MARINA VON ATZINGEN DOS REIS

**“Expressão, purificação e caracterização
de proteínas de superfície de
Leptospira interrogans”.**

Tese apresentada ao Programa de Pós-
Graduação Interunidades em
Biotecnologia (USP / Instituto Butantan /
IPT), para obtenção do título de Doutor
em Biotecnologia.

São Paulo
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Área de concentração: Biotecnologia

Orientadora: Dra. Ana Lúcia Tabet Oller do Nascimento

São Paulo
2009

DADOS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
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Atzingen, Marina von.

Expressão, purificação e caracterização de proteínas de superfície de *Leptospira interrogans* / Marina von Atzingen. -- São Paulo, 2009.

Orientador: Ana Lúcia Tabet Oller do Nascimento.

Tese (Doutorado) – Universidade de São Paulo. Instituto de Ciências Biomédicas. Programa de Pós-Graduação Interunidades em Biotecnologia USP/IPT/Instituto Butantan. Área de concentração: Biotecnologia. Linha de pesquisa: Desenvolvimento de vacinas.

Versão do título para o inglês: Expression, purification and characterization of surface proteins from *Leptospira interrogans*.

Descritores: 1. *Leptospira* 2. Genoma 3. Proteínas recombinantes 4. Vacinas I. Nascimento, Ana Lúcia Tabet Oller do II. Universidade de São Paulo. Instituto de Ciências Biomédicas. Programa de Pós-Graduação em Biotecnologia. III. Título.

ICB/SBIB13/2009

UNIVERSIDADE DE SÃO PAULO
Programa de Pós-Graduação Interunidades em Biotecnologia
Universidade de São Paulo, Instituto Butantan, Instituto de Pesquisas Tecnológicas

Candidato(a): Marina von Atzingen.

Título da Tese: Expressão, purificação e caracterização de proteínas de superfície de *Leptospira interrogans*.

Orientador(a): Ana Lúcia Tabet Oller do Nascimento.

A Comissão Julgadora dos trabalhos de Defesa da Tese de Doutorado, em sessão pública realizada a/...../....., considerou

() Aprovado(a)

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CERTIFICADO

Certificamos que o Protocolo para uso de animais em experimentação nº 286/06, sobre o projeto intitulado "Obtenção de soros hiperimunes contra proteínas recombinantes de *Leptospira interrogans* em camundongos e hamsters", sob a responsabilidade de Patrícia Antonia Estima Abreu e Ana Lúcia Tabet Oller do Nascimento está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro (COBEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO INSTITUTO BUTANTAN (CEUAIB) em reunião de 14/03/2006.

São Paulo, 18 de outubro de 2006.

Dra. Denise V. Tambourgi
Presidente da CEUAIB

De acordo:

Dr. Otávio Azevedo Mercadante
Diretor do Instituto Butantan

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CERTIFICADO

Certificamos que o Projeto intitulado "Expressão, purificação e caracterização de proteínas de superfície de *Leptospira interrogans*", protocolo nº 449/08, sob a responsabilidade de Ana Lúcia Tabet Oller do Nascimento e Marina von Atzingen dos Reis, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS DO INSTITUTO BUTANTAN (CEUAIB) em reunião de **12/03/2008**.

"We certify that the Research entitled "Expression, purification and characterization of surface proteins of *Leptospira interrogans*", protocol nº 449/08, under the responsibility of Ana Lúcia Tabet Oller do Nascimento and Marina von Atzingen dos Reis, is in agreement with the Ethical Principles in Animal Research, adopted by the Brazilian College of Animal Experimentation, and was approved by the ETHICAL COMMITTEE FOR ANIMAL RESEARCH of BUTANTAN INSTITUTE in the meeting of 03/12/2008".

Vigência do Projeto: 02/2008 – 03/2009	Nº de animais/espécie
	45 / camundongo Balb/c
	10 / hamster Golden

São Paulo, 17 de março de 2008.

p/ Dra. Denise V. Tambourgi
Presidente da CEUAIB

De acordo:

Dr. Otávio Azevedo Mercadante
Diretor do Instituto Butantan

UNIVERSIDADE DE SÃO PAULO
Faculdade de Medicina Veterinária e Zootecnia

Comissão Bioética

CERTIFICADO

Certificamos que o Projeto intitulado "Clonagem e expressão de candidatos vacinais contra a leptospirose, identificados no projeto genoma. *Leptospira interrogans*, sorovar Copenhageni", protocolado sob o nº1114/2007, utilizando 1000 (um mil) hamsters, sob a responsabilidade do Prof. Dr. Silvio Arruda Vasconcellos, está de acordo com os princípios éticos de experimentação animal da Comissão de Bioética da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 19/09/07.

We certify that the Research "Cloning and expression of leptospiral vaccine candidates identified by genome project. *Leptospira interrogans* serovar Copenhageni", utilizing 1000 (one thousand) hamsters, protocol number 1114/2007, under the responsibility Prof. Dr. Silvio Arruda Vasconcellos, agree with Ethical Principles in Animal Research adopted by Bioethic Commission of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 09/19/07.

São Paulo, 20 de setembro de 2007

Prof. Dr. José Luis Bernardino Merusse
Presidente da Comissão de Bioética
FMVZ/USP

Aos meus pais, Vitor e Yara, responsáveis
pela minha formação desde o principio;

Ao meu esposo, José Edson, pelo incentivo
e apoio constante durante esta etapa
acadêmica.

Agradecimentos

Aproveito para registrar meus agradecimentos àqueles e às instituições que de várias maneiras contribuíram para a realização dessa Tese de Doutorado.

À Dra. Ana Lúcia Tabet Oller do Nascimento pela orientação, confiança e incentivo, fundamentais para o bom desenvolvimento desta tese e para minha formação.

À Dra. Márcia Gamberini e ao Dr. Ricardo M. Gómez pela amizade e ajuda inestimável durante o Projeto Piloto Funcional do Genoma da *L. interrogans* e minha iniciação científica que serviram de base para o desenvolvimento técnico dessa tese.

Ao professor Dr. Sílvio A. Vasconcellos, Ms. Amane P. Gonçalves, Zenáide M. de Moraes e Gisele do Laboratório de Zoonose da Faculdade de Medicina Veterinária da USP pela colaboração com as culturas de leptospiros e ensaios de imunoproteção em hamster.

À professora Dra. Eliete C. Romero do Instituto Adolfo Lutz pela colaboração com os soros de pacientes.

À Dra. Beatriz G. Guimarães e doutoranda Priscila O. Giuseppe do Centro de Biologia Molecular Estrutural do Laboratório Nacional de Luz Síncrotron (LNLS) pela colaboração com os ensaios de dicroísmo circular.

À Dra. Patrícia A. E. Abreu e Dra. Tatiane R. Oliveira pela colaboração com a imunização e sangria dos camundongos.

À professora Dra. Toshie Kawano e Alexsander S. Souza do Departamento de Parasitologia, Instituto Butantan, pelo auxílio no microscópio confocal.

Ao professor Dr. Thales de Brito, Dirce M. C. Lima e Eduardo do Instituto de Medicina Tropical, e À Dra. Ângela S. Barbosa do Laboratório de Bacteriologia do Instituto Butantan pela colaboração científica.

Ao Laboratório de Biotecnologia Molecular II, Centro de Biotecnologia do Instituto Butantan pelo inestimável auxílio e convivência diária, que fortifica a verdadeira amizade: Daiane, Daniel, Denise, Fernanda, Gabriela, Mariana, Mônica Larucci, Monica Watanabe, Raquel, Rosane, Sarai e Tatiane.

Aos pesquisadores, funcionários e estudantes do Centro de Biotecnologia, pela ajuda e amizade durante todos esses anos.

Aos meus pais, Vitor e Yara, meu esposo, José Edson, e familiares pelo incentivo, torcida e apoio constante durante minha formação.

À coordenação do curso de Pós-Graduação Interunidades em Biotecnologia da Universidade de São Paulo / Instituto Butantan / Instituto de Pesquisas Tecnológicas, e a todos professores que contribuíram para a minha formação.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela concessão da bolsa de doutorado direto e financiamento do projeto, bem como ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e a Fundação Butantan, sem os quais este trabalho não seria viável.

“Comece fazendo o que é necessário,
depois o que é possível, e de repente
você estará fazendo o impossível”.

São Francisco de Assis.

Resumo

Atzingen MV. Expressão, purificação e caracterização de proteínas de superfície de *Leptospira interrogans* [Tese de Doutorado em Biotecnologia]. São Paulo: Instituto de Ciências Biomédicas da Universidade de São Paulo; 2009.

Leptospirose é uma zoonose mundial causada por espécies patogênicas do gênero *Leptospira*. O sequenciamento genômico da *L. interrogans* sorovar Copenhageni e os avanços das análises bioinformáticas permitiram a identificação de novos candidatos vacinais e novos fatores de virulência. Assim, foram selecionados 15 genes que codificam proteínas hipotéticas conservadas preditas de superfície de *L. interrogans* sorovar Copenhageni. A conservação e a expressão desses genes foram confirmadas por PCR e RT-PCR em seis sorovares de *L. interrogans* predominantes no Brasil. As proteínas recombinantes foram clonadas em vetor de expressão de *E. coli* em fusão com seis resíduos de histidina e expressas no sistema induzível por NaCl (*E. coli* BL21 SI). A purificação por cromatografia de afinidade a metal foi bem sucedida, entretanto, somente sete proteínas foram purificadas na ausência do agente desnaturante (rLIC10368, rLIC10494, rLIC11207, rLIC11935, rLIC12690, rLIC12730 e rLIC12922). As proteínas rLIC10494, rLIC11207, rLIC12730 e rLIC12922 foram identificadas na superfície de leptospirosas vivas através de imunofluorescência de fase líquida. As proteínas rLIC10494, rLIC11207, rLIC11935, rLIC12730, rLIC12922, rLIC20214 apresentaram reatividade com anticorpos presentes em soros de pacientes com leptospirose. Através de um ensaio de adesão por ELISA, identificamos uma nova adesina de leptospira codificada pelo gene LIC10368, denominada Lsa21 (*Leptospiral surface adhesin of 21kDa*), que interage fortemente com laminina, colágeno IV e fibronectina plasmática, de uma maneira dose dependente (Atzingen *et al.*, 2008). Usando a metodologia de Western blotting, identificamos mais nove possíveis adesinas que interagem com laminina, fibronectina plasmática e celular e colágeno IV. Estas adesinas, juntamente com a Lsa21, que é provavelmente um novo fator de virulência, devem estar envolvidas na patogênese da leptospirose. Nossos ensaios de imunização e desafio demonstraram que a proteína rLIC12730 conferiu proteção significativa contra infecção letal de *L. interrogans* em hamsters. A

imunoproteção conferida a estes animais é provavelmente via resposta Th2 revelada pelo aumento de título de anticorpos após a dose de reforço. Embora, mais estudos sejam necessários, nossos dados sugerem que a proteína rLIC12730 é um promissor candidato na prevenção da leptospirose.

Palavras chave: *Leptospira*. Genoma. Proteína recombinante. Vacina.

Abstract

Atzingen MV. Expression, purification and characterization of surface proteins from *Leptospira interrogans* [Doctoral thesis in Biotechnology]. São Paulo: Instituto de Ciências Biomédicas da Universidade de São Paulo; 2009.

Leptospirosis is a worldwide zoonosis caused by a pathogenic *Leptospira*. The whole-genome sequences of *L. interrogans* serovar Copenhageni and the bioinformatic tools allow us to search for novel candidate antigens suitable for improved vaccines against leptospirosis and to identify new virulent factors. We focused on fifteen genes encoding for conserved hypothetical proteins predicted to be exported to the outer membrane. The genes were amplified by PCR from six predominant pathogenic serovars in Brazil. The DNA sequences of the chosen genes were cloned into pDEST17TM, an *E. coli* vector, and the recombinant proteins were expressed in fusion with 6xHis-tag at N-terminus. Eleven proteins were expressed in NaCl-*E. coli* BL21 (SI)-induced cultures and purified by metal affinity chromatography, being seven of them recovered without the presence of urea (rLIC10368, rLIC10494, rLIC11207, rLIC11935, rLIC12690, rLIC12730 and rLIC12922). The proteins rLIC10494, rLIC11207, rLIC12730, rLIC12922 were shown to be surface-exposed by liquid-phase immunofluorescence assays with living organisms. The proteins rLIC10494, rLIC11207, rLIC11935, rLIC12730, rLIC12922, rLIC20214 were recognized by antibodies present in sera from human patients diagnosed with leptospirosis. By ELISA-attachment assay, we have identified a leptospiral protein encoded by LIC10368, named Lsa21, that binds strongly to laminin, collagen IV, and plasma fibronectin, in dose-dependent fashion (Atzingen *et al.*, 2008). By western blotting assay, we have further identified nine novel probable adhesins that interact to laminin, plasma and cellular fibronectin and collagen IV. These adhesins, in addition to Lsa21, which is probably a novel virulence factor, might be involved in leptospiral pathogenesis. Our results with the immunization/challenge assays showed that the recombinant protein rLIC12730 afforded significant protection against lethal leptospiral inoculation in hamsters. The immunoprotection conferred to these animals is probable via Th2 response as revealed by the increase in antibody titers during subsequent boosters. Although

more studies are needed, our data suggest that rLIC12730 is promising candidate for prevention of leptospirosis.

Keywords: *Leptospira*. Genome. Recombinant protein. Vaccine.

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Lista de Abreviaturas e Siglas

2YT	meio de cultura contendo 1% de extrato de levedura, 1,6% de peptona e 0,5% de NaCl
2YT-ON	meio de cultura contendo 1% de extrato de levedura, 1,6% de peptona
Amp	ampicilina 100 µg/ml
BSA	albumina de soro bovino
CD	Dicroísmo Circular
cDNA	DNA complementar
Da	Dalton
ddNTP	2', 3'didesoxirribonucleotídeo
DNA	ácido desoxirribonucleico
dNTP	desoxirribonucleotídeo
DO	Densidade Ótica
DTT	Ditiotreitrol
DUF	domínio de função desconhecida
EDTA	Ácido Etilenodiamino Tetracético
ELISA	<i>enzyme-linked immunosorbent assay</i>
EMJH	meio de cultura Ellinghausen-McCullough-Johnson-Harris
FITC	Isotiocianato de fluoresceína
IgG	imunoglobulina G
IgM	imunoglobulina M
IPTG	isopropil-β-galatosídeo
Kb	kilobase
kDa	kiloDalton
LPS	lipopolissacarídeo
MAT	teste de microaglutinação
Mb	megabase
mOsM	miliosmol
MS	Ministério da Saúde
OPD	o-fenilenodiamina
PAGE/SDS	eletroforese em gel de poliacrilamida contendo SDS

pb	pares de bases
PBS	solução fosfato salina, pH 7,4
PBS-T	tampão PBS acrescido de Tween-20 0,05%
PCR	Reação de Polimerase em Cadeia
pI	ponto isoelétrico
RBS	sítio de ligação de ribossomo
RNA	ácido ribonucleico
RNA _m	RNA mensageiro
RNA _r	RNA ribossomal
RT	transcriptase reversa
SDS	sulfato dodecil de sódio
SINAN	Sistema de Informação de Agravos de Notificação
SVE-SP	Sistema de Vigilância Epidemiológica do Estado de São Paulo
SVS	Secretaria de Vigilância em Saúde
TA	temperatura de anelamento
TAE	Tris-acetato 40 mM, EDTA 1 mM
TBE	Tris-borato 45 mM, EDTA 1 mM
TBS	Tris 10 mM (pH 7,4), NaCl 150 mM, PMSF 1 mM
TLR	Toll-like receptor

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1 Introdução

Em 20 de janeiro de 1.915, Inada e Ido anunciaram a descoberta do agente causador da doença de Weil, uma nova espécie de *Spirochaeta* denominada *Spirochaeta icterohaemorrhagiae japonica*. Em 1.918, Noguchi considerou que as características morfológicas justificavam a criação de um novo gênero, *Leptospira*, a ser incluído na ordem *Spirochaetales*. Muitos outros sorovares antigenicamente distintos foram identificados pelo mundo, e essa primeira leptospira recebeu uma nova denominação, *Leptospira interrogans* sorovar *Icterohaemorrhagiae* (Kobayashi, 2001b).

Atualmente, a leptospirose é considerada uma zoonose mundial que acomete várias espécies de animais, incluindo humanos, causada por espécies patogênicas do gênero *Leptospira*. Possui um amplo espectro de manifestações clínicas, variando da infecção subclínica (forma anictérica) a uma síndrome severa de infecção multissistêmica com alto índice de mortalidade, denominada doença de Weil (Faine *et al.*, 1999; Plank e Dean, 2000).

1.1 Taxonomia e classificação

A ordem *Spirochaetales* compreende importantes patógenos humanos incluindo *Treponema pallidum*, causador da sífilis, e *Borrelia burgdorferi*, causador da doença de Lyme. A Figura 1 apresenta um esquema simplificado da taxonomia dessa ordem.

Antes de 1989, as leptospirosas eram classificadas em duas espécies, *L. interrogans* e *L. biflexa*, que compreendiam as linhagens patogênicas e não-patogênicas, respectivamente. As linhagens isoladas eram classificadas em sorovares para facilitar o estudo epidemiológico da doença em humanos e animais numa certa região geográfica (Levett, 2001; 2007).

Figura 1 – Taxonomia das espiroquetas. De modo simplificado, este esquema foca os gêneros de importância clínica. As doenças causadas ou associadas a sua presença estão marcadas em vermelho.

A classificação em sorovares é fundamentada em testes de aglutinação microscópica, empregando soros fornecidos pelos centros de referência da Organização Mundial da Saúde (OMS). A diversidade dos sorovares é devida à heterogeneidade de carboidratos da cadeia lateral dos lipopolissacarídeos (LPS) da membrana externa da leptospira. Os sorovares mais patogênicos ao homem e prevalentes nos centros urbanos são o Copenhageni e Icterohaemorrhagiae (Faine *et al.*, 1999; Levett, 2001; Bharti *et al.*, 2003).

A variação genética observada dentro do gênero *Leptospira* implicou numa nova classificação baseada na hibridização de DNA. Nesse novo sistema foram reconhecidas 16 espécies, que incluem sorovares patogênicos e não-patogênicos (Levett, 2001; 2007; Bharti *et al.*, 2003). A classificação por genotipagem é motivo de controvérsia para alguns microbiologistas, por ser considerada incompatível com o sistema de sorovares utilizados por clínicos e epidemiologistas (Levett, 2001; Bharti *et al.*, 2003).

1.2 Aspectos biológicos e morfológicos das leptospiiras

As leptospiiras são bactérias móveis espiraladas (espiroquetas), geralmente possuem 0,1 μm de diâmetro por 6 a 20 μm de comprimento. A amplitude helicoidal é de aproximadamente 0,1 a 0,15 μm , e o comprimento de onda é de aproximadamente 0,5 μm . As extremidades são finas e encurvadas na forma de gancho. As leptospiiras podem sofrer alterações morfológicas durante subcultivo *in vitro*, o que pode ser restaurado através de inoculação em hamster. As leptospiiras não se coram pela coloração de Gram, mas podem ser coradas por impregnação por sais de prata, ou observadas diretamente em solução através de microscópio de campo escuro ou contraste de fase (Faine *et al.*, 1999; Levett, 2001; Bharti *et al.*, 2003).

Possuem duas membranas organizadas de modo semelhante às bactérias gram-negativas, sua membrana citoplasmática e parede celular de peptidoglicano estão intimamente associadas e são circundadas por uma membrana externa. A membrana externa contém diversas lipoproteínas e LPS. Os LPS de leptospiiras diferem dos encontrados em enterobactérias devido a uma alteração na composição do lipídeo A, o que resulta em atividade imune específica, incluindo menor endotoxicidade e ativação do TLR 2 ao invés do TLR 4 (Werts *et al.*, 2001; Que-Gewirth *et al.*, 2004; Schroder *et al.*, 2008). As lipoproteínas são as proteínas mais predominantes na membrana das espiroquetas e apresentam-se ancoradas através da porção amino-terminal por ligação covalente a um ácido graxo constituinte da membrana. Devido à localização na superfície da bactéria, as lipoproteínas desempenham diversas funções essenciais à adaptação e interação com o meio ambiente (Haake, 2000; Haake e Matsunaga, 2002; Cullen *et al.*, 2005). A Figura 2 apresenta o modelo da estrutura da membrana da leptospira e disposição das lipoproteínas mais conhecidas.

Figura 2 – Modelo da estrutura da membrana de leptospira. Leptospiras possuem duas membranas, uma membrana externa (ME) e uma membrana citoplasmática ou interna (MI). Como as bactérias Gram-negativas a parede de peptidoglicano (PG) é intimamente associada a MI. A superfície da leptospira apresenta lipopolissacarídeos (LPS) justapostos. Abaixo da superfície encontramos a proteína citoplasmática, GroEL, e o endoflagelo periplasmático (EF). A MI contém lipoproteínas como a LipL31 e proteínas transmembrana como a peptidase sinal (SP) e a ImpL36. A ME contém lipoproteínas incluindo LipL41, LipL32 e LipL36, e proteínas transmembrana incluindo a porina OmpL1.

FONTE: Adaptado de Nascimento *et al.*, 2004b.

Dois flagelos de estrutura complexa estão inseridos no espaço periplasmático entre as duas membranas. Estes flagelos garantem a forma helicoidal da célula além de conferir motilidade. As leptospiras exibem movimentos rápidos e rotatórios, sobretudo nas extremidades, mantendo a parte central fixa (Trueba *et al.*, 1992; Levett, 2001; Schroder *et al.*, 2008).

Leptospiras são bactérias aeróbicas obrigatórias que apresentam ótimo crescimento em temperatura de 28 a 30 °C. Produzem tanto catalase como oxidase. Crescem em meios simples enriquecidos com vitaminas (B2 e B12), ácidos graxos de cadeia longa e sais de amônio. Ácidos graxos de cadeia longa são utilizados como única fonte de carbono e são metabolizadas através da β -oxidação (Faine *et al.*, 1999; Levett, 2001).

1.3 A leptospirose

A infecção humana resulta da exposição direta ou indireta à urina de animais infectados. A penetração da leptospira ocorre ativamente através de mucosas (ocular, digestiva, respiratória e genital), de pele lesionada (incluindo pequenos arranhões) ou da pele íntegra, quando ocorre permanência por tempo prolongado com água contaminada. Após a penetração, as leptospiplas percorrem as vias linfáticas e sanguíneas, atingindo túbulos renais, fígado cérebro e olhos, onde persistem e se multiplicam (Faine *et al.*, 1999).

O tempo de incubação da doença até o surgimento dos sintomas pode ser de algumas horas até 28 dias, sendo em média de sete a 15 dias. O curso clínico pode ser dividido em três fases. Durante a primeira semana ocorre a fase febril. Durante a segunda semana ocorre a fase ictérica, enquanto a terceira fase ocorre nas semanas subsequentes e é denominada fase convalescente. O quadro clínico inicial da leptospirose é febre alta com calafrios, cefaleia, mal estar geral grave, lombalgia, dor muscular acentuada nos membros inferiores e congestão conjuntival, sendo estes últimos mais característicos (Faine *et al.*, 1999; Kobayashi, 2001a).

De cinco a 15% dos pacientes desenvolvem a doença de Weil (Faine *et al.*, 1999; Ko *et al.*, 1999). Icterícia, hemorragia e proteinúria formam a tríade de sinais característicos, entretanto não são sinais precoces da doença. A icterícia torna-se visível na maioria dos pacientes entre o quarto e sexto dia, acentuando-se na segunda semana da doença. Os pacientes com doença de Weil apresentam icterícia de moderada a grave, com duração entre três e seis semanas. A hemorragia ocorre especialmente nos casos graves da doença. Hemorragia subcutânea, tais como petéquias e manchas purpúreas, são mais comumente observadas, mas hemorragia gengival e palatal, epistaxe, hemorragia no trato gastrointestinal, hemorragia conjuntival, hemorragia genital e hematúria também ocorrem (Kobayashi, 2001a). Os sintomas neurológicos incluem cefaleia severa e insônia na maioria dos pacientes. Perturbação de consciência, delírio e rigidez do pescoço são observados na maioria das infecções graves. Os sintomas do sistema digestivo incluem anorexia e constipação na maioria dos pacientes. Náusea e vômito são observados nos pacientes mais graves (Faine *et al.*, 1999; Kobayashi, 2001a).

1.4 Epidemiologia

A epidemiologia da leptospirose humana reflete a relação entre humanos e animais cronicamente infectados (Vinetz, 2001). Os roedores são os principais reservatórios da doença, albergam as leptospiros nos rins e as eliminam vivas no meio ambiente, contaminando água, solo e alimentos. Dentre os roedores domésticos (*Rattus norvegicus*, *Rattus rattus* e *Mus musculus*), grande importância deve se dispensar ao *R. norvegicus*, portador clássico da *L. interrogans* sorogrupo Icterohaemorrhagiae, a mais patogênica ao homem (Faine *et al.*, 1999; Ko *et al.*, 1999).

A leptospirose acomete indivíduos de todas as idades e ambos os sexos. Entretanto homens jovens são mais frequentemente infectados por aceitarem trabalhos não especializados e de alto-risco, e assim a leptospirose assume caráter ocupacional. Pessoas que trabalham em ambientes alagadiços (plantação de arroz, sistema de esgoto, minas, locais em construção) ou mesmo em locais úmidos e infestados de roedores (cozinhas, restaurantes, supermercados, indústria alimentícia) são frequentemente acometidas (Kobayashi, 2001a).

No Brasil, a leptospirose é uma doença endêmica (SVS, 2005), com pico epidêmico nos meses quentes e úmidos, pois a leptospira depende de umidade para sobreviver e alcançar o hospedeiro (Faine *et al.*, 1999; Kobayashi, 2001a; Vinetz, 2001). Nas capitais e áreas metropolitanas, a disseminação é favorecida por enchentes, aglomeração populacional de baixa renda em condições inadequadas de saneamento e alta infestação de roedores infectados (Ko *et al.*, 1999). No período de 2.001 a 2.003, o local provável de infecção de 55% dos casos relatados era o domicílio (SVS, 2005).

A leptospirose urbana ocorre em todas as regiões brasileiras. Segundo dados do Sistema de Informação de Agravos de Notificação (SINAN - <http://dtr2004.saude.gov.br/sinanweb/novo/>) do Ministério da Saúde, foram relatados 15.962 casos no Brasil no período de janeiro de 2.004 a dezembro de 2.008 (em média, 3.192 casos/ano), sendo 10% dos casos ocorridos na região Norte; 20%, Nordeste; 38%, Sudeste; 31%, Sul e 1% na região Centro-Oeste. Nesse período foram informados 1.766 óbitos (em média, 353 óbitos/ano), sendo a letalidade em

torno de 11%. O maior índice de casos relatados encontra-se no estado de São Paulo, 3.617 casos (59% dos casos ocorridos na região sudeste e 22% dos casos ocorridos no Brasil), com coeficiente médio de incidência de 1,86 para cada 100.000 habitantes segundo dados do Sistema de Vigilância Epidemiológica do Estado de São Paulo (SVE-SP - http://www.cve.saude.sp.gov.br/htm/cve_lepto.html).

Do total de casos confirmados, 77% foram hospitalizados, o que demonstra que o sistema de vigilância sofre um importante grau de subnotificação, captando os casos moderados e graves. Estima-se que esses casos correspondam apenas a 40% do total de indivíduos infectados (SVS, 2005). A amplitude dos sintomas observados, principalmente na fase inicial da leptospirose, e a semelhança com outras doenças tropicais acarretam subdiagnóstico clínico da leptospirose, contribuindo com a subnotificação (Faine *et al.*, 1999; Ko *et al.*, 1999; Plank e Dean, 2000; Levett, 2001). Como consequência, ocorre negligência justificável de políticas públicas preventivas, como controle de roedores, melhoria das condições higiênico-sanitárias da população, sobretudo em regiões superpovoadas e periféricas (Barcellos e Sabroza, 2000).

Quando diagnosticada, o tratamento envolve combinação de antibióticos e terapia de suporte. Os casos graves necessitam de hospitalização imediata e, se apresentarem complicações renais, respiratórias ou hemorrágicas, deverão ser encaminhados a hospitais que disponham de capacidade para realizar procedimentos como diálise e terapia intensiva. A média de internação é de sete dias (SVS, 2005). Assim, a leptospirose representa um problema econômico para o sistema de saúde pública (Faine *et al.*, 1999; Ko *et al.*, 1999).

A leptospirose também representa um importante problema econômico para a pecuária, uma vez que outros mamíferos, como gado bovino, porcos, ovelhas, cabras e cavalos também são suscetíveis. Estes animais sofrem aborto, natimortalidade, nascimento prematuro, infertilidade, redução no ganho de peso, mastite e morte prematura. Esses eventos acarretam decréscimo na produção de leite e carne (Faine *et al.*, 1999).

O impacto econômico, a dificuldade de instalar as medidas de controle ambiental e a abundância de animais reservatórios (domésticos ou silvestres), indica o desenvolvimento de vacinas como a estratégia de prevenção mais promissora

(Levett, 2001; Nascimento *et al.*, 2004a; Nascimento *et al.*, 2004b; Palaniappan *et al.*, 2007).

1.5 Vacinas profiláticas

As vacinas veterinárias disponíveis atualmente empregam bactérias inativadas ou preparações de membrana de leptospiros patogênicos e parecem induzir resposta protetora a partir da produção de anticorpos contra LPS de leptospiros (Faine *et al.*, 1999; Levett, 2001). Contudo, essas vacinas falham em induzir proteção a longo prazo contra infecção, o que requer administração anual ou semestral (Bolin *et al.*, 1989a; Bolin *et al.*, 1989b); também não promovem proteção cruzada contra leptospiros de sorogrupos diferentes das incluídas na preparação da vacina. O grande número de sorovares (mais de 200) e o alto custo da produção de uma vacina multissorovar competente são os maiores limitantes no desenvolvimento de protocolos de imunização que empregam preparados de células ou membranas (Levett, 2001).

Em países como China e Cuba já existem vacinas aprovadas para uso em humanos, com as mesmas características das vacinas de uso veterinário (Yan *et al.*, 2003; Martinez *et al.*, 2004). Porém, o emprego de preparações com bactérias inativadas acarreta efeitos colaterais, como febre e dor no local da aplicação. Por esse motivo, alguns indivíduos não aceitam ser imunizados.

Neste sentido, a busca de antígenos conservados que, mesmo purificados, tenham efeito imunoprotetor, é imprescindível. As proteínas de superfície da célula bacteriana, afiguram-se excelentes candidatos vacinais por estarem envolvidas na interação das bactérias com a célula hospedeira (Cullen *et al.*, 2003; Palaniappan *et al.*, 2007).

1.6 Vacinologia reversa

A abordagem convencional para identificação de candidatos vacinais depende do cultivo *in vitro* do patógeno e isolamento dos antígenos por processos bioquímicos. Além de demorado, esse método limita-se ao estudo de patógenos cultiváveis, e geralmente, os antígenos isolados são os mais abundantes e, às vezes, não conferem proteção eficiente ou são muito variáveis entre as linhagens infectantes. O sequenciamento genômico e os avanços das análises bioinformáticas revolucionaram o campo da pesquisa de vacinas, uma vez que permitem a identificação de candidatos vacinais independente de suas características de expressão (Rappuoli, 2000; Adu-Bobie *et al.*, 2003; Gamberini *et al.*, 2005; Koizumi e Watanabe, 2005; Serruto e Rappuoli, 2006; Medini *et al.*, 2008).

Este método foi empregado pela primeira vez no desenvolvimento de uma vacina contra a *Neisseria meningitidis* sorogrupo B. Considerando que antígenos de superfície são mais susceptíveis ao reconhecimento de anticorpos e, assim, melhores candidatos, foram selecionados 600 genes, dos quais 28 revelaram-se antígenos protetores, sendo alguns conservados em diversas linhagens (Pizza *et al.*, 2000). Após três anos do término do sequenciamento do genoma da *N. meningitidis* sorogrupo B, três proteínas iniciaram a fase de testes clínicos, sendo uma delas, uma lipoproteína, GNA1870 (Massignani *et al.*, 2003; Koeberling *et al.*, 2007).

Esta mesma estratégia foi empregada com sucesso na busca de candidatos vacinais contra infecção por *Streptococcus pneumoniae* (Wizemann *et al.*, 2001), *Porphyromonas gingivalis* (Ross *et al.*, 2001), *Chlamydia pneumoniae* (Montigiani *et al.*, 2002), *Streptococcus pyogenes* (Reid *et al.*, 2001; 2002), entre outros.

1.7 Sequenciamento genômico de *Leptospira* spp.

Atualmente, três espécies de *Leptospira* foram sequenciadas: *L. interrogans* sorovar Lai (Ren *et al.*, 2003) e sorovar Copenhageni (Nascimento *et al.*, 2004a;

Nascimento *et al.*, 2004b), *L. borgpetersenii* sorovar Hardjo-bovis (Bulach *et al.*, 2006) e *L. biflexa* sorovar Patoc (Picardeau *et al.*, 2008).

A maioria das infecções são causadas pela *L. interrogans* ou *L. borgpetersenii* e abrangem 48% dos sorovares patogênicos descritos (Brenner *et al.*, 1999). Embora apresentem características clínicas semelhantes, a transmissão da *L. borgpetersenii* ocorre somente através de contato direto com indivíduo infectado. A comparação desses genomas permitiu identificar diferenças relevantes quanto a sua organização sendo que a redução do genoma de *L. borgpetersenii* deve ser responsável pela limitação de sobrevivência fora do hospedeiro (Bulach *et al.*, 2006).

L. biflexa é uma espiroqueta saprofítica de vida-livre presente em ambientes aquáticos. A análise comparativa de seu genoma tornou evidente que representa um excelente modelo para estudo da evolução das leptospiros. A comparação do genoma da *L. biflexa* com as duas espécies patogênicas revelou que dois terços de seus genes são ortólogos. Os genomas de *L. biflexa* e *L. borgpetersenii* são iguais em tamanho (3,9 Mb), enquanto, *L. interrogans* possui um genoma maior (4,6 Mb), provavelmente por ter adquirido informações gênicas para sobreviver tanto no meio ambiente como em mamíferos hospedeiros. Um conjunto de 1.431 genes considerados patogênicos-específicos, não possui ortólogos na *L. biflexa*, foi identificado. Cerca de 62% desses genes não possuem função conhecida, sugerindo uma patogênese única para a leptospirose (Picardeau *et al.*, 2008).

A análise *in silico* das sequências do genoma da *L. interrogans* sorovar Copenhageni identificou muitas lipoproteínas e proteínas transmembrana que podem estar envolvidas na patogênese e proteção imunológica. Dentre as lipoproteínas, 174 são hipotéticas, ou seja, são proteínas ainda não identificadas em nenhum outro organismo (Nascimento *et al.*, 2004b).

1.8 Candidatos vacinais e fatores de virulência de *Leptospira* spp.

Em leptospiros, os mecanismos de patogenicidade não são bem definidos. Um número crescente de prováveis fatores de virulência tem sido sugerido, mas em poucas exceções seu papel na patogênese está bem definido (Levett, 2001;

Palaniappan *et al.*, 2007). Diversos grupos de pesquisa têm buscado proteínas relacionadas a patogenicidade ou virulência, que tenham amplo espectro de conservação entre os principais sorovares patogênicos ao homem, sendo o foco mantido sobre as proteínas de membrana e lipoproteínas (Haake *et al.*, 1999; Branger *et al.*, 2001; Guerreiro *et al.*, 2001; Palaniappan *et al.*, 2002; Koizumi e Watanabe, 2003; Artiushin *et al.*, 2004; Gamberini *et al.*, 2005; Verma *et al.*, 2006; Chang *et al.*, 2007; Seixas *et al.*, 2007b; Faisal *et al.*, 2008). A identificação dessas proteínas poderá servir de base para o desenvolvimento de uma vacina que seja eficiente contra a leptospirose e que confira proteção a longo prazo.

Haake *et al.* (1999) mostraram o efeito sinérgico entre duas proteínas de membrana externa, OmpL1 e LipL41, na proteção contra a leptospirose em hamsters. A combinação OmpL1 + LipL41 apresentou 71% de proteção, enquanto isoladamente conferiram apenas 42% e 23%, respectivamente (Haake *et al.*, 1999). A proteína de membrana OmpL1 apresentou proteção cruzada (25-62,5%) em desafio empregando *L. interrogans* sorovar Lai e Autumnalis e *L. borgpetersenii* sorovar Bullum em porquinhos da Índia (Dong *et al.*, 2008).

As proteínas de membrana externa da família das proteínas bacterianas semelhantes a imunoglobulina (Big, “*bacterial immunoglobulin-like*”), denominadas LigA e LigB (Lig, “*leptospiral immunoglobulin-like*”), também mostraram-se potenciais candidatos vacinais (Koizumi e Watanabe, 2004; Palaniappan *et al.*, 2006; Silva *et al.*, 2007). Koizumi e Watanabe (2004) empregaram camundongos C3H/HeJ, que são suscetíveis a leptospirose devido a deficiência em produzir resposta contra LPS via TLR 4, vacinados com as proteínas LigA e LigB clonadas a partir de *L. interrogans* sorovar Manilae em um ensaio de desafio e obtiveram 90% de proteção (Koizumi e Watanabe, 2004). Em outro estudo, hamsters imunizados com rLigA sobreviveram a infecção com *L. interrogans* sorovar Pomona, não apresentaram alteração morfológica significativa em seus rins nem foi possível isolar leptospiros através de cultura (Palaniappan *et al.*, 2006). O fragmento LigANI que corresponde aos seis domínios semelhantes a imunoglobulina da região C-terminal da proteína LigA conferiu imunoproteção de 67-100% em hamsters desafiados com *L. interrogans* sorovar Copenhageni, apesar da proteção significativa foi possível isolar leptospiros dos rins desses animais (Silva *et al.*, 2007). A proteína LigB foi truncada em três: rLigBcon (região conservada), rVarB1 e rVarB2 (região variável 1 e 2).

Quando comparados em ensaio de imunoproteção em hamster, o fragmento rLigBcon conferiu a melhor proteção (71%) e menor gravidade nas lesões histopatológicas (Yan *et al.*, 2008).

A abundante lipoproteína LipL32 já foi empregada como antígeno vacinal em diversas estratégias de vacinação (Seixas *et al.*, 2007a; Seixas *et al.*, 2007b). Hamster imunizados com o bacilo Calmette-Guerin recombinante (rBCG) expressando LipL32 apresentaram de 32-55% de proteção contra desafio letal de *L. interrogans* serovar Copenhageni. A necropsia dos rins não revelou alterações histológicas características da leptospirose (Seixas *et al.*, 2007a).

O laboratório de Genoma Funcional do Centro de Biotecnologia do Instituto Butantan iniciou em 2002 um Projeto Piloto no qual mais de 200 genes de *Leptospira* foram clonados e avaliados quanto a possibilidade de serem bons candidatos vacinais (Gamberini *et al.*, 2005). No entanto, nem todos foram completamente avaliados e, a partir deste grupo, selecionamos para o presente trabalho 15 genes que codificam proteínas hipotéticas preditas serem expressas na superfície: seis codificam prováveis lipoproteínas (LIC10368, LIC10494, LIC10498, LIC11207, LIC12217, LIC12690), cinco codificam proteínas de membrana externa (LIC11506, LIC11668, LIC11935, LIC12225, LIC20214), dois codificam proteínas integrais de membrana (LIC12144 e LIC13101) e dois codificam proteínas hipotéticas conservadas (LIC12730, LIC12922).

2 Objetivo

O objetivo deste trabalho é caracterizar novas proteínas de superfície de *L. interrogans* através da expressão de proteínas recombinantes em *Escherichia coli*. Avaliar a conservação entre diferentes sorovares, a localização celular da proteína nativa correspondente, a imunorreatividade frente a soro de paciente diagnosticados com leptospirose e hamster infectados experimentalmente, a interação com componentes de matriz extracelular, a imunoproteção em modelo animal (hamster).

3 Materiais e Métodos

3.1 Bactérias

3.1.1 *Leptospira* spp.

Culturas de *Leptospira* spp de diversos sorovares são mantidas no laboratório de Zoonoses da Faculdade de Medicina Veterinária da USP em meio de cultura Ellinghausen-McCullough-Johnson-Harris (EMJH, DifcoTM, Le Pont de Claix, França) enriquecido com soro de coelho (10%), L-asparagina (0,015%), piruvato de sódio (0,001%), cloreto de cálcio (0,001%), cloreto de magnésio (0,001%), peptona (0,03%) e extrato de carne (0,02%) (EMJH modificado) a 28 °C (Turner, 1970).

As linhagens de *L. biflexa* sorovar Patoc (Patoc 1), *L. interrogans* sorovar Canicola (Hond Utrecht IV e LO4), *L. interrogans* sorovar Copenhageni (M-20 e FIOCRUZ L1-130), *L. interrogans* sorovar Grippotyphosa (Moskva V), *L. interrogans* sorovar Hardjo (Hardjoprajitno), *L. interrogans* sorovar Icterohaemorrhagiae (RGA), *L. interrogans* sorovar Pomona (Pomona e LPF) foram empregadas em nossos estudos.

Algumas linhagens são mantidas virulentas (LO4, LPF e FIOCRUZ L1-130) através de passagem em hamster (Faine *et al.*, 1999). Hamsters recém desmamados são injetados com 500 µl de cultura de *L. interrogans* virulentas na diluição de 10⁸ células / ml via intraperitoneal. Estes animais devem ser acompanhados diariamente até o aparecimento dos sintomas da doença, como perda de peso e mobilidade, o que ocorre em aproximadamente cinco dias, então os animais são sacrificados em câmara de CO₂. Os rins e fígados são retirados, macerados e cultivados em meio EMJH modificado semi-sólido (ágar 2%) a 28 °C e sub-cultivados em meio EMJH modificado líquido a 28 °C por no máximo duas passagens para a manutenção da virulência das leptospiros.

3.1.2 *E. coli*

Bactéria *E. coli* DH5 α TM (Invitrogen Corporation, Carlsbad, CA, EUA) foi empregada para clonagem. Seu genótipo é [*F*- Φ 80/*lacZ* Δ M15 Δ (*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rk-, m k+) *phoA supE44 thi-1 gyrA96 relA1* λ].

A expressão de proteínas recombinantes foi realizada preferencialmente em *E. coli* BL21 SI (Invitrogen) que possui o gene da T7 RNA polimerase sob ação do promotor responsivo a osmolaridade, promotor *proU*, e é induzível pela adição de NaCl (Bhandari e Gowrishankar, 1997). Seu genótipo é [*F*-, *ompT*, *lon*, *hsdS* (*rb-mb*-), *gal*, *dcm*, *endA1*, *proUp::T7RNAP::malQ-lac-Z*].

Outras duas linhagens de *E. coli* também foram utilizadas para expressão, BL21 StarTM (DE3) pLysS (Invitrogen) e C43 (DE3) (Avidis, Saint-Beauzire, França), que possuem o gene da T7 RNA polimerase sob controle do promotor *lacUV5*, sendo induzível pela adição de isopropil- β -galatosídeo (IPTG) (Studier *et al.*, 1990). O genótipo da BL21 StarTM (DE3) pLysS (Invitrogen) é *F ompT hsdS_B(r_B⁻m_B⁻) gal dcm rne131 (DE3) pLysS (Cam^R)* (Lopez *et al.*, 1999) e da C43 (DE3) (Avidis) é *F ompT hsdS_B(r_B⁻m_B⁻) gal dcm (DE3)* (Miroux e Walker, 1996).

3.2 Vetores de clonagem e expressão

O vetor pENTR/D-TOPO (Invitrogen) possui dois sítios para reação de recombinação sítio-específica no vetor de expressão (*attL1* e *attL2*) e sítios para clonagem direcional. Possui uma sequência *rrnB* de terminação de transcrição para prevenir expressão residual do inserto de DNA de interesse em *E. coli*, gene de resistência a kanamicina (20 μ g/ml) como marcador de seleção em *E. coli* e, origem de replicação do vetor pUC para garantir alto número de cópia do plasmídeo, como mostrado na Figura 3.

Figura 3 – Mapa do vetor de clonagem pENTR/D-TOPO (Invitrogen) e suas características.
C, fita complementar.

FONTE: Invitrogen.

O vetor pDEST17 (Invitrogen) foi desenvolvido para expressão de proteínas heterólogas em fusão com seis resíduos de histidina na porção N-terminal, o que permite a purificação da proteína expressa em colunas de afinidade a metal, como por exemplo, Ni²⁺. Este vetor contém o promotor de T7, sítio de ligação de ribossomo (RBS), códon ATG de iniciação, sequência codificante para seis histidinas e gene de resistência a ampicilina (100 µg/ml) como marcador de seleção em *E. coli*. Dois sítios de recombinação, attR1 e attR2, flanqueiam o gene de resistência a cloranfenicol e são trocados pelas sequências, attL1 e attL2, presentes no pENTR/D-TOPO (Invitrogen) pela reação com a enzima *LR Clonase* (Invitrogen). Sequências de DNA clonadas no vetor pDEST17 (Invitrogen) podem ser expressas pela ação da T7 RNA polimerase em *E. coli*. O mapa do vetor pDEST17 (Invitrogen) está apresentado na Figura 4.

	Localização
Promotor T7	21-40
Sítio de ligação do ribossomo (RBS)	86-92
ATG de iniciação	101-103
6xHis tag	113-130
<i>attR1</i>	140-264
Gene de resistência a cloranfenicol (C ^{Mr})	373-1032
<i>ccdB</i> gene	1374-1679
<i>attR2</i>	1720-1844
T7 região de terminação da transcrição	1855-1983
<i>bla</i> promotor	2471-2569
Gene de resistência a ampicilina	2570-3430
Origem pBR322	3575-4248
<i>ROP</i> ORF	4619-4810 (C)

Figura 4 – Mapa do vetor de expressão pDEST17 (Invitrogen) e suas características. C, fita complementar.

FONTE: Invitrogen.

3.3 Soros

Os soros de indivíduos previamente diagnosticados com leptospirose através do teste de microaglutinação (MAT) pertencem a uma soroteca e foram cedidos pela Dra. Eliete C. Romero do Instituto Adolfo Lutz (ANEXO A).

3.4 Análise *in silico* das características das proteínas selecionadas

Os candidatos vacinais foram selecionados a partir do genoma da *L. interrogans* sorovar Copenhageni disponível no servidor público <http://aeg.lbi.ic.unicamp.br/world/lic/> (Nascimento *et al.*, 2004b). A conservação dos genes foi avaliada através da análise comparativa das sequências de aminoácidos preditas com auxílio do programa Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> (Altschul *et al.*, 1990; Altschul *et al.*, 1997). A presença de domínios comuns a outras proteínas foi avaliada com auxílio dos programas SMART - <http://smart.embl-heidelberg.de/> (Schultz *et al.*, 1998; Letunic *et al.*, 2006) e Pfam - <http://pfam.sanger.ac.uk/search> (Finn *et al.*, 2006). Análise de predição de localização celular foi realizada com o auxílio dos programas PSORT - <http://psort.nibb.ac.jp/form.html> (Nakai e Kanehisa, 1991; Nakai e Horton, 1999) e P-Classifier - <http://protein.bii.a-star.edu.sg/localization/gram-negative/index.html> (Wang *et al.*, 2005). A presença de sinal de clivagem ou lipidação foi avaliada com auxílio do programa LipoP - <http://www.cbs.dtu.dk/services/LipoP/> (Juncker *et al.*, 2003).

3.5 Extração de DNA de culturas de *Leptospira* spp.

O DNA genômico foi extraído por fenol-clorofórmio com pequenas modificações no método descrito por Maloy (1989). Alíquotas de 3 ml de cultura com cinco dias de crescimento foram centrifugadas 30 minutos a 11.500 g (4 °C). O sobrenadante foi descartado e o precipitado ressuspenso em 467 µl de tampão Tris-EDTA. Foram adicionados 30 µl de SDS 10% e 120 µg de proteinase K. A solução foi homogeneizada e incubada por uma hora a 37 °C. Foi adicionado igual volume de solução fenol-clorofórmio (1:1) e a solução foi homogeneizada por agitação vigorosa. A solução foi centrifugada 5 minutos a 11.500 g e a fase aquosa transferida a um novo tubo. Foi repetida a extração por adição de fenol-clorofórmio e recolhida a fase aquosa em um novo tubo. Seguiu-se a adição de 0,1 volumes de acetato de sódio

3 M (pH 5.2) e homogeneização. Adicionou-se 0,6 volumes de isopropanol e a solução foi invertida até a formação de precipitados de DNA. A solução foi centrifugada 15 minutos a 11.500 g. O sobrenadante foi descartado e ao precipitado adicionado 1 ml de etanol 70%. A solução foi invertida duas vezes e centrifugada 5 minutos a 11.500 g. O sobrenadante foi descartado e o precipitado seco a temperatura ambiente e, posteriormente, ressuspenso em 50 µl de tampão Tris-EDTA (Maloy, 1989).

A qualidade do DNA extraído foi avaliada por eletroforese em gel de agarose 1% em tampão Tris-acetato 40 mM, EDTA 1 mM (TAE) corado com brometo de etídeo. O DNA obtido foi quantificado em espectrofotômetro a 260 nm e 280 nm.

3.5.1 Reação de Polimerase em Cadeia (PCR)

A reação de amplificação foi realizada com DNA genômico 50 ng e *Taq DNA polimerase* (Invitrogen) num volume final de 25 µl, conforme protocolo básico recomendado pelo fabricante. A PCR foi realizada em termociclador seguindo a rotina: 94 °C por 4 minutos, 35 ciclos de: (a) 94 °C por 50 segundos, (b) temperatura de anelamento por 50 segundos, (c) 72 °C por 1 minuto, finalizando com 72 °C por 7 minutos e conservando a 4 °C.

Os oligonucleotídeos sintéticos utilizados para a amplificação dos genes foram desenhados com base nas sequências de *L. interrogans* sorovar Copenhageni com o auxílio do programa *Generunner*® - <http://www.generunner.net/> (Tabela 1). A temperatura de anelamento (TA) dos oligonucleotídeos foi determinada 5 °C abaixo da temperatura média na qual metade das moléculas tem suas pontes de hidrogênio dissociadas (T_m).

A avaliação do tamanho dos insertos clonados foi realizada através de eletroforese em gel de agarose 1% em tampão TAE corado com brometo de etídeo.

Tabela 1 – Sequência de oligonucleotídeos empregada para amplificação dos genes nos diversos sorovares.

Gene ID	Oligonucleotídeo sintético	TA	Inserto
LIC10368	F: 5' CGGCACTCAATTTTCTAGAGATCA 3' R: 5' CTTCGCAACTTGTGGATAAGG 3'	55 °C	591 pb
LIC10494	F: 5' CACCACTGCTAGGGCTGCAGAAA 3' R: 5' GAATTGGTTTCGGTGGAAAGTTGTAGTC 3'	63 °C	330 pb
LIC10498	F: 5' CACCAAATCCAGCGCTACGGGTG 3' R: 5' GATTTCCAGTACAATTTGGGCCGG 3'	64 °C	383 pb
LIC11207	F: 5' CACCACTCTTGCAGCTGTTTCCACC 3' R: 5' GTTTTAGGAGTTGTTCTTGGTGGTCCTG 3'	66 °C	366 pb
LIC11506	F: 5' CACCCCAAGTTCTGTTTTATCTCAAGC 3' R: 5' GTTCCGGATTCTGCGCCTAC 3'	64 °C	412 pb
LIC11668	F: 5' CACCCGGTACCATTACTAAACCGACTC 3' R: 5' CTAAAGCTTCTAGTACATTCGGGGCAACTAC 3'	66 °C	380 pb
LIC11935	F: 5' CACCCCTTCCCAAACGATCGAA 3' R: 5' CACTTCGTAACCTATGTGAGTTGCCATC 3'	65 °C	397 pb
LIC12144	F: 5' CACCGGATTAGATGCTAATGTAGCTTATC 3' R: 5' CTGAATACTCTTGAAGATATTGGAATAATAAC 3'	62 °C	352 pb
LIC12217	F: 5' CACCATTTCGGCTTGTTCGGGAG 3' R: 5' CCAAGATTACCTGATTTTGCGGCAG 3'	65 °C	367 pb
LIC12225	F: 5' CACCTTGTATCAAGAAATTGCATTGAG 3' R: 5' CGAAATTTAACTGAGAATCCAGCCATC 3'	55 °C	290 pb
LIC12690	F: 5' CACCTTTTCTAACTTCGCGGACTC 3' R: 5' GTATAAGTTCCTGGAGACCAATTGAGAG 3'	62 °C	399 pb
LIC12730	F: 5' CACCAGTTCTGACGGACTTCCCAA 3' R: 5' GTACCGGACTGTAAATCTTGACTTCCAC 3'	66 °C	325 pb
LIC12922	F: 5' CACCGAATCACTCAACAGAGTCATTGC 3' R: 5' GAGGGACCGCAATTTTAACTGAAGTAG 3'	66 °C	389 pb
LIC13101	F: 5' CACCAAAGGAGGAAGTTCCTTTGC 3' R: 5' GTTGTATTGGAATAAGAGCAGCACCAG 3'	66 °C	396 pb
LIC20214	F: 5' CACCCGTGCACGTGTTTTTTCCCG 3' R: 5' GTAGCAGTCAATCCGTCAAAGTGAAAC 3'	66 °C	522 pb

NOTAS: Gene ID: identificação do gene no genoma *L. interrogans* sorovar Copenhageni; Abreviaturas (em inglês): F, “forward”; R, “reverse”; TA, temperatura de anelamento; pb, pares de base.

3.6 Extração de RNA de culturas de *Leptospira spp.*

Alíquotas de 20 ml de cultura com cinco dias de crescimento foram centrifugadas 30 minutos a 11.500 g (4 °C). O sobrenadante foi descartado. O sedimento foi ressuspenso em 2 ml de *Trizol* (Invitrogen) e centrifugado 10 minutos a 11.500 g (4 °C). O sobrenadante foi transferido a um novo tubo, onde foi adicionado 400 µl de clorofórmio. A solução foi homogeneizada através de agitação manual por 15 segundos, incubada 3 minutos a temperatura ambiente e, a seguir, centrifugada por 15 minutos a 11.500 g (4 °C). A fase aquosa (superior) foi então transferida a um novo tubo, onde foi adicionado 1 ml de isopropanol. A solução foi invertida seis vezes, incubada 10 minutos a temperatura ambiente e a seguir centrifugada 10 minutos a 11.500 g (4 °C). O sobrenadante foi descartado e ao precipitado foi adicionado 2 ml de etanol 75%. A solução foi invertida duas vezes e centrifugada 5 minutos a 11.500 g (4 °C). O sobrenadante foi descartado e o precipitado seco a temperatura ambiente e, posteriormente, ressuspenso em 40 µl de água livre de RNase.

A qualidade do RNA extraído foi avaliada por eletroforese em gel de 1% agarose em tampão Tris-borato 45 mM, EDTA 1 mM (TBE) corado com brometo de etídeo. O RNA obtido foi quantificado em espectrofotômetro a 260 nm e 280 nm.

3.6.1 Reação de Transcrição Reversa (RT-PCR)

Uma alíquota de 2 µg de RNA foi tratada com *Dnase_I Amplification Grade* (Invitrogen) e tampão fornecido pelo fabricante em um volume final de 20 µl. Após 15 minutos de incubação a temperatura ambiente, foi adicionado EDTA 2,5 mM e aquecido a 65 °C por 10 minutos.

Em cada fração foi adicionado random hexamers 25 ng (Invitrogen) e desoxirribonucleotídeos (dNTPs) 1 mM, incubada por 5 minutos a 65 °C e transferida ao gelo por pelo menos 1 minuto. Adicionou-se MgCl₂ 5 mM, DTT 10 mM, *RNase OUT* 40 U (Invitrogen), *SuperScript III RT* 200 U (Invitrogen) e tampão fornecido pelo

fabricante para um volume final de 22 µl. A reação foi incubada em termociclador a 25 °C por 10 minutos, 50 °C por 50 minutos, 85 °C por 5 minutos e gelo. Em seguida as amostras foram tratadas com *RNase H* 1 U (Invitrogen) a 37 °C por 20 minutos e armazenadas a -20 °C.

Foi preparada uma reação de PCR semelhante a descrita anteriormente exceto o DNA genômico que foi substituído por 2 µl do produto da transcrição reversa. Como controle da qualidade e quantidade de transcritos foi elaborado um par de oligonucleotídeos para o RNAr 16S (5'CAAGTCAAGCGGAGTAGCAATACTCAGC 3'; 5'GATGGCAACATAAGGTGAGGGTTGC 3', TA 63 °C, inserto 1.050 pb).

3.7 Clonagem no vetor pDEST17

As construções no vetor pENTR/D-TOPO (Invitrogen) foram clonadas durante o Projeto Piloto Funcional do Genoma da *L. interrogans* e cedidas gentilmente para nosso projeto. Os genes foram amplificados a partir do DNA genômico extraído de *L. interrogans* sorovar Copenhageni empregando oligonucleotídeos sintéticos, cujas sequências estão na Tabela 2. A correta orientação é obtida através da adição de quatro bases no oligonucleotídeo forward (CACC) que anela com a sequência complementar presente nesse vetor de clonagem. Todos os genes foram clonados sem a sequência codificadora para o peptídeo sinal.

O inserto de DNA clonado no vetor pENTR/D-TOPO (Invitrogen) foi transferido por recombinação para o vetor de expressão em *E. coli*, pDEST17 (Invitrogen), pela ação da *LR Clonase* (Invitrogen). Cada reação foi executada num volume final de 10 µl contendo 150 ng do clone selecionado em pENTR/D-TOPO, 150 ng de vetor pDEST17 (Invitrogen), tampão *LB Reaction* (Invitrogen), e *LR Clonase Enzyme Mix* (Invitrogen), fornecidos pelo fabricante. A mistura foi incubada uma hora a temperatura ambiente, a seguir adicionado 1 µl de proteinase K fornecida pelo fabricante e incubada 10 minutos a 37 °C; como descrito no kit *Gateway™ LR Clonase™ Enzyme Mix* (Invitrogen).

Tabela 2 – Sequência de oligonucleotídeos empregados para amplificação e clonagem dos genes no vetor pENTR/D-TOPO.

Gene ID	Oligonucleotídeo sintético	Inserto
LIC10368	F: 5' CACCG ATGAAAAAAAGAAAATGAATTGAG 3' R: 5' AACGCGATTCATAGAGAGCG 3'	591 pb
LIC10494	F: 5' CACCA CTGCTAGGGCTGCAGAAA 3' R: 5' ACTTTGAGAGCTTCGTCTCGT 3'	795 pb
LIC10498	F: 5' CACCA AAATCCAGCGCTACGGGTG 3' R: 5' GAATCACGGCGCACTTGG 3'	2.814 pb
LIC11207	F: 5' CACCA CTCTTGCAGCTGTTTCCACC 3' R: 5' CTTAGTTACAAGCTCCCGAAGC 3'	1.003 pb
LIC11506	F: 5' CACCC CAAGTTCTGTTTTATCTCAAGC 3' R: 5' ATGCGGAGGGAGAGATTTAG 3'	932 pb
LIC11668	F: 5' CACCC GGTACCATTACTAAACCGACTC 3' R: 5' GTTGGACCGGAAGGATTGTAA 3'	1.284 pb
LIC11935	F: 5' CACCC CTTCCCAAACGATCGAA 3' R: 5' TTCGAAACAGTAGGTAGCTTTG 3'	1.563 pb
LIC12144	F: 5' CACCG GATTAGATGCTAATGTAGCTTATC 3' R: 5' CGAGTCAAAGTATAAAATTCGC 3'	2.008 pb
LIC12217	F: 5' CACCA TTTCGGCTTGTTCCGGAG 3' R: 5' ATCGCACCCGTCCAGATAG 3'	2.151 pb
LIC12225	F: 5' CACCT TGTATCAAGAAATTGCATTGAG 3' R: 5' GCTGGTTCTGTTTTCTCCATT 3'	2.170 pb
LIC12690	F: 5' CACCT TTTCTAACTTCGCGGACTC 3' R: 5' CTATTGTTCCACACAAAGAATGC 3'	2.445 pb
LIC12730	F: 5' CACCA GTTCTGACGGACTTCCCAA 3' R: 5' TCTTGCGAATGAGTTGATCC 3'	2.137 pb
LIC12922	F: 5' CACCG AATCACTCAACAGAGTCATTGC 3' R: 5' ATCAATCTAAATGAAACGTCTCTTC 3'	949 pb
LIC13101	F: 5' CACCA AAAGGAGGAAGTTCCTTTGC 3' R: 5' TTAAATCGTTCACATCGGAAG 3'	3.720 pb
LIC20214	F: 5' CACCC GTGCACGTGTTTTTCCCG 3' R: 5' TCCGCCTTTTATGGTTTCG 3'	2.496 pb

NOTAS: Gene ID: identificação do gene no genoma *L. interrogans* sorovar Copenhageni; Abreviaturas (em inglês): F, “forward”; R, “reverse”; pb, pares de base; **CACC** sequência para clonagem direcional no pENTR/D-TOPO.

Bactérias *E. coli* DH5 α TM (Invitrogen) tornadas competentes (Hanahan, 1983) foram transformadas com 5 μ l da mistura da reação de recombinação seguindo o

protocolo: incubação 30 minutos em gelo, choque térmico (42 °C) por 2 minutos, incubação 5 minutos em gelo. Foram adicionados 300 µl de meio de cultura contendo 1% de extrato de levedura, 1,6% de peptona e 0,5% de NaCl (2YT) à bactéria. Essa mistura foi incubada 50 minutos a 37 °C, a seguir plaqueada em meio 2YT sólido (ágar 1,5%) contendo 100 µg/ml de ampicilina e incubada 16 horas a 37 °C (Sambrook e Russell, 2001).

Três clones de cada transformação foram inoculados em 6 ml de meio 2YT contendo 100 µg/ml de ampicilina (2YT-Amp), incubados 16 horas a 37 °C e a busca do clone correto contendo o inserto com tamanho esperado foi realizada por PCR com 5 µl da cultura como molde e os oligonucleotídeos 5`GAGACCACAACGGTTTCCC 3` e 5`CCTCGAATCAACCACTTTGTAC 3` que se anelam com ao vetor pDEST17 (Invitrogen) na posição 40 a 59 e 1.833 a 1.855 da fita complementar, respectivamente. A minipreparação foi realizada com kit Illustra PlasmidPrep Mini Spin (GE Healthcare, Buckinghamshire, Reino Unido) conforme protocolo básico.

3.7.2 Sequenciamento das construções

Os clones positivos foram sequenciados utilizando os oligonucleotídeos 5' TAATACGACTCACTATAGGG 3' e 5' CAGCAGCCAACTCAGTTCCT 3', que se anelam ao vetor pDEST17 (Invitrogen) na posição 21 a 41 e 1.879 a 1.899 da fita complementar, respectivamente, e o sequenciador automático *ABI PRISM_(R) 3100 Genetic Analyzer* (Applied Biosystems, Warrington, Reino Unido) empregando o método da terminação da cadeia (Sanger *et al.*, 1977). Este método emprega o uso de 2', 3'-didesoxirribonucleotídeos (ddNTPs) marcados por fluoróforos que interrompem a amplificação aleatoriamente e possibilitam a leitura automatizada através de um feixe de laser gerando um cromatograma.

Conforme recomendação da Applied Biosystems, a reação de amplificação dos fragmentos a serem sequenciados foi realizada num volume final de 20 µl contendo DNA molde 500 ng, 3,2 pmoles de oligonucleotídeo e 2 µl do tampão *BigDye®* (Applied Biosystems) que contém: ddNTPs marcados com corantes

fluorescentes específicos, dNTPs e Taq DNA polimerase. A reação foi realizada em termociclador seguindo a rotina de 40 ciclos de a) 94 °C por 10 segundos; b) 52 °C por 20 segundos; c) 60 °C por 4 minutos. A seguir, a mistura foi precipitada com isopropanol 75% e centrifugada 50 minutos a 11.500 g a temperatura ambiente. O precipitado de DNA foi lavado com 70% etanol, seco a vácuo em centrifuga e ressuspensão para aplicação em 15 µl de *formamida HiDi* (Applied Biosystems).

Os cromatogramas obtidos foram analisados com o auxílio dos programas públicos: Chromas -<http://www.mb.mahidol.ac.th/pub/chromas/chromas.htm> e Blast 2 - <http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi> (Altschul *et al.*, 1990; Altschul *et al.*, 1997).

3.8 Expressão de proteínas recombinantes em *E. coli*

Bactérias *E. coli* BL21 SI (Invitrogen) tornadas competentes (Hanahan, 1983) foram transformadas com 50 ng da construção pDEST17 (Invitrogen) e plaqueadas em meio 2YT-ON (sem adição de NaCl) sólido contendo 100 µg/ml de ampicilina e incubada 16 horas a 30 °C.

Um clone foi inoculado em meio 2YT-ON-Amp e incubado 16 horas a 30 °C sob agitação. A cultura foi diluída 1:20 e o crescimento celular foi acompanhado até atingir uma densidade ótica a 600 nm (DO_{600nm}) de 0,6-0,8. A indução da expressão da proteína recombinante foi realizada pela adição de NaCl 300 mM por três horas.

Alíquotas antes e depois da indução foram colhidas e centrifugadas 20 minutos a 3.200 g. O sedimento bacteriano induzido foi ressuspensão em tampão de lise com pH diferente do ponto isoelétrico (pI) da proteína recombinante contendo NaCl 500 mM, Tris-HCl 50 mM e Triton x-100® 0,1% e submetido a pressão no *French Pressure* (Spectronic Instruments, Garforth, Reino Unido) ou sonicação 60 Hz por 5 minutos. A suspensão clarificada por centrifugação, o sedimento obtido lavado até cinco vezes com tampão contendo ureia 1 M, NaCl 500 mM, Tris-HCl 50 mM e Triton x-100® 0,1%, e ressuspensão em tampão desnaturante contendo ureia 8 M, NaCl 500 mM e Tris-HCl 50 mM. Alíquotas do sobrenadante do lisado celular, das lavagens do sedimento e do sedimento solubilizado foram analisadas

por eletroforese em gel de poliacrilamida 12% contendo SDS (PAGE/SDS 12%) (Sambrook e Russell, 2001).

3.8.1 Expressão em outras linhagens de *E. coli*

Bactérias *E. coli* BL21 StarTM (DE3) pLysS (Invitrogen) e C43 (DE3) (Avidis) foram tornadas competentes (Hanahan, 1983), transformadas com 50 ng da construção pDEST17, plaqueadas em meio 2YT-Amp sólido e incubadas 16 horas a 37 °C. Um clone foi inoculado em meio 2YT-Amp e incubado 16 horas a 37 °C sob agitação. A cultura foi diluída 1:20 e o crescimento celular foi acompanhado até atingir uma DO_{600nm} de 0,6-0,8. A indução da expressão da proteína recombinante foi realizada pela adição de IPTG 1 mM por três horas. Alíquotas antes e depois da indução foram analisadas por PAGE/SDS 12%.

3.9 Purificação das proteínas recombinantes

A proteína recombinante, quando expressa no sobrenadante do lisado bacteriano, foi purificada no próprio tampão de lise. Quando expressa no precipitado do lisado bacteriano, a proteína recombinante foi purificada no tampão desnaturante ou após diluição por gotejamento lento (0,1 ml / minuto) em 2 L de tampão contendo NaCl 500 mM e Tris-HCl 50 mM (pH diferente do pI da proteína recombinante).

A proteína recombinante diluída foi adsorvida em fluxo de 1 ml/min em coluna de 5 ml contendo resina de afinidade metálica (*“Chelating Sepharose Fast Flow”*, GE, Uppsala, Suécia) preparada com NiSO₄ 300 mM conforme instruções do fabricante. A resina foi submetida a lavagens sucessivas com 50 ml da solução de diluição acrescida de imidazol em concentrações crescentes (5, 20 e 40 mM). A proteína recombinante foi eluída com 15 ml da solução de diluição acrescida de imidazol 1 M e coletadas a cada 1 ml. Todas as etapas do processo de purificação foram analisadas por PAGE/SDS 12%.

A concentração de proteína total foi estimada por ensaio descrito por Bradford (1976) empregando até 20 µl da amostra diluída no reagente *Coomassie (Bradford) Protein Assay* (Pierce, Rockford, IL, EUA) (Bradford, 1976; Compton e Jones, 1985). A intensidade da reação foi analisada em espectrofotômetro a 595 nm (Labsystems iEMS, Victoria, Austrália). Os resultados obtidos foram comparados com uma curva de albumina de soro bovino (BSA) como padrão.

3.9.1 Diálise

Membranas semi-permeáveis com poros de 12.000 Da foram preenchidas com cerca de 10 ml da proteína purificada (0,1 mg / ml) em tampão de eluição foram dialisados contra 1 L de solução contendo NaCl 500 mM, Tris-HCl 50 mM, EDTA 1 mM e glicina 0,1% (pH diferente do pI da proteína recombinante) sob agitação a 4 °C. Após 12 horas, o tampão foi trocado por 1 L de solução fosfato-salina (PBS, pH 7,4) e glicina 0,1%. Esta etapa foi realizada duas vezes. A amostra foi fracionada a cada 1 ml, congelada a -80 °C e liofilizada.

3.10 Dicroísmo circular

Espectros de dicroísmo circular (CD) foram captados em polarímetro Jasco J-810 (*“circular dichroism system”*, Japan Spectroscopic, Tokyo, Japan) do Laboratório Nacional de Luz Sincrotron (Campinas, SP). As amostras de proteínas foram diluídas para uma concentração final de 10 µM e dialisadas contra tampão Na-fosfato 10 mM (pH 7,4). Os dados foram tomados em cubetas de 1 mm de caminho óptico a 0,5 nm / seg (20 °C) com um *“bandwidth”* de 1 nm. Os espectros de CD foram expressos em termos de elipcidade molar residual:

$$[\Phi] = \Phi \text{ (mdeg)} / 10 \times \mathbf{C}(\text{M}) \times \mathbf{I}(\text{cm})$$

onde Φ é elipcidade, $\mathbf{C}$ é a concentração da proteína e $\mathbf{I}$ é o caminho ótico (Kelly *et al.*, 2005).

Como parâmetro de comparação, foi realizada análise de predição de estrutura teórica com o auxílio do programa público PSIPRED -<http://bioinf.cs.ucl.ac.uk/psipred/> (Jones, 1999).

3.11 Imunização de camundongos para obtenção de soro hiperimune

Dez camundongos BALB/c fêmeas (18 a 22 g) foram imunizados subcutaneamente com 10 µg de cada proteína recombinante ou PBS (controle negativo) em suspensão contendo *Alhydrogel* (2% Al(OH)₃, Brenntag Biosector, Frederikssund, Dinamarca) como adjuvante. Foram realizadas duas imunizações com intervalos de 15 dias.

Os animais foram sangrados via plexo retro-orbital sete dias após o término das imunizações. O sangue coletado foi mantido a 37 °C por 30 minutos e, posteriormente, centrifugado a 800 g por 15 minutos. Após a centrifugação o sobrenadante foi cuidadosamente coletado e armazenado a -20 °C.

3.11.1 Titulação dos anticorpos policlonais

Esses soros foram avaliados através da técnica de ELISA (*enzyme-linked immunosorbent assay*). Para adsorção dos antígenos, placas de micro-diluição foram expostas a 100 µl / poço de solução tampão carbonato/bicarbonato 0,1 M (pH 9,6) contendo 500 ng de proteína recombinante purificada e incubadas por uma noite a 4 °C. As placas foram lavadas três vezes com tampão PBS acrescido de Tween-20 0,05% (PBS-T) e bloqueadas com 200 µl / poço de PBS-T contendo leite em pó desnatado 10% por uma hora a 37 °C. As placas foram lavadas três vezes com PBS-T.

Diluições seriadas dos soros foram preparadas em 100 µl / poço de tampão PBS contendo leite em pó desnatado 1% e Tween-20 0,01% (tampão de diluição) e incubadas por uma hora a 37 °C. As placas foram lavadas três vezes com PBS-T. A

seguir, foram adicionados 100 µl / poço de tampão de diluição contendo anticorpo de cabra anti-IgG de camundongo conjugado com peroxidase (Sigma, diluição 1:10.000) e incubado por uma hora a 37 °C. As placas foram lavadas três vezes com PBS-T.

A reação foi revelada com o substrato da peroxidase, o-fenilenodiamina (OPD), na presença de traços de H₂O₂ e interrompida após 15 minutos pela adição de H₂SO₄. A sua intensidade foi analisada em espectrofotômetro a 492 nm (Labsystems iEMS). O título de anticorpos foi considerado como a maior diluição do soro capaz de resultar uma absorbância a 492 nm superior a 0,1.

3.12 Obtenção de extrato proteico de *Leptospira*

Culturas de leptospiras de sete a 10 dias tiveram sua concentração celular estimada através do uso de câmara de Petroff-Hausser em microscópio de campo escuro. Ao atingirem 10⁸ células/ml, estas culturas foram centrifugadas 30 minutos a 8.000 g (4 °C) e o sedimento lavado três vezes com tampão PBS acrescido de MgCl₂ 5 mM.

Uma fração do sedimento bacteriano foi ressuspensa em PBS acrescido de SDS 10% em um volume 100 vezes inferior ao volume da cultura. Esta alíquota foi empregada como extrato total.

3.12.1 Fracionamento com Triton X-114

O fracionamento das proteínas de *Leptospira* por Triton X-114 foi realizado segundo método descrito por Cunningham *et al.* (1988) com pequenas modificações. O sedimento bacteriano foi ressuspenso em tampão Tris 10 mM (pH 7,4), NaCl 150 mM, PMSF 1 mM gelado (TBS) acrescido de 2% de Triton X-114 (Sigma-Aldrich Corporate, St Louis, MO, EUA) em um volume 15 vezes inferior ao volume da cultura. A solução foi homogeneizada e incubada 4 horas a 4 °C com agitação

esporádica. Após esse período, o material insolúvel foi removido por centrifugação por 10 minutos a 8.800 g à 4 °C (P1). O sobrenadante (S1) foi coletado em frasco novo e congelado a -20 °C.

Após 24 horas, o sobrenadante (S1) foi descongelado lentamente em gelo, homogeneizado por inversão e incubado 10 minutos a 37 °C. Nessa etapa, forma-se uma fase aquosa (S2 - proteínas citoplasmáticas) e outra rica em Triton X-114 (proteínas de membrana). A solução foi centrifugada 3 minutos a 3.000 g (temperatura ambiente) e o sobrenadante (S2) reservado. À fase rica em detergente foi adicionado tampão TBS gelado contendo 0,06% Triton X-114 (Sigma). A suspensão foi homogeneizada, incubada 10 minutos em gelo, transferida para um banho-maria a 37 °C por 10 minutos, centrifugada 3 minutos a 3.000 g (temperatura ambiente) e o sobrenadante (S3) reservado. Foi adicionado a fase rica em detergente tampão TBS gelado contendo 0,06% Triton X-114 (Sigma). A suspensão foi homogeneizada, incubada 10 minutos em gelo e centrifugada 10 minutos a 15.800 g (0 °C) para remover o material insolúvel (P2 - proteínas protoplasmáticas).

O sobrenadante recuperado (S4) foi incubado 10 minutos a 37 °C, centrifugado 3 minutos a 3.000 g (temperatura ambiente) e o sobrenadante (S5) reservado. A fase rica em detergente possui um volume 10 vezes inferior ao volume de tampão TBS acrescido.

Alíquotas de todas as frações obtidas foram precipitadas com três volumes de acetona gelada e incubadas 30 minutos a -80 °C. As proteínas foram recuperadas por centrifugação 15 minutos a 15.800 g (0 °C). O sobrenadante foi descartado. O sedimento seco a temperatura ambiente e ressuspenso em tampão PBS em um volume 1.000 vezes inferior ao volume da cultura. As frações obtidas, bem como o extrato total, foram avaliadas por PAGE/SDS 12%.

3.12.2 Detecção das proteínas de leptospiros por Western blotting

Uma alíquota de cada preparado proteico foi separada por PAGE/SDS 12% e transferida para membrana de nitrocelulose (*Hybond ECL*, GE) em sistema semi-

úmido (GE) por uma hora e 30 minutos a 0,8 mA por cm² em Tris-Glicina acrescido de 1,85 % de SDS (tampão de transferência).

A eficiência da transferência foi avaliada por coloração de *Ponceau S* (Sigma). As proteínas foram descoradas com tampão PBS-T sob agitação e a membrana bloqueada com PBS-T contendo leite em pó desnatado 5% e BSA 2,5% (tampão de bloqueio) por 12 horas a 4 °C. A membrana foi lavada três vezes por 10 minutos sob agitação com PBS-T e incubada por três horas com anticorpo policlonal específico para cada proteína recombinante teste (diluição na qual absorbância a 492 nm igual a 2,0) em tampão de bloqueio. A membrana foi lavada e incubada por uma hora com anticorpo de cabra anti-IgG de camundongo conjugado com peroxidase (Sigma, diluição 1:5.000) em tampão de bloqueio. A membrana foi novamente lavada e as frações detectadas através da utilização do kit ECL (GE) conforme instruções do fabricante. Filmes de raio-X (Kodak, Cedex, França) foram expostos à membrana por tempos curtos (1 a 10 minutos) e em seguida revelados.

3.13 Imunofluorescência em fase líquida

Alíquotas de 2,5 ml de cultura com sete dias de crescimento foram centrifugadas 15 minutos a 11.500 g. O sobrenadante foi descartado e o sedimento lavado duas vezes com 2,5 ml de tampão PBS (NaCl 50 mM). O sedimento foi ressuspenso em 200 µl de tampão PBS (NaCl 50 mM) contendo 2 µg de iodeto de propídio e incubado 45 minutos a 37 °C no escuro. As células foram centrifugadas 15 minutos a 11.500 g e lavadas duas vezes com 1 ml de tampão PBS (NaCl 50 mM). O sedimento foi ressuspenso em 200 µl de tampão PBS (NaCl 50 mM) gelado contendo com anticorpo policlonal específico para cada proteína recombinante teste (diluição 1:50) e incubado 30 minutos em gelo. As células foram centrifugadas 15 minutos a 11.500 g e lavadas duas vezes com 1 ml de tampão PBS (NaCl 50 mM). O sedimento foi ressuspenso em 100 µl de tampão PBS (NaCl 50 mM) gelado contendo com anticorpo de cabra anti-IgG de camundongo conjugado com isotiocianato de fluoresceína (FITC, Sigma, diluição 1:50) e incubado 30 minutos em gelo no escuro. As células foram centrifugadas 15 minutos a 11.500 g

e lavadas duas vezes com 1 ml de tampão PBS (NaCl 50 mM). O precipitado foi ressuspenso em 50 µl de tampão PBS (NaCl 50 mM) gelado acrescido de uma gota do reagente *ProLong Gold antifade* (Invitrogen).

Imagens foram captadas em microscópio de imunofluorescência confocal LSM 510 META (Carl Zeiss Inc., Alemanha) do Departamento de Parasitologia do Instituto Butantan (São Paulo, SP). Empregamos aumento de 600 vezes (FITC, excitação 488 nm, emissão 500-550 nm; Iodeto de propídio, excitação 543 nm, emissão 612-619 nm).

3.14 Reatividade das proteínas com soro de indivíduos com leptospirose

3.14.1 Adsorção do soro em extrato de *E. coli*

O extrato bacteriano da mesma linhagem empregada na expressão das proteínas recombinantes foi obtido a partir cultura saturada de *E. coli*. A cultura foi centrifugada 20 minutos a 3.200 g (4 °C), o sedimento bacteriano foi ressuspenso em 1/10 do volume inicial com PBS-T e lisado por sonicação a 60 Hz por 5 minutos. Os debris foram removidos por centrifugação e o sobrenadante aliquoteado e armazenado à -20 °C.

Os soros-teste foram diluídos em tampão contendo extrato bacteriano 25%. Esta solução foi incubada por uma hora a temperatura ambiente e empregada nos imunoenaios (Western blotting ou ELISA).

3.14.2 ELISA com soro de indivíduos com leptospirose

Para adsorção dos antígenos, placas de micro-diluição foram expostas a 50 µl / poço de solução tampão carbonato/bicarbonato 0,1 M (pH 9,6) contendo 200 ng de proteína recombinante purificada e incubadas por uma noite a

temperatura ambiente. As placas foram lavadas três vezes com PBS-T e bloqueadas com 200 µl / poço de PBS-T contendo leite em pó desnatado 5% e BSA 2,5% (tampão de bloqueio) por duas horas a 37 °C.

A solução de bloqueio foi descartada e às placas foram adicionados 50 µl / poço de tampão de bloqueio contendo o soro-teste diluído 1:100 em duplicata. As placas foram incubadas por duas horas a temperatura ambiente e, em seguida, lavadas seis vezes com PBS-T. Foram adicionados as placas 50 µl / poço de tampão de bloqueio contendo o anticorpo de cabra anti-IgG de humano conjugado com peroxidase (Sigma, diluição 1:5.000) por uma hora a temperatura ambiente. As placas foram lavadas três vezes com PBS-T. A reação foi revelada o substrato OPD como descrito anteriormente (item 3.11.1 Titulação dos anticorpos policlonais).

Foram realizados ensaios com soros de seis indivíduos normais e de 28 indivíduos previamente diagnosticados infectados com leptospirose (ANEXO A). O valor de *cut-off* foi calculado através da média das absorbâncias obtidas a 492 nm para os soros normais mais três desvios padrões.

3.14.3 Western blotting

Frações contendo em média 2 µg de cada proteína recombinante purificadas da fração insolúvel do lisado de *E. coli* em tampão contendo ureia 8 M foram separadas por PAGE/SDS 12% e transferidas para membrana de nitrocelulose (*Hybond ECL*, GE) em sistema semi-úmido (GE) como descrito anteriormente. A membrana foi bloqueada e incubada por três horas com soro-teste diluído em tampão de bloqueio. A seguir, a membrana foi lavada e incubada com soro de cabra anti-IgG humano conjugado com peroxidase (Sigma, diluição 1:5.000) em tampão de bloqueio. As frações foram detectadas através do kit ECL (GE) e filmes de raio-X (Kodak) como descrito anteriormente (item 3.12.2 Detecção das proteínas de leptospiros por Western blotting).

3.15 Ensaio de adesão a componentes de matriz extracelular

3.15.1 por ELISA

Os experimentos de adesão a componentes de matriz extracelular imobilizados foram realizados segundo método descrito por Cameron *et al.* (2003). Componentes de matriz extracelular (1 µg de laminina, colágeno I e IV, fibronectina plasmática e celular, BSA e fetuína - Sigma) em PBS foram adsorvidos em placas de microdiluição por três horas a 37 °C.

As placas foram lavadas três vezes com PBS-T e bloqueadas com tampão PBS contendo BSA 1% por uma hora a 37 °C e por uma noite a 4 °C. A solução de bloqueio foi descartada e às placas foram adicionados 100 µl / poço de solução tampão PBS contendo 1 µg proteína recombinante purificada. As placas foram incubadas uma hora e 30 minutos a 37 °C e, em seguida, lavadas seis vezes com PBS-T. Foram adicionados 100 µl / poço de PBS contendo anticorpo policlonal específico para cada proteína recombinante teste (diluição na qual absorbância a 492 nm igual a 1,0) e incubada por uma hora a 37 °C. As placas foram lavadas três vezes com PBS-T. A seguir, foram adicionados 100 µl / poço de PBS contendo anticorpo de cabra anti-IgG de camundongo conjugado com peroxidase (Sigma, diluição 1:5.000) por uma hora a 37 °C. As placas foram lavadas três vezes com PBS-T. A reação foi revelada o substrato OPD como descrito anteriormente (item 3.11.1 Titulação dos anticorpos policlonais).

Para cada proteína teste, quatro poços foram adsorvidos com determinado componente de matriz extracelular, sendo que um deles não foi adicionado proteína recombinante a fim de mensurar a reatividade basal (controle branco) (Cameron, 2003; Barbosa *et al.*, 2006).

3.15.2 por Western blotting

Foram realizados experimentos de adesão a componente de matriz extracelular por Western blotting segundo método descrito por Stevenson (2007). Neste ensaio, empregamos membranas de nitrocelulose (*Hybond ECL*, GE) contendo 2 µg de cada proteína recombinante. As membranas foram incubadas com PBS-T contendo BSA 5% por uma noite a 4 °C. As membranas foram, então, lavadas com PBS-T e incubadas por uma hora com 1 µg / ml de componente de matriz (laminina, colágeno I e IV, fibronectina celular e plasmática - Sigma) em PBS-T. As membranas foram novamente lavadas e incubadas por uma hora com soro de coelho ou camundongo específico contra o componente de matriz empregado (Sigma, diluição 1:1.000) em PBS-T, novamente lavadas e, então incubadas com soro de cabra anti-IgG de coelho ou camundongo conjugado com peroxidase (Sigma, 1:10.000) em PBS-T. Após lavagem as frações reativas foram detectadas através do kit ECL (GE) e filmes de raio-X (Kodak) conforme descrito anteriormente. Como controle da especificidade da reação do anticorpo, suprimimos o componente de matriz extracelular (Stevenson *et al.*, 2007).

3.15.3 Tratamento da laminina com metaperiodato

Laminina (1 µg) em tampão acetato de sódio 50 mM (pH 5) foi tratada com concentrações crescentes de metaperiodato de sódio (5-100 mM) por 15 minutos a 4 °C no escuro. A adesão das proteínas recombinantes foi avaliada como descrito anteriormente.

3.16 Imunização de hamsters para ensaio de imunoproteção

Os ensaios de imunoproteção foram realizados no laboratório de Zoonoses da Faculdade de Medicina Veterinária da USP. Grupos de quatorze hamsters machos recém-desmamados (80 a 120 g) foram imunizados subcutaneamente no dorso com 50 µg de proteína recombinante, vacina comercial *Farrow Sure B* (Pfizer, Platte City, MO, EUA, diluição 1:40), bacterina ou PBS (controle negativo) em suspensão contendo *Alhydrogel* (Brenntag) como adjuvante. Foram realizadas duas imunizações com intervalos de 15 dias. Dois animais foram sangrados via punção cardíaca 15 dias após cada imunização. Os soros foram avaliados por ELISA semelhante ao descrito para soro hiperimune produzido em camundongo. Após a diluição do soro na placa de microdiluição, incubava-se com anticorpo de coelho anti-IgG de hamster (Sigma, diluição 1:5.000) e a seguir, com anticorpo de cabra anti-IgG de coelho conjugado com peroxidase (Sigma, diluição 1:5.000). A reação foi revelada com OPD como descrito anteriormente.

3.16.1 Preparo da bacterina

A bacterina foi preparada conforme descrito por Silva *et al.* (2007). Cultura de *L. interrogans* sorovar Copenhageni (FIOCRUZ L1-130) virulenta recém isolada de animal infectado teve sua concentração celular estimada através do uso de câmara de Petroff-Hausser em microscópio de campo escuro. Esta cultura foi centrifugada 20 minutos a 8.000 g (temperatura ambiente) e o sedimento lavado duas vezes com tampão PBS acrescido de MgCl₂ 5 mM. O sedimento foi ressuspenso em PBS e incubadas 20 minutos a 56 °C. Alíquotas foram armazenadas a -20 °C. Cada dose de imunização para os hamsters continha 10⁹ leptospiplas inativadas (Silva *et al.*, 2007).

3.16.2 Desafio

No 30º dia após a 1ª imunização, os animais foram infectados por inoculação intraperitoneal de 200 µl de cultura de *L. interrogans* sorovar Copenhageni (FIOCRUZ L1-130) virulenta com apenas três passagens em cultura. Através de contagem em câmara de Petroff-Hausser em microscópio de campo escuro, a dose foi ajustada para 2×10^5 células / animal. Os animais foram observados por 21 dias e os sobreviventes foram sacrificados. Foi coletado sangue via punção cardíaca e seu soro avaliado por MAT. Os rins dos animais sobreviventes foram coletados, macerados, suspensos em solução salina (diluição 1:10 e 1:100) e cultivados em meio EMJH modificado semi-sólido a 28 °C para avaliação da presença de leptospiros após 6 semanas de observação.

A análise de significância estatística foi realizada com auxílio do programa One-Way ANOVA - <http://faculty.vassar.edu/lowry/VassarStats.html>.

3.16.3 Teste de microaglutinação (MAT)

O ensaio de MAT foi realizado no laboratório de Zoonoses da Faculdade de Medicina Veterinária da USP conforme descrito por Faine *et al.* (1999). Empregamos uma bateria de 22 sorovares de *Leptospira* spp como antígeno: Andaman, Australis, Autumnalis, Bataviae, Butembo, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Shermani, Tarassovi e Wolffi. Todas as linhagens são mantidas em meio EMJH modificado a 28 °C

Um teste é determinado positivo quando há um aumento de pelo menos quatro vezes na microaglutinação de amostras de soro pareadas. O sorovar que apresentar um título de aglutinação de 50% na maior diluição é considerado o provável sorotipo predominante. O teste é considerado negativo se não houver microaglutinação com nenhum dos 22 sorovares listados acima.

4 Resultados e Discussão

4.1 Análise das características das proteínas selecionadas

As vacinas anti-leptospirose hoje disponíveis são constituídas de bactérias inativadas e induzem a produção de anticorpos anti-LPS que protegem somente contra os sorovares presentes na preparação (Faine *et al.*, 1999; Levett, 2001). Sonrier *et al.* (2000) demonstraram que extrato proteico induz proteção contra desafio heterólogo. Desde então, iniciou-se a busca de proteínas antigênicas que sejam conservadas nas espécies patogênicas de *Leptospira* (Plank e Dean, 2000; Sonrier *et al.*, 2000; Palaniappan *et al.*, 2007).

Atualmente, três espécies de *Leptospira* spp foram sequenciadas: *L. interrogans* sorovar Lai (Ren *et al.*, 2003) e sorovar Copenhageni (Nascimento *et al.*, 2004a; Nascimento *et al.*, 2004b), *L. borgpetersenii* sorovar Hardjo-bovis (Bulach *et al.*, 2006) e *L. biflexa* sorovar Patoc (Picardeau *et al.*, 2008). Selecionamos para o presente trabalho 15 genes de *L. interrogans* sorovar Copenhageni e através da análise comparativa das sequências de aminoácidos preditas, verificou-se que todas as proteínas são altamente conservadas em *Leptospira* spp e não possuem função esclarecida (Tabela 3). O LIC12730 é, inclusive, encontrado em *Treponema* spp com mais de 20% de identidade.

O estudo de proteínas de membrana é importante porque estas estão em contato com o hospedeiro e contribuem para a sobrevivência no meio ambiente. Inclusive, algumas proteínas de membrana externa são reguladas de forma diferenciada nas condições *in vivo* e *in vitro* (Lo *et al.*, 2006; Matsunaga *et al.*, 2007). Todas as proteínas selecionadas possuem sítio de clivagem e devem ser encaminhadas a membrana da leptospira (Tabela 3). Sete (LIC10368, LIC10494, LIC10498, LIC11207, LIC12144, LIC12217, LIC12690) possuem, adicionalmente, sítio de lipidação e provavelmente são ancoradas na membrana através do lipídeo.

Tabela 3 – Resumo da análise *in silico* da conservação dos genes em *Leptospira spp* e localização celular.

Gene ID	Descrição do produto gênico	Conservação (identidade)	Predição de localização celular (probabilidade)
LIC10368	Provável lipoproteína hipotética sem domínio conhecido	Lai (100%)	membrana externa (>70%)
LIC10494	Provável lipoproteína hipotética sem domínio conhecido	Lai (99%)	membrana externa (>75%)
LIC10498	Provável lipoproteína hipotética com domínio de função desconhecida DUF1566	Lai (100%)	membrana externa (>80%)
LIC11207	Provável lipoproteína hipotética com domínio de função desconhecida DUF1565	Lai (98%) LBH (45 - 62%)	membrana externa (>40%)
LIC11506	Proteína de membrana externa hipotética sem domínio conhecido	Lai (96%) LBH (61%)	membrana externa (>30%)
LIC11668	Proteína de membrana externa hipotética sem domínio conhecido	Lai (99%) LBH (81%) LBP (33%)	membrana externa (>35%)
LIC11935	Proteína de membrana externa hipotética sem domínio conhecido	Lai (99%) LBP (37%)	membrana externa (>30%)
LIC12144	Proteína integral de membrana hipotética com domínio de função desconhecida DUF1420	Lai (99%)	membrana interna (>50%)
LIC12217	Provável lipoproteína hipotética com domínio de ligação a cobre	Lai (99%) LBH (84%)	membrana interna (>40%)
LIC12225	Proteína de membrana externa hipotética sem domínio conhecido	Lai (99%) LBP (33%)	membrana externa (>40%)
LIC12690	Provável lipoproteína hipotética com domínio de função desconhecida DUF1554	Lai (99%)	membrana externa (>30%)
LIC12730	Proteína hipotética com domínio TPR e mais 4 repetições NHL	Lai (100%) LBH (90%) LBP (37%)	membrana externa (80%)
LIC12922	Proteína hipotética sem domínio de função conhecida	Lai (100%) LBH (89%) LBP (48%)	membrana interna (>30%)
LIC13101	Proteína integral de membrana hipotética com domínio DUF529 e 5 domínio de repetição semelhante a alfa-integrina (FG-GAP)	Lai (79%)	membrana externa (80%)
LIC20214	Proteína de membrana externa hipotética com domínio de receptor dependente de TonB	Lai (100%) LBH (84%) LBP (53%)	membrana externa (100%)

NOTAS: Gene ID: identificação do gene no genoma *L. interrogans* sorovar Copenhageni; Abreviaturas: Lai: *L. interrogans* sorovar Lai; LBH: *L. borgpetersenii* sorovar Hardjo-bovis; LBP: *L. biflexa* sorovar Patoc.

A análise da sequência ainda nos permitiu encontrar para alguns dos genes selecionados, alguns possíveis parálogos dentro do genoma da *L. interrogans* sorovar Copenhageni:

- LIC10498 possui um possível parálogo LIC10497 (34% de identidade) e dois domínios de função desconhecida DUF1566;
- LIC11207 possui quatro possíveis parálogos: LIC10365 (50% de identidade), LIC10774 (38%), LIC10821 (50%) e LIC11030 (34%), todos possuem o domínio de função desconhecida DUF1565. O gene LIC10365 codifica uma proteína capaz de ativar o endotélio, sendo assim uma potencial molécula efetora da resposta inflamatória (Vieira *et al.*, 2007);
- LIC11506 possui um possível parálogo LIC13238 (26% de identidade) que codifica uma proteína hipotética sem domínio conhecido;
- LIC12690 possui identidade (17 a 23%) com proteínas da família Len (*Leptospiral endostatin-like proteins*) (Stevenson *et al.*, 2007). Todas possuem o domínio de função desconhecida DUF1554, que partilha características estruturais com endostatina de mamíferos. LenA e LenB se ligam ao fator H humano. Todas as proteínas Len se ligam a laminina e fibronectina, exceto a LenA que só adere ao primeiro componente (Barbosa *et al.*, 2006; Stevenson *et al.*, 2007). Estas características sugerem que estas proteínas juntas facilitem a invasão e colonização de tecidos hospedeiros, enquanto a adesão ao fator H permite a proteção contra a resposta imune durante o processo de infecção (Stevenson *et al.*, 2007);
- LIC13101 possui dois possíveis parálogos: LIC12259 e LIC10021 (54% e 29% de identidade, respectivamente). Ambas possuem mais de 6 repetições FG-GAP. Proteínas com função de alfa-integrina possuem 7 repetições ou mais desse domínio FG-GAP e são potenciais adesinas. Como característica adicional é um pseudogene na *L. borgpetersenii* sorovar Hardjo (Bulach *et al.*, 2006).

4.2 Análise da conservação dos genes entre os diversos sorovares de *Leptospira*

As leptospiros são classificadas em sorovares de acordo com a composição antigênica de seus lipopolissacarídeos. Essa diversidade se revela no amplo número de espécies de mamíferos que servem como hospedeiro reservatório e em mais de 200 sorovares descritos (Haake e Matsunaga, 2005). Dessa forma, a busca por proteínas conservadas entre os sorovares é indispensável para a obtenção de bons antígenos para diagnóstico ou preparações vacinais. Com essa finalidade, foram escolhidos seis sorovares de *L. interrogans* (espécie patogênica) e o sorovar Patoc de *L. biflexa* (espécie saprófita). A escolha dos sorovares foi baseada nos isolados em humanos (sorovares Canicola, Copenhageni, Grippotyphosa, Icterohaemorrhagiae, Pomona) ou gado (sorovar Hardjo) mais prevalentes no Brasil (Pereira *et al.*, 2000; Romero e Yasuda, 2006; Thompson *et al.*, 2006).

A conservação foi avaliada por PCR empregando oligonucleotídeos sintetizados com base na sequência do genoma da *L. interrogans* sorovar Copenhageni e o DNA genômico de cada um dos sorovares. Na Figura 5, observa-se o padrão de amplificação dos 15 genes deste trabalho. Embora, alguns genes estejam presentes em *L. biflexa* sorovar Patoc, estes oligonucleotídeos não foram eficientes em amplificá-los, provavelmente devido a baixa similaridade entre as sequências.

Os genes LIC10368, LIC10494, LIC11506, LIC11668, LIC12144, LIC12225, LIC12690 e LIC13101 não foram amplificados a partir do DNA genômico de *L. interrogans* sorovar Grippotyphosa. Este sorovar é mais comumente encontrado em marsupiais (Bharti *et al.*, 2003) e este resultado sugere que existam diferenças consideráveis entre o seu genoma e o dos demais sorovares. O gene LIC11506 somente foi amplificado a partir do DNA genômico dos sorovares Copenhageni e Icterohaemorrhagiae, que são do mesmo sorogrupo e compartilham características antigênicas.

Figura 5 – Análise da conservação dos genes selecionados em sorovares de *L. interrogans* prevalentes no Brasil e em *L. biflexa* sorovar Patoc através de eletroforese em gel de agarose 1% corado com brometo de etídeo. Coluna 1, *L. biflexa* sorovar Patoc, colunas de 2 a 7, *L. interrogans* sorovares Canicola (2), Copenhageni (3), Grippotyphosa (4), Hardjo (5), Icterohaemorrhagiae (6) e Pomona (7).

4.3 Análise da expressão dos genes entre os diversos sorovares de *Leptospira*

Leptospiras mantidas em cultura por longo período tendem a perder a virulência, isto é, não são capazes de promover a infecção. A linhagem LPF de *L. interrogans* sorovar Pomona é mantida virulenta através de passagens em hamsters (Faine *et al.*, 1999; Nally *et al.*, 2004) e foi adicionada neste experimento no intuito de avaliar se há alteração na expressão dos genes. Os RNAs extraídos foram submetidos a RT-PCR, empregando os mesmos oligonucleotídeos do estudo de

conservação mais um par para o RNAr 16S, que serve como controle da qualidade e quantidade de transcritos. O perfil eletroforético dos produtos obtidos está apresentado na Figura 6.

Figura 6 – Análise da expressão dos genes selecionados em sorovares de *L. interrogans* prevalentes no Brasil e em *L. biflexa* sorovar Patoc através de eletroforese em gel de agarose 1% corado com brometo de etídeo. Coluna 1, *L. biflexa* sorovar Patoc, de 2 a 8, *L. interrogans* sorovares Canicola (2), Copenhageni (3), Grippotyphosa (4), Hardjo (5), Icterohaemorrhagiae (6), Pomona (7) e Pomona linhagem LPF virulenta (8). RT+: transcriptase reversa presente na reação; RT-: transcriptase reversa omitida.

Somente os genes LIC10498 e LIC12217 foram amplificados a partir do cDNA da *L. interrogans* sorovar Grippotyphosa, sugerindo haver diferenças consideráveis entre seu fenótipo e os dos demais sorovares estudados. Os genes LIC11506, LIC12144 e LIC12690 não apresentaram amplificação a partir dos DNA

genômico do sorovar Pomona, assim não foi possível avaliar se há ou não alteração de sua expressão entre linhagens virulentas ou não.

Entre os genes avaliados, LIC10368, LIC10498 e LIC13101 se destacaram por apresentarem aumento em sua expressão na linhagem mantida virulenta, enquanto os genes LIC11207, LIC12225 e LIC20214 apresentaram redução na expressão na linhagem mantida virulenta. Para assegurar esta variação de expressão se faz necessária a avaliação por uma técnica quantitativa como Real Time PCR, mas este resultado dá indício que os três primeiros genes são regulados negativamente na adaptação ao meio de cultura, enquanto os três últimos são regulados positivamente.

Outros genes de leptospira também exibem diferenças na expressão durante o processo de infecção e quando as condições de cultivo são alteradas. Com intuito de entender as alterações ocorridas na adaptação da leptospira ao meio ambiente ou ao hospedeiro, Lo *et al* (2006) avaliaram as alterações transcricionais ocorridas em culturas mantidas a 20 °C, 30 °C, 37 °C e 39 °C. Dentre diversos genes, constataram que LIC11207, LIC11506 e LIC13101 foram regulados positivamente na condição fisiológica (37 °C e 39 °C). No mesmo empenho, Matsunaga *et al* (2007) avaliaram a regulação gênica em culturas com baixa osmolaridade (67 mOsM) ou acrescidas de NaCl para obter uma osmolaridade equivalente a fisiológica (300 mOsM). Igualmente identificaram diversos genes interessantes e, dentre eles, LIC11207, LIC11935, LIC12217 e LIC12225 que foram regulados negativamente na condição fisiológica. Os resultados obtidos a respeito da regulação do gene LIC11207 nas condições analisadas são conflitantes (Lo *et al.*, 2006; Matsunaga *et al.*, 2007). Nosso experimento avalia duas linhagens de *L. interrogans* sorovar Pomona, uma mantida virulenta e outra não, ambas sob as mesmas condições de cultivo, o que permite detectar alterações entre o estado de virulência, diferentemente desses estudos que simulavam o processo de infecção. De qualquer modo os dados destes estudos mostram que a expressão dos genes LIC11207 e LIC13101 é sensível as diferentes condições testadas.

4.4 Caracterização da regulação da expressão do gene LIC10368

O gene LIC10368 se destacou neste primeiro ensaio por expressar fortemente somente na linhagem LPF virulenta. Visando avaliar o efeito da atenuação em cultura através de passagens *in vitro*, empregamos duas linhagens virulentas de *L. interrogans* sorovar Pomona (LPF) e sorovar Canicola (LO4) isoladas de animais infectados e passadas em cultura somente uma vez (P1), três vezes (P3), quatro vezes (P4) ou muitas vezes (HP, *high-passage*). O RNA dessas culturas foi extraído e submetido a RT-PCR, o perfil eletroforético está apresentado na Figura 7. Observamos significativa redução da expressão do gene LIC10368, em P3 e P4, indicando que existe uma regulação negativa desse gene durante cultivo *in vitro* e assim, uma associação entre virulência da *Leptospira* e expressão do gene LIC10368 (Atzingen *et al.*, 2008).

Figura 7 - Análise da expressão do gene LIC10368 em *L. interrogans* durante atenuação em cultura através de eletroforese em gel de agarose 1% corado com brometo de etídeo. O RNA foi extraído de linhagens virulentas de *L. interrogans* (A) sorovar Pomona linhagem LPF e (B) sorovar Canicola linhagem LO4 isoladas de animais infectados e passadas em cultura somente uma vez (P1), três vezes (P3), quatro vezes (P4) ou muitas vezes (HP, *high-passage*). RT+: transcriptase reversa presente na reação; RT-: transcriptase reversa omitida; M: padrão de tamanho molecular. O gráfico a direita representa a leitura densitométrica da intensidade das bandas.

FONTE: Atzingen *et al.*, 2008.

As condições de cultivo foram alteradas para avaliar se fatores ambientais, como osmolaridade e temperatura, poderiam influenciar na regulação do gene LIC10368. Cultura de *L. interrogans* sorovar Pomona (LPF) isoladas de animais infectados foi inoculada em meio EMJH contendo somente 1% de soro de coelho. Após sete dias de crescimento, as leptospiplas foram ressuspensas em meio EMJH fresco contendo 1% de soro de coelho (controle) acrescido de NaCl 120 mM (mimetizando a condição fisiológica, 300 mOsM) ou EMJH modificado (10% de soro de coelho, condição padrão de cultivo). O RNA dessas culturas foi extraído e submetido a RT-PCR, o perfil eletroforético está apresentado na Figura 8. Os transcritos do gene LIC10368 foram detectados somente após incubação com meio acrescido de NaCl ou em meio EMJH modificado que contém mais sais e soro, demonstrando que a expressão do gene LIC10368 foi regulada positivamente pela osmolaridade do meio de cultura (Atzingen *et al.*, 2008).

Figura 8 – Análise da regulação por osmolaridade da expressão do gene LIC10368 em *L. interrogans* de eletroforese em gel de agarose 1% corado com brometo de etídeo. Cultura de *L. interrogans* sorovar Pomona (LPF) com sete dias de crescimento foi ressuspensa em meio EMJH fresco contendo 1% de soro de coelho (1%) acrescido de NaCl 120 mM (1% + NaCl) ou EMJH modificado (10% EM). RT+: transcriptase reversa presente na reação; RT-: transcriptase reversa omitida; M: padrão de tamanho molecular. O gráfico a direita representa a leitura densitométrica da intensidade das bandas.

FONTE: Atzingen *et al.*, 2008.

A temperatura é outro sinal do meio ambiente que afeta a expressão de proteínas bacterianas. No intuito de avaliar essa influência, cultura de *L. interrogans* sorovar Pomona (LPF) isoladas de animais infectados foi inoculada em meio EMJH modificado e incubada a 20 °C, 29 °C e 37 °C por sete dias. A fim de analisar a regulação ocorrida na mudança de temperatura, culturas com sete dias de

crescimento foram transferidas por 16 horas de 29 °C para 37 °C e de 37 °C para 39 °C. Essas mudanças simulam o estágio inicial da infecção ou um estágio febril. O RNA dessas culturas foi extraído e submetido a RT-PCR, o perfil eletroforético está apresentado na Figura 9. Quando comparamos as culturas 20 °C, 29 °C e 37 °C, observamos um aumento gradual da expressão do gene LIC10368. A mudança de temperatura de 29 °C para 37 °C não afetou a expressão do gene LIC10368, enquanto a mudança de 37 °C para 39 °C parece reduzir a expressão desse gene (Atzingen *et al.*, 2008).

Figura 9 - Análise da regulação por temperatura da expressão do gene LIC10368 em *L. interrogans* de eletroforese em gel de agarose 1% corado com brometo de etídeo. Cultura de *L. interrogans* sorovar Pomona (LPF) foi incubada a 20 °C, 29 °C e 37 °C por sete dias. Culturas com sete dias de crescimento foram transferidas por 16 horas de 29 °C para 37 °C e de 37 °C para 39 °C. RT+: transcriptase reversa presente na reação; RT-: transcriptase reversa omitida; M: padrão de tamanho molecular. O gráfico a direita representa a leitura densitométrica da intensidade das bandas.

FONTE: Atzingen *et al.*, 2008.

A passagem *in vitro* atenua a expressão do gene LIC10368 enquanto as condições fisiológicas favorecem. Estes resultados em conjunto sugerem um papel na patogenicidade da *L. interrogans* (Atzingen *et al.*, 2008).

4.5 Confirmação da clonagem no pDEST17

Durante o Projeto Piloto do Genoma Funcional da *L. interrogans* diversos genes foram amplificados e clonados no vetor de entrada pENTR/D-TOPO (Invitrogen). A confirmação da recombinação no vetor de expressão pDEST17

(Invitrogen) foi realizada por PCR e alíquotas desses produtos foram analisadas através de eletroforese em gel de agarose 1%, como mostrado na Figura 10.

Figura 10 – Análise da eficiência da clonagem através de eletroforese em gel de agarose 1% corado com brometo de etídeo. Insertos de DNA amplificados por PCR a partir do vetor pDEST17 (Invitrogen) após a reação de recombinação. **M** indica o padrão de tamanho molecular em pares de base (pb), **1** - LIC10368 (675 pb); **2** - LIC10494 (879 pb); **3** - LIC10498 (2.919 pb); **4** - LIC11207 (1.087 pb); **5** - LIC11506 (1.016 pb); **6** - LIC11668 (1.368 pb); **7** - LIC11935 (1.647 pb); **8** - LIC12144 (2.092 pb); **9** - LIC12217 (2.235 pb); **10** - LIC12225 (2.254 pb); **11** - LIC12690 (2.529 pb); **12** - LIC12730 (2.140 pb); **13** - LIC12922 (1.033 pb); **14** - LIC13101 (3.804 pb); **15** - LIC20214 (2.580 pb).

O sequenciamento de todas as construções confirmou a clonagem quanto à fase de leitura. As regiões 5' e 3' dos cromatogramas estão apresentados em ANEXO B. Na construção pDEST17-LIC10498 houve a inserção de nucleotídeos que resultam na adição de sete aminoácidos na região N-terminal da proteína recombinante sem afetar a fase de leitura.

4.6 Avaliação da expressão das proteínas recombinantes em *E.coli*

Entre os diversos sistemas para produção de proteína heteróloga, a bactéria Gram-negativa *E. coli* continua sendo o mais atrativo. Isto se deve a habilidade de crescer rapidamente e alcançar alta densidade em substrato de baixo custo, a genética bem caracterizada, a existência de um amplo número de vetores de clonagem e cepas mutantes (Baneyx, 1999). Neste trabalho empregamos a cepa

BL21 SI (Invitrogen) que sendo induzível pela adição de NaCl ao meio de cultivo garante um custo ainda menor para a produção de proteínas recombinantes (Bhandari e Gowrishankar, 1997).

Na Tabela 4 estão descritos o resultado dos cálculos de massa molecular e *pi* teórico das proteínas recombinantes que foram realizados a partir da composição de aminoácidos, utilizando o programa

ProtParam - <http://www.expasy.org/tools/protparam.html> (Gasteiger *et al.*, 2005).

Tabela 4 – Massa molecular e *pi* teórico das proteínas codificadas pelos genes selecionados.

Gene ID	Massa molecular (Da)	<i>pi</i> teórico
LIC10368	20992.5	7.05
LIC10494	25869.8	7.87
LIC10498	102459.9	5.28
LIC11207	38015.2	6.58
LIC11506	37881.1	7.78
LIC11668	49520.4	7.62
LIC11935	60437.6	6.61
LIC12144	77758.1	9.48
LIC12217	73472.0	7.63
LIC12225	78079.2	6.32
LIC12690	94490.8	6.16
LIC12730	77415.1	9.29
LIC12922	40073.5	6.59
LIC13101	126397.7	4.57
LIC20214	95290.6	8.63

NOTA: Gene ID: identificação do gene no genoma *L. interrogans* sorovar Copenhageni.

A expressão das proteínas recombinantes na linhagem bacteriana BL21 SI (Invitrogen) foi bem sucedida para 11 das 15 proteínas recombinantes e sua solubilidade foi avaliada. Todas as frações foram analisadas por PAGE/SDS 12% e estão apresentadas na Figura 11. Os resultados obtidos mostraram expressão significativa dessas proteínas em comparação com o controle não induzido. A proteína rLIC10494 apresentou migração próximo ao marcador de 30 kDa, diferentemente da massa molecular teórica (25,8 kDa).

Figura 11 – Análise das proteínas recombinantes expressas em *E. coli* BL21 SI (Invitrogen) através de PAGE/SDS 12%. A - rLIC10368; B - rLIC10494; C - rLIC10498; D - rLIC11207; E - rLIC11668; F - rLIC11935; G - rLIC12690; H - rLIC12730; I - rLIC12922; J - rLIC13101; L - rLIC20214. NI - clone não induzido; I - clone induzido com NaCl 300mM. S - fase solúvel do lisado e P - fase insolúvel. M - padrão de massa molecular (em kDa). As setas indicam a massa molecular esperada para cada proteína recombinante. As proteínas foram coradas com azul de Coomassie.

Em comparação com a fração solúvel do lisado bacteriano, todas as proteínas recombinantes foram expressas na fase insolúvel na forma de corpúsculo de inclusão (Figura 11). A formação de corpúsculos de inclusão em sistemas de expressão de proteínas recombinantes é resultado de um desequilíbrio entre a agregação e solubilização das proteínas *in vivo* durante o processo de dobramento de sua estrutura. As proteínas recombinantes insolúveis chegam a contribuir com 50 a 95% do material proteico dos corpúsculos de inclusão e sua degradação por proteases é limitada (Sorensen *et al.*, 2004).

A solubilização de proteínas expressas em corpúsculos de inclusão requer o uso de agentes desnaturantes como ureia ou guanidina, contudo a remoção desses agentes e renaturação da proteína nem sempre é bem sucedida. A agregação de proteínas recombinantes pode ser minimizada através do controle de parâmetros como temperatura, taxa de expressão entre outros (Sorensen *et al.*, 2004).

Com o intuito de reduzir a taxa de expressão e assim favorecer a purificação das proteínas recombinantes na forma solúvel foi realizado uma cinética de expressão através da redução da concentração de agente indutor para 50 e 25% da concentração padrão. As proteínas rLIC10494, rLIC12730 e rLIC12922 que estavam sendo expressas tanto na forma solúvel como insolúvel tiveram aumento da expressão na fração solúvel quando empregado metade do agente indutor. Já as proteínas rLIC10498, rLIC11668, rLIC11935 e rLIC12690, quando houve redução do indutor, não foram expressas sequer na fração insolúvel, mostrando que este sistema de expressão não permite a solubilização *in vivo* destas proteínas (dados não mostrados).

Quatro dos genes selecionados (LIC11506, LIC12144, LIC12217 e LIC12225) não apresentaram expressão significativa na linhagem BL21 SI (Invitrogen) e foram avaliados em outras duas linhagens de *E. coli*: BL21 StarTM (DE3) pLysS (Invitrogen) e C43 (DE3) (Avidis), que são mais adequadas a expressão de proteínas tóxicas ou de baixa expressão. As linhagens StarTM possuem mutação na RNase E que está envolvida na maturação do RNAr e na degradação do RNAm contribuindo para o aumento de transcritos (Lopez *et al.*, 1999). O plasmídeo pLysS codifica a T7 lisozima que reduz a expressão basal do gene de interesse e assim permite o crescimento celular. Dessa maneira, a linhagem BL21 StarTM (DE3) pLysS (Invitrogen) reúne em si duas características que permitem

o aumento da expressão. A linhagem C43 (DE3) (Avidis) possui uma mutação ainda não caracterizada que previne a morte celular associada à expressão de proteínas recombinantes tóxicas (Miroux e Walker, 1996).

A análise de expressão das proteínas foi feita em PAGE/SDS 12% e através de purificação por cromatografia de afinidade a metal; contudo, não foi possível observar expressão significativa das proteínas recombinantes com a massa molecular esperada (dados não mostrados). Os genes LIC12217 e LIC12144 possuem quatro e treze hélices transmembranas, respectivamente, o que reduz a eficiência de expressão em *E. coli* (Pizza *et al.*, 2000). Nenhum domínio que justificasse a não expressão dos genes LIC12225 e LIC11506 foi encontrado através de análise de bioinformática.

4.7 Avaliação da purificação das proteínas recombinantes

A expressão das proteínas recombinantes contendo uma sequência de seis histidinas permite a purificação através de resina de afinidade metálica, pois as histidinas se ligam fortemente em níquel, via anel imidazólico, permitindo a eluição com altas concentrações de imidazol (competidor).

O processo de purificação foi eficiente, no entanto, as proteínas revelaram-se muito instáveis em altas concentrações provavelmente por haver isoformas em diferentes conformações estruturais. Houve necessidade de diluí-las a concentrações inferiores a 100 ng / μ l. A remoção do agente desnaturante (ureia) não foi bem sucedida, para as proteínas rLIC10498, rLIC11668, rLIC13101 e rLIC20214 ocorrendo precipitação da proteína recombinante durante o processo.

As proteínas rLIC10494, rLIC12730 e rLIC12922 foram purificadas a partir da fração solúvel do lisado bacteriano, a fração purificada e dialisada contém em média 50 ng/ μ l. As proteínas rLIC10368, rLIC11207, rLIC11935 e rLIC12690 foram purificadas a partir da fração insolúvel do lisado bacteriano, a fração purificada e dialisada contém no máximo 50 ng / μ l e é muito instável ao armazenamento a -80 °C ou liofilização. A Figura 12 apresenta as proteínas após o processo de purificação.

Figura 12 – Avaliação das proteínas recombinantes purificadas através de PAGE/SDS 12%.
1 - LIC10494 (30 kDa); **2** - LIC11935 (60,4 kDa); **3** - LIC12730 (77,4 kDa); **4** - LIC12922 (40,0 kDa); **5** - LIC10368 (20,9 kDa); **6** - LIC11207 (38,0 kDa); **7** - LIC12690 (94,5 kDa).
M - padrão de massa molecular (em kDa). As proteínas foram coradas com azul de Coomassie.

4.8 Avaliação dos espectros de dicroísmo circular

Dicroísmo circular (CD) é uma técnica reconhecida para avaliação da estrutura de proteínas em solução. Os diferentes tipos de estrutura secundária regulares encontrados em proteínas solúveis dão origem a espectros de CD característicos conforme demonstrado na Figura 13 (Kelly *et al.*, 2005). Peptídeos com estrutura tipo alfa-hélice (linha sólida) apresentam intenso sinal negativo em 208 e 222 nm e forte sinal positivo em 192 nm. Peptídeos com estrutura tipo folha-beta anti-paralela (tracejado longo) apresentam forte sinal positivo a 198 nm e sinal negativo em torno de 215 nm. Porém os peptídeos de estrutura irregular (tracejado curto) apresentam intenso sinal negativo a 198 nm (Johnson, 1990).

Figura 13 – Espectro de CD associado a vários tipos de estrutura secundária. Linha sólida, alfa-hélice; tracejado longo, folha-beta anti-paralela; linha pontilhada, volta-beta tipo 1; tracejado cruzado, 3,1-hélice estendida ou hélice poli (pro) II; tracejado curto, estrutura irregular.

FONTE: Kelly *et al.*, 2005.

As proteínas recombinantes purificadas foram liofilizadas, reconstituídas e dialisadas contra solução tampão Na-fosfato 10 mM (pH 7,4) a 4 °C e mantidas sob refrigeração até o momento da coleta dos espectros de CD. Foram tomados três espectros de cada proteína recombinante e as médias resultantes estão representadas na Figura 14.

De acordo com os espectros obtidos, as proteínas rLIC10368, rLIC10494 e rLIC12922 possuem predominância de estrutura alfa hélice, uma vez que em 208 e 222 nm apresentam picos negativos e um pico positivo em 192 nm. Enquanto a proteína rLIC12730 possui predominância de estrutura tipo folha-beta, com um pico negativo em torno de 215 nm. Todos os resultados são condizentes com a predição de estrutura teórica realizada a partir da composição de aminoácidos, utilizando o programa PSIPRED - <http://bioinf.cs.ucl.ac.uk/psipred/> (Jones, 1999) e apresentadas no ANEXO C.

Figura 14 – Espectro de CD das proteínas recombinantes após liofilização e reconstituição. Foram utilizadas amostras de proteínas recombinantes (10 μ M) em tampão Na-fosfato (pH 7,4). **A** - rLIC10368; **B** - rLIC10494; **C** - rLIC12730; **D** - rLIC12922.

A avaliação dos espectros de CD nos permitiu obter sinais característicos de estrutura secundária o que nos dá segurança para prosseguir com experimentos de caracterização de função e imunogenicidade das proteínas em estudo.

4.9 Avaliação dos soros hiperimunes obtidos em camundongos

A imunização de camundongos com as proteínas recombinantes purificadas permite a obtenção de soro hiperimune homólogo que é indispensável para ensaios como Western blotting ou imunofluorescência possibilitando a detecção da proteína nativa equivalente. Todos os soros coletados tiveram seus anticorpos quantificados por ELISA e seus resultados estão resumidos na Figura 15.

Figura 15 – Presença de anticorpos nos soros dos camundongos imunizados. Os animais foram imunizados subcutaneamente com duas doses contendo 10 µg de proteína recombinante ou PBS (controle negativo) em *Alhydrogel* (Brenntag). Os soros obtidos foram recolhidos num *pool* e seus títulos determinados por ELISA contra a proteína homóloga (500 ng).

Os títulos obtidos foram de 1:163.840 para o soro anti-rLIC10368, 1:40.960 para o soro anti-rLIC10494, 1:20.480 para o soro anti-rLIC11207; 1:81.920 para o soro anti-rLIC11935; 1:10.240 para o soro anti-rLIC12690; 1:81.920 para o soro anti-rLIC12730 e 1:163.840 para o soro anti-rLIC12922. Estes valores demonstram que as proteínas são imunogênicas e que o protocolo é adequado para obtenção de soro hiperimune.

4.10 Ensaio de localização celular.

A membrana externa de *Leptospira* spp. contém grande variedade de lipoproteínas e sua identificação e caracterização é fundamental para compreensão da patogênese da leptospirose. Um método prático e confiável é a solubilização

seletiva empregando detergentes não iônicos como Triton X-114 (Haake e Matsunaga, 2005). Este detergente remove a membrana externa sem causar danos à estrutura bilipídica da membrana citoplasmática (Cunningham *et al.*, 1988; Haake *et al.*, 1991).

Durante o processo de solubilização o detergente não-iônico substitui as moléculas de lipídeos que estão em contato com o domínio hidrofóbico das proteínas integrais de membrana e formam micelas solúveis. Essas micelas condensam-se em temperaturas superiores a 20 °C (“*cloud point*”) enquanto outras proteínas hidrofílicas permanecem na fase aquosa (Bordier, 1981). Existem também proteínas constituintes do citoesqueleto, ou cilindro protoplasmático, que são insolúveis em Triton X-114 e formam um agregado que pode ser separado a 0 °C por centrifugação (Cunningham *et al.*, 1988; Haake *et al.*, 1991).

Com o objetivo de confirmar a localização celular já predita *in silico*, realizamos o fracionamento dos extratos proteicos totais de *L. interrogans* sorovar Copenhageni e *L. biflexa* sorovar Patoc com Triton X-114 (Sigma). A identificação das proteínas nativas foi realizada através de Western blotting contendo o lisado total, as frações da extração Triton X-114 e proteína recombinante purificada (controle positivo) imobilizados em membrana de nitrocelulose e empregando o soro policlonal contra proteína recombinante produzido em camundongo (diluição 1:100). No entanto, não houve reatividade com os extratos proteicos na massa molecular esperada, somente contra a proteína recombinante específica (dados não mostrados).

No entanto, o soro anti-rLIC10494 identificou uma proteína com massa molecular duas vezes maior do que esperado (Figura 16). Cunningham *et al* (1988) observaram resultado semelhante para uma proteína de 47 kDa de *T. pallidum*, e sugeriram que a proteína encontra-se ligada a algum componente insolúvel em Triton X-114 (Cunningham *et al.*, 1988).

Figura 16 – Identificação de uma proteína no extrato de *L. interrogans* sorovar Copenhageni pelo soro anti-rLIC10494. Filme de raio-X sensibilizado pela reação da peroxidase com o substrato (ECL, GE) revelando a reatividade do soro anti-rLIC10494 à uma proteína no extrato de *L. interrogans*. Extratos de *L. biflexa* sorovar Patoc linhagem Patoc 1 (colunas de 1 a 4) e de *L. interrogans* sorovar Copenhageni linhagem M20 (colunas de 5 a 8) sendo: lisado total (1 e 5); fase aquosa (2 e 6); fração insolúvel em Triton X-114 (3 e 7); fase rica em Triton X-114 (4 e 8); 9 - padrão de massa molecular; 10 - rLIC10494 (200 ng). A seta indica a massa molecular esperada.

Então, decidimos empregar a técnica de imunofluorescência de fase líquida que permite detectar na superfície da bactéria viva as proteínas nativas correspondentes. Como controle, empregamos soro contra duas proteínas muito abundantes em leptospira e bem caracterizadas: GroEL, uma proteína citoplasmática e seu anticorpo não deve marcar se as células estiverem integras, e LipL32, uma proteína de superfície (Cullen *et al.*, 2005). Esse experimento nos permitiu identificar quatro proteínas expressas na superfície de leptospiros (LIC10494, LIC11207, LIC12730 e LIC12922) na Figura 17 apresentamos as melhores imagens obtidas.

Figura 17 – Imunofluorescência de fase líquida empregando *L. interrogans* sorovar Copenhageni (M20). Leptospiras vivas com DNA marcado com iodeto de propídeo (em vermelho), foram incubadas com soro anti-proteína recombinante e, a seguir, com anti-IgG de camundongo conjugado com FITC (em verde). **A** - anti-rLIC10494; **B** - anti-rLIC11207; **C** - anti-rLIC12730; **D** - anti-rLIC12922; **E** - somente PBS (controle negativo); **F** - anti-rLipL32 (controle positivo); **G** - anti-rGroEL (controle negativo). Magnitude x 600.

A presença da proteína rLIC10368 foi demonstrada na superfície da linhagem virulenta de *L. interrogans* sorovar Pomona (LPF) através de imunofluorescência indireta empregando leptospiros fixadas com glutaraldeído 25% e formalina 10% (Atzingen *et al.*, 2008). A Figura 18 apresenta uma das imagens captadas.

Figura 18 – Identificação de uma proteína na superfície de *L. interrogans* sorovar Pomona fixada pelo soro anti-rLIC10368 através de imunofluorescência indireta. *L. interrogans* sorovar Pomona linhagem LPF foi incubada com soro anti-rLIC10368 e, a seguir, com anti-IgG de camundongo conjugado com FITC (em verde). Magnitude x 1.260.

FONTE: Atzingen *et al.*, 2008.

4.11 Análise da reatividade de soro de paciente diagnosticado com leptospirose

Durante o processo de infecção, os patógenos sensibilizam o organismo infectado com diversos antígenos. O mesmo ocorre na leptospirose, que apresenta duas fases clínicas, iniciando-se com septicemia e progredindo para a fase imune onde ocorre produção de anticorpos e excreção de leptospiros pela urina (De Brito *et al.*, 2006). Neste segundo estágio é possível diagnosticar a infecção através do teste de microaglutinação (MAT). Este teste é baseado na reatividade com antígenos LPS da membrana da leptospira, e dessa forma, permite determinar o sorovar infectante. Através de microscópio de campo escuro observar-se a aglutinação de leptospiros

com os anticorpos presentes no soro de indivíduos infectados (Guerreiro *et al.*, 2001).

No intuito de identificar se alguma das proteínas em estudo é expressa durante o processo de infecção e pode ser aplicada como antígeno num kit diagnóstico, investigamos a reatividade com soros de 28 pacientes através de ELISA e Western blotting. Como controle positivo da reação antígeno-anticorpo, empregamos a LipL32 é um bom reagente com soro de paciente com leptospirose (Haake *et al.*, 2000; Flannery *et al.*, 2001).

Nos ensaios de ELISA, os soros tiveram sua reatividade avaliada quanto ao tipo de anticorpo produzido, IgG e IgM, e os resultados estão ilustrados na Figura 19 e 20 respectivamente.

Os resultados obtidos com a proteína rLipL32 são coerentes com o observado por Flannery *et al* (2001), LipL32 é um antígeno que apresenta maior reatividade a soros da fase clínica. As proteínas recombinantes LIC10494, LIC11207, LIC11935, LIC12730 e LIC12922 apresentaram menor reatividade o que as excluiria da confecção de um kit para diagnóstico. No entanto, este experimento sugere que esses antígenos são expressos em algum momento da infecção e seu envolvimento no processo de patogênese deve ser investigado.

Figura 19 – Análise da presença de anticorpos IgG específicos nos soros de indivíduos diagnosticados com leptospirose contra proteínas recombinantes através ELISA. Soros de 28 indivíduos na fase subclínica da leptospirose (A), e soros dos mesmos indivíduos na fase clínica (B), foram analisados quanto a presença de anticorpos específicos IgG. Os soros foram testados na diluição de 1:100, em duplicata. O valor de *cut-off* está representado por uma linha horizontal.

Figura 20 – Análise da presença de anticorpos IgM específicos nos soros de indivíduos diagnosticados com leptospirose contra proteínas recombinantes através ELISA. Soros de 28 indivíduos na fase subclínica da leptospirose (A), e soros dos mesmos indivíduos na fase clínica (B), foram analisados quanto a presença de anticorpos específicos IgM. Os soros foram testados na diluição de 1:100, em duplicata. O valor de *cut-off* está representado por uma linha horizontal

O ensaio de ELISA permite a manutenção da estrutura conformacional das proteínas, e assim, a reatividade observada deve-se a anticorpos presentes no soro

que reconhecem epítomos estruturados, como os que podem ser encontrados na superfície da bactéria. No entanto, o indivíduo infectado também produz anticorpos contra epítomos lineares ou intracelulares que podem ser apresentados após a fagocitose das leptospirosas. Estes anticorpos são melhor avaliados através de Western blotting, e com esse propósito elegemos um grupo de 11 pacientes representativos de quatro sorovares diferentes (Copenhageni, Icterohaemorrhagiae, Tarassovi e Autumnalis) dos quais haviam amostras de soros coletadas na fase subclínica e clínica (em cinza, ANEXO A).

Foi preparado um *pool* com soros de cada fase, bem como, com soros de indivíduos normais e foram avaliados quanto a presença de anticorpos IgG, estes resultados estão apresentados no ANEXO D e E.

As proteínas rLIC10494 (coluna 2 ANEXO D) e rLIC20214 (coluna 5 ANEXO E) demonstraram ser imunorreativas tanto a soros da fase clínica como da fase subclínica. Estes antígenos foram, então, avaliados com os soros individualmente (diluição 1:100) e a proporção de respondedores pode ser observada na Tabela 5 e Figura 21. Ambos mostraram-se interessantes uma vez que são reativos a soros de ambas as fases diferentemente da LipL32.

Tabela 5 – Proporção de soros respondedores aos antígenos testados

Antígenos	Soros de indivíduos diagnosticados com leptospirose		Soro de indivíduos normais
	Fase subclínica	Fase clínica	
rLIC10494	7/11 (63%)	7/11 (63%)	2/8 (25%)
rLIC20214	2/11 (18%)	3/11 (27%)	1/8 (12%)
rLipL32	1/11 (9%)	7/11 (63%)	1/8 (12%)

Figura 21 – Análise da reatividade das proteínas recombinantes LIC10494 e LIC20214 com o soro de pacientes diagnosticados com leptospirose. Membranas contendo as proteínas recombinantes imobilizadas foram avaliadas com soro de 8 indivíduos normais (N1 a N8) e 11 pacientes (P1 a P11) da fase aguda (-) e da fase convalescente (+) da leptospirose. Acima estão apresentados filmes de raio-X sensibilizados pela reação da peroxidase com o substrato (ECL) revelando a reatividade dos anticorpos IgG dos soros as proteínas recombinantes, sendo 1 - rLIC10494; 2 - rLIC20214; 3 - rLipL32 (controle positivo). M indica o padrão de massa molecular. Os asteriscos indicam a massa molecular esperada.

4.12 Análise da reatividade de soro de hamsters infectados experimentalmente

Quando empregamos soros de humanos diagnosticados com leptospirose é difícil determinar a quanto tempo houve a infecção e a carga de bactéria infectante. O uso de soros de hamsters infectados experimentalmente permite precisar estes parâmetros. Além do mais, estes soros estão disponíveis, uma vez que para manutenção das culturas virulentas é necessária a passagem em animais.

Coletamos soro de 56 hamsters infectados com *L. interrogans* sorovar Copenhageni (FIOCRUZ L1-130) e 21 dias de infecção. Investigamos a reatividade desses soros quanto a presença de anticorpos específicos IgG através de ELISA semelhante ao utilizado para soros humanos (exceto pelo anticorpo de detecção anti-hamster). Os resultados estão ilustrados na Figura 22, obteve-se 70% de respondedores para rLipL32, 38% para rLIC10494, 17% para rLIC11935 e rLIC12922, 8% para rLIC11207 e rLIC12730.

Figura 22 – Análise da reatividade dos soros de hamsters infectados experimentalmente com leptospirose contra proteínas recombinantes através ELISA. Hamsters infectados experimentalmente com *L. interrogans* sorovar Copenhageni (FIOCRUZ L1-130) foram sangrados após 21 dias. Estes soros foram analisados quanto a presença de anticorpos específicos IgG na diluição de 1:100, em duplicata. O valor de *cut-off* está representado por uma linha horizontal.

Esses resultados confirmam a expressão destes antígenos durante a infecção e sugerem um possível papel na patogenicidade.

4.13 Adesão a componentes de matriz extracelular.

Os mecanismos pelos quais a *Leptospira* spp. invade e coloniza os tecidos hospedeiros são pouco conhecidos. A interação de patógenos a componentes de matriz extracelular contribui com a colonização primária dos tecidos do hospedeiro. Sob condições normais, os componentes de matriz extracelular não estão expostos a bactérias. Os patógenos podem acessar esses componentes após trauma do tecido seguido de injúria mecânica ou química ou consequência de uma infecção bacteriana através da ação de toxinas e enzimas líticas (Ljungh *et al.*, 1996).

A aderência aos tecidos do hospedeiro é mediada por proteínas expostas a superfície expressas pelos microorganismos durante a infecção. Nosso grupo descreveu a primeira proteína de leptospira ligante a laminina, Lsa24 (Barbosa *et al.*, 2006), e outras adesinas foram relatadas LigA e LigB (Choy *et al.*, 2007); proteínas da família Len (Stevenson *et al.*, 2007); LipL32 (Hauk *et al.*, 2008; Hoke *et al.*, 2008).

Laminina é uma importante componente da membrana basal e fibronectina é um dos principais componentes da matriz extracelular e do soro, ambas são glicoproteínas e cujos sítios de interação com célula ou bactéria caracterizados. Enquanto, colágeno possui múltiplos sítios de interação ao longo de sua estrutura. Em nossos estudos acrescentamos BSA e fetuína como controles, empregamos o método descrito por Cameron (2003) e nossos resultados estão apresentados na Figura 23.

Figura 23 – Adesão das proteínas recombinantes a componentes de matriz extracelular imobilizados. Os poços foram revestidos com laminina, colágeno I e IV, fibronectina celular e plasmática e as proteínas controle, BSA e fetuina. A adesão das proteínas recombinantes aos componentes foi avaliada por um ensaio baseado na técnica de ELISA. Os resultados estão apresentados como magnitude de adesão em relação ao controle negativo (BSA), arbitrariamente ajustado para 1. Os dados representam uma média e o desvio padrão de um ensaio em triplicata.

A proteína codificada pelo gene LIC10368 se destacou pela aderência a laminina, colágeno IV e fibronectina plasmática. A especificidade dessa interação foi avaliada de forma quantitativa e uma adesão dose-dependente foi observada em razão da concentração da proteína recombinante (Figura 24A).

O papel dos carboidratos da laminina na interação com a proteína recombinante LIC10368 foi investigado através do tratamento da laminina com metaperiodato. Esse reagente oxida os grupamento hidroxil dos carboidratos sem prejudicar a estrutura da cadeia polipeptídica (Woodward *et al.*, 1985). O efeito da oxidação foi dose-dependente e uma redução significativa da adesão da LIC10368 a laminina (~65%) foi observada com 100 mM de periodato (Figura 24B).

Figura 24 – Análise da especificidade da interação da proteína rLIC10368 a componente de matriz extracelular. (A) Ligação da proteína rLIC10368 a BSA, laminina, fibronectina plasmática e colágeno IV em função da concentração de proteína recombinante. BSA foi incluído como controle negativo. **(B)** Contribuição dos resíduos de açúcar para a interação da LIC10368 com a laminina. Cada ponto representa a média do valor da absorbância a 492 nm mais o desvio padrão de três experimentos independentes.

FONTE: Atzingen *et al.*, 2008.

Esses resultados nos permitiram caracterizar a proteína codificada pelo gene LIC10368 como uma nova adesina de leptospira, Lsa21 (*Leptospiral surface adhesin of 21kDa*) (Atzingen *et al.*, 2008).

Essa técnica, entretanto, permite trabalhar somente com proteínas solúveis. Então, decidimos investigar através de Western blotting se as onze proteínas expressas seriam capazes de interagir com componentes de matriz solúveis, semelhante ao realizado por Stevenson *et al* (2008). Empregamos Lsa21, como controle positivo e, BSA, como controle negativo. Como controle adicional, suprimimos o componente de matriz teste e esse resultado foi empregado para distinguir a interação inespecífica do anticorpo (dados não mostrados).

Na Figura 25 apresentamos o resultado destes ensaios que permitiram identificar 9 novas prováveis adesinas: LIC10498 (coluna 3), LIC13101 (coluna 10) e LIC20214 (coluna 12) que interagem com laminina e fibronectina (plasmática e celular), LIC11207 (coluna 4) e LIC11935 (coluna 6) que interagem com fibronectina plasmática e laminina, LIC11668 (coluna 5), LIC12690 (coluna 7) e LIC12730 (coluna 8) que interagem com laminina, fibronectina (plasmática e celular) e colágeno tipo IV, LIC12922 (coluna 9) que interage com colágeno IV.

Figura 25 – Adesão dos componentes de matriz extracelular solúveis às proteínas recombinantes imobilizadas em membrana de nitrocelulose. **A** - Membrana contendo proteínas recombinantes imobilizadas e coradas com Ponceau; **B** a **F** - filmes de raio-X sensibilizados pela reação da peroxidase com o substrato (ECL) revelando a adesão dos componentes de matriz às proteínas recombinantes, sendo: **B** - fibronectina celular; **C** - fibronectina plasmática; **D** - laminina; **E** - colágeno tipo I; **F** - colágeno tipo IV. **Coluna 1** - rLsa21 (controle positivo); **2** - rLIC10494; **3** - rLIC10498; **4** - rLIC11207; **5** - rLIC11668; **6** - rLIC11935; **7** - rLIC12690; **8** - rLIC12730; **9** - rLIC12922; **10** - rLIC13101; **11** - rLIC20214; **12** - BSA (controle negativo). **M** - padrão de massa molecular. Os asteriscos indicam a massa molecular esperada.

Investigamos também a participação dos carboidratos da laminina nessa interação. Quando oxidamos a laminina com metaperiodato 200 mM todas as interações foram abolidas, como demonstrado na Figura 26, sugerindo a participação dos carboidratos da laminina na interação com estas adesinas.

Figura 26 – Contribuição dos resíduos de açúcar para a interação das proteínas recombinantes com a laminina. **A** - Membrana contendo proteínas recombinantes imobilizadas e coradas com Ponceau; **B** e **C** - filmes de raio-X sensibilizados pela reação da peroxidase com o substrato (ECL) revelando a adesão da laminina às proteínas recombinantes, sendo: **B** - laminina não tratada e **C** - laminina tratada com metaperiodato 200mM. **Coluna 1** - Lsa21 (controle positivo); **2** - rLIC10494; **3** - rLIC10498; **4** - rLIC11207; **5** - rLIC11668; **6** - rLIC11935; **7** - rLIC12690; **8** - rLIC12730; **9** - rLIC12922; **10** - rLIC13101; **11** - rLIC20214; **12** - BSA (controle negativo). **M** - padrão de massa molecular. Os asterísticos indicam a massa molecular esperada.

4.14 Avaliação do ensaio de imunoproteção em hamster.

Vacinas representam uma estratégia de prevenção a doenças tropicais negligenciadas, como a leptospirose, e de promoção da redução da pobreza com melhor custo-benefício (Silva *et al.*, 2007). As vacinas comerciais disponíveis consistem em leptospirosas inativadas por calor ou quimicamente. Vacinas deste caráter produzem apenas uma resposta imune de curta duração (cerca de seis meses), não conferem proteção contra sorovares não contidos na preparação, bem como causam reações adversas indesejadas. Proteínas de membrana externa são interessantes por serem conservadas entre os sorovares (Haake *et al.*, 1999).

A fim de verificar se alguma das proteínas recombinantes confere resposta imunológica protetora contra infecção de *L. interrogans* sorovar Copenhageni em hamster produzimos um lote de proteínas recombinantes com cerca de 1,5 mg numa

concentração mínima de 0,4 $\mu\text{g} / \mu\text{l}$. Realizamos dois ensaios independentes empregando as proteínas rLIC10494, rLIC12730 e rLIC12922, e como controle de proteção, a vacina comercial *Farrow Sure B* (Pfizer) na dose equivalente para o peso do animal e uma vacina de bactéria inativada por calor (bacterina). Hamsters machos recém desmamados foram distribuídos em grupos de 14 animais conforme Figura 27.

Figura 27 – Distribuição de animais no ensaio de imunoproteção. SC - inoculação subcutânea; IP - inoculação intraperitoneal.

Os animais foram sangrados 15 dias após cada imunização e a resposta humoral foi acompanhada por ELISA. Os títulos obtidos após a primeira imunização foram equivalentes a 1:25.600 para o soro anti-rLIC10494, 1:3.200 para o soro anti-rLIC12730, 1:200 para o soro anti-rLIC12922. Os títulos obtidos após a segunda imunização foram equivalentes a 1:102.400 para o soro anti-rLIC10494, 1:51.200 para o soro anti-rLIC12730, 1:800 para o soro anti-rLIC12922. Com exceção da rLIC12922, as preparações foram capazes de estimular uma resposta humoral (Th2) significativa revelada pelo aumento de título de anticorpos após a dose de reforço.

Os hamsters foram desafiados com 2×10^5 *L. interrogans* sorovar Copenhageni (FIOCRUZ L1-130) por via intraperitoneal e acompanhados diariamente por 21 dias. A Figura 28 apresenta a média de proteção alcançada nesse período de observação. Os animais sobreviventes foram sacrificados, e seus rins coletados para cultura e isolamento de leptospiros. Com exceção dos grupos vacinados com vacina comercial ou bacterina, todos os hamster continham leptospiros em seus rins. Os soros dos hamsters também foram avaliados por MAT. Nos grupos vacinados com vacina comercial ou bacterina, cerca de 40-50% dos animais foram positivos com títulos entre 1:100 e 1:400. Nos grupos vacinados com os antígenos teste, todos os animais foram positivos com títulos entre 1:400 e 1:800.

Figura 28 – Sobrevivência dos hamsters imunizados com as proteínas recombinantes e desafiados com *L. interrogans* sorovar Copenhageni virulenta. Os animais foram desafiados com 2×10^5 células em PBS duas semanas após o término da imunização. Os animais foram monitorados quanto a morbidade por 21 dias. Cada ponto representa a média de dois experimentos independentes.

Esses dados em conjunto, nos permitem avaliar que as vacinas em teste conferiram imunidade parcial capaz de atenuar a gravidade da doença e prolongar a sobrevivência. No entanto, para concluir se haveria cura, estes animais deveriam ser observados por um período maior de dias.

Na Tabela 6 estão apresentados de forma resumida os dados de proteção conferida por nossos antígenos nesses dois experimentos. A fim de comparar os

grupos vacinados com o grupo controle (PBS) foi realizada uma análise de significância estatística com auxílio do programa One-Way ANOVA (<http://faculty.vassar.edu/lowry/VassarStats.html>). Esse resultado demonstra que a proteção conferida pela proteína rLIC12730 foi significativa, e embora, mais estudos sejam necessários, sugerem que é um promissor candidato na prevenção da leptospirose.

Tabela 6 – Proteção conferida através de imunização com proteína recombinante contra desafio letal em hamster.

vacina	% Proteção (sobreviventes/total)			
	Experimento 1	Experimento 2	média	Análise estatística
Vacina comercial	100% (8/8)	90% (9/10)	95%	P < 0,01
Bacterina	88% (7/8)	80% (8/10)	84%	P < 0,01
PBS	0% (0/8)	20% (2/10)	10%	----
rLIC10494	29% (2/7)	50% (5/10)	40%	NS
rLIC12730	38% (3/8)	50% (5/10)	44%	P < 0,05
rLIC12922	29% (2/7)	30% (3/10)	30%	NS

NOTA: Análise de significância estatística realizada com auxílio do programa One-Way ANOVA (<http://faculty.vassar.edu/lowry/VassarStats.html>). NS: diferença não significativa.

5 Conclusões

A conservação e a expressão dos genes selecionados foram confirmadas por PCR e RT-PCR em seis sorovares de *L. interrogans* (espécie patogênica) mantidas em cultura. O gene LIC10368 destacou-se por ter uma expressão elevada em leptospiros virulentas.

Onze proteínas recombinantes foram expressas em fusão com uma sequência de seis resíduos de histidina no sistema induzível por NaCl (*E. coli* BL21 SI), sendo três na forma solúvel. A purificação por cromatografia de afinidade a metal foi bem sucedida, entretanto, somente sete foram purificadas na ausência do agente desnaturante (rLIC10368, rLIC10494, rLIC11207, rLIC11935, rLIC12690, rLIC12730 e rLIC12922). Quatro dessas proteínas tiveram sua estrutura secundária avaliada por dicroísmo circular e de acordo com os espectros obtidos as proteínas rLIC10368, rLIC10494 e rLIC12922 possuem predominância de estrutura alfa hélice, enquanto a proteína rLIC12730 possui predominância de estrutura tipo folha-beta. A imunização de camundongos demonstrou que as proteínas foram imunogênicas. O ensaio de localização celular através de imunofluorescência de fase líquida permitiu confirmar que quatro proteínas são expressas na superfície de leptospiros vivos (rLIC10494, rLIC11207, rLIC12730 e rLIC12922).

A reatividade das proteínas recombinantes a soros de pacientes diagnosticados com leptospirose foi avaliada através das técnicas de ELISA e Western blotting. As proteínas rLIC10494, rLIC11207, rLIC11935, rLIC12730, rLIC12922 foram avaliadas por ELISA e apresentaram baixa reatividade com anticorpos presentes em soros de pacientes com leptospirose. Através da metodologia de Western blotting, todas as onze proteínas expressas foram avaliadas. Dentre estas merecem destaque a proteína rLIC10494 que reagiu com 63% dos soros de ambas fases clínicas e a proteína rLIC20214 que reagiu com 18% dos soros da fase subclínicas e 27% dos soros da fase clínica, sugerindo a participação dessas proteínas na patogênese da leptospirose.

A reatividade das proteínas recombinantes a soros de hamsters infectados experimentalmente foi avaliada por ELISA e obteve-se 38%, de respondedores para

rLIC10494, 17% para rLIC11935 e rLIC12922, 8% para rLIC11207 e rLIC12730, confirmando a expressão destes antígenos durante a infecção.

Através de um ensaio de adesão por ELISA, identificamos uma nova adesina de leptospira, Lsa21 (*Leptospiral surface adhesin of 21 kDa*), que interage fortemente com laminina, colágeno IV e fibronectina plasmática de maneira específica e dose dependente. O efeito da oxidação da laminina foi dose-dependente e uma redução significativa da adesão da LIC10368 a laminina (~65%) foi observada com 100 mM de periodato (Atzingen *et al.*, 2008).

Usando a metodologia de Western blotting, identificamos mais 9 novas prováveis adesinas (LIC10498, LIC11207, LIC11668, LIC11935, LIC12690, LIC12730, LIC12922, LIC13101 e LIC20214) que interagem com laminina, fibronectina plasmática e celular. A oxidação da laminina com metaperiodato 200 mM aboli as interações das proteínas recombinantes a laminina.

Nossos ensaios de imunização e desafio demonstraram que a proteína rLIC12730 conferiu proteção significativa (38-50%, $P < 0,05$) contra infecção letal de *L. interrogans* em hamsters. A imunoproteção conferida a estes animais é provavelmente via resposta Th2 revelada pelo aumento de título de anticorpos após a dose de reforço. Embora, mais estudos sejam necessários, nossos dados sugerem que a proteína rLIC12730 é um promissor candidato na prevenção da leptospirose.

Nossos estudos identificaram novos antígenos de *L. interrogans* e certamente são de grande contribuição para a compreensão da biologia e patogenicidade dessa espiroqueta. Esse conhecimento servirá de base para o desenvolvimento de uma nova vacina e/ou kit para sorodiagnóstico da doença.

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Anexos

ANEXO A – Soros humanos empregados nos imunoensaios.

Pacientes	MAT	Pacientes	MAT
1	negativo Autumnalis 1/25600	15	negativo Ictero/Copenhageni 1/3200
2	negativo Ictero/Copenhageni 1/6400	16	negativo Autumnalis 1/1600
3	negativo Autumnalis 1/6400	17	negativo Ictero/ Copenhageni 1/1600
4	negativo Copenhageni 1/1600	18	negativo Autumnalis 1/800
5	negativo Copenhageni 1/1600	19	negativo Copenhageni 1/1600
6	negativo Cynopteri 1/3200	20	negativo Inconclusivo 1/6400
7	negativo Ictero 1/800	21	negativo Ictero 1/6400
8	negativo Copenhageni 1/3200	22	negativo Autumnalis 1/800
9	negativo Ictero 1/6400	23	negativo Tarassovi
10	negativo Ictero 1/3200	24	negativo Inconclusivo 1/400
11	negativo Copenhageni 1/800	25	negativo Ictero 1/1600
12	negativo Copenhageni 1/3200	26	negativo Inconclusivo 1/800
13	negativo Ictero 1/6400	27	negativo Copenhageni 1/3200
14	negativo Ictero 1/1600	28	negativo Ictero 1/3200

NOTA: Em cinza, os onze soros empregados na confecção do *pool*/ empregado no ensaio de Western blotting.

ANEXO B – Cromatogramas dos sequenciamentos das construções pDEST17 (Invitrogen) contendo os insertos de DNA dos genes selecionados

LIC 10368

LIC 10494

LIC 10498

NOTA: O retângulo assinala a sequência C ACC e o círculo sinaliza o códon de terminação do gene. A sequência grifada no cromatograma do sequenciamento do gene LIC10498 indica a sequência de nucleotídeos que foi inserida acidentalmente durante o processo de clonagem do gene.

Continua

ANEXO B – Continuação.

LIC 11207

LIC 11506

LIC 11668

Continua

ANEXO B – Continuação.

LIC 11935

LIC 12144

LIC 12217

Continua

ANEXO B – Continuação.

LIC 12225

LIC 12690

LIC 12730

Continua

ANEXO B – Continuação.

LIC 12922

LIC 13101

LIC 20214

ANEXO C – Predição de estrutura teórica das proteínas recombinantes através do servidor público PSIPRED

rLIC10368

Continua

rLIC10494

ANEXO C – Continuação.

rLIC12730

Continua

ANEXO C – Continuação rLIC12730

Continua

rLIC12922

ANEXO D – Análise da reatividade das proteínas recombinantes com o soro de pacientes diagnosticado com leptospirose.

NOTA: À esquerda, membranas contendo proteínas recombinantes imobilizadas e coradas com Ponceau, e à direita, filmes de raio-X sensibilizados pela reação da peroxidase com o substrato (ECL) revelando a reatividade dos anticorpos IgG dos soros às proteínas recombinantes. Os soros foram testados na forma de *pool* (diluição 1:500), sendo: A - 6 soros de indivíduos normais, B - 11 soros de pacientes da fase aguda da leptospirose e C - 11 soros de pacientes da fase convalescente. 1 - rLIC10368; 2 - rLIC10494; 3 - rLIC11207; 4 - rLIC11668; 5 - rLIC12922; 6 - rLipL32 (controle positivo). M indica o padrão de massa molecular.

ANEXO E – Análise da reatividade das proteínas recombinantes com o soro de pacientes diagnosticado com leptospirose.

NOTA: À esquerda, membranas contendo proteínas recombinantes imobilizadas e coradas com Ponceau, e à direita, filmes de raio-X sensibilizados pela reação da peroxidase com o substrato (ECL) revelando a reatividade dos anticorpos IgG dos soros às proteínas recombinantes. Os soros foram testados na forma de *pool* (diluição 1:500), sendo: A - 6 soros de indivíduos normais, B - 11 soros de pacientes da fase aguda da leptospirose e C - 11 soros de pacientes da fase convalescente. 1 - rLIC10498; 2 - rLIC11935; 3 - rLIC12690; 4 - rLIC12730; 5 - rLIC20214; 6 - rLIC13101; 7 - rLipL32 (controle positivo). M indica o padrão de massa molecular.

ANEXO F – Artigos de Periódicos

- Artigo 1 Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, Romero EC, Leite LC, Ho PL e Nascimento AL. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. *FEMS Microbiol Lett.* 2005; 244, 305-313.
- Artigo 2 Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, Moraes ZM, Vasconcellos SA e Nascimento AL. A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun.* 2006; 74, 6356-6364.
- Artigo 3 Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima DM, Abreu PA e Nascimento AL. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol.* 2008; 8, 70.
- Artigo 4 Gómez RM, Vieira ML, Schattner M, Malaver E, Watanabe MM, Barbosa AS, Abreu PA, de Moraes ZM, Cifuentes JO, Atzingen MV, Oliveira TR, Vasconcellos SA e Nascimento AL. Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein endothelial cells (HUVECS) and express during infection. *Microb Pathog.* 2008; 45(5-6):315-22.

Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis

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Received 11 January 2005; received in revised form 31 January 2005; accepted 2 February 2005

First published online 16 February 2005

Edited by M.Y. Galperin

Abstract

Leptospirosis is an important global human and veterinary health problem. Humans can be infected by exposure to chronically infected animals and their environment. An important focus of the current leptospiral research is the identification of outer membrane proteins (OMPs). Due to their location, leptospiral OMPs are likely to be relevant in host–pathogen interactions, hence their potential ability to stimulate heterologous immunity. The existing whole-genome sequence of *Leptospira interrogans* serovar Copenhageni offers a unique opportunity to search for cell surface proteins. Predicted genes encoding potential surface proteins were amplified from genomic DNA by PCR methodology and cloned into an *Escherichia coli* expression system. The partially purified recombinant proteins were probed by Western blotting with sera from human patients diagnosed with leptospirosis. Sixteen proteins, out of a hundred tested, were recognized by antibodies present in human sera. Four of these proteins were conserved among eight serovars of *L. interrogans* and absent in the non-pathogenic *Leptospira biflexa*. These proteins might be useful for the diagnosis of the disease as well as potential vaccine candidates.

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Keywords: *Leptospira interrogans*; Surface protein; Cloning and expression; Vaccine; Leptospirosis

1. Introduction

Leptospirosis, an emerging infectious disease, is a worldwide zoonosis of human and veterinary concern. Caused by spirochaetes of the genus *Leptospira*, the disease presents greater incidence in tropical and subtropical regions [1,2]. The transmission of leptospirosis has been associated with exposure of individuals in close proximity to wild or farm animals [3]. Recently the

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disease became prevalent in cities with sanitation problems and large population of urban rodent reservoirs, which contaminate the environment through their urine [4]. The incidence of leptospirosis remains underestimated in part due to the broad spectrum of signs and symptoms that patients may present. Children primarily show fever, vomiting, headache, diarrhea, abdominal and generalized muscle pain, whereas adults have fever, headache, anorexia, muscle pain and constipation [4,5]. Five percent to 15% of the cases evolve more severely presenting hemorrhages with renal and hepatic failure, a condition known as Weil's syndrome [4], with a mortality rate of 5–40%. Leptospirosis has also a great economic impact in the agricultural industry since the disease affects the livestock inducing abortions, stillbirths, infertility, reduced milk production and death [3,4].

Currently available veterinarian vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. These types of vaccine confer protective responses through induction of antibodies against leptospiral lipopolysaccharide [6]. However, these vaccines fail to induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation. There is a large number of pathogenic serovars (>200) which imposes a major limitation to the production of a multi-serovar component vaccine and to the development of immunization protocols based on whole cell or membrane preparations. In humans, a prototype vaccine has been tested in China but children under 14 years were not protected [7]. A vaccine licensed for human is still long awaited [4].

The advent of whole-genome sequencing has made an impressive impact in the microbial field landscape. The complete genomic sequence of *Neisseria meningitidis* serogroup B offered a new strategy for the identification of vaccine candidates [8]. This landmark approach, now called reverse vaccinology, has been applied in the last few years revolutionizing the vaccine research area [9,10]. The design of vaccines is based on bioinformatic tools for the prediction of potential antigens *in silico*, hence narrowing down the universe to be tested. In addition, this approach has the advantage of revealing proteins independently of their abundance and without the need of growing the microorganism *in vitro* [11].

In the present study, we describe 16 new leptospiral membrane-associated proteins selected from the genome of *L. interrogans* serovar Copenhageni [12,13]. The rationale for the choice of these predicted coding sequences is that surface-associated molecules are potential targets for inducing immune responses in animals and may serve as vaccines against disease and/or for use in diagnostic tests.

2. Materials and methods

2.1. Bacteria

L. interrogans serovar Copenhageni sequenced strain (Fiocruz L1-130) was provided by Dr. A.I. Ko (CPqGM/FIOCRUZ/MS). The strain was isolated as described [12]. *L. interrogans* serovars Canicola, Icterohaemorrhagiae, Copenhageni, Bratislava, Hardjo, Autumnalis, Pomona, Pyrogenes, Grippotyphosa and *Leptospira biflexa* serovar Patoc were maintained in one of our laboratories (S.A.V., Faculdade de Medicina Veterinária, Universidade de São Paulo, SP, Brazil).

2.2. In silico identification of surface proteins

Protein coding genes from the *L. interrogans* genome sequence were identified using GeneMark and Glimmer [14]. The PSORT program [15] (<http://psort.nibb.ac.jp/>) was then used to predict the localization of the coded proteins within the bacterium. Public and custom sequence-specific search algorithms were used for identification of sequence motifs including signal peptides, lipoprotein cleavages sites and transmembrane domains (<http://www.cbs.dtu.dk/services/TMHMM>) [16] and (<http://www.cbs.dtu.dk/services/SignalP>) [17]. In addition, putative proteins, homologous to surface proteins previously characterized as virulence factors in other organisms, were searched for by blast analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>) [18]. In this work, the predicted coding sequences are referred to according to their genome nomenclature, LIC [12].

2.3. Cloning, expression and purification

Cloning techniques were performed according to Sambrook et al. [19]. For the expression of the selected predicted coding sequences the Gateway® (Invitrogen) cloning and expression system was used. Each selected DNA sequence was amplified by PCR from *Leptospira* genomic DNA using Pfx DNA polymerase (Invitrogen) and primers specially designed following the manufacturer recommendations. In this system, the PCR products are first cloned into pENTR TOPO vector (Invitrogen). The correct orientation is obtained by adding four bases to the forward primer (CACC) which anneals to a complementary overhang in the cloning vector (GTGG). In Table 1, there is a list of primers for PCR amplification of 17 coding sequences which became of special interest in this report. When necessary, DNA bands were extracted from agarose gel using Concert purification system (Gibco BRL) following the manufacture's protocol. The sequence of the cloned leptospira DNA insert was confirmed by sequencing and transferred by recombination reaction by LR Clonase (Invitrogen) to the *Escherichia coli* expression vector

Table 1

Sequence of the primers employed for DNA amplification and molecular mass of native and expressed recombinant proteins

Gene id genome nomenclature*	Primers for PCR amplification	Molecular mass (kDa)	
		Native	Recombinant
LIC10054	F: CACCACGTCTTGTGCGTCGGTAGAG R: CCAAGTATTCTATTTATACGTCCGAG	35.6	35.1
LIC10091	F: CCATGGGACTCGAGACGCCTCCTCTAAAGATCC R: CTCCATGGTCATTTCAAACCTTCTACGGGGC	40.6	39.0
LIC10508	F: ACCATGGGATCCGCTCTTTTGGTTGATCCAGAG R: GAATTCCTAACAACAGGACCTTCACAT	23.0	18.5
LIC11947	F: CACCCCTTCGAGGTTGGAAATCG R: AATCGATGGATCACGTTACG	20.1	22.8
LIC10561	F: CACCAAGAAGGATTCCAACGATGATG R: TCTCTGCTTGACAGCCGAC	33.1	32.0
LIC10765	F: CACCGAAAGTCCCGTAAGGTTCAAA R: TGCAGGAGTTCCACATTTTA	16.6	15.4
LIC11271	F: CACCAATCGACTTTTCACTGAGTTTCTT R: CGAAAGTATCAAGAAGAACCCTA	30.9	29.2
LIC11574	F: CACCATCATTCCTTCGGGAAGTGAC R: CCATTCTCTGTTGTTTGATCCC	21.2	20.6
LIC12518	F: CACCCCGTGTCTTTTGGTTTAGAT R: TTCCAACAAATCGAATCATCT	15.8	15.9
LIC13131	F: CACCACGTCTCAAAGTTACGCTTCAG R: TTCTCACCATCCAGCTCGG	23.0	21.9
LIC10380	F: CACCATGGGCGCTTTTAATCGG R: CGGAACTAGGGAACCTTTCAAC	20.9	21.9
LIC12099	F: CACCACCAATGTGTTTGGTATAGCG R: CAGCGTTTTGTGATAAAATTAAC	52.7	53.8
LIC12228	F: CACCAAACCTGGATATGGAATGGC R: TACAGAGGTAGAAGCGTTAGAAG	17.3	18.4
LIC13306	F: CACCTTTGCACAATCCAAAGAGAAATG R: TCATTTCGGAACCGGATGAC	20.1	21.2
LIC10191	F: CACCGAGCCTTCAACGCAAGAGCAA R: AACGTAAGACGTTGAGTTGCCACA	18.7	20.6
LIC13008	F: CACCATGCGTGCTGTAGTAGAGAAAC R: GTCGACATTGGCAGAATTTACG	15.3	15.2
LIC11352	F: CACCGGTGCTTTCGGTGCTGCTG R: ATTACTAGTCGCGTCAGAAGC	29.6	30.2

* LIC: *Leptospira interrogans* Copenhageni.

pDEST17. The vector containing the correct DNA sequence was cloned into *E. coli* BL21(DE3) (Novagen) for protein expression as previously described [20]. The production of recombinant proteins was achieved by the addition of isopropyl β -D-thiogalactoside (IPTG) to the medium. The pDEST17 vector allows the expression of recombinant proteins with 6 $\times$ His-tag at the N-terminus. Most of the assays were performed in high throughput scale with 96 samples each round. The first screening to check the protein expression was done by SDS-PAGE with total protein extracts from each clone. Proteins from expressing clones were purified from the pellets of the cell extracts, by solubilization in 8 M urea, followed by metal affinity chromatography using QIAfilter 96 plates Ni-nitrilotriacetic acid resin superflow (Qiagen) as described by the manufacturer. In brief, contaminants were washed away with 10 vol. of binding buffer, 20 mM Tris, 500 mM NaCl, containing 5 or 20 mM imidazole. Recombinant protein samples were

eluted with 5 vol. of the same buffer but with 1 M imidazole. Eluted fractions were analyzed by SDS-PAGE, through 12–15% (wt/vol) acrylamide concentration according to the expected molecular mass of the proteins.

2.4. Mice antisera against recombinant proteins

Groups of 5, 6–8 weeks female BALB/c mice, (Institute Butantan, São Paulo, SP, Brazil) were immunized subcutaneously with approximately 15 μ g of each recombinant protein with complete Freund's adjuvant. After 21 days, mice were boosted with the same amount of protein in incomplete Freund's adjuvant. Twenty-eight days following the booster the animals were bled and immune titers were determined by antibody capture endpoint enzyme-linked immunosorbent assay (ELISA). The control group was inoculated with PBS and adjuvant.

2.5. Recognition of recombinant proteins by sera from leptospirosis patients

Cell pellets of induced bacteria as well as purified proteins were separated by 12–15% SDS-PAGE and electrotransferred to nitrocellulose membrane for Western blotting assay. Membranes were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% nonfat dry milk; after which they were incubated with individual human serum (microscopic agglutination test – MAT – titre of 25,000, Instituto Adolfo Lutz, Sao Paulo, SP, Brazil) from convalescent patients diagnosed with leptospirosis at 1:100 dilution. Goat anti-human IgG peroxidase-conjugate was used as secondary antibody at 1:1000 dilution in PBS-T. Proteins reacting with the antisera were detected by reaction with DAB and H₂O₂ or with ECL Kit (Amersham).

2.6. Recognition of proteins from leptospiral extracts by sera from mice immunized with recombinant proteins

Bacterial cultures of several leptospiral serovars were centrifuged and the cell pellets were resuspended and washed three times by centrifugation with PBS containing 5 mM MgCl₂. The cell pellets were then resuspended in PBS containing 10% SDS and a sample from each serovar sample was applied on a 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with serum obtained from mice immunized with each of the recombinant proteins.

2.7. Nucleotide sequence Accession Numbers

The sequences of the two chromosomes of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 have

been deposited in the GenBank database under Accession Nos. [AE016823](#) and [AE016824](#). The accession numbers for public data base for each protein sequence analyzed in this work are listed in [Table 2](#). The proteins can also be accessed by the genome nomenclature for the gene locus, LIC number (*Leptospira interrogans* Copenhageni).

3. Results

3.1. Identification of vaccine candidates from the leptospiral genome: in silico analysis

Our rationale for the selection of the predicted coding sequences is that surface associated molecules are potential targets for inducing immune responses in the host [8–11]. These sequences could be identified in the genome database by one or more sequence motifs commonly found in known surface proteins from other bacteria. A primary screening was performed using the Psort program [<http://psort.nibb.ac.jp/>] to identify putative proteins with predicted cellular localization from the inner to the outer bacterial membrane. Genes encoding proteins with known cytoplasmic functions were excluded. In addition, we searched for exportation signal peptides, transmembrane domains, lipoprotein signatures and homologies to known surface proteins [12]. The *in silico* approach resulted in a large number of genes covering ~20% of the total number of predicted proteins in the genome. From these sequences we focused the selection mainly on hypothetical, unknown proteins, having either signal peptide sequences or lipobox motifs [12].

Table 2

Name, feature and accession number of the proteins reactive to sera of leptospirosis patients

Locus id	Protein name*	Feature	Protein Accession No.
LIC10054	MPL36	Lipoprotein, probable**	AAS68691
LIC10091	LipL40	Lipoprotein, probable**	AAS68725
LIC10508	LipL23	Lipoprotein, probable**	AAS69129
LIC11947	LipL22	Lipoprotein, probable**	AAS70529
LIC10561	OMPL31	Leptospira conserved hypothetical protein**	AAS69182
LIC10765	MPL17	Leptospira conserved hypothetical protein	AAS69382
LIC11271	OMPL30	Leptospira conserved hypothetical protein	AAS69877
LIC11574	OMPL22	Leptospira conserved hypothetical protein	AAS70170
LIC12518	OMPL16	Leptospira conserved hypothetical protein	AAS71083
LIC13131	MPL21	Leptospira conserved hypothetical protein	AAS71676
LIC10380	OMPL21	Conserved hypothetical protein	AAS69003
LIC12099	LipL53	Conserved hypothetical protein**	AAS70670
LIC12228	OMPL17	Conserved hypothetical protein	AAS70800
LIC13306	OMPL20	Conserved hypothetical protein	AAS71848
LIC10191	OMPAL21	Membrane protein, peptidoglycan associated (OmpA-like)**	AAS68819
LIC13008	OMPL15	Hypothetical protein	AAS71558
LIC11352	LipL32	Lipoprotein LipL32	AAS69953

* MP, Lip and OMP stand for membrane protein, lipoprotein and outer membrane protein, respectively; OMPA stands for OMPA-like domain; L, refers to *Leptospira*, while the numbers are related to the molecular mass of the protein.

** Probable lipoproteins according to criteria described in Nascimento et al. [12] and Haake [22].

3.2. Cloning and expression of vaccine candidates

Oligonucleotides for PCR amplifications (Table 1) were designed from the genome without the signal peptide sequences, which typically contain 18–28 nucleotides, as described in experimental procedures. From 206 selected coding sequences (see Table 1, supplementary data), more than 97% were amplified. The correct sequences were confirmed by DNA sequencing and 175 genes (84%) were successfully cloned into pENTR. The DNA inserts were transferred by recombination from pENTR to pDEST17 expression vector. This *E. coli* vector expresses the recombinant proteins with six histidine residues at the N-terminus which allows a rapid purification of the protein by metal chelating chromatography. We have expressed and purified 150 recombinant proteins with this system. A representative set of SDS-PAGE containing cell extracts from induced *E. coli* BL21(DE3) cultures expressing the recombinant proteins is shown in Fig. 1. Based on the results obtained with the immune assays (see below), 16 proteins were found to be of special interest and the data related to them are highlighted along this report. Some of these proteins are indicated in Fig. 1.

The majority of the recombinant proteins were found to be insoluble in the pellets of bacterial cell lysates, as evaluated by SDS-PAGE (not shown). The inclusion bodies were isolated, urea-solubilized and purified by metal affinity. The purified proteins were grouped according to their molecular mass and analyzed by SDS-PAGE. A representative set of these gels is shown in Fig. 2, in which numbered lanes refer to proteins reactive to sera of leptospirosis patients (see below).

3.3. Screening recombinant proteins for reactivity with leptospiral antisera

Purified proteins were screened for reactivity by immunoblotting with pooled sera from patients diagnosed with leptospirosis. The recombinant lipoprotein LipL32 was used as a positive control since it was shown to be immunogenic and highly conserved among *Leptospira* pathogenic serovars [20,21]. A total of 16 proteins out of a hundred tested were recognized by sera from leptospirosis's convalescent patients (Fig. 3). Table 2 summarizes features of these sera-reactive recombinant proteins.

3.4. Features of the proteins recognized by antibodies

The recombinant proteins listed in Table 2 were recognized by human sera from patients diagnosed with leptospirosis. They are all predicted to be membrane proteins with a signal peptide of 20–36 amino acids, as indicated by the software described in Bendtsen et al. [17]. Seven of these proteins (indicated by ** in Table 2) are probably new leptospiral lipoproteins according to the criteria previously described [12,22]. The signal peptides regions are characterized by a higher proportion of hydrophobic amino acids, a lipoprotein signal peptidase and a cysteine to be lipidated [22]. No specific function could be attributed to these putative lipoproteins. LIC10054 encodes a rare lipoprotein domain to which homologs were already found in other microorganisms according to GeneBank database [<http://www.ncbi.nlm.nih.gov/BLAST/>] [18]. Interestingly, the protein named OMPL15 (LIC13008) did not match any sequence in the available protein databases and should be considered for further studies on protein function. The protein encoded by LIC10191 has been

Fig. 1. Protein expression analysis by SDS-PAGE. Representative gels showing the cell extracts of BL21(DE3) *E. coli* transformed with different expression vectors. Proteins are assembled according to their expected molecular mass. Lane numbers refer to sera-reactive proteins: (1) OMPL16, (2) OMPL15, (3) MPL17, (4) LipL22, (5) OMPL31 and (6) LipL53. M indicates the protein molecular mass marker.

Fig. 2. Purification of recombinant proteins. Representative SDS-PAGE containing samples of purified proteins obtained by high throughput metal affinity chromatography purification system. Proteins are assembled in the gels according to their expected molecular mass. Lane numbers refer to sera-reactive proteins: (1) OMPL15, (2) MPL17, (3) OMPL16, (4) OMPL30, (5) MPL21, (6) OMPL21, (7) LipL22, and (8) OMPL22. **M** indicates the protein molecular mass marker.

recently characterized from *L. interrogans* serovar Manilae, strain UP-MMC, as a novel lipoprotein with an OmpA domain [23]. Three proteins out of the 16 were found to be hypothetical proteins conserved among other microorganisms, while five were hypothetical proteins conserved only among *Leptospira* species (Table 2), according to the currently available databases.

3.5. Conservation of antigenic proteins among leptospiral serovars

Protein expression in the most prevalent pathogenic serovars of *L. interrogans* is an important requirement for leptospiral vaccine candidates. In order to character-

ize the conservation of the selected proteins we employed protein extracts from several *L. interrogans* serovars: Canicola, Icterohaemorrhagiae, Copenhageni, Bratislava, Hardjo, Autumnalis, Pomona, Pyrogenes, Grippotyphosa and the nonpathogenic strain *L. biflexa* serovar Patoc. Cell extracts were prepared from cultures of the different serovars and Western blot analysis was performed with polyclonal sera from mice immunized with each recombinant protein. The serological cross-reactivity showed a high degree of conservation among four out of 10 proteins tested (Fig. 4). Interestingly, none of these four proteins were present in the non-pathogenic *L. biflexa* strain, suggesting that they may be relevant for pathogenesis. Most of the proteins reac-

Fig. 3. Recognition of recombinant proteins by leptospirosis human serum. Proteins blotted into nitrocellulose membranes were probed with serum (1:100 dilution) from individual covalent patient diagnosed with leptospirosis (microscopic agglutination test – MAT – titre of 25,000, Instituto Adolfo Lutz, Sao Paulo, SP, Brazil). Anti-human IgG-peroxidase conjugate was used as the secondary antibody and the bands were developed with DAB/H₂O₂. Lanes: 1. LipL32 (30.2); 2. LipL40 (39.0); 3. LipL23 (18.5); 4. OMPL20 (21.2); 5. OMPL15 (15.2); 6. OMPL31 (32.0); 7. OMPL16 (15.9); 8. LipL53 (53.8); 9. MPL21 (21.9); 10. MPL17 (15.4); 11. OMPL22 (20.6); 12. OMPL21 (21.9); 13. OMPL30 (29.2); 14. OMPL17 (18.4); 15. LipL22 (22.8); 16. MPL36 (35.1) and 17. OMPAL21 (20.6). Numbers in parenthesis are the molecular mass of the recombinant proteins. **M** indicates the protein molecular mass marker.

Fig. 4. Conservation of proteins among pathogenic leptospires. Whole cell lysates were prepared from representative strains of *L. interrogans* serovars. Proteins from the lysates were separated by electrophoresis, transferred into nitrocellulose membranes and probed with mice antisera raised against the recombinant proteins, as indicated by protein name and LIC number. Serovars in lanes are: 1. Canicola; 2. Icterohaemorrhagiae; 3. Copenhageni; 4. Bratislava; 5. Hardjo; 6. Autumnalis; 7. Pomona; 8. Pyrogenes; 9. Grippotyphosa; 10. *L. biflexa* sv Patoc; 11. respective purified recombinant protein (positive control).

tive with human leptospirosis serum shared roughly comparable expression levels among the different serovars tested, but LIC10508 and LIC10561 were more variable.

4. Discussion

Considering that *L. interrogans* has an AT rich genome, with a 35% G + C content, [12], which imposes certain difficulties for primer design, it was above expectations to have amplified more than 97% of the 206 coding sequences, as well as to have expressed and purified more than 80% of the cloned products. By screening with immune sera from leptospirosis patients 16 proteins were identified as potential vaccine candidates or to be used in diagnosis. The fact that these proteins react with infected human sera not only shows their immune reactive properties, but also strongly suggests that these proteins are expressed in the course of human infection. Interestingly, one of them (OMPL15) did not match at all any protein sequence in the public databases and deserves further studies to characterize its origin and function.

The central mechanism in pathogenesis of leptospirosis, as in other spirochetal diseases such as Lyme disease and syphilis, is the ability of the pathogens to disseminate widely within the host during the early stage of

infection [4]. Although surface-associated proteins that play a role in virulence have not been yet identified, it is assumed that they may mediate interactions that enable entry and dissemination through host tissues. Putative outer membrane proteins would be accessible to the immune response during host infection and therefore, constitute targets for immune protection through mechanisms such as antibody-dependent phagocytosis and killing mediated by complement. In this regard, a major limitation in the field of leptospirosis has been to identify membrane associated proteins through conventional biochemical and molecular methods. For instance, before the complete genome sequence of a leptospira was known, only 10 lipoproteins had been characterized through isolation of membrane fractions [24–29]. Using the genome approach, we have identified 174 novel putative lipoproteins [12]. In addition, the identified leptospiral proteins may also have diagnostic applications. At present, rapid and efficient diagnostic tests are not available for use in human and veterinary leptospirosis [1,2,4]. A proportion of these membrane proteins will be antigens expressed during infection and should be recognized by the host immune system. Therefore, they may serve as the basis to develop antigen-capture detection strategies or recombinant protein-based serologic tests.

Our results corroborate previous studies showing the advantages of using whole genome approach [10,11] for

the identification of novel vaccine antigens at a reasonable cost and time compared with traditional strategies.

We believe this work represents an important contribution to the leptospiral field. The proteins described herein may certainly be exploited for the establishment of a much-needed kit for diagnosis of leptospirosis. Furthermore, these proteins may provide an optimal basis for the development of a new and effective vaccine that would help reduce the burden of leptospirosis.

Acknowledgements

This work has benefited from Grants from FAPESP, CNPq, PADCT-FINEP and Fundação Butantan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.femsle.2005.02.004](https://doi.org/10.1016/j.femsle.2005.02.004).

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A Newly Identified Leptospiral Adhesin Mediates Attachment to Laminin[▽]

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Received 21 March 2006/Returned for modification 26 April 2006/Accepted 16 August 2006

Pathogenic leptospires have the ability to survive and disseminate to multiple organs after penetrating the host. Several pathogens, including spirochetes, have been shown to express surface proteins that interact with the extracellular matrix (ECM). This adhesin-mediated binding process seems to be a crucial step in the colonization of host tissues. This study examined the interaction of putative leptospiral outer membrane proteins with laminin, collagen type I, collagen type IV, cellular fibronectin, and plasma fibronectin. Six predicted coding sequences selected from the *Leptospira interrogans* serovar Copenhageni genome were cloned, and proteins were expressed, purified by metal affinity chromatography, and characterized by circular dichroism spectroscopy. Their capacity to mediate attachment to ECM components was evaluated by binding assays. We have identified a leptospiral protein encoded by LIC12906, named Lsa24 (leptospiral surface adhesin; 24 kDa) that binds strongly to laminin. Attachment of Lsa24 to laminin was specific, dose dependent, and saturable. Laminin oxidation by sodium metaperiodate reduced the protein-laminin interaction in a concentration-dependent manner, indicating that laminin sugar moieties are crucial for this interaction. Triton X-114-solubilized extract of *L. interrogans* and phase partitioning showed that Lsa24 was exclusively in the detergent phase, indicating that it is a component of the leptospiral membrane. Moreover, Lsa24 partially inhibited leptospiral adherence to immobilized laminin. This newly identified membrane protein may play a role in mediating adhesion of *L. interrogans* to the host. To our knowledge, this is the first leptospiral adhesin with laminin-binding properties reported to date.

Leptospira interrogans, the causative agent of leptospirosis, is a highly invasive spirochete that efficiently colonizes target organs after penetrating the host, presumably via small abrasions or breaches of the surface integument (11). Leptospire invasiveness is attributed to its ability to multiply in blood, adhere to endothelial and epithelial cells, and penetrate into tissues. Protection against the host's innate immune mechanisms is achieved by its capacity to escape complement, notably the alternative pathway (25). Conversely, the nonpathogenic, saprophytic leptospires belonging to the species *Leptospira biflexa* are rapidly cleared from the bloodstream by phagocytosis (9, 43).

The mechanisms by which *L. interrogans* invades and colonizes the host are poorly understood, since very few virulence factors contributing to the pathogenesis of the disease have been identified (24). It has been well documented over the past decades that interaction of pathogens with the extracellular matrix (ECM) might play a primary role in the colonization of host tissues. The extracellular matrix of mammals is composed of two main classes of macromolecules: glycosaminoglycans (GAGs), usually found covalently linked to proteins in the form of proteoglycans, and fibrous proteins with both struc-

tural and adhesive functions, such as collagens, elastin, fibronectin, and laminin. Besides serving as a scaffold to stabilize the physical structure of tissues, the ECM plays an important role in regulating eukaryotic cell adhesion, differentiation, migration, proliferation, shape, and function. Under normal conditions, ECM is not exposed to bacteria. Pathogens may gain access to the ECM components after a tissue trauma following a mechanical or chemical injury or as a consequence of bacterial infection through the activity of toxins and lytic enzymes (23). Long-lasting infections may occur if microorganisms reach the subepithelial tissues.

Adherence to host tissues is mediated by surface-exposed proteins expressed by the microorganisms during infection. Among spirochetes, *Borrelia* spp. have been shown to express a 47-kDa outer membrane protein (OMP) named BBK32 that confers attachment to fibronectin (33, 34) as well as to the GAGs dermatan sulfate and heparin (12), two surface lipoproteins (DbpA and DbpB) that interact with decorin, a dermatan sulfate proteoglycan that is associated with and "decorates" collagen fibers (4, 16), and another GAG-binding adhesin termed Bgp (31, 32). *Treponema pallidum*, the causative agent of syphilis, also interacts with the ECM through outer surface proteins: Tp0155 and Tp0483 show specific attachment to fibronectin (6), and Tp0751 binds to a variety of laminin isoforms (5, 7). The only putative leptospiral adhesin identified to date is a 36-kDa fibronectin-binding protein isolated from the outer sheath of a virulent variant of pathogenic leptospires (26). A family of surface-exposed leptospiral proteins (lepto-

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[▽] Published ahead of print on 5 September 2006.

spiral immunoglobulin-like, or *Lig*, proteins) with a structural organization that resembles that of bacterial adhesins has also been described (24, 30), but it remains to be confirmed whether the *Lig* proteins play a role in host cell attachment and invasion.

Whole-genome sequencing analysis of *L. interrogans* allowed identification of a repertoire of putative leptospiral surface proteins categorized as nonspecific porins, specific channels for nutrient acquisition, efflux channels, adhesins, S-layer glycoproteins, peripheral membrane proteins, and surface maintenance proteins (28). Of the 263 predicted genes encoding potential surface-exposed integral membrane proteins, 250 were previously unknown (29). Besides serving as targets for the host's immune system, it is possible that a number of proteins encoded by those genes mediate the initial adhesion to host cells.

In the present study, our goal was to identify *L. interrogans* ECM-binding proteins. For this purpose, five putative outer membrane proteins, selected via bioinformatics on the *L. interrogans* genome (28, 29, 37), and the previous characterized lipoprotein Loa22 (21) were expressed as recombinant proteins, and their capacity to mediate attachment to various ECM components was evaluated. We have identified and characterized a leptospiral membrane protein, named Lsa24 (leptospiral surface adhesin, 24 kDa) that binds strongly to laminin, a major adhesive glycoprotein of ECM and the basement membrane. Lsa24 was tested for the ability to competitively inhibit attachment of intact *L. interrogans* to laminin, and a moderate inhibition was achieved with in vitro experiments.

MATERIALS AND METHODS

ECM components. All macromolecules, including the control proteins fetuin and bovine serum albumin (BSA), were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin-1 and collagen type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was derived from human foreskin fibroblasts, plasma fibronectin was isolated from human plasma, and collagen type I was isolated from rat tail.

Leptospira strains and culture. A virulent strain of *L. interrogans* serovar Pomona and a culture-attenuated strain of *L. interrogans* serovar Copenhageni (Laboratório de Zoonoses, Faculdade de Medicina Veterinária e Zootecnia, Universidade São Paulo, São Paulo, Brazil) were used in the assays. The virulent strain is maintained by iterative passages in hamsters. Both strains were cultured at 29°C under aerobic conditions in liquid EMJH medium (Difco) with 10% rabbit serum enriched with L-asparagine, sodium pyruvate, calcium chloride, and magnesium chloride. For the ECM-binding assays, cells were harvested by centrifugation at $11,600 \times g$ for 30 min and gently washed in sterile phosphate-buffered saline (PBS) twice. The leptospires were resuspended in PBS, and bacterial density was determined using dark-field microscopy and a Petroff-Hausser chamber.

In silico selection of outer membrane proteins. Cellular localization of the proteins was predicted by the PSORT program (<http://psort.nibb.ac.jp>) (27). Sequence motifs, including signal peptides, lipoprotein cleavage sites, and transmembrane domains, were searched for on public web servers (<http://www.cbs.dtu.dk/services/SignalP>) (20, 22) and (<http://www.cbs.dtu.dk/services/TMHMM>) (3).

Cloning, expression, and purification of recombinant proteins. Predicted coding sequences LIC10009, LIC10191, LIC10494, LIC11947, LIC12730, and LIC12906 were amplified by PCR from total *L. interrogans* genomic DNA (strain Fioeruz L1-130) using the primer pairs listed in Table 1. The Gateway (Invitrogen) system was used for the cloning and expression of proteins encoded by LIC10191, LIC10494, LIC11947, and LIC12730. PCR products were first cloned into a pENTR TOPO vector. Directional cloning was ensured by the addition of four bases to the forward primers (CACC) which annealed to a complementary overhang in the cloning vector (GTGG). DNA inserts were then transferred by recombination to the *Escherichia coli* expression vector pDEST17 using LR Clonase (Invitrogen). PCR fragments corresponding to LIC10009 and LIC12906 genes were cloned into the pGEM T-Easy vector (Promega) and transformed

TABLE 1. Coding sequence-specific oligonucleotides used for DNA amplification and molecular masses of expressed recombinant proteins

Gene name ^a	Primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC10009	F, CGCGCTCGAGAAAAAGGAAGAA; R, AAGCTTTTATTGAAGAATAATTCC	26.5
LIC10191 ^b	F, CACCGAGCCTTCAACGCAAGAGCAA; R, AACGTAAGACGTTGAGTTGCCACA	23.8
LIC10494	F, CACCACTGCTAGGGCTGCAGAAA; R, ACTTTGAGAGCTTCGTCTCGT	25.8
LIC11947 ^b	F, CACCCCTCGAGGTTGGAAATCG; R, AATCGATGGATCACGTTACG	22.8
LIC12730	F, CACCACTTCTGACGGACTTCCCAA; R, TCTTGCGAATGAGTTGATCC	77.4
LIC12906	F, CTCGAGTATTCTGTGGGGATAAA; R, CCATGGTAGAAATCAAACATCGCC	23.7

^a *Leptospira interrogans* serovar Copenhageni (LIC) genes.

^b Previously published by Gamberini and colleagues (14).

into *E. coli* DH5 α . Following digestion with restriction enzymes (XhoI/HindIII for LIC10009 and XhoI/NcoI for LIC12906), fragments were subcloned into the *E. coli* expression vector pAE, which has been previously described (2, 36). All constructs were verified by DNA sequencing with appropriate vector-specific primers. Expression and purification of the resulting six-His-tagged recombinant proteins were performed as previously described (1). Protein refolding was achieved by multistep dialysis against 20 mM Tris-HCl (pH 8.0) and 0.5 M NaCl, gradually decreasing the urea concentration. Renatured proteins were extensively dialyzed against PBS for 24 to 48 h, and samples were analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All purified recombinant proteins were passed through columns containing immobilized polymyxin B (Detoxi-Gel AffinityPak prepacked columns; Pierce, Rockford, IL) for removal of residual *E. coli* lipopolysaccharide (LPS).

Circular dichroism spectroscopy. Purified recombinant proteins were dialyzed against sodium phosphate buffer (pH 7.4). Circular dichroism (CD) spectroscopy measurements were performed at 20°C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1-mm-path-length cell at 0.5-nm intervals. The spectra were presented as an average of five scans recorded from 190 to 260 nm.

Antisera against recombinant proteins. Ten female BALB/c mice (4 to 6 weeks old) were immunized subcutaneously with 10 μ g of recombinant proteins. Aluminum hydroxide was used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same protein preparation. Negative control mice were injected with PBS. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers.

Binding of recombinant proteins to ECM. Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol (5) with some modifications. Briefly, ELISA plate wells (Nunc-Immuno plate, MaxiSorp surface) were coated with 1 μ g of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, BSA (nonglycosylated attachment-negative control protein), and fetuin (highly glycosylated attachment-negative control protein) in 100 μ l of PBS for 2 h at 37°C. The wells were washed three times with PBS-0.05% Tween 20 (PBST) and then blocked with 200 μ l of 1% BSA for 1 h at 37°C, followed by an overnight incubation at 4°C. One microgram of recombinant protein was added per well in 100 μ l of PBS, and protein was allowed to attach to the different substrates for 1 h 30 min at 37°C. After washing six times with PBST, bound proteins were detected by adding an appropriate dilution of mouse antiserum in 100 μ l of PBS. Dilutions of mouse antisera against each recombinant protein were as follows: LIC10009, 1:5,000; LIC10191 (Loa22), 1:10,000; LIC10494, 1:5,000; LIC11947, 1:2,000; LIC12730, 1:5,000; LIC12906 (Lsa24), 1:2,000. Incubation proceeded for 1 h, and after three washes with PBST, 100 μ l of a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G in PBS was added per well for 1 h. All incubations took place at 37°C. The wells were washed three times, and *o*-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5.0) plus 0.01% H₂O₂ was added. The reaction was allowed to proceed for 15 min and was then interrupted by the addition of 50 μ l of 8 M H₂SO₄. The absorbance at 492

TABLE 2. Features, accession numbers, and probable locations of recombinant proteins

Gene	Protein accession no.	Feature	Probable location ^b
LIC10009	AAS68646	Lipoprotein probable	79% outer membrane, 70% inner membrane
LIC10191	AAS68819	Lipoprotein, OmpA-like (Loa22) ^a	93% periplasmic space, 25% outer membrane
LIC10494	AAS69115	Lipoprotein, probable	79% outer membrane, 70% inner membrane
LIC11947	AAS70529	Lipoprotein, probable	79% outer membrane, 70% inner membrane
LIC12730	AAS71287	Conserved hypothetical protein	93% outer membrane, 23% periplasmic space
LIC12906	AAS71459	Lipoprotein, probable (Lsa24)	28% inner membrane

^a Characterized by Koizumi and Watanabe (21).^b Predicted by PSORT (27).

nm was determined in a microplate reader (Multiskan EX; Labsystems Uis-science). For determination of dose-dependent attachment of Lsa24 to laminin, protein concentrations varying from 0 to 1,000 nM in PBS were used. Binding was also evaluated as a function of varied laminin amounts from 0 to 1 µg. For statistical analyses, the attachment of Lsa24 to laminin was compared to its binding to BSA and to all ECM macromolecules by Student's two-tailed *t* test. A *P* value less than 0.05 was considered statistically significant.

Metaperiodate treatment of laminin. Microtiter wells were coated with 1 µg of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three times with 50 mM sodium acetate buffer, pH 5.0, and immobilized laminin was treated with different sodium metaperiodate concentrations (5 to 100 mM) in the same buffer for 15 min at 4°C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 µl of 1% BSA for 1 h at 37°C. Binding of Lsa24 (1 µg in PBS per well) to periodate-treated laminin was assessed as outlined above.

***L. interrogans* ECM-binding assays.** Adhesion of intact *L. interrogans* to ECM components was performed according to a protocol described for spirochetes (33). Briefly, individual wells of Lab-Tek II chamber slides (Nalge Nunc International) were coated with 4 µg of laminin, cellular and plasma fibronectin, collagens I and IV, and the attachment negative control protein BSA, in PBS. After 16 h of incubation at 4°C, wells were washed with PBS and blocked for 2 h with 1% BSA in PBS. After washing the wells with PBS, 5×10^7 leptospires diluted in PBS were added to each well. Following a 2-h incubation at 29°C, wells were gently washed with PBS (six times for 5 min each time), and the attached spirochetes were visualized by dark-field microscopy. The number of leptospires in six 400× fields was enumerated, and the average number of bacteria per field was estimated.

Inhibition experiments. In vitro inhibition experiments were performed using the same protocol described for *L. interrogans* ECM-binding assays, except for the addition of 20 µg of the recombinant proteins Lsa24 and Loa22 (used as a negative control) to the blocking solution. Quantification of inhibition was performed essentially as described by Guo and colleagues (16). ELISA plate wells were coated with 1 µg of laminin, blocked and washed as already described, and then incubated with 0.5 µg of Lsa24 diluted in 100 µl PBS. Control wells were incubated with PBS. After washing with PBS, the wells were incubated with 50 µl of a suspension containing 2×10^8 spirochetes ml⁻¹ PBS–0.1% BSA (PBSB) for 1 h at 37°C. Unattached organisms were removed after three washes with PBSB. The wells were then incubated for 1 h with 100 µl of a 1:10,000 dilution of anti-LipL32 serum in PBS. After three washes with PBSB, the wells were incubated for 1 h with 100 µl of a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G in PBS. All incubations were performed at 37°C. After washing, the reactions were developed as described above and the absorbance at 492 nm was taken.

Isolation of leptospiral OMPs by TX-114 extraction. OMPs were extracted according to a previously described method (18). In brief, leptospires cultured as outlined above were washed in PBS–5 mM MgCl₂ and then extracted in the presence of 2% Triton X-114 (TX-114; Sigma-Aldrich), 150 mM NaCl, 10 mM

Tris-HCl pH 8.0, and 1 mM EDTA at 4°C for 2 h. The insoluble material was removed by centrifugation at $17,000 \times g$ for 10 min. After centrifugation, 20 mM CaCl₂ was added to the supernatant. Phase separation was performed by warming the supernatant at 37°C and subjecting it to centrifugation for 10 min at $1,000 \times g$. Three distinct fractions became apparent: the aqueous phase, the TX-114 phase, and the insoluble pellet. The detergent phase was precipitated with acetone. All fractions were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes for immunological analysis with Lsa24 antiserum.

Protein Data Bank accession numbers. The public database accession numbers for each protein sequence analyzed in this work are listed in Table 2. Proteins can also be accessed by their genome nomenclature for the gene locus and LIC numbers (*Leptospira interrogans* serovar Copenhageni).

RESULTS

Selection of putative surface proteins from genome sequences. With the ECM-binding studies in mind, our rationale for protein selection was mostly based on cellular localization, since surface proteins are potential targets for mediating adhesion to host. We focused on six proteins: four probable outer membrane proteins, according to genome annotation (29), one lipoprotein having a C-terminal OmpA consensus domain (17, 21, 37), and one protein annotated as hypothetical (29). Except for the previously characterized lipoprotein Loa22 (21), the proteins are new, of unknown function, and could be expressed and purified in our laboratory in concentrations and purities suitable to perform the binding assays. Gene locus identification, protein accession number, features, and computational predictions concerning cellular location of these proteins are listed in Table 2.

Expression, purification, and characterization of recombinant proteins. Oligonucleotides for PCR amplifications (Table 1) were designed from the genome, excluding the signal peptide sequences. The amplified coding sequences were cloned and expressed as full-length proteins in *E. coli*. Recombinant proteins were purified by metal chelation chromatography, and an aliquot of each protein was analyzed by SDS-PAGE (Fig. 1). As we can see from this figure, most of the contaminants were washed away and all proteins are represented as single major bands. Structural integrity of the purified proteins was assessed by CD spectroscopy. As depicted in Fig. 2, the minima at 208 and 222 nm and the maximum at 192 nm in the CD spectrum showed the high α -helical secondary structure con-

FIG. 1. Purification of recombinant proteins: SDS-PAGE (15%) of purified recombinant proteins obtained by metal affinity chromatography. Proteins encoded by LIC10009 (lane 1), LIC10191, known as protein Loa22 (lane 2), LIC11947 (lane 3), LIC12730 (lane 4), LIC10494 (lane 5), LIC12906, named Lsa24 (lane 6) are shown. Lane M, molecular mass standard.

FIG. 2. Circular dichroism spectra of the recombinant proteins. CD spectra of proteins encoded by LIC10009, LIC10191 (Loa22), LIC11947 (predominant α -helical secondary structure), LIC10494 (both α -helical and β -strand), and LIC12730 and LIC12906 (Lsa24) (predominant signal of β -strand) are shown. Far-UV CD spectra are presented as an average of five scans recorded from 190 to 260 nm. ϕ , molar ellipticity.

tent of the recombinant protein encoded by LIC11947. LIC10009-encoded protein and Loa22 also seemed to have a predominance of α -helices, while the protein encoded by LIC10494 may have both α -helices and β -sheets in its secondary structure composition. LIC12730-encoded protein and Lsa24 presented a predominant signal of β -strands, with minimum ellipticities around 215 nm. The experimental data confirmed the secondary structure content previously predicted by computational analysis (<http://bioinf.cs.ucl.ac.uk/psipred/>).

Adhesion of recombinant proteins to ECM components. Laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, and the control proteins BSA and fetuin were immobilized on microdilution wells, and recombinant protein attachment was assessed by an ELISA-based method. As shown in Fig. 3, Lsa24 exhibited a significant level of attachment to laminin, 40-fold above the background level seen with immobilized BSA. Binding of Lsa24 to laminin was also significant compared to its binding to collagen IV ($P < 0.05$) and cellular fibronectin ($P < 0.05$) (Table 3). No binding was observed when wells were coated with collagen type I or with the highly glycosylated control protein fetuin. A moderate binding to collagen type I, collagen type IV, and plasma fibronectin was observed with Loa22 (Fig. 3), but optical density values registered at 492 nm were too low to prompt further investigation (Table 3). No specific binding to the target ECM macromolecules was detected with proteins encoded by LIC10009, LIC10494, LIC11947, and LIC12730 (Fig. 3). In fact, LIC10009 encoded protein bound to all coating macromolecules, including the control proteins BSA and fetuin. It seems that those interactions are more due to a general stickiness of the protein or, alternatively, due to residual contaminants not fully eliminated during the purification step rather than to specific interactions. Data are presented as the mag-

nitude of attachment over the negative control protein BSA (arbitrarily set equal to 1) to emphasize specificity of attachment to the ECM macromolecules.

The interaction between Lsa24 and laminin was also assessed on a quantitative basis as illustrated in Fig. 4. A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant protein (0 to 1,000 nM) were allowed to adhere to a fixed laminin amount (1 μ g). The saturation level was reached at a protein concentration of 500 nM (Fig. 4A). Binding was also evaluated as a function of laminin concentration for a given recombinant protein concentration (1 μ g). As shown in Fig. 4B, the degree of attachment increased proportionally as a function of laminin concentration and reached its maximum level at 1 μ g of laminin. Increasing the laminin or protein concentration had no effect on the Loa22-laminin interaction (Fig. 4). This recombinant protein was included in our experiments as a negative control.

All recombinant proteins used in the binding assays presented above were treated with polymyxin B to ensure removal of residual *E. coli* LPS that could eventually interfere with the assays. In fact, we compared absorbance values before and after polymyxin treatment and found that Loa22, Lsa24, and the protein encoded by LIC11947 showed a partial reduction in their optical density values after treatment, suggesting a possible involvement of LPS in mediating attachment to ECM (data not shown).

Effects of laminin oxidation on protein binding. In order to address the role of laminin sugar moieties in the interaction with Lsa24, laminin was oxidized by increasing concentrations of sodium metaperiodate, ranging from 5 to 100 mM. The effect of periodate concentration on the binding is shown in Fig. 5. Laminin oxidation was performed at 4°C for 15 min. This mild treatment ensures cleavage of vicinal carbohydrate

FIG. 3. Binding of recombinant proteins to ECM components. Wells were coated with 1 μ g of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, and the control proteins BSA and fetuin. Recombinant protein attachment to those ECM macromolecules was assessed by an ELISA-based assay. One microgram of recombinant protein was added per well. Optical densities were taken at 492 nm. Specific profiles of adhesion to the various coating macromolecules are shown as magnitude of attachment (fold) over the negative control protein BSA, arbitrarily set equal to 1. Data represent the mean $\pm$ standard error of three independent experiments.

hydroxyl groups while the polypeptide chain structure remains intact (44). The oxidation effect was dose dependent, and a considerable reduction (72%) in Lsa24 attachment to meta-periodate-treated laminin was observed at a 10 mM concentration of periodate (Fig. 5). These results indicate that laminin sugar residues are critical for the interaction of Lsa24 with this major ECM glycoprotein.

***L. interrogans* attachment to ECM macromolecules.** The ability of intact *L. interrogans* to adhere to various ECM proteins was evaluated by dark-field microscopy. Leptospires attached to chamber slides coated with laminin at an average density of 40 organisms per 400 $\times$ field (Fig. 6A). Slides coated with collagen I, collagen IV, cellular fibronectin, or plasma fibronectin bound approximately 100 to 140 leptospores per 400 $\times$ field, as shown in Fig. 6B to E, respectively. In contrast,

control slides coated with BSA bound fewer than five organisms per 400 $\times$ field (Fig. 6F).

Inhibition of *L. interrogans* attachment to laminin by Lsa24. The effect of recombinant Lsa24 on leptospiral adherence to laminin was first examined by in vitro inhibition experiments (Fig. 7). Laminin-coated chamber slides were preincubated with Lsa24 for 2 h before whole *L. interrogans* cells were added. Attached leptospores were visualized by dark-field microscopy. The addition of Lsa24 reduced leptospire binding to laminin-coated slides (Fig. 7B). Attachment was not affected by prior incubation with the negative control protein Loa22 (data not shown). The inhibitory effect exerted by Lsa24 on leptospiral adherence was quantified by an ELISA-like method. Laminin-coated microtiter wells were incubated with 0.5 μ g of Lsa24 prior to the addition of 1×10^7 *L. interrogans*. Wells were

TABLE 3. Binding of Lsa24 and Loa22 to ECM components

Recombinant protein	Binding to ECM component ^a						
	Laminin	Collagen I	Collagen IV	Fibronectin		Fetuin	BSA
				Cellular	Plasma		
Lsa24	0.75 $\pm$ 0.028	0.03 $\pm$ 0.027	0.26 $\pm$ 0.183	0.24 $\pm$ 0.043	0.17 $\pm$ 0.063	0.03 $\pm$ 0.002	0.02 $\pm$ 0.010
Loa22	0.03 $\pm$ 0.021	0.17 $\pm$ 0.029	0.14 $\pm$ 0.062	0.08 $\pm$ 0.041	0.14 $\pm$ 0.064	0.03 $\pm$ 0.031	0.02 $\pm$ 0.011

^a Wells were coated with 1 μ g of each ECM component, and 1 μ g of recombinant protein was added per well. Values are optical densities at 492 nm (95% level of confidence). The attachment of Lsa24 to laminin was compared to the attachment of the protein to BSA by the two-tailed *t* test ($P < 0.05$). Binding of Lsa24 to laminin was also significant compared to its binding to all ECM proteins mentioned above ($P < 0.05$).

FIG. 4. Binding of Lsa24 to laminin as a function of protein and laminin concentration. (A) Binding of laminin (1 µg) with recombinant protein (concentration ranging from 0 to 1,000 nM); (B) recombinant protein (1 µg) with laminin (amount ranging from 0 to 1 µg). Each point represents the mean absorbance value at 492 nm $\pm$ the standard deviation of three independent experiments. Loa22 was included as a negative control since it showed no specific attachment to laminin.

FIG. 5. Sugar moiety contribution to the laminin-Lsa24 interaction. Immobilized laminin was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark. Mean absorbance values at 492 nm ($\pm$ the standard deviations of three independent experiments) were compared to those obtained with untreated laminin (0 mM). The percent reduction in protein attachment to metaperiodate-treated laminin as a function of periodate concentration is indicated above each bar.

FIG. 6. *L. interrogans* attachment to ECM macromolecules. Glass slides coated with laminin (A), collagen I (B), collagen IV (C), cellular fibronectin (D), plasma fibronectin (E), or BSA (F) were incubated with 5×10^7 leptospires (*L. interrogans* serovar Pomona). Attachment was visualized using dark-field microscopy.

probed with anti-LipL32 serum, based on the fact that LipL32 is a major outer membrane leptospiral protein (18). Lsa24 caused a modest but significant reduction in the number of leptospires adhering to laminin ($P < 0.05$) (Fig. 7C). The experiment was repeated three times with similar results.

Cellular localization of Lsa24. To determine whether Lsa24 is localized at the membrane, we performed an extraction of the *L. interrogans* outer membrane with the nonionic detergent Triton X-114. The three fractions derived from TX-114-extracted material after incubation at 37°C were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with Lsa24 antiserum. Lsa24 was found to be completely absent from the detergent-insoluble pellet fraction but

FIG. 7. Inhibition of *L. interrogans* attachment to laminin by Lsa24. Chamber slides were coated with 4 μ g of laminin for 16 h. The slides were blocked with BSA, and 5×10^7 leptospires were added to each chamber. (A) No protein added to the blocking solution; (B) 20 μ g of Lsa24 was added to the blocking solution to test for its ability to competitively inhibit attachment of *L. interrogans* to laminin. Adherence was visualized by dark-field microscopy. (C) *Leptospira* attachment to the substrate was quantified by ELISA. Laminin-coated microtiter wells were incubated with 0.5 μ g of Lsa24 for 1 h 30 min prior to the addition of 1×10^7 leptospires. Wells were probed with anti-LipL32 serum. Data are expressed as the mean $A_{492} \pm$ the standard error of three independent experiments, each performed in triplicate. Significance was assessed by comparison with the no-protein wells by Student's two-tailed *t* test (*, $P < 0.05$).

could be readily detected in the TX-114 detergent phase, indicating that this protein is a component of the leptospiral membrane (Fig. 8).

DISCUSSION

It is well known that pathogen adhesion to and colonization of host tissue is an early and critical event in the infection process. Although a number of putative spirochete virulence factors involved in toxin production, attachment, and immunity have been suggested, the role of the majority of those factors in pathogenesis remains uncertain. To date, a single leptospiral adhesin displaying fibronectin-binding properties has been described (26). Of unknown identity, this protein is specifically expressed on the surface of virulent *L. interrogans* serovar Icterohaemorrhagiae. In this work, we report for the first time an *L. interrogans* protein, named Lsa24, that binds strongly to laminin, a constituent of the basement membrane underlying both the epithelium and the endothelium. Lsa24 was expressed in *E. coli* as a 24-kDa full-length recombinant protein. The purified protein exhibited a single major band in SDS-PAGE suitable for ECM-binding assays. The structural integrity of the purified protein was assessed by CD spectroscopy, which re-

vealed a predominant molecule population of β -strands. The presence of Lsa24 in the hydrophobic, detergent phase of *L. interrogans* Triton X-114-solubilized extract points out its localization at the bacterial membrane, most likely at the outer membrane, as previously shown for LipL32 (17, 18).

Laminin-1, classically referred as laminin, is a large, flexible protein composed of three very long polypeptide chains (α , β , and γ) arranged in the shape of an asymmetric cross and held together by disulfide bonds. A characteristic feature of laminin is its high carbohydrate content (13 to 15%) (13). Laminin recognition may play a role in the pathogenicity of microorganisms: virulence and adherence of *Trichomonas vaginalis* and *Trichomonas fetus* to epithelial cells is greatly increased in the presence of laminin (38). Based on an in vivo model for the study of fungal pathogenicity, Vicentini and colleagues demonstrated that the binding of *Paracoccidioides brasiliensis* to laminin enhanced the fungal pathogenesis (42).

Helicobacter pylori, particularly hemagglutinating strains, has been shown to bind specifically to this ECM component (41). Initial recognition and binding of laminin occur through surface-exposed lipopolysaccharide (40). Subsequently, a more specific interaction with a 25-kDa outer membrane adhesin on the bacterial surface occurs (41). To rule out the presence of residual *E. coli* LPS that could eventually interfere with our ECM-binding assays, all recombinant proteins used in our studies were treated with polymyxin B. A dose-dependent specific and saturable binding of Lsa24 to immobilized laminin was observed, fulfilling the properties of a typical receptor-ligand interaction. Metaperiodate oxidation of laminin caused significant reduction in the binding activity, strongly suggesting the involvement of laminin carbohydrate moieties in the interaction. This finding is in agreement with the previously published data pointing to a crucial role of laminin carbohydrate groups in the interaction with pathogens (5, 8, 10, 15, 35, 39, 41).

To date, no universal laminin-binding motif has been described within the known adhesins. Laminin-binding proteins do not share amino acid sequence similarity and vary in mo-

FIG. 8. Cellular localization of Lsa24. Triton X-114 fractions of *L. interrogans* serovar Copenhageni organisms were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with Lsa24 antiserum. Fractions analyzed were the whole organism (lane 1), Triton X-114-insoluble pellet (lane 2), aqueous phase (lane 3), and detergent phase (lane 4).

lecular size (5). Lsa24 seems to have no significant similarity with sequences currently available, including those from other spirochetes. In a recent report, Cameron and colleagues, using synthetic peptides, identified 10 amino acid residues present in a *T. pallidum* laminin-binding protein that are essential for attachment to laminin: amino acids P⁹⁸V⁹⁹Q¹⁰⁰T¹⁰¹, W¹²⁷I¹²⁸, and T¹⁸²A¹⁸³I¹⁸⁴S¹⁸⁵ (7). Alignment of the Lsa24 amino acid sequence to this *T. pallidum* protein revealed no similarity (data not shown). Since no consensus laminin-binding motif has been described to date, it is possible that laminin-interacting sites harbored by Lsa24 might be specific and still need to be defined. Of all the ECM components tested, adhesiveness to laminin suggests that this highly glycosylated protein is the major ECM target for Lsa24.

In a previous report, Ito and Yanagawa demonstrated that virulent strains of *L. interrogans* attached to the ECM of mouse fibroblast cells (19). Moreover, it was suggested that leptospiral attachment to ECM correlates with virulence, since virulent lines of *L. interrogans* attached to ECM more effectively than intermediate virulent and avirulent lines of the same strains (19). From these results one may anticipate that *Leptospira* possesses proteins capable of adhering to several ECM components. We have demonstrated for the first time that this is indeed the case, as *L. interrogans* showed attachment to laminin, collagen type I, collagen type IV, cellular fibronectin, and plasma fibronectin.

As expected, Lsa24 partially inhibited attachment of intact *L. interrogans* to immobilized laminin. The inhibitory effect exerted by the recombinant protein was moderate and can be justified by the existence of additional *L. interrogans*-binding proteins contributing to the leptospiral adherence to laminin.

In conclusion, we have identified a leptospiral protein in the *L. interrogans* serovar Copenhageni genome sequence that exhibits attachment to laminin. Considering that leptospires are highly invasive microorganisms, there might be several other adhesins involved in the initial steps leading to infection. Studies concerning the underlying molecular mechanisms involved in adhesion are currently under way. Uncovering bacterium-host interactions at a molecular level not only will assist our understanding of the host physiology but also will facilitate the search for vaccine targets against leptospirosis.

ACKNOWLEDGMENTS

We express our deep gratitude to Frank H. Quina (Instituto de Química, Universidade de São Paulo) for a critical reading of the manuscript.

This work has benefited from grants from FAPESP, CNPq, and Fundação Butantan.

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Editor: J. B. Bliska

Research article

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Lsa2I, a novel leptospiral protein binding adhesive matrix molecules and present during human infection

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Published: 29 April 2008

Received: 27 September 2007

BMC Microbiology 2008, 8:70 doi:10.1186/1471-2180-8-70

Accepted: 29 April 2008

This article is available from: <http://www.biomedcentral.com/1471-2180/8/70>

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Abstract

Background: It has been well documented over past decades that interaction of pathogens with the extracellular matrix (ECM) plays a primary role in host cell attachment and invasion. Adherence to host tissues is mediated by surface-exposed proteins expressed by the microorganisms during infection. The mechanisms by which pathogenic leptospires invade and colonize the host remain poorly understood since few virulence factors contributing to the pathogenesis of the disease have been identified. Whole-genome sequencing analysis of *L. interrogans* allowed identification of a repertoire of putative leptospiral surface proteins.

Results: Here, we report the identification and characterization of a new leptospiral protein that exhibits extracellular matrix-binding properties, called as Lsa2I (leptospiral surface adhesin, 21 kDa). Compatible with its role in adhesion, the protein was shown to be surface-exposed by indirect immunofluorescence. Attachment of Lsa2I to laminin, collagen IV, and plasma fibronectin was specific and dose dependent. Laminin oxidation by sodium metaperiodate reduced the protein-laminin interaction in a concentration-dependent manner, indicating that laminin sugar moieties are crucial for this interaction. The gene coding for Lsa2I is present in pathogenic strains belonging to the *L. interrogans* species but was not found in the saprophytic *L. biflexa* serovar Patoc strain Patoc I. Loss of gene expression occurs upon culture attenuation of pathogenic strains. Environmental factors such as osmolarity and temperature affect Lsa2I expression at the transcriptional level. Moreover, anti-Lsa2I serum labeled liver and kidney tissues of human fatal cases of leptospirosis.

Conclusion: Our data suggest a role of Lsa2I in the pathogenesis of leptospirosis.

Background

Leptospirosis, a worldwide zoonotic infection, is an important human and veterinary health problem. Caused by spirochaetes of the genus *Leptospira*, the disease presents greater incidence in tropical and subtropical regions [1,2]. The transmission of leptospirosis has been associated with exposure of individuals near to wild or farm animals [3]. Recently, the disease has been prevalent in cities with sanitation problems and a large population of urban rodent reservoirs, which contaminate the environment through their urine [4]. In the host, leptospirosis has a biphasic clinical presentation beginning with a septicemic followed by an immune phase with antibody production and urinary excretion of leptospires. Children primarily show fever, vomiting, headache, diarrhea, abdominal and generalized muscle pain, whereas adults have fever, headache, anorexia, muscle pain and constipation [4,5]. The most severe form of leptospirosis, known as Weil's syndrome, seen in 5 to 15% of patients, is a multisystemic febrile illness, chiefly with hepatic, renal and pulmonary involvement and a mortality rate of 5 to 40% [4]. Leptospirosis also has a great economic impact in the agricultural industry because the disease affects livestock inducing abortions, stillbirths, infertility, reduced milk production and death [3,4].

The advent of whole-genome sequencing has greatly impacted on the microbial field with the development of new large-scale technologies, such as bioinformatics. This approach has the advantage of revealing proteins independently of their abundance and without the need of culturing the microorganism *in vitro* [6]. Functional genomic studies, including transcription profiles, gene cloning, protein expression and characterization complement the *in silico* analysis and help in understanding the bacterial pathogenesis. The genome of *L. interrogans* serovar Copenhageni has been sequenced and *in silico* analysis identified more than 200 predicted outer membrane proteins [7,8]. These proteins are potential targets for inducing immune responses during host infection and therefore, constitute targets for immune protection through mechanisms such as antibody-dependent phagocytosis and killing mediated by complement. In addition, it is possible that some of these membrane proteins mediate the initial adhesion to host cells [9-11]. Leptospiral adhesins have been described: a 36-kDa fibronectin-binding protein of unknown identity isolated from the outer sheath of a virulent variant of pathogenic leptospires [11], a 24-kDa laminin-binding protein named Lsa24 [9]/LfhA [12], LigA and LigB proteins [10]. More recently, Lsa24 [9]/LfhA [12] was shown to belong to a paralog family designated *Leptospira* endostatin-like proteins (Len) [13]. Some proteins of the Len family were capable to bind host fibronectin [13]. Fibronectin binding activity has been also shown with LigA and LigB proteins [10] and a

domain of the LigB protein that contributes to this binding has recently been described [14].

Several predicted surface-coding sequences were selected from the genome of *L. interrogans* serovar Copenhageni and are under study in our laboratory [8,9,15]. In the present study, we focused on a novel hypothetical protein of unknown function, encoded by the gene LIC10368. The gene was cloned and the protein expressed using *E. coli* as a heterologous host system. The recombinant protein of 21 kDa was purified and its capacity to mediate attachment to various extracellular matrix (ECM) components was evaluated. We have found that this novel leptospiral protein is a surface exposed adhesin that binds strongly to laminin, fibronectin (plasma) and collagen IV. The gene coding for Lsa21 (leptospiral surface adhesin of 21 kDa) is expressed in low passage virulent Fiocruz L1-130, LPF and LO4 strains of *L. interrogans*, and is regulated by osmolarity and temperature. Anti-Lsa21 serum labeled liver and kidney tissues of human fatal cases of leptospirosis. Our data suggest a role of Lsa21 in pathogenesis and virulence.

Results

Cloning, expression and purification of recombinant protein

The choice of predicted surface-CDS was mostly based on cellular localization since surface proteins are potential targets for mediating adhesion to host. Thus, the LIC10368 CDS was predicted to be an outer membrane protein (93.1%) according to PSORT program [16]. The LipoP server predicted LIC10368 CDS to be a lipoprotein with a cleavage site for signal peptidase II at amino acids 18-19 [17]. It is a hypothetical protein with no known domain by BLAST [18,19] and PFAM analysis [20]. Identical predicted coding sequence of LIC10368 was identified in *L. interrogans* serovar Lai [21] but absent in *L. borgpetersenii* [22] and *L. biflexa* [23] genome sequences. The gene was amplified, without the signal peptide sequence, and the DNA insert cloned and expressed as a full-length protein in *E. coli*. Recombinant protein was expressed with 6XHis tag at the N-terminal, purified by metal chelation chromatography, and an aliquot of each step of the process was analyzed through SDS-PAGE (Fig. 1A). The expected protein band of 21 kDa is shown in NaCl-*E. coli* BL21 (SI)-induced culture, in insoluble form, as inclusion bodies (Fig. 1A, lane 5). Although the Lsa21 protein presented low expression level, it was consistently recovered from the column as single major band (Fig. 1A, lane 6). Structural integrity of the purified protein was assessed by circular dichroism (CD) spectroscopy. As depicted in Fig. 1B, the recombinant protein may encompass a mixture of both α -helices and β -sheets in its secondary structure composition. The experimental data obtained with Lsa21 is similar to the secondary structure

Figure 1
Analysis of Lsa2I recombinant protein from NaCl-induced *E. coli* BL21-SI by SDS-PAGE and Circular dichroism spectrum. In **A**, SDS-15% PAGE showing lane 1 – molecular weight protein marker (kDa), lane 2 – non-induced culture, lane 3 – induced culture, lanes 4 and 5 – supernatant and inclusion body pellet after bacterial cell lysis and centrifugation, respectively, lane 6 – purified protein eluted from Ni²⁺ – charged chelating sepharose column with 1 M imidazole. In **B**, CD spectrum of Lsa2I protein depicting both α -helical and β -sheets in its secondary structure composition. Far-UV CD spectrum is presented as an average of five scans recorded from 190 to 260 nm.

content of the native protein predicted by computational analysis [24].

Distribution and expression of LIC10368 gene among leptospire strains

The presence of LIC10368 gene in five pathogenic strains and in one saprophytic strain was examined by PCR with a pair of primers designed according to *L. interrogans* serovar Copenhageni genome sequences. A 540-bp DNA fragment covering almost the entire LIC10368 predicted CDS was amplified by PCR in all five strains belonging to the pathogenic species *L. interrogans* serovars: Canicola, Copenhageni, Hardjo, Icterohaemorrhagiae and Pomona. No amplification product was detected in the non-pathogenic *L. biflexa* serovar Patoc strain Patoc 1 (Fig. 2A). Fragments excised from the gel were purified, cloned into the pGEM-T Easy vector (Promega), and inserts were sequenced. Multiple sequence alignment of the deduced amino acid sequences of the five serovars harboring the LIC10368 gene revealed high conserved identity among the tested strains (not shown).

The ability of both high- (>200) and low- (< 3) passage *in vitro* cultured leptospires to express LIC10368 was assessed by PCR amplification of reversely transcribed total RNA. Significant levels of gene product could be

detected in all three low-passage strains tested (Fig. 2B), whereas among the high-passage strains detectable amounts of LIC10368 transcripts were only observed for the Hond Utrecht IV strain (serovar Canicola). Integrity of total RNA used in RT-PCR experiments was assured by the presence of a 1,042-bp 16S ribosomal cDNA fragment in all samples (Fig. 2B).

Regulation of LIC10368 by environmental cues

It is well known that prolonged culture promotes leptospiral virulence attenuation [25], and it has been suggested that expression of virulence factors may be downregulated upon sequential *in vitro* culture passages [26]. To evaluate the effect of culture-attenuation by *in vitro* passages on LIC10368 expression, the virulent low-passage strains *L. interrogans* serovar Pomona strain LPF, *L. interrogans* serovar Canicola strain LO4, and the reference high-passage strains *L. interrogans* serovar Pomona strain Pomona, *L. interrogans* serovar Canicola strain Hond Utrecht IV were grown to late-log phase and collected for RNA isolation. RT-PCR analyses of LIC10368 transcripts were performed, and a significant reduction of gene expression was already observed after 4 (strain LPF) or 3 (strain LO4) passages, thus indicating that LIC10368 expression is rapidly reduced with sequential *in vitro* culture passages. Amplified products were not detected in

Figure 2

Distribution and expression of LIC10368 gene in saprophytic and pathogenic leptospires. (A) Genomic DNA from *L. biflexa* Patoc and from five serovars belonging to the pathogenic species *L. interrogans* was subjected to PCR analysis with LIC10368 specific primers designed according to *L. interrogans* serovar Copenhageni genome sequences. The expected size of the PCR product is 540 bp. No DNA was added to the negative control reaction (-). **(B)** RT-PCR analysis of LIC10368 transcripts in high-passage *L. interrogans* strains and in the low-passage LO4 (Canicola), Fiocruz LI-130 (Copenhageni), LPF (Pomona) strains. Reactions were performed with the same primer pairs mentioned above. Samples quantity and integrity were verified by amplification of a 1042-bp 16S ribosomal cDNA fragment. RT+: reverse transcriptase present; RT -: reverse transcriptase omitted; M: molecular mass markers.

control reactions lacking reverse transcriptase, ruling out DNA contamination.

As our results pointed to a correlation between virulence and LIC10368 expression, we decided to examine whether environmental factors, such as osmolarity and temperature could influence LIC10368 regulation at the transcriptional level. All RT-PCR assays were performed with the virulent, low-passage *L. interrogans* serovar Pomona strain LPF. Induction of LIC10368 expression by osmolarity was assessed by growing cultures at 29°C in EMJH supplemented with 1% rabbit serum and resuspending them in fresh EMJH medium or in EMJH containing 120 mM NaCl. The addition of 120 mM NaCl to the medium mimics physiological conditions (~300 mosmol per liter) encountered by leptospires upon entry into the host [26]. Cultures were incubated for 24 h and examined for gene expression. LIC10368 expression was also evaluated in a culture grown under our standard laboratory conditions. As a positive control, we included RT-PCR data for the *lipL53* gene (LIC12099) (Fig. 3B). This gene codes for a predicted lipoprotein reactive with sera from patients with leptospirosis [15] and was shown to be strongly up-regulated by sodium chloride in a recently published paper [27]. As shown in Fig. 3B, LIC10368 expression appears to be regulated by the osmolarity of the growth medium, because transcripts were detected only upon incubation with additional NaCl, or with a rich EMJH medium containing more serum and salts. Similar up regulation was observed with *lipL53* expression, as previously detected by microarrays analysis [27].

Temperature is another important environmental signal that may affect protein expression in bacteria. The transition of temperatures from ambient to mammalian body (37° and later 39°C or higher during the febrile stage) has been correlated with changes in the expression of virulence determinants in many pathogens [28]. Moreover, it has been demonstrated that *L. interrogans* also regulates protein synthesis in response to *in vitro* temperature changes [29,30]. Therefore, we compared LIC10368 gene expression patterns of cultures grown at 20°C, 29°C and 37°C, reflecting ambient temperatures in the environment, growth under laboratory conditions, and mammalian host body core temperature. Transcriptional analyses were also performed with cultures subjected to temperature upshifts from 29°C to 37°C and from 37°C to 39°C during an overnight period to simulate conditions experienced by leptospires in the early stages of infection and during febrile stage. LIC10368 transcript levels gradually increased when cultures grown at 20°C, 29°C and 37°C were compared (Fig. 3C). An overnight upshift from 29°C to 37°C had no effect on LIC10368 expression, while an upshift from 37° to 39°C seems to have down-regulated gene expression (Fig. 3C). Our results indicate that tem-

perature is another critical parameter involved in the control of LIC10368 gene expression. Densitometric readings provided information regarding accumulation of LIC10368 transcripts (Fig. 3A, B, C).

Cellular localization of Lsa21 protein

Indirect immunofluorescence was used to examine whether Lsa21 protein is surface-exposed. A similar protocol has been previously employed to evaluate the capability of *Leptospira* to bind factor H [12]. The low-passage *L. interrogans* serovar Pomona strain LPF reacted with antiserum directed against recombinant Lsa21 protein, but no staining was observed with the *L. biflexa* serovar Patoc strain Patoc 1 (Fig. 4). Pre-immune serum failed to elicit a positive signal in both strains (data not shown).

Adhesion of Lsa21 protein to ECM components

As Lsa21 protein is suggested to be surface-exposed and might play a role in virulence, we queried whether it could mediate host colonization by adhering to extracellular matrix proteins. Therefore, we examined its interaction with laminin, collagen type I, collagen type IV, cellular fibronectin, and plasma fibronectin. BSA and fetuin were included as negative controls and binding assays were performed by an ELISA-based assay [9]. As shown in Fig. 5, Lsa21 protein bound to all immobilized ECM macromolecules tested, but no interaction was detected with BSA or fetuin. As a negative control, we have included another recombinant protein, rLIC10191, known as Loa22 [31], and recently described as a virulence factor in *Leptospira* species [32]. A stronger interaction was observed with laminin, collagen IV and plasma fibronectin. This interaction was also assessed on a quantitative basis as illustrated in Fig. 6A. A dose-dependent binding was observed when increasing concentrations of the recombinant protein (0 to 2,000 nM) were allowed to adhere to a fixed laminin, collagen IV or fibronectin concentration (1 µg).

Protein binding is affected by laminin oxidation

To investigate the role of laminin carbohydrate moieties in the interaction with Lsa21 protein, laminin was oxidized by increasing concentrations of sodium metaperiodate (5 to 100 mM) for 15 min at 4°C. The mild treatment ensures cleavage of vicinal carbohydrate hydroxyl groups, but the polypeptide chain structure remains intact [33]. Oxidation effect was dose-dependent and a significant reduction (~65%) in Lsa21 protein attachment to metaperiodate-treated laminin was observed at a 100 mM concentration of periodate (Fig. 6B). These data indicate that laminin sugar residues are critical for the interaction of Lsa21 protein with this major ECM glycoprotein.

Figure 3

Regulation of LIC10368 expression by environmental factors. (A) RT-PCR analysis of LIC10368 transcripts upon culture-attenuation. RNA was extracted from low- and high-passage *L. interrogans* strains. Above, low-passage *L. interrogans* serovar Pomona strain LPF (passages 1 and 4, P1 and P4) and high-passage *L. interrogans* serovar Pomona strain Pomona (HP). Below, low-passage *L. interrogans* serovar Canicola strain LO4 (passages P1 and P3) and high-passage *L. interrogans* serovar Canicola strain Hond Utrecht IV (HP). **(B)** Regulation of LIC10368 transcript levels by osmolarity. Cultures of *L. interrogans* serovar Pomona strain LPF grown in EMJH with 1% rabbit serum were centrifuged and resuspended in fresh EMJH medium or in EMJH containing 120 mM NaCl. Cultures were incubated for 24 h before being harvested for RNA isolation. RNA was also obtained from a culture grown under our standard laboratory conditions (10% rabbit serum supplemented with amino acids and salts). LIC10368 transcripts obtained from cultures with 1% serum (1%), 1% serum + 120 mM NaCl (1% + NaCl) and 10% serum + amino acids and salts referred as enriched medium, EM (10% EM). 16S ribosomal cDNA fragments (1,042-bp) were co-migrated in all lanes. LIC12099 (*lipL53* gene) transcripts were included as a positive control (1%, 1% + NaCl). **(C)** The effect of temperature on LIC10368 transcript levels was assessed by culturing leptospires at 20°C, 29°C and 37°C. Additional cultures grown at 29°C and at 37°C were shifted overnight to 37°C and to 39°C, respectively, in order to simulate conditions encountered by bacteria upon entry into the host and in a febrile stage. Optical densities of LIC10368 and LIC12099 transcripts were normalized for each sample with corresponding 16S ribosomal densitometry data to obtain the relative expression levels. RT+: reverse transcriptase present in the reaction; RT-: reverse transcriptase omitted; M: molecular mass markers.

L. interrogans (LPF)

L. biflexa (Patoc 1)

Figure 4

Indirect immunofluorescence staining of intact fixed leptospires. *L. interrogans* serovar Pomona strain LPF and *L. biflexa* serovar Patoc strain Patoc 1 were incubated with mouse anti-Lsa21 serum, followed by incubation with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody. Magnification $\times 1,260$.

Sequence comparison between Lsa21 and leptospiral adhesions

To investigate whether Lsa21 protein shared a sequence similarity or a common domain with other previously identified leptospiral ECM-binding proteins [9,10,13] we proceeded with a sequence analysis using Clustal X program and a tree-display NJ plot [34,35]. The calculated tree derived from sequence alignment is depicted in Fig. 7 and clearly shows that Lsa21 sequence is unrelated to both Len protein family and LigA/LigB proteins. Contrasting with the Len and Lig proteins that divide a common branch inside their family, Lsa21 appears alone on its branch.

Lsa21 is expressed during human leptospiral infection

IHC (immunohistochemistry) analysis showed essentially similar results in all 5 patients diagnosed with Weil's syndrome. It is necessary to point out that, due to the relatively short interval between autopsy and the patients' death, tissues were better preserved, and classical findings such as liver-plate disarray, although present, were not prominent. Leptospiral Lsa21 antigen was frequently detected as red deposits in the cytoplasm of isolated macrophages along the sinusoidal lining (Kupffer cells and circulating macrophages) (Fig. 7A). Similar deposits were rarely observed in macrophages of the portal inflamma-

tory infiltrate. Lsa21 antigen was also observed focally on the sinusoidal side of the membrane of hepatocytes and as small granules in the underneath cytoplasm (Fig. 7A). Lsa21 protein was infrequently seen over endothelial cells of portal vessels and supra-hepatic branches. In the kidney Lsa21 antigen was present in the luminal side of epithelial cells of the distal nephron, particularly distal tubules and collecting ducts (Fig. 7B, 7C). As observed in liver cells, antigen granules were also identified in the cytoplasm below the cellular membrane. No definite Lsa21 expression was detected in epithelial cells of the proximal tubules. Lsa21 deposit in the cytoplasm of macrophages of the focal interstitial inflammatory infiltrate was rarely observed (see Fig. 8).

Discussion

The characterization of leptospiral outer membrane proteins represents an important step toward the understanding of leptospirosis pathogenicity. To date, there has been little functional analysis of *Leptospira* membrane proteins, in spite of their unquestionable relevance to host-pathogen interactions. Bioinformatic analysis of the genome sequences of *L. interrogans* serovar Copenhageni revealed more than 200 predicted outer membrane proteins that merit further studies [7,8]. In this report, we have characterized one of those hypothetical proteins, a surface lept-

Figure 5
Binding of Lsa21 recombinant protein to ECM components. Wells were coated with 1 µg of laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, and the control proteins BSA and fetuin. Recombinant Lsa21 and Loa22 proteins attachment to those ECM macromolecules was assessed by an ELISA-based assay. One microgram of recombinant protein was added per well. Optical densities were taken at 492 nm. Data represent the mean ± standard error of three independent experiments. For statistical analysis, the attachment of Lsa21 to ECM macromolecules was compared to its binding to BSA by the Student's two tailed t- test (*, $P < 0.01$).

ospiral adhesin of 21 kDa, named Lsa21 that may play a role in pathogenesis.

The LIC10368 expression is mostly detected in low-passage virulent strains (Figs. 2B, 3A). Culture attenuation has also been reported for the LigA and B proteins, leptospiral antigens recognized during the acute host infection [26,36]. Although a larger sample set will be required for definite conclusion, our findings suggest a correlation between Lsa21 protein and virulence.

The LIC10368 gene is up regulated by osmolarity, another distinguishing feature shared with *ligA* and *B* genes [26]. The addition of 120mM NaCl to the culture medium reproduces the host's serum osmolarity (~300 mosM), thus providing a more physiological environment for leptospiral growth. In fact, EMJH-salt supplementation enhanced Lsa21 expression (Fig. 3B). It is worth mentioning that several predicted outer membrane proteins currently under study in our laboratory are responsive to

physiologic osmolarity (unpublished data). It is not surprising because 6% of the *L. interrogans* genes are susceptible to osmoregulation [27]. Osmotic control of gene expression has been reported as an environmental cue associated with virulence in a variety of pathogens [28]. In *V. cholerae*, optimum expression of cholera toxin and *Tcp pilli* occurs within an osmolarity range that probably represents that of mucosal secretions [37]. Transcription levels of *invA*, an *S. Typhimurium* invasion gene, have also been reported to be considerably higher on media with high osmolarity [38]. Moreover, genes controlling the *P. aeruginosa* capsule, an important *Pseudomonas* virulence factor, are also subjected to osmoregulation [39].

Temperature induction of LIC10368 expression was also observed (Fig. 3C). Maximal mRNA levels were detected at 37°C, the body core temperature of most mammalian species. Intriguingly, LIC10368 is not represented among the leptospiral genes found to be up-regulated in response to temperature or physiologic osmolarity, as assessed by whole-genome microarrays [27,29]. This could be explained by the fact that LIC10368 expression rapidly decreases after three or four passages in culture medium (Fig. 3A). In addition, optimum expression might occur when both physiological parameters (mammalian body core temperature and host's serum osmolarity) are combined.

Lsa21 protein exhibits extracellular matrix-binding properties. It is thus possible that it may play a role in the attachment to host tissues. Several leptospiral adhesins have been described, including a 36-kDa fibronectin-binding protein [11], a 24-kDa laminin-binding protein named Lsa24 [9]/LfhA [12], LigA and LigB proteins [10]. Recently, it has been demonstrated that *L. interrogans* contain five additional paralogs of Lsa24 [9]/LfhA [12], designated as Len-like proteins [13]. The Len proteins were all found to bind laminin and in addition LenB, LenC, LenD, LenE and LenF have shown affinity to bind host fibronectin [13].

Lsa21 protein exhibits a broader spectrum binding profile because it interacts with laminin, collagen IV and fibronectin. Similarly, other adhesins, namely the Lig proteins [10], have been reported to bind to different ECM macromolecules. In fact, attachment of *Leptospira* to several ECM macromolecules, including laminin, collagen I, collagen IV, cellular fibronectin, and plasma fibronectin was previously shown [9]. Interestingly, Lsa21 shares neither sequence similarity nor a common domain with the Len-like and the Lig proteins. Emp, a cell surface protein of *Staphylococcus aureus*, strongly interacts with fibronectin, fibrinogen, collagen, and vitronectin [40]. The outer membrane protein YadA of *Yersinia enterocolitica* has been shown to bind to laminin, fibronectin, and several types

Figure 6

(A) Lsa21 binds to ECM components in a dose-dependent manner. Binding of Lsa21 to laminin, collagen IV and plasma fibronectin, as a function of protein concentration (0 to 2,000 nM). Each point represents the mean absorbance value at 492 nm $\pm$ the standard deviation of three independent experiments. BSA was included as a negative control. **(B) Sugar moiety contribution to the laminin-Lsa21 interaction.** Immobilized laminin was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark. Each point represents the mean absorbance value at 492 nm $\pm$ the standard deviation of three independent experiments. Reduction in attachment compared to the level of attachment to untreated laminin was statistically significant: in each case (*), the *P* value, as measured by the Student two-tailed *t*-test, was ≤ 0.004 .

of collagens [41-43]. *Enterococcus faecalis* adhesin Ace mediates attachment to laminin, and to collagens I and IV [44]. Finally, the *Haemophilus influenzae* Hap autotransporter protein exhibits the same binding profile of Lsa21 protein: it interacts with laminin, fibronectin and collagen IV [45]. Chemical disruption of laminin carbohydrate moieties by sodium metaperiodate caused significant reduction in the binding activity of Lsa21 protein, thus indicating the sugar moiety involvement in interactions between this recombinant protein and ECM macromolecules. Possibly Lsa21 protein interacts with a particular structural epitope shared by the ECM components mentioned above. A good candidate would be complex sugars of glycosylated proteins. During infection, injury of wall vessels exposes a repertoire of adhesive glycoproteins that constitute major targets for initial adherence of pathogens [46]. The extracellular matrix-binding properties of Lsa21 together with the data of indirect immunofluorescence suggest that this protein is surface-exposed.

The presence of Lsa21 antigen as demonstrated by IHC in cells, chiefly macrophages, of the inflammatory infiltrate both in human liver and kidney is expected because lept-

ospirosis is an acute septicemic disease involving several organs and blood vessels. Liver-plate disarray is a pathological finding described in human and experimental leptospirosis and in the human liver, its prominence is closely related to the interval between patient's death and the autopsy, thus indicating that post-mortem changes might accentuate the lesion. In any circumstance, liver-plate disarray supports speculation that cell membrane lesion might play an important role in leptospirosis pathogenesis [47]. Although cross-reactivity between anti-Lsa21 serum and other leptospiral proteins is not excluded, immunohistochemical detection of leptospiral antigens on the sinusoidal side of human hepatocytes is an infrequent finding in human leptospirosis when standard whole bacterial polyclonal sera were used [47]. The fact that Lsa21 anti-serum stained hepatocyte membranes more often might strengthen the possible role of cell membrane lesion in the pathogenesis of the disease.

Tubulo interstitial nephritis is the main manifestation of acute renal failure in leptospirosis and the renal injury is usually associated with polyuria. Hypokalemia appear frequently with an elevated urinary fractional excretion of

Figure 7

Sequence comparison between Lsa21 and leptospiral proteins. Unrooted phylogenetic tree of predicted amino acid sequences of the Len family, LigA/LigB and Lsa21 proteins. The tree was generated by Clustal X program and displayed by NJ plot. Branch lengths are depicted.

potassium, possibly due to proximal tubular lesions that is expressed both in human and experimental leptospirosis [47-50]. The IHC detection of the leptospiral antigenic protein Lsa21 on the epithelial membrane of the distal nephron, particularly distal tubules and collecting ducts, might be, up to a certain point, unexpected when a functional correlation is attempted. However, it is known that in a normal state, urine is concentrated because of the combined functions of Henle's loop and the collecting

duct. Therefore, we might speculate that the Lsa21 deposits detected by IHC chiefly in collecting ducts might be interfering with the renal water absorption in leptospirosis, in spite of the absence of definite morphological alterations. Altogether these results strongly suggest a role of this protein in the pathogenesis of the disease because no reactivity was seen in non-leptospirosis human fatal cases.

Figure 8

(A) Leptospirosis, human liver: Lsa21 is present as red deposits in the cytoplasm of isolated macrophages along the sinusoidal lining (arrow heads). Similar deposits are observed focally on the apical sinusoidal plasma membrane of hepatocytes and as small granules in the underneath cytoplasm (arrows). IHC and light hematoxylin counterstain; **(B) Leptospirosis, human kidney:** Lsa21 antigen is present over the luminal side of distal tubules. Red granules are also seen in the cell cytoplasm below. Notice the absence of Lsa21 antigen in the proximal tubules. Glomerulus present is apparently normal. IHC and light hematoxylin counterstain; **(C) Leptospirosis, human kidney:** Lsa21 antigen is present at the luminal side of collecting ducts as red granules over the epithelial cells and in the cell cytoplasm below; IHC and light hematoxylin counterstain. In **A**, **B**, and **C**: original magnification $\times 300$.

Conclusion

We describe a novel leptospiral surface adhesin of 21 kDa, Lsa21, shown for the first time in human fatal cases of the disease, a finding that should contribute to our understanding of the molecular mechanisms of leptospiral pathogenesis.

Methods

Leptospira strains and culture conditions

The non-pathogenic *L. biflexa* serovar Patoc strain Patoc 1, the high-passage *L. interrogans* serovar Canicola reference strains Hond Utrecht IV, *L. interrogans* serovar Copenhageni strain M-20, *L. interrogans* serovar Hardjo strain Hardjoprajtino, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona, the virulent strains of *L. interrogans* serovar Pomona strain LPF, *L. interrogans* serovar Canicola strain LO4 and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were cultured at 29°C under aerobic conditions in liquid EMJH medium (Difco®- USA) with 10% rabbit serum, enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%), calcium chloride (wt/vol: 0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol: 0.03%) and meat extract (wt/vol: 0.02%). Virulence of the *L. interrogans* serovar Pomona strain LPF, *L. interrogans* serovar Canicola LO4 and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 is maintained by iterative pas-

sages in Golden Syrian hamsters. Animals were injected with 10 to 10⁵ leptospires inocula from low and high passage cultures. Hamsters were monitored during 21 days. No significant difference in mortality taxes was observed when animals were inoculated with "no-passage" or "3-4" passages of *Leptospira* cultures and no death was observed after animal inoculation with high passage cultures. Regulation of LIC10368 expression by environmental factors such as temperature and osmolality was evaluated in the low-passage *L. interrogans* serovar Pomona strain LPF. The effect of temperature on LIC10368 transcript levels was assessed by culturing leptospires at 20°C, 29°C and 37°C. Additional cultures grown at 29°C and at 37°C were shifted overnight to 37°C and to 39°C, respectively, to simulate conditions encountered by bacteria upon entry into the host and in a febrile stage. Induction of LIC10368 expression by osmolality was examined by centrifugation of cultures grown at 29°C in EMJH supplemented with 1% rabbit serum followed by resuspension in fresh EMJH medium or in EMJH containing 120 mM NaCl. Cultures were incubated for 24 h before being harvested for RNA isolation.

'In silico' identification and characterization of the protein

Predicted coding sequence (CDS) LIC10368 was selected from the *L. interrogans* serovar Copenhageni genome

sequences based on its cellular localization prediction by PSORT program [16]. Sequence motifs, including signal peptides, lipoprotein cleavage sites and transmembrane domains were searched using specific softwares [17-20,51,52]. Sequence analysis was performed with Clustal X [35] and a tree displayed program by the Neighbour-Joining method [34].

Cloning, expression and purification of recombinant protein

Predicted CDS LIC10368 was amplified by the PCR from total *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA using the primer pairs 5'-GATGAAAAAAGAAAATGAATTGAG and 5'-AACGCGATTCATAGAGAGCG. PCR product was cloned into pENTR TOPO vector (Invitrogen) followed by transfer/recombination of DNA insert into the *E. coli* expression vector pDEST17 using the LR Clonase (Invitrogen). The construct was verified by DNA sequencing with appropriate vector-specific primers. Protein expression was achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotically induced promoter *proU* [53]. The cells were harvested by centrifugation, the bacterial pellets resuspended in 30 ml lysis buffer (500 mM NaCl, 20 mM Tris-HCl, and 0.1% Triton X-100, pH 8.0) and lysed by French Pressure (Aminco). The insoluble fraction was washed 3 times with 30 ml of buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM β mercaptoethanol, 1 M urea and 1% Triton X-100) before solubilization with 50 ml of buffer containing 20 mM Tris-HCl, 500 mM NaCl, 5 mM β mercaptoethanol, and 6 M guanidine-Cl. Protein refolding was achieved by 500 $\times$ dilution with 50 mM Tris-HCl (pH 9.0) and 500 mM NaCl. The refolded protein was then purified through metal chelating chromatography in a Sepharose fast flow column (GE Healthcare), extensively dialyzed against phosphate-buffered saline (PBS) pH 7.4, 0.1% (wt/vol) glycine solution for 24 – 48 h and fractions were analyzed in 15% SDS-PAGE.

Circular Dichroism spectroscopy

CD spectroscopy measurements were performed at 20°C in a Jasco J-810 Spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm path length cell at 0.5 nm intervals. The spectrum is presented as an average of five scans recorded from 190 to 260 nm.

DNA isolation and PCR analysis

Leptospira cultures were harvested by centrifugation at 11,500 g for 30 min and gently washed in sterile PBS twice. Genomic DNA was isolated from the pellets with a guanidine-detergent lysing solution (DNAzol® Reagent, Invitrogen), according to manufacturer's instructions. A

540-bp LIC10368 DNA fragment was amplified using oligonucleotides LIC10368-F: 5'-GATGAAAAAAGAAAATGAATTGAG and R: 5'-CTTCGCAACTTGTGGATAAGG and a 1450-bp LIC12099 DNA fragment was amplified using oligonucleotides LP14-F: 5'-CACCACCAATGTGTTTGGTATAGCG and LP14-R: 5'-CAGCGTTTTGTGATAAAATTAAC designed according to *L. interrogans* serovar Copenhageni genome sequences (GenBank accession AE016823). PCR was performed in a reaction volume of 25 μ l containing 100 ng of genomic DNA, 1 $\times$ PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 20 pmol of each specific primer, 200 μ M of each dNTP, and 2.5 U Taq DNA Polymerase (Invitrogen). Cycling conditions were: 94°C, 5 min, followed by 35 cycles at 94°C, 50 sec, 60°C (LIC10368)/55°C (LIC12099), 50 sec, 72°C, 1 min 30 sec, and a final extension cycle of 7 min at 72°C. Amplicons were loaded on a 1.5% agarose gel for electrophoresis and visualization with ethidium bromide. Gel-purified bands (Concert Rapid Gel Extraction System – Gibco BRL, Life Technologies) were cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) and the products were sequenced with the primers M13F (5'-GTTTCCCAGTCACGA) and M13R (5'-CAGGAAACAGCTATGAC) on an ABI Prism 3730 $\times$ 1 sequencer (SeqWright, Houston, Tex.). Multiple sequence alignment was performed with Clustal W [54].

RNA extraction and RT-PCR analysis

Leptospira cultures were harvested by centrifugation at 11,500 g for 30 min. For reverse transcription (RT)-PCR, total RNA was isolated from leptospires cultured as previously mentioned by the acid guanidinium thiocyanate phenol-chloroform method using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations. One microgram of RNA from each sample was treated with 1 U of DNase I (Invitrogen) for 15 min at RT. DNase I was inactivated by the addition of 1 μ l of 25 mM EDTA solution followed by an incubation at 65°C for 10 min. DNase-treated RNAs were reversely transcribed using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). One tenth of RT products were amplified in a 25 μ l reaction mix using oligonucleotides LIC10368-F/LIC10368-R or LP14-F/LP14-R as described above. In experiments designed to evaluate regulation of LIC10368 expression by environmental factors (osmolarity and temperature), 30 PCR cycles, instead of 35, were employed. Samples quantity and integrity were verified by amplification of a 1,042 bp 16S ribosomal cDNA fragment using oligomers 16S-F 5'-CAAGTCAAGCGGAGTAGCAATACTCAGC and 16S-R 5'-GATGGCAACATAAGGTGAGGGTTGC. Cycling conditions were: 94°C, 5 min, followed by 30 cycles at 94°C, 50 sec, 62°C, 50 sec, 72°C, 1 min 30 sec, and a final extension cycle of 7 min at 72°C. All samples were tested with and without reverse transcriptase to rule out genomic DNA contamination. PCR-

amplified products were loaded onto 1.5% agarose gels for electrophoresis and visualized by ethidium bromide staining. Direct sequencing was performed to confirm the identity of RT-PCR products. Densitometric readings were taken using Image-Quant 2.1 software (Amersham Pharmacia Biotech, Piscataway, NJ). Optical densities of LIC10368 transcripts were normalized for each sample with corresponding 16S ribosomal densitometry data to obtain the relative expression levels.

Antiserum

Ten female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 µg of Lsa21 protein. The recombinant protein was adsorbed in 10% (vol/vol) of Alhydrogel (2% Al(OH)₃, Biosector, formerly Superfos Biosector, Denmark), used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same protein preparation. Negative-control mice were injected with PBS. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers.

Immunofluorescence

The non-pathogenic *L. biflexa* serovar Patoc strain Patoc 1 and the virulent *L. interrogans* serovar Pomona strain LPF were grown to late log phase and collected by centrifugation at 11,500 g for 30 min. The cell pellets were washed three times with phosphate-buffered saline (PBS) (pH 7.4) and resuspended in a fixing solution containing 25% glutaraldehyde, 10% formalin, and PBS (pH 7.4), essentially according to the methodology described in Verma et al. [12]. Ten microliters of 1×10^9 leptospire per ml suspensions were applied to glass slides, which were then placed on ice for 1 h. After two washes with PBS, blocking was performed using 2% (wt/vol) BSA in PBS for 1 h. Slides were washed twice with PBS and incubated with a 1:50 dilution of mouse anti-Lsa21 protein serum or with preimmune serum for 1 h at room temperature (RT) in a humidifying chamber. After three washes with PBS, slides were incubated with a 1:200 dilution of a fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Sigma) for 1 h at RT. Slides were washed three times with PBS and mounting was performed in the antifading agent Mowiol (Calbiochem) with 2.5% DABCO (Sigma). A coverslip was added, and the slide was left overnight at 4°C before visualization by confocal microscopy (Carl Zeiss, Inc., Jena, Germany). Images were obtained with LSM 510 Meta software and the objective used was C-Apochromat 63×, scan zoom 2.0.

Binding of Lsa21 protein to ECM

All macromolecules, including the control proteins fetuin and BSA, were purchased from Sigma Chemical Co. (St.

Louis, Mo.). Laminin-1 and collagen Type IV were from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma, cellular fibronectin was from human foreskin fibroblasts, plasma fibronectin was from human plasma and collagen Type I was from a rat tail. Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol Briefly, ELISA plate wells (Nunc-Immuno Plate MaxiSorp Surface) were coated with 1 µg of laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, BSA (nonglycosylated attachment-negative control protein) and fetuin (highly glycosylated attachment-negative control protein) in 100 µl of PBS for 2 h at 37°C. The wells were washed three times with PBS-0.05% Tween 20 (PBST) and then blocked with 200 µl of 1% BSA for 1 h at 37°C followed by an overnight incubation at 4°C. One microgram of recombinant protein was added per well in 100 µl of PBS and protein was allowed to attach to the different substrates for 1 h 30 min at 37°C. After washing six times with PBST, bound protein was detected by adding 100 µl of a 1:5,000 dilution of mouse anti-Lsa21 serum in PBS. Incubation proceeded for 1 h and after three washes with PBST, 100 µl of a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) in PBS were added per well for 1 h. All incubations took place at 37°C. The wells were washed three times, and *o*-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5.0) plus 0.01% H₂O₂ was added. The reaction was allowed to proceed for 15 min and was then interrupted by the addition of 50 µl of 8 M H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (Labsystems Uniscience, Multiskan EX). For determination of dose-dependent attachment of Lsa21 protein to laminin, collagen IV and plasma fibronectin, protein concentrations varying from 0 to 2,000 nM in PBS were used.

For statistical analyses, the attachment of Lsa21 protein to ECM macromolecules was compared to its binding to BSA by the Student's two-tailed *t*-test. A *P* value less than 0.01 was considered statistically significant.

Metaperiodate treatment of laminin

Laminin oxidation was performed as described elsewhere [9]. Briefly, microtitre wells were coated with 1 µg of laminin in 50 mM sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three times with 50 mM sodium acetate buffer, pH 5.0, and immobilized laminin was treated with different sodium metaperiodate concentrations (5 – 100 mM) in the same buffer for 15 min at 4°C in the dark. After three washes with 50 mM sodium acetate buffer, wells were blocked with 200 µl of 1% BSA for 1 h at 37°C. Binding of Lsa21 protein (1 µg in PBS per well) to periodate-treated laminin was assessed as outlined above.

Human samples

Five patients, three males and two females, were autopsied with a diagnosis of Weil's syndrome. The age of the four patients ranged from 20 to 29 years old and one patient was 67 years old. Illness duration averaged seven days with a sudden onset manifested by headache, malaise, muscle pains and high fever. They developed jaundice, palpable enlarged liver, acute renal failure and a hemorrhagic syndrome. Epidemiological data showed that patients had had contact with contaminated water, mainly from floods and sewage. Main clinico-epidemiological data of the patients are in Table 1. Of note, two cases (4 and 5) were part of our previous publication [47]. The illness was usually of short duration and this, associated with delayed clinical diagnosis, contributed to the lack of some important confirmatory laboratorial tests. In fatal human cases and in farm animals, diagnosis by immunohistochemistry methods are specific and also offer clues to the understanding of the pathogenesis of the disease [47]. Special care was taken in selecting autopsies with typical macro and microscopical findings described in leptospirosis and performed after an average of up to five to six hours of death. Liver and kidney samples, which are more representative of the disease, were routinely fixed in formalin, embedded in paraffin and stained hematoxylin-eosin. Liver and kidney of five non-leptospirotic patients were obtained from autopsies performed within a close post-mortem interval as compared to leptospirotic patients and used as controls. Leptospiral antigens were detected by an immunohistochemical assay

using polyclonal rabbit anti-serum as previously described [47] with positive results in all five leptospirotic patients. Controls were negative.

Immunohistochemical assay

IHC studies were performed to assess the presence and localization of leptospiral antigens as detected by the mouse anti-Lsa21 serum (1:1,000 dilution). Kidney and liver 3 µm sections were analyzed using a standard immunohistochemistry protocol [47] with EnVision labeled polymer-AP mouse/rabbit K 4018 [55]. Antigen retrieval was performed by steamer incubation of the sections in citrate pH 6.0. Staining was completed with liquid permanent Red Chromogen K0640 [55]. All specimens were then lightly counterstained with hematoxylin. This method represents a better choice for diagnosis than the usual silver stains to detect the microorganisms particularly when we are dealing with human autopsies.

All animal studies were approved by the Ethics Committee of the Instituto Butantan (Sao Paulo, Brazil). Autopsies were performed at the Department of Pathology, University of Sao Paulo, School of Medicine (Sao Paulo, Brazil) and are routinely used for diagnosis, teaching and research purposes. All protocols used were verbally approved by the Ethics Committee of the University of Sao Paulo (São Paulo, Brazil).

Table 1: Leptospirosis: clinico-epidemiological and laboratory data

Case number/gender/age [56]	Clinical and epidemiological information	Illness duration(days)	Laboratory data available(leptospirosis)
1/m/29	Contact with contaminated water; fever, headache, vomiting, muscular pain, jaundice. Pulmonary hemorrhage. Hemodynamic and hydroelectrolytic disturbances, atrial fibrillation, acute renal failure. Hepatomegaly.	14	Seroagglutination and ELISA IgM positive
2/f/22	Contact with sewage and rats. Fever, muscular pain and respiratory failure (pulmonary hemorrhage). Jaundice. Hepatomegaly.	4	None available
3/m/29	Contact with contaminated water (floods). Fever, muscular pain and respiratory failure (pulmonary hemorrhage). Jaundice. Hepatomegaly. Acute renal failure.	6	None available
4*/m/20	Fever, muscular pain for the last 5 days, jaundice, bipalpebral edema, acute renal failure, pulmonary hemorrhage, abdominal pain and vomits, low platelet count.	5	None available
5*/f/67	Contact with rats and contaminated water, fever and muscular pain for the last 8 days. Jaundice, conjunctival hemorrhage, dyspnea and hemorrhagic sputum, acute renal failure.	8	None available

m: male; f: female; * cases previously published by De Brito et al. 2006 [47].

Authors' contributions

MA carried out molecular cloning, protein expression and purification. AB carried out molecular genetic studies and helped to draft the manuscript. TB designed and carried out immunohistochemical assays. SV carried out organism isolation and virulence maintenance. DL participated in the histochemical assays. ZM participated in organism isolation and experimental animal infection. PA carried out RNA isolation and immunofluorescence studies. AN selected the studied sequence, conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

Acknowledgements

We wish to express our deep gratitude to Drs. Jim Hesson and Roxane M. F. Piazza for helpful discussions and a critical reading of the manuscript. This research was supported by FAPESP, CNPq, Fundação Butantan and a grant from LIM 06 FMUSP (T. De Brito).

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Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein endothelial cells (HUVECS) and express during infection

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ARTICLE INFO

Article history:

Received 20 March 2008

Received in revised form 4 July 2008

Accepted 6 August 2008

Available online 20 August 2008

Keywords:

Leptospira interrogans

Recombinant proteins

HUVECS

ICAM-1

E-selectin

ABSTRACT

Cell adhesion molecules (CAMs) are surface receptors present in eukaryotic cells that mediate cell–cell or cell–extracellular matrix interactions. Vascular endothelium stimulation *in vitro* that lead to the upregulation of CAMs was reported for the pathogenic spirochaetes, including rLIC10365 of *Leptospira interrogans*. In this study, we report the cloning of LIC10507, LIC10508, LIC10509 genes of *L. interrogans* using *Escherichia coli* as a host system. The rationale for selecting these sequences is due to their location in *L. interrogans* serovar Copenhageni genome that has a potential involvement in pathogenesis. The genes encode for predicted lipoproteins with no assigned functions. The purified recombinant proteins were capable to promote the upregulation of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on monolayers of human umbilical vein endothelial cells (HUVECS). In addition, the coding sequences are expressed in the renal tubules of animal during bacterial experimental infection. The proteins are probably located at the outer membrane of the bacteria since they are detected in detergent-phase of *L. interrogans* Triton X-114 extract. Altogether our data suggest a possible involvement of these proteins during bacterial infection and provide new insights into the role of this region in the pathogenesis of *Leptospira*.

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1. Introduction

The genus *Leptospira* consists of a genetically heterogeneous group of pathogenic and saprophytic species. Pathogenic *Leptospira* are the etiologic agent of leptospirosis, a worldwide zoonosis of human and veterinary concern [1,2]. The transmission of leptospirosis has been associated with exposure of individuals close to wild or farm animals [3]. Recently, the disease became prevalent in cities with sanitation problems and large population of urban rodent reservoirs, which contaminate the environment through their urine [4]. Fever, chills, headache, and severe myalgias characterize the early phase of the disease. Progression to multi-organ system complications occurs in 5–15% of cases, with mortality rates of 5–40% [4,5]. Leptospirosis has also a great economic impact in the

agricultural industry since the disease affects the livestock inducing abortions, stillbirths, infertility, reduced milk production and death [4].

The central mechanism in pathogenesis of leptospirosis, as in other spirochetal diseases, is the ability of the pathogens to disseminate widely within the host during the early stage of infection [4]. In this regard, it is assumed that surface-associated proteins elicit several activities, including adhesion, antigenicity and host cell stimulation [6]. Cell adhesion molecules (CAMs) are surface receptors present in eukaryotic cells that mediate cell–cell or cell–extracellular matrix interactions [7–9]. Stimulation of the vascular endothelium *in vitro* that lead to the upregulation of CAMs was reported for the pathogenic spirochaetes, *Borrelia* sp and *Treponema* sp [10–14]. Recently, we showed that the recombinant protein rLIC10365 of *Leptospira interrogans* promoted upregulation of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on human umbilical vein endothelial cells (HUVECS) [15].

In this work, we report the cloning of the three genes, LIC10507, LIC10508 and LIC10509, protein expression, purification and characterization of these predicted outer membrane proteins of *L.*

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interrogans. The rationale to study these selected coding sequences rely on their location in *L. interrogans* serovar Copenhageni genome that has a potential involvement in pathogenesis [16]. This region has several notable features, some of them suggesting that it may have been acquired through horizontal transfer: (1) it has a relatively high GC content of 38.5% when compared to 35% of the genome; (2) there are two distinct transposases related to IS3 family and a nuclease gene similar to Staphylococcal thermonuclease; and (3) a large number of predicted genes are outer or cytoplasmic membrane proteins or lipoproteins with internal repeat sequence [16]. In addition, we have previously demonstrated that LIC10508 recombinant protein was recognized by antibodies present in patient's serum, suggesting its expression during infection [17]. The three recombinant proteins (rLIC10507, rLIC10508 and rLIC10509) were expressed in *Escherichia coli* BL21 SI strain and purified by metal affinity chromatography. The recombinant proteins promoted upregulation of ICAM-1 and E-selectin on monolayers of HUVECS and are expressed in the renal tubules of animals during bacterial experimental infection, suggesting their involvement in cell adhesion interactions and pathogenesis.

2. Results and discussion

The genes encoding LIC10507, LIC10508 and LIC10509 were identified in the chromosome I by analysis of the probable open-reading frames; the genes are contained in a large region of ~45 kb (position 574512–619577) of the genome of *L. interrogans* serovar Copenhageni that has distinguished features, including several membrane-associated proteins, higher G + C content (~38%) and flanked by insertion sequences (IS) [16,18]. BLAST analysis [19,20] of these coding sequences show that they are hypothetical proteins, with some similarities: LIC10509 has 54% and 41% identity with LIC10508 and LIC10507, respectively. A high similar coding sequence, LA3724, is present in a related region of *L. interrogans* serovar Lai [21] (Fig. 1A) with 98% identity with LIC10509, 54% identity with LIC10508 and 41% identity with LIC10507. However, no putative conserved domain has been detected within these sequences. ClustalW sequence alignments of all these coding sequences are shown in Fig. 1B. No similar coding sequences were identified in both sequenced strains of *L. borgpetersenii* [22]. The three genes are also absent in the genome of the non-pathogenic, saprophytic strain *L. biflexa* [23]. The coding sequences are predicted to be outer membrane proteins according to PSORT [24], having a signal peptide type II (SpII) with a cleavage site between amino acids 24–25 (LIC10507) and 19–20 (LIC10508, LIC10509), when analyzed by LipoP [25].

The recombinant prokaryotic expression of plasmids pAE-LIC10507, pAE-LIC10508, pAE-LIC10509 were designed to exclude the signal peptide regions that are cleaved during *in vivo* native protein maturation and lipidation, and to express N-terminal 6 × His-tags that allow rapid medium scale purification by metal affinity chromatography. The recombinant proteins were expressed in *E. coli* BL21 SI induced cultures, with the expected sizes, of approximately 22 kDa, observed for rLIC10508 and rLIC10509 but not for rLIC10507 (Fig. 2A). A higher molecular mass protein band was observed with this rLIC10507 in PAGE-SDS (Fig. 2A). The reason for this migration is unknown since all the cloned genes were shown to have the correct sequences and the three plasmid constructs were similar. The recombinant proteins were expressed somewhat in insoluble and soluble forms and were purified from their supernatants by loading onto a Ni²⁺-charged chromatographic column. In each case, the purified recombinant protein was recovered from the column with 1 M imidazole as a major protein band, as shown in Fig. 2A. To further confirm that these proteins are indeed his-tag recombinants we carried out immunoblotting and

probed them with his-tag antibodies. The data is depicted in Fig. 2B and, as we can see, is similar to the one obtained with the purified proteins (Fig. 2A). The protein bands of low molecular mass seen in rLIC10507 and rLIC10509 lanes are probably due to some protein degradation.

The instability of certain recombinant proteins either from the purification process or during storage has been one of the main concerns as with some proteins it may hinder their immunogenicity and immunoprotection activities [26]. The secondary structures of the purified recombinant proteins were therefore evaluated by circular dichroism (CD) spectroscopy. As illustrated in Fig. 3A, all recombinant proteins showed secondary conformation, as none of the spectra presents a flat line, characteristic of denatured non-structured form. The percentage of secondary structure content of each recombinant protein is shown in Fig. 3B where we can see that the three proteins have a predominance of random secondary structures in addition to α -helical and β -sheet contents. The data showing that secondary structures of the three recombinant proteins were maintained after purification process rendered them suitable for further biological characterizations.

To determine whether LIC10507, LIC10508 and LIC10509 proteins are located at the membrane, we carried out an extraction of the *L. interrogans* outer membrane with the non-ionic detergent Triton X-114 (TX-114). The three fractions derived from TX-114 extracted-material were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with each recombinant protein antiserum. The proteins were completely absent from the detergent-insoluble pellet fraction, but could be readily detected in the TX-114 detergent-phase (Fig. 4). The presence of LIC10507, LIC10508 and LIC10509 in the hydrophobic, detergent-phase of *L. interrogans* TX-114 solubilization extract suggests their location at the bacterial membrane, most likely at the outer membrane, as previously shown for other leptospiral proteins [27,28].

Protein expression and conservation of LIC10507, LIC10508 and LIC10509 among *Leptospira* strains was assessed with total protein extracts from *L. interrogans* pathogenic serovars: Canicola, Hardjo, Icterohaemorrhagiae, Copenhageni and the non-pathogenic strain *L. biflexa* serovar Patoc. Cell extracts were prepared from cultures of the different strains and Western blot analysis was performed with polyclonal sera from mice immunized with each recombinant protein. The serological cross-reactivity showed conservation of LIC10508 and LIC10509 proteins in all pathogenic strains tested (Fig. 5A), while LIC10507 protein is absent in serovar Hardjo. Additionally, in a previous study, the LIC10508 protein was shown to be also present in other pathogenic serovars [17]. In agreement with the *in silico* search, none of these proteins were identified in the non-pathogenic *L. biflexa* strain, suggesting that they may be relevant for pathogenesis. The higher molecular mass protein bands detected within the serovar Copenhageni (Fig. 5A, lane 5) by the sera against the three proteins might be explained by linkage of the proteins to a subsurface structure such as peptidoglycan or to a very high molecular mass protein present in this serovar given that leptospiral proteins comes from total bacterial extract. Cross-reactivity between protein's sera is one possible explanation for the double reactive bands displayed in this blotting, probably due to their sequence similarities (Fig. 1B). This possibility was confirmed when we probed the three blotted recombinants with mouse antiserum raised against each protein (Fig. 5B). As illustrated, the anti-rLIC10507 was capable to react with its homologous rLIC10507, rLIC10508 and rLIC10509 proteins. The same behavior was observed with rLIC10508 and rLIC10509 antisera.

We have previously identified a laminin-binding adhesin [28] and more recently, we have found a novel leptospiral protein that binds strongly to laminin, fibronectin and collagen IV [29]. We examined the interaction of rLIC10507, rLIC10508 and rLIC10509 proteins with laminin, collagen type I, collagen type IV, cellular

Cell adhesion molecules (CAMs) are employed by various cells in host defense against infection [9]. E-selectin and ICAM-1 adhesion molecules have been shown to be upregulated upon activation of endothelium by the pathogenic spirochaetes *Borrelia* sp [10–12], *Treponema* sp [13,14] and by the recombinant protein rLIC10365 of

To evaluate whether the LIC10507, LIC10508 and LIC10509 proteins are expressed during infection, kidney tissues obtained after 14 days p.i. of experimentally infected guinea pigs were probed with serum raised against each recombinant protein.

Fig. 3. Circular dichroism spectra of the recombinant proteins. A. CD spectra of the proteins rLIC10507, rLIC10508 with predominant signal of β -strand and rLIC10509 with predominant α -helical secondary structure. Far-UV CD spectra are presented as an average of five scans recorded from 180 to 260 nm. B. Percentage of secondary structure content of each recombinant protein as calculated by Dicroprot software K2D method (Section 4).

L. interrogans proteins located at the renal tubular lumen stained positive with antiserum of rLIC10507 (Fig. 7A), rLIC10508 (Fig. 7B) and rLIC10509 (Fig. 7C), while no reactivity was seen when kidney tissues from non-infected animals were probed with each antiserum (Fig. 7D, with anti-rLIC10507). Because serum cross-reactivity observed within the three proteins, serological distinction between them will not be feasible. In any event, the immunohistochemistry data obtained with LIC10507, LIC10508, LIC10509 proteins show that at least one of them, like other leptospiral

proteins studied to date, such as OmpL1, LipL41, LipL32 and rLIC10365 [15,30], are expressed by *Leptospira* within the renal tubules of experimentally infected guinea pig.

3. Conclusions

The results obtained with rLIC10507, rLIC10508, rLIC10509 show that these predicted lipoproteins are located at the membrane of the bacteria, most probably at the outer membrane. Due to their sequence similarity and therefore the cross-reactivity between their antiserum, we can only assume that at least one of them is expressed during bacterial infection and promote upregulation of cellular adhesion molecules. Taken together, our data point out to a possible function of these proteins in leptospiral pathogenesis and reinforce the attributes that the coding sequences in this distinguished region might be involved in bacterial infection.

4. Materials and methods

4.1. In silico evaluation of LIC10507, LIC10508, LIC10509

Cellular localization of the proteins was predicted by the PSORT program, <http://psort.nibb.ac.jp> [24]. Determination of signal peptides, lipoprotein cleavage sites, and transmembrane domains, were based on the public web servers, <http://www.cbs.dtu.dk/services/SignalP/> and <http://www.cbs.dtu.dk/services/LipoP/> and <http://www.cbs.dtu.dk/services/TMHMM> [25,31,32]. Multiple sequence alignment was performed with Clustal W program [33] [<http://www.ebi.ac.uk/clustalw/>].

4.2. Bacteria, plasmids and DNA recombinant techniques

Strains of *L. interrogans* serovars Canicola (Hond Utrecht IV), Copenhageni (M-20, Fiocruz L1-130), Hardjo (Hardjoprajtino), Icterohaemorrhagiae (RGA) and *L. biflexa* serovar Patoc strain Patoc 1, were cultured at 29 °C under aerobic conditions in liquid EMJH medium (Difco®-USA) with 10% rabbit serum, enriched with L-asparagine (wt/vol: 0.015%), sodium pyruvate (wt/vol: 0.001%),

Fig. 4. Cellular localization of LIC10507, LIC10508, LIC10509 coding sequences-Triton X-114 fractions of *L. interrogans* serovar Copenhageni bacteria were separated by SDS-PAGE, transferred to a nitrocellulose membrane and probed with the respective antiserum. Fractions analyzed were the whole organism (1), Triton X-114 insoluble pellet (2), aqueous-phase (3) and detergent-phase (4).

Fig. 5. Conservation of LIC10507, LIC10508 and LIC10509 proteins among pathogenic leptospires and immunoblotting. A. Whole cell lysates were prepared from strains of *L. interrogans* serovars. Proteins from the lysates were separated by electrophoresis, transferred into nitrocellulose membranes and probed with each recombinant protein antiserum. Lanes are: 1 – *L. biflexa* sv Patoc; 2 – *L. interrogans* sv Canicola; 3 – *L. interrogans* sv Hardjo; 4 – *L. interrogans* sv Icterohaemorrhagiae; 5 – *L. interrogans* sv Copenhageni; 6 – respective purified recombinant protein (positive control). B. Blotted recombinant proteins rLIC10507, rLIC10508, rLIC10509 probed with mouse antiserum raised against each of them.

calcium chloride (wt/vol: 0.001%), magnesium chloride (wt/vol: 0.001%), peptone (wt/vol: 0.03%) and meat extract (wt/vol: 0.02%). Total protein extract of each *L. interrogans* serovars and of *L. biflexa* was performed as described in Gamberini et al. [17]. Amplification of the predicted coding sequences of LIC10507, LIC10508 and LIC10509 were performed by the polymerase chain reaction (PCR) from *L. interrogans* serovar Copenhageni genomic DNA using the primer pairs: LIC10507F: CGGGATCCAAAAAGAGTCAAGAA-GAATTGG and LIC10507R: GGTACCCTACTCGAGACAGCCAGGACCTTC; LIC10508F: CCGGGATCCAAAAAGAGCAAAGAAGAAATT and LIC10508R: GGTACCCTACTCGAGACAACCAGGACCTTC; LIC10509F: CCGGGATCCAAAAAGAGCAAAGAAG and LIC10509R: GGTACCC-TACTCGAGACAGCCAGGACCTTC. The three gene sequences were amplified without the signal peptide tag. The purified fragments were cloned into pGEM-T easy vector (Promega) according to manufacturer guidelines. *E. coli* DH5 α competent cells were transformed with the ligation products. Recombinant colonies were searched for the correct inserts by restriction analysis. The constructions were digested using BamHI and KpnI restriction enzymes, and the released inserts were cloned in to pAE, an *E. coli* expression vector [34], previously digested with the same enzymes. Cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA) with appropriate vector-specific T7 (F: TAATACGACTCACTATAGGG) and pAE (R: CAGCAGCCAACCTCAGTTCCT) primers. BL21 SI *E. coli* (Invitrogen) competent cells were transformed with the pAE constructions and grown overnight at 30 °C.

4.3. Expression and purification of rLIC10507, rLIC10508 and rLIC10509

Ampicillin resistant clones were inoculated into 5 ml 2YT/on – amp (Luria–Bertani without NaCl) medium. The expression of the recombinant proteins was induced by the addition of 300 mM NaCl to the culture medium. Cells were harvested by centrifugation, resuspended in binding buffer pH 8.0 (20 mM Tris–HCl and 500 mM NaCl) containing 0.1% Triton X-100, and lysed by French Pressure (Spectronic Instruments). The soluble and insoluble fractions were separated by centrifugation at 10,000 $\times$ g for 15 min. Inclusion bodies (rLIC10507) were washed with binding buffer (containing 1 mM β -mercaptoethanol, 1 M urea, and 1% Triton X-100) and then dissolved in binding buffer (containing 8 M urea, and 5 mM β -mercaptoethanol) for 24 h at RT. For refolding, the bacterial pellet suspensions were diluted with 2 L of binding buffer containing 5 mM imidazole, followed by incubation for 24 h at RT. The soluble fractions of the rLIC10508 and rLIC10509 were diluted in binding buffer containing 5 mM imidazole and loaded into the column of Ni²⁺-charged Sepharose beads (GE Healthcare). The contaminants were washed away with low imidazole concentration, and His-tag recombinant proteins were eluted with the same buffer but containing 1 M imidazole. Imidazole was eliminated by successive dialysis against binding buffer containing decreasing imidazole concentrations. Expression and purification of rLIC12906 has been previously published [28].

Fig. 6. Induction of E-selectin and ICAM-1 expression on HUVECs by rLIC10507, rLIC10508 and rLIC10509 proteins. Confluent monolayers of HUVECs were stimulated for 1 h with rLIC10507 (white bars), rLIC10508 (gray bars), rLIC10509 (black bars) at the indicated concentrations, and 50 µg/ml of rLIC12906, as a negative control (black squares), with 7 µg/ml of polymyxin B. LPS 1 µg/ml with or without 7 µg/ml of polymyxin B (horizontal and vertical stripes, respectively) was used as a positive control. Then medium was removed and cells were cultured with RPMI-1640 supplemented with 10% FBS. A. E-selectin expression was evaluated 4 h post stimulation. B. ICAM-1 expression was evaluated 12 h post stimulation. Data shown are mean $\pm$ SEM of four independent experiments.

4.4. Circular Dichroism spectroscopy

CD spectroscopy measurements were performed at 20 °C in a Jasco J-810 Spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using 1 mm path length cell at 0.5 nm intervals. The spectra were presented as an average of five scans recorded from 180 to 260 nm. Spectra data was submitted to Dicroprot software, <http://dicroprot-pbil.ibcp.fr/>, using K2D method for the secondary structure content estimation [35,36].

4.5. Antisera against rLIC10507, rLIC10508 and rLIC10509

Ten female BALB/c mice (18–22 g) were immunized subcutaneously with 10 µg of the recombinants LIC10507, LIC10508 and LIC10509 with 10% (vol/vol) of Alhydrogel (2% Al (OH)₃, Biosector, Denmark) as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same protein preparations. One week after each immunization, the mice were bled from the retro-orbital plexus and the pooled sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for determination of antibody titers. The control group was inoculated with PBS and adjuvant.

4.6. Immunoblotting

This was performed as described by Neves et al. [37]. The membrane containing the three blotted rLIC10507, rLIC10508, rLIC10509 proteins was washed with PBS-T and incubated with anti-His-tag (1:3000), anti-rLIC10507, anti-rLIC10508, anti-rLIC10509 mouse serum, respectively, all at 1:5000 dilution, in 5% non-fat dry milk-PBS-T. The sera were previously treated with a suspension of *E. coli* BL21 SI cell lysate/5% non-fat dry milk for 1 h. After the washings, the membrane was incubated with anti-mouse IgG (HRP)-conjugate (1:5000) in 5% non-fat dry milk-PBS-T for 1 h. The bands were revealed with ECL reagents (GE Healthcare).

4.7. Recognition of proteins in leptospiral extracts by sera from mice immunized with recombinant proteins

Bacterial cultures of several leptospiral serovars were harvested by centrifugation, washed three times with PBS containing 5 mM MgCl₂ and resuspended in PBS containing 10% SDS. A sample from each serovar was applied on a 10% SDS-PAGE, the proteins were transferred to nitrocellulose membrane and probed with serum obtained from mice immunized with each of the recombinant proteins, as described above.

4.8. ECM adherence assays

All macromolecules, including the control proteins fetuin and BSA, were purchased from Sigma Chemical Co. (St. Louis, MO). Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to Barbosa et al. [28]. Briefly, ELISA plate wells (Nunc-Immuno Plate MaxiSorp Surface) were coated with 1 µg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, BSA (non-glycosylated attachment-negative control protein) and fetuin (highly glycosylated attachment-negative control protein) in 100 µl of PBS for 2 h at 37 °C. The wells were washed three times with PBS-0.05% Tween 20 (PBST) and then blocked with 200 µl of 1% BSA for 1 h at 37 °C followed by an overnight incubation at 4 °C. One microgram of recombinant protein was added per well in 100 µl

Fig. 7. Immunohistochemistry. Antigens recognition on leptospires within the renal tubular lumen of kidney tissue obtained at 14 days postinfection with virulent *L. interrogans* after with rLIC10507 (A), rLIC10508 (B) and rLIC10509 (C). No reactivity was observed when each of the antiserum was employed in kidney from uninfected animal, shown in (D) with rLIC10507 antiserum. Antigens were detected with hematoxylin counterstaining. Bar: 10 µm.

of PBS and protein was allowed to attach to the different substrates for 1 h 30 min at 37 °C. After washing six times with PBST, bound proteins were detected by adding an appropriate dilution of mouse antiserum in 100 µl of PBS. Incubation proceeded for 1 h and after three washes with PBST, 100 µl of a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) in PBS were added per well for 1 h. All incubations took place at 37 °C. The wells were washed three times and *o*-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5.0) plus 0.01% H₂O₂ was added. The reaction was allowed to proceed for 15 min and was then interrupted by the addition of 50 µl of 8 M H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (Labsystems Uniscience, Multiskan EX).

4.9. Isolation of leptospiral outer membrane proteins (OMPs) by Triton X-114 extraction

OMPs were extracted according to a previously described method [38,39]. In brief, leptospires cultured as outlined above were washed in PBS containing 5 mM MgCl₂ and then extracted in the presence of 2% Triton X-114 (Sigma-Aldrich), 150 mM NaCl, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA at 4 °C for 2 h. The insoluble material was removed by centrifugation at 17,000 × *g* for 10 min. After centrifugation, 20 mM CaCl₂ was added to the supernatant. Phase separation was performed by warming the supernatant at 37 °C and subjecting it to centrifugation for 10 min at 1000 × *g*. Three distinct fractions became apparent: the aqueous phase, the TX-114 phase and the insoluble pellet. The detergent-phase was precipitated with acetone. All fractions were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes for immunological analysis with the recombinant protein antisera.

4.10. In vivo detection of LIC 10507, LIC10508, LIC10509 by immunohistochemistry

To detect LIC10507, LIC10508, LIC10509 encoded proteins in kidney tissues, three guinea pigs of 180 g were inoculated intraperitoneally with 0.5 ml of PBS (pH 7.2) containing 10^{2.5} bacteria (*L. interrogans* serovar Copenhageni), sacrificed 2 weeks post infection (p.i.) when animals presented symptoms, e.g. weight loss, lethargy [4], and their kidneys were harvested and processed for routine histology. Mock infected animals were used as controls. After rehydration, the tissue Pro-Bond Plus slides were heated three times for 5 min in a 10 mM citrate buffer in a microwave oven. The sections were then cooled and immersed in 3% H₂O₂ for 15 min to inhibit endogenous peroxidase activity. To block non-specific antigen sites, sections were incubated with PBS with 5% of normal goat serum for 20 min at room temperature. Then, slides were incubated with the above described primary polyclonal murine antiserum diluted 1:100 for 1 h at room temperature. After several washes with PBS, specimens were incubated with secondary antibody (goat anti-mouse immunoglobulin) conjugated to peroxidase-labeled dextran polymer (DAKO EnVision) for 20 min at room temperature and again washed with PBS. Diaminobenzidine/hydrogen peroxidase substrate was incubated for 2–10 min to reach the appropriate intensity and slides were rinsed with distilled water to stop the staining reaction. Immunostained sections were counterstained with hematoxylin for 1 min, bathed under tap water, rinsed with distilled water and dehydrated in increasing ethanol concentrations followed by xylene (each treatment, 5 min). Finally, the slides were mounted with a coverslip in a non-aqueous permanent mounting medium and observed in a Leica photomicroscope.

4.11. *Limulus* amebocyte lysate assay (LAL-test)

The chromogenic *Limulus* amebocyte lysate assay for endotoxin activity of the protein samples was performed using the QCL-1000 kit (Bio-Whittaker Inc., Walkersville, MD), according the manufacturer's instructions. LPS content of our samples by this test were estimated to be 1.2 × 10⁻⁴ µg/ml (rLIC10507), 0.10 µg/ml (rLIC10508), 0.86 µg/ml (rLIC10509) and 0.88 µg/ml (rLIC12906).

4.12. Endothelial cell culture

Endothelial cells were obtained from human umbilical veins (HUVECs) by collagenase (GIBCO) digestion according to the method of Jaffe et al. [40]. Cells were grown to confluence in 25 cm² tissue culture flasks that were precoated with 1% gelatin (Sigma). The growth medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (GIBCO), 90 µg/ml heparin (GIBCO), 50 µg/ml ECGS (endothelial cell growth supplement) (Sigma), 2 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma) at 37 °C in a humidified 5% CO₂ incubator. Confluent HUVECs were passaged with 0.05% trypsin + 0.02% EDTA and were routinely used between the first and third passage. Cultured cells were identified as endothelial by their morphology and vWF antibody binding. All experiments were performed in the presence of 7 µg/ml polymyxin B (Sigma) to rule out lipopolysaccharide interference.

4.13. Expression of E-selectin and ICAM-1

HUVECs were incubated with the recombinant proteins at the indicated concentrations for 1 h in medium RPMI-1640. The protein was removed by washing, and endothelial cells were further incubated in RPMI 1640 with 10% FBS. ICAM-1 expression was assessed after 12 h. HUVECs were detached by treatment with a 0.25% trypsin and 0.02% EDTA solution and incubated in the dark, at 4 °C for 15 min, with PE-mouse anti-human CD54 (clone HA58, BD Pharmingen). For E-selectin expression, cells were harvested after 4 h, combined with a primary anti-CD62E monoclonal antibody (MoAb) (clone 1.2B6 Immunotech) and a secondary FITC-conjugated fragment F(ab')₂ anti-mouse IgG (Immunotech). For non-specific binding, anti-CD54 or anti-CD62E were replaced by a correspondent concentration of irrelevant isotype-matched IgG1. After labeling, cells were washed, fixed with 1% paraformaldehyde, and analyzed by flow cytometry in a FACScan cytofluorimeter (Becton Dickinson, Mountain View, CA). Appropriate settings of forward and side scatter gates were used to examine 10,000 cells per experiment. The percentage of positive cells was determined by the thresholds set using isotypic controls.

4.14. Nucleotide sequence accession numbers

The accession numbers for public data base for each protein sequence analyzed in this work are AAS69128 (LIC10507), AAS69129 (LIC10508) and AAS69130 (LIC10509). The proteins can also be accessed by the genome nomenclature for the gene locus, LIC number (*Leptospira interrogans* Copenhageni).

All animal studies were approved by the Ethics Committee of the Faculty of Exact Sciences, University of La Plata, Argentina and of the Instituto Butantan, Sao Paulo, Brazil.

Acknowledgements

This work was supported by FAPESP, CNPq, and Fundação Butantan, Brazil; CABBIO 19/04 and PICT 13768, Argentina. M.L.V. has an MSc fellowship from FAPESP (Brazil) and M.M.W. had

a fellowship from PIBIC/CNPq. E.M. and J.O.C. have a fellowship from FONCyT (Argentina).

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