Amazon basin (Dias and Coura, 1985).

The high frequency of HBV, together with the presence of HDV markers, showed that these viruses represent the etiologic factors of these diseases. The BSL also support the time of the increased circulation of HDV in the northern region of South America by showing an exponential growth between 1950s and 1970s. This agrees with other studies that reported the presence of HDV infection since 1950s as an unusual type of hepatitis in the Amazon region of South America where, severe, often fatal, acute and chronic type D hepatitis occurs among indigenous people in Brazil, Peru Venezuela, and Colombia (Gomes-Gouvêa et al., 2009; Bensabath et al., 1987; Dias and Coura, 1985; Hadler et al., 1992; Popper, 1983). Coincidentally, the period between 1950 and 1970 coincides with the years were Brazilian and Colombian population growths reach their maximum rates (http://www.census.gov/ipc/prod/wp96/wp96005.pdf). The presence of HDV infection before this time was much less relevant, probably because these Amazon regions had a very low number of inhabitants, living in small villages dispersed in the jungle, with very low contact between them, making difficult the spreading of this agent. Consequently, the increase in the viral genetic variability observed in this period may be a consequence of the increased number of susceptible hosts in this area, as well as the rate of contact among them.

Different reasons are possibly related to the decrease in HDV after 1970. First, it is possible to associate the slope down of HDV/3

in the BSL in the end of 1970s to the high mortality caused by fulminant hepatitis, when HDV/3 infection was common (Buitrago et al., 1986; Hadler et al., 1992; Popper, 1983). Second, in 1976 and 1979, Brazil implemented an epidemiological program in Sena Madureira (Acre state) and in Boca do Acre counties (Amazon state) to control both HBV and HDV infections (Bensabath and Dias, 1983). The most important fact associated with the decrease found in HDV infection was the introduction of HBV vaccination programs. Colombia and Brazil were the first countries to introduce universal vaccination against HBV in Latin America (de la Hoz et al., 2005). In Brazil, vaccination started in September 1989 in Labrea city, where the infection was prevalent and a significant reduction in anti-HDV was reported thereafter (Braga et al., 1998; Braga, 2004). In the Amazon basin, Colombia started the vaccination program against HBV in 1992. Overall, after 2000, vaccination was introduced in most Latin American countries using different vaccination policies (Tambini et al., 1998). Therefore, it is plausible that the decreasing rate of HBV spread may be influencing the HDV growth. Unfortunately, since there are few HBV genotype F sequences reported specifically from the Amazon region in South America, it was not possible to reconstruct the BSL for HBV/F of this region.

Finally, it is noteworthy that the first treatment for HDV (IFN-α) started in the mid 1980s (Rizzetto et al., 1986) and PEG-IFN-α was implemented for the HDV treatment in 2006 (Erhardt et al., 2006; Niro et al., 2006). Consequently, the use of effective treatment for HDV may have had an impact on the HDV/3 spread, but it is hard to evaluate its impact on populations resident in the Amazon rainforest.

4.4. HDV/3 vs other HDV genotypes

The topology of the HDV MCC tree showed that HDV/3 is the most divergent of the 8 HDV genotypes with a high significant posterior probability. This topology raises different questions in relation of HDV/3 to the other genotypes: (1) did HDV/3 experience a distinct introduction in the human population? (2) Is the route of transmission of HDV/3 different from the other genotypes?

Concerning HDV origin, there are two main non self excluding hypotheses for it: (1) as HDV shares similarities in terms of genome structure and mechanisms of replication with plant viroids or virusoids (Taylor and Pelchat, 2010), it was proposed that it might have originated from RNA pathogens infectious from the vegetable world; (2) HDV originated from host mRNA precursors with ribozyme activity (Taylor and Pelchat, 2010).

Since there are different hypothesis for HDV origin, it will be interesting to compare sequences from non-coding region of the several different HDV genotypes to viroids, virusoids and self-cleaving ribozymes sequences in eukariotyc organisms. The differences found in the phylogenetic tree between HDV/3 and the other genotypes raises the hypothesis that this genotype might have a different origin from the remaining genotypes. Summing up with its different geographical distribution, we might speculate a particular event associated to its origin in the Amazon rainforest (Rizzetto, 1990; Viana et al., 2005).

Concerning the possibility of different routes for each HDV genotype, in industrialized countries, HDV in risk populations include illicit drug users and individuals exposed to blood or blood products (Pascarella and Negro, 2010). This is the case of Asian countries where HDV/2 and HDV/4 genotypes are found in intravenous drug addicts with HBV infection (Wedemeyer and Manns, 2010). HDV generally is not considered a typically sexually transmitted disease but in countries such as Taiwan this route is the predominant way of transmission (Liaw et al., 1990). For HDV/3, studies in the Amazon region on prevalence of HBV and HDV showed that family members are reservoirs for transmission of infection by HDV (Wedemeyer and Manns, 2010). In this way, the chances of contamination from an extrafamilial source expressed by highly divergent isolates (Niro et al., 1999) and the sequence similarity in most families units indicate a single source of infection providing evidence that HDV infection is probably mostly transmitted within the families (Viana et al., 2005). This hypothesis has been supported with the HDV distances between genotypes and within genotypes (Table s1a and s1b) since the HDV/3 has the greatest distance when compared with other genotypes but has the shortest distance within of the sequences of the same HDV/3 group.

Finally, the geographical region where the HDV/3 is prevalent is smaller than the other regions in the world where others delta genotypes circulate. Consequently, it could also influence on the low intra-genetic variability existing within HDV/3 in relation to the other HDV genotypes.

5. Conclusions

In conclusion, the results obtained here show the importance of continuing to undertake studies about the dynamics of HDV. Also, this study shows the co-infection of HBV genotype F and HDV genotype 3 in the Colombian Amazon region, similar to other Amazon regions from Brazil and Venezuela. Finally, we suggest that the measures implemented to control HBV transmission had an impact on the control of HDV/3 spreading in South America, especially after the important rise in this infection associated with a huge mortality during the 1950s up to the 1970s. The differences found among HDV/3 and the other HDV genotypes concerning its diversity raises the hypothesis of a different origin and/or a different transmission route.

Disclosures

No conflict of interest in relation to this study.

Acknowledgments

This study was previously presented as a poster in 2010 in the EASL monothematic conference on Delta Hepatitis, Istanbul, Turkey. We thank the collaboration of the Amazon Public Health Laboratory of Leticia, Amazonas, especially Dra. Luz Mila Murcia Montaño, coordinator of the Amazon Public Health, for providing the samples for the study. 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, CNPq and Pontificia Universidad Javeriana, Bogotá, Colombia.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2011.05.020.

References

Alvarado Mora, M.V., Romano, C.M., Gomes-Gouvêa, 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.

Alvarado Mora, M.V., Romano, C.M., Gomes-Gouvêa, 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.

Arauz-Ruiz, P., Norder, H., Robertson, B.H., Magnius, L.O., 2002. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J. Gen. Virol. 83, 2059–2073.

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. Sao Paulo 25, 182–194.

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.

Blitz, L., Pujol, F.H., Swenson, P.D., Porto, L., Atencio, R., Araujo, M., Costa, L., Monsalve, D.C., Torres, J.R., Fields, H.A., Lambert, S., Van Geyt, C., Norder, H., Magnius, L.O., Echevarria, J.M., 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.

Bonino, F., Negro, F., Baldi, M., Brunetto, M.R., Chiaberge, E., Capalbo, M., Maran, E., Lavarini, C., Rocca, N., Rocca, G., 1987. The natural history of chronic delta hepatitis. Prog. Clin. Biol. Res. 234, 145–152.

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.

Braga, W.S., Melo, H., Cossate, M.D.B., Castilho, M.C., Souza, R.A.B., Brasil, L.M., Fonseca, J.C.F., 1998. Prevalência de marcadores sorológicos dos vírus da hepatite B e Delta em população assintomática: estudo do impacto do uso da vacina contra hepatite B em áreas hiperendêmicas, Itamarati-Amazonas, Vale do rio Juruá. Rev. Soc. Bras. Med. Trop. 31, 31.

Braga, W.S., 2004. Hepatitis B and D virus infection within Amerindians ethnic groups in the Brazilian Amazon: epidemiological aspects. Rev. Soc. Bras. Med. Trop. 37 (2), 9–13.

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.

Chao, Y.C., Chang, M.F., Gust, I., Lai, M.M., 1990. Sequence conservation and divergence of hepatitis delta virus RNA. Virology 178, 384–392.

Chao, Y.C., Tang, H.S., Hsu, C.T., 1994. Evolution rate of hepatitis delta virus RNA isolated in Taiwan. J. Med. Virol. 43, 397–403.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.

de la Hoz, F., Perez, L., Wheeler, J.G., de Neira, M., Hall, A.J., 2005. Vaccine coverage with hepatitis B and other vaccines in the Colombian Amazon: do health worker knowledge and perception influence coverage? Trop. Med. Int. Health 10, 322–329.

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.

Devesa, M., Pujol, F.H., 2007. Hepatitis B virus genetic diversity in Latin America. Virus Res. 127, 177–184.

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.

Dias, L.B., Coura, J.R., 1985. Labrea hepatitis Review study of hepatic viscerotomies from 1934 to 1940. Rev. Inst. Med. Trop. Sao Paulo 27, 242–248.

Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7, 214.

Erhardt, A., Gerlich, W., Starke, C., Wend, U., Donner, A., Sagir, A., Heintges, T., Haussinger, D., 2006. Treatment of chronic hepatitis delta with pegylated interferon-alpha2b. Liver Int. 26, 805–810.

Espinal, C., 1998. Perfil Epidemiológico de la Hepatitis B y D en Colombia. Biomédica 18, 216–249.

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., Wendl, M.C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8, 175–185.

Fan, W., Shi, B., Wei, H., Du, G., Song, S., 2010. Comparison of hepatitis B X gene mutation between patients with hepatocellular carcinoma and patients with chronic hepatitis B. Virus Genes.

Gomes-Gouvêa, 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.

Ivaniushina, V., Radjef, N., Alexeeva, M., Gault, E., Semenov, S., Salhi, M., Kiselev, O., Deny, P., 2001. Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia, Russia. J. Gen. Virol. 82, 2709–2718.

Hadler, S.C., Alcala de Monzon, M., Rivero, D., Perez, M., Bracho, A., Fields, H., 1992. Epidemiology and long-term consequences of hepatitis delta virus infection in the Yucpa Indians of Venezuela. Am. J. Epidemiol. 136, 1507–1516.

Hadziyannis, S.J., Papaioannou, C., Alexopoulou, A., 1991. The role of the hepatitis delta virus in acute hepatitis and in chronic liver disease in Greece. Prog. Clin. Biol. Res. 364, 51–62.

Hadziyannis, S.J., 1997. Review: hepatitis delta. J. Gastroenterol. Hepatol. 12, 289–298.

Hadziyannis, S.J., 2011. Natural history of chronic hepatitis B in Euro-Mediterranean and African Countries. J. Hepatol., in press, doi:10.1016/j.jhep.2010.12.030.

Imazeki, F., Omata, M., Ohto, M., 1990. Heterogeneity and evolution rates of delta virus RNA sequences. J. Virol. 64, 5594–5599.

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.

Kwok, S., Higuchi, R., 1989. Avoiding false positives with PCR. Nature 339, 237–238.

Lavanchy, D., 2005. Worldwide epidemiology of HBV infection, disease burden, and vaccine prevention. J. Clin. Virol. 34 (Suppl 1), S1–3.

Le Gal, F., Gault, E., Ripault, M.P., Serpaggi, J., Trinchet, J.C., Gordien, E., Deny, P., 2006. Eighth major clade for hepatitis delta virus. Emerg. Infect. Dis. 12, 1447–1450.

Lee, C.M., Bih, F.Y., Chao, Y.C., Govindarajan, S., Lai, M.M., 1992. Evolution of hepatitis delta virus RNA during chronic infection. Virology 188, 265–273.

Liaw, Y.F., Chiu, K.W., Chu, C.M., Sheen, I.S., Huang, M.J., 1990. Heterosexual transmission of hepatitis delta virus in the general population of an area endemic for hepatitis B virus infection: a prospective study. J. Infect. Dis. 162, 1170–1172.

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.

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.

Makino, S., Chang, M.F., Shieh, C.K., Kamahora, T., Vannier, D.M., Govindarajan, S., Lai, M.M., 1987. Molecular cloning and sequencing of a human hepatitis delta (delta) virus RNA. Nature 329, 343–346.

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.

Niro, G.A., Casey, J.L., Gravinese, E., Garrubba, M., Conoscitore, P., Sagnelli, E., Durazzo, M., Caporaso, N., Perri, F., Leandro, G., Facciorusso, D., Rizzetto, M., Andriulli, A., 1999. Intrafamilial transmission of hepatitis delta virus: molecular evidence. J. Hepatol. 30, 564–569.

Niro, G.A., Ciancio, A., Gaeta, G.B., Smedile, A., Marrone, A., Olivero, A., Stanzione, M., David, E., Brancaccio, G., Fontana, R., Perri, F., Andriulli, A., Rizzetto, M., 2006. Pegylated interferon alpha-2b as monotherapy or in combination with ribavirin in chronic hepatitis delta. Hepatology 44, 713–720.

Prieto, F., Rojas, D., 2003. Situación semestral de la hepatitis B, Colombia. Programa ITS/sida, Instituto Nacional de Salud. Biomédica 8, 2–11.

Popper, H., 1983. Concerning particularly delta agent infection, chronic hepatitis, and relation of hepatitis B infection to hepato-cellular carcinoma. Prog. Clin. Biol. Res. 143, 397–410.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Pascarella, S., Negro, F., 2010. Hepatitis D virus: an update. Liver Int. 31, 7–21.

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.

Rizzetto, M., 1990. Hepatitis delta: the virus and the disease. J. Hepatol. 11 (Suppl. 1), S145–148.

Rizzetto, M., 2000. Hepatitis D: virology, clinical and epidemiological aspects. Acta Gastroenterol. Belg. 63, 221–224.

Rizzetto, M., Rosina, F., Saracco, G., Bellando, P.C., Actis, G.C., Bonino, F., Smedile, A., Trinchero, P., Sansalvadore, F., Pintus, C., et al., 1986. Treatment of chronic delta hepatitis with alpha-2 recombinant interferon. J. Hepatol. 3 (Suppl. 2), S229–233.

Romano, C.M., Zanotto, P.M., Holmes, E.C., 2008. Bayesian coalescent analysis reveals a high rate of molecular evolution in GB virus C. J. Mol. Evol. 66, 292–297.

Romano, C.M., de Carvalho-Mello, I.M., Jamal, L.F., de Melo, F.L., Iamarino, A., Motoki, M., Pinho, J.R., Holmes, E.C., de Andrade Zanotto, P.M., 2010. Social networks shape the transmission dynamics of hepatitis C virus. PLoS One 5 (6), e11170.

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, 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.

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., 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.

Tambini, G., Suane Mune, K.S., Raad, J., 1998. Hepatitis B: situacion mundial y regional. Biomedica 18, 169–174.

Taylor, J., Pelchat, M., 2010. Origin of hepatitis delta virus. Future Microbiol. 5, 393–402.

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.

Viana, S., Parana, 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.

Watanabe, H., Nagayama, K., Enomoto, N., Chinzei, R., Yamashiro, T., Izumi, N., Yatsuhashi, H., Nakano, T., Robertson, B.H., Nakasone, H., Sakugawa, H., Wata-

nabe, M., 2003. Chronic hepatitis delta virus infection with genotype IIb variant is correlated with progressive liver disease. J. Gen. Virol. 84, 3275–3289.
Wedemeyer, H., Manns, M.P., 2010. Epidemiology, pathogenesis and management of hepatitis D: update and challenges ahead. Nat. Rev. Gastroenterol. Hepatology 7, 31–40.
Wu, J.C., Chen, C.M., Sheen, I.J., Lee, S.D., Tzeng, H.M., Choo, K.B., 1995. Evidence of transmission of hepatitis D virus to spouses from sequence analysis of the viral genome. Hepatology 22, 1656–1660.
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 (Pt 5), 1105–1113.
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 "I". PLoS One 5 (2), e9297.
Zehender, G., De Maddalena, C., Giambelli, C., Milazzo, L., Schiavini, M., Bruno, R., Tanzi, E., Galli, M., 2008. Different evolutionary rates and epidemic growth of hepatitis B virus genotypes A and D. Virology 380, 84–90.
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.
Zhou, Y., Holmes, E.C., 2007. Bayesian estimates of the evolutionary rate and age of hepatitis B virus. J. Mol. Evol. 65, 197–205.

CAPÍTULO 4: O VÍRUS GB-C (GBV-C) NA COLÔMBIA

4. INTRODUÇÃO

4.1 Características gerais do vírus GB-C

O vírus GB-C (GBV-C) ou vírus da hepatite G (HGV) é um vírus envelopado, com genoma fita simples de RNA de aproximadamente 9,3 Kb com polaridade positiva, pertencente à família *Flaviviridae* (Stapleton *et al.,* 2011).

A forma de transmissão principal do GB-C é por via parenteral (Moaven *et al.,* 1996; Feucht *et al.,* 1996; Thomas *et al.,* 1997; Casteling *et al.,* 1998; Frey *et al.,* 2002), especialmente por transfusões sanguíneas (Simons *et al.,* 1995; Alter *et al.,* 1997) ou de hemoderivados (Jarvis *et al.,* 1996; Feucht *et al.,* 1997). Devido a esta forma de transmissão. o vírus possui alta prevalência entre os usuários de drogas injetáveis (Stark *et al.,* 1996) assim como nos politransfundidos (Linnen et al., 1996), hemofílicos (Yamada-Osaki et al., 1998) e hemodialisados (Masuko *et al.,* 1996). A transmissão entre homens que fazem sexo com homens tem sido relatada como uma forma eficaz de transmissão (Berzsenyi *et al.,* 2005) e evidências de transmissão intrafamiliar foram determinadas com base na análise de sequências do genoma do vírus (Pinho *et al.,* 1999; Seifried *et al.,* 2004).

O genoma do GBV-C é similar ao genoma do HCV na sua organização estrutural sendo que possui duas regiões não traduzidas nos estremos 5' e 3', duas proteínas estruturais (E1 e E2) e cinco proteínas não estruturais: NS2, NS3, NS4b, NS5a e NS5b (Pessoa *et al.,* 1998; Kudo *et al.,* 1997). Estas proteínas desempenham a função de protease, helicase e RNA polimerase RNA-dependente. Da mesma forma, o sequenciamento das regiões E1 e E2 do GBV-C mostraram que não possuem sitos hipervariáveis, ao contrário do HCV que apresenta três regiões hipervariaveis na região E2 (Stapleton *et al.,* 2004). Uma outra característica importante do GBV-C é a ausência da proteína do capsídeo no inicio da poliproteina viral (Simons *et al.,* 1996).

4.2 Epidemiologia e caracterização dos genótipos do vírus GB-C na Colômbia.

A distribuição geográfica do GBV-C está relacionada com a co-evolução do vírus com os humanos durante a migração ao longo da história, sugerindo que o GBV-C é um vírus antigo (Smith *et al.,* 1997; Naito *et al.,* 2001). O genótipo 1 é encontrado na África Ocidental (Muerhoff *et al.,* 1996); genótipo 2 (sub-classificado em 2a e 2b), nos Estados Unidos e na Europa (Muerhoff *et al.,* 1997); genótipo 3 na Ásia (Mukaide *et al.,* 1997; Katayama *et al.,* 1998); genótipo 4, em Mianmar e Vietnam (Naito *et al.,* 1999); genótipo 5 na África do Sul (Tucker *et al.,* 1999); e genótipo 6, na Indonésia (Muerhoff *et al.,*

2006). Na América do Sul, os genótipos 1, 2a, 2b e 3 foram encontrados (Konomi *et al.,* 1999; Oubina *et al.,* 1999; Loureiro *et al.,* 2002; Ramos Filho *et al.,* 2004).

Vários estudos têm sido realizados para avaliar sua prevalência em grupos de pacientes com doenças crônicas do fígado. Em 1999, foi realizado um trabalho no Brasil onde foi procurado o RNA do GBV-C em dois grupos de pacientes; pacientes com hepatite não A-E e pacientes com hepatite C detectando o RNA do GBV-C em 13 dos 137 (9,5%) pacientes com hepatite não A-E e em 8 de 44 (18,2%) dos pacientes com hepatite C (Pinho *et al.,* 1999). Em outro estudo realizado no Rio de Janeiro em um grupo de 87 doadores de sangue. encontrou-se o RNA do GBV-C em 9 (10%) dos 87 doadores e em 15 (13%) num grupo de 113 pacientes com doenças hepáticas crônicas (Lampe *et al.,* 1998). Um estudo mais recente realizado em Taiwan mostrou que a prevalência do RNA do GBV-C em pacientes crônicos com hepatite B ou hepatite C foi de 7,7% e 17,3% respectivamente (Yang *et al.,* 2006).

Quanto ao diagnostico pela técnica de PCR, em um trabalho realizado no ano 2006, foi analisado o efeito da seleção de primers para diagnostico do vírus amplificando as regiões 5'UTR, NS3, NS5A e E2, no qual encontrou-se que a melhor região para diagnóstico é a 5'UTR, já que apresenta maior sensibilidade quando amplificada por esta técnica (Souza *et al.,* 2006). Da mesma forma, a escolha da região do genoma que pode gerar a melhor classificação por genótipo tem sido muito discutida nos últimos anos. Os primeiros estudos que

tentaram estabelecer grupos filogenéticos para o GB-C usando as regiões NS3 e NS5B não mostraram associação filogenética consistente, não conseguindo separar os diferentes genótipos de diferentes partes do mundo (Kao *et al.,* 1996; Smith *et al.,* 1997). Por causa da dificuldade de associar regiões codificantes com analise filogenética, em 1996, Muerhoff e colaboradores propuseram 5´UTR para a genotipagem de isolados de diferentes partes do mundo, o que tem sido aplicado em vários trabalhos publicados que realizaram a genotipagem desse vírus. 5´UTR se caracteriza por ser altamente conservada, como ocorre no genoma do HCV. A maioria dos estudos que utilizam a amplificação de 5´UTR para realizar análises filogenéticas apresentam uma ótima correlação entre os clusters encontrados e a origem geográfica do vírus (Muerhoff *et al.,* 2006).

Na Colômbia, uma alta prevalência de GBV-C RNA e a presença do genótipo 3 foram encontrados entre os índios nativos da Colômbia das etnias Wayuu, Kamsa e grupos Inga (Tanaka *et al.,* 1998). Este foi o único estudo préviopublicado sobre os genótipos do GBV-C que circulam na Colômbia.

A fim de identificar a frequência de GBV-C na Colômbia, foram analisadas: 1) 408 amostras de doadores de sangue positivos para anti-HCV (n=250) e positivos para HBsAg (n=158) provenientes de Bogotá e ii) 99 amostras provenientes de povos indígenas do estado do Amazonas. Um fragmento de 5'UTR do GBV-C foi amplificado e sequenciado para realizar a identificação dos genótipos circulantes na população **(Alvarado-Mora *et al.,***

2011i). Dessa forma, foi encontrado que das 158 amostras HBsAg positivas, 5,06% (n=8) foram positivas para GBV-C RNA e das 250 amostras anti-HCV positivas, 3,20% (n=8) foram positivas para GBV-C RNA. Entre as 99 amostras testadas provenientes de Letícia, 7,70% (n=7) foram positivas para GBV-C RNA. Sendo assim, as amostras positivas foram sequenciadas e 18/23 sequências foram utilizadas para determinar o genótipo encontrado: na população dos doadores o genótipo 2a (41.6%) foi o mais frequente, seguido do 1 (33.3%), 3 (16.6%) e o 2b (8.3%). Da mesma forma todas as amostras positivas de Letícia foram genótipo 3.

A presença do genótipo 3 como a mais frequente foi também relatado em países como Venezuela e Bolívia (Konomi *et al.,* 1999; Loureiro *et al.,* 2002). Dessa forma, os resultados obtidos podem se juntar a hipótese previamente estabelecida de que a presença deste genótipo, característico de populações asiáticas, em populações indígenas da América do Sul, sugere que esse genótipo seja antigo (Smith *et al.,* 1997), sendo provavelmente introduzido no nosso continente junto com as primeiras migrações humanas provenientes da Ásia através do Estreito de Bering (Gonzáles-Perez *et al.,* 1997; Tanaka *et al.,* 1998; Konomi *et al.,* 1999; Loureiro *et al.,* 2002). De qualquer forma, essa conclusão deve ser avaliada com mais detalhes quando houver um número maior de sequências disponíveis nos bancos de dados.

4.3 Referências

1. Alter HJ, Nakatsuji Y, Melpolder J, Wages J, Wesley R, Shih JWK, Kim JP. The incidence of transfusion associated hepatites G vírus infection and its relation to liver disease. *NEJM.* 1997; 336: 747-754.

2. **Alvarado-Mora MV, Botelho L, Nishiya A, Azevedo R, Gomes-Gouvea MS, Gutierrez MF, Carrilho F, Pinho JR**. Frequency and genotypic distribution of GB vírus C (GBV-C) among Colombian population with Hepatitis B (HBV) or Hepatitis C (HCV) infection. ***Virology J.*** **2011i; 8:345.**

3. Berzsenyi MD, Bowden DS, Bailey MJ, White C, Coghlan P, et al. Male to male sex is associated with a high prevalence of exposure to GB virus C. *J Clin Virol.* 2005; 33: 243-246.

4. Casteling A, Song E, Sim J, Blaauw D, Heyns A, et al. GB virus C prevalence in blood donors and high risk groups for parenterally transmitted agents from Gauteng, South Africa. *J Med Virol.* 1998; 55: 103-108.

5. Feucht HH, Zollner B, Polywka S, Laufs R. Vertical transmission of hepatitis G. *Lancet*. 1996; 347: 615-616.

6. Feucht HH, Fischer L, Sterneck M, Broelsch CE, Laufs R. GB virus C transmission by blood products. *Lancet.* 1997; 349: 435.

7. Frey SE, Homan SM, Sokol-Anderson M, Cayco MT, Cortorreal P, et al. Evidence for probable sexual transmission of the hepatitis g virus. *Clin Infect Dis*. 2002; 34: 1033-1038.

8. Gonzalez-Perez MA, Norder H, Bergstrom A, Lopez E, Visona KA, et al. High prevalence of GB virus C strains genetically related to strains with Asian origin in Nicaraguan hemophiliacs. *J Med Virol.* 1997; 52: 149-155.

9. Katayama K, Kageyama T, Fukushi S, Hoshino FB, Kurihara C, et al. Full-length GBV-C/HGV genomes from nine Japanese isolates: characterization by comparative analyses. *Arch Virol.* 1998; 143: 1063-1075.

10. Kao JH, Chen PJ, Hsiang SC, Chen W, Chen DS. Phylogenetic analysis of GB virus C: comparison of isolates from Africa, North America and Taiwan. *J Infect Dis.* 1996: 174:410-413.

11. Konomi N, Miyoshi C, La Fuente Zerain C, Li TC, Arakawa Y, et al. Epidemiology of hepatitis B, C, E, and G virus infections and molecular analysis of hepatitis G virus isolates in Bolivia. *J Clin Microbiol.* 1999; 37: 3291-3295.

12. Kudo T, Morishima T, Shibata M. Hepatitis G infection. *N Engl J Med* 1997; 337: 276-277

13. Jarvis LM, Davidson F, Hanley JP, Yap PL, Ludlam CA, Simmonds P. Infection with hepatitis G virus among recipients of plasma products. *Lancet.* 1996, 348:1352-1355.

14. Lampe E, de Oliveira JM, Pereira JL, Saback FL, Yoshida CF, Niel C. Hepatitis G virus (GBV-C) infection among Brazilian patients with chronic liver disease and blood donors. *Clin Diagn Virol.* 1998, 9:1-7.

15. Linnen J, JR J, Zhang-Keck ZY, Fry KE, Krawczynski KZ, et al. Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent. *Science.* 1996, 271: 505-508.

16. Loureiro CL, Alonso R, Pacheco BA, Uzcategui MG, Villegas L, et al. High prevalence of GB virus C/hepatitis G virus genotype 3 among autochthonous Venezuelan populations. *J Med Virol.* 2002; 68: 357-362.

17. Masuko K, Mitsui T, Iwano K, Yamazaki C, Okuda K, et al. Infection with hepatitis GB virus C in patients on maintenance hemodialysis. *NEJM.* 1996. 334; 1485-1490.

18. Moaven LD, Hyland CA, Young IF, Bowden DS, McCaw R, et al. Prevalence of hepatitis G virus in Queensland blood donors. *Med J Aust.* 1996; 165: 369-371.

19. Muerhoff AS, Simons JN, Leary TP, Erker JC, Chalmers ML, et al. Sequence heterogeneity within the 5'-terminal region of the hepatitis GB

virus C genome and evidence for genotypes. *J Hepatol.* 1996; 25: 379-384.

20. Muerhoff AS, Smith DB, Leary TP, Erker JC, Desai SM, et al. Identification of GB virus C variants by phylogenetic analysis of 5'-untranslated and coding region sequences. *J Virol.* 1997; 71: 6501-6508.

21. Muerhoff AS, Dawson GJ, Desai SM. A previously unrecognized sixth genotype of GB virus C revealed by analysis of 5'-untranslated region sequences. *J Med Virol.* 2006; 78: 105-111.

22. Mukaide M, Mizokami M, Orito E, Ohba K, Nakano T, et al. Three different GB virus C/hepatitis G virus genotypes. Phylogenetic analysis and a genotyping assay based on restriction fragment length polymorphism. *FEBS Lett.* 1997; 407: 51-58.

23. Naito H, Win KM, Abe K. Identification of a novel genotype of hepatitis G virus in Southeast Asia. *J Clin Microbiol.* 1999; 37: 1217-1220.

24. Naito H, Abe K. Genotyping system of GBV-C/HGV type 1 to type 4 by the polymerase chain reaction using type-specific primers and geographical distribution of viral genotypes. *J Virol Methods.* 2001; 91: 3-9.

25. Oubina JR, Mathet V, Feld M, Della Latta MP, Ferrario D, et al. Genetic diversity of GBV-C/HGV strains among HIV infected-IVDU and blood donors from Buenos Aires, Argentina. *Virus Res.* 1999; 65: 121-129.

26. Pessoa MG, Terrault NA, Detmer J, Kolberg J, Collins M, Hassoba HM, Wright TL. Quantitation of hepatitis G and C viruses in the liver: evidence that hepatitis G virus is not hepatotropic. *Hepatology* 1998; **27**: 877-880.

27. Pinho JR, Zanotto PM, Ferreira JL, Sumita LM, Carrilho FJ, et al. High prevalence of GB virus C in Brazil and molecular evidence for intrafamilial transmission. *J Clin Microbiol.* 1999; 37:1634-1637.

28. Ramos Filho R, Carneiro MA, Teles SA, Dias MA, Cardoso DD, et al. GB virus C/hepatitis G virus infection in dialysis patients and kidney transplant recipients in Central Brazil. *Mem Inst Osw Cruz.* 2004; 99: 639-643.

29. Stapleton JT, Williams CF, Xiang J. GB virus type C: a beneficial infection? *J Clin Microbiol.* 2004; 42: 3915-3919.

30. Stapleton JT, Foung S, Muerhoff AS, Bukh J, Simmonds P. The GB viruses: a review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus Pegivirus within the family *Flaviviridae*. *J Gen Virol.* 2011; 92: 233-246.

31. Seifried C, Weber M, Bialleck H, Seifried E, Schrezenmeier H, et al. High prevalence of GBV-C/HGV among relatives of GBV-C/HGV-positive blood donors in blood recipients and in patients with aplastic anemia. *Transfusion.* 2004; 44: 268-274.

32. Simons JN, Pilot-Matias TJ, Leary TP, Dawson GJ, Desai SM, et al. Identification of two flavivirus-like genomes in the GB hepatitis agent. *PNAS.* 1995; 92: 3401-3405.

33. Simons JN, Desai SM, Schultz DE, Lemon SM, Mushahwar IK. Translation initiation in GB virus A and C evidence for internal ribossome entry and implications for genome organization. *J Virol.* 1996; 70:6126-6135.

34. Smith DB, Cuceanu N, Davidson F, Jarvis LM, Mokili JL, et al. Discrimination of hepatitis G virus/GBV-C geographical variants by analysis of the 5' non-coding region. *J Gen Virol.* 1997; 78 (7): 1533-1542.

35. Souza IE, Allen JB, Xiang J, Klinzman D, Diaz R, Zhang S, Chaloner K, Zdunek D, Hess G, Williams CF, Benning L, Stapleton JT. Effect of primer selection on estimates of GB virus C (GBV-C) prevalence and response to antiretroviral therapy for optimal testing for GBV-C viremia. *J Clin Microbiol.* 2006; 44(9): 3105-13.

36. Stark K, Bienzle U, Hess G, Engel AM, Hegenscheid B, et al. Detection of hepatitis G virus genome among injection drug user, homosexual and bisexual men and blood donors. *J Infec Dis.* 1996: 174; 1320-1323.

37. Tanaka Y, Mizokami M, Orito E, Ohba K, Nakano T, et al. GB virus C/hepatitis G virus infection among Colombian native Indians. *Am J Trop Med Hyg.* 1998; 59: 462-467.

38. Thomas DL, Nakatsuji Y, Shih JW, Alter HJ, Nelson KE, et al. Persistence and clinical significance of hepatitis G virus infections in injecting drug users. *J Infect Dis*. 1997; 176: 586-592.

39. Tucker TJ, Smuts H, Eickhaus P, Robson SC, Kirsch RE. Molecular characterization of the 5' non-coding region of South African GBV-C/HGV isolates: major deletion and evidence for a fourth genotype. *J Med Virol.* 1999; 59: 52-59.

40. Yang JF, Dai CY, Chuang WL, Lin WY, Lin ZY, Chen SC, et al. Prevalence and clinical significance of HGV/GBV-C infection in patients with chronic hepatitis B or C. *Jpn J Infect Dis.* 2006; 59(1):25-30.

41. Yamada-Osaki M, Sumazaki R, Kajiwara Y, Miyakawa T, Shirahata A. Natural course of HGV infection in haemophiliacs. *B J Haem.* 1998; 102;616-621.

VIROLOGY JOURNAL

RESEARCH Open Access

Frequency and genotypic distribution of GB virus C (GBV-C) among Colombian population with Hepatitis B (HBV) or Hepatitis C (HCV) infection

Mónica V Alvarado-Mora[1*], Livia Botelho[1], Anna Nishiya[2], Raymundo A Neto[3], Michele S Gomes-Gouvêa[1], Maria F Gutierrez[4], Flair J Carrilho[1] and João RR Pinho[1]

Abstract

Background: GB virus C (GBV-C) is an enveloped positive-sense ssRNA virus belonging to the *Flaviviridae* family. Studies on the genetic variability of the GBV-C reveals the existence of six genotypes: genotype 1 predominates in West Africa, genotype 2 in Europe and America, genotype 3 in Asia, genotype 4 in Southwest Asia, genotype 5 in South Africa and genotype 6 in Indonesia. The aim of this study was to determine the frequency and genotypic distribution of GBV-C in the Colombian population.

Methods: Two groups were analyzed: i) 408 Colombian blood donors infected with HCV (n = 250) and HBV (n = 158) from Bogotá and ii) 99 indigenous people with HBV infection from Leticia, Amazonas. A fragment of 344 bp from the 5' untranslated region (5' UTR) was amplified by nested RT PCR. Viral sequences were genotyped by phylogenetic analysis using reference sequences from each genotype obtained from GenBank (n = 160). Bayesian phylogenetic analyses were conducted using Markov chain Monte Carlo (MCMC) approach to obtain the MCC tree using BEAST v.1.5.3.

Results: Among blood donors, from 158 HBsAg positive samples, eight 5.06% (n = 8) were positive for GBV-C and from 250 anti-HCV positive samples, 3.2%(n = 8) were positive for GBV-C. Also, 7.7% (n = 7) GBV-C positive samples were found among indigenous people from Leticia. A phylogenetic analysis revealed the presence of the following GBV-C genotypes among blood donors: 2a (41.6%), 1 (33.3%), 3 (16.6%) and 2b (8.3%). All genotype 1 sequences were found in co-infection with HBV and 4/5 sequences genotype 2a were found in co-infection with HCV. All sequences from indigenous people from Leticia were classified as genotype 3. The presence of GBV-C infection was not correlated with the sex (p = 0.43), age (p = 0.38) or origin (p = 0.17).

Conclusions: It was found a high frequency of GBV-C genotype 1 and 2 in blood donors. The presence of genotype 3 in indigenous population was previously reported from Santa Marta region in Colombia and in native people from Venezuela and Bolivia. This fact may be correlated to the ancient movements of Asian people to South America a long time ago.

Background

GB virus C/Hepatitis G virus (GBV-C/HGV) is an enveloped, positive-sense, single-strand RNA virus belonging to the family *Flaviviridae* with a genomic size of about 9.3 Kb [1]. It is genomic organization mainly consists of a large open reading frame (ORF) that encodes a single polyprotein precursor in which the structural (E1 and E2) and nonstructural proteins (NS2 to NS5B) are positioned at the N-terminal and C-terminal end, respectively [2]. It was first identified in 1995 in serum from individuals with idiopathic hepatitis [3]. Although it was initially identified as a possible etiological agent of viral hepatitis in humans, and despite its similarity in genome structure with hepatitis C virus (HCV), in contrast to HCV, GBV-C does not appear to be a hepatotrophic virus neither

* Correspondence: monica.viviana@usp.br
[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
Full list of author information is available at the end of the article

replicates in hepatocytes nor causes acute or chronic hepatitis [4-6].

GBV-C can be transmitted parenterally through blood and derivates transfusion, intravenous drug use, hemodialysis and vertical transmission [7-10]. There is extensive evidence that GBV-C is transmitted by sexual and percutaneous routes and is frequently found in populations at risk for blood-borne or sexually transmitted viruses [5,11]. Male to male sex has been reported as an effective way of transmission [12] and intrafamilial transmission has been determined based on the sequences analysis [13,14]. GBV-C has not been associated with any particular disease despite numerous investigations. Alteration in the host's cellular immune response to HIV seems to be responsible for a protective effect of GBV-C but the exact mechanism to it still have to be defined. In contrast, GBV-C infection does not appear to have any effect on chronic liver disease due to HCV or HBV [12].

GBV-C infection is relatively common and has a worldwide distribution. At least 1 to 4% of healthy blood donors have GBV-C RNA [1,5,7]. Most people clear the virus and develop antibodies to the E2 envelope glycoprotein. Furthermore, infection is common in the normal population with up to 12.9% prevalence among paid blood donors in the United States of America [15], 11% to 14% in West Africa or South Africa [8], and as high as 37% among HIV-infected individuals [16]. GBV-C incidence amongst of patients with HCV infection varies from 11 to 24% [17-19]. Further, little work has been done on coinfection of GBV-C and HBV but no significant effect of this co-infection was reported [12].

The geographical distribution of GBV-C is related to the coevolution of the viruses with human during the migration along the history, suggesting that GBV-C is an ancient virus [20,21]. Genotype 1 is found in West Africa [22], genotype 2 (sub-classified as both 2a and 2b) in the United States and Europe [23], genotype 3 in Asia [24-26], genotype 4 in Myanmar and Vietnam [27], genotype 5 in South Africa [28] and genotype 6 in Indonesia [29]. In South America, the genotypes 1, 2a, 2b and 3 have been reported [30-34]. In Colombia, a high prevalence of GBV-C RNA and presence of genotype 3 were found among Colombian native Indians from Wayuu, Kamsa and Inga ethic groups [34].

The aim of this study was determined the frequency of GBV-C RNA and the GBV-C genotypes circulating in Colombia. This is the first study that characterizes the presence of GBV-C among blood donors in coinfection with HCV and HBV in Colombia.

Materials and methods

Study Population

In order to identified the frequency of GBV-C in Colombia, i) 408 samples from Colombian blood donors positive for anti-HCV (n = 250) and positive for HBsAg (n = 158) using third generation ELISA in the blood bank of Cruz Roja Colombiana in Bogota city, Colombia, and ii) 99 samples from indigenous people with HBV infection from Leticia, Amazonas were obtained for this study. These samples were collected between 2003 and 2007 and the presence of HCV RNA and HBV DNA were previously reported by our group [35,36].

The protocol of this study was approved from Ethical Committees from the Pontificia Universidad Javeriana, Bogotá, Colombia and University of São Paulo Medical School, São Paulo, Brazil. All patients have signed an informed consent before joining the study.

GBV-C RNA Extraction

To avoid false-positive results, rigorous procedures used for nucleic acid amplification diagnostic techniques were followed [37]. HCV-RNA extraction was carried out from 140 ml of serum using QIAamp Viral RNA Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. The synthesis of the complementary DNA (cDNA) was carried out immediately after RNA extraction.

Synthesis of the complementary DNA (cDNA)

Reverse transcriptase reaction was performed using the Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) and random primers. The final volume of the reaction was 60 ml in the following concentrations: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 450 ng random primers, 30 U RNAse enzyme inhibitor (RNaseOUT™), and 300 U MMLV-RT. Samples were submitted to the following temperature cycles: 70°C for 10 min, 25°C for 15 min, 37°C for 60 min, and 95°C for 15 min in a thermocycler (Eppendorf Mastercycler 1, Eppendorf, Hamburg, Germany).

Polymerase chain reaction (PCR)

A fragment of 344 bp from 5' untranslated region (5'UTR) was amplified by nested RT PCR [28,38]. Amplification consisted of 40 cycles for first and second round of PCR, with the following incubation times and temperatures: 94°C 30 s, 50°C 30 s and 72°C 30s for the first round and 94°C 30s, 60°C 30 s and 72°C 30s for the second round.

GBV-C Sequencing

Amplified cDNA was purified using the ChargeSwitch PCR Clean-Up Kit. Sequencing was done in an ABI Prism 3500 Automatic Sequencer (Applied Biosystems, Foster City, CA) using dideoxy nucleoside triphosphates (ddNTPs) containing fluorescent markers (Big Dye1

Terminator v3.1 Cycle Sequencing Ready Reaction Kit-Applied Biosystems).

The consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using the SEQUENCHER software (Gene Codes Corporation Ann Arbor, Michigan, United States of America).

Phylogenetic Analysis

The sequences obtained in this work were genotyped by phylogenetic reconstructions using reference sequences from each GBV-C genotype obtained from GenBank (n = 160). Sequences were aligned and edited using Clustal X [39] and Se-AL (available at: http://tree.bio.ed.ac.uk/software/seal/) softwares respectively. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3 [40]. The dataset was analyzed under relaxed uncorrelated lognormal and relaxed uncorrelated exponential molecular clock using the best model of nucleotide substitution (GTR+G+I) chosen by MODELTEST [41] and 20 million generations were sufficient to obtain the convergence of parameters. The molecular clock that best fitted the data was chosen by Bayes factor (BF) comparison. The maximum clade credibility (MCC) tree was obtained from summarizing the 20,000 substitution trees after excluding 10% of burn-in using Tree Annotator v.1.5.3 [40]. Phylogenetic trees were visualized in FigTree v.1.2.2 (available at: http://tree.bio.ed.ac.uk/software/figtree/).

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 variations in the presence of GBV-C RNA adjusted for age group, sex and co-infection group (Anti-HCV or HBsAg).

Results

Detection of GBV-C RNA

Among Colombian blood donors, from 158 HBsAg positive samples, 5.06% (n = 8) were positive for GBV-C RNA and from 250 anti-HCV positive samples, 3.2% (n = 8) were positive for GBV-C. Among 99 indigenous people from Leticia (n = 99), 7.7% (n = 7) GBV-C positive samples were found (Table 1).

Table 1 Geographical origins and epidemiological data from 23 Colombian GBV-C infected patients

Patient	Origin	Clinical Status	Sex	Age	HBV	HCV	Genotype
1035	Bogotá	Blood donor	F	26	Positive	Negative	1
1087	Bogotá	Blood donor	F	28	Positive	Negative	1
1123	Bogotá	Blood donor	M	50	Positive	Negative	2a
1183	Bogotá	Blood donor	F	30	Positive	Negative	3
1196	Bogotá	Blood donor	F	30	Positive	Negative	1
1206	Bogotá	Blood donor	F	22	Positive	Negative	1
1075	Bogotá	Blood donor	M	41	Positive	Negative	No sequence
1174	Bogotá	Blood donor	F	23	Positive	Negative	No sequence
2002	Bogotá	Blood donor	F	30	Negative	Positive	2a
2117	Bogotá	Blood donor	M	63	Negative	Positive	2a
2136	Bogotá	Blood donor	M	63	Negative	Positive	2a
2238	Bogotá	Blood donor	M	59	Negative	Positive	2b
2221	Bogotá	Blood donor	F	21	Negative	Positive	2a
2033	Bogotá	Blood donor	M	37	Negative	Positive	3
2244	Bogotá	Blood donor	F	33	Negative	Positive	No sequence
2259	Bogotá	Blood donor	M	23	Negative	Positive	No sequence
02	Leticia	Native Ticuna	F	67	Positive	Negative	3
95	Leticia	Native Ticuna	F	54	Positive	Negative	No sequence
98	Leticia	Native Yaua	M	51	Positive	Positive	3
113	Leticia	Native Yaua	F	23	Positive	Negative	3
120	Leticia	Native Ticuna	M	43	Positive	Negative	3
148	Leticia	Native Ticuna	F	23	Positive	Negative	3
174	Leticia	Native Ticuna	F	26	Positive	Negative	3

Phylogenetic Analysis

Eighteen out of 23 sequences with good quality were used for phylogenetic analysis. The phylogenetic tree constructed with the GBV-C sequences (n = 160) is shown in Figure 1. This analysis revealed the presence of the following GBV-C genotypes among blood donors: 2a (41.6%), 1 (33.3%), 3 (16.6%) and 2b (8.3%). All genotype 1 sequences were found in co-infection with HBV and 4/5 sequences genotype 2a were found in co-infection with HCV. All sequences from indigenous people from Leticia were classified as genotype 3. The presence of GBV-C infection among blood donors group and indigenous people was not correlated with sex ($p = 0.43$), age ($p = 0.38$) or origin of the samples ($p = 0.17$). The Colombian GBV-C sequences were deposited in the

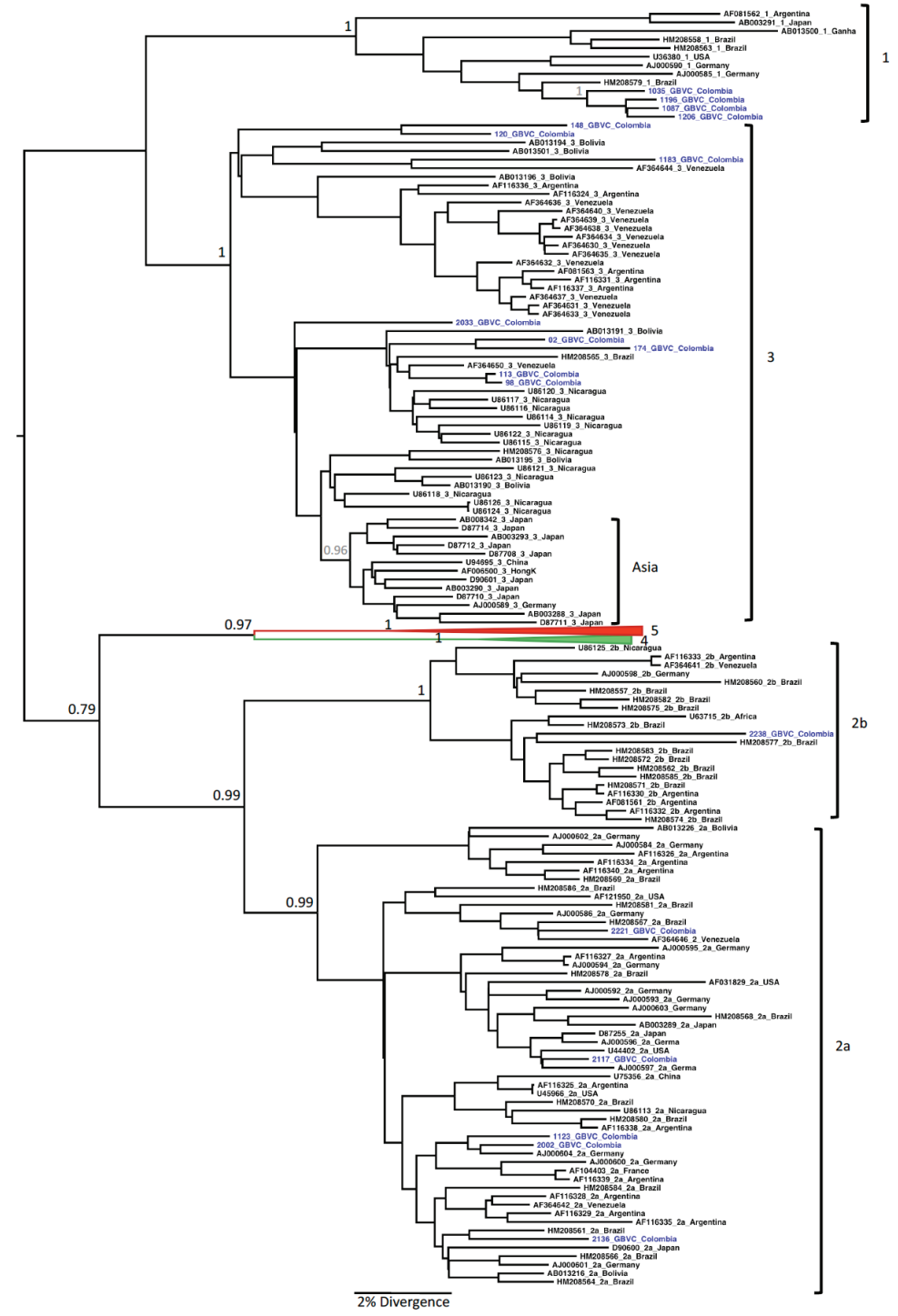


Figure 1 The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 160 sequences with 344 bp of GB virus C strains. The posterior probabilities of the key nodes are depicted above the respective nodes. Samples obtained from Colombian blood donors (n = 12) and Amerindian population (n = 6) were analyzed together with other worldwide strains. Genotype 4 and genotype 5 branches were collapsed.

GenBank database under accession numbers JF832366 to JF832383.

Discussion

In this study, we determined the frequency and genotypic characteristics of GBV-C virus in Colombia. Among Colombian blood donors, from 158 HBsAg positive samples, eight (5.06%) were positive for GBV-C and from 250 anti-HCV positive samples, eight (3.2%) were positive for GBV-C. This is the first report showing the frequency of GBV-C in HBV and HCV positive blood donors in Colombia.

There are few studies that reported GBV-C frequency in blood donors populations. In Salvador and in Rio de Janeiro, Brazil it was reported 10% of frequency of GBV-C among blood donors [42,43]. Also, in São Paulo, Brazil it was reported a high prevalence among blood donors with normal and elevated ALT levels: 5.2% (5/95) and 6.5% (5/76), respectively [14]. Furthermore, the prevalence of GBV-C was 9.7% among 545 blood donors in São Paulo [44] and 8.3% in 1.039 healthy individuals [45].

In Iranian volunteer blood donors the prevalence of GBV-C was around 1% [46]. A study of prevalence of GBV-C among northeastern Thai blood donors carrying HBsAg and anti-HCV revealed a higher frequency of GBV-C RNA (10% and 11%, respectively) in the co-infected when compared with the controls [47]. In United States, GBV-C prevalence in blood donors was reported ranging from 0.8% to 12.9% [48,49]. In Turkey the prevalence of GBV-C was 14% in hemodialysis patients and 5% in blood donors [50]. Also, in Thailand the GBV-C RNA positivity among blood donors was 4.8% [51]. In France, among 306 HCV RNA-positive donors, 19.3% were GBV-C RNA positive [52]. In Egypt, El-Zayadi et al., [53] found 12.2% of GBV-C prevalence among blood donors and GBV-C coinfection in HBV and HCV infected patients were 7.6 and 64.9%, respectively. GBV-C infection is generally more common in groups with risk factors for percutaneous and sexual transmission of infectious agents [54].

The presence of GBV-C infection in our study was not correlated with sex ($p = 0.43$), age ($p = 0.38$) or origin ($p = 0.17$). These results are correlated with the previous study performed among three different Indian groups from Colombia (Wayuu, Inga and Kamsa) where no significant differences were found [34].

A phylogenetic analysis revealed the presence of GBV-C genotypes 2a, 1, 3 and 2b among Colombian blood donors. There are few studies that reported GBV-C genotypes among blood donor populations in the world. In Bolivia, among blood donors, it was found that the major genotype was genotype 3 followed by genotype 2 [32]. In Shanghai, China, genotype 3 was the most prevalent [55]. In Martinique, Césaire et al., [56], reported genotypes 2a, 1 and 2b among blood donors.

Furthermore, all genotype 1 sequences from Colombian blood donors were found in co-infection with HBV and 4/5 sequences from blood donors genotype 2a were found in co-infection with HCV, it was not found a significant association between the presence of HBV or HCV co-infection and GBV-C genotype ($p = 0.431$). These results are similar to other studies where no significant differences of HBV, HCV and GBV-C infection rates were found [57].

Phylogenetic analysis showed that genotype 3 is the most common in Leticia, Amazon region of Colombia. The finding of an Asian GBV-C genotype in the Americas was first suggested by the analysis of 5'UTR hemophiliac patients from nine locations in Nicaragua [58], Colombian Amerindians [34] and Bolivia [32]. Similarity between Nicaraguan and Asian GBV-C genotype 3 strains indicates that these strains in the region presumably have an Amerindian origin [58].

In Colombia, from 163 native Indians, 6.1% ($n = 10$) were positive for GBV-C RNA and it was concluded that the incidence of GBV-C infection in native Indians tended to be high compared with the general population [34]. Furthermore, most Colombian native Indians harbored an Asian GBV-C genotype.

In an Amerindian population from Venezuela, a high prevalence of GBV-C genotype 3 was observed, ranging from 5% (9 out of 162) in the West to 25% (14 out of 56) in the south region of the country [33]. Whereas GBV-C genotypes 1, 2 and 3 were presented in Venezuela, genotype 3 (Asian genotype) was found infecting Amerindians and rural population [33].

Together with other studies, our results corroborate the hypothesis that GBV-C is an old virus [21], having been probably introduced in the America continent with the fist men coming thought the Bering Strait [32-34,58]. It is generally believed that Colombian native Indians migrated from Asia to Colombia approximately 12,000 years ago and were isolated from other people for religious reasons [59]. On the other hand, since HTLV-1 or HTLV-2 may have been brought from Asia to Colombia together with the first human migrants [60], GBV-C/HGV apparently followed the same route [34]. These results are correlated with the evolutionary analysis of GBV-C performed by Suzuki et al., [61], that assumed that this virus diverged 100.000 years ago. However, further analysis needs to be performer to test this hypothesis. Actually, more sequences of GBV-C genotype 3 need to be reported with sampling date, allowing to estimate a specific substitution rate for this genotype that is needed for a more detailed phylogeographic analysis.

Conclusions

In conclusion, this is the first study that reported the GBV-C frequency and the distribution of genotypes among Colombian blood donors. The result presented indicated the circulation of the genotype 3 among Amerindian population in Colombia and blood donors.

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.

Author details

[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]São Paulo Blood Bank, São Paulo, Brazil. [3]Department of Pathology, School of Medicine, University of São Paulo, São Paulo, Brazil. [4]Laboratory of Virology, Department of Microbiology, Pontificia Javeriana University, Bogotá, Colombia.

Authors' contributions

MVAM conducted the phylogenetic and evolutionary analysis, drafted the manuscript and participated in its design and coordination. LB, AN and MSGG participated in the PCR amplification and sequencing process. RAN conducts the statistical analysis. FJC and MFG participated in the design of the study. JRRP participated in the elaboration of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 23 May 2011 Accepted: 11 July 2011 Published: 11 July 2011

References

1. Stapleton JT, Foung S, Muerhoff AS, Bukh J, Simmonds P: **The GB viruses: a review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus Pegivirus within the family Flaviviridae.** *J Gen Virol* 2011, **92**:233-246.
2. Leary TP, Muerhoff AS, Simons JN, Pilot-Matias TJ, Erker JC, Chalmers ML, Schlauder GG, Dawson GJ, Desai SM, Mushahwar IK: **Sequence and genomic organization of GBV-C: a novel member of the flaviviridae associated with human non-A-E hepatitis.** *J Med Virol* 1996, **48**:60-67.
3. Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK: **Isolation of novel virus-like sequences associated with human hepatitis.** *Nat Med* 1995, **1**:564-569.
4. Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J: **Lack of evidence for hepatitis G virus replication in the livers of patients coinfected with hepatitis C and G viruses.** *J Virol* 1997, **71**:7804-7806.
5. Alter HJ: **G-pers creepers, where'd you get those papers? A reassessment of the literature on the hepatitis G virus.** *Transfusion* 1997, **37**:569-572.
6. Zhu WF, Yin LM, Li P, Huang J, Zhuang H: **Pathogenicity of GB virus C on virus hepatitis and hemodialysis patients.** *World J Gastroenterol* 2003, **9**:1739-1742.
7. Moaven LD, Hyland CA, Young IF, Bowden DS, McCaw R, Mison L, Locarnini SA: **Prevalence of hepatitis G virus in Queensland blood donors.** *Med J Aust* 1996, **165**:369-371.
8. Casteling A, Song E, Sim J, Blaauw D, Heyns A, Schweizer R, Margolius L, Kuun E, Field S, Schoub B, Vardas E: **GB virus C prevalence in blood donors and high risk groups for parenterally transmitted agents from Gauteng, South Africa.** *J Med Virol* 1998, **55**:103-108.
9. Thomas DL, Nakatsuji Y, Shih JW, Alter HJ, Nelson KE, Astemborski JA, Lyles CM, Vlahov D: **Persistence and clinical significance of hepatitis G virus infections in injecting drug users.** *J Infect Dis* 1997, **176**:586-592.
10. Feucht HH, Zollner B, Polywka S, Laufs R: **Vertical transmission of hepatitis G.** *Lancet* 1996, **347**:615-616.
11. Bjorkman P, Naucler A, Winqvist N, Mushahwar I, Widell A: **A case-control study of transmission routes for GB virus C/hepatitis G virus in Swedish blood donors lacking markers for hepatitis C virus infection.** *Vox Sang* 2001, **81**:148-153.
12. Berzsenyi MD, Bowden DS, Roberts SK: **GB virus C: insights into co-infection.** *J Clin Virol* 2005, **33**:257-266.
13. Seifried C, Weber M, Bialleck H, Seifried E, Schrezenmeier H, Roth WK: **High prevalence of GBV-C/HGV among relatives of GBV-C/HGV-positive blood donors in blood recipients and in patients with aplastic anemia.** *Transfusion* 2004, **44**:268-274.
14. Pinho JR, Zanotto PM, Ferreira JL, Sumita LM, Carrilho FJ, da Silva LC, Capacci ML, Silva AO, Guz B, Goncales FL Jr, Goncales N, Buck G, Meyers G, Bernardini P: **High prevalence of GB virus C in Brazil and molecular evidence for intrafamilial transmission.** *J Clin Microbiol* 1999, **37**:1634-1637.
15. Dawson GJ, Schlauder GG, Pilot-Matias TJ, Thiele D, Leary TP, Murphy P, Rosenblatt JE, Simons JN, Martinson FE, Gutierrez RA, Lentino JR, Pachucki C, Muerhoff AS, Widell A, Tegtmeier G, Desai S, Mushahwar IK: **Prevalence studies of GB virus-C infection using reverse transcriptase-polymerase chain reaction.** *J Med Virol* 1996, **50**:97-103.
16. Tillmann HL, Manns MP: **GB virus-C infection in patients infected with the human immunodeficiency virus.** *Antiviral Res* 2001, **52**:83-90.
17. Di Bisceglie AM: **Hepatitis G virus infection: a work in progress.** *Ann Intern Med* 1996, **125**:772-773.
18. Tanaka E, Alter HJ, Nakatsuji Y, Shih JW, Kim JP, Matsumoto A, Kobayashi M, Kiyosawa K: **Effect of hepatitis G virus infection on chronic hepatitis C.** *Ann Intern Med* 1996, **125**:740-743.
19. Feucht HH, Zollner B, Polywka S, Knodler B, Schroter M, Nolte H, Laufs R: **Prevalence of hepatitis G viremia among healthy subjects, individuals with liver disease, and persons at risk for parenteral transmission.** *J Clin Microbiol* 1997, **35**:767-768.
20. Naito H, Abe K: **Genotyping system of GBV-C/HGV type 1 to type 4 by the polymerase chain reaction using type-specific primers and geographical distribution of viral genotypes.** *J Virol Methods* 2001, **91**:3-9.
21. Smith DB, Cuceanu N, Davidson F, Jarvis LM, Mokili JL, Hamid S, Ludlam CA, Simmonds P: **Discrimination of hepatitis G virus/GBV-C geographical variants by analysis of the 5' non-coding region.** *J Gen Virol* 1997, **78(Pt 7)**:1533-1542.
22. Muerhoff AS, Simons JN, Leary TP, Erker JC, Chalmers ML, Pilot-Matias TJ, Dawson GJ, Desai SM, Mushahwar IK: **Sequence heterogeneity within the 5'-terminal region of the hepatitis GB virus C genome and evidence for genotypes.** *J Hepatol* 1996, **25**:379-384.
23. Muerhoff AS, Smith DB, Leary TP, Erker JC, Desai SM, Mushahwar IK: **Identification of GB virus C variants by phylogenetic analysis of 5'-untranslated and coding region sequences.** *J Virol* 1997, **71**:6501-6508.
24. Okamoto H, Nakao H, Inoue T, Fukuda M, Kishimoto J, Iizuka H, Tsuda F, Miyakawa Y, Mayumi M: **The entire nucleotide sequences of two GB virus C/hepatitis G virus isolates of distinct genotypes from Japan.** *J Gen Virol* 1997, **78(Pt 4)**:737-745.
25. Mukaide M, Mizokami M, Orito E, Ohba K, Nakano T, Ueda R, Hikiji K, Iino S, Shapiro S, Lahat N, Park YM, Kim BS, Oyunsuren T, Rezieg M, Al-Ahdal MN, Lau JY: **Three different GB virus C/hepatitis G virus genotypes. Phylogenetic analysis and a genotyping assay based on restriction fragment length polymorphism.** *FEBS Lett* 1997, **407**:51-58.
26. Katayama K, Kageyama T, Fukushi S, Hoshino FB, Kurihara C, Ishiyama N, Okamura H, Oya A: **Full-length GBV-C/HGV genomes from nine Japanese isolates: characterization by comparative analyses.** *Arch Virol* 1998, **143**:1063-1075.
27. Naito H, Win KM, Abe K: **Identification of a novel genotype of hepatitis G virus in Southeast Asia.** *J Clin Microbiol* 1999, **37**:1217-1220.
28. Tucker TJ, Smuts H, Eickhaus P, Robson SC, Kirsch RE: **Molecular characterization of the 5' non-coding region of South African GBV-C/HGV isolates: major deletion and evidence for a fourth genotype.** *J Med Virol* 1999, **59**:52-59.
29. Muerhoff AS, Dawson GJ, Desai SM: **A previously unrecognized sixth genotype of GB virus C revealed by analysis of 5'-untranslated region sequences.** *J Med Virol* 2006, **78**:105-111.
30. Oubina JR, Mathet V, Feld M, Della Latta MP, Ferrario D, Verdun R, Libonatti O, Fernandez J, Carballal G, Sanchez DO, Quarleri JF: **Genetic diversity of GBV-C/HGV strains among HIV infected-IVDU and blood donors from Buenos Aires, Argentina.** *Virus Res* 1999, **65**:121-129.

31. Ramos Filho R, Carneiro MA, Teles SA, Dias MA, Cardoso DD, Lampe E, Yoshida CF, Martins RM: **GB virus C/hepatitis G virus infection in dialysis patients and kidney transplant recipients in Central Brazil.** *Mem Inst Oswaldo Cruz* 2004, **99**:639-643.
32. Konomi N, Miyoshi C, La Fuente Zerain C, Li TC, Arakawa Y, Abe K: **Epidemiology of hepatitis B, C, E, and G virus infections and molecular analysis of hepatitis G virus isolates in Bolivia.** *J Clin Microbiol* 1999, **37**:3291-3295.
33. Loureiro CL, Alonso R, Pacheco BA, Uzcategui MG, Villegas L, Leon G, De Saez A, Liprandi F, Lopez JL, Pujol FH: **High prevalence of GB virus C/hepatitis G virus genotype 3 among autochthonous Venezuelan populations.** *J Med Virol* 2002, **68**:357-362.
34. Tanaka Y, Mizokami M, Orito E, Ohba K, Nakano T, Kato T, Kondo Y, Ding X, Ueda R, Sonoda S, Tajima K, Miura T, Hayami M: **GB virus C/hepatitis G virus infection among Colombian native Indians.** *Am J Trop Med Hyg* 1998, **59**:462-467.
35. Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Carrilho FJ, Pinho JR: **Molecular characterization, distribution, and dynamics of hepatitis C virus genotypes in blood donors in Colombia.** *J Med Virol* 2010, **82**:1889-1898.
36. Alvarado Mora MV, Romano CM, Gomes-Gouvea MS, Gutierrez MF, Botelho L, Carrilho 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.
37. Kwok S, Higuchi R: **Avoiding false positives with PCR.** *Nature* 1989, **339**:237-238.
38. Jarvis LM, Davidson F, Hanley JP, Yap PL, Ludlam CA, Simmonds P: **Infection with hepatitis G virus among recipients of plasma products.** *Lancet* 1996, **348**:1352-1355.
39. 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.
40. Drummond AJ, Rambaut A: **BEAST: Bayesian evolutionary analysis by sampling trees.** *BMC Evol Biol* 2007, **7**:214.
41. Posada D, Crandall KA: **MODELTEST: testing the model of DNA substitution.** *Bioinformatics* 1998, **14**:817-818.
42. Lyra AC, Pinho JR, Silva LK, Sousa L, Saraceni CP, Braga EL, Pereira JE, Zarife MA, Reis MG, Lyra LG, Silva LC, Carrilho FJ: **HEV, TTV and GBV-C/HGV markers in patients with acute viral hepatitis.** *Braz J Med Biol Res* 2005, **38**:767-775.
43. Lampe E, de Oliveira JM, Pereira JL, Saback FL, Yoshida CF, Niel C: **Hepatitis G virus (GBV-C) infection among Brazilian patients with chronic liver disease and blood donors.** *Clin Diagn Virol* 1998, **9**:1-7.
44. Levi JE, Contri DG, Lima LP, Takaoka DT, Garrini RH, Santos W, Fachini R, Wendel S: **High prevalence of GB virus C/hepatitis G virus RNA among Brazilian blood donors.** *Rev Inst Med Trop Sao Paulo* 2003, **45**:75-78.
45. Ribeiro-dos-Santos G, Nishiya AS, Nascimento CM, Bassit L, Chamone DF, Focaccia R, Eluf-Neto J, Sabino EC: **Prevalence of GB virus C (hepatitis G virus) and risk factors for infection in Sao Paulo, Brazil.** *Eur J Clin Microbiol Infect Dis* 2002, **21**:438-443.
46. Ramezani A, Gachkar L, Eslamifar A, Khoshbaten M, Jalilvand S, Adibi L, Salimi V, Hamkar R: **Detection of hepatitis G virus envelope protein E2 antibody in blood donors.** *Int J Infect Dis* 2008, **12**:57-61.
47. Barusruk S, Urwijitaroon Y: **High prevalence of HGV coinfection with HBV or HCV among northeastern Thai blood donors.** *Southeast Asian J Trop Med Public Health* 2006, **37**:289-293.
48. Dawson GJ, Schlauder GG, Pilot-Matias TJ, Thiele D, Leary TP, Murphy P, Rosenblatt JE, Simons JN, Martinson FE, Gutierrez RA, Lentina JR, Pachucki C, Muerhoff AS, Widell A, Tegtmeier G, Desai S, Mushahwar IK: **Prevalence studies of GB virus-C infection using reverse transcriptase-polymerase chain reaction.** *J Med Virol* 1996, **50**:97-103.
49. Linnen J, Wages J Jr, Zhang-Keck ZY, Fry KE, Krawczynski KZ, Alter H, Koonin E, Gallagher M, Alter M, Hadziyannis S, Karayiannis P, Fung K, Nakatsuji Y, Shih WK, Young L, Piatak M Jr, Hoover C, Fernandez J, Chen S, Chao-Zou JC, Morris T, Hyams K, Ismay S, Lifson J, Hess G, Foung S, Thomas H, Bradley D, Margolis H, Kim J: **Molecular cloning and disease association of hepatitis G virus: a transfusion-transmissible agent.** *Science* 1996, **271**:505-508.
50. Hanci SY, Cevahir N, Kaleli I, Hanci V: **Investigation of hepatitis G virus prevalence in hemodialysis patients and blood donors in Denizli, Turkey.** *Mikrobiyol Bul* 2008, **42**:617-625.
51. Wiwanitkit V: **Hepatitis G, hepatitis SEN, hepatitis TT and hepatitis TT-like viruses: New emerging hepatitis viruses in pediatric patients.** *J Ped Infec Dis* 2006, **1**:83-88.
52. Bouchardeau F, Laperche S, Pillonel J, Elghouzzi MH, Maisonneuve P, Tirtaine C, Boiret E, Razer A, Girault A, Beaulieu MJ, Courouce AM: **GB virus type C/HGV markers in HCV RNA-positive French blood donors: correlation with HCV genotypes and risk factors.** *Transfusion* 2000, **40**:875-878.
53. El-Zayadi AR, Abe K, Selim O, Naito H, Hess G, Ahdy A: **Prevalence of GBV-C/hepatitis G virus viraemia among blood donors, health care personnel, chronic non-B non-C hepatitis, chronic hepatitis C and hemodialysis patients in Egypt.** *J Virol Methods* 1999, **80**:53-58.
54. Giret MT, Miraglia JL, Sucupira MC, Nishiya A, Levi JE, Diaz RS, Sabino EC, Kallas EG: **Prevalence, Incidence Density, and Genotype Distribution of GB Virus C Infection in a Cohort of Recently HIV-1-Infected Subjects in Sao Paulo, Brazil.** *PLoS One* 2011, **6**:e18407.
55. Ding X, Mizokami M, Kang LY, Cao K, Orito E, Tanaka Y, Ueda R, Sasaki M: **Prevalence of TT virus and GBV-C infections among patients with liver diseases and the general population in Shanghai, China.** *Virus Genes* 1999, **19**:51-58.
56. Cesaire R, Martial J, Maier H, Kerob-Bauchet B, Bera O, Duchaud E, Brebion A, Pierre-Louis S: **Infection with GB virus C/hepatitis G virus among blood donors and hemophiliacs in Martinique, a Caribbean island.** *J Med Virol* 1999, **59**:160-163.
57. Ling BH, Zhuang H, Cui YH, An WF, Li ZJ, Wang SP, Zhu WF: **A cross-sectional study on HGV infection in a rural population.** *World J Gastroenterol* 1998, **4**:489-492.
58. Gonzalez-Perez MA, Norder H, Bergstrom A, Lopez E, Visona KA, Magnius LO: **High prevalence of GB virus C strains genetically related to strains with Asian origin in Nicaraguan hemophiliacs.** *J Med Virol* 1997, **52**:149-155.
59. Cavalli-Sforza LL: **Genes, people and languages.** *Sci Am* 1991, **265**:72-78.
60. Zaninovic V, Sanzon F, Lopez F, Velandia G, Blank A, Blank M, Fujiyama C, Yashiki S, Matsumoto D, Katahira Y: **Geographic independence of HTLV-I and HTLV-II foci in the Andes highland, the Atlantic coast, and the Orinoco of Colombia.** *AIDS Res Hum Retroviruses* 1994, **10**:97-101.
61. Suzuki Y, Katayama K, Fukushi S, Kageyama T, Oya A, Okamura H, Tanaka Y, Mizokami M, Gojobori T: **Slow evolutionary rate of GB virus C/hepatitis G virus.** *J Mol Evol* 1999, **48**:383-389.

doi:10.1186/1743-422X-8-345
Cite this article as: Alvarado-Mora *et al.*: Frequency and genotypic distribution of GB virus C (GBV-C) among Colombian population with Hepatitis B (HBV) or Hepatitis C (HCV) infection. *Virology Journal* 2011 **8**:345.

MEDICINA
USP

Of.0165/11-CPGE

São Paulo, 2 de junho de 2011

Senhor Coordenador,

Com referência à solicitação da aluna **Monica Viviana Alvarado Mora**, de apresentação de tese na forma de compilação de artigos, consultamos a Pró-Reitoria de Pós-graduação sobre o assunto, que nos informou não haver, a princípio, qualquer objeção, lembrando que a interessada deve apresentar o trabalho numa sequência lógica, que demonstre cabalmente a sua contribuição original ao estado da arte do tema tratado.

Aluísio Augusto Cotrim Segurado
Presidente da Comissão de Pós-Graduação

Ilmo. Sr.
Prof. Dr. FLAIR JOSÉ CARRILHO
Coordenador do Programa de Pós-graduação em
Gastroenterologia Clínica

Comissão de Pós-Graduação
Rua Teodoro Sampaio, 115, – 1° andar, Prédio do Instituto Oscar Freire, CEP 05405 - 000 – São Paulo
Fones: (11)3061-8203/8204/8205/8209/8214 – Fax: 3061.8215