RENAN FRANCISCO DOMINGOS

**CARACTERIZAÇÃO DE DUAS PROTEÍNAS
DE *Leptospira interrogans* NA PATOGÊNESE
DA LEPTOSPIROSE**

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

São Paulo
2014

RENAN FRANCISCO DOMINGOS

**CARACTERIZAÇÃO DE DUAS PROTEÍNAS
DE *Leptospira interrogans* NA PATOGÊNESE
DA LEPTOSPIROSE**

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

Área de Concentração: Biotecnologia

Orientador: Dra. Ana Lucia Tabet Oller do Nascimento

Versão original

São Paulo
2014

DADOS DE CATALOGAÇÃO NA PUBLICAÇÃO (CIP)
Serviço de Biblioteca e Informação Biomédica do
Instituto de Ciências Biomédicas da Universidade de São Paulo

© reprodução total

Domingos, Renan Francisco.

Caracterização de duas proteínas de *Leptospira interrogans* na patogênese da leptospirose / Renan Francisco Domingos. -- São Paulo, 2014.

Orientador: Profa. Dra. Ana Lucia 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: Genômica funcional.

Versão do título para o inglês: Characterization of two proteins of *Leptospira interrogans* in pathogenesis of leptospirosis.

1. *Leptospira* 2. Leptospirose 3. Proteínas recombinantes 4. Patogênese I. Nascimento, Profa. Dra. Ana Lucia Tabet Oller do II. Universidade de São Paulo. Instituto de Ciências Biomédicas. Programa de Pós-Graduação Interunidades em Biotecnologia USP/IPT/Instituto Butantan III. Título.

ICB/SBIB083/2014

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

Candidato(a): Renan Francisco Domingos.

Título da Tese: Caracterização de duas proteínas de *Leptospira interrogans*
na patogênese da leptospirose.

Orientador(a): Profa. Dra. Ana Lucia Tabet Oller do Nascimento.

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

☐ **Aprovado(a)**

☐ **Reprovado(a)**

Examinador(a): Assinatura:
Nome:
Instituição:

Examinador(a): Assinatura:
Nome:
Instituição:

Examinador(a): Assinatura:
Nome:
Instituição:

Examinador(a): Assinatura:
Nome:
Instituição:

Presidente: Assinatura:
Nome:
Instituição:

COMISSÃO DE ÉTICA NO USO DE ANIMAIS
INSTITUTO BUTANTAN

Av. Dr. Vital Brazil, 1500, CEP 05503-900, São Paulo, SP, Brazil
Telefone: (55) (011) 2627-9585 - Fax: (55) (011) 2627-9505
ceuaib@butantan.gov.br

CERTIFICADO

Certificamos que o projeto intitulado "Expressão e caracterização de duas proteínas de superfície de *Leptospira interrogans*", protocolo nº 599/09, sob a responsabilidade de Ana Lucia Tabet Oller do Nascimento e Renan Francisco Domingos - que envolve a criação e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica - está de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto 6.899, de 15 de julho de 2009 e de normas complementares, bem como 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 9/10/2013.

Vigência do Projeto:	Nº de animais/espécie	Observação
05/2009 - 07/2014 Laboratório de Biotecnologia	125 camundongos Balb/c (F); 30 hamsters Golden (F)	Alteração de vigência

São Paulo, 10 de outubro de 2013

Dr. Marcelo L. Santoro
Coordenador da CEUAIB

Aos meus amigos que sempre estão
comigo e à minha família que amo
tanto, em especial aos meus pais José e
Fátima, meus irmãos Michelle e Pablo,
meus cunhados Edinaldo e Thaise,
minha sobrinha Isabela, e minha
grande companheira Marcela. O apoio
e amor de vocês que me fortalece a
cada dia.

Muito obrigado!

AGRADECIMENTOS

À Deus, pela força de sempre e pelas pessoas que colocou no meu caminho.

À Dra. Ana Lúcia Tabet Oller do Nascimento pela orientação e pela paciência durante meu aprendizado, e pela confiança em meu trabalho. Serei muito agradecido sempre, pois com certeza cresci como profissional e como pessoa.

À minha co-orientadora Dra. Monica Larucci Vieira, pelo incentivo e pela orientação, principalmente nos primeiros anos e a todos outros colegas que passaram no Laboratório de Biotecnologia Molecular II – Centro de Biotecnologia, do Instituto Butantan, em especial Gabriela Hase de Siqueira pelo grande auxílio. Vocês foram muito mais que companheiros de trabalho, vocês se tornaram grandes amigos.

À professora Dra. Eliete Romero e Ivy Alves de Jesus do Instituto Adolfo Lutz pela colaboração com os soros de pacientes.

À equipe do professor Dr. Sílvio Vasconcellos e Zenaide Maria de Moraes do Laboratório de Zoonose da Faculdade de Medicina Veterinária da USP pela colaboração com as culturas de leptospiras e ensaios de imunoproteção em hamster.

À Alexsander Souza do Departamento de Parasitologia do Instituto Butantan, pelo auxílio com o microscópio confocal.

À minha família, em especial meus pai, irmãos, cunhados, sobrinha e minha namorada, minha grande companheira, por me apoiarem sempre, por andarem ao meu lado quando preciso e pelo imenso amor.

À todos os meus amigos que considero parte da minha família.

Aos meus professores da Universidade Presbiteriana Mackenzie.

À coordenação do curso de Pós-Graduação Interunidades em Biotecnologia da Universidade de São Paulo / Instituto Butantan / Instituto de Pesquisas Tecnológicas, e a todos os professores do programa 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 à Fundação Butantan.

“A persistência é o menor caminho do êxito”.

(Charles Chaplin)

RESUMO

DOMINGOS, R. F. **Caracterização de duas proteínas de *Leptospira interrogans* na patogênese da leptospirose.** 2014. 276 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014.

Leptospirose é uma zoonose altamente disseminada causada por espécies patogênicas do gênero *Leptospira*. Os roedores são os principais reservatórios da doença nos centros urbanos. A doença apresenta um amplo quadro de manifestações clínicas em humanos, variando desde febre, calafrios, dor de cabeça e muscular, até uma condição mais severa, conhecida como síndrome de Weil, caracterizada por hemorragia, falência renal e icterícia. 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. Dessa forma, foram selecionados através de bioinformática dois genes de *L. interrogans* sorovar Copenhageni, LIC11834 e LIC12253, os quais foram submetidos a ensaios de presença do DNA genômico e RNA mensageiro em diferentes espécies e sorovares de *Leptospira*. Através desses ensaios, observamos que o gene LIC12253 foi o mais presente entre os sorovares testados. Os genes foram clonados em vetor de expressão pAE, com adição de 6 resíduos de histidina na região N-terminal, e submetidos a testes de expressão em *E. coli* BL21 SI. Após a expressão, as proteínas recombinantes foram purificadas por cromatografia de afinidade a metal, e submetidas a ensaio de dicroísmo circular, o qual confirmou que ambas as proteínas estavam estruturadas. Por meio de testes de imunogenicidade em camundongos, ambas as proteínas mostraram-se altamente imunogênicas, apresentando altos títulos de anticorpos, porém não foram capazes de promover resposta imune celular. Em ensaios de localização das proteínas nativas (imunofluorescência de fase líquida e proteólise com proteinase K) pode-se observar a presença destas proteínas nativas na membrana externa de *Leptospira*. Ensaios de reatividade com soros de pacientes diagnosticados com leptospirose mostraram que há reconhecimento das proteínas recombinantes por anticorpos presentes nesses soros, sugerindo que essas proteínas são expressas durante a infecção. Em ensaios de adesão a componentes da matriz extracelular e componentes do soro e plasma humano, a proteína rLIC11834 apresentou ligação à laminina, sendo nomeada de Lsa33 (*Leptospiral Surface Adhesin*). Lsa33 liga-se também ao plasminogênio, ao C4bp e ao fibrinogênio de forma dose-dependente. Quando ligado ao plasminogênio, esta proteína recombinante, na presença de ativador específico, é capaz de converter o plasminogênio em plasmina. Já a proteína rLIC12253 apresentou ligação à laminina, sendo chamada de Lsa25, e ao C4bp de forma dose-dependente. Ensaios de imunização e desafio demonstraram que ambas as proteínas recombinantes não apresentaram proteção significativa contra infecção letal de *L. interrogans* em hamsters. Vale ressaltar que Lsa33 foi a primeira proteína descrita de *Leptospira* que se liga à laminina, ao plasminogênio, ao C4bp e ao fibrinogênio *in vitro*. Assim, acreditamos que estas proteínas multifuncionais possam interagir com proteínas do hospedeiro e ter participação na patogênese da doença.

Palavras-chave: *Leptospira*. Leptospirose. Proteínas recombinantes. Patogênese.

ABSTRACT

DOMINGOS, R. F. **Characterization of two proteins of *Leptospira interrogans* in the pathogenesis of leptospirosis.** 2014. 276 p. Ph. D. thesis (Biotechnology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2014.

Leptospirosis is a widespread zoonosis caused by pathogenic species of the genus *Leptospira*. Rodents are the main reservoirs of the disease in the urban areas. The disease presents a broad spectrum of clinical manifestations in humans, ranging from fever, chills, muscular pain and headaches, to a more severe condition, known as Weil syndrome, characterized by hemorrhage, renal failure and jaundice. The genomic sequencing and the advances of bioinformatics analysis allowed the identification of new vaccine candidates and new virulence factors. Therefore, two genes from *L. interrogans* serovar Copenhageni, LIC11834 and LIC12253 were selected through bioinformatics and subjected to assays for the presence of genomic DNA and mRNA in different species and serovars of *Leptospira*. These assays found that the gene LIC12253 was the most present among the tested serovars. The genes were then cloned in the expression vector pAE, with addition of 6xHis-tag at N-terminus region, and subjected to expression tests in strain of *E. coli* BL21 SI. After the expression, the recombinant proteins were purified by metal affinity chromatography, and subjected to circular dichroism, which confirmed that both proteins presented secondary structures. Immunogenicity tests in mice showed that both proteins are immunogenic, with high antibodies titers, but don't induce cellular immune response. Localization assays of the native proteins on the leptospires performed by liquid phase immunofluorescence and proteolysis with proteinase K demonstrated the presence of the proteins on the surface of *Leptospira*. Reactivity assays with sera of patients diagnosed with leptospirosis showed that the recombinant proteins could be recognized by their antibodies, suggesting that they are expressed during infection. Adhesion assays to the extracellular matrix components, human serum and plasma components, showed that the protein rLIC11834 binds to laminin and was called Lsa33 (*Leptospiral Surface Adhesin*). In addition, Lsa33 interacts to plasminogen, to C4bp and to fibrinogen in a dose-dependent manner. In the presence of the specific activator, plasminogen bound to this protein can be converted into plasmin. The protein rLIC12253 showed binding to laminina, named Lsa25, and to C4bp in a dose-dependent manner. Immunization and challenge assays showed that both recombinant proteins don't afford protection against lethal infection of *L. interrogans* in hamsters. It's noteworthy that Lsa33 was the first protein described to bind to laminin, plasminogen, C4bp and fibrinogen *in vitro*. Thus, we believe that these multifunctional proteins may interact with host proteins and may play a role in leptospiral pathogenesis.

Keywords: *Leptospira*. Leptospirosis. Recombinant proteins. Pathogenesis.

LISTA DE FIGURAS

Figura 1 - Taxonomia	23
Figura 2 - Representação esquemática da parede celular de <i>Leptospira</i> spp.	25
Figura 3 - Transmissão da leptospirose em animais e humanos	28
Figura 4 - Mapa do vetor de clonagem pGEM-T Easy	43
Figura 5 - Mapa do vetor de clonagem e expressão pAE.....	45
Figura 6 - Esquema do ensaio de desafio	62
Figura 7 - Filograma das sequências de aminoácidos codificadas pelos genes (A) LIC11834 e (B) LIC12253.	64
Figura 8 - Análise da presença genômica para os genes LIC11834 e LIC12253.....	66
Figura 9 - Análise da presença dos transcritos para os genes LIC11834 e LIC12253.....	67
Figura 10 - DNA de interesse amplificado através de reação de PCR.....	68
Figura 11 - Insertos de DNA obtidos por digestão do DNA plasmidial (pGEM-T Easy/inserto)	69
Figura 12 - Cassete de expressão do vetor pAE	70
Figura 13 - Insertos de DNA obtidos por digestão do DNA plasmidial (pAE/inserto)	71
Figura 14 – Cromatogramas dos sequenciamentos da região 5' das construções (A) pAE-LIC11834 e (B) pAE-LIC12253	72
Figura 15 - Análise da expressão das proteínas recombinantes	74
Figura 16 - Análise da purificação da proteína recombinante rLIC11834.....	75
Figura 17 - Análise da purificação da proteína recombinante rLIC12253.....	75
Figura 18 - Quantificação das proteínas recombinantes por comparação com curva de BSA	76
Figura 19 - Espectros de dicroísmo circular das proteínas recombinantes	78
Figura 20 - Reconhecimento de anticorpos específicos pelas proteínas recombinantes	80
Figura 21 - Reconhecimento de diferentes concentrações de anticorpos específicos pelas proteínas recombinantes	81
Figura 22 - Avaliação de linfoproliferação	82

Figura 23 - Avaliação da resposta de citocinas resultante da imunização de camundongos com as proteínas recombinantes após estímulo <i>in vitro</i> .	83
Figura 24 - Análise da reatividade de soros de indivíduos diagnosticados para leptospirose contra (A) rLIC11834, (B) rLIC12253 e (C) rLIC11834 + rLIC12253	86
Figura 25 - Análise da reatividade de soros de indivíduos diagnosticados com outras doenças contra (A) rLIC11834 e (B) rLIC12253	89
Figura 26 - Reconhecimento das proteínas codificadas pelas sequências LIC11834 e LIC12253 em <i>L. interrogans</i> por seus respectivos anticorpos por imunofluorescência	91
Figura 27 - Ensaio de acessibilidade a protease pelas proteínas de <i>L. interrogans</i> codificadas pelos genes LIC11834 e LIC12253	92
Figura 28 - Caracterização da ligação das proteínas rLIC11834 e rLIC12253 aos componentes da MEC e do plasma	94
Figura 29 - Análise da especificidade da interação das proteínas recombinantes a laminina	95
Figura 30 - Ligação das proteínas recombinantes a componentes do soro humano e do plasma	97
Figura 31 - Análise da especificidade da interação das proteínas recombinantes ao PLG, ao C4bp e ao Fg	98
Figura 32 - Inibição da interação do PLG à Lsa33 pelo AAC	100
Figura 33 - Geração de plasmina pelo PLG ligado à Lsa33	101
Figura 34 - Tratamento da laminina, do C4bp e do Fg com metaperiodato de sódio	102
Figura 35 - Efeito da proteína recombinante Lsa33 na formação do coágulo de fibrina induzido por trombina	103
Figura 36 - Efeito na ligação das proteínas recombinantes aos componentes pela incubação destas proteínas recombinantes com antissoro específico	104
Figura 37 - Efeito na ligação das proteínas recombinantes aos componentes por desnaturação proteica	105
Figura 38 - Inibição da ligação de <i>L. interrogans</i> à laminina e aos componentes do soro e do plasma pela Lsa33	107
Figura 39 - Inibição da ligação de <i>L. interrogans</i> à laminina e ao C4bp pela Lsa25	108
Figura 40 - Interferência entre os componentes na ligação à Lsa33	110
Figura 41 - Interferência entre os componentes na ligação à Lsa25	111

Figura 42 - Curva de sobrevivência dos hamsters imunizados com a proteínas recombinantes Lsa33 e Lsa25 e desafiados com *L. interrogans* sorovar Kennewicki cepa Pomona Fromm 113

LISTA DE QUADROS

Quadro 1 – Oligonucleotídeos referentes aos genes LIC11834 e LIC12253	39
Quadro 2 – Oligonucleotídeos utilizados para o sequenciamento	46

LISTA DE TABELAS

Tabela 1 - Resumo das características preditas por análise de bioinformática para os genes em estudo.....	65
Tabela 2 - Massa molecular e ponto isoelétrico (PI) teórico das proteínas recombinantes.....	73
Tabela 3 - Títulos de anticorpos policlonais obtidos após imunização de camundongos com as proteínas recombinantes	79
Tabela 4 - Títulos dos soros dos pacientes diagnosticados com leptospirose nas quais ainda há reatividade com as proteínas recombinantes	88
Tabela 5 - Constantes de dissociação para as curvas de dose-dependência da laminina detectadas com antissoro específico	96
Tabela 6 - Constantes de dissociação para as curvas de dose-dependência do PLG e Fg – Lsa33	99

LISTA DE ABREVIATURAS E SIGLAS

2YT/ON - meio de cultura contendo 1% de extrato de levedura, 1,6% de peptona

AAC - ácido aminocaproico

Abs - absorbância

Brdu - bromodesoxiuridina

BSA - albumina do soro bovino

C4bp - C4 *protein binding*

CD - dicroísmo circular

ConA - concanavalina A

Da - dalton

ddNTP - didesoxirribonucleotídeo

DNA - ácido desoxirribonucleico

dNTP - desoxirribonucleotídeo

DO - densidade óptica

EDTA - ácido etilenodiamino tetracético

ELISA - *Enzyme Linked Immunosorbent Assay*

EMJH - Ellinghausen-McCullough/Johnson-Harris

Fg - fibrinogênio

FIOCRUZ - fundação Oswaldo Cruz

FITC - isotiocianato de fluoresceína

His - histidina

HIV - vírus de imunodeficiência humana

IFN - interferon

Ig - imunoglobulina

IHA - ensaio de hemaglutinação indireta

IL- interleucina

IMT/USP - Instituto de Medicina Tropical / Universidade de São Paulo

IPTG - isopropil- β -tiogalactopiranosídeo

Kb - kilobase

K_D - constante de dissociação

LB - meio Luria-Bertani contendo 0,5% de extrato de levedura, 1% tripton e 1% NaCl

LIC - *Leptospira interrogans* sorovar Copenhageni

LPHS - síndrome hemorrágica pulmonar associada à leptospirose

LPS - lipopolissacarídeo

MAT - teste de microaglutinação

MEC - matriz extracelular

OMP - proteína de membrana externa

OMS – organização mundial de saúde

OPD - cloridrato de o-fenilenodiamina

pb - pares de base

PBS - fosfato salina

PBS/T - fosfato salina contendo 0,05% Tween 20

PCR - reação em cadeia da polimerase

pH - potencial hidrogeniônico

PI - ponto isoelétrico

PK - proteinase K

PLG - plasminogênio

PMSF – fenilmetilsulfonylfluor

RNA - ácido ribonucleico

RNAr - ácido ribonucleico ribossômico

rpm - rotações por minuto

RPMI - meio *Roswell Park Memorial Institute*

RT - transcriptase reversa

RT-PCR - reação em cadeia da polimerase com transcrição reversa

SDS - dodecil sulfato de sódio

TAE - tris-acetato EDTA

Tb - trombina

TLR - *Toll-like receptor*

TNF - fator de necrose tumoral

uPA - ativador de PLG tipo uroquinase

X-Gal - 5-bromo-4-cloro-indolil- β -D-galactopiranosideo

SUMÁRIO

1 INTRODUÇÃO	22
1.1 Taxonomia e classificação das leptospiras	22
1.2 Aspectos biológicos e morfológicos das leptospiras	24
1.3 Epidemiologia da leptospirose	26
1.4 Leptospirose humana	27
1.5 Diagnóstico e tratamento	29
1.6 Sequenciamento genômico	30
1.7 Patogenicidade e Fatores de Virulência	31
1.8 Modelos experimentais de animais para leptospirose	33
1.9 Vacinas	33
2 OBJETIVO	36
3 MATERIAIS E MÉTODOS	37
3.1 Bactérias	37
3.2 Soros de pacientes com leptospirose	37
3.3 Soros de doenças infecciosas não relacionadas à leptospirose	38
3.4 Análise por bioinformática	38
3.5 Construção de oligonucleotídeos	39
3.6 Estudo da presença dos genes dentre diferentes cepas de <i>Leptospira</i> por PCR	39
3.6.1 Extração de DNA genômico de culturas de <i>Leptospira</i> spp.	39
3.6.2 Avaliação por PCR	40
3.7 Estudo da presença dos transcritos dentre diferentes cepas de <i>Leptospira</i> por PCR	41
3.7.1 Extração de RNA de culturas de <i>Leptospira</i> spp.	41
3.7.2 Tratamento com DNase	41
3.7.3 Reação de Transcrição Reversa (RT-PCR)	41
3.7.4 Avaliação por PCR	42
3.8 Amplificação das sequências genômicas para clonagem	42
3.9 Clonagem no vetor pGEM-T Easy (Promega®)	42
3.10 Clonagem no vetor de expressão pAE	44
3.11 Sequenciamento automático dos clones	46
3.12 Avaliação da expressão das proteínas recombinantes	46
3.12.1 Estudo de indução das proteínas recombinantes	46
3.12.2 Estudo da solubilidade das proteínas recombinantes	47
3.13 Solubilização e renaturação da proteína rLIC12253	47
3.14 Purificação e diálise das proteínas recombinantes	47

3.15 Análise da estrutura e estabilidade das proteínas recombinantes por espectroscopia circular (CD)	48
3.16 Preparação do extrato de <i>E. coli</i>	48
3.17 Imunização de camundongos	48
3.18 Ensaio de linfoproliferação e liberação de citocinas	49
3.19 Teste de reconhecimento do anticorpo policlonal IgG pelas proteínas recombinantes por ELISA, após imunização de camundongos	50
3.20 Teste de reconhecimento do anticorpo policlonal IgG pelas proteínas recombinantes por <i>Imunoblotting</i>, após imunização de camundongos	51
3.21 Detecção de anticorpos IgG e IgM humanos em soro de pacientes diagnosticados com leptospirose por ELISA	52
3.22 Detecção de anticorpos IgG humanos em soro de pacientes com diagnóstico de outras doenças por ELISA	52
3.23 Ensaio de localização da proteína nativa	52
3.23.1 Imunofluorescência em fase líquida (<i>L – IFA</i>)	53
3.23.2.1 Ensaio de acessibilidade com <i>Proteinase K</i>	53
3.23.2.2 <i>ELISA para detecção da localização celular</i>	54
3.24 Ensaio de adesão aos componentes da matriz extracelular e componente do plasma por ELISA	54
3.24.1 <i>Análise da interação destes componentes às proteínas recombinantes</i>	54
3.24.2 <i>Curvas de dose-resposta</i>	55
3.25 Ensaio de adesão a componentes do soro humano e componente do plasma por ELISA	55
3.25.1 <i>Análise da interação destes componentes às proteínas recombinantes</i>	55
3.25.2 <i>Curvas de dose-resposta</i>	55
3.25.3 <i>Inibição da ligação da proteína recombinante <i>Lsa33 (rLIC11834)</i> ao PLG pelo ácido 6-aminocaproico (AAC)</i>	56
3.25.4 <i>Avaliação da atividade enzimática da plasmina</i>	57
3.26 Tratamento dos componentes glicoproteicos com metaperiodato de sódio	57
3.27 Ensaio de inibição da formação do coágulo de fibrina	58
3.28 Ensaio de inibição da ligação das proteínas recombinantes aos componentes pela incubação destas proteínas com antissoro específico	58
3.29 Ensaio de inibição da ligação das proteínas recombinantes aos componentes por desnaturação térmica destas proteínas	58
3.30 Inibição da ligação das leptospirosas vivas aos componentes	59
3.31 Interferência entre os componentes na ligação às proteínas recombinantes	59
3.32 Ensaio de imunoproteção	60
3.32.1 <i>Cálculo da <i>DL</i>₅₀ (dose letal)</i>	60
3.32.2 <i>Preparo de bacterina para ensaios de imunoproteção</i>	60
3.32.3 <i>Ensaio de desafio</i>	61

4 RESULTADOS E DISCUSSÃO	63
4.1 Análise por bioinformática	63
4.2 Estudo da presença dos genes dentre diferentes cepas de <i>Leptospira</i> por PCR	66
4.3 Estudo da presença dos transcritos dentre diferentes cepas de <i>Leptospira</i> por PCR	67
4.4 Amplificação das sequências genômicas para clonagem	68
4.5 Clonagem no vetor pGEM-T Easy (<i>Promega</i> ®)	68
4.6 Clonagem no vetor de expressão pAE	69
4.7 Sequenciamento automático dos clones positivos pAE/inserto	71
4.8 Avaliação da expressão das proteínas recombinantes	72
4.9 Purificação e diálise das proteínas recombinantes	74
4.10 Análise da estrutura e estabilidade das proteínas recombinantes por espectroscopia circular (CD)	77
4.11 Avaliação da imunogenicidade das proteínas recombinantes em camundongos	79
4.12 Detecção de anticorpos IgG e IgM humanos em soro de pacientes diagnosticados com leptospirose por ELISA	85
4.13 Avaliação de reação cruzada dos anticorpos IgG humanos de soro de pacientes com diagnóstico de outras doenças por ELISA	88
4.14 Ensaios de localização da proteína nativa	88
4.14.1 Imunofluorescência em fase líquida (L – IFA)	90
4.14.2 Ensaio de acessibilidade com Proteinase K (PK)	92
4.15 Ensaio de adesão das proteínas recombinantes aos componentes da matriz extracelular e componente do plasma por ELISA	92
4.15.1 Análise da interação destes componentes às proteínas recombinantes	93
4.15.2 Curvas de dose-resposta	94
4.16 Ensaio de adesão das proteínas recombinantes a componentes do soro humano e componente do plasma por ELISA	96
4.16.1 Análise da interação destes componentes às proteínas recombinantes	96
4.16.2 Curvas de dose-resposta	97
4.17 Inibição da ligação da proteína recombinante Lsa33 ao PLG pelo ácido 6-aminocaproico (AAC)	99
4.18 Avaliação da atividade enzimática da plasmina	100
4.19 Tratamento dos componentes glicoproteicos com metaperiodato de sódio	101
4.20 Ensaio de inibição da formação do coágulo de fibrina	102
4.21 Ensaio de inibição da ligação das proteínas recombinantes aos componentes pela incubação destas proteínas com antissoro específico	104
4.22 Ensaio de inibição da ligação das proteínas recombinantes aos componentes por desnaturação térmica destas proteínas recombinantes	105
4.23 Inibição da ligação das leptospiras vivas aos componentes que se ligam às proteínas recombinantes	106

4.24 Interferência entre os componentes na ligação às proteínas recombinantes	108
4.25 Ensaio de imunoproteção	111
5 CONCLUSÃO	116
REFERÊNCIAS	117
APÊNDICES	128
APÊNDICE A – ATZINGEN, M.V. et al. Identification and Characterization of Novel Adhesins in <i>Leptospira</i> . In: Antonio Mendez Vilas, Ed. (Org.). Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology , v. 1, p. 704-713, 2010.....	129
APÊNDICE B – ATZINGEN, M.V. et al. Evaluation of immunoprotective activity of six leptospiral proteins in the hamster model of leptospirosis. The Open Microbiology Journal , v. 6, p. 79-87, 2012.....	139
APÊNDICE C – VIEIRA, M. L. et al. Plasminogen Binding Proteins and Plasmin Generation on the Surface of <i>Leptospira</i> spp.: The Contribution to the Bacteria-Host Interactions. Journal of Biomedicine and Biotechnology (Online) , v. 2012, p. 1-17, 2012.	148
APÊNDICE D – DOMINGOS, R. F. et al. Features of two proteins of <i>Leptospira</i> interrogans with potential role in host-pathogen interactions. BMC Microbiology (Online) , v. 12, p. 50, 2012.....	165
APÊNDICE E – OLIVEIRA, R. et al. Adhesins of <i>Leptospira</i> interrogans mediate the interaction to fibrinogen and inhibit fibrin clot formation in vitro. PloS. Negl. Trop. Dis. , v. 7, n. 8, p. e2396, 2013.....	181
APÊNDICE F – VIEIRA, M. L. et al. Leptospiral extracellular matrix adhesins as mediators of pathogen-host interactions. FEMS Microbiol. Lett. , v. 352, n. 2, p. 129-139, 2014.....	195
APÊNDICE G – DOMINGOS, R. F. et al. Mapeamento de genomas bacterianos: sequenciamento e funcional de leptospiros. Biotecnologia Aplica à Saúde: Fundamentos e Aplicações , Editores: Rodrigo Resende e Carlos Ricardo Soccol, Ed. Blucher S/A. Capítulo de livro aceito para publicação.	207
APÊNDICE H – OLIVEIRA, R. et al. The intermediate and C-terminus regions of leptospiral adhesin Lsa66 are responsible for the binding with plasminogen and extracellular matrix components. Artigo submetido (em revisão).....	239
APÊNDICE I – DOMINGOS, R. F. et al. Resumo dos resultados da proteína rLIC11089 (em processo de confecção de artigo).	276

1 INTRODUÇÃO

A leptospirose é uma zoonose de importância global, pois está presente em todos os continentes (exceto na Antártica) sendo considerada uma das principais doenças infecciosas emergentes, com aumento considerável de números de casos em países do sudeste asiático e América Latina. É uma doença causada por um grupo de bactérias patogênicas pertencentes ao gênero *Leptospira*, a qual apresenta diversos sintomas clínicos. Entre estes, a doença (ou síndrome) de Weil (ADLER; DE LA PENA MOCTEZUMA, 2010; KO et al., 1999; LOMAR; DIAMENT; TORRES, 2000).

Em 1886, o alemão Adolf Weil relatou uma síndrome clínica caracterizada por esplenomegalia, icterícia e nefrite, a qual foi comumente referida como doença de Weil (LEVETT, 2001). Porém, foi só entre os anos de 1914 e 1915 que as leptospiros foram identificadas como causadoras da doença de Weil em mineradores de carvão do Japão pelos pesquisadores Inada e Ido (FAINE et al., 1999).

1.1 Taxonomia e classificação das leptospiros

A família *Leptospiraceae* pertencente à ordem *Spirochetales* possui dois gêneros: *Leptospira* e *Leptonema*, este último com apenas uma espécie saprofítica. Esta ordem também possui outra família, *Spirochaetaceae*, de grande importância para saúde pública, pois possui representantes patogênicos causadores de algumas doenças como a sífilis e a doença de Lyme (Figura 1) (SCHRODER et al., 2008).

Figura 1 – Taxonomia

O gênero *Leptospira* pertence à família *Leptospiraceae* da ordem *Spirochetales*, a qual possui outra família, *Spirochaetaceae*, composta por organismos também de grande importância para saúde pública.

Fonte: Modificado de Schoder et al. (2008).

A classificação e a nomenclatura das espécies de *Leptospira* é algo complexo. Há dois diferentes sistemas de classificação: um baseado na patogenicidade da cepa e outro na homologia genética (VIJAYACHARI; SUGUNAN; SHRIRAM, 2008).

A primeira classificação divide as leptospirosas em duas espécies sorológicas distintas: com a maioria das espécies patogênicas (ou supostamente patogênicas) classificadas em *interrogans*. E todas as outras classificadas em *biflexa*, que contém as cepas saprofíticas (BHARTI et al., 2003; PLANK; DEAN, 2000).

Atualmente, a classificação mais aceita é a genética, baseada na hibridização do ácido desoxirribonucleico (DNA), a qual indica a existência de pelo menos 19 espécies (13 patogênicas e 6 saprofíticas) (BRENNER et al., 1999; LEVETT, 2001), sendo sete destas espécies, *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri* e *L. alexanderi*, as principais causadores da leptospirose (AHMED et al., 2006).

Todas as espécies reconhecidas de *Leptospira* são classificadas em 24 sorogrupos e cerca de 250 sorovares com base na expressão dos lipopolissacarídeos (LPS) expostos na

superfície (ADLER; DE LA PENA MOCTEZUMA, 2010). As diferenças estruturais dos carboidratos que compõem a cadeia lateral dos LPS determinam a diversidade antigênica entre os numerosos grupos de sorovares. Os sorovares que contém sobreposição de determinantes antigênicos são classificados em um sorogrupo maior (LEVETT, 2001).

Além disso, uma análise filogenética do gene *16S ácido ribonucleico ribossomal* (RNAr) sugere que espécies de *Leptospira* se dividem em três grupos distintos: patogênicas, intermediárias e saprofíticas (LEVETT et al., 2006; PEROLAT et al., 1998).

Nesta classificação, o grupo das espécies patogênicas possui 8 espécies (*L. interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, e *L. alstonii*), enquanto o grupo das espécies saprofíticas possui 7 espécies (*L. biflexa*, *L. wolbachii*, *L. kmetyi*, *L. meyeri*, *L. vanthielii*, *L. terpstrae*, e *L. yanagawae*) e forma o ramo mais afastado filogeneticamente. Outro ramo evolucionário possui 5 espécies e são chamadas intermediárias (*L. inadai*, *L. broomii*, *L. fainei*, *L. wolffii*, *L. licerasiae*), o qual contém espécies de patogenicidade moderada (BHARTI et al., 2003; KO; GOARANT; PICARDEAU, 2009; LEVETT, 2001).

1.2 Aspectos biológicos e morfológicos das leptospiras

Leptospiras são bactérias espiroquetas finas, helicoidais, móveis, com 6 – 20 µm de comprimento por 0,1 µm de diâmetro. Possui terminações em gancho, semelhantes a um ponto de interrogação e, por isso inicialmente se deu a nomenclatura de *interrogans* para as bactérias patogênicas (HAAKE et al., 2000; KO; GOARANT; PICARDEAU, 2009; LEVETT, 2001).

As superfícies das leptospiras possuem estruturas características tanto de bactérias Gram-positivas quanto de Gram-negativas (Figura 2). A membrana dupla e a presença de LPS são características de bactérias Gram-negativas, enquanto que a íntima associação da membrana plasmática com a parede celular composta de peptídeoglicano é uma característica semelhante à de bactérias Gram-positivas (HAAKE et al., 2000; KO; GOARANT; PICARDEAU, 2009; LEVETT, 2001).

Os LPS constituem o principal antígeno para as leptospiras e são relativamente não tóxicos para células ou animais, sendo 12 vezes menos letal para camundongos que os LPS de *Escherichia coli*, possivelmente devido às características incomuns do lipídeo A da leptospira (FAINE et al., 1999). A membrana das leptospiras também é composta por proteínas

oxidação. O meio líquido mais comumente usado é o Ellinghausen–McCullough/Johnson–Harris (EMJH), que contém ácido oleico, albumina sérica e polisorbato, porém, outros meios já foram descritos, como Fletcher, Korthoff, Noguchi, e Stuart para o cultivo das leptospiros (FAINE et al., 1999; LEVETT, 2001). Para meio semissólido, adiciona-se agar em baixas concentrações (0,1% - 0,2%) (ADLER; DE LA PENA MOCTEZUMA, 2010).

As culturas de leptospiros são mantidas por repetidas passagens em meio líquido, porém, por razões desconhecidas, com perda de virulência, ou podem ser mantidas em meio semissólido ágar contendo hemoglobina. Para armazenamento por longos períodos de tempo o uso de nitrogênio líquido apresenta bons resultados e é o método indicado para manutenção da virulência (ADLER; DE LA PENA MOCTEZUMA, 2010).

Algumas cepas patogênicas, como a *L. interrogans*, podem sobreviver em ambientes com baixa concentração de nutrientes, como solos úmidos e água fresca por longos períodos, tendo concentração de sal, pH e viscosidade como fatores críticos (TRUEBA et al., 2004). Porém, outras cepas patogênicas, como a *L. borgpetersenii* não sobrevivem fora do hospedeiro (BULACH et al., 2006). Além disso, as leptospiros também formam biofilmes com objetivo de sobreviver em diversos nichos ambientais (RISTOW et al., 2008).

1.3 Epidemiologia da leptospirose

A leptospirose é uma zoonose de ampla distribuição geográfica, ocorrendo tanto em áreas rurais, quanto em áreas urbanas de regiões tropicais, subtropicais e temperadas. Está associada à exposição ocupacional, turismo e eventos esportivos em países desenvolvidos. Já em países em desenvolvimento, onde ocorrem os principais surtos da doença (com destaque para países do sudeste asiático e da América do Sul), está associada a condições higiênico-sanitárias precárias (BENSCHOP et al., 2009; DESAI et al., 2009; MORGAN et al., 2002; STERN et al., 2010).

Um amplo espectro de animais domésticos, sinantrópicos e selvagens servem como reservatórios para as leptospiros (praticamente todos os mamíferos), pois albergam as bactérias nos túbulos proximais renais eliminando-as vivas constantemente no ambiente pela urina, contaminando água, solo, alimentos e outros animais. Nos centros urbanos, os principais animais reservatórios são os roedores, em especial *Rattus norvegicus*, rato de esgoto, *Rattus Rattus*, rato preto, e *Mus musculus*, camundongo (FAINE et al., 1999). Porém, alguns roedores, como hamsters e porquinhos-da-índia, são bastante suscetíveis à leptospirose. Anfíbios, cobras e peixes também foram mostrados como animais com potencial

de albergar leptospiros (MINETTE, 1983), mas a significância destes achados em relação à transmissão da doença é desconhecida (LANGSTON; HEUTER, 2003).

No Brasil, a leptospirose é uma doença endêmica, ocorrendo durante todos os meses e com coeficiente médio de incidência anual de 1,9/100.000 habitantes. Porém, se torna epidêmica nos grandes centros urbanos nos períodos de grandes chuvas, devido a inundações, redes de saneamento precárias, condições inapropriadas de moradia e infestações de roedores infectados, que propiciam a disseminação das leptospiros no ambiente, e predispõem o contato humano com águas contaminadas (BRASIL, 2009).

No país, a doença tem grande importância social e econômica devido à sua alta incidência e o grande número de internações, o que resulta em alto custo hospitalar e perdas de dias de trabalho (BRASIL, 2009).

1.4 Leptospirose humana

O homem é o hospedeiro terminal e acidental da leptospirose. A infecção ocorre pelo contato com a urina de animais infectados de forma direta ou indireta (contato com águas ou solos contaminados). A penetração do micro-organismo ocorre através da pele com lesões, da pele íntegra quando imersa em água por longo período de tempo, ou das mucosas (a transmissão inter-humana é rara e de pouca relevância epidemiológica) (BRASIL, 2009) (Figura 3). O início da doença em humanos é variável, um dia a quatro semanas após exposição, sendo mais frequente entre cinco a quatorze dias, e a infecção nos sobreviventes pode durar meses (PLANK; DEAN, 2000).

Figura 3 - Transmissão da leptospirose em animais e humanos

O homem é o hospedeiro terminal e acidental da leptospirose. A infecção, tanto em humanos, como em animais, ocorre pelo contato com a urina de animais infectados de forma direta ou indireta.

Fonte: Modificado de Adler; de la Pena Moctezuma (2010).

A leptospirose pode apresentar um espectro de manifestações clínicas, dependendo da cepa ou do sorovar envolvido na infecção, e da idade e condição imunológica do hospedeiro. Dessa forma, pode apresentar desde quadros assintomáticos e oligossintomáticos, até formas graves associadas a manifestações fulminantes. Muitos casos ocorrem de forma rápida e autolimitada, apresentando sintomas comuns a outras doenças, como febre, dor de cabeça, náuseas e vômitos, mialgia e, menos comum, erupções cutâneas, o que dificulta o diagnóstico da doença. A síndrome de Weil é a forma severa da doença e apresenta esplenomegalia, icterícia, meningites, disfunção hepática e renal, e colapso vascular (aproximadamente 15% dos pacientes evoluem para a forma severa e 4 a 50% destes vão a óbito) (ADLER; DE LA PENA MOCTEZUMA, 2010; BRASIL, 2009; KO; GOARANT; PICARDEAU, 2009; LEVETT, 2001).

Recentemente, a síndrome hemorrágica pulmonar associada à leptospira (LPHS, *leptospiral pulmonary haemorrhagic syndrome*) tem sido relatada como outra manifestação clínica severa da leptospirose. Apresenta sintomas inespecíficos como dor no peito e tosse, e pode evoluir para insuficiência respiratória aguda, hemorragia maciça, ou síndrome de angústia respiratória do adulto. Estes sintomas aparecem geralmente entre o quarto e o sexto dia da doença, pode levar a óbito em até 72 horas, e apresenta índices de mortalidade de 30-70% (BRASIL, 2009; FAINE et al., 1999; NICODEMO et al., 1997; TOYOKAWA; OHNISHI; KOIZUMI, 2011; YERSIN et al., 2000).

1.5 Diagnóstico e tratamento

O diagnóstico da leptospirose é dificultado devido à diversidade de sintomas apresentado pelo paciente, dependendo, por tanto, de uma série de ensaios clínicos. Os métodos empregados para o diagnóstico estão divididos em duas categorias: evidências indiretas (detecção de anticorpos) e evidências diretas (que incluem a detecção da bactéria ou de componentes da bactéria) (FAINE et al., 1999; LEVETT, 2001).

Para a detecção de anticorpos pode-se empregar o ensaio de hemaglutinação indireta (IHA), o ensaio imunoenzimático (ELISA) para detecção de anticorpos IgM, e o teste de microaglutinação (MAT).

O MAT é o teste padrão ouro recomendado pela Organização Mundial de Saúde (OMS) e, sendo assim, o mais utilizado. Apesar disso, apresenta baixa sensibilidade na fase inicial da doença (fase aguda), já que anticorpos contra antígenos de leptospira só são detectados alguns dias após a exposição (14 – 21 dias, fase crônica), e requer a manutenção de culturas vivas de diferentes espécies e sorovares, o que dificulta a execução do ensaio, sendo realizado somente em laboratórios de referência. Neste teste, os soros pareados dos pacientes, ou seja, uma primeira amostra (fase aguda) não reagente e uma segunda amostra mais tardia (fase crônica), são submetidos à reação com suspensões de diferentes espécies e sorovares de leptospirosas vivas para detecção de anticorpos por aglutinação. O teste se mostra positivo quando há aumento de quatro vezes ou mais nos títulos entre as duas amostras (quando não houver disponibilidade de duas amostras, um título maior ou igual a 800 confirma o diagnóstico) (BHARTI et al., 2003; BRASIL, 2009; FAINE et al., 1999; LEVETT, 2001).

As leptospirosas ou alguns componentes da bactéria podem ser detectados na urina ou em tecidos por cultura, microscopia de campo escuro, imuno-histoquímica e Reação em

Cadeia da Polimerase (PCR) (BHARTI et al., 2003; BRASIL, 2009; FAINE et al., 1999; LEVETT, 2001).

Recentemente, têm sido propostos alguns protocolos usando a técnica de PCR para diagnóstico da leptospirose como uma alternativa ao MAT. Alguns genes específicos, como *secY*, *rrs*, *flaB*, *LigB*, *LipL32*, *rrl* e *LA3521*, foram utilizados como alvos e amplificados por PCR a partir de amostras do liquor e da urina de suspeitos de leptospirose (BROWN et al., 1995; FONSECA et al., 2006; KOIZUMI; KAWABATA; WATANABE, 2003; KOSITANONT et al., 2007; MERIEN et al., 1992).

O diagnóstico da leptospirose por PCR vem sendo considerado o método mais rápido para detecção de DNA de leptospiros. Porém, o uso deste método de forma generalizada vem dificultando a aplicação do mesmo devido aos riscos de contaminação por DNA exógeno e falso-positivos, além de relatos de sensibilidade variável (CHAGAS-JUNIOR et al., 2012).

Um novo método diagnóstico da leptospirose foi divulgado por pesquisadores da Fundação Oswaldo Cruz (FIOCRUZ) e tem por objetivo ser mais rápido que os testes tradicionais. Este método utiliza-se somente de uma gota de sangue do paciente suspeito, que é colocada em uma pequena plataforma contendo uma fita com o antígeno da bactéria *LigA*, *leptopiral immunoglobulin-like A*, seguido de uma solução reveladora. A coloração rosa indica positividade e é observado entre 15 a 20 minutos (testes foram realizados com mais de 1.100 amostras de sangue e no total apresentaram sensibilidade de 85% na fase aguda da doença) (FUNDAÇÃO OSWALDO CRUZ, 2010).

Quando a leptospirose é diagnosticada, o tratamento se dá com uso de antibióticos como Amoxicilina e Doxiciclina para fase aguda, e Penicilina G Cristalina, Ampicilina e Ceftriaxona ou Cefotaxima para fase crônica. A antibioticoterapia é indicada em qualquer período da doença, mas sua eficácia é maior nos primeiros dias do início dos sintomas. O uso de antibióticos também é indicado como quimioprofilaxia para aqueles indivíduos que se expõem em áreas de alto risco endêmico. Além disso, nos casos graves, os quais há complicações renais, respiratórias ou hemorrágicas, os pacientes devem realizar procedimentos como diálise e internações em unidades de terapia intensiva (BRASIL, 2009; FAINE et al., 1999; LEVETT, 2001).

1.6 Sequenciamento genômico

A primeira espécie de leptospira sequenciada foi a *L. interrogans* sorovar Lai e, logo após, sorovar Copenhageni. Esta espécie representa o principal agente etiológico da maior

parte das infecções humanas, o que explica a importância do sequenciamento da mesma. O genoma das leptospiiras é composto por dois cromossomos circulares. No sorovar Lai, o cromossomo I possui 4.333.241 pb e o cromossomo II 358.943 pb, já no sorovar Copenhageni, o cromossomo I possui 4.277.185 pb, enquanto que o II 350.181 pb (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b; REN et al., 2003; VIJAYACHARI; SUGUNAN; SHRIRAM, 2008).

Outra espécie patogênica sequenciada foi a *L. borgpetersenii*. Esta espécie é de grande importância para pecuária, já que infecta rebanhos de suínos, de equinos e, principalmente, de bovinos. O genoma da *L. borgpetersenii* é aproximadamente 700.000 pb menor que o genoma da *L. interrogans*, o que deve influenciar diretamente na sobrevivência das espécies. Enquanto que a *L. interrogans* é capaz de sobreviver em ambientes úmidos, fora do hospedeiro, a *L. borgpetersenii* parece evoluir em direção a um ciclo de transmissão restrita entre hospedeiros (ADLER; DE LA PENA MOCTEZUMA, 2010; BULACH et al., 2006).

A espécie *L. biflexa* foi a primeira espécie saprofítica de vida livre sequenciada. O cromossomo I possui 3.603.977 pb e o cromossomo II possui 277.995 pb. Além destes, nesta espécie também há um terceiro replicon denominado p74, o qual parece ser essencial para a sobrevivência da espécie saprofítica, pois possui genes constitutivos do metabolismo basal que são encontrados no cromossomo I das leptospiiras patogênicas. Análises comparativas entre as sequências das leptospiiras patogênicas com a *L. biflexa* mostrou que há presença de 61% dos genes em ambas as espécies. Sendo assim, há genes comuns às espécies patogênicas que estão ausentes na saprofítica e parecem estar relacionados à patogênese (PICARDEAU et al., 2008).

Recentemente, mais duas espécies tiveram os seus genomas completamente sequenciados: a espécie patogênica *L. santarosai*, e a espécie *L. licerasiae*, que foi a primeira espécie de patogênese intermediária sequenciada. A análise da sequência genômica desta última foi de grande importância para melhor entendimento da evolução das leptospiiras e mostrou que a *L. licerasiae* geneticamente está mais relacionada com as espécies patogênicas do que com a espécie saprofítica *L. biflexa* (CHOU et al., 2012; RICALDI et al., 2012).

1.7 Patogenicidade e Fatores de Virulência

Os mecanismos moleculares de patogenicidade e virulência de leptospiiras são pouco conhecidos. Porém, com a disponibilidade das sequências genômicas, o desenvolvimento de ferramentas genéticas, e o uso de modelos animais, estes mecanismos estão cada vez mais

sendo descobertos (ADLER; DE LA PENA MOCTEZUMA, 2010; BULACH et al., 2006; CHOU et al., 2012; NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b; PICARDEAU et al., 2008; REN et al., 2003; RICALDI et al., 2012).

O primeiro fator de virulência geneticamente definido foi a Loa22, uma lipoproteína com domínio tipo OmpA. *Leptospiras* mutantes para o gene *loa22* perderam a virulência em hamsters e porquinhos-da-índia, animais suscetíveis à leptospirose (RISTOW et al., 2007).

Porém, apesar do pouco conhecimento, há outros candidatos a fatores de virulência identificados, como os LPS, hemolisinas, adesinas e proteínas de membrana externa (OMPs).

Os LPS de leptospira são reconhecidos por células murinas por receptores Toll-like receptors2 (TLR2) e 4 (TLR4) resultando na ativação do sistema imune. Porém, em células humanas, os LPS são reconhecidos somente por TLR4 possivelmente devido à composição incomum do lipídeo A e, dessa forma, provoca falha na ativação do sistema imune (QUEGEWIRTH et al., 2004; WERTS et al., 2001). Além disso, foi observado perda de virulência em leptospiras mutantes de LPS em modelo de hamster (MURRAY et al., 2009).

Tem sido relatada a presença de hemolisinas em diversos sorovares de leptospiras. Sendo assim, uma gama de genes que codificam esfingomielinases foi identificada em diferentes espécies. Porém, foram encontrados estes genes somente em espécies patogênicas, o que pode sugerir papel destas proteínas na patogênese, apesar de já ter sido relatado que não promovem hemólise em humanos (ADLER; DE LA PENA MOCTEZUMA, 2010; BULACH et al., 2006).

Foi demonstrada a capacidade de aderência de diversas espécies de leptospiras a componentes da matriz extracelular, como laminina, colágeno tipo I, colágeno tipo IV e fibronectina (ATZINGEN et al., 2008; BARBOSA et al., 2006). Desde então, estudos estão sendo dirigidos para identificar OMPs capazes de se ligar a estes componentes e auxiliar na adesão das bactérias aos tecidos dos hospedeiros. Diversas adesinas já foram caracterizadas, como as proteínas LigA e LigB (CHOY et al., 2007), proteínas da família Len (STEVENSON et al., 2007), Lsa21 (ATZINGEN et al., 2008), LipL32 (HOKE et al., 2008), Lp95 (ATZINGEN et al., 2009), TlyC (CARVALHO et al., 2009), LipL53 (OLIVEIRA et al., 2010), OmpL37 (PINNE; CHOY; HAAKE, 2010), Lsa63 (VIEIRA et al., 2010b), Lsa66 (OLIVEIRA et al., 2011), Lsa20 (MENDES et al., 2011), Lsa30 (SOUZA et al., 2012), Lsa23, Lsa26, Lsa36 (SIQUEIRA et al., 2013), Lsa44, e Lsa45 (FERNANDES et al., 2013), entre outras (VIEIRA et al., 2014).

Leptospiras são capazes de se ligar ao plasminogênio (PLG), uma protease do hospedeiro, converter em sua forma ativa (plasmina) e, sendo assim, auxiliar na penetração

tecidual e invasão (VIEIRA et al., 2010a; VIEIRA et al., 2009b). Dessa forma, várias proteínas que se ligam ao PLG e são capazes de converter em plasmina foram identificadas (DOMINGOS et al., 2012; FERNANDES et al., 2013; MENDES et al., 2011; SIQUEIRA et al., 2013; VERMA et al., 2010; VIEIRA et al., 2010a).

Foi verificada também a capacidade de evasão do sistema complemento pelas leptospiros. A ligação das leptospiros aos reguladores do sistema complemento, Fator H e C4 binding protein (C4bp) inibe o ataque deste sistema facilitando a disseminação das bactérias e colonização dos órgãos dos hospedeiros. Algumas proteínas capazes de se ligarem a estes reguladores já foram descritas, como LenA e LenB (Len A também conhecida por LfhA e Lsa24), ligantes do Fator H, e LcpA, ligantes do C4bp (BARBOSA et al., 2009; BARBOSA et al., 2010; CASTIBLANCO-VALENCIA et al., 2012; DOMINGOS et al., 2012; MERI et al., 2005; SIQUEIRA et al., 2013; STEVENSON et al., 2007; VERMA et al., 2010; VERMA et al., 2006).

1.8 Modelos experimentais de animais para leptospirose

Modelos animais experimentais têm sido usados para testar candidatos vacinais, bem como para elucidar respostas imunes dos hospedeiros e patologia da leptospirose. Hamsters e porquinhos-da-índia são suscetíveis à infecção aguda por leptospiros e, por isso, são comumente utilizados em testes de desafio. Camundongos e ratos são resistentes à infecção e, por isso, utilizados como modelos para estudos de infecção crônica persistente. Porém, camundongos com mutações específicas, como no gene *tlr4*, também vem sendo usados como modelos para infecção aguda, já que foi demonstrado que o reconhecimento de antígenos de leptospiros por TLR-4 protege contra a infecção letal (FAINE et al., 1999; HAAKE, 2006; JOST et al., 1986; NALLY et al., 2005).

O hamster *Golden Syrian* é um modelo bem caracterizado para infecção aguda por leptospiros. Apresenta sintomas semelhantes aos humanos e, portanto, é o mais explorado. Esse modelo é utilizado para determinar a infectividade das leptospiros, manter culturas virulentas, e em ensaios de desafio (HAAKE, 2006; SILVA et al., 2007; SILVA et al., 2008).

1.9 Vacinas

As vacinas contra leptospirose disponíveis hoje no mercado são compostas de bactérias inativadas ou preparações de membrana de leptospiros patogênicos. A maior parte

delas é para uso veterinário, porém alguns países utilizam também para uso humano (ADLER; DE LA PENA MOCTEZUMA, 2010; GAMBERINI et al., 2005; KO; GOARANT; PICARDEAU, 2009). Dentre estes países estão Japão (KOIZUMI; WATANABE, 2005; YANAGIHARA et al., 2007), Cuba (MARTINEZ et al., 2004; SANCHEZ et al., 2002) e França, os quais utilizam preparações vacinais baseadas em leptospiros inativadas (LAURICHESSE et al., 2007; RODRIGUEZ-GONZALEZ et al., 2004), e China, na qual utiliza uma vacina baseada em preparação de membrana externa de *Leptospira* spp. (YAN et al., 2003).

Porém, estas vacinas causam efeitos adversos, como dor no local da aplicação, náusea e febre, não induzem resposta cruzada, já que conferem proteção dirigida contra os LPS que são os determinantes antigênicos dos mais de 250 sorovares de *Leptospira* spp., e não induzem memória, sendo necessária revacinação entre 6 meses a 1 ano (BHARTI et al., 2003; HAAKE; MATSUNAGA, 2010).

Dessa forma, novas estratégias estão sendo desenvolvidas para a produção de uma vacina mais eficaz, capaz de induzir resposta cruzada e células de memória. As proteínas de membrana externa (OMPs), devido à interação direta com o hospedeiro e, se conservadas, são proteínas tidas como principais alvos para compor uma vacina de componentes definidos (SRIVASTAVA, 2006).

Com o desenvolvimento da tecnologia do DNA recombinante, os avanços obtidos com os projetos genomas de membros da ordem Spirochaetales como *Borrelia burgdorferi* (FRASER et al., 1997), *Treponema pallidum* (FRASER et al., 1998), *L. interrogans* sorovar Lai (REN et al., 2003), *L. interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b), *L. borgpetersenii* sorovar Hardjo-bovis (BULACH et al., 2006), *L. biflexa* sorovar Patoc (PICARDEAU et al., 2008), *L. santarosai* (CHOU et al., 2012) e *L. licerasiae* (RICALDI et al., 2012), assim como as análises proteômicas (VIEIRA et al., 2009a), é possível aplicar a Vacinologia Reversa (RAPPUOLI, 2000), que se trata do isolamento a partir de análises *in silico*, clonagem e sequenciamento dos genes que codificam antígenos imunologicamente relevantes de *Leptospira* spp.. Esta estratégia já foi empregada de modo satisfatório para preparações vacinais contra alguns patógenos, como *Neisseria meningitidis* sorogrupo B (PIZZA et al., 2000) e *Streptococcus pneumoniae* (WIZEMANN et al., 2001).

Após o sequenciamento do genoma da *Leptospira interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b), nosso grupo realizou um Projeto Piloto Funcional que resultou na identificação de 23 proteínas de membrana reativas

com anticorpos presentes em soros de pacientes diagnosticados com a doença (GAMBERINI et al., 2005).

Assim sendo, para este projeto, selecionou-se através de análise *in silico* dois genes para expressão e caracterização de proteínas: LIC11834 e LIC12253 (LIC: *Leptospira interrogans* sorovar Copenhageni).

A obtenção e caracterização de proteínas envolvidas na patogenicidade de *L. interrogans* deverão fornecer dados importantes para o conhecimento da infecção e também na identificação de antígenos protetores para o desenvolvimento futuro de uma vacina e/ou sorodiagnóstico.

2 OBJETIVO

O presente trabalho tem por objetivo estudar genes de *Leptospira interrogans* e caracterizar as proteínas recombinantes respectivas, para avaliar o papel das mesmas na patogenicidade da doença.

3 MATERIAIS E MÉTODOS

3.1 Bactérias

Cepas patogênicas atenuadas de *Leptospira* foram utilizadas no presente estudo, tais como: *L. interrogans* sorovar Canicola cepa Hound Utrech IV, *L. interrogans* sorovar Copenhageni cepa M 20, *L. interrogans* sorovar Hardjo cepa Hardjo-prajitno, *L. interrogans* sorovar Icterohaemorrhagiae cepa RGA, *L. interrogans* sorovar Pomona cepa Pomona, *L. borgpetersenii* sorovar Whitcombi cepa Whitcomb, *L. kirschneri* sorovar Cynoptery cepa 3522C, *L. kirschneri* sorovar Grippotyphosa cepa Moskva V, *L. santarosai* sorovar Shermani cepa 1342 K, *L. noguchii* sorovar Panama cepa CZ 214 e a cepa não patogênica *L. biflexa* sorovar Patoc cepa Patoc.

As bactérias foram cultivadas a 28 °C sob condições aeróbias em meio EMJH líquido (Difco®, BD, Franklin Lakes, NJ, USA) (ELLINGHAUSEN; MCCULLOUGH, 1965; JOHNSON; ROGERS, 1967) com 10% (v/v) de soro de coelho, enriquecido com L-asparagina (m/v: 0,001%), cloreto de magnésio (m/v: 0,001%), peptona (m/v: 0,03%) e extrato de carne (m/v: 0,001%). A cepa virulenta *L. interrogans* sorovar Kennewicki cepa Pomona Fromm também foi utilizada (ensaio de desafio). As culturas de *Leptospira* spp. são mantidas na Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brasil. As leptospirosas se duplicam aproximadamente a cada 16 horas e seu crescimento é lento no isolamento primário, de modo que as culturas são mantidas por até cerca de 13 semanas antes de serem descartadas (LEVETT, 2001). As leptospirosas de subcultura são cultivadas por em média 14 dias, e em seguida são novamente subcultivadas.

Bactérias *E. coli* DH5 α TM (Invitrogen Corporation, Carlsbad, CA, EUA) foram empregadas para clonagem. Para expressão de proteínas recombinantes empregamos *E. coli* BL21 SI (Invitrogen) que possui o gene da T7 RNA polimerase sob ação do promotor responsivo a osmolaridade, promotor *proU*, induzível pela adição de NaCl (BHANDARI; GOWRISHANKAR, 1997).

3.2 Soros de pacientes com leptospirose

Amostras de soros de pacientes com diagnóstico laboratorial de leptospirose confirmado foram obtidas da soroteca do Instituto Adolfo Lutz de São Paulo, SP, Brasil, e do Instituto Adolfo Lutz de Santos, SP, Brasil.

3.3 Soros de doenças infecciosas não relacionadas à leptospirose

Amostras de soros de pacientes com doença de Chagas foram cedidas pelo Laboratório de Protozoologia do Instituto de Medicina Tropical da Universidade de São Paulo (IMT/USP). Soros de pacientes diagnosticados com vírus de imunodeficiência humana (HIV) e dengue foram doados pelo Laboratório de Virologia do IMT/USP. Amostras de pacientes com malária foram recebidas do Núcleo de Estudos em Malária do IMT/USP. Todos os soros de pacientes foram doados para fins de pesquisa científica.

3.4 Análise por bioinformática

Os genes foram selecionados a partir do genoma de *L. interrogans* sorovar Copenhageni, disponível no servidor público *Leptospira - Genome Project* (<http://aeg.lbi.ic.unicamp.br/world/lic/>, NASCIMENTO et al., 2004b). Esta seleção teve como base a predição da localização celular das proteínas hipotéticas codificadas por estes genes, que foi feita através do programa PSORT (<http://psort.nibb.ac.jp/>, NAKAI; HORTON, 1999; NAKAI; KANEHISA, 1991). As sequências foram submetidas à análise pelo programa LipoP (<http://www.cbs.dtu.dk/services/LipoP/>, JUNKER et al., 2003), que analisa a presença de uma sequência consenso denominada lipobox, na qual a ocorrência de um resíduo de cisteína indica que a proteína é lipídada (WU et al., 1996). Os programas SMART (<http://smart.embl-heidelberg.de/>, LETUNIC; DOERKS; BORK, 2012; SCHULTZ et al., 1998) e PFAM (<http://www.sanger.ac.uk/Software/Pfam/>, PUNTA et al., 2012) foram usados para pesquisar domínios estruturais e funcionais.

Os genes ainda foram analisados pelo programa BLAST - *Basic Local Alignment Search Tool* (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>, ALTSCHUL et al., 1990; ALTSCHUL et al., 1997), que analisa as similaridades destas sequências com outras depositadas no banco de dados do Gene Bank.

A filogenia das sequências codificadas pelos genes selecionados para o estudo foi realizada pelo programa de alinhamento múltiplo ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>, LARKIN et al., 2007).

As características físicas e químicas das proteínas preditas codificadas pelos genes em estudo foram avaliadas utilizando a ferramenta ProtParam (<http://www.expasy.org/tools/protparam.html>, GASTEIGER, 2006).

3.5 Construção de oligonucleotídeos

Os oligonucleotídeos *forward* (F) e *reverse* (R) foram desenhados no programa Generunner, contendo os sítios de restrição para as enzimas *XhoI*/*NcoI* para ambas sequências (sem sequência peptídeo sinal) (Quadro 1).

Quadro 1 - Oligonucleotídeos referentes aos genes LIC11834 e LIC12253

LIC11834	<p>Oligonucleotídeo F 5'CTCGAGGATCTACAAGGTGGGGTTTTTAC3' XhoI</p> <p>Oligonucleotídeo R 5'CCATGGTTACTGAGGTTTTACTTGGTCC3' NcoI</p>
LIC12253	<p>Oligonucleotídeo F 5'CTCGAGGAGGAGAAACCGGACGATAC 3' XhoI</p> <p>Oligonucleotídeo R 5'CCATGGTTAGGGAAGACTTCTAACACATC3' NcoI</p>

Notas: Sublinhado: sequências dos sítios de restrição correspondentes.

Abreviaturas em inglês: F - *forward*. R - *reverse*.

3.6 Estudo da presença dos genes dentre diferentes cepas de *Leptospira* por PCR

3.6.1 Extração de DNA genômico de culturas de *Leptospira* spp.

Para extração do DNA das cepas bacterianas (patogênicas: *L. interrogans* sorovares Canicola, Copenhageni, Hardjo, Icterohaemorrhagiae e Pomona; *L. borgpetersenii* sorovar Whitcombi; *L. kirshnery* sorovares Grippothyphosa e Cynoptery; *L. santarosai* sorovar Shermani; *L. noguchi* sorovar Panama; não patogênica: *L. biflexa* sorovar Patoc), alíquotas de 3 mL das culturas com 5 dias de crescimento foram centrifugadas por 2 minutos a 13.000 rpm em temperatura ambiente. Ressuspendeu-se o pellet em 467 µL de tampão TE e adicionou-se 30 µL de 10% SDS e 3 µL de 20 mg/mL de proteinase K. Incubou-se por 1 hora a 37 °C.

Após essa etapa, volume igual de fenol-clorofórmio foi adicionado e misturado bem por inversão. Centrifugou-se a 14.000 rpm por 5 minutos e coletou-se a fase superior, na qual encontra-se o DNA. Repetiu-se esse procedimento.

Houve adição de 1/10 do volume de acetato de sódio. Misturou-se. Houve adição também de 0,6 do volume de isopropanol e misturou-se gentilmente até precipitação do DNA. Incubou-se por 1 hora a 20 °C. Centrifugou-se então por 20 minutos a 11.000 rpm à 4 °C e descartou o sobrenadante (o isopropanol).

O DNA foi lavado com 1 mL de 70% etanol por 30 segundos (11.000 rpm por 10 minutos à 4 °C). Desprezado o etanol, adicionou-se 50 µL de tampão TE para ressuspender o DNA e armazenou-se à -20 °C.

A comprovação da extração do DNA foi verificada por eletroforese em gel de agarose 1% em tampão TAE (450 mM Tris-Acetato e 10mM EDTA).

Após essa etapa, diluiu-se o DNA de algumas cepas para padronização da concentração da mesma (nova eletroforese em gel de agarose 1% em tampão TAE).

3.6.2 Avaliação por PCR

As presenças dos genes LIC11834 e LIC12253 foram avaliadas nas cepas de leptospiras citadas acima através de reação de PCR, a qual se utilizou 1X tampão de PCR; 0,2 mM dNTP's (*mix* dATP, dTTP, dGTP e dCTP); 2 mM MgCl₂; 0,2 mM de cada oligonucleotídeo (F e R); 2,5 U Taq polimerase; 100 ng de DNA genômico das cepas bacterianas, para um volume final de 50 µL. As temperaturas de anelamento utilizadas foram determinadas de acordo com o programa Generunner → 57 °C e 56 °C para LIC11834 e LIC12253, respectivamente. A reação foi realizada em termociclador, seguindo a rotina de 35 ciclos: a) 94 °C por 50 segundos; b) 57 °C e 56 °C (dependendo do gene) por 50 segundos; c) 72 °C por 3 minutos. Porém, antes se realizou PCR com oligonucleotídeos para o gene de expressão 16s RNAr (16S F: GCGTCTTAAACATGCAAG; 16S R: ACTTAACCCAACATCTCACG), o qual serve como controle interno (controle positivo para a presença de DNA).

A amplificação das sequências de interesse foi verificada através de eletroforese em gel de agarose 1% em tampão TAE.

3.7 Estudo da presença dos transcritos dentre diferentes cepas de *Leptospira* por PCR

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

Alíquotas de 20 mL de cultura (mesmos sorovares citados no item 3.6.1) com 5 dias de crescimento foram centrifugadas por 30 minutos a 6.000 rpm (4 °C). Ressuspendeu-se o precipitado com 2 mL de Trizol (Invitrogen) e centrifugou-se novamente por 10 minutos a 12.000 rpm (4 °C). O sobrenadante foi transferido para tubos novos e adicionou-se 200 µL de clorofórmio por mL de Trizol. Houve agitação vagarosa por 15 segundos e descansou por 2 minutos. Centrifugou-se por 15 minutos a 12.000 rpm (4 °C). A fase aquosa (superior) foi transferida para novos tubos e adicionou-se 500 µL de isopropanol por mL de Trizol. Agitou-se 6x vagarosamente e incubou-se por 10 minutos a temperatura ambiente. Logo após, uma nova centrifugação foi realizada: 10 minutos a 12.000 rpm (4 °C). O sobrenadante foi descartado e adicionou-se 1 mL de 75% etanol. Inverteu-se 2x e centrifugou-se por 5 minutos a 12.000 rpm (4 °C). O sobrenadante foi descartado e secou-se por 5 minutos. Por fim, os pellets foram ressuspensos com 40 µL de H₂O-RNase free e congelados em -70 °C.

3.7.2 Tratamento com DNase

Após dosagem dos RNAs extraídos por espectrofotometria a 260 nm e 280 nm, utilizou-se 2 µg de RNA de cada sorovar e adicionou-se 2 µL de tampão DNase 10x, 2 µL DNase I e H₂O-RNase free (ajustou-se para 20 µL total) (Invitrogen). Incubou-se por 15 minutos a temperatura ambiente. 2 µL de 25 mM EDTA foi adicionado e aqueceu-se por 10 minutos a 65 °C. E, então, dividiu-se em 2 alíquotas cada amostra (10 µL cada).

3.7.3 Reação de Transcrição Reversa (RT-PCR)

Em cada tubo foi adicionado 0,5 µL random hexamers, 1 µL 10 mM dNTPmix e 0,5 µL H₂O-RNase free (Invitrogen). Incubou-se por 5 minutos a 65 °C, logo após em gelo por 1 minuto e adicionou-se 10 µL do mix RT+ (com enzima transcriptase reversa) ou RT- (sem enzima transcriptase reversa) (Invitrogen) → Mix: 2 µL tampão RT 10x, 4 µL 25 mM MgCl₂, 2 µL 0,1 M DTT, 1 µL RNase OUT, 1 µL enzima transcriptase reversa ou 1 µL água.

Um ciclo de amplificação foi realizado para obter o DNA complementar (cDNA): 10 minutos a 25 °C, 50 minutos a 50 °C, 5 minutos a 85 °C e finalizando em 4 °C.

Por fim adicionou-se 1 µl RNase H, incubou-se por 20 minutos a 37 °C e armazenou-se a -20 °C.

3.7.4 Avaliação por PCR

A presença dos transcritos foi avaliada da mesma forma utilizada para avaliar a presença de DNA genômico (seção 3.6.2) utilizando, inclusive, as mesmas cepas e os mesmos oligonucleotídeos.

3.8 Amplificação das sequências genômicas para clonagem

As sequências dos genes LIC11834 e LIC12253 foram amplificadas novamente para posterior clonagem através de PCR a partir do DNA genômico de *L. interrogans* sorovar Copenhageni (M - 20).

A reação de PCR foi realizada nas mesmas condições utilizadas para os estudos de prevalência genômica e do transcrito (seção 3.6.2).

Os insertos foram amplificados sem a sequência peptídeo sinal. A amplificação e o tamanho das sequências de interesse foram verificados através de eletroforese em gel de agarose 1% em tampão TAE. Os insertos de interesse foram purificados através do *GFX PCR DNA and Gel Band Purification Kit* (GE Healthcare) e os produtos obtidos foram quantificados por espectrofotometria a 260 nm e 280 nm.

3.9 Clonagem no vetor pGEM-T Easy (Promega®)

Os insertos de DNA obtidos foram clonados no vetor pGEM-T Easy (Promega Corporation, Madison, WI, EUA) (Figura 4). Os produtos de ligação foram utilizados para transformar bactérias *E. coli* DH5α previamente tornadas competentes pelo método descrito por Mandel e Higa (1970).

Para a transformação foram utilizados 50 ng do produto de ligação para 50 µL de bactéria competente. Deixou-se por 30 minutos em banho de gelo e depois se submeteu a amostra a um choque térmico por 2 minutos a 42 °C. Retornou-se ao banho de gelo por mais 5 minutos e acrescentou-se 350 µL de meio de cultura Luria Bertani – LB (0,5% extrato de levedura, 1% triptona e 1% NaCl). Incubou-se a 37 °C, por 1 hora, sob agitação. A amostra foi plaqueada em meio de cultura contendo 100 µg/mL de ampicilina, 100 mM IPTG

(isopropil- β -D-tiogalactopiranosídeo) e 50 mg/mL X-gal (5-bromo-4-cloro-3-indolil- β -D-galactopiranosídeo). Incubou-se a 37° C por aproximadamente 16 horas.

O vetor pGEM-T Easy possui uma quebra de sequência com extremidades 3'T sobressalentes, o que possibilita a ligação do *amplicon*, uma vez que algumas polimerases acrescentam adenina à extremidade 3' do fragmento amplificado, possibilitando o anelamento e posterior ligação do arcabouço açúcar-fosfato. A presença do inserto interrompe o gene *LacZ*, o qual é essencial para a degradação do substrato cromogênico X-gal. Assim, pode-se selecionar colônias positivas para o inserto, já que estas são deficientes para a degradação, ficando incolores, ao contrário das colônias negativas para o inserto que se tornam azuis, devido a degradação do substrato.

As colônias positivas selecionadas foram inoculadas em 3 mL de meio de cultura mais 100 μ g/mL de ampicilina e incubadas a 37 °C, sob agitação, por aproximadamente 16 horas.

Figura 4 - Mapa do vetor de clonagem pGEM-T Easy

f1ori: origem de replicação. Amp^r: gene de resistência a ampicilina. lacZ: gene para metabolismo de lactose (β -galactosidase).

Fonte: Promega (2012).

Para obtenção do DNA plasmidial (pGEM-T Easy/inserto) destes inóculos utilizou-se o kit *Plasmid prepmini spin* (GE Healthcare) seguindo as orientações do fabricante. Foi verificada a presença de insertos nos plasmídeos obtidos, através de eletroforese em gel de

agarose 1% em tampão TAE, após a digestão dos mesmos com suas respectivas enzimas de restrição.

3.10 Clonagem no vetor de expressão pAE

Os insertos de DNA correspondentes a cada gene foram retirados do vetor pGEM-T Easy após digestão com as respectivas enzimas de restrição e clonados no vetor de expressão em *E. coli* pAE (Figura 5) (RAMOS et al., 2004), o qual também foi digerido com as mesmas enzimas de restrição, e, após serem submetidos à eletroforese em gel de agarose 1% em tampão TAE, foram purificados pelo kit *GFX PCR DNA and Gel Band Purification* (GE Healthcare).

O vetor pAE foi desenvolvido para expressar a proteína recombinante contendo 6 resíduos de histidina (6xHis) na região N-terminal e é um sistema de alta expressão que é controlado pelo promotor do fago T7, sendo que a expressão da proteína recombinante só ocorre na presença da T7 RNA polimerase.

A ligação dos insertos ao vetor pAE foi realizada em uma reação contendo inserto:vetor em uma proporção 5:1 em massa de DNA. Deixou-se o inserto e o vetor em contato por 5 minutos a uma temperatura de 42 °C à 45 °C e depois incubou-se em banho de gelo por 5 minutos. Acrescentou-se 5 U da T4 DNA ligase (Fermentas) e incubou-se por aproximadamente 16 horas na temperatura de 14 °C à 16 °C. Também foram feitos controles negativos para cada reação de ligação (omitindo-se o inserto).

Figura 5 - Mapa do vetor de clonagem e expressão pAE

PT7: promotor. 6xHis: seis resíduos de histidina. RBS: sítio de ligação ao ribossomo. ATG: códon de início da transcrição. T_{term}: região de terminação de transcrição. F1ori: origem de replicação. Amp^R: gene de resistência à ampicilina.

Fonte: Ramos et al. (2004).

Cada construção foi, então, usada para transformar bactérias *E. coli* DH5 α previamente tornadas competentes, as quais foram semeadas em placas com meio de cultura e antibiótico apropriados e incubadas a 37 °C por 16 horas. As colônias resistentes foram selecionadas, inoculadas em 3 mL de meio de cultura com 100 μ g/mL de ampicilina e incubadas a 37 °C, sob agitação, por aproximadamente 16 horas.

Com estes inóculos foi realizada uma análise por fenol-clorofórmio, a fim de confirmar a presença de inserto no vetor (clones positivos): foram centrifugados 300 μ L dos inóculos a 12.000 rpm, por 3 minutos. O sobrenadante foi descartado e o sedimento formado foi ressuscitado em 50 μ L de água deionizada. Adicionou-se a amostra 20 μ L de tampão de carregamento 10X (0,19% azul de bromofenol, 0,42% xileno cianol e 50% glicerol) e 28 μ L de solução fenol-clorofórmio 1:1. Homogenizou-se a amostra com o auxílio do *vortex*. A amostra foi centrifugada a 12.000 rpm, por 3 minutos, e a fase superior foi analisada por eletroforese em gel de agarose 1% em tampão TAE.

Os DNAs plasmidiais (pAE/inserto) foram isolados por mini-preparação utilizando o kit *Plasmid prepmini spin* (GE Healthcare) e analisados em gel de agarose 1% em tampão TAE, após digestão pelas enzimas de restrição específicas, afim de verificar a presença do inserto.

3.11 Sequenciamento automático dos clones

Os clones positivos foram sequenciados utilizando os oligonucleotídeos (Quadro 2) que se anelam ao vetor pAE 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).

Quadro 2 - Oligonucleotídeos utilizados para o sequenciamento

T7 Oligonucleotídeo Promotor	5'TAATACGACTCACTATAGGG3'
T7 Oligonucleotídeo Terminador Reverso (19-mer)	5'TATGCTAGTTATTGCTCAG3'

Conforme recomendação da Applied Biosystems, a reação de amplificação dos fragmentos a serem sequenciados foi realizada num volume final de 20 µL contendo 500 ng de DNA molde, 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 8.000 rpm a temperatura ambiente. O precipitado de DNA foi lavado com 70% etanol, seco a vácuo em centrífuga 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 (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>).

3.12 Avaliação da expressão das proteínas recombinantes

3.12.1 Estudo de indução das proteínas recombinantes

Bactérias competentes *E. coli* BL21 SI (induzidas por NaCl) foram transformadas com os plasmídeos recombinantes e cultivadas em meio 2YT/ON (1% extrato de levedura; 1,6% triptona; sem adição de NaCl) contendo 100 µg/mL de ampicilina e incubadas a 30 °C, sob agitação, por aproximadamente 16 horas. Após este período, as culturas saturadas foram diluídas (1:25) em um novo meio e incubadas até atingir uma densidade óptica a 600 nm

(DO) entre 0,6 e 0,8. Induziu-se então adicionando diferentes concentrações de NaCl (3, 30 e 300 mM) e incubando as culturas, novamente, a mesma temperatura por um período de 3 horas. A seguir, as bactérias foram centrifugadas (5.000 rpm/15 minutos/4 °C) e os sedimentos ressuspensos em tampão de lise (20 mM Tris-HCl pH 8; 200 mM NaCl; 200 µg/mL lisozima; 2 mM PMSF e 1% Triton X-100).

3.12.2 Estudo da solubilidade das proteínas recombinantes

As bactérias foram lisadas por sonicação em banho de gelo e centrifugadas novamente (5.000 rpm/15 minutos/4 °C). As alíquotas do sobrenadante foram reservadas. Os sedimentos formados foram ressuspensos em tampão de desnaturação (20 mM Tris-HCl pH 8; 0,5 M NaCl; 8 M ureia; 5 mM Imidazol; 1 mM β -mercaptoetanol), e as alíquotas destes também foram reservadas para análise em eletroforese em gel de poliacrilamida/12% SDS (sulfato dodecil de sódio – SDS-PAGE).

3.13 Solubilização e renaturação da proteína rLIC12253

A proteína rLIC12253, por expressar na forma insolúvel como corpúsculo de inclusão, foi solubilizada em 30 mL de tampão de desnaturação. Para diluição do agente desnaturante (ureia), preparou-se 2 L de solução de renaturação (Tris-HCl 20 mM pH 8; NaCl 0,5 M) e gotejou-se o sobrenadante nesta solução. Verificando-se que não ocorreu precipitação pode-se, então, purificar a proteína.

3.14 Purificação e diálise das proteínas recombinantes

As proteínas recombinantes rLIC11834 e rLIC12253 foram purificadas utilizando-se resina *Chelating Sepharose* (GE Healthcare) carregada com Ni^{2+} , a qual foi previamente equilibrada com tampão contendo 20 mM Tris-HCl pH 8; 0,5M NaCl. As lavagens foram feitas com soluções contendo este tampão mais 5, 20, 40 e 60 mM imidazol. A proteína foi eluída com o tampão descrito acima mais 1M imidazol.

As frações de todas as etapas da purificação foram coletadas e analisadas em gel 12% SDS-PAGE. As alíquotas que continham as proteínas recombinantes rLIC11834 e rLIC12253 foram dialisadas contra PBS e 0,1% Glicina a 4 °C por aproximadamente 16 horas (com 3 trocas de tampão). Após esta diálise, a concentração das proteínas recombinantes foi avaliada

em gel 12% SDS-PAGE, através da comparação destas com concentrações pré-estabelecidas de albumina (BSA – *Bovine Serum Albumin*) (comparação densitométrica das bandas) utilizando o *software Carestream* (Gel Logic 2200 Pro).

3.15 Análise da estrutura e estabilidade das proteínas recombinantes por espectroscopia circular (CD)

Para tal análise realizou-se uma diálise com as proteínas recombinantes em tampão sódio-fosfato (Na_2HPO_4 0,5 M; NaH_2PO_4 1 M) à 4 °C com 3 trocas deste tampão. Após essa diálise, realizou-se a leitura por espectroscopia circular. Os espectros de dicroísmo circular (CD) foram captados em polarímetro J-810 (Jasco). Os dados foram tomados em cubetas de 1 cm de caminho óptico.

Os espectros de CD foram expressos em termos de elipcidade molar residual $[\Phi] = \Phi$ (mdeg) / 10 X C(M) X I(cm), na qual Φ é elipcidade, C é a concentração da proteína e I é o caminho ótico (KELLY et al., 2005).

3.16 Preparação do extrato de *E. coli*

Para preparação do extrato de *E. coli* foi utilizada a mesma cepa pela qual a proteína recombinante foi expressa. A cepa selvagem foi inoculada em 400 mL de meio 2YT/ON por 16 horas a 37 °C. Todo o volume de cultura saturada foi centrifugado a 5.000 rpm por 20 minutos. O sedimento foi ressuspensionado em 40 mL de tampão de lise (20 mM Tris-HCl pH 8; 200 mM NaCl; 200 µg/mL lisozima; 2 mM PMSF e 1% Triton X-100) e lisado em banho de gelo, por sonicação.

O extrato foi utilizado em experimentos nos quais era necessário neutralizar anticorpos contra proteínas de *E. coli*.

3.17 Imunização de camundongos

Foram realizadas 2 imunizações com 10 µg da proteína recombinante por animal mais 12% adjuvante *Alhydrogel* (2% $\text{Al}(\text{OH})_3$, Brenntag Biosector, Frederikssund, Dinamarca) para rLIC11834 e 3 imunizações para rLIC12253 com 10 µg da proteína recombinante por animal mais 12% adjuvante *Alhydrogel*. Cada imunização ocorreu em intervalos de 15 dias e os animais usados foram camundongos BALB/C fêmeas com 18 a 22 g (grupos de 10

camundongos). Os animais foram imunizados no dorso via subcutânea. O grupo controle foi imunizado com PBS e adjuvante. Os animais foram sangrados via plexo retro-orbital 15 dias após cada dose de imunização. O sangue coletado foi incubado a 4 °C durante aproximadamente 16 horas e centrifugado por 5 minutos a 5.000 rpm. O soro foi cuidadosamente coletado e armazenado a -20 °C.

Quinze dias após a última imunização, os camundongos foram sacrificados em câmara de CO², higienizados com etanol 70% e seus baços foram removidos por incisão na lateral esquerda para cultivo dos esplenócitos.

3.18 Ensaio de linfoproliferação e liberação de citocinas

Após sacrifício dos animais, os baços foram assepticamente removidos e depositados em meio RPMI (Roswell Park Memorial Institute Medium-RPMI-1640, contendo 2 mM L-glutamina, 100 IU/mL of Penicilina, 100 µg/mL de Streptomicina e 50 µg/mL of Gentamicina). Os baços foram macerados e centrifugados por 10 minutos a 1.100 rpm (4 °C). O sobrenadante foi descartado e foi adicionado 1 mL de água gelada estéril à fase sedimentada (lise dos eritrócitos); após este procedimento, as suspensões foram novamente centrifugadas.

O sobrenadante resultante foi descartado e os esplenócitos foram ressuspensos em 1 mL de RPMI suplementado com 10% soro fetal bovino. As células foram contadas após marcação com 0,4% azul de tripano e, então, distribuídas em triplicata em uma placa de cultura celular de 96 poços (Costar, Corning), na quantidade de 5×10^5 células/poço.

Os esplenócitos foram tratados com 5 µg/mL do mitógeno Concanavalina A (ConA – controle positivo, Sigma - Aldrich), 5 µg/mL de cada proteína recombinante ou foram destituídos de estímulo (apenas meio – controle negativo). Foi feita a cultura destas células por 48 horas a 37 °C e 5% CO₂, em atmosfera umidificada, e a taxa de proliferação celular foi determinada em função da replicação do DNA, medida pela incorporação de bromodesoxiuridina (BrdU), um análogo de base nitrogenada timina, por um kit de ELISA colorimétrico-BrdU (Roche Diagnostic, Indianapolis, IN).

As células foram incubadas por 2 horas com BrdU para permitir a marcação do DNA genômico. Em seguida, foi adicionado anticorpo anti-BrdU conjugado com peroxidase (1:100) e incubou-se por 90 minutos. Solução de substrato para a enzima foi adicionada e incubada à temperatura ambiente por 15 minutos; a reação enzimática foi parada pela adição

de H_2SO_4 1 M e a DO foi determinada em leitor de microplacas (TP-reader, Thermo) usando o comprimento de onda de 450 nm.

Para análise estatística, primeiramente foi avaliada a diferença entre as médias do tratamento no qual as células foram estimuladas com a proteína, entre o grupo imunizado e grupo PBS (controle negativo), e se, caso houver diferença significativa, a análise estatística é então feita dentro do grupo dos animais imunizados entre o tratamento com proteína e apenas meio, pelo *Student's two tailed t test*.

Para avaliação das citocinas secretadas pelos esplenócitos extraídos dos camundongos, as células foram cultivadas em placa de cultura celular de 24 poços, cada qual contendo 5×10^6 células, em triplicata. Após 48 horas de cultura, a placa foi centrifugada e o sobrenadante resultante da cultura celular foi coletado e armazenado em curto prazo a -20°C para mensuração das citocinas liberadas. Níveis de interleucina-4 (IL-4), interleucina-10 (IL-10), interleucina-12 (IL-12), interferon gama ($\text{IFN-}\gamma$) e fator de necrose tumoral alfa ($\text{TNF-}\alpha$) foram avaliados por ELISA utilizando kits comerciais e as respectivas recomendações do fabricante (PeproTech, Colonia Narvarte, México). Os resultados foram expressos em pg/mL, em função de uma curva padrão realizada com amostras fornecidas pelo próprio kit.

Para análise estatística, primeiramente foi avaliada a diferença entre as médias do tratamento no qual as células foram estimuladas com a proteína, entre o grupo imunizado e grupo PBS (controle negativo). Sendo detectada diferença significativa, a análise estatística foi então feita dentro do grupo dos animais imunizados entre o tratamento com proteína e apenas meio, pelo *Student's two tailed t test*.

3.19 Teste de reconhecimento do anticorpo policlonal IgG pelas proteínas recombinantes por ELISA, após imunização de camundongos

A detecção de anticorpos IgG anti-rLIC11834 e anti-rLIC12253 após as imunizações foi feita por ensaio imunoenzimático ELISA conforme descrito por Neves et al., 2007. A concentração das proteínas recombinantes foi ajustada para 5 $\mu\text{g/mL}$ em tampão carbonato 0,05 M pH 9,6 e, em seguida, foram adicionados 50 μL por poço em uma placa de 96 poços (Costar® High binding, Corning Incorporated, EUA), compondo um total de 250 ng de proteína recombinante por poço. As placas foram incubadas por aproximadamente 16 horas a temperatura ambiente. Após incubação, as placas foram lavadas com PBS/Tween 0,05% (PBS/T). Incubou-se então com solução de bloqueio (PBS/T adicionado de 5% leite desnatado desidratado e 2,5% BSA), 200 μL por poço, durante 2 horas a 37°C . Foi feita uma

diluição seriada (1:200 a 1:6.553.600) dos soros de camundongos em solução de bloqueio acrescida de 25% de extrato de *E. coli*, evitando a interferência de proteínas contaminantes presentes na amostra dos recombinantes (GRUBER; ZINGALES, 1995). Incubou-se estes soros por 1 hora para que ocorresse a adsorção dos anticorpos inespecíficos. Em seguida, adicionou-se 50 µL das amostras adsorvidas por poço e incubou-se a temperatura ambiente por 1 hora. Após essa etapa, lavou-se com PBS/T e adicionou-se 50 µL por poço de solução de bloqueio contendo anti-IgG de camundongo conjugado com peroxidase (Sigma - Aldrich) na diluição 1:5.000 por 1 hora a temperatura ambiente. A placa foi lavada novamente com PBS/T e a reação enzimática foi revelada pela adição de 1 mg/mL de OPD (δ -phenylenediamina, Sigma - Aldrich) diluído em tampão fosfato-citrato pH 5 contendo 0,03% peróxido de hidrogênio (100 µL por poço). Após 15 minutos de incubação no escuro, a reação enzimática foi interrompida pela adição de 50 µL de solução de H₂SO₄ 4 N.

A DO foi determinada usando o comprimento de onda de 492 nm. Os valores de DO obtidos foram subtraídos do valor do controle negativo da placa (poço com antígeno e sem incubação com soro dos camundongos). O título individual foi determinado como sendo o inverso da maior diluição do soro com valor da DO maior ou igual a 0,1.

3.20 Teste de reconhecimento do anticorpo policlonal IgG pelas proteínas recombinantes por *Imunoblotting*, após imunização de camundongos

As proteínas recombinantes purificadas foram submetidas à eletroforese em gel 12% SDS-PAGE, eletro-transferidas para membranas de nitrocelulose (GE Healthcare) em sistema semi-úmido (GE Healthcare) por cerca de 1 hora (0,8 mA por cm²) e, então, as membranas foram coradas com ponceau (Merck) e incubadas com solução de bloqueio por aproximadamente 16 horas.

As membranas foram lavadas com PBS/T e incubadas com antissoro específico (1:1.000) por 1 hora a temperatura ambiente. Logo após, a membrana foi lavada novamente e incubada com anti-IgG de camundongo conjugado a peroxidase (1:5.000) por 1 hora a temperatura ambiente. Após nova lavagem, a reação foi revelada utilizando ECL (GE Healthcare) em câmera escura e filme de raio-x (Kodak).

Repetiu-se essa metodologia, porém, além da diluição 1:1.000 dos anticorpos, realizou-se também diluição 1:500 e, para rLIC12253, também 1:300, modificando o tempo e a temperatura de incubação desses anticorpos para todas diluições (3 horas a 37 °C).

3.21 Detecção de anticorpos IgG e IgM humanos em soro de pacientes diagnosticados com leptospirose por ELISA

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 amostras de 23 soros correspondentes à fase inicial da doença, com resultado MAT negativo, e amostras de 34 soros correspondentes à fase convalescente da doença, com resultado MAT positivo por meio de ELISA. O teste foi realizado utilizando as proteínas separadas ou de forma conjunta para análise de efeito sinérgico. Após revestir as placas de ELISA com as proteínas recombinantes por 16 horas, aplicou-se as amostras de soros de pacientes (1:400), e avaliou-se a reatividade usando anti-IgG e anti-IgM humano conjugado com peroxidase (Sigma - Aldrich) na diluição 1:5.000. A revelação da placa ocorreu como descrito na seção 3.19.

O valor do *cut-off* foi calculado através da média das absorbâncias obtidas para 11 soros de indivíduos saudáveis e um *pool* de amostras de soro normal comercial (Sigma - Aldrich) mais três desvios padrões. Foram consideradas amostras positivas os soros dos pacientes com leptospirose que apresentaram valores maiores do que o estabelecido pelo *cut-off*.

Após esta etapa, uma nova placa de ELISA foi revestida com ambas as proteínas recombinantes e adicionou-se um *pool* dos soros de pacientes que reagiram com estas proteínas em uma diluição seriada, tanto MAT negativo, quanto MAT positivo. Avaliou-se utilizando anti-IgG humano conjugado com peroxidase (Sigma - Aldrich) na diluição 1:5.000. Revelou-se então como descrito na seção 3.19.

Para estes experimentos foram feitas incubações prévias dos soros com extrato de *E. coli* na concentração de 25%, evitando a interferência de proteínas contaminantes presentes na amostra dos recombinantes.

3.22 Detecção de anticorpos IgG humanos em soro de pacientes com diagnóstico de outras doenças por ELISA

Este ensaio ocorreu como o descrito anteriormente (item 3.21), porém utilizando soro de pacientes diagnosticados com outras doenças, as quais apresentam sintomas semelhantes à leptospirose: dengue (n = 13), malária (n = 11), doença de Chagas (n = 20) e HIV (n = 15).

3.23 Ensaios de localização da proteína nativa

3.23.1 Imunofluorescência em fase líquida (L – IFA)

Alíquotas de 2,5 mL de cultura de *L. interrogans* sorovar Copenhageni (M-20) por tratamento, com sete dias de crescimento ($\sim 10^9$ células/mL), foram centrifugadas 15 minutos a 10.000 rpm. Os sobrenadantes foram descartados e o sedimentos lavados 2 vezes com 2,5 mL de tampão PBS na concentração final de 50 mM NaCl. Os sedimentos foram ressuspensos em 200 μ L de tampão PBS na concentração final de 50 mM NaCl contendo 2 μ g de iodeto de propídio e incubados 45 minutos a 37 °C no escuro. As células foram centrifugadas 15 minutos a 10.000 rpm e lavadas 2 vezes com 1 mL de tampão PBS na concentração final de 50 mM NaCl. Os sedimentos foram ressuspensos em 200 μ L de tampão PBS na concentração final de 50 mM NaCl gelado contendo antissoros produzidos em camundongos contra rLIC11834, rLIC12253, LipL32 (HAAKE et al., 2000), GroEL (HAAKE; MATSUNAGA, 2002), e PBS (1:50) e incubados 30 minutos em gelo. As células foram centrifugadas 15 minutos a 10.000 rpm e lavadas 2 vezes com 1 mL de tampão PBS na concentração final de 50 mM NaCl. Os sedimentos foram ressuspensos em 100 μ L de tampão PBS na concentração final de 50 mM NaCl gelado contendo anticorpo anti-IgG de camundongo conjugado com isotiocianato de fluoresceína (FITC, 1:50, Sigma - Aldrich) e incubados 30 minutos em gelo no escuro. As células foram centrifugadas 15 minutos a 10.000 rpm e lavadas 2 vezes com 1 mL de tampão PBS na concentração final de 50 mM NaCl. Os sedimentos foram ressuspensos em 50 μ L de tampão PBS na concentração final de 50 mM NaCl 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). Foi empregado aumento de 600 vezes (FITC, excitação 488 nm, emissão 500-550 nm; Iodeto de propídio, excitação 543 nm, emissão 612-619 nm).

3.23.2.1 Ensaio de acessibilidade com Proteinase K

Alíquotas de 5 mL de cultura de *L. interrogans* sorovar Copenhageni (M-20) por tratamento ($\sim 10^8$ células/mL) foram centrifugadas a 10.000 rpm por 15 minutos à temperatura ambiente, e lavadas 1 vez com PBS na concentração final de 50 mM NaCl. Ressuspendeu-se então em 5 mL de PBS na concentração final de 50 mM NaCl acrescido com 25 μ g/mL de

proteínase K (PK, Sigma - Aldrich). Todas as alíquotas foram incubadas por 0 a 5 h (37 °C) antes da adição de 100 mM PMSF, o qual foi utilizado no intuito de parar a atividade da PK. As suspensões foram centrifugadas a 10.000 rpm por 5 minutos, lavadas 2 vezes com PBS na concentração final de 50 mM NaCl e ressuspensas em 1 mL PBS na concentração final de 50 mM NaCl para análise por ELISA utilizando antissoros anti-rLIC11834 e anti-rLIC12253.

3.23.2.2 ELISA para detecção da localização celular

As leptospiros foram imobilizadas em microplacas e incubadas à temperatura ambiente por 16 horas. Lavou-se 3 vezes com PBS na concentração final de 50 mM NaCl e bloqueou-se com PBS na concentração final de 50 mM NaCl adicionado de 5% leite desnatado desidratado e 1% BSA por 2 horas a 37 °C. Incubou-se, então, por mais 2 horas a 37 °C com antissoros produzidos em camundongos contra rLIC11834, rLIC12253, LipL32 (HAAKE et al., 2000) ou DnaK (STAMM et al., 1991) (utilizou-se a diluição na qual a DO é igual a 1 quando testou-se estes antissoros contra suas respectivas proteínas recombinantes). As leptospiros foram lavadas 3 vezes com PBS na concentração final de 50 mM NaCl e incubadas com 50 µL por poço de anti-IgG de camundongo conjugado com peroxidase (1:5.000) em PBS na concentração final de 50 mM NaCl por 1 hora a 37 °C. Os poços foram lavados 3 vezes novamente com PBS na concentração final de 50 mM NaCl e revelados como descrito no item 3.19, porém, para este experimento, a reação foi interrompida após 5 minutos.

3.24 Ensaio de adesão aos componentes da matriz extracelular e componente do plasma por ELISA

3.24.1 Análise da interação destes componentes às proteínas recombinantes

Componentes de matriz extracelular (MEC) (1 µg), laminina, colágeno tipo I e tipo IV, fibronectina plasmática, fibronectina celular, elastina e vitronectina (Sigma - Aldrich), além de gelatina (Difco) e Fetuína (Sigma - Aldrich) em PBS foram adsorvidos em placa de microdiluição e incubados por 3 horas a 37 °C. Logo após, foi feita 3 lavagens com PBS/T e bloqueio com PBS/T adicionado de 10% leite desnatado desidratado. Incubou-se por 1 hora a temperatura ambiente e por mais 16 horas a 4 °C. Bloqueio desprezado, incubou-se com proteína recombinante (1 µg) por 2 horas a 37 °C. Depois de 6 lavagens com PBS/T, as placas foram incubadas com anticorpo policlonal específico para cada proteína recombinante

(utilizou-se a diluição na qual a DO é igual a 1 quando testou-se estes antissoros contra suas respectivas proteínas recombinantes) ou com anticorpo monoclonal anti-polihistidina (1:200). Realizou-se nova lavagem com PBS/T e adicionou-se anticorpo anti-IgG de camundongo conjugado com peroxidase (1:5.000). A reação foi revelada com como descrito anteriormente (subseção 3.19). Para cada proteína teste, quatro poços foram adsorvidos com determinado componente, sendo que a um deles não foi adicionado proteína recombinante a fim de mensurar a reatividade basal (controle branco) (BARBOSA et al., 2006; CAMERON, 2003).

3.24.2 Curvas de dose-resposta

Laminina (1 µg/poço), além de gelatina, diluídos em PBS, foram adsorvidos em placas de ELISA e incubados por 3 horas a 37 °C. Logo após, foram realizadas 3 lavagens com PBS/T e bloqueio por 2 horas a 37 °C com PBS/T adicionado de 10% leite desnatado desidratado. Incubou-se por 1 hora a temperatura ambiente e por mais 16 horas a 4 °C. Bloqueio desprezado, incubou-se com concentrações crescentes das proteínas recombinantes (0-6 µM) em tampão PBS (100 µL/poço). A avaliação das proteínas ligadas ao componente foi realizada como descrito no item acima. Os dados dos testes foram utilizados para calcular a constante de dissociação (K_D) de acordo com o método descrito por Pathirana et al. (2006) e Lin et al. (2009), com base na equação: $A = [\text{proteína}] A_{\text{max}} / (K_D + [\text{Proteína}])$, onde A é a absorbância a uma determinada concentração de proteína, A_{max} é a absorbância máxima para o leitor de placas de ELISA (equilíbrio), [proteína] é a concentração de proteína e K_D é a constante de dissociação do equilíbrio para uma dada absorbância em uma dada concentração de proteína.

3.25 Ensaio de adesão a componentes do soro humano e componente do plasma por ELISA

3.25.1 Análise da interação destes componentes às proteínas recombinantes

Os componentes do soro e do plasma humano (1 µg), plasminogênio (PLG, Calbiochem), fator H (Calbiochem), C4bp (Complement Technology), e Fibrinogênio (Fg, Sigma - Aldrich), além de gelatina (Difco) e fetuína (Sigma - Aldrich), diluídos em PBS foram adsorvidos em placa de microdiluição e incubados por 3 horas a 37 °C. Logo após, foram realizadas 3 lavagens com PBS/T e bloqueio por 2 horas a 37 °C com PBS/T

adicionado de 10% leite desnatado desidratado. Incubou-se por 1 hora a temperatura ambiente e por mais 16 horas a 4 °C. A solução de bloqueio foi desprezada, seguida de incubação com a proteína recombinante (1 µg) por 2 horas a 37 °C. Depois de 6 lavagens com PBS/T, as placas foram incubadas com anticorpo policlonal específico para cada proteína recombinante (utilizou-se a diluição na qual a DO é igual a 1 quando testou-se estes antissoros contra suas respectivas proteínas recombinantes) ou com anticorpo monoclonal anti-polihistidina (1:200). Realizaram-se novas lavagens com PBS/T e adicionou-se anticorpo anti-IgG de camundongo conjugado com peroxidase (1:5.000). A reação foi revelada como descrito anteriormente na subseção 3.19. Para cada proteína teste, quatro poços foram adsorvidos com determinado componente, sendo que a um deles não foi adicionado proteína recombinante a fim de mensurar a reatividade basal (controle branco) (BARBOSA et al., 2006; CAMERON, 2003).

3.25.2 Curvas de dose-resposta

Componentes do soro humano (1 µg de PLG e de C4bp), 1 µg de Fg, além de gelatina, diluídos em PBS, foram adsorvidos em placas de ELISA e incubados por 3 horas a 37 °C. Logo após, foram realizadas 3 lavagens com PBS/T e bloqueio por 2 horas a 37 °C com PBS/T adicionado de 10% leite desnatado desidratado. Incubou-se por 1 hora a temperatura ambiente e por mais 16 horas a 4 °C. Bloqueio desprezado, incubou-se com concentrações crescentes das proteínas recombinantes (0-4 µM) em tampão PBS (100 µL/poço). A avaliação das proteínas ligadas aos componentes foi realizada como descrito no item acima. Os dados dos testes foram utilizados para calcular a constante de dissociação (K_D) de acordo com o método descrito por Pathirana et al. (2006) e Lin et al. (2009), com base na equação: $A = \frac{[proteína] A_{max}}{K_D + [Proteína]}$, onde A é a absorbância a uma determinada concentração de proteína, A_{max} é a absorbância máxima para o leitor de placas de ELISA (equilíbrio), [proteína] é a concentração de proteína e K_D é a constante de dissociação do equilíbrio para uma dada absorbância em uma dada concentração de proteína.

3.25.3 Inibição da ligação da proteína recombinante Lsa33 (rLIC11834) ao PLG pelo ácido 6-aminocaproico (AAC)

O AAC (2 mM e 20 mM, Sigma - Aldrich), juntamente ao PLG, foi adicionado à proteína recombinante previamente adsorvida em placa de ELISA. O PLG capturado foi detectado como descrito acima, utilizando-se anticorpo policlonal específico para a proteína

recombinante. Para controle utilizou-se apenas o PLG em contato com a proteína recombinante Lsa33.

3.25.4 Avaliação da atividade enzimática da plasmina

Proteínas recombinantes (1 µg) Lsa33 e Lsa63 (VIEIRA et al., 2010b), e BSA, diluídas em PBS, foram incubadas (100 µL/poço) por 16 horas a 4 °C. Logo após, foi realizada uma lavagem com PBS/T e um bloqueio por 2 horas a 37 °C com PBS/T adicionado de 10% leite desnatado desidratado. Incubou-se então 1 µg de PLG em tampão PBS (100 µL/poço) por 2 horas a 37 °C. As placas foram lavadas com PBS/T e, em seguida, adicionou-se 4 ng/poço de ativador de PLG tipo uroquinase (uPA, Sigma - Aldrich). O substrato específico para a plasmina D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (Sigma - Aldrich) também foi adicionado na concentração final de 0,4 mM diluído em PBS (100 µL/poço). As placas foram então incubadas por 16 horas a 37 °C e a degradação do substrato foi mensurada através de leitor de microplacas no comprimento de onda de 405 nm.

3.26 Tratamento dos componentes glicoproteicos com metaperiodato de sódio

Laminina, C4bp e Fg (250 ng/poço) foram incubados em tampão acetato de sódio 50 mM (pH 5) durante 16 horas a 4 °C em uma placa de ELISA. Após 3 lavagens com tampão acetato de sódio, os componentes foram tratados com concentrações crescentes de metaperiodato de sódio (5, 10, 25, 50 e 100 mM) por 1 hora a temperatura ambiente no escuro (apenas em um dos poços não houve tratamento com metaperiodato de sódio = 0 mM). Lavou-se com tampão acetato de sódio. Foi realizado um bloqueio com PBS adicionado de 1% BSA por 1 hora a 37 °C. Desprezado o bloqueio, incubou-se com (50 µL/poço) 0,5 µg/poço das proteínas recombinantes por 1 hora e 30 minutos a 37 °C em PBS. Lavou-se com tampão acetato de sódio. Utilizando anticorpo policlonal específico para cada proteína recombinante (utilizou-se a diluição na qual a DO é igual a 1 quando testou-se estes antissoros contra suas respectivas proteínas recombinantes) em PBS (100 µL/poço), incubou-se por 1 hora a 37 °C. Uma nova lavagem foi feita. Adicionou-se anticorpo anti-IgG de camundongo conjugado com peroxidase (1:5000) por 1 hora a 37 °C (100 µL/poço). Realizou-se mais uma lavagem e, então, a adesão das proteínas recombinantes foi avaliada como descrito na seção 3.19.

3.27 Ensaio de inibição da formação do coágulo de fibrina

Um mg/mL de Fg diluído em 0,5 mL de solução de PBS foi incubada com 3 μ M da proteína recombinante Lsa33 (concentração na qual há saturação da ligação com o Fg) por 2 horas a 37 °C. Após incubação, 90 μ L da solução contendo Fg e a proteína Lsa33 foi transferida para placa de ELISA adicionando 10 μ L/poço de trombina (10 U/mL, Tb, Sigma - Aldrich). A formação do coágulo de fibrina foi monitorada em leitor de microplacas no comprimento de onda de 595 nm a cada 1 minuto durante 10 minutos, e por mais 35 minutos durante 5 minutos. A solução contendo apenas Fg com ou sem adição de trombina foram utilizadas como controles positivo e negativo do ensaio, respectivamente.

3.28 Ensaio de inibição da ligação das proteínas recombinantes aos componentes pela incubação destas proteínas com antissoro específico

As proteínas recombinantes (1 μ g) foram incubadas com os antissoros específicos para cada proteína (utilizou-se a diluição na qual a DO é igual a 1 quando testou-se estes antissoros contra suas respectivas proteínas recombinantes) por 2 horas a 37 °C, e, então, adicionadas em placas de ELISA previamente imobilizadas com os componentes. Após lavagem com PBS/T, as placas foram incubadas com anticorpo monoclonal anti-poli-histidina (1:200) por 2 horas a 37 °C, seguido de nova lavagem e incubação com anticorpo anti-IgG de camundongo conjugado com peroxidase (1:5.000) por 1 hora a 37 °C. A placa foi lavada novamente e a reação enzimática foi revelada como descrito no item anterior.

Para as análises estatísticas, a média dos valores de absorbância obtidos pela ligação das proteínas previamente tratadas com antissoros específicos aos componentes foi comparada com a média dos valores de absorbância obtidos pela ligação das proteínas as quais não sofreram tal tratamento, pelo *Student's two tailed t test*.

3.29 Ensaio de inibição da ligação das proteínas recombinantes aos componentes por desnaturação térmica destas proteínas

As proteínas recombinantes (1 μ g) foram aquecidas a 96 °C por 10 minutos, para que ocorresse a desnaturação, e, então, adicionadas em placas de ELISA previamente imobilizadas com os componentes. Após lavagem com PBS/T, as placas foram incubadas com antissoro específico para cada proteína (utilizou-se a diluição na qual a DO é igual a 1

quando testou-se estes antissoros contra suas respectivas proteínas recombinantes) por 2 horas a 37 °C, seguido de nova lavagem e incubação com anticorpo anti-IgG de camundongo conjugado com peroxidase (1:5.000) por 1 hora a 37 °C. A reação foi revelada como descrito na seção acima.

Para as análises estatísticas, a média dos valores de absorbância obtidos pela ligação das proteínas desnaturadas aos componentes foi comparada com a média dos valores de absorbância obtidos pela ligação das proteínas não desnaturadas, pelo *Student's two tailed t test*.

3.30 Inibição da ligação das leptospiiras vivas aos componentes

Para verificar se as proteínas recombinantes ligadas aos componentes são capazes de reduzir a interação destes com as leptospiiras, imobilizou-se 1 µg de cada componente por poço de uma placa de ELISA, a qual foi bloqueada com PBS/T adicionado de 10% leite desnatado desidratado. Então, os poços foram incubados por 90 minutos à 37 °C com massas crescentes das proteínas recombinantes. Após 3 lavagens com PBS/T, foram adicionados 100 µL/poço de 4×10^7 bactérias vivas (*L. interrogans* sorovar Copenhageni (M-20)) e incubou-se por 90 minutos a 37 °C. Após lavagem, a detecção das leptospiiras ligadas foi feita de forma indireta por meio da incubação com antissoro anti-LipL32 (1:4.000), pelo fato da proteína LipL32 ser o antígeno majoritário da membrana externa de *Leptospira* spp. (HAAKE et al., 2000). Em seguida adicionou-se anti-IgG de camundongo conjugado com peroxidase (1:5.000) por 1 hora a 37 °C. A reação foi revelada como descrito na seção anterior.

Para as análises estatísticas, os valores de absorbância obtidos quando os componentes foram incubados com as proteínas recombinantes foram comparados com às situações na qual não houve incubações, ou seja, o máximo de ligação das leptospiiras aos componentes, pelo *Student's two tailed t test*.

3.31 Interferência entre os componentes na ligação às proteínas recombinantes

Para elucidar se uma proteína recombinante se liga a seus componentes por meio de sítios distintos, foi feito um ensaio de interferência. Placas de ELISA foram incubadas com 1 µg da proteína recombinante por 16 horas a 4 °C. Em seguida, as placas foram lavadas 1 vez com PBS/T e bloqueadas com PBS/T adicionado de 10% leite desnatado desidratado por 2 horas a 37 °C. A cada poço, foi adicionado uma massa fixa de um dos componentes (1 µg)

juntamente com massas crescentes do componente com o qual se deseja verificar interferência (0 - 1 µg). Os componentes foram deixados interagir por 2 horas a 37 °C com a proteína recombinante imobilizada e a detecção da ligação foi feita com o uso de anticorpos que reconhecem especificamente os componentes (aqueles imobilizados com concentração fixa), os quais foram previamente titulados e empregados em uma diluição que fornece uma DO 492 nm igual a 1.

As reações foram reveladas como descrito acima, e as análises estatísticas foram feitas comparando-se os valores de absorbância obtidos nas incubações com diferentes massas do competidor em relação aos valores obtidos na situação na qual foi omitido este competidor, pelo *Student's two tailed t test*.

3.32 Ensaio de imunoproteção

3.32.1 Cálculo da DL_{50} (dose letal)

Para calcular a DL_{50} , leptospiros virulentas recém-isoladas (*L. interrogans* sorovar Kennewicki cepa Pomona Fromm) foram contadas em câmara de Petroff-Hausser utilizando microscopia de campo escuro, e, em seguida, diluídas em séries de 10 vezes (10^{-1} - 10^{-10} /mL). Cada grupo de hamster foi imunizado com uma diluição. Os animais foram observados por aproximadamente 21 dias, e ao final desse período os animais mortos foram contabilizados. A última diluição em que 50% dos animais morreram foi multiplicada por 100 para obtenção da diluição a ser usada no ensaio de imunoproteção (dose do desafio = $DL_{50} \times 100$).

3.32.2 Preparo de bacterina para ensaios de imunoproteção

A bacterina foi preparada conforme descrito por Silva et al. (2007). Em resumo, foi determinada a densidade celular de cultura de *L. interrogans* sorovar Kennewicki cepa Pomona Fromm virulenta recém isolada de animal infectado. Após a contagem, a dose do inóculo foi ajustada para 10^9 leptospiros/animal. A cultura foi centrifugada durante 20 minutos a 5.000 rpm, à temperatura ambiente, e o sedimento foi lavado 2 vezes com tampão PBS na concentração final de 50 mM NaCl. O sedimento foi ressuspensionado em PBS e incubado por 20 minutos a 56 °C para inativação das bactérias, e as alíquotas foram armazenadas a -20 °C.

3.32.3 Ensaio de desafio

Os ensaios de imunoproteção foram realizados no laboratório de Zoonoses da Faculdade de Medicina Veterinária da USP. Grupos de 14 hamsters *Golden Syrian* machos recém-desmamados (80 a 120 gramas) foram imunizados pela via subcutânea no dorso com 50 µg de proteína recombinante, dose de bacterina (controle positivo) ou PBS (controle negativo) em suspensão contendo *Aldydrogel* (2% Al(OH)₃) na concentração final de 12% como adjuvante.

Foram realizadas duas imunizações no intervalo de 15 dias e ao final da segunda imunização, dois animais de cada grupo foram sangrados via punção cardíaca. Os soros foram avaliados por ELISA, semelhante ao descrito para a seção 3.19.

Duas semanas após a segunda imunização, os animais foram infectados por inoculação intraperitoneal de 200 µL de cultura de *L. interrogans* sorovar Kennewicki cepa Pomona Fromm virulenta recém-isolada, sendo inoculadas 4×10^7 leptospiplas/animal.

Os animais inoculados foram observados quanto ao aparecimento dos sintomas de leptospirose por 21 dias, e ao final deste período os sobreviventes foram sacrificados. O sangue foi coletado via punção cardíaca e o soro foi avaliado quanto à presença de anticorpos. Os rins dos animais sobreviventes foram coletados, macerados, ressuspensos em solução salina (na diluição de 1:10 e 1:100) e cultivados em meio EMJH modificado semissólido a 28 °C para avaliação da presença de leptospiplas após 6 semanas de observação (Figura 6).

A análise de significância estatística foi realizada com auxílio do programa *GraphPad Prism* versão 3.0, utilizando o método Kaplan e Meier, 1958.

Figura 6 - Esquema do ensaio de desafio

Esquema da metodologia usada para o ensaio de imunoproteção.

4 RESULTADOS E DISCUSSÃO

4.1 Análise por bioinformática

O estudo de proteínas de membrana de *Leptospira* spp. é importante devido ao contato íntimo que estas têm com o ambiente na qual as bactérias estão inseridas, e com as proteínas do hospedeiro, contribuindo para a sobrevivência bacteriana. 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). Dessa forma, a escolha dos genes se baseou pela predita localização das proteínas nas leptospiros.

O gene LIC11834, segundo o programa PSORT (NAKAI; HORTON, 1999), codifica uma predita lipoproteína de membrana externa. Análise no BLAST (ALTSCHUL et al., 1990; ALTSCHUL et al., 1997) revelou a presença desta proteína nas leptospiros patogênicas já sequenciadas *L. interrogans* sorovar Lai (similaridade igual a 99%), *L. borgpetersenii* sorovar Hardjo-bovis (similaridade igual a 87%), *L. licerasiae* sorovar Varillal (similaridade igual a 60%), *L. santarosai* sorovar Shermani (similaridade igual a 34%), e 31% de similaridade com uma sequência identificada na espécie saprofítica *L. biflexa* sorovar Patoc (além da presença também em outras espécies, as quais ainda não apresentam seus genomas completos). Segundo o programa LipoP (JUNCKER et al., 2003), esta sequência possui peptídeo sinal de exportação entre os aminoácidos 1 e 21, e sítio spII, o qual indica lipidação. De acordo com o programa SMART, o gene LIC11834 possui domínio FecR (entre aminoácidos 60 a 162), que segundo o programa PFAM, está envolvido na regulação do transporte de dicitrato de ferro e, provavelmente, reconhece dicitrato de ferro no periplasma.

O gene LIC12253, segundo o programa PSORT (NAKAI; HORTON, 1999), codifica uma predita proteína de membrana externa. A análise do BLAST (ALTSCHUL et al., 1990; ALTSCHUL et al., 1997) revelou a presença desta proteína nas leptospiros patogênicas já sequenciadas *L. interrogans* sorovar Lai (similaridade igual a 100%), *L. borgpetersenii* sorovar Hardjo-bovis (similaridade igual a 77%), *L. licerasiae* sorovar Varillal (similaridade igual a 48%) e 39% de similaridade com uma sequência identificada na espécie saprofítica *L. biflexa* sorovar Patoc (além da presença também em outras espécies, as quais ainda não apresentam seus genomas completos). Segundo o programa LipoP (JUNCKER et al., 2003), possui peptídeo sinal de exportação entre os aminoácidos 1 e 20, e sítio spI. E por último, de acordo com o programa PFAM, o gene LIC12253 possui um domínio de função desconhecida entre os aminoácidos 58 e 164: DUF1566 (*Protein of unknown function*).

A partir das análises de similaridade pelo BLAST (ALTSCHUL et al., 1990; ALTSCHUL et al., 1997) - análises comparativas das sequências de aminoácidos preditas), foi verificado que as proteínas são altamente conservadas em diversas cepas de *Leptospira*, como pode ser observado claramente nos filogramas gerados pelo programa CLUSTALW2 (Figura 7A e 7B) (LARKIN et al., 2007).

Figura 7 - Filograma das sequências de aminoácidos codificadas pelos genes (A) LIC11834 e (B) LIC12253

B

Em destaque nos filogramas estão as espécies que já possuem o genoma totalmente sequenciado.

A tabela 1 resume as principais características preditas dos genes de estudo.

Tabela 1 - Resumo das características preditas por análise de bioinformática para os genes em estudo

Gene ID ¹	Descrição	Sítio de clivagem	Similaridade com outras espécies já sequenciadas ²
LIC11834	Lipoproteína predita	21 - 22	Lai (99%) LBH (87%) LlicsVM (60%) LSS (34%) LBP (31%)
LIC12253	Proteína de membrana predita	20 - 21	Lai (100%) LBH (77%) LlicsVM (48%) LBP (39%)

Notas: Gene ID - identificação do gene no genoma. ¹<http://aeg.lbi.ic.unicamp.br/world/lic>.
²<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi/>.

Abreviaturas: LIC – *L. interrogans* sorovar Copenhageni. Lai - *L. interrogans* sorovar Lai. LBH - *L. borgpetersenii* sorovar Hardjo-bovis. LlicsVM - *L. licerasiae* sorovar Varillal. LSS - *L. santarosai* sorovar Shermani. LBP - *L. biflexa* sorovar Patoc.

4.2 Estudo da presença dos genes dentre diferentes cepas de *Leptospira* por PCR

Devido à grande variedade da composição antigênica entre os diversos sorovares de *Leptospira* (LEVETT, 2001), a análise da presença dos genes é extremamente importante, pois pode indicar expressão de possíveis antígenos candidatos para produção de uma vacina. A presença dos genes foi avaliada utilizando-se espécies patogênicas e uma saprofítica de *Leptospira*. (Figura 8).

Figura 8 - Análise da presença genômica para os genes LIC11834 e LIC12253

Gel de agarose 1% apresentando (1) o gene 16S rRNA usado como controle interno, além da análise da presença dos genes (2) LIC11834 e (3) LIC12253 em espécies patogênicas (*L. interrogans*, *L. borgpetersenii*, *L. kirshnery*, *L. noguchi* and *L. santarosai*) e na não patogênica *L. biflexa* após PCR. O controle negativo, o qual não contém DNA, está indicado por (-).

O gene LIC12253 foi detectado em todos os sorovares testados, incluindo na espécie saprofítica *L. biflexa*, na qual se verifica uma banda, apesar de sua baixa intensidade, e na *L. santarosai*, a qual não apresentou similaridade significativa com a sequência deste gene encontrada na *L. interrogans* sorovar Copenhageni através da predição pelo BLAST.

Já o gene LIC11834 não foi detectado na *L. santarosai* e nem na *L. biflexa*, dado que pode ser explicado pela baixa similaridade destas sequências com a sequência deste gene encontrada na *L. interrogans* sorovar Copenhageni. Portanto, de acordo com os resultados obtidos, pode-se afirmar que entre ambos os genes testados mostraram-se bastante conservados nas diferentes cepas de leptospiros testadas.

4.3 Estudo da presença dos transcritos dentre diferentes cepas de *Leptospira* por PCR

Como já dito no item acima, a análise da conservação dos genes é importante no sentido de indicar expressão de possíveis antígenos potenciais para produção de uma vacina. A realização do ensaio de prevalência dos transcritos acaba por completar estes dados, já que, se o gene é transcrito, há grande possibilidade de haver uma tradução. Quanto maior o número de espécies que expressam esses genes, maior será o espectro de proteção deste contra diferentes leptospiros. Esta presença dos transcritos foi avaliada utilizando-se espécies patogênicas de *Leptospira*, além da espécie não patogênica *L. biflexa* sorovar Patoc (Figura 9).

Figura 9 - Análise da presença dos transcritos para os genes LIC11834 e LIC12253

Gel de agarose 1% apresentando (1) o gene 16S rRNA usado como controle interno, além da análise da presença dos transcritos (2) LIC11834 e (3) LIC12253 em espécies patogênicas (*L. interrogans*, *L. borgpetersenii*, *L. kirshnery*, *L. noguchi* and *L. santarosai*) e a não patogênica *L. biflexa* após PCR. O controle negativo, o qual não contém DNA, está indicado por (-).

Foi verificada expressão do gene LIC12253 em todas as espécies e sorovares testados, exceto na espécie saprofítica *L. biflexa*, na qual não se verifica banda, apesar de ter sido detectada presença do gene nesta espécie. Este dado evidencia a presença do transcrito para o gene LIC12253 entre as diferentes espécies e sorovares. Já a expressão do gene LIC11834 foi detectada apenas nos sorovares da espécie *L. interrogans*, embora o gene tenha sido identificado também nas outras espécies testadas.

4.4 Amplificação das sequências genômicas para clonagem

As sequências de DNA correspondentes aos genes LIC11834 e LIC12253 foram obtidas por PCR a partir do DNA genômico de *L. interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004a) utilizando os oligonucleotídeos descritos no Quadro 1.

As bandas de DNA obtidas através da amplificação foram analisadas em gel de agarose 1% e apresentaram os tamanhos esperados de 885 e 651 pb para os genes LIC11834 e LIC12253, respectivamente. A figura 10 demonstra os produtos obtidos da reação de PCR, os quais foram, em seguida, purificados a partir do gel de agarose.

Figura 10 - DNA de interesse amplificado através de reação de PCR

Gel de agarose 1% apresentando (marcador molecular - 1) *1Kb Plus DNA Ladder* (Invitrogen) e as bandas obtidas após PCR, as quais apresentaram os tamanhos esperados. (2) LIC11834, (3) LIC12253.

4.5 Clonagem no vetor pGEM-T Easy (*Promega*[®])

O inserto de DNA obtido pela reação de PCR foi utilizado para clonagem em vetor pGEM-T Easy no sítio de clonagem existente para produtos de PCR. Este vetor é utilizado para otimizar as reações de digestão com as enzimas específicas de cada clonagem. O vetor é fornecido pelo fabricante pronto para uso, preparado a partir da digestão do pGEM-5Zf (+) com *EcoR* V e adição de timidinas nas extremidades 3' obtidas. Estas timidinas possibilitam a ligação de produtos de PCR, pois estes, normalmente, possuem uma adenosina em cada extremidade 3', adicionada pela atividade transferase terminal da *Taq* polimerase.

Após transformação em bactérias competentes e extração de DNA, os plasmídeos referentes aos clones foram digeridos com as enzimas de restrição correspondentes para certificação da presença de insertos (Figura 11).

Figura 11 - Insertos de DNA obtidos por digestão do DNA plasmidial (pGEM-T Easy/inserto)

Gel de agarose 1% apresentado (marcador molecular – 1) *1Kb Plus DNA Ladder*, (2 e 4) o pGEM-T Easy contendo inserto gene LIC11834, (3 e 5) o pGEM-T Easy digerido com as enzimas XhoI/NcoI e as bandas correspondentes ao gene LIC11834, (6 e 8) o pGEM-T Easy contendo inserto gene LIC12253, (7 e 9) e o pGEM-T Easy digerido com as enzimas XhoI/NcoI e as bandas correspondentes ao gene LIC12253.

4.6 Clonagem no vetor de expressão pAE

Os insertos correspondentes aos genes LIC11834 e LIC12253 foram removidos dos plasmídeos recombinantes (pGEM-T Easy/inserto) após digestão com as enzimas de restrição (XhoI e NcoI) e clonados no vetor de expressão em *E. coli*, pAE (RAMOS et al., 2004), o qual também foi digerido com as mesmas enzimas de restrição. Este foi desenvolvido para expressar a proteína recombinante contendo 6 resíduos de histidina na região N-terminal. O vetor pAE possui códon ATG de início de transcrição, gene de resistência a ampicilina e sinal de terminação de transcrição. É um sistema de alta expressão que é controlado pelo promotor do fago T7, sendo que a expressão da proteína recombinante só ocorre na presença da T7 RNA polimerase (STUDIER et al., 1990).

A figura 12 mostra o cassete de expressão do vetor pAE com o códon de início (ATG), que é seguido pelas bases nitrogenadas que codificam resíduos de histidinas (CAT/CAC) e, logo após, a sequência codificadora do inserto.

Figura 12 - Cassete de expressão do vetor pAE

PT7: promotor T7. RBS: sítio de ligação ao ribossomo. ATG: códon de início da transcrição. 6xHis: seis resíduos de histidina. Inserto: presença dos genes LIC11834 ou LIC12253. T7term: região de terminação de transcrição.

Por meio da análise dos plasmídeos por fenol-clorofórmio foram selecionados os inóculos que possuíam clones positivos. Após a obtenção do DNA plasmidial (pAE/inserto) destes inóculos, os clones foram digeridos com as enzimas de restrição correspondentes para certificação da presença de inserto (Figura 13).

Figura 13 - Insetos de DNA obtidos por digestão do DNA plasmidial (pAE/inserito)

Após análise plasmidial por fenol-clorofórmio, os clones positivos foram purificados e os plasmídeos obtidos foram digeridos com as enzimas apropriadas. Gel de agarose 1% apresentado (marcador molecular – 1) *1Kb Plus DNA Ladder*, (2 e 4) o pAE contendo inserto gene LIC11834, (3 e 5) o pAE digerido com as enzimas XhoI/NcoI e as bandas correspondentes ao gene LIC11834, (6 e 8) o pAE contendo inserto gene LIC12253, (7 e 9) e o pAE digerido com as enzimas XhoI/NcoI e as bandas correspondentes ao gene LIC12253.

4.7 Sequenciamento automático dos clones positivos pAE/inserito

O método para o sequenciamento 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.

Assim sendo, essa leitura revelou as sequências de bases de nucleotídeos referente aos insertos de DNA para os genes LIC11834 e LIC12253 no vetor de expressão pAE, como pode ser visto na figura 14, na qual observa-se o início de cada sequência. A sequência codificadora no vetor de expressão pAE foi confirmada através do programa BLAST (ALTSCHUL et al., 1990; ALTSCHUL et al., 1997).

Figura 14 - Cromatogramas dos sequenciamentos da região 5' das construções (A) pAE-LIC11834 e (B) pAE-LIC12253

ATG: códon de início da transcrição. 6xHis: seis resíduos de histidina. XhoI: sítio de restrição inserido nos oligonucleotídeos de amplificação. LIC11834 e LIC12253: início de cada gene.

4.8 Avaliação da expressão das proteínas recombinantes

A *E. coli* é considerada um dos sistemas mais eficientes de expressão heteróloga de proteínas devido a habilidade de crescimento fácil e rápido, e ao grande conhecimento que se tem de sua genética (BANEYX, 1999). Dessa forma, para obtenção das proteínas recombinantes realizou-se um estudo de indução utilizando a cepa *E. coli* BL21 SI (*Salt Induced*), que possui o gene T7 RNA polimerase integrado ao genoma sob o controle do promotor *proU* induzível por NaCl (BHANDARI; GOWRISHANKAR, 1997).

As bactérias *E. coli* BL21 SI foram transformadas e cultivadas até a fase log para indução da expressão dos plasmídeos recombinantes em diferentes concentrações de indutor (3, 30 e 300 mM NaCl). Após indução, as bactérias foram lisadas por sonicação em banho de

gelo e as frações proteicas obtidas (sobrenadante e sedimento, correspondentes às frações solúveis e insolúveis, respectivamente) analisadas em gel 12% SDS-PAGE.

A proteína recombinante rLIC11834 foi expressa na forma solúvel, enquanto que a rLIC12253 foi expressa na forma insolúvel como corpúsculo de inclusão.

A figura 15 apresenta os resultados da análise de expressão das proteínas recombinantes na cepa *E. coli* BL21 SI, sendo possível observar rLIC11834 com massa molecular aparente de ~33 kDa presente no sobrenadante do lisado bacteriano, após indução com 300mM de NaCl, e rLIC12253 com massa molecular aparente de ~24 kDa na fração insolúvel do lisado bacteriano, após indução com 300 mM de NaCl. Essas massas moleculares apresentadas no gel estão de acordo com as massas moleculares calculadas pelo programa ProtParam (GASTEIGER, 2006) tendo como base a composição dos aminoácidos (Tabela 2).

Tabela 2 - Massa molecular e ponto isoelétrico (PI) teórico das proteínas recombinantes

Gene ID ¹	Massa molecular (Da)	PI teórico
LIC11834	33132.0	5.28
LIC12253	24074.7	5.25

Notas: Gene ID - identificação do gene no genoma. ¹<http://aeg.lbi.ic.unicamp.br/world/lic>.

Figura 15 - Análise da expressão das proteínas recombinantes

Gel 12% SDS-PAGE apresentando bandas das proteínas recombinantes com os tamanhos esperados de ~33 e ~24 kDa, correspondendo a (A) rLIC11834 e (B) rLIC12253, respectivamente, visualizadas por *Comassie blue*. (1) Massa molecular kDa. A expressão das proteínas recombinantes foi analisada em diferentes concentrações de NaCl → 300 mM (2 e 3, solúvel e insolúvel, respectivamente); 30 mM (4 e 5 solúvel e insolúvel, respectivamente); 3 mM (6 e 7 solúvel e insolúvel, respectivamente). As setas indicam rLIC11834 expressa na forma solúvel (A – linha 2), e rLIC12253 expressa na forma insolúvel (B – linha 3).

4.9 Purificação e diálise das proteínas recombinantes

A purificação das proteínas recombinantes foi realizada por cromatografia de afinidade utilizando resina de afinidade a metal tratada com níquel em sua forma catiônica. Como o vetor de expressão pAE adiciona uma sequência de seis resíduos de histidina na região N-terminal da proteína recombinante, esta se liga fortemente ao níquel via anel imidazólico, ao passo que os contaminantes proteicos da cultura se ligam fracamente, sendo lavados com tampão contendo baixa concentração de imidazol. A proteína recombinante por sua vez, é eluída por meio de um tampão contendo alta concentração de imidazol.

Assim sendo, a purificação da proteína rLIC11834 foi realizada a partir do sobrenadante obtido após lise bacteriana, já que se trata de uma proteína expressa na forma solúvel. As lavagens e as frações eluídas foram analisadas por gel 12% SDS-PAGE como demonstra a figura 16.

Figura 16 - Análise da purificação da proteína recombinante rLIC11834

Gel 12% SDS-PAGE apresentando (A) as lavagens e (B) eluição da proteína recombinante. (1) Massa molecular kDa, (2) fração não induzida, (3) induzida, fração anterior à passagem na coluna, (4) fração não ligada, (L) frações das lavagens com concentrações crescentes de imidazol (5, 20, 40 e 60 mM), (E) frações eluídas com alta concentração de imidazol (1 M).

Já a proteína rLIC12253, por ser expressa sob a forma insolúvel, foi solubilizada em tampão de desnaturação e, dessa maneira, pode ser purificada, após gotejamento em solução de renaturação (para diluição da ureia, já que se trata de um desnaturante). As lavagens e as frações eluídas foram analisadas por gel 12% SDS-PAGE, como demonstra a figura 17.

Figura 17 - Análise da purificação da proteína recombinante rLIC12253

Gel 12% SDS-PAGE apresentando (A) as lavagens e (B) eluição da proteína recombinante. (1) Massa molecular kDa, (2) fração não induzida, (3) induzida, fração anterior à passagem na coluna, (4) fração não ligada, (L) frações das lavagens com concentrações crescentes de imidazol (5, 20, 40 e 60 mM), (E) frações eluídas com alta concentração de imidazol (1 M).

As alíquotas que continham as proteínas recombinantes foram dialisadas contra tampão apropriado: PBS e 0,1% Glicina. Para a proteína rLIC11834, acrescentou-se ainda 2mM PMSF, o qual inibe ação de proteases (para evitar degradação da mesma, já que após a primeira purificação desta, verificou-se pequena banda de degradação).

Após diálise, a concentração das proteínas recombinantes foi estimada através da comparação destas com concentrações pré-estabelecidas de BSA (densitometria) através do *software Carestream* (Gel Logic 2200 Pro). Como esperado, bandas majoritárias de ~33 e ~24 kDa correspondentes as proteínas rLIC11834 e rLIC12253, respectivamente, foram observadas em gel 12% SDS-PAGE (Figura 18).

Figura 18 - Quantificação das proteínas recombinantes por comparação com curva de BSA

Gel 12% SDS-PAGE apresentando diferentes concentrações das proteínas recombinantes (A) rLIC11834 e (B) rLIC12253, e de BSA. (1) Massa molecular kDa, (2) 3µl da proteína recombinante, (3) 5µl da proteína recombinante, (4) 10µl da proteína recombinante, BSA em concentrações crescente (0,5; 1; 1,5; 2; 2,5; 3µg).

Após quantificação, verificou-se que a proteína recombinante rLIC11834 se encontrava na concentração de 0,6µg/µL e a proteína recombinante rLIC12253 se encontrava na concentração de 1µg/µL.

4.10 Análise da estrutura e estabilidade das proteínas recombinantes por espectroscopia circular (CD)

O CD é uma técnica para o estudo dos elementos de estrutura secundária das proteínas (α -hélices, estruturas β e estrutura randômica), os quais contribuem na formação da sua estrutura terciária. Esta técnica está baseada no fato de que moléculas com centros assimétricos (quirais) absorvem a luz circularmente polarizada para a direita diferentemente da luz circularmente polarizada para a esquerda provocando um desvio no ângulo da polarização da luz incidente para a direita ou esquerda (elipcidade), que é medido pelo polarímetro. Os cromóforos que originam os espectros de CD em peptídeos e proteínas são as ligações amida, os resíduos aromáticos e as pontes dissulfeto (GREENFIELD, 2006).

Proteínas com predomínio de α -hélice apresentam dois picos negativos (aproximadamente 208 e 222 nm) e um pico positivo (aproximadamente 193 nm), e proteínas com predomínio de folhas- β antiparalelas apresentam um pico negativo em aproximadamente 218 nm e um pico positivo em 195 nm. Proteínas com estruturas randômicas, ou seja, não ordenadas, apresentam sinal muito baixo e um pico negativo próximo a 195 nm (GREENFIELD, 2006).

As proteínas recombinantes purificadas foram dialisadas contra solução tampão sódio-fosfato 10 mM (pH 7,4) a 4°C e mantidas sob refrigeração até o momento da coleta dos espectros de dicroísmo circular, os quais podem ser vistos na figura 19 , além de uma curva padrão (KELLY; JESS; PRICE, 2005) para posterior análise.

Figura 19 - Espectros de dicroísmo circular das proteínas recombinantes

Através da análise dos dicroísmos circulares (em comparação com a curva padrão (P) foi possível observar, para ambas as proteínas recombinantes, sinais característicos das estruturas secundárias (tanto α -hélice, quanto folha- β), porém com predomínio de α -hélice devido ao ponto máximo do espectro de 192 nm e ao ponto mínimo do espectro de 222 nm para (A) rLIC11834 e de 208nm para (B) rLIC12253.

Fonte (curva padrão): Kelly, Jess e Price (2005).

Os espectros de dicroísmo circular, quando comparados com a curva padrão, nos permitiram obter sinais característicos das estruturas secundárias, revelando presença tanto de α -hélice, quanto de folha- β em ambas as proteínas, com predominância de α -hélice. Esses dados foram importantes, pois nos deu segurança para prosseguir com experimentos de caracterização de função e imunogenicidade das proteínas em estudo.

4.11 Avaliação da imunogenicidade das proteínas recombinantes em camundongos

Um grupo de animais foi imunizado com 2 doses de proteína recombinante rLIC11834, enquanto um outro grupo com 3 doses de proteína recombinante rLIC12253, e sangrados via plexo retro-orbital após cada dose. Os soros obtidos foram avaliados quanto à presença de anticorpos anti-rLIC11834 e anti-rLIC12253. Os títulos de anticorpos policlonais presentes no soro dos animais de cada grupo foram avaliados por ELISA.

O grupo imunizado com a proteína rLIC12253 só apresentou títulos de anticorpos após a segunda dose, por isso a necessidade de uma dose a mais, quando comparado com a rLIC11834, a qual já apresentou altos títulos após a primeira dose. O grupo controle (PBS), por sua vez, não apresentou quaisquer títulos, assim como o soro dos animais pré-ímmes (dados não mostrados). O maior título de anticorpos obtidos em pelo menos um animal de cada grupo após cada imunização é apresentado na tabela 3.

Tabela 3 - Títulos de anticorpos policlonais obtidos após imunização de camundongos com as proteínas recombinantes

Proteína recombinante	1ª sangria	2ª sangria	3ª sangria
rLIC11834	409.600	6.553.600	-
rLIC12253	0	12.800	204.800

Como mostra a tabela 3, todas as proteínas recombinantes mostraram um aumento dos títulos de anticorpos entre as imunizações, apresentando, ao final, títulos significativos.

Para demonstrar a especificidade de anticorpos anti-rLIC11834 e anti-rLIC12253, além do ELISA, realizou-se *Western blotting* no qual foi possível a visualização do anticorpo ligado à proteína recombinante. Realizou-se primeiramente o ensaio com anticorpo específico (1:1000) por 1 hora a temperatura ambiente (Figura 20).

Figura 20 – Reconhecimento de anticorpos específicos pelas proteínas recombinantes

(A) Anti-rLIC11834 e (B) Anti-rLIC12253 foram detectados pelo contato com proteínas recombinantes específicas (marcador molecular indicado por KDa).

Verificou-se sinal fraco para rLIC12253, provavelmente devido ao menor título apresentado pelo anticorpo específico. Portanto, repetiu-se o ensaio, porém, além da diluição dos anticorpos 1:1000, realizou-se diluição 1:500 e, para rLIC12253, também 1:300 e modificou-se o tempo e temperatura de incubação desses anticorpos (3 horas a 37°C) (Figura 21).

Figura 21 – Reconhecimento de diferentes concentrações de anticorpos específicos pelas proteínas recombinantes

(A) Anti-rLIC11834 e (B) Anti-rLIC12253 foram detectados pelo contato com proteínas recombinantes específicas (marcador molecular indicado por KDa).

Como pode-se analisar na figura 21, houve aumento do sinal dependente da concentração do anticorpo: quanto maior a concentração, mais forte o sinal. E também pode-se verificar, juntamente com os resultados de análise dos títulos de anticorpos policlonais por ELISA, que se tratam de proteínas imunogênicas.

Após as imunizações, os camundongos foram sacrificados, os baços foram extraídos e processados, para subsequente cultura celular e análise da linfoproliferação e determinação de citocinas.

As células de todos os grupos se mostraram viáveis, o que é observado pela alta proliferação dos esplenócitos estimulados com o mitógeno ConA, o qual é uma lectina (proteína ligante de carboidrato) com alto potencial de induzir mitose.

Nenhuma das proteínas recombinantes induziu uma resposta celular, já que não foi observada proliferação dos esplenócitos quando estes foram estimulados *in vitro* pelos antígenos (Figura 22). Ou seja, não houve estimulação da linfoproliferação pelas proteínas recombinantes.

Figura 22 - Avaliação de linfoproliferação

A proliferação de linfócitos foi analisada a partir de células de baço de camundongos imunizados com rLIC1184 (A) ou com rLIC12253 (B), ou ainda animais controle, imunizados com PBS. As células foram cultivadas e, então, estimuladas com meio (controle negativo), mitógeno ConA (controle positivo, 5 µg/mL) ou proteína recombinante (5 µg/mL). A resposta de proliferação foi medida pelo ensaio colorimétrico ELISA-BrdU. Os valores representam o valor de Abs $\pm$ desvio padrão de dois experimentos independentes. As comparações para análise estatística foram feitas pelo *Student's two tailed t test* entre as médias do tratamento no qual as células foram estimuladas com a proteína, entre o grupo imunizado e grupo PBS.

Para mensuração das citocinas liberadas com objetivo de discriminar a indução de uma resposta imune celular (Th1) ou humoral (Th2) pelas proteínas recombinantes, níveis de IL-4, IL-10, IL-12, IFN- γ e TNF- α foram avaliados por ELISA utilizando kits comerciais. IL-4 e IL-10 foram utilizadas como marcadores de resposta imune Th2, enquanto que IL-12, IFN- γ e TNF- α foram utilizadas como marcadores de resposta Th1. (Figura 23).

Mastócitos e células T CD4+, após ativação da resposta imune adaptativa, são os principais produtores de IL-4, estimulando o desenvolvimento de células Th2. Macrófagos e linfócitos Th2 são os principais produtores de IL-10, o qual atua inibindo os próprios macrófagos, além de ser uma citocina moduladora, restringindo e impedindo uma resposta imune excessiva e danos colaterais (SABAT et al., 2010). Células Th1 CD4+, T CD8+ e NK (*natural killer*) produzem IFN- γ , a qual atua tanto na imunidade inata quanto na imunidade adaptativa ativando macrófagos e estimulando a proliferação de mais células Th1. IFN- γ ainda aumenta a síntese de TNF- α por macrófagos estimulados por LPS, e este atua nas células do endotélio vascular e leucócitos estimulando o recrutamento de neutrófilos e monócitos para o local da infecção. Células apresentadoras de antígenos, principalmente durante uma resposta às infecções por bactérias intracelulares e vírus, produzem IL-12, a qual

estimula a diferenciação de células T CD4⁺ em células Th1 e a produção de IFN- γ (AMSEN; SPILIANAKIS; FLAVELL, 2009).

Figura 23 - Avaliação da resposta de citocinas resultante da imunização de camundongos com as proteínas recombinantes após estímulo *in vitro*

B

Células de baço foram isoladas dos camundongos imunizados com rLIC11834 (A) e rLIC12253 (B), e cultivadas em placa de cultura de 24 poços. Após 48 horas, o sobrenadante da cultura foi coletado e as citocinas IL-4, IL-10, IL-12, IFN- γ e TNF- α foram dosadas por um kit de ELISA. Os valores de absorbância obtidos foram comparados com os valores de uma curva padrão contendo concentrações conhecidas de cada citocina. Para análise estatística, primeiramente foi avaliada a diferença entre as médias do tratamento no qual as células foram

estimuladas com a proteína, entre o grupo imunizado e grupo PBS (controle negativo). Sendo detectada diferença significativa, a análise estatística foi então feita dentro do grupo dos animais imunizados entre o tratamento proteína e meio pelo *Student's two tailed t test*, e o p-valor mostrado no gráfico se refere a esta última análise (* $P < 0,05$).

O grupo imunizado com rLIC11834 apresentou um aumento significativo para citocina IL-10 após estimulação com o antígeno. Nenhum outro aumento nas concentrações de citocinas foi observado.

Assim sendo, analisando todos os resultados, os dados indicam que as proteínas recombinantes estimulam apenas resposta humoral, já que, além de produzirem consideráveis títulos de anticorpos policlonais, não promoveram a linfoproliferação, e quanto à liberação de citocinas, observou-se aumento na produção de IL-10 pela rLIC11834, indicando resposta Th2, apesar de estudos indicarem presença de ambas respostas para outras proteínas recombinantes e para o uso do adjuvante *Alhydrogel* (FERNANDES et al., 2013; FERNANDES et al., 2012; REED et al., 2009).

4.12 Detecção de anticorpos IgG e IgM humanos em soro de pacientes diagnosticados com leptospirose por ELISA

A identificação de antígenos de *Leptospira* spp. que possam ser reconhecidos na fase inicial da doença, como as proteínas Lp29 (NEVES et al., 2007), MPL17 e MPL21 (OLIVEIRA et al., 2008) poderão facilitar o diagnóstico e tratamento precoce da doença.

Para analisar 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 amostras de 23 soros correspondentes à fase inicial da doença, com resultado MAT negativo, e amostras de 34 soros correspondentes à fase convalescente da doença, com resultado MAT positivo através de ELISA. O teste foi realizado utilizando tanto as proteínas separadas, quanto as utilizando de forma conjunta, para análise de efeito sinérgico. O MAT é o método referência para diagnóstico da leptospirose (FAINE et al., 1999; LEVETT, 2001), porém é uma técnica que apresenta baixa sensibilidade na fase inicial da doença, já que anticorpos contra antígenos da leptospira não são detectados nos primeiros dias após a exposição (CUMBERLAND; EVERARD; LEVETT, 1999). Além disso, por se tratar de um ensaio sorovar específico, os laboratórios que realizam MAT necessitam manter culturas vivas de diferentes sorovares de *Leptospira* (LEVETT, 2003), o que dificulta a execução do ensaio.

Os soros tiveram sua reatividade avaliada quanto ao tipo de anticorpo produzido, IgG e IgM. Inicialmente estabeleceu-se o valor de *cut-off* a partir de 11 amostras de soros de indivíduos normais que relataram não terem entrado em contato com a doença e que foram diagnosticados com resultado MAT negativo, além de um *pool* de amostras de soros normais comercial (Sigma - Aldrich). Para este ensaio foram feitas incubações prévias dos soros com extrato de *E. coli* na concentração de 25%, para evitar a interferência de proteínas contaminantes desta bactéria presentes na amostra dos recombinantes.

Resultados obtidos quando se utilizou anticorpos anti-IgM humano foram semelhantes para ambas as proteínas: 3 e 0% de reatividade para MAT positivo e negativo, respectivamente (Figura 24). Já a proteína rLIC12253 mostrou 48 e 46% de reatividade para MAT positivo e negativo, respectivamente, quando houve utilização de anticorpos anti-IgG humano (Figura 24B), enquanto a proteína rLIC11834 mostrou 12 e 8% de reatividade para MAT positivo e negativo, respectivamente (Figura 24A). A análise do efeito com ambas as proteínas em conjunto foi verificada para IgG e mostrou certo sinergismo, já que houve 58% de reatividade para MAT positivo, como pode ser visto na figura 24C.

Figura 24 - Análise da reatividade de soros de indivíduos diagnosticados para leptospirose contra (A) rLIC11834, (B) rLIC12253 e (C) rLIC11834 + rLIC12253

A

B **C**

Amostras de soros foram analisados quanto à presença de anticorpos IgG e IgM específicos por ELISA e são apresentados como gráfico de pontos de dispersão para valores individuais. Linhas horizontais representam o *cut-off* calculado a partir de soros de indivíduos que relataram não terem leptospirose e um soro comercial. (A) rLIC11834. (B) rLIC12253. (C) rLIC11834 + rLIC12253.

Estes dados sugerem que a rLIC12253 seja mais reativa do que a rLIC11834, e que houve efeito sinérgico pela presença de ambas proteínas, com aumento de 10% no reconhecimento de soros de pacientes.

Por uma diluição seriada do *pool* dos soros dos pacientes diagnosticados com leptospirose que reagiram com as proteínas recombinantes, verificou-se qual é a maior diluição que ainda gera reatividade com as proteínas recombinantes (Tabela 4).

Tabela 4 - Títulos dos soros dos pacientes diagnosticados com leptospirose nas quais ainda há reatividade com as proteínas recombinantes

Proteína recombinante	Soros MAT-	Soros MAT+
rLIC11834	400	1.600
rLIC12253	400	6.400

Notas: MAT - Teste de microaglutinação.

Por meio destes dados observa-se que é possível detectar a reatividade com as proteínas recombinantes pelos soros de pacientes com leptospirose acima da diluição 1:400 (diluição utilizada no experimento anterior) para os indivíduos correspondentes à fase convalescente da doença.

4.13 Avaliação de reação cruzada dos anticorpos IgG humanos de soro de pacientes com diagnóstico de outras doenças por ELISA

Devido aos sintomas clínicos inespecíficos da leptospirose (OLIVEIRA et al., 2008), avaliou-se a reatividade das proteínas recombinantes com amostras de soros de pacientes com outras doenças febris, utilizando soro de indivíduos com dengue (n = 13), malária (n = 11), doença de Chagas (n = 20) e HIV (n = 15), doenças comuns no Brasil (Figura 25).

Figura 25 - Análise da reatividade de soros de indivíduos diagnosticados com outras doenças contra (A) rLIC11834 e (B) rLIC12253

Amostras de soros foram analisados quanto à presença de anticorpos IgG específicos por ELISA e são apresentados como gráfico de pontos de dispersão para valores individuais. Linhas horizontais representam o *cut-off* calculado a partir de soros de indivíduos que relataram não terem leptospirose e um soro comercial. (A) rLIC11834. (B) rLIC12253.

Como pode ser observado, não houve reatividade com ambas as proteínas recombinantes, conferindo, portanto, 100% de especificidade para as mesmas.

4.14 Ensaios de localização da proteína nativa

Proteínas expostas na membrana externa são importantes na relação patógeno-hospedeiro: servem como alvos potenciais para o sistema imunológico do hospedeiro, e estão relacionadas aos processos de adesão e colonização da bactéria.

A partir do sequenciamento genômico de espécies patogênicas de *Leptospira* (BULACH et al., 2006; CHOU et al., 2012; NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b; REN et al., 2003; RICALDI et al., 2012), análises *in silico* identificaram diversas proteínas com predição para presença na superfície desta bactéria. Para se ter uma ideia, há mais de 200 proteínas com predição para localização na superfície da *L. interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b).

Os genes LIC11834 e LIC12253 codificam proteínas com probabilidade de estarem associadas à membrana externa, porém, é necessária confirmação experimental.

Sendo assim, duas técnicas foram empregadas:

4.14.1 Imunofluorescência em fase líquida (L – IFA)

A técnica de L-IFA permite detectar na superfície da bactéria viva as proteínas nativas correspondentes (Figura 26) pelo reconhecimento do anticorpo policlonal específico para cada proteína e posterior incubação com iodeto de propídio e anti-IgG de camundongo conjugado com FITC. Como controle, foi empregado soro contra duas proteínas abundantes em leptospira e bem caracterizadas: Groel, uma proteína citoplasmática, e, portanto, seu anticorpo não deve marcar quando as células se encontram integras, e LipL32, uma proteína de superfície, além do soro dos camundongos imunizados com PBS (CULLEN et al., 2005).

Figura 26 - Reconhecimento das proteínas codificadas pelas sequências LIC11834 e LIC12253 em *L. interrogans* por seus respectivos anticorpos por imunofluorescência

Ensaio de imunofluorescência em fase líquida (L - IFA) foi realizado sob microscopia confocal utilizando leptospiros vivas em contato com antissoro para LIC11834, LIC12253, LipL32 (controle para proteína exposta na membrana), GroEL (controle para proteína citoplasmática) e soro de camundongos imunizados com PBS. (A) DNA das leptospiros foram identificadas por coloração com iodeto de propídio. (B) Anticorpos secundários conjugados (FITC) foram usados para detectar os anticorpos primários ligados. (C) Co-localização mostrado em imagens fundidas.

Verificou-se que as proteínas em estudo possivelmente são expressas na superfície de leptospiros, já que observou o sinal em verde, o qual indica marcação da proteína pelo FITC. Além disso, conclui-se que o ensaio foi válido, pois observou-se que as bactérias estavam íntegras, já que todas as células apresentaram a coloração avermelhada, a qual indica

marcação do DNA das bactérias pelo iodeto de propídio, além da não marcação da proteína Groel pelo FITC.

Para confirmação destes resultados, um novo ensaio foi realizado:

4.14.2 Ensaio de acessibilidade com *Proteinase K* (PK)

Este método vem sendo utilizado para investigar se proteínas de leptospiros estão expostas na membrana externa a partir da digestão com PK (MENDES et al., 2011; OLIVEIRA et al., 2011; PINNE; HAAKE, 2009). A PK é uma protease não específica que cliva as partes acessíveis das proteínas que estão expostas na superfície de leptospiros intactas. Neste ensaio, leptospiros vivas foram tratadas com PK por diferentes períodos de tempo e a presença das proteínas nativas correspondentes aos genes LIC11834 e LIC12253 na superfície das leptospiros foi detectada pela adição de anticorpos específicos (Figura 27).

Figura 27 - Ensaio de acessibilidade a protease pelas proteínas de *L. interrogans* codificadas pelos genes LIC11834 e LIC12253

Leptospiros viáveis foram incubadas com 25 µg/mL de PK nos tempos indicados (horas) e imobilizadas em microplaca. Os anticorpos contra as proteínas recombinantes rLIC11834, rLIC12253, LipL32 e DnaK foram adicionados. Após incubação, adicionou-se também o anti-IgG conjugado e a reação foi revelada pelo substrato de peroxidase OPD. As leituras foram realizadas a 492 nm. As barras representam a média de absorbância $\pm$ o desvio padrão de três repetições para cada proteína e são representativos de três experimentos independentes. Para análise estatística, o sinal foi comparado entre o início da reação (hora 0) e as horas de tratamento com PK por *Student's two tailed t test* (* $P < 0,05$).

Como se observa, as proteínas codificadas pelos genes LIC11834 e LIC12253, além do controle positivo LipL32, após 1 hora de incubação com a PK, já mostrou uma redução significativa do sinal em relação ao tempo 0, indicando que as proteínas foram susceptíveis ao tratamento. Como esperado, não foi observada alteração na detecção da proteína citoplasmática, DnaK, usada como controle negativo do ensaio. Assim sendo, estes resultados, juntamente com os do item anterior (seção 4.14.1), sugerem que as proteínas codificadas pelos genes LIC11834 e LIC12253 estão presentes na membrana externa da *L. interrogans* sorovar Copenhageni.

4.15 Ensaio de adesão das proteínas recombinantes aos componentes da matriz extracelular e componente do plasma por ELISA

4.15.1 Análise da interação destes componentes às proteínas recombinantes

Os mecanismos pelos quais as leptospiros invadem e colonizam os tecidos hospedeiros são pouco conhecidos. A interação de patógenos a certos componentes contribui para a colonização primária destes tecidos. Sob condições normais, os componentes da MEC não estão expostos a bactérias. Os patógenos podem acessar esses componentes após trauma do tecido seguido de injúria mecânica ou química, ou ainda consequência de uma infecção bacteriana através da ação de toxinas e enzimas líticas (LJUNGH et al., 1996).

Nosso grupo descreveu a primeira proteína de leptospira ligante a laminina, Lsa24 , e, logo após, outras adesinas foram relatadas, como LigA e LigB (CHOY et al., 2007), proteínas da família Len (STEVENSON et al., 2007), Lsa21 (ATZINGEN et al., 2008), LipL32 (HOKE et al., 2008) Lp95 (ATZINGEN et al., 2009), TlyC (CARVALHO et al., 2009), LipL53 (OLIVEIRA et al., 2010), OmpL37 (PINNE; CHOY; HAAKE, 2010), Lsa63 (VIEIRA et al., 2010b), Lsa66 (OLIVEIRA et al., 2011), Lsa20 (MENDES et al., 2011), OmpL1 (FERNANDES et al., 2012), Lsa30 (SOUZA et al., 2012), Lsa23, Lsa26, Lsa36 (SIQUEIRA et al., 2013), Lsa44 e Lsa45 (FERNANDES et al., 2013), entre outras (VIEIRA et al., 2014).

Neste sentido, investigou-se a ligação das proteínas em estudo com os seguintes componentes: laminina, fibronectina celular e plasmática, colágeno tipo I e tipo IV, elastina, e vitronectina, por ELISA. Acrescentou-se gelatina e fetuína como controles negativo (Figura 28).

Figura 28 - Caracterização da ligação das proteínas rLIC11834 e rLIC12253 aos componentes da MEC e do plasma

Poços foram revestidos com 1 μ g de laminina, colágeno do tipo I, colágeno do tipo IV, fibronectina celular, fibronectina do plasma, elastina, vitronectina, e proteínas de controle negativo (gelatina e fetúina). As proteínas recombinantes foram adicionadas (1 μ g/poço) e a ligação analisada por ELISA. A ligação foi analisada utilizando (A) anticorpos policlonais contra cada proteína, e utilizando (B) anticorpo monoclonal anti – polihistidina. Os dados representam a média $\pm$ o desvio padrão de três experimentos independentes. Para análise estatística, a ligação das proteínas recombinantes aos componentes foi avaliada por comparação com a sua ligação a gelatina por *Student's two tailed t test* (* P <0,05; ** P <0,005).

Como é possível observar, há adesão à laminina por ambas as proteínas. Desta forma, estas proteínas foram nomeadas: Lsa33 (rLIC11834) e Lsa25 (rLIC12253) (*Leptospiral surface adhesin* de 33 e 24 KDa, respectivamente; esta última foi chamada de Lsa25 porque já há uma outra proteína nomeada Lsa24 (BARBOSA et al., 2006; DOMINGOS et al., 2012).

4.15.2 Curvas de dose-resposta

A especificidade destas interações foi avaliada de forma quantitativa e uma curva de adesão dose-resposta foi feita em razão da concentração da proteína recombinante (Figura 29), sendo possível calcular a constante de dissociação (K_D) quando detectado com antissoro específico.

Figura 29 - Análise da especificidade da interação das proteínas recombinantes a laminina

Os experimentos de dose - dependência da ligação das proteínas recombinantes a laminina foram detectados utilizando (A) anticorpos policlonais contra cada proteína e (B) anticorpo monoclonal anti – polihistidina. (1) Lsa33 e (2) Lsa25. Cada ponto foi realizado em triplicata, e expresso como o valor médio de absorbância a 492 nm $\pm$ erro padrão para cada ponto. Gelatina foi incluída como controle negativo.

As K_D descritas (Tabela 5) foram calculadas a partir dos dados de ELISA para as proteínas recombinantes que atingiram o equilíbrio, o qual ocorreu a partir da concentração de 3 μ M para ambas as proteínas. Esses valores são semelhantes à de outras proteínas recombinantes já descritas, tais como: LenB, TlyC, OmpL37, Lsa20 e Lsa30 (CARVALHO et al., 2009; MENDES et al., 2011; PINNE; CHOY; HAAKE, 2010; SOUZA et al., 2012; STEVENSON et al., 2007).

Tabela 5 - Constantes de dissociação para as curvas de dose-dependência da laminina detectadas com antissoro específico

Proteína recombinante	K_D
rLIC11834	367,59 ± 248,73
rLIC12253	415,46 ± 203,88

Notas: K_D - Constante de dissociação.

A ligação à laminina pode promover aderência das leptospiras aos tecidos do hospedeiro.

4.16 Ensaio de adesão das proteínas recombinantes a componentes do soro humano e componente do plasma por ELISA

4.16.1 Análise da interação destes componentes às proteínas recombinantes

O dano às células pela ativação do sistema complemento pode ser prevenido por um número de proteínas regulatórias do complemento, incluindo fator H e C4bp. Sendo assim, vários patógenos de importância médica e veterinária produzem uma ou mais proteínas de membrana externa que se ligam a estes componentes e, portanto, se tornam protegidos ao ataque do sistema complemento. Já foi demonstrado que a leptospira é um destes organismos, já que se liga tanto ao fator H (MERI et al., 2005), regulador da via alternativa, quanto ao C4bp (BARBOSA et al., 2009), regulador das vias clássicas e das lecitinas do sistema complemento. Outras proteínas de leptospira que se ligam à estes componentes já foram identificadas, como a LfhA (VERMA et al., 2006), a LcpA (BARBOSA et al., 2010) e a Lsa23 (SIQUEIRA et al., 2013).

Também já foi demonstrada a interação das leptospiras com o PLG (VIEIRA et al., 2009b), a qual tem sido sugerida como uma atividade que contribui significativamente para a virulência de patógenos por facilitar a disseminação bacteriana. Através da captura do PLG do hospedeiro em sua superfície, seguida pela ativação em plasmina, a bactéria se torna um organismo proteolítico, uma atividade que contribui para a degradação de componentes da matriz extracelular, penetração tecidual e invasão (LAHTENMAKI; KUUSELA; KORHONEN, 2001).

Além disso, já foi identificado alguns patógenos que interagem com o Fg, como *Staphylococcus aureus* (MOREILLON et al., 1995), e a espiroqueta *Treponema denticola* (BAMFORD et al., 2007). O Fg é uma glicoproteína plasmática e uma das principais proteínas envolvidas na cascata de coagulação sanguínea, sendo um componente essencial ao final desta cascata para formar a rede de fibrina, e, portanto, é essencial na cicatrização de feridas e na reparação de tecidos (BAMFORD et al., 2007; CHOY et al., 2011; LIN et al., 2011).

Nesse sentido, investigou se as proteínas recombinantes Lsa33 e Lsa25 seriam capazes de interagir com os reguladores do sistema complemento, com a enzima inativa PLG, e com um dos componentes da cascata de coagulação, o Fg.

Como se observa na figura 30 ocorre adesão ao PLG e ao Fg pela Lsa33 e ao C4bp por ambas as proteínas.

Figura 30 - Ligação das proteínas recombinantes a componentes do soro humano e do plasma

Um µg de PLG, fator H, C4bp, Fg, e proteínas de controlo negativo (gelatina e fetuína), foram imobilizados em placas de ELISA e incubados com as proteínas recombinantes Lsa33 ou Lsa25 (1 µg/poço). A ligação foi analisada utilizando (A) anticorpos policlonais contra cada proteína, e utilizando (B) anticorpo monoclonal anti – polihistidina. Os dados representam a média ± o desvio padrão de três experimentos independentes. Para análise estatística, a ligação das proteínas recombinantes aos componentes foi avaliada por comparação com a sua ligação a gelatina por *Student's two tailed t test* (* P < 0,05; ** P < 0,005).

4.16.2 Curvas de dose-resposta

A especificidade dessas interações foi avaliada de forma quantitativa e uma adesão dose-dependente foi observada em razão da concentração das proteínas recombinantes (Figura

31), sendo possível calcular o K_D (somente para as curvas do PLG e do Fg, já que apresentaram saturação da ligação).

Figura 31 - Análise da especificidade da interação das proteínas recombinantes ao PLG, ao C4bp e ao Fg

Os experimentos de dose - dependência da ligação das proteínas recombinantes ao PLG (1), ao C4bp (2) e ao Fg (3), foram realizados utilizando anticorpos policlonais contra cada proteína (A) e anticorpo monoclonal anti – polihistidina (B). Cada ponto foi realizado em triplicata, e expresso como o valor médio de absorbância a 492 nm $\pm$ erro padrão para cada ponto. Gelatina foi incluída como controle negativo.

Os valores de K_D descritos (Tabela 6) foram calculados a partir dos dados de ELISA para a proteína recombinante Lsa33, a qual atingiu o equilíbrio a partir das concentrações 0,25 μ M e 2 μ M para interação ao PLG detectado com anticorpo policlonal específico e com anti-polihistidina, respectivamente, e 2 μ M para interação ao Fg detectado tanto com anticorpo policlonal específico e quanto com anti-polihistidina.

Tabela 6 - Constantes de dissociação para as curvas de dose-dependência do PLG e Fg – Lsa33

PLG	K _D
Antissoro	23,53 ± 4,66
Anti-his	75,23 ± 54,23
Fg	K _D
Antissoro	128 ± 89,91
Anti-his	130,66 ± 104,86

Notas: K_D - Constante de dissociação.

Vale ressaltar o baixo valor de K_D para o PLG, indicando uma forte ligação entre este componente e a proteína Lsa33, semelhante ao verificado por outras proteínas recombinantes, tais como Lsa20 (MENDES et al., 2011), Lsa23, Lsa26, Lsa36 (SIQUEIRA et al., 2013), Lsa44, e Lsa45 (FERNANDES et al., 2013).

4.17 Inibição da ligação da proteína recombinante Lsa33 ao PLG pelo ácido 6-aminocaproico (AAC)

Já foi demonstrado que os domínios *kringle* do PLG medeiam a interação com resíduos de lisina em receptores de diversas bactérias (LAHTEENMAKI; KUUSELA; KORHONEN, 2001). Este mesmo efeito também foi observado para as leptospiros (VIEIRA et al., 2009b). Neste sentido, avaliou-se a participação dos resíduos de lisina da Lsa33 na interação com o PLG pelo acréscimo de AAC, um derivado e análogo de lisina, à reação de ligação proteína recombinante-PLG.

Como pode ser observado na figura 32, houve redução significativa na interação da Lsa33 com o PLG pela a adição de 2 mM AAC (68%), o que sugere participação dos resíduos de lisina da proteína recombinante Lsa33 nesta interação.

Figura 32 – Inibição da interação do PLG à Lsa33 pelo AAC

A ligação da proteína recombinante Lsa33 ao PLG foi verificada na ausência (0 mM) e na presença de AAC (2 e 20 mM). Cada ponto foi realizado em triplicata e expresso como a média dos valores de absorbância a 492 nm $\pm$ o desvio padrão de cada ponto. Os dados são representativos de dois experimentos independentes. Para análise estatística, a ligação da proteína recombinante ao PLG com adição ao AAC foi avaliada por comparação com a ligação sem AAC por *Student's two tailed t test* (** P < 0,005).

4.18 Avaliação da atividade enzimática da plasmina

Na presença de um ativador, o PLG aderido à superfície de *L. interrogans* gera plasmina enzimaticamente ativa (VIEIRA et al., 2009b). Assim sendo, alguns autores mostraram este mesmo efeito através da ligação de algumas proteínas recombinantes ao PLG (MENDES et al., 2011; OLIVEIRA et al., 2011; SOUZA et al., 2012; VERMA et al., 2010; VIEIRA et al., 2010a). Portanto, verificou se o PLG ligado à Lsa33 gera atividade proteolítica: placas de ELISA foram adsorvidas com Lsa33 e incubadas com PLG. Adicionou-se o ativador de PLG tipo uroquinase (uPA) e o substrato cromogênico específico para avaliar a atividade da plasmina.

Como demonstrado pela figura 33, o PLG ligado à Lsa33 pode ser convertido em plasmina, como demonstrado pela atividade proteolítica da plasmina gerada. Os controles do experimento, onde um dos componentes da reação (PLG, uPA ou o substrato cromogênico) foi omitido, ou a reação empregando Lsa63, já que se trata de uma proteína que não se liga ao PLG (VIEIRA et al., 2010b), ou BSA (controles negativos), não mostraram atividade enzimática.

Figura 33 - Geração de plasmina pelo PLG ligado à Lsa33

A geração de plasmina pelo PLG ligado à Lsa33 foi avaliada indiretamente, por ELISA. A proteína imobilizada (1µg/poço) recebeu os seguintes tratamentos: plasminogênio+uPA+Substrato específico, ou controles nos quais um dos componentes foi omitido. BSA e proteína Lsa63 foram empregadas como controle negativo. As barras representam o valor médio de absorbância no comprimento de onda de 405 nm, como uma medida da clivagem do substrato, $\pm$ o desvio padrão de quatro replicatas e são representativas de dois experimentos independentes. Significância estatística em comparação ao BSA avaliada por *Student's two tailed t test* (* $P < 0,05$). PLG: plasminogênio. uPA: ativador do PLG tipo uroquinase. S: substrato cromogênico específico para a plasmina.

Dessa forma, esse dado sugere que a proteína Lsa33 tenha um papel na patogênese e na invasão da bactéria nos tecidos, pois já foi demonstrado que vários patógenos empregam proteases do hospedeiro para degradar componentes da MEC e penetrar nos tecidos. Este sistema já foi descrito para outras espiroquetas como *Borrelia* spp. e *T. denticola* (COLEMAN; ROEMER; BENACH, 1999; LAHTENMAKI; KUUSELA; KORHONEN, 2001).

4.19 Tratamento dos componentes glicoproteicos com metaperiodato de sódio

O papel dos carboidratos da laminina, do C4bp, e do Fg, na interação com as proteínas recombinantes foi investigado através do tratamento destes componentes com metaperiodato de sódio. Esse reagente oxida o grupamento hidroxil dos carboidratos sem prejudicar a estrutura da cadeia polipeptídica (WOODWARD et al., 1985).

Para a proteína Lsa25, a oxidação dos resíduos de carboidratos da laminina e do C4bp provocou uma redução na interação com estes componentes, efeito não verificado para a

proteína Lsa33, que também foi avaliada na ligação ao Fg e também não apresentou redução na ligação (Figura 34).

Figura 34 - Tratamento da laminina, do C4bp e do Fg com metaperiodato de sódio

Laminina (A), C4bp (B) e Fg (C) imobilizados foram tratados com metaperiodato de sódio (5 - 100 mM) durante 15 minutos a 4° C no escuro antes da interação com as proteínas Lsa33 e Lsa25. Os valores de absorvância a 492 nm $\pm$ o desvio padrão de três experimentos independentes foram comparados com aqueles obtidos com os componentes não tratados (0 mM) por *Student's two tailed t test* (* P < 0,05).

De acordo com os dados obtidos, os resíduos de carboidratos da laminina e do C4bp são importantes na ligação destes componentes à proteína Lsa25, mas sem completa participação neste processo.

4.20 Ensaio de inibição da formação do coágulo de fibrina

Recentemente, a proteína LigB de leptospiros foi demonstrada como ligante ao Fg, sendo capaz de inibir a formação do coágulo de fibrina e a agregação plaquetária, sugerindo que há nas leptospiros um mecanismo de regulação da coagulação sanguínea do hospedeiro, o

que poderia explicar os quadros hemorrágicos observados em casos clínicos de leptospirose (CHOY et al., 2011; LIN et al., 2011).

Assim sendo, avaliou-se a possível interferência na formação do coágulo de fibrina pela proteína Lsa33. Portanto, realizou-se um ensaio no qual o Fg solúvel pré-incubado com Lsa33 induziu a conversão para fibrina insolúvel na reação catalisada pela Tb. A formação do coágulo foi detectada pelo aumento da densidade ótica por um período de 45 minutos. Assim como ocorreu na presença da proteína LigB (CHOY et al., 2011; LIN et al., 2011), os resultados apresentados pela figura 35 mostram inibição na formação do coágulo de fibrina exercido pela presença da proteína Lsa33. Esta inibição foi estatisticamente significativa já a partir de 2 minutos, com redução de 44% na formação do coágulo ao final do ensaio.

Os dados obtidos sugerem que esta proteína pode estar envolvida então na patogenicidade das leptospirosas, já que, ao se ligar ao Fg, a Lsa33 provocou inibição da conversão deste componente em fibrina, interferindo no processo de coagulação e facilitando a disseminação da bactéria.

Figura 35 - Efeito da proteína recombinante Lsa33 na formação do coágulo de fibrina induzido por trombina

Após pré-incubação do Fg (1 mg/mL) com a proteína Lsa33 (3 μ M) por 2 horas a 37 °C, adicionou-se Tb (10 U/mL) para análise da formação do coágulo, o qual foi quantificada pelo aumento da densidade ótica durante 45 minutos. Os dados são representativos de dois experimentos independentes. Cada ponto foi realizado em triplicata, e expresso como o valor médio de absorbância a 595 nm $\pm$ desvio padrão para cada ponto (A). Porcentagem da formação do coágulo de fibrina após 45 minutos. Barras representam a absorbância a 595 nm $\pm$ o desvio padrão de três replicatas e são representativas de dois experimentos independentes (B). Significância estatística em relação ao controle positivo avaliada por *Student's two tailed t test* (* $P < 0,05$). Fb – fibrinogênio; Tb – trombina.

4.21 Ensaio de inibição da ligação das proteínas recombinantes aos componentes pela incubação destas proteínas com antissoro específico

Foi avaliada a possível inibição da interação entre as proteínas recombinantes e seus ligantes por meio da incubação das proteínas com antissoro gerado contra cada uma destas.

A pré-incubação da proteína recombinante Lsa33 com seu antissoro específico inibiu totalmente a interação entre a proteína e os componentes (Figura 36A). Este efeito inibitório da ligação exercido pela pré-incubação da proteína com seu antissoro específico também foi verificado para a proteína Lsa25 e o C4bp. Somente a interação entre a proteína Lsa25 e a laminina ainda se manteve de forma significativa mesmo após o tratamento, apesar de ter ocorrido também certo efeito inibitório considerável na ligação (Figura 36B). Estes dados sugerem que alguns epítomos antigênicos contidos nestas proteínas recombinantes estão envolvidos na interação com os componentes ou localizados próximos das regiões de interação.

Figura 36 - Efeito na ligação das proteínas recombinantes aos componentes pela incubação destas proteínas recombinantes com antissoro específico

As proteínas recombinantes Lsa33 (A) e Lsa25 (B) foram incubadas com os antissoros específicos e então adicionadas à placa de ELISA para interação com os componentes previamente imobilizados: laminina, PLG, C4bp e Fg. A interação foi detectada pela incubação com anticorpo monoclonal anti-His conjugado com peroxidase. A média dos valores de absorvância obtidos pela ligação das proteínas previamente tratadas com antissoros específicos aos componentes foi comparada com a média dos valores de absorvância obtidos pela ligação das proteínas as quais não sofreram tal tratamento, pelo *Student's two tailed t test* (* $P < 0,05$, ** $P < 0,005$).

4.22 Ensaio de inibição da ligação das proteínas recombinantes aos componentes por desnaturação térmica destas proteínas recombinantes

A importância da estrutura das proteínas recombinantes na interação com os componentes também foi avaliada por tratamento por fervura das mesmas para que ocorresse a desnaturação, e, então, incubação com o componente de interesse, para verificar a ligação.

Para ambas as proteínas recombinantes, houve uma redução expressiva e significativa nas interações com a laminina e com o C4bp, o que evidencia a importância estrutural das proteínas nas interações com estes componentes, ou seja, os domínios de ligação são preferencialmente conformacionais (Figuras 37A e 37B).

Verificou-se reduções significativas também nas interações entre Lsa33 e PLG e Lsa33 e Fg (Figura 37A), porém foram pouco expressivas, sugerindo que estas interações se dá principalmente por domínios lineares.

Figura 37 - Efeito na ligação das proteínas recombinantes aos componentes por desnaturação proteica

As proteínas recombinantes Lsa33 (A) e Lsa25 (B) foram fervidas por 10 minutos e então adicionadas à placa de ELISA para interação com os componentes previamente imobilizados. A detecção da ligação foi realizada por antissoro específico contra cada proteína recombinante. As médias da ligação foram comparadas com a situação onde as recombinantes não foram aquecidas, considerada o máximo de ligação, pelo *Student's two tailed t test* (* $P < 0,05$).

4.23 Inibição da ligação das leptospiiras vivas aos componentes que se ligam às proteínas recombinantes

Já foi demonstrado que algumas proteínas recombinantes (BARBOSA et al., 2006; MENDES et al., 2011; OLIVEIRA et al., 2011; VIEIRA et al., 2010b) exibem um efeito inibitório na ligação de leptospiiras à macromoléculas. Assim sendo, realizou-se um experimento similar, no qual os componentes imobilizados em placas de ELISA foram incubados com concentrações crescentes das proteínas recombinantes Lsa33 e Lsa25 (0 a 10 µg), e subsequentemente com as leptospiiras.

Os dados mostram que a adição de concentrações crescentes de Lsa33 reduz a ligação de leptospiiras às moléculas de laminina, de PLG e de Fg, de forma significativa (já a partir de 1,25 µg) e dose-dependente (Figuras 38A, 38B e 38C). O mesmo efeito também é visto pela adição de concentrações crescentes de Lsa25, o qual reduziu a ligação de leptospiiras às moléculas de laminina de forma significativa (já a partir de 1,25 µg) e dose-dependente (Figura 39A). Porém, estas reduções não foram totais ao fim do experimento, sendo considerada redução moderada, o que pode ser explicado pela redundância funcional apresentada pela leptospira, a qual possui uma série de receptores já descritos para a laminina e para o PLG, e possivelmente também para o Fg, apesar de até o momento somente dois ligantes de leptospira para este componente foram descritos.

Não foi observado redução da ligação somente ao incubar as leptospiiras ao C4bp pré-incubado com as proteínas (Figuras 38D e 39B), sugerindo que estas proteínas na superfície da bactéria tenham pouca participação efetiva na interação com o C4bp, ou ainda que a proteína recombinante e a nativa possuam avidéz diferente de ligação a este componente. Este efeito de não inibição da ligação das leptospiiras também foi observado por outros autores, como no caso da proteína recombinante OmpL37, que se liga à elastina, e não foi capaz de inibir a adesão das leptospiiras a este mesmo componente (PINNE; CHOY; HAAKE, 2010).

Figura 38 - Inibição da ligação de *L. interrogans* à laminina e aos componentes do soro e do plasma pela Lsa33

Os componentes laminina (A), PLG (B), Fg (C), e C4bp (D) foram adsorvidos em placas de ELISA e incubados com concentrações crescentes de Lsa33 (0 a 10 µg). *Leptospiras* vivas (100 µL/poço de 4×10^7 *L. interrogans* sorovar Copenhageni) foram adicionadas. A quantificação das leptospiras ligadas foi realizada indiretamente por anticorpos anti-LipL32 produzidos em camundongo. Dados representativos de dois experimentos independentes. A significância das reduções foi feita pelo *Student's two tailed t test* (* P < 0,05, ** P < 0,005).

Figura 39 - Inibição da ligação de *L. interrogans* à laminina e ao C4bp pela Lsa25

Os componentes laminina (A) e C4bp (B) foram adsorvidos em placas de ELISA e incubados com concentrações crescentes de Lsa25 (0 a 10 µg). *Leptospiras* vivas (100 µL/poço de 4×10^7 *L. interrogans* sorovar Copenhageni) foram adicionadas. A quantificação das leptospiras ligadas foi realizada indiretamente por anticorpos anti-LipL32 produzidos em camundongo. Dados representativos de dois experimentos independentes. A significância das reduções foi feita pelo *Student's two tailed t test* (* $P < 0,05$).

4.24 Interferência entre os componentes na ligação às proteínas recombinantes

A proteína Lsa33 apresentou ligação significativa à laminina, ao PLG, ao Fg e ao C4bp. Assim sendo, foi avaliado se esses componentes competem pelo mesmo sítio de ligação à Lsa33 ou interferem de algum modo na interação.

Os ensaios de interferência na ligação foram realizados na presença de uma massa fixa de um dos componentes e massas crescentes do outro (do competidor - 0 a 1 µg). Os resultados (Figuras 40A, 40B, 40C e 40D) mostram que há interferência na ligação na presença de laminina e PLG, laminina e Fg, e laminina e C4bp de modo dose-dependente, tanto quando há adição da laminina, diminuindo assim a ligação dos outros componentes a Lsa33, quanto quando há adição dos outros componentes, diminuindo assim a ligação da laminina a Lsa33, sugerindo que ambas as moléculas competem pelo mesmo sítio de ligação na proteína recombinante ou uma molécula, quando ligada à proteína recombinante, interfere de alguma forma na acessibilidade do outro componente a esta mesma proteína recombinante. E um comportamento similar foi observado também na presença de PLG e Fg (Figuras 40B e 40C).

Pode-se verificar que Fg e C4bp apresentam sítios de ligação distintos (Figuras 40C e 40D), uma vez que a adição de massas crescentes de um componente não interferiu na ligação do outro à proteína recombinante imobilizada. Já o PLG interferiu na ligação do C4bp à proteína recombinante Lsa33 (Figura 40B), sugerindo que, uma vez ligado à proteína, o PLG interfere na acessibilidade do C4bp à proteína recombinante. Porém, este efeito não é observado na presença de uma massa fixa de PLG e massas crescentes de C4bp, pois, apesar de uma redução da ligação, esta não é significativa, provavelmente devido à menor afinidade do C4bp a esta proteína recombinante (verificado pelo experimento de ligação de dose-dependência do C4bp à Lsa33 apresentado no item 4.16.2), o que poderia explicar também a redução significativa da ligação da laminina à Lsa33 na presença de somente 1µg de C4bp (Figura 40D).

Figura 40 - Interferência entre os componentes na ligação à Lsa33

A proteína recombinante Lsa33 foi imobilizada (1 µg/poço) e incubada com um componente com massa fixa (1 µg em 100 µL PBS) acrescido de massas crescentes de outro componente, com o qual se deseja verificar a competição. Em (A) tem-se o experimento feito com massa fixa de PLG, Fg e C4bp e massas crescentes de laminina, em (B) massa fixa de laminina, Fg e C4bp, e massas crescentes de PLG, em (C) massa fixa de laminina, PLG e C4bp, e massas crescentes de Fg, e em (D) massa fixa de laminina, PLG e Fg, e massas crescentes de C4bp. A detecção foi feita com o uso de anticorpo contra o componente que possui massa fixa, utilizando uma diluição que apresentou OD 492 nm igual a 1 em experimentos de titulação. A significância estatística foi calculada pelo *Student's two tailed t test* por meio da comparação com o tratamento apenas contendo o componente com concentração fixa (* $P < 0,05$, ** $P < 0,005$).

Já a proteína Lsa25 apresentou ligação significativa à laminina e ao C4bp. Assim sendo, foi avaliado se esses componentes competem pelo mesmo sítio de ligação à Lsa25 ou interferem de algum modo na interação.

O ensaio de interferência na ligação foi realizado na presença de uma massa fixa de um dos componentes e massas crescentes do outro (do competidor - 0 a 1 µg), e os resultados mostram que a laminina e o C4bp apresentam sítios de ligação distintos (Figuras 41A e 41B),

uma vez que a adição de massas crescentes de um componente não interferiu na ligação do outro à proteína recombinante imobilizada.

Figura 41 - Interferência entre os componentes na ligação à Lsa25

A proteína recombinante Lsa25 foi imobilizada (1 μg/poço) e incubada com um componente com massa fixa (1 μg em 100 μL PBS) acrescido de massas crescentes de outro componente, com o qual se deseja verificar a competição. Em (A) tem-se o experimento feito com massa fixa de C4bp e massas crescentes de laminina, e em (B) massa fixa de laminina, e massas crescentes de C4bp. A detecção foi feita com o uso de anticorpo contra o componente fixo, utilizando uma diluição que apresentou OD 492 nm igual a 1 em experimentos de titulação. A significância estatística foi calculada pelo *Student's two tailed t test* por meio da comparação com o tratamento apenas contendo o componente com concentração fixa.

4.25 Ensaio de imunoproteção

A identificação de proteínas imunogênicas de *Leptospira* como candidatos vacinais tornou-se o principal foco na pesquisa com leptospiros (HAAKE et al., 2000; HAAKE et al., 1999; OLIVEIRA et al., 2010; PALANIAPPAN et al., 2006), já que as vacinas representam importante estratégia de prevenção de doenças com melhor custo-efetivo (SILVA et al., 2007). As vacinas contra a leptospirose disponíveis no mercado são basicamente veterinárias e compostas de bactérias inativadas ou preparações de membrana de leptospiros patogênicos (ADLER; DE LA PENA MOCTEZUMA, 2010; ATZINGEN et al., 2010; KO; GOARANT; PICARDEAU, 2009). Apenas alguns países possuem vacinas contra a leptospirose licenciadas para uso humano (ADLER; DE LA PENA MOCTEZUMA, 2010; KOIZUMI; WATANABE, 2005). Estas vacinas conferem proteção induzindo a produção de anticorpos contra os LPS presentes na membrana das leptospiros, o que produz apenas uma resposta imune de curta duração. Além disso, é importante ressaltar que existem variações da

composição dos carboidratos do LPS das leptospiras baseado na expressão diferenciada destes LPS de superfície, que resulta em cerca de 250 sorovares de leptospiras patogênicas (EVANGELISTA; COBURN, 2010; KOIZUMI; WATANABE, 2005). Sendo assim, as vacinas disponíveis conferem imunidade sorovar-específica, não sendo capazes de proteger contra sorovares não inclusos em sua composição (ADLER; DE LA PENA MOCTEZUMA, 2010; ATZINGEN et al., 2010; GAMBERINI et al., 2005; KO; GOARANT; PICARDEAU, 2009; KOIZUMI; WATANABE, 2005).

Por causa das limitações encontradas nas vacinas contra a leptospirose disponíveis atualmente no mercado, têm sido desenvolvidas novas estratégias para a identificação de antígenos que sejam conservados e capazes de conferir proteção contra diferentes sorovares.

Devido a sua localização, proteínas associadas à membrana externa são importantes na relação patógeno-hospedeiro por estarem envolvidas nos processos de adesão e colonização, além de serem alvos potenciais para o sistema imunológico do hospedeiro (ATZINGEN et al., 2010; CAO et al., 2011; GAMBERINI et al., 2005; KOIZUMI; WATANABE, 2005).

Vários grupos de pesquisa vem testando a atividade imunoprotetora de proteínas associadas à membrana externa, como por exemplo, OmpL1 e LipL41 (HAAKE et al., 1999), LipL32 (BRANGER et al., 2005; LUCAS et al., 2011; SEIXAS et al., 2007), LigA e LigB (CAO et al., 2011; KOIZUMI; WATANABE, 2005; PALANIAPPAN et al., 2006; SILVA et al., 2007; YAN et al., 2009) e proteínas OmpA-like (YAN et al., 2010).

Nosso grupo também vem testando a atividade de proteínas recombinantes com probabilidade de serem expostas à superfície, e demonstrado que algumas são capazes de conferir proteção parcial em animais desafiados com dose letal de leptospiras, como rLIC12730, rLIC10494 e rLIC12922 (ATZINGEN et al., 2010).

Portanto para avaliar a atividade imunoprotetora das proteínas Lsa33 e Lsa25, hamsters foram imunizados com duas doses destas proteínas recombinantes e desafiados com 4×10^7 de *L. interrogans* sorovar Kennewicki cepa Pomona Fromm virulenta. Os hamsters foram utilizados como modelo animal deste ensaio por serem altamente suscetíveis a infecção por leptospiras, e por exibirem características que mimetizam a infecção severa em humanos (HAAKE, 2006).

Para analisar a resposta humoral dos animais imunizados com as proteínas Lsa33 e Lsa25, além daqueles imunizados com PBS, dois hamsters de cada grupo foram sangrados via punção cardíaca, 15 dias após a segunda imunização. A média dos títulos de anticorpos obtidos após as imunizações com Lsa33 foi de 1:6.400 para o experimento 1 e 1:1.600 para o experimento 2, e com Lsa25 foi de 1:800 para ambos os experimentos. Também foram

analisados os soros obtidos dos animais que sobreviveram ao desafios, e a média dos títulos de anticorpos dos soros dos animais imunizados com Lsa33 para o experimento 2 foi de 1:3.200, apresentando ligeiro aumento em comparação com a titulação antes do desafio (não houveram sobreviventes para o experimento 1 para proteína Lsa33), e a média dos títulos de anticorpos dos soros obtidos dos animais imunizados com Lsa25 para o experimento 1 foi de 1:800, se mantendo igual à titulação antes do desafio, e para o experimento 2 foi de 1:400, apresento ligeiro declínio em comparação com a titulação antes do desafio. Embora a razão para o declínio dos títulos de anticorpos ser desconhecida, pode-se especular que ocorreu uma mudança na resposta da subclasse IgG, ou por se tratar de animais não isogênicos, a resposta imune pode ser diferente (ATZINGEN et al., 2012). Não foi detectada a produção de anticorpos nos animais imunizados com PBS em nenhum dos experimentos realizados.

A figura 42 mostra a curva de sobrevivência dos hamsters imunizados com as proteínas recombinantes Lsa33 e Lsa25, bacterina e PBS e desafiados com as leptospiras virulentas.

Figura 42 - Curva de sobrevivência dos hamsters imunizados com a proteínas recombinantes (A) Lsa33 e (B) Lsa25 e desafiados com *L. interrogans* sorovar Kennewicki cepa Pomona Fromm

B

Cinética de sobrevivência dos animais imunizados com as proteínas Lsa33 (A), Lsa25 (B), bacterina e PBS, em dois experimentos distintos. Hamsters foram imunizados com 50 µg da proteína recombinante por dose, totalizando duas doses no intervalo de 15 dias cada, sendo então desafiados por inoculação intraperitoneal com leptospiros virulentas e acompanhados por 21 dias quanto à morte ou aparecimento dos sintomas típicos de leptospirose.

Os resultados mostram que a proteína Lsa33 conferiu proteção parcial de 41,5% aos animais desafiados com dose letal de leptospiros virulentas no experimento 2, enquanto que não houveram sobreviventes no experimento 1. A bacterina foi capaz de conferir 100% de proteção em ambos os experimentos, e os animais imunizados com PBS apresentaram 8,3% e 25% de sobreviventes para os experimentos 1 e 2, respectivamente. A análise da cinética das curvas de sobrevivência pelo método de Kaplan e Meyer (1958) revelou que, apesar da proteção conferida por Lsa33 no segundo experimento, esta não foi significativa (experimento 1: $P = 0,3996$; experimento 2: $P = 0,4880$; * $P < 0,05$ é considerado estatisticamente significativo).

Os resultados mostram também que a proteína Lsa25 conferiu proteção modesta de 20% aos animais desafiados com dose letal de leptospiros virulentas no experimento 1. A bacterina foi capaz de conferir 100% de proteção, enquanto que não houveram sobreviventes no grupo PBS. No segundo experimento, 30% dos animais imunizados com Lsa25 sobreviveram. A bacterina novamente conferiu 100% de proteção, enquanto que 20% dos animais imunizados com PBS sobreviveram. A análise da cinética das curvas de sobrevivência pelo método de Kaplan e Meyer (1958) revelou que, apesar da proteção conferida por Lsa25, esta não foi significativa em ambos os experimentos (experimento 1: $P = 0,064$; experimento 2: $P = 0,3784$; * $P < 0,05$ é considerado estatisticamente significativo).

A existência de sobreviventes no grupo de animais não vacinados e as dificuldades encontradas na reprodução de resultados em ensaios de desafio tem sido descritos pelo nosso grupo e por outros grupos de pesquisa (ATZINGEN et al., 2010; BRANGER et al., 2001; PALANIAPPAN et al., 2006).

Os hamsters que sobreviveram ao ensaio foram sacrificados e tiveram seus rins coletados para cultura e isolamento de leptospiros. Foi verificado que o efeito da bacterina foi esterilizante em todos os experimentos. Em contrapartida, as proteínas Lsa33 e Lsa25 apresentaram um efeito parcialmente esterilizante, uma vez que foram detectadas leptospiros nos rins de alguns sobreviventes.

Esses resultados demonstram que a proteção conferida pelas proteínas em estudo não foram significativas, e que todos os experimentos podem ser considerados válidos, pois a proteção conferida pela bacterina foi significativa em relação ao PBS.

5 CONCLUSÃO

Os resultados sugerem que as proteínas nativas correspondentes às proteínas Lsa33 e Lsa25 deste estudo são expressas na membrana externa da *Leptospira interrogans* sorovar Copenhageni e estão sendo possivelmente expressas durante a infecção no hospedeiro humano.

As proteínas recombinantes não apresentaram atividade imunoprotetora, porém mostraram ligação à laminina, o que pode auxiliar na adesão ao hospedeiro, e ao C4bp, o que pode auxiliar na evasão imune. Além disso, a proteína Lsa33 mostrou ainda ligação ao PLG, que foi convertido a plasmina e, portanto, pode auxiliar na penetração dos tecidos, e ao Fg, o que pode interferir no processo de coagulação sanguínea e disseminação das leptospiros.

Dessa forma, acreditamos que estas proteínas multifuncionais podem interagir com proteínas do hospedeiro e ter participação na patogênese da doença. Vale ressaltar que Lsa33 foi a primeira proteína descrita que se ligou à laminina, ao PLG, ao C4bp e ao Fg *in vitro*.

REFERÊNCIAS*

- ADLER, B.; DE LA PENA MOCTEZUMA, A. Leptospira and leptospirosis. **Vet. Microbiol.**, v. 140, n. 3-4, p. 287-296, 2010.
- AHMED, N. et al. Multilocus sequence typing method for identification and genotypic classification of pathogenic Leptospira species. **Ann. Clin. Microbiol Antimicrob.**, v. 5, p. 28, 2006.
- ALTSCHUL, S. F. et al. Basic local alignment search tool. **J. Mol. Biol.**, v. 215, n. 3, p. 403-410, 1990.
- _____. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Res.**, v. 25, n. 17, p. 3389-3402, 1997.
- AMSEN, D.; SPILIANAKIS, C. G.; FLAVELL, R. A. How are T(H)1 and T(H)2 effector cells made? **Curr. Opin. Immunol.**, v. 21, n. 2, p. 153-160, 2009.
- ATZINGEN, M. V. et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. **BMC Microbiol.**, v. 8, p. 70, 2008.
- _____. Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. **J. Infect.**, v. 59, n. 4, p. 264-276, 2009.
- _____. Characterization of leptospiral proteins that afford partial protection in hamsters against lethal challenge with Leptospira interrogans. **J. Med. Microbiol.**, v. 59, pt. 9, p. 1005-1015, 2010.
- _____. Evaluation of immunoprotective activity of six leptospiral proteins in the hamster model of leptospirosis. **Open Microbiol. J.**, v. 6, p. 79-87, 2012.
- BAMFORD, C. V. et al. The chymotrypsin-like protease complex of Treponema denticola ATCC 35405 mediates fibrinogen adherence and degradation. **Infect. Immun.**, v. 75, n. 9, p. 4364-4372, 2007.
- BANEYX, F. Recombinant protein expression in Escherichia coli. **Curr. Opin. Biotechnol.**, v. 10, n. 5, p. 411-421, 1999.
- BARBOSA, A. S. et al. A newly identified leptospiral adhesin mediates attachment to laminin. **Infect. Immun.**, v. 74, n. 11, p. 6356-6364, 2006.
- _____. Immune evasion of leptospira species by acquisition of human complement regulator C4BP. **Infect. Immun.**, v. 77, n. 3, p. 1137-1143, 2009.

* De acordo com:

ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. **NBR 6023:** Informação e documentação: referências: elaboração. Rio de Janeiro, 2002.

_____. Functional characterization of LcpA, a surface-exposed protein of *Leptospira* spp. that binds the human complement regulator C4BP. **Infect. Immun.**, v. 78, n. 7, p. 3207-3216, 2010.

BENSCHOP, J. et al. Sero-prevalence of leptospirosis in workers at a New Zealand slaughterhouse. **N. Z. Med. J.**, v. 122, n. 1307, p. 39-47, 2009.

BHANDARI, P.; GOWRISHANKAR, J. An *Escherichia coli* host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. **Journal of Bacteriology**, v. 179, p. 4403-4406, 1997.

BHARTI, A. R. et al. Leptospirosis: a zoonotic disease of global importance. **Lancet. Infect. Dis.**, v. 3, n. 12, p. 757-771, 2003.

BRANGER, C. et al. Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. **Infect. Immun.**, v. 73, n. 7, p. 4062-4069, 2005.

_____. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. **Infect. Immun.**, v. 69, n. 11, p. 6831-6838, 2001.

BRASIL. Ministério da Saúde. Secretaria de Vigilância em Saúde. **Guia leptospirose: diagnóstico e manejo clínico**. Brasília, 2009. 32 p.

BRENNER, D. J. et al. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. **Int. J. Syst. Bacteriol.**, v. 49, pt 2, p. 839-858, 1999.

BROWN, P. D. et al. Evaluation of the polymerase chain reaction for early diagnosis of leptospirosis. **J. Med. Microbiol.**, v. 43, n. 2, p. 110-114, 1995.

BULACH, D. M. et al. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. **Proc. Natl. Acad. Sci. U S A**, v. 103, n. 39, p. 14560-14565, 2006.

CAMERON, C. E. Identification of a *Treponema pallidum* laminin-binding protein. **Infect. Immun.**, v. 71, n. 5, p. 2525-2533, 2003.

CAO, Y. et al. Evaluation of novel fusion proteins derived from extracellular matrix binding domains of LigB as vaccine candidates against leptospirosis in a hamster model. **Vaccine**, v. 29, n. 43, p. 7379-7386, 2011.

CARVALHO, E. et al. Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. **FEBS Lett.**, v. 583, n. 8, p. 1381-1385, 2009.

CASTIBLANCO-VALENCIA, M. M. et al. Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. **J. Infect. Dis.**, v. 205, n. 6, p. 995-1004, 2012.

CHAGAS-JUNIOR, A. D. et al. Detection and quantification of *Leptospira interrogans* in hamster and rat kidney samples: immunofluorescent imprints versus real-time PCR. **PLoS One**, v. 7, n. 2, p. e32712, 2012.

CHOU, L. F. et al. Sequence of *Leptospira santarosai* serovar Shermani genome and prediction of virulence-associated genes. **Gene**, v. 511, n. 2, p. 364-370, 2012.

CHOY, H. A. et al. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. **Infect. Immun.**, v. 75, n. 5, p. 2441-2450, 2007.

_____. The multifunctional LigB adhesin binds homeostatic proteins with potential roles in cutaneous infection by pathogenic *Leptospira interrogans*. **PLoS One**, v. 6, n. 2, p. e16879, 2011.

COLEMAN, J. L.; ROEMER, E. J.; BENACH, J. L. Plasmin-coated borrelia Burgdorferi degrades soluble and insoluble components of the mammalian extracellular matrix. **Infect. Immun.**, v. 67, n. 8, p. 3929-3936, 1999.

CULLEN, P. A. et al. Surfaceome of *Leptospira* spp. **Infect. Immun.**, v. 73, n. 8, p. 4853-4863, 2005.

CUMBERLAND, P.; EVERARD, C. O.; LEVETT, P. N. Assessment of the efficacy of an IgM-elisa and microscopic agglutination test (MAT) in the diagnosis of acute leptospirosis. **Am. J. Trop. Med. Hyg.**, v. 61, n. 5, p. 731-734, 1999.

DESAI, S. et al. Resurgence of field fever in a temperate country: an epidemic of leptospirosis among seasonal strawberry harvesters in Germany in 2007. **Clin. Infect. Dis.**, v. 48, n. 6, p. 691-697, 2009.

DOMINGOS, R. F. et al. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. **BMC Microbiol.**, v. 12, p. 50, 2012.

ELLINGHAUSEN, H. C., JR.; MCCULLOUGH, W. G. Nutrition of *Leptospira Pomona* and Growth of 13 Other Serotypes: Fractionation of Oleic Albumin Complex and a Medium of Bovine Albumin and Polysorbate 80. **Am. J. Vet. Res.**, v. 26, p. 45-51, 1965.

EVANGELISTA, K. V.; COBURN, J. *Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune responses. **Future Microbiol.**, v. 5, n. 9, p. 1413-1425, 2010.

FAINE, S. et al., Eds. **Leptospira and Leptospirosis**. MediSci. Melbourne, Australia, MediScied. 1999.

FERNANDES, L. G. et al. Functional and immunological evaluation of two novel proteins of *Leptospira* spp. **Microbiology**, 2013.

_____. OmpL1 is an extracellular matrix- and plasminogen-interacting protein of *Leptospira* spp. **Infect. Immun.**, v. 80, n. 10, p. 3679-3692, 2012.

FONSECA, C. D. A. et al. Leptospira DNA detection for the diagnosis of human leptospirosis. **J. Infect.**, v. 52, n. 1, p. 15-22, 2006.

FRASER, C. M. et al. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. **Nature**, v. 390, n. 6660, p. 580-586, 1997.

_____. Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. **Science**, v. 281, n. 5375, p. 375-388, 1998.

FUNDAÇÃO OSWALDO CRUZ. **Diagnóstico rápido para leptospirose**. Rio de Janeiro, 2010. Disponível em: < <http://www.bio.fiocruz.br/index.php/produtos/reativos/testes-rapidos/dppr-leptospirose> > Acesso em: 10 set. 2012.

GAMBERINI, M. et al. Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. **FEMS Microbiol. Lett.**, v. 244, n. 2, p. 305-313, 2005.

GASTEIGER, J. Chemoinformatics: a new field with a long tradition. **Anal. Bioanal. Chem.**, v. 384, n. 1, p. 57-64, 2006.

GREENFIELD, N. J. Using circular dichroism spectra to estimate protein secondary structure. **Nat. Protoc.**, v. 1, n. 6, p. 2876-2890, 2006.

GRUBER, A.; ZINGALES, B. Alternative method to remove antibacterial antibodies from antisera used for screening of expression libraries. **Biotechniques**, v. 19, p. 28-30, 1995.

HAAKE, D. A. Hamster model of leptospirosis. **Curr. Protoc. Microbiol.**, v. Chapter 12, p. Unit 12E 12, 2006.

HAAKE, D. A. et al. The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. **Infect. Immun.**, v. 68, n. 4, p. 2276-2285, 2000.

HAAKE, D. A.; MATSUNAGA, J. Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. **Infect. Immun.**, v. 70, n. 9, p. 4936-4945, 2002.

_____. *Leptospira*: a spirochaete with a hybrid outer membrane. **Mol. Microbiol.**, 2010.

HAAKE, D. A. et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. **Infect. Immun.**, v. 67, n. 12, p. 6572-6582, 1999.

HOKE, D. E. et al. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. **Infect. Immun.**, v. 76, n. 5, p. 2063-2069, 2008.

JOHNSON, R. C.; ROGERS, P. Metabolism of leptospires. II. The action of 8-azaguanine. **Can. J. Microbiol.**, v. 13, n. 12, p. 1621-1629, 1967.

JOST, B. H. et al. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. **J. Med. Microbiol.**, v. 22, n. 3, p. 269-275, 1986.

JUNCKER, A. S. et al. Prediction of lipoprotein signal peptides in Gram-negative bacteria. **Protein Sci.**, v. 12, n. 8, p. 1652-1662, 2003.

KAPLAN, E. L.; MEIER, P. Nonparametric estimation from incomplete observations. **Journal of the American Statistical Association**, v. 53, p. 457-481, 1958.

KELLY, S. M.; JESS, T. J.; PRICE, N. C. How to study proteins by circular dichroism. **Biochim. Biophys. Acta.**, v. 1751, n. 2, p. 119-139, 2005.

KO, A. I. et al. Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. **Lancet.**, v. 354, p. 820-825, 1999.

KO, A. I.; GOARANT, C.; PICARDEAU, M. Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen. **Nat. Rev. Microbiol.**, v. 7, n. 10, p. 736-747, 2009.

KOIZUMI, N.; KAWABATA, H.; WATANABE, H. Probable laboratory contamination of clinical specimens with *Leptospira meyeri*. **Microbiol. Immunol.**, v. 47, n. 4, p. 305-306, 2003.

KOIZUMI, N.; WATANABE, H. Leptospirosis vaccines: past, present, and future. **J. Postgrad. Med.**, v. 51, n. 3, p. 210-214, 2005.

KOSITANONT, U. et al. Detection and differentiation between pathogenic and saprophytic *Leptospira* spp. by multiplex polymerase chain reaction. **Diagn. Microbiol. Infect. Dis.**, v. 57, n. 2, p. 117-122, 2007.

LAHTENMAKI, K.; KUUSELA, P.; KORHONEN, T. K. Bacterial plasminogen activators and receptors. **FEMS Microbiol. Rev.**, v. 25, n. 5, p. 531-552, 2001.

LANGSTON, C. E.; HEUTER, K. J. Leptospirosis. A re-emerging zoonotic disease. **Vet. Clin. North. Am. Small Anim. Pract.**, v. 33, n. 4, p. 791-807, 2003.

LARKIN, M. A. et al. Clustal W and Clustal X version 2.0. **Bioinformatics**, v. 23, n. 21, p. 2947-2948, 2007.

LAURICHESSE, H. et al. Safety and immunogenicity of subcutaneous or intramuscular administration of a monovalent inactivated vaccine against *Leptospira interrogans* serogroup Icterohaemorrhagiae in healthy volunteers. **Clin. Microbiol. Infect.**, v. 13, n. 4, p. 395-403, 2007.

LETUNIC, I.; DOERKS, T.; BORK, P. SMART 7: recent updates to the protein domain annotation resource. **Nucleic Acids Res.**, v. 40, n. Database issue, p. D302-305, 2012.

LEVETT, P. N. Leptospirosis. **Clin. Microbiol. Rev.**, v. 14, n. 2, p. 296-326, 2001.

_____. Usefulness of serologic analysis as a predictor of the infecting serovar in patients with severe leptospirosis. **Clin. Infect. Dis.**, v. 36, n. 4, p. 447-452, 2003.

LEVETT, P. N. et al. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. **Int. J. Syst. Evol. Microbiol.**, v. 56, n. Pt 3, p. 671-673, 2006.

LIN, Y. P. et al. Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. **J. Biol. Chem.**, v. 284, n. 29, p. 19380-19391, 2009.

_____. Leptospira immunoglobulin-like protein B (LigB) binding to the C-terminal fibrinogen alphaC domain inhibits fibrin clot formation, platelet adhesion and aggregation. **Mol. Microbiol.**, v. 79, n. 4, p. 1063-1076, 2011.

LJUNGH, A.; MORAN, A. P.; WADSTROM, T. Interactions of bacterial adhesins with extracellular matrix and plasma proteins: pathogenic implications and therapeutic possibilities. **FEMS Immunology & Medical Microbiology**. v. 16, p. 117-126, 1996.

LO, M. et al. Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. **Infect. Immun.**, v. 74, p. 5848-5859, 2006.

LOMAR, A. V.; DIAMENT, D.; TORRES, J. R. Leptospirosis in Latin America. **Infect. Dis. Clin. North. Am.**, v. 14, n. 1, p. 23-39, vii-viii, 2000.

LUCAS, D. S. et al. Recombinant LipL32 and LigA from *Leptospira* are unable to stimulate protective immunity against leptospirosis in the hamster model. **Vaccine**, v. 29, n. 18, p. 3413-3418, 2011.

MANDEL, M.; HIGA, A. Calcium-dependent bacteriophage DNA infection. **J. Mol. Biol.**, v. 53, n. 1, p. 159-162, 1970.

MARTINEZ, R. et al. [Efficacy and safety of a vaccine against human leptospirosis in Cuba]. **Rev. Panam. Salud Publica**, v. 15, n. 4, p. 249-255, 2004.

MATSUNAGA, J. et al. Response of *Leptospira interrogans* to physiologic osmolarity: relevance in signaling the environment-to-host transition. **Infect. Immun.**, v. 75, p. 2864-2874, 2007.

MENDES, R. S. et al. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. **Infect. Immun.**, v. 79, n. 11, p. 4657-4667, 2011.

MERI, T. et al. Regulation of complement activation at the C3-level by serum resistant leptospires. **Microb. Pathog.**, v. 39, n. 4, p. 139-147, 2005.

MERIEN, F. et al. Polymerase chain reaction for detection of *Leptospira* spp. in clinical samples. **J. Clin. Microbiol.**, v. 30, n. 9, p. 2219-2224, 1992.

MINETTE, H. P. Leptospirosis in poikilothermic vertebrates. A review. **Int. J. Zoonoses**, v. 10, n. 2, p. 111-121, 1983.

MOREILLON, P. et al. Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. **Infect. Immun.**, v. 63, n. 12, p. 4738-4743, 1995.

MORGAN, J. et al. Outbreak of leptospirosis among triathlon participants and community residents in Springfield, Illinois, 1998. **Clin. Infect. Dis.**, v. 34, n. 12, p. 1593-1599, 2002.

MURRAY, G. L. et al. Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. **Infect. Immun.**, v. 77, n. 2, p. 810-816, 2009.

NAKAI, K.; HORTON, P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. **Trends Biochem. Sci.**, v. 24, n. 1, p. 34-36, 1999.

NAKAI, K.; KANEHISA, M. Expert system for predicting protein localization sites in gram-negative bacteria. **Proteins**, v. 11, n. 2, p. 95-110, 1991.

NALLY, J. E. et al. Lethal infection of C3H/HeJ and C3H/SCID mice with an isolate of *Leptospira interrogans* serovar copenhageni. **Infect. Immun.**, v. 73, n. 10, p. 7014-7017, 2005.

NASCIMENTO, A. L. et al. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. **J. Bacteriol.**, v. 186, n. 7, p. 2164-2172, 2004a.

_____. Genome features of *Leptospira interrogans* serovar Copenhageni. **Braz. J. Med. Biol. Res.**, v. 37, n. 4, p. 459-477, 2004b.

NEVES, F. O. et al. Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis. **Arch. Microbiol.**, v. 188, n. 5, p. 523-532, 2007.

NICODEMO, A. C. et al. Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. **Am. J. Trop. Med. Hyg.**, v. 56, n. 2, p. 181-187, 1997.

OLIVEIRA, R. et al. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. **PLoS One**, v. 6, n. 7, p. e21962, 2011.

OLIVEIRA, T. R. et al. Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays. **Clin. Vaccine Immunol.**, v. 15, n. 11, p. 1715-1722, 2008.

_____. LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. **Microbes Infect.**, v. 12, n. 3, p. 207-217, 2010.

PALANIAPPAN, R. U. et al. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. **Infect. Immun.**, v. 74, n. 3, p. 1745-1750, 2006.

PATHIRANA, R. D. et al. Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*. **Microbiology**, v. 152, n. Pt 8, p. 2381-2394, 2006.

PEROLAT, P. et al. *Leptospira fainei* sp. nov., isolated from pigs in Australia. **Int. J. Syst. Bacteriol.**, v. 48 Pt 3, p. 851-858, 1998.

PICARDEAU, M.; BRENOT, A.; SAINT GIRONS, I. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa* *flaB* results in non-motile mutants deficient in endoflagella. **Mol. Microbiol.**, v. 40, n. 1, p. 189-199, 2001.

PICARDEAU, M. et al. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. **PLoS One**, v. 3, n. 2, p. e1607, 2008.

PINNE, M.; CHOY, H. A.; HAAKE, D. A. The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. **PLoS Negl. Trop. Dis.**, v. 4, n. 9, p. e815, 2010.

PINNE, M.; HAAKE, D. A. A comprehensive approach to identification of surface-exposed, outer membrane-spanning proteins of *Leptospira interrogans*. **PLoS One**, v. 4, n. 6, p. e6071, 2009.

PIZZA, M. et al. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. **Science**, v. 287, n. 5459, p. 1816-1820, 2000.

PLANK, R.; DEAN, D. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. **Microbes Infect.**, v. 2, n. 10, p. 1265-1276, 2000.

PROMEGA. **pGEM®-T and pGEM®-T Easy Vector Systems Protocol**. Disponível em: <<http://www.promega.com/resources/protocols/technical-manuals/0/pgem-t-and-pgem-t-easy-vector-systems-protocol/>>. Acesso em: 20 ago. 2012.

PUNTA, M. et al. The Pfam protein families database. **Nucleic Acids Res.**, v. 40, n. Database issue, p. D290-301, 2012.

QUE-GEWIRTH, N. L. et al. A methylated phosphate group and four amide-linked acyl chains in *leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR2. **J. Biol. Chem.**, v. 279, n. 24, p. 25420-25429, 2004.

RAMOS, C. R. et al. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. **Braz. J. Med. Biol. Res.**, v. 37, n. 8, p. 1103-1109, 2004.

RAPPUOLI, R. Reverse vaccinology. **Curr. Opin. Microbiol.**, v. 3, n. 5, p. 445-450, 2000.

REED, S. G. et al. New horizons in adjuvants for vaccine development. **Trends Immunol.**, v. 30, n. 1, p. 23-32, 2009.

REN, S. X. et al. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. **Nature**, v. 422, n. 6934, p. 888-893, 2003.

REYES, E. A. et al. [Expression in *Escherichia coli* of the *gspd*(I) gene of the type II secretion system in *Leptospira borgpetersenii* serovariety *hardjo*]. **Rev. Cubana Med. Trop.**, v. 57, n. 1, p. 45-46, 2005.

RICALDI, J. N. et al. Whole genome analysis of *Leptospira licerasiae* provides insight into leptospiral evolution and pathogenicity. **PLoS Negl. Trop. Dis.**, v. 6, n. 10, p. e1853, 2012.

RISTOW, P. et al. The OmpA-like protein Loa22 is essential for leptospiral virulence. **PLoS Pathog.**, v. 3, n. 7, p. e97, 2007.

_____. Biofilm formation by saprophytic and pathogenic leptospires. **Microbiology**, v. 154, n. Pt 5, p. 1309-1317, 2008.

RODRIGUEZ-GONZALEZ, I. et al. [Efficacy of Spirolept vaccine against human leptospirosis as estimated by passive protection of laboratory rodents]. **Med. Mal. Infect.**, v. 34, n. 5, p. 196-200, 2004.

SABAT, R. et al. Biology of interleukin-10. **Cytokine Growth Factor Rev.**, v. 21, n. 5, p. 331-344, 2010.

SANCHEZ, R. M. et al. [Reactogenicity and immunogenicity of Cuban trivalent inactivated vaccine against human leptospirosis in different vaccination schedules]. **Rev. Cubana Med. Trop.**, v. 54, n. 1, p. 37-43, 2002.

SANGER, F.; NICKLEN, S.; COULSON, A. R. DNA sequencing with chain-terminating inhibitors. **Proceedings of the National Academy of Sciences of the United States of America**, v. 74, p. 5463-5467, 1977.

SCHRODER, N. W. et al. Immune responses induced by spirochetal outer membrane lipoproteins and glycolipids. **Immunobiology**, v. 213, n. 3-4, p. 329-340, 2008.

SCHULTZ, J. et al. SMART, a simple modular architecture research tool: identification of signaling domains. **Proc. Natl. Acad. Sci. U S A**, v. 95, n. 11, p. 5857-5864, 1998.

SEIXAS, F. K. et al. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. **Vaccine**, v. 26, n. 1, p. 88-95, 2007.

SHANG, E. S. et al. The rare outer membrane protein, OmpL1, of pathogenic *Leptospira* species is a heat-modifiable porin. **Infect. Immun.**, v. 63, n. 8, p. 3174-3181, 1995.

SILVA, E. F. et al. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. **Vaccine**, v. 25, n. 33, p. 6277-6286, 2007.

_____. Characterization of virulence of *Leptospira* isolates in a hamster model. **Vaccine**, v. 26, n. 31, p. 3892-3896, 2008.

SIQUEIRA, G. H. et al. Characterization of Three Novel Adhesins of *Leptospira interrogans*. **Am. J. Trop. Med. Hyg.**, 2013.

SOUZA, N. M. et al. Lsa30, a novel adhesin of *Leptospira interrogans* binds human plasminogen and the complement regulator C4bp. **Microb. Pathog.**, v. 53, n. 3-4, p. 125-134, 2012.

SRIVASTAVA, S. K. Prospects of developing leptospiral vaccines for animals. **Indian J. Med. Microbiol.**, v. 24, n. 4, p. 331-336, 2006.

STAMM, L. V. et al. Heat shock response of spirochetes. **Infect. Immun.**, v. 59, n. 4, p. 1572-1575, 1991.

STERN, E. J. et al. Outbreak of leptospirosis among Adventure Race participants in Florida, 2005. **Clin. Infect. Dis.**, v. 50, n. 6, p. 843-849, 2010.

STEVENSON, B. et al. Leptospira interrogans endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. **PLoS One**, v. 2, n. 11, p. e1188, 2007.

STUDIER, F. W. et al. Use of T7 RNA polymerase to direct expression of cloned genes. **Methods Enzymol.**, v. 185, p. 60-89, 1990.

TOYOKAWA, T.; OHNISHI, M.; KOIZUMI, N. Diagnosis of acute leptospirosis. **Expert Rev. Anti. Infect. Ther.**, v. 9, n. 1, p. 111-121, 2011.

TRUEBA, G. et al. Cell aggregation: a mechanism of pathogenic Leptospira to survive in fresh water. **Int. Microbiol.**, v. 7, n. 1, p. 35-40, 2004.

VERMA, A. et al. Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. **Infect. Immun.**, v. 78, n. 5, p. 2053-2059, 2010.

_____. LfhA, a novel factor H-binding protein of Leptospira interrogans. **Infect. Immun.**, v. 74, n. 5, p. 2659-2666, 2006.

VIEIRA, M. L. et al. In vitro identification of novel plasminogen-binding receptors of the pathogen Leptospira interrogans. **PLoS One**, v. 5, n. 6, p. e11259, 2010a.

_____. Lsa63, a newly identified surface protein of Leptospira interrogans binds laminin and collagen IV. **J. Infect.**, v. 60, n. 1, p. 52-64, 2010b.

_____. Leptospiral extracellular matrix adhesins as mediators of pathogen-host interactions. **FEMS Microbiol. Lett.**, 2014.

_____. Proteome analysis of Leptospira interrogans virulent strain. **Open Microbiol. J.**, v. 3, p. 69-74, 2009a.

_____. Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. **Infect. Immun.**, v. 77, n. 9, p. 4092-4101, 2009b.

VIJAYACHARI, P.; SUGUNAN, A. P.; SHRIRAM, A. N. Leptospirosis: an emerging global public health problem. **J. Biosci.**, v. 33, n. 4, p. 557-569, 2008.

WERTS, C. et al. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. **Nat. Immunol.**, v. 2, n. 4, p. 346-352, 2001.

WIZEMANN, T. M. et al. Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. **Infect. Immun.**, v. 69, n. 3, p. 1593-1598, 2001.

WOODWARD, M. P.; YOUNG JR., W. W.; BLOODGOOD, R. A. Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. **The Journal of Immunology Methods**, v. 78, p. 143-153, 1985.

WU, W. et al. [16S rRNA gene PCR-SSCP analysis of the reference strains from 15 serovars (14 serogroups) of pathogenic leptospires in China]. **Hua Xi Yi Ke Da Xue Xue Bao**, v. 27, n. 1, p. 17-20, 1996.

YAN, W. et al. Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. **Vaccine**, v. 28, n. 11, p. 2277-2283, 2010.

_____. Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. **Microbes Infect.**, v. 11, n. 2, p. 230-237, 2009.

YAN, Y. et al. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to leptospira. **J. Chin. Med. Assoc.**, v. 66, n. 4, p. 224-230, 2003.

YANAGIHARA, Y. et al. Current status of leptospirosis in Japan and Philippines. **Comp. Immunol. Microbiol Infect. Dis.**, v. 30, n. 5-6, p. 399-413, 2007.

YERSIN, C. et al. Pulmonary haemorrhage as a predominant cause of death in leptospirosis in Seychelles. **Transactions of the Royal Society of Tropical Medicine and Hygiene**. v. 94, p. 71-16, 2000.

APÊNDICES – Artigos Publicados em Periódicos, Capítulo de Livro e Manuscrito Submetido

- APÊNDICE A** – ATZINGEN, M.V. et al. Identification and Characterization of Novel Adhesins in *Leptospira*. In: Antonio Mendez Vilas, Ed. (Org.). **Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology**, v. 1, p. 704-713, 2010.
- APÊNDICE B** – ATZINGEN, M.V. et al. Evaluation of immunoprotective activity of six leptospiral proteins in the hamster model of leptospirosis. **The Open Microbiology Journal**, v. 6, p. 79-87, 2012.
- APÊNDICE C** – VIEIRA, M. L. et al. Plasminogen Binding Proteins and Plasmin Generation on the Surface of *Leptospira* spp.: The Contribution to the Bacteria-Host Interactions. **Journal of Biomedicine and Biotechnology (Online)**, v. 2012, p. 1-17, 2012.
- APÊNDICE D** – DOMINGOS, R. F. et al. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. **BMC Microbiology (Online)**, v. 12, p. 50, 2012.
- APÊNDICE E** – OLIVEIRA, R. et al. Adhesins of *Leptospira interrogans* mediate the interaction to fibrinogen and inhibit fibrin clot formation in vitro. **PLoS. Negl. Trop. Dis.**, v. 7, n. 8, p. e2396, 2013.
- APÊNDICE F** – VIEIRA, M. L. et al. Leptospiral extracellular matrix adhesins as mediators of pathogen-host interactions. **FEMS Microbiol. Lett.**, v. 352, n. 2, p. 129-139, 2014.
- APÊNDICE G** – DOMINGOS, R. F. et al. Mapeamento de genomas bacterianos: sequenciamento e funcional de leptospiros. **Biociência Aplicada à Saúde: Fundamentos e Aplicações**, Editores: Rodrigo Resende e Carlos Ricardo Soccol, Ed. Blucher S/A. Capítulo de livro aceito para publicação.
- APÊNDICE H** – OLIVEIRA, R. et al. The intermediate and C-terminus regions of leptospiral adhesin Lsa66 are responsible for the binding with plasminogen and extracellular matrix components. Artigo submetido (em revisão).
- APÊNDICE I** – DOMINGOS, R. F. et al. Resumo dos resultados da proteína rLIC11089 (em processo de confecção de artigo).

Identification and Characterization of Novel Adhesins in *Leptospira*

M. V. Atzingen¹, G. M. Cerqueira¹, M. L. Vieira^{1,2}, R. Oliveira^{1,2}, T. R. Oliveira¹, R. F. Domingos^{1,2}, A. T. Barros¹, R. S. Mendes^{1,2} and Ana L. T. O. Nascimento^{1,2*}

¹Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, Sao Paulo, SP, Brazil;

²Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Sao Paulo, Brazil;

* corresponding author

Leptospirosis is a worldwide zoonosis of human and veterinary concern, caused by pathogenic spirochetes of the genus *Leptospira*. This work reviews the ability of six putative leptospiral outer membrane proteins to interact with extracellular matrix (ECM) molecules. The chosen genes, LIC10314, LIC10368, LIC12099, LIC12895, LIC12906 and LIC12690, were selected from the genome sequences of *L. interrogans* serovar Copenhageni mostly based on their cellular localization. The purified proteins were evaluated for their binding capacity by ELISA, except for LIC12690 coding sequence that was tested under denaturing conditions by Western blotting. Binding characterization and specificity of the proteins to ECM components were also studied. Our data show that the attachment of the recombinant proteins to ECM molecules was specific, dose-dependent and saturable, fulfilling the properties of a typical receptor-ligand interaction. These novel identified membrane proteins may play a role in mediating adhesion of *L. interrogans* to the hosts and therefore in bacterial pathogenesis.

Keywords: *Leptospira*, recombinant proteins, adhesins.

1. Introduction

Spirochetes are motile, helically shaped bacteria including the genera *Leptospira*, *Leptonema*, *Borrelia* and *Treponema*. *Borrelia* and *Treponema* are the causative agents of Lyme disease, relapsing fever and syphilis, respectively. *Leptospira* consists of a genetically diverse group of pathogenic and non-pathogenic or saprophytic species [1-3]. Leptospirosis is a widespread zoonotic disease: transmission to humans occurs through contact with domestic or wild animal reservoirs or an environment contaminated by their urine. Infection produces a wide spectrum of clinical manifestations. The early-phase of illness is characterized by fever, chills, headache and severe myalgias. Disease progresses in 5 to 15 % of the clinical infections to produce severe multisystem complications such as jaundice, renal failure and hemorrhagic manifestations [3, 4]. In developed countries, leptospirosis is associated with recreational activities [1] while in developing countries it produces large urban epidemics with mortality mainly during the rainy season [5]. Leptospirosis also represents a major economic problem producing abortions, stillbirths, infertility, failure to thrive, reduced milk production and death in animals such as cows, pigs, sheep, goats, horses and dogs [3]. Environmental control measures are difficult to implement because of the long-term survival of pathogenic leptospires in soil and water and the abundance of wild and domestic animal reservoirs [1, 3]. *Leptospira* are classified according to serovar status - more than 250 pathogenic serovars have been identified. Structural heterogeneity in lipopolysaccharide moieties appears to be the basis for the large degree of antigenic variation observed among serovars [1].

Currently available veterinarian vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospires. These types of vaccine confer protective responses through, but not exclusively, the induction of antibodies against leptospiral lipopolysaccharide [6, 7]. However, these vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation. The large number of pathogenic serovars imposes a major limitation to the production of a multi-serovar vaccine and to the development of immunization protocols based on whole cell or membrane preparations.

Leptospira invasiveness is attributed to its ability to disseminate widely within the host during the early stage of infection but, the mechanisms associated with this invasion are poorly understood [8]. Due to their location, surface-associated proteins are likely to be relevant in host-pathogen interactions, hence their potential to elicit several activities, including adhesion. The interaction of pathogens with the extracellular matrix (ECM) has been well documented [9]. 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 structural 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 [9]. Long-lasting infections may occur if microorganisms reach the subepithelial tissues.

Among spirochetes, *Borrelia* species has been shown to express a 47-kDa outer membrane protein named BBK32 that confers attachment to fibronectin [10, 11] and another GAG-binding adhesin termed Bgp [12, 13]. *Treponema*

pallidum, the causative agent of syphilis, also interacts with ECM through outer-surface proteins: Tp0155 and Tp0483 showed specific attachment to fibronectin [14] and Tp0751 bound to a variety of laminin isoforms [15, 16]. When we have started these investigations, the only putative leptospiral adhesin identified was a 36-kDa fibronectin-binding protein isolated from the outer sheath of a virulent variant of pathogenic leptospires [17].

Whole-genome sequencing of four pathogenic strains of *Leptospira* have been accomplished; two serovars of *L. interrogans*, Lai [18] and Copenhageni [19, 20] and two strains of *L. borgpetersenii* [21]. To date, only one saprophytic strain *L. biflexa* serovar Patoc, has been sequenced [22]. Genome analysis of *L. interrogans* serovar Copenhageni 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, or surface-maintenance proteins [20]. Of the 263 predicted genes encoding potential surface-exposed integral membrane proteins, 250 were previously unknown [19]. 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.

This work reviews the data of six putative leptospiral outer membrane proteins and their ability to interact with the ECM molecules, laminin, collagen Type I, collagen Type IV, cellular fibronectin and plasma fibronectin. The genes were selected from the genome sequences of *L. interrogans* serovar Copenhageni mostly based on their predicted cellular localization. The chosen genes, LIC10314, LIC10368, LIC12099, LIC12895, LIC12906 and LIC12690, were cloned and proteins expressed in *Escherichia coli* host system. The proteins were named Lsa63 (LIC10314) [23], Lsa21 (LIC10368) [24], Lsa27 (LIC12895) [25], Lsa24 (LIC12906) [26], LipL53 (LIC12099) [27] and Lp95 (LIC12690) [28], where Lsa stands for Leptospiral surface adhesin, LipL for lipoprotein of *Leptospira* and Lp for Leptospiral protein. The interaction of the recombinant proteins with ECM components and the binding characterization are presented. Our data suggest a possible involvement of these proteins in the cell adhesion interaction.

2. Materials and Methods

2.1 ECM components.

All macromolecules, including the control protein fetuin, BSA and ovalbumin, 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. Antibodies used to detect ECM components were from Sigma Chemical Co. (St. Louis, Mo.), produced in rabbits against the ECM components described above.

2.2 Bioinformatics characterization of the proteins.

Predicted coding sequences (CDSs) were selected from the *L. interrogans* serovar Copenhageni genome sequences [20] based on their cellular localization predictions by PSORT program, <http://psort.nibb.ac.jp> [29, 30] and CELLO programs, <http://cello.life.nctu.edu.tw/> [31, 32]. The SMART, <http://smart.embl-heidelberg.de/> [33, 34] and PFAM, <http://www.sanger.ac.uk/Software/Pfam/> [35] web servers were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs. The predicted lipobox sequence was evaluated by LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [36] and SpLip [37] programs. Sequence analysis was performed with ClustalX [38] and a tree displayed program by the Neighbour-Joining method [39].

2.3 DNA recombinant techniques.

Amplification of the CDSs was performed by PCR from total *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA using complementary primer pairs listed on Table 1. The gene sequences were amplified without the signal peptide tag, predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The sequences coding for Lsa21, LipL53 and Lp95 were cloned into pENTR-TOPO vector (Invitrogen) followed by transfer/recombination of DNA insert into the *E. coli* expression vector pDEST17 (Invitrogen) using the LR Clonase (Invitrogen). The sequences coding for Lsa63, Lsa27 and Lsa24 were cloned into pGEM-T easy vector (Promega) and subcloned into the pAE expression vector [40]. Both vector pDEST17 and pAE allow the expression of recombinant proteins with a minimal 6 x His-tag at the N-terminus. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA).

2.4 Expression and purification of recombinant protein.

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* [41]. *E. coli* BL21 SI containing recombinant plasmids (except for Lsa27) was grown at 30 °C in Luria-Bertani (LB) broth without NaCl and with 100 µg/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6 to 0.8 was reached. Recombinant protein synthesis was induced by the addition of

300 mM NaCl for three hours. The recombinant protein Lsa27 was obtained from *E. coli* BL21 Star (DE3) pLys. Bacteria were grown at 37 °C in LB broth with 100 µg/ml ampicillin and 50 µg/ml chloramphenicol until the log-phase was reached, and induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for three hours. The cells were harvested by centrifugation, the bacterial pellets were resuspended in sonication buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 200 µg/ml of lysozyme, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 % Triton X-100) and lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). The bacteria cell lysates were cleared by centrifugation at 3,000 × g for 15 min at 4 °C. The recombinant proteins Lsa63 and Lsa27 were purified from the supernatant fraction loaded onto Ni²⁺-charged Sepharose beads. The proteins were extensively washed with buffer containing 20 mM Tris-HCl, pH 8.0, 200 mM NaCl and increasing concentrations of imidazole (5 to 60 mM), except for Lsa27, which was washed with 5 mM imidazole and eluted with wash buffer containing 1 M imidazole. The rLsa27 protein was purified twice to remove contaminating *E. coli* proteins. The recombinant proteins Lsa21, LipL53, Lsa24 and Lp95 were purified from insoluble fraction after solubilization with 8 M urea, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl and addition of 10% glycerol for protein LipL53. Proteins refolding were achieved by 500 × dilution with 50 mM Tris-HCl, pH 8.0, and 500 mM NaCl, except for LipL53, which was refolded by decreasing concentrations of urea during the purification through metal chelating chromatography. All recombinant proteins were extensively dialyzed against phosphate-buffered saline (PBS), pH 7.4, 0.1 % (wt/vol) glycine solution (at the proportion of 10 ml of protein per 1,000 ml of buffer) with at least five changes of buffer every 4 h for 48 h.

2.5 Binding of recombinant proteins to ECM by ELISA.

Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol [15] with some modifications. 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 proteins were detected by adding an appropriate dilution of mouse antiserum in 100 µl of PBS. Dilution of mouse antiserum against each recombinant protein was: Lsa24, 1:2,000; Lsa63, 1:750; Lsa21, 1:5,000; LipL53, 1:25,600; Lsa27, 1:200. Incubation proceeded for 1 h, and after three washes with PBST, 100 µl 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 µl of 4 N H₂SO₄. The absorbance at 492 nm was taken in a microplate reader (Multiskan EX; Labsystems Uniscience). For determination of dose-dependent attachment of recombinant proteins to ECM components, protein concentrations varying from 0 to 2,000 nM in PBS were used.

2.6 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 recombinant protein (1 µg in PBS per well) to periodate-treated laminin was assessed as outlined above.

2.7 ECM ligand-binding assays by Western blot.

Aliquots (1 µg) of each recombinant protein and negative control protein BSA were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5 % BSA in PBS-T (0.05 % Tween 20), then incubated for 1 h at room temperature with either murine laminin, human fibronectin (plasma, 70 kDa proteolytic fragment and cellular), calf collagen type I or human collagen type IV at different concentrations in PBS-T. Following extensive washing with PBS-T, membranes were incubated for 1 h with rabbit polyclonal antibodies specific for either ECM component (diluted 1:1,000). Finally, the membranes were washed with PBS-T and incubated for 1 h with anti-rabbit horseradish peroxidase-conjugated (diluted 1:5,000, Sigma). Bound antibodies were detected using ECL reagent kit chemiluminescence substrate (GE Healthcare). Metaperiodate treatment was performed by diluting 1 µg/ml of laminin in 50 mM sodium acetate buffer, pH 5.0, with 200 mM sodium metaperiodate just before the use on ligand-binding assay.

2.8 Statistical analysis.

All results are expressed as means $\pm$ SEM. Student's paired t test was used to determine the significance of differences between means, and *P* lower than 0.05 was considered as statistically significant.

2.9 Protein Data Bank accession numbers.

The public database accession numbers for each protein sequence analyzed in this work are listed in Table 1. Proteins can also be accessed by their genome nomenclature for the gene locus and LIC numbers (*Leptospira interrogans* serovar Copenhageni).

3. Results and Discussion

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: LIC10368, LIC12895, LIC12906 and LIC12690 are putative lipoproteins with SpII lipoprotein signal peptide (signal peptidase II), consistent with LipoP program [36], LIC10314 is a conserved hypothetical protein with P83 domain [23, 42] and LIC12099 is a putative outer membrane protein with a Beta propeller repeat [27]. All predicted coding sequences are membrane proteins according to PSORT [30] and CELLO [31, 32] programs. P83 domain, Beta propeller repeats and a Domain of Unknown Function – DUF-1554 were identified by BLAST analysis [43] in LIC10314, LIC12099 and LIC12906, LIC12690, respectively. Table 1 summarizes gene locus identification, protein given name, protein accession number, genome annotation feature and the sequence of primers used for cloning.

Table 1. Gene locus, protein name, NCBI reference sequence, features, sequence of the primers employed for DNA amplification.

Gene locus ¹	Recombinant protein given name	NCBI reference sequence number ²	Genome annotation features	Sequence of primers for PCR amplification ³
LIC10314	Lsa63 [23]	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	F: 5' <u>GGATCCT</u> TATTTTCTCAGGAAAG (BamH I) R: 5' <u>GGTACCCT</u> AAGGTTTAATTTTTTTT (Kpn I)
LIC10368	Lsa21 [24]	YP_000355	Putative lipoprotein	F: 5' CACCGATGAAAAAAGAAAAATGAATTGAG R: 5' AACGCGATTCATAGAGAGCG
LIC12099	LipL53 [27]	YP_002033	Outer membrane protein with Beta-propeller repeat	F: 5' CACCACCAATGTGTTTGGTATAGCG R: 5' CAGCGTTTTGTGATAAAATTAAC
LIC12895	Lsa27 [44]	YP_002811	Putative lipoprotein	F: 5' <u>GGATCCT</u> GAAATATACGAA (EcoRI) R: 5' <u>GAATTC</u> TTACTGTCTCCTC (BamHI)
LIC12906	Lsa24 [26]	YP_002822	Probable Lipoprotein with a domain of unknown function (DUF1554)	F: 5' <u>CGCGCT</u> CGAGAAAAAGGAAGAA (Xho I) R: 5' <u>AAGCTT</u> TATTGAAGAATAATTCC (Nco I)
LIC12690	Lp95 [28]	YP_002611	Probable Lipoprotein with a domain of unknown function (DUF1554)	F: 5' CACCTTTTCTAACTTCGCGGACTC R: 5' CTATTGTTCCACACAAAGAATGC

¹ <http://aeg.lbi.ic.unicamp.br/world/lic/> [20]

² <http://www.ncbi.nlm.nih.gov/protein/>

³ Underlined nucleotides indicate restriction sites

The amplified coding sequences were cloned and expressed as full-length proteins in *E. coli*. Recombinant proteins were purified by metal chelating chromatography and an aliquot of each protein was analyzed through SDS-PAGE: lane 1, Lsa63; lane 2, Lsa21; lane 3, LipL53; lane 4, Lsa27 and lane 5, Lsa24 (Fig. 1A). As we can see from this figure, most of the contaminants were washed away and all proteins are represented as single major bands. Due to difficulties associated with Lp95 solubility, we decided to generate three fragments corresponding to the N-terminal, C-terminal and overlap portions of the LIC12690 gene (Fig. 1B). The DNA fragments were amplified and the inserts cloned into *E. coli* expression vector, pAE [40]. Recombinant proteins were expressed with 6 x His-tag at the N-terminal, purified by metal chelating chromatography. The recombinant protein fragments were consistently recovered from the column as single major band indicating that most of the contaminants had been removed (Fig. 1C) but, the three portions, as the whole protein, presented poor solubility in PBS. The proteins were employed to immunize mice and the antiserum raised was used to characterize the proteins.

Live immunofluorescence assay using the immuno serum against each protein confirmed the exposed cellular localization of Lsa63 [23], Lsa21[24], Lsa27 [25] and LipL53 [27]. The localization of LipL53 protein was strengthened by proteolysis assessment studies on intact leptospire. In the case of Lsa24, Triton X-100 assay confirmed its membrane localization because the protein remained in the detergent phase [26]. No conclusive experimental data was obtained with the Lp95 protein.

Fig. 1. (A) Analysis of recombinant protein purification: SDS - PAGE of purified protein eluted from Ni^{+2} - charged chelating Sepharose column with 1 M imidazole. Lane M, molecular mass protein marker, positions of standards are indicated to the left (in kDa); lane 1, Lsa63 (63.0 kDa); lane 2, Lsa21 (20.9 kDa); lane 3, LipL53 (54.0 kDa); lane 4, Lsa27 (27.0 kDa); lane 5, Lsa24 (26.5 kDa). (B) Schematic representation of Lp95. Shows the signal peptide (amino acids 1 to 16), the DUF1554 domain (amino acids 664 to 807), N-terminal domain (amino acids 18 to 464), C-terminal domain (amino acids 468 to 841), and the overlap domain (amino acids 274 to 610). (C) SDS - PAGE of purified Lp95 and fractions. Lane 1, Lp95 full length (94.5 kDa); lane 2, N-terminal fragment (51.9 kDa); lane 3, C-terminal fragment (43.5 kDa); lane 4, overlap fragment (39.7 kDa). Protein bands were visualized by Coomassie blue staining.

Lsa63 protein expression and conservation among *Leptospira* strains were assessed with total protein extracts from *L. interrogans* pathogenic serovars Canicola, Copenhageni, Icterohaemorrhagiae, Pomona, *L. borgpetersenii* serovars Castelonis and Whitcombi, *L. kirshneri* serovars Cynoptery and Grippotyphosa, *L. santarosai* serovar Shermani and the non-pathogenic strain *L. biflexa* serovar Patoc. The data depicted in Fig. 2 shows that Lsa63 protein is conserved in all pathogenic strains and absent in the saprophytic non-pathogenic strain [23]. On the other hand, Lsa27 was only conserved in *L. interrogans* serovar Copenhageni and Icterohaemorrhagiae [25]. Lp95 transcript was identified serovar Canicola, Copenhageni, Icterohaemorrhagiae and Hardjo of *L. interrogans* [28]. Lsa21 and LipL53 were present only in low passage, virulent strain of *L. interrogans* at the normal growth temperatures (28 °C) [24, 27]. However, upon shifted of attenuated cultures of pathogenic strains from 28 °C to 37 °C and to 39 °C, LipL53 transcript was observed, suggesting that this protein is up regulated by normal body core temperature and fever conditions [27]. The gene encoded for Lsa24 was conserved among serovars of *L. interrogans* (Neves, F. O., unpublished results).

Fig. 2 Conservation of Lsa63 among leptospira strains. Whole cell lysates and Lsa63 were separated by SDS-PAGE, transferred to membranes and probed with recombinant protein antiserum followed by anti-mouse IgG conjugated to peroxidase. Reactivity was detected by ECL kit and X-ray films exposition. (A) Coomassie blue stained gel and (B) western blot of duplicate gel transferred to membrane. M: protein molecular mass marker (positions shown on the left).

To evaluate the adhesion of recombinant proteins to ECM molecules, 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 assay. The protein Lsa24 exhibited a significant level of attachment to laminin; the binding of Lsa24 to laminin was also significant compared to its binding to collagen IV ($P < 0.05$) and cellular fibronectin ($P < 0.05$) (Fig. 3A). No binding was observed when wells were

coated with collagen Type I or with the highly glycosylated control protein fetuin (Fig. 3A) [26]. Similar to Lsa24, Lsa27 is also laminin-binding protein (Fig. 3B) [25]. The other proteins show a broader range binding activity. Lsa63 binded laminin and collagen IV [23], Lsa21 interacted with laminin, collagen IV and plasma fibronectin [24] and LipL53 showed adhesion to laminin, collagen IV, plasma and cellular fibronectin [27]. The behavior of Lsa63, Lsa21 and LipL53 proteins is similar to other reported adhesins, namely Len family protein [45], LigA and LigB proteins [46], LipL32 [47, 48] and TlyC [49] that have been shown to attach to more than one 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 [26]. Emp, a cell surface protein of *Staphylococcus aureus*, strongly interacts with fibronectin, fibrinogen, collagen, and vitronectin [50]. The outer membrane protein YadA of *Yersinia enterocolitica* has been shown to bind to laminin, fibronectin, and several types of collagens [51-53]. *Enterococcus faecalis* adhesin Ace mediates attachment to laminin, and to collagens I and IV [54]. Finally, the *Haemophilus influenzae* Hap autotransporter protein exhibits the same binding profile of Lsa21 protein: it interacts with laminin, fibronectin and collagen IV [55].

Fig. 3. Binding characteristics of recombinant proteins to ECM components. One microgram of laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, and the control protein fetuin were adsorbed to microtiter plates and incubated with 1 µg of each recombinant protein for 2 h at 37 °C. After washing, the attachments to ECM components were detected with mouse serum. (A) Specific profiles of adhesion of Lsa24 to the ECM macromolecules are shown as magnitude of attachment (fold) over the negative control protein BSA, arbitrarily set equal to 1. (B) Lsa63, Lsa21, LipL53 and Lsa27 attachments to ECM components. Optical densities were obtained at 492nm and the magnitude of the binding represented by bars corresponding to the mean absorbance value of three replicates $\pm$ the standard deviation. All statistical analyses were by Student's two-tailed t-test, and the attachment of Lsa63 to ECM was compared to its binding to fetuin (*, $P < 0.00005$; **, $P < 0.0005$); and the attachment of Lsa21, LipL53 and Lsa27 were compared to its binding to BSA (#, $P < 0.01$; §, $P < 0.05$; Θ, $P < 0.05$, respectively).

A previous study indicated that all Len proteins, but Len A, bind laminin and fibronectin [45]. We thus examined whether the DUF1554 (*len*-motif) contribute to the adhesion of the Lp95 and which fragments were involved in the binding. We have employed blotting instead of ELISA based assay due to the poor solubility of the protein. The Lp95 and each protein fragment were mixed up with SDS buffer, subjected onto SDS-PAGE and transferred to nitrocellulose membranes. Blotted proteins were stained Ponceau S (Fig. 4A and 4C) then examined for their ability to bind soluble laminin, fibronectin (plasma and cellular), collagen type I and IV.

Our data with affinity blotting shows that Lp95 (Fig. 4, lane 2), C-terminal fragment (Fig. 4, lane 4), overlap fragment (Fig. 4, lane 5) bind laminin (I), plasma fibronectin (II) fibronectin proteolytic fragment of 70 kDa (III) and cellular fibronectin (IV); the N-terminal fragment (Fig. 4, lane 3) binds laminin (I) and cellular fibronectin (IV). The binding with all the fragments was dependent on ECM concentration ranging from 0.5 to 2.0 µg. ECM-binding protein Lsa21 [24] was used as positive control (Fig. 4D, lane 8) while rLIC10494, previously shown to be non reactive with ECM components [26] was added as a negative control (Fig. 4D, lane 9). The assays with proteolytic fragments of fibronectin indicated that the 70 kDa fragment could account for part of the binding by the Lp95 and its fragments observed with intact fibronectin (Fig. 4II and 4III, lanes 2-5). No ECM binding was detected with BSA protein (Fig. 4, lane 6), employed as nonspecific binding control, although a weak band appeared when the highest laminin concentration (2.0 µM) was employed. No significant binding of Lp95 and fragments to collagen type I or type IV were detected (data not shown). 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 [56].

Fig. 4. Ligand affinity blot analyses of Lp95 with purified extracellular matrix components. In (A and C), protein bands were visualized by Ponceau staining. In (B and D) nitrocellulose membranes containing immobilized recombinant protein were incubated with ECM components and the binding was revealed with specific antibodies. (I) laminin, (II) plasma fibronectin, (III) 70 kDa proteolytic fragment of plasma fibronectin, (IV) cellular fibronectin. ECM concentrations ranged from 2 μ g to 0.5 μ g. Lane 1 and 7, molecular weight protein marker (in kDa); lane 2, Lp95 full length (94.5 kDa); lane 3, N-terminal fragment (51.9 kDa); lane 4, C-terminal fragment (43.5 kDa); lane 5, overlap fragment (39.7 kDa); lane 6, BSA (negative control), lane 8, Lsa21, lane 9, rLIC10494. Positions of molecular mass standards are indicated to the left (in kDa).

The interaction between the Lsa63, Lsa27, Lsa21, Lsa24 and LipL53 recombinant proteins and ECM molecules was also assessed on a quantitative basis as illustrated in Fig. 5. A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant protein (0 – 2,000 nM) were allowed to adhere to a fixed laminin concentration (1 μ g), fulfilling the properties of a typical receptor-ligand interaction. Saturation level was reached at a protein concentration of 700 nM – 800 nM for the proteins Lsa27 and Lsa24, respectively (Fig. 5), but for the other proteins, no saturation was reached up to 1,000 nM.

Fig. 5. Recombinant proteins bind to ECM components in a dose-dependent manner. The binding of recombinant proteins to ECM were assayed as a function of protein concentration. Each point represents the mean absorbance value at 492 nm $\pm$ the standard deviation of three independent experiments.

Metaperiodate oxidation of laminin caused significant reduction in the binding activity, strongly suggesting the involvement of laminin carbohydrate moieties in the interaction. All the proteins in this study, but Lsa63, showed a decrease in laminin-binding when the reaction was performed with metaperiodate (data not shown). This finding is in agreement with the previously published data pointing to a crucial role of laminin carbohydrate groups in the interaction with pathogens [15, 57-61].

To investigate whether the identified proteins presented sequence similarity with other published spirochetal ECM-binding proteins [62-66], we carried out sequence alignment analysis using Clustal X program and a tree-display NJ plot [38, 39]. The calculated dendrogram derived from sequence alignments is pictured in Fig. 6 and shows that ECM-binding leptospiral proteins do not share either sequence similarity or conserved domains with other reported spirochetal adhesins.

Fig. 6. Sequence comparison between spirochetal ECM binding proteins. Unrooted phylogenetic tree of predicted amino acid sequences of ECM binding proteins Lsa63 [23], Lsa21 [24], LipL53 [27], Lsa27 [25], Lp95 [28], LenA/Lsa24 [26,45], LenB-F [45], LipL32 [47,48], TlyC [49], LigA and LigB [46] of *Leptospira*; TP0136 [67], TP0751 [15], TP0155 and TP0483 [14] of *Treponema*; Bgp [12], ErpX [66] and BBK32 [10] of *Borrelia*. The tree was generated by Clustal X program and displayed by NJ plot. Branch lengths are depicted.

In conclusion, we have identified several leptospiral proteins in the *L. interrogans* serovar Copenhageni genome sequences that exhibit attachment to ECM components. 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 underway. Uncovering bacteria–host interactions at a molecular level will not only assist our understanding of the host physiology, but will facilitate the search for vaccine targets against leptospirosis.

Acknowledgements: This research was supported by FAPESP, CNPq and Fundação Butantan, Brazil. MLV, MVA, TRO, RSM, RFD, GMC, ATB and RO have fellowships from FAPESP.

References

- [1] Levett PN. Leptospirosis. *Clin Microbiol Rev.* 2001; 14: 296-326.
- [2] Farr RW. Leptospirosis. *Clin Infect Dis.* 1995; 21: 1-6; quiz 7-8.
- [3] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and leptospirosis. Melbourne, Australia: MediSci. 1999.
- [4] Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, et al. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis.* 2003; 3: 757-771.
- [5] Romero EC, Bernardo CC, Yasuda PH. Human leptospirosis: a twenty-nine-year serological study in Sao Paulo, Brazil. *Rev Inst Med Trop Sao Paulo.* 2003; 45: 245-248.
- [6] de la Pena-Moctezuma A, Bulach DM, Kalambaheti T, Adler B. Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. *FEMS Microbiol Lett.* 1999; 177: 319-326.
- [7] Adler B, de la Pena Moctezuma A. *Leptospira* and leptospirosis. *Vet Microbiol.* 2010; 140 (3-4): 287-96.
- [8] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and Leptospirosis. Melbourne, Australia MediSci. 1999.
- [9] Ljungh A, Moran AP, Wadstrom T. Interactions of bacterial adhesins with extracellular matrix and plasma proteins: pathogenic implications and therapeutic possibilities. *FEMS Immunol Med Microbiol.* 1996; 16: 117-126.
- [10] Probert WS, Johnson BJ. Identification of a 47 kDa fibronectin-binding protein expressed by *Borrelia burgdorferi* isolate B31. *Mol Microbiol.* 1998; 30: 1003-1015.
- [11] Probert WS, Kim JH, Hook M, Johnson BJ. Mapping the ligand-binding region of *Borrelia burgdorferi* fibronectin-binding protein BBK32. *Infect Immun.* 2001; 69: 4129-4133.

- [12] Parveen N, Leong JM. Identification of a candidate glycosaminoglycan-binding adhesin of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol Microbiol*. 2000; 35: 1220-1234.
- [13] Parveen N, Caimano M, Radolf JD, Leong JM. Adaptation of the Lyme disease spirochaete to the mammalian host environment results in enhanced glycosaminoglycan and host cell binding. *Mol Microbiol*. 2003; 47: 1433-1444.
- [14] Cameron CE, Brown EL, Kuroiwa JM, Schnapp LM, Brouwer NL. *Treponema pallidum* fibronectin-binding proteins. *J Bacteriol*. 2004; 186: 7019-7022.
- [15] Cameron CE. Identification of a *Treponema pallidum* laminin-binding protein. *Infect Immun*. 2003; 71: 2525-2533.
- [16] Cameron CE, Brouwer NL, Tisch LM, Kuroiwa JM. Defining the interaction of the *Treponema pallidum* adhesin Tp0751 with laminin. *Infect Immun*. 2005; 73: 7485-7494.
- [17] Merien F, Truccolo J, Baranton G, Perolat P. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar icterohaemorrhagiae. *FEMS Microbiol Lett*. 2000; 185: 17-22.
- [18] Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, et al. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature*. 2003; 422: 888-893.
- [19] Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, et al. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J Bacteriol*. 2004; 186: 2164-2172.
- [20] Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, et al. Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz J Med Biol Res*. 2004; 37: 459-477.
- [21] Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, et al. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci U S A*. 2006; 103: 14560-14565.
- [22] Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, et al. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS One*. 2008; 3: e1607.
- [23] Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, et al. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect*. 2010; 60: 52-64.
- [24] Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol*. 2008; 8: 70.
- [25] Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM, et al. A newly identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol*. 2009; 58: 1275-1282.
- [26] Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, et al. A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun*. 2006; 74: 6356-6364.
- [27] Oliveira TR, Longhi MT, Goncalves AP, de Moraes ZM, Vasconcellos SA, et al. LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. *Microbes Infect*. 2010; 12: 207-217.
- [28] Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, et al. Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *J Infect*. 2009; 59 (4):264-76.
- [29] Nakai K, Horton P. PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci*. 1999; 24: 34-36.
- [30] Nakai K, Kanehisa M. Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins*. 1991; 11: 95-110.
- [31] Yu CS, Lin CJ, Hwang JK. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Sci*. 2004; 13: 1402-1406.
- [32] Yu CS, Chen YC, Lu CH, Hwang JK. Prediction of protein subcellular localization. *Proteins*. 2006; 64: 643-651.
- [33] Letunic I, Copley RR, Pils B, Schultz J, et al. SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res*. 2006; 34: D257-260.
- [34] Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A*. 1998; 95: 5857-5864.
- [35] Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, et al. Pfam: clans, web tools and services. *Nucleic Acids Res*. 2006; 34: D247-251.
- [36] Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, et al. Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci*. 2003; 12: 1652-1662.
- [37] Setubal JC, Reis M, Matsunaga J, Haake DA. Lipoprotein computational prediction in spirochaetal genomes. *Microbiology*. 2006; 152: 113-121.
- [38] Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res*. 1997; 25: 4876-4882.
- [39] Perriere G, Gouy M. WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie*. 1996; 78: 364-369.
- [40] Ramos CR, Abreu PA, Nascimento AL, Ho PL. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res*. 2004; 37: 1103-1109.
- [41] Bhandari P, Gowrishankar J. An *Escherichia coli* host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. *J Bacteriol*. 1997; 179: 4403-4406.
- [42] Rossler D, Eiffert H, Jauris-Heipke S, Lehnert G, Preac-Mursic V, et al. Molecular and immunological characterization of the p83/100 protein of various *Borrelia burgdorferi* sensu lato strains. *Med Microbiol Immunol*. 1995; 184: 23-32.
- [43] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990; 215: 403-410.
- [44] Longhi MT, Oliveira TR, Romero EC, Goncalves AP, Moraes ZM, et al. A novel identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol*. 2009; 58 (10): 1275-82.
- [45] Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, et al. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE*. 2007; 2: e1188.
- [46] Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, et al. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun*. 2007; 75: 2441-2450.

- [47] Hoke DE, Egan S, Cullen PA, Adler B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. *Infect Immun*. 2008; 76: 2063-2069.
- [48] Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, et al. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun*. 2008; 76: 2642-2650.
- [49] Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, et al. Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS Lett*. 2009; 583 (8): 1381-5.
- [50] Hussain M, Becker K, von Eiff C, Schrenzel J, Peters G, et al. Identification and characterization of a novel 38.5-kilodalton cell surface protein of *Staphylococcus aureus* with extended-spectrum binding activity for extracellular matrix and plasma proteins. *J Bacteriol*. 2001; 183: 6778-6786.
- [51] Flugel A, Schulze-Koops H, Heesemann J, Kuhn K, Sorokin L, et al. Interaction of enteropathogenic *Yersinia enterocolitica* with complex basement membranes and the extracellular matrix proteins collagen type IV, laminin-1 and -2, and nidogen/entactin. *J Biol Chem*. 1994; 269: 29732-29738.
- [52] Schulze-Koops H, Burkhardt H, Heesemann J, von der Mark K, Emmrich F. Plasmid-encoded outer membrane protein YadA mediates specific binding of enteropathogenic yersiniae to various types of collagen. *Infect Immun*. 1992; 60: 2153-2159.
- [53] Terti R, Skurnik M, Vartio T, Kuusela P. Adhesion protein YadA of *Yersinia* species mediates binding of bacteria to fibronectin. *Infect Immun*. 1992; 60: 3021-3024.
- [54] Nallapareddy SR, Qin X, Weinstock GM, Hook M, Murray BE. Enterococcus faecalis adhesin, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. *Infect Immun*. 2000; 68: 5218-5224.
- [55] Fink DL, Buscher AZ, Green B, Fernsten P, St Geme JW. The *Haemophilus influenzae* Hap autotransporter mediates microcolony formation and adherence to epithelial cells and extracellular matrix via binding regions in the C-terminal end of the passenger domain. *Cell Microbiol*. 2003; 5: 175-186.
- [56] Ito T, Yanagawa R. Leptospiral attachment to extracellular matrix of mouse fibroblast (L929) cells. *Vet Microbiol*. 1987; 15: 89-96.
- [57] Carneiro CR, Postol E, Boilesen C, Brentani RR. Participation of glycosylation sites in the binding of *Staphylococcus aureus* to laminin. *Braz J Med Biol Res*. 1993; 26: 689-697.
- [58] Crouch ML, Alderete JF. *Trichomonas vaginalis* interactions with fibronectin and laminin. *Microbiology*. 1999; 145 (Pt 10): 2835-2843.
- [59] Ghosh A, Bandyopadhyay K, Kole L, Das PK. Isolation of a laminin-binding protein from the protozoan parasite *Leishmania donovani* that may mediate cell adhesion. *Biochem J*. 1999; 337 (Pt 3): 551-558.
- [60] Tanskanen J, Saarela S, Tankka S, Kalkkinen N, Rhen M, et al. The *gaf* fimbrial gene cluster of *Escherichia coli* expresses a full-size and a truncated soluble adhesin protein. *J Bacteriol*. 2001; 183: 512-519.
- [61] Valkonen KH, Wadstrom T, Moran AP. Identification of the N-acetylneuraminylactose-specific laminin-binding protein of *Helicobacter pylori*. *Infect Immun*. 1997; 65: 916-923.
- [62] Grosskinsky S, Schott M, Brenner C, Cutler SJ, Kraiczy P, et al. *Borrelia recurrentis* employs a novel multifunctional surface protein with anti-complement, anti-opsonic and invasive potential to escape innate immunity. *PLoS One*. 2009; 4: e4858.
- [63] Fuchs H, Wallich R, Simon MM, Kramer MD. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proc Natl Acad Sci U S A*. 1994; 91: 12594-12598.
- [64] Lagal V, Portnoi D, Faure G, Postic D, Baranton G. *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. *Microbes Infect*. 2006; 8: 645-652.
- [65] Rossmann E, Kraiczy P, Herzberger P, Skerka C, Kirschfink M, et al. Dual binding specificity of a *Borrelia hermsii*-associated complement regulator-acquiring surface protein for factor H and plasminogen discloses a putative virulence factor of relapsing fever spirochetes. *J Immunol*. 2007; 178: 7292-7301.
- [66] Brissette CA, Haupt K, Barthel D, Cooley AE, Bowman A, et al. *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. *Infect Immun*. 2009; 77: 300-306.
- [67] Brinkman MB, McGill MA, Pettersson J, Rogers A, Matejkova P, et al. A novel *Treponema pallidum* antigen, TP0136, is an outer membrane protein that binds human fibronectin. *Infect Immun*. 2008; 76: 1848-1857.

Evaluation of Immunoprotective Activity of Six Leptospiral Proteins in the Hamster Model of Leptospirosis

Marina V. Atzingen¹, Mônica L. Vieira^{1,3}, Rosane Oliveira^{1,3}, Renan F. Domingos^{1,3}, Renata S. Mendes^{1,3}, Aline T. Barros¹, Amane P. Gonçalves², Zenaide M. de Moraes², Silvio A. Vasconcellos² and Ana L. T. O Nascimento^{1,3,*}

¹Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil

²Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia, USP, Avenida Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, São Paulo, SP, Brazil

³Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Avenida Prof. Lineu Prestes, 1730, 05508-900, São Paulo, SP, Brazil

Abstract: Leptospirosis is a worldwide zoonosis caused by pathogenic *Leptospira*. The whole-genome sequence of *L. interrogans* serovar Copenhageni together with bioinformatics tools represent a great opportunity to search for novel antigen candidates that could be used as subunit vaccine against leptospirosis. We focused on six genes encoding for conserved hypothetical proteins predicted to be exported to the outer membrane. The genes were amplified by PCR from *Leptospira interrogans* genomic DNA and were cloned and expressed in *Escherichia coli*. The recombinant proteins tagged with N-terminal hexahistidine were purified by metal-charged chromatography. The immunization of hamsters followed by challenge with lethal dose of virulent strain of *Leptospira* showed that the recombinant proteins Lsa21, Lsa66 and rLIC11030 elicited partial protection to animals. These proteins could be used combined or in a mixture with novel adjuvants in order to improve their effectiveness.

Keywords: *Leptospira interrogans*, leptospirosis, recombinant protein, vaccine.

INTRODUCTION

Leptospirosis is a highly spread zoonosis of human and veterinary concern. The disease is caused by pathogenic species of the genus *Leptospira* with a higher incidence in tropical and subtropical countries, where the conditions for transmission are particularly appropriate [1, 2]. Transmission to humans occurs by direct or indirect contact to water or soil contaminated with the urine of chronically infected mammals. In the urban surroundings, due to sanitation problems and large population of urban rodent reservoirs, the disease spreads and become prevalent [3]. Flu-like symptoms, such as fever, chills, headache, and severe myalgias take place in the early phase of the disease, making clinical diagnostic very difficult. Multi-organ system complications may occur in 5 - 15 % of the cases with 5 - 40 % mortality rates [1, 4].

Presently there are veterinarian vaccines derived from inactivated whole cell preparations of pathogenic leptospires. These vaccines afford protection most probably through the

induction of antibodies against leptospiral lipopolysaccharide [2, 5, 6]. Therefore, they do not confer protection against leptospiral serovars not included in the preparation and as polysaccharide antigens, do not induce long-term protection. Human vaccines are available in Cuba [7] and China [8] with the same limitations of the veterinarian ones. The large number of pathogenic serovars (> 200) represents a major limitation to the production of a multi-serovar component vaccine. A cost-effective vaccine preparation against leptospirosis has long been pursued [3].

Due to their location, outer membrane proteins (OMPs) can participate of host-pathogen interactions and have become the focus of many research groups. OMPs have been shown to be surface-exposed and expressed during infection of the mammalian host [9, 10]. Several recombinant protein have been evaluated as vaccine candidates, such as, outer membrane protein OmpL1, lipoprotein LipL41 [11], hemolysis-associated protein 1 (Hap1) / lipoprotein LipL32 [12-14], immunoglobulin-like protein [15-18] and predicted outer membrane proteins [19]. Moreover, Yan *et al.* [20] have shown that leptospiral proteins with an OmpA-like domain conferred partial protection against lethal infection of leptospira. Recently, the evaluation of several recombinant proteins as potential antigens against leptospirosis were published but the results were not very encouraged [21]. Cell-

*Address correspondence to this author at the Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil; Tel: (5511) 37220019; Fax: (5511) 37261505; E-mail: tabet@butantan.gov.br

mediated immunity has also been explored [22] and pathogenic leptospires have been shown to stimulate *in vivo* the production of type 1 cytokines involved in cellular immunity in hamster model [23].

In the present work, we evaluated the immune response promoted by six recombinant proteins in hamsters. Four of them were previously characterized, LIC10368 [24], LIC11030 [25], LIC10258 [26] and LIC12253 [27], but their immune protective activity has not been tested by our research group. LIC10821 and LIC10672 are hypothetical coding sequences identified by bioinformatics tools in the genome sequences of the *L. interrogans* serovar Copenhageni. LIC10672 has also been identified by proteomics studies with virulent leptospiral strain [28]. Our results showed that the recombinant proteins elicited partial protection against lethal challenge with *L. interrogans* serovar Kennewicki strain Pomona Fromm in hamsters.

MATERIALS AND METHODOLOGY

Leptospira Strains and Culture Conditions

The non-pathogenic *L. biflexa* (serovar Patoc strain Patoc 1) and the pathogenic *L. interrogans* (serovars Copenhageni strain M-20, Canicola strain Hond Utrecht IV, Hardjo strain Hardjoprajitno, Icterohaemorrhagiae strain RGA and Pomona strain Pomona), *L. borgpetersenii* serovar Whitcombi strain Whitcomb, *L. kirshneri* (serovars Cynopteri strain 3522C and Grippotyphosa strain Moskva V), *L. santarosai* serovar Shermani strain 1342K, *L. noguchi* serovar Panama strain CZ214 and the virulent strain of *L. interrogans* serovar Kennewicki strain Pomona Fromm (strain LPF) and serovar Copenhageni (strain Fiocruz L1-130) were cultured at 28 °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 %) [29]. Virulence of the *L. interrogans* serovar Kennewicki strain Pomona Fromm (strain LPF) and serovar Copenhageni (strain Fiocruz L1-130) was maintained by iterative passages in Golden Syrian hamsters [1]. The animals were infected with 10⁴ leptospires and sacrificed after the appearance of symptoms, such as loss of weight and mobility (approximately 5 days post-infection). Kidneys were removed and macerated and the leptospires recovered through culture [1].

'In silico' Identification and Characterization of the Protein

Predicted coding sequences (CDSs) were selected from the *L. interrogans* serovar Copenhageni genome sequences [30] based on their cellular localization prediction by CELLO program, <http://cello.life.nctu.edu.tw/> [31, 32]. SMART, <http://smart.embl-heidelberg.de/> [33, 34] and PFAM, <http://www.sanger.ac.uk/Software/Pfam/> [35] web servers were used to search for predicted functional and structural domains within the amino acid of the selected CDS. The predicted lipobox sequence was evaluated by LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [36] program.

DNA Isolation and PCR Analysis

Leptospira cultures were harvested by centrifugation at 11,500 X g for 30 min and gently washed in sterile PBS twice. Genomic DNA was isolated from the pellets by guanidine-detergent lysing method using DNAzol® Reagent (Invitrogen), according to manufacturer's instructions. The DNA fragments were amplified using oligonucleotides designed according to *L. interrogans* serovar Copenhageni genome sequences (GenBank accession AE016823) (Table 1). PCR was performed in a reaction volume of 25 µl containing 100 ng of genomic DNA, 1 X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2 mM MgCl₂, 20 pmol of each specific primer, 200 µM 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, 62 °C, 50 sec, 72 °C, 1 min 30 sec, and a final extension cycle of 7 min at 72 °C. PCR amplified products were loaded on a 1 % agarose gel for electrophoresis and visualization with ethidium bromide.

Cloning, Expression and Purification of Recombinant Proteins

Amplification of the CDSs was performed by PCR from total *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA using complementary primer pairs listed on Table 1. The gene sequences were amplified without the signal peptide tag, predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) [37]. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA). Cloning, expression and purification of the recombinant proteins LIC10368 (Lsa21), LIC10258 (Lsa66), LIC11030 and LIC12253 have been previously described [24-27]. The coding sequences for LIC10821 and LIC10672 were cloned into pGEM-T easy vector (Promega) and subcloned into the pAE expression vector [38] that allows the expression of recombinant proteins with a minimal 6 x His-tag at the N-terminus. 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* [39]. *E. coli* BL21 SI containing recombinant plasmids were grown at 30 °C in Luria-Bertani broth without NaCl and with 100 µg/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6 to 0.8 was reached. Recombinant protein synthesis was induced by the addition of 300 mM NaCl. After three hours, the cells were harvested by centrifugation, the bacterial pellets resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 100 µg/ml of lysozyme, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 % Triton X-100). The bacterial cell pellets were lysed on ice with the aid of an ultrasonic cell disruptor (Sonifier 450, Branson, USA). The bacterial lysate was centrifuged at 3,000 X g for 10 min at 4 °C. The pellets were resuspended in buffer containing 8 M urea; 20 mM Tris-HCl, pH8.0; 500 mM NaCl. The proteins were purified through Ni²⁺-charged beads of chelating fast-flow chromatographic column (GE Healthcare). The rLIC10821 and rLIC10672 were refolded on-column by gradually removing urea (8-0M). The contaminants were washed away with low imidazole concentration and the recombinant proteins were eluted in 20 mM Tris-HCl, pH8.0; 500 mM NaCl and 500 mM imidazole. The efficiency of the purifications was evaluated by 12 % SDS-PAGE. The puri-

Table 1. Gene Locus, Protein name, NCBI Reference Sequence, Features, Gene Conservation, Sequence of the Primers Employed for DNA Amplification, and Molecular Mass of Expressed Recombinant Proteins

Gene Locus ¹	Recombinant Protein given Name	NCBI Reference Sequence Number ²	Description/Function	Conservation (Identity) ³	Sequence of primers for PCR amplification	Recombinant Protein Molecular Mass
LIC10368	Lsa21 ^b	YP_000355	Putative lipoprotein	Lai (100 %)	F: 5' CACCGATGAAAAAAGAAAAATGAATTGAG 3' R: 5' AACGCGATTCATAGAGAGCG 3'	21 kDa
LIC10258	Lsa66 ^a	YP_000249	Hypothetical protein with ompA domain	Lai (99 %) LBH (83 %)	F: 5' GGATCCGAAGCCTTCTCACCCAATTG 3' (BamH I) R: 5' CCATGGTTAAAGTGAAAGATAAAAATCGATTTC 3' (Nco I)	65 kDa
LIC11030	rLIC11030 ^c	YP_001000	Putative lipoprotein with a domain of unknown function (DUF1565)	Lai (99 %)	F: 5' CTCGAGCCAATTCTTTTCGATCCAAATC 3' (XhoI) R: 5' AAGCTTTCAATCCTCTACTGCAGCCC 3' (HindIII)	37 kDa
LIC10821	rLIC10821	YP_000798	Putative lipoprotein with a domain of unknown function (DUF1565)	Lai (100 %)	F: 5' CTCGAGTGTGCCAACGAAAAACG 3' (XhoI) R: 5' AAGCTTTCAAGGATTACAAGGTTTAG 3' (HindIII)	37 kDa
LIC12253	Lsa25 ^d	YP_002188	Putative lipoprotein with a domain of unknown function (DUF1566)	Lai (100 %) LBH (77 %) LBP (39 %)	F: 5' CTCGAGGAGGAGAAACCGGACGATAC 3' (XhoI) R: 5' CCATGGTTAGGGAAGACTTCTAACACATC 3' (NcoI)	24 kDa
LIC10672	rLIC10672	YP_000656	hypothetical protein	Lai (100 %) LBH (95 %)	F: 5' CCGGATCCCAGAATAAAG 3' (BamHI) R: 5' GGGGTACCTCAGATGTCG 3' (KpnI)	23 kDa

¹<http://aeg.lbi.ic.unicamp.br/world/lic/> [30, 41].²<http://www.ncbi.nlm.nih.gov/protein/>.³Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> [42, 43].^aPreviously published by Oliveira *et al* [26].^bPreviously published by Atzingen *et al* [24].^cPreviously published by Mendes *et al* [25].^dPreviously published by Felix *et al*. [21]; Domingos *et al.*, [27].Lai: *L. interrogans* serovar Lai [44]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [45]; LBP: *L. biflexa* serovar Patoc [46].

fied recombinant proteins were extensively dialyzed against PBS, pH 7.4, glycine solution (wt/vol: 0.1 %), at the proportion of 10 ml of protein per 1,000 ml of buffer, with at least five changes of buffer every 4 h for 48 h.

ELISA for Detection of Hamster IgG Antibodies

Hamster immunoglobulin G (IgG) antibodies against recombinant proteins were detected by ELISA. The plates were covered with 250 ng/well of each protein. Total IgG was evaluated by serial dilution of hamster's sera followed by the addition of goat anti-hamster IgG peroxidase conjugated (1:5,000; KPL, Gaithersburg, MD, USA).

Hamster Immunization and Challenge Studies

Twelve male Golden Syrian hamsters (6 - 8 weeks old) were immunized subcutaneously with 50 µg of recombinant protein adsorbed in 10 % Alhydrogel (2% Al(OH)₃), used as adjuvant. One booster injection was given after two-weeks

with the same preparation of recombinant protein (50 µg). Negative-control group of hamsters were injected with PBS in 10% Alhydrogel. As a positive control, a group of hamsters was immunized with killed whole-leptospire (bacterin vaccine), prepared as described by Silva *et al.* [17]. In brief, washed pellets of *L. interrogans* serovar Kennewicki strain Pomona Fromm strain LPF cultures were heat-inactivated at 56 °C for 20 min, resuspended in PBS and stored at -20 °C until use. Hamsters were immunized with a dose of 10⁹ inactivated leptospire in 10% Alhydrogel on days 0 and 14. Two hamsters of each group were bled, before the challenge, by cardiac puncture and the sera were analyzed by ELISA for the determination of antibody titers. The ELISA titer was considered to be the last dilution of serum that registered an optical density of 0.10. Two weeks after the second immunization, groups of 10 hamsters were challenged with an intraperitoneal inoculum of 3-5 X 10⁷ leptospire assessed in a Petroff-Hausser counting chamber (approx. 400-10,000 LD₅₀) from the same *L. interrogans* serovar Kennewicki

strain Pomona Fromm, strain LPF, but in this case freshly isolated from liver of infected Golden Syrian hamster and no culture passaged. Hamsters were monitored daily for clinical signs of leptospirosis and euthanized when clinical signs of terminal disease appeared. For the challenges, LD₅₀ was calculated as follows: hamsters were infected intraperitoneally with 10-fold serial dilutions. Inocula of 10⁵–10⁰ organisms were tested. Animals were monitored daily for clinical symptoms until 28 days post-infection. We then estimated how many virulent leptospires per injected hamster led to ca. 50% mortality, a dose therefore used for our LD₅₀ experiments. Negative control animals were injected with the same volume of sterile EMJH media.

Ethics of Animal Experimentation

All animal studies were approved by the Ethic Committees of the Instituto Butantan and of the Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, Brazil.

Statistical Analysis

The statistical analysis was performed by using GraphPad Prism 3.00 (GraphPad software Inc.) which calculates survival fractions using the product limit or Kaplan-Meier method [40]. Logrank test (equivalent to Mantel-Haenszel test) was used to compare survival curves. All *P*-values were two-tailed and a *P* value of < 0.05 was considered to indicate statistical significance.

RESULTS

Bioinformatics Analysis

The genes encoding LIC10368, LIC10258, LIC11030, LIC10821, LIC12253 and LIC10672 were identified by analysis of the genome sequences of the chromosome I of *L. interrogans* serovar Copenhageni and each one is presented as a single copy [30, 41]. The CDS LIC12253 and LIC10258 are predicted to be outer membrane proteins, while CDS LIC10368, LIC11030, LIC10821 and LIC10672 are predicted to be extracellular based on CELLO web server [31, 32]. PFAM and SMART programs found no putative conserved domain within the LIC10368 and LIC10672 CDS; the LIC10821 and LIC11030 presented a DUF1565 (domain of unknown function), the LIC12253 presented a DUF1566 domain, and the LIC10258 presented an OmpA-family protein domain. Blast analysis showed that the CDS LIC10368 has no homology with other known bacterial proteins; LIC10258 has partial identity (25%) with other spirochetal proteins; LIC12253, LIC10821 and LIC11030 present 30% similarity with other bacterial proteins due to the presence of DUF1566 or DUF1565 domain, respectively; CDS LIC10672 has partial identity (20-30%) with other bacterial proteins (chaperone Skp of *G. lovleyi*, ferredoxin of *T. oceanii*, transposase of *C. botulinum*) [42, 43]. The Lipop program predicted LIC10368, LIC11030 and LIC10821 CDS as putative lipoproteins, with a cleavage site for signal peptidase II at amino acids 18-19, 17-18, 17-18, respectively [36]. Similar putative coding sequences LIC10368, LIC10258, LIC11030, LIC10821, LIC12253 and LIC10672 were found in *L. interrogans* serovar Lai (99 - 100 % identity with LA0419, LA0301, LA3064, LA3340, LA1508 and LA3522, respectively) [44]. LIC10258, LIC12253 and LIC10672 CDS were also identified in the genome sequences of *L.*

borgpetersenii serovar Hardjo (77 - 95% identity with LBL0511, LBL1929 and LBL2387, respectively) [45]. LIC10368, LIC11030 and LIC10821 CDS were not identified in *L. borgpetersenii* [45]. Only LIC12253 CDS were identified in the genome sequences of *L. biflexa* serovar Patoc (39 % identity with LEPBI_I0560) [46]. Table 1 summarizes the features of the selected proteins and gene conservation within the sequenced genomes.

Distribution and Expression of Genes Among *Leptospira* Strains

The presence of the genes LIC10368 (Lsa21), LIC10258 (Lsa66) and LIC11030 were reported previously [24-26] having DNA fragments amplified only in *L. interrogans* (serovars Canicola, Copenhageni, Hardjo, Icterohaemorrhagiae and Pomona). LIC12253 gene was amplified in all pathogenic strains and a less intense band was detected in the saprophytic strain [27]. The presence of the novel genes LIC10821 and LIC10672 was examined by PCR in ten pathogenic and in one saprophytic strain of *Leptospira*, using a pair of primers designed according to *L. interrogans* serovar Copenhageni genome sequences (Table 1). DNA fragments were amplified in all ten strains of pathogenic species of *L. interrogans*, except that LIC10821 was not amplified in the *L. interrogans* serovar Canicola and *L. santarosai* serovar Shermani. In the case of LIC10672 gene no significant amplification product was detected in the non pathogenic strain Patoc 1 (*L. biflexa* serovar Patoc) (Fig. 1). Template integrity was attested by 16S DNA amplification.

Cloning, Expression and Purification of Recombinant Proteins

The genes were amplified, without the signal peptide sequence, and the DNA insert cloned and expressed as a full-length protein in *E. coli*. Recombinant proteins were ex-

Fig. (1). Distribution of genes among saprophytic and pathogenic leptospires. Genomic DNA from *L. biflexa* Patoc and from ten serovars belonging to the pathogenic species of *Leptospira* were subjected to PCR analysis with specific primers designed according to *L. interrogans* serovar Copenhageni genome sequences. (A) Amplification of 16S DNA shows template integrity; (B) LIC10821 and (C) LIC10672. No DNA was added to the negative control reaction (-).

pressed with 6 x His-tag at the N-terminus and purified by metal chelating chromatography. Purified recombinant proteins appeared as a single band by SDS-PAGE analysis with Coomassie staining (Fig. 2).

Antibody Response Induced by Immunization with Recombinant Proteins

To assess whether hamsters immunized with the recombinant antigens (Lsa21, Lsa66, rLIC11030, rLIC10821, Lsa25 and rLIC10672) and PBS (control) promoted IgG antibody response against the antigens, sera were collected from the animals on days 30th (prior) and 51th (after) the challenge. Two animals were bled by cardiac puncture and euthanatized. The antibodies were analyzed by ELISA using the recombinant proteins as antigen probes. The data obtained for each protein are depicted in Fig. (3) and refer to assay 1. Two weeks after the second immunization (day 30), hamsters produced an antibody response with titers equivalent to 1:400 of anti-Lsa21, 1:200 of anti-Lsa66, 1:400 of anti-rLIC11030, 1:200 of anti-rLIC10821, 1:800 of anti-Lsa25 and 1:1,600 of anti-rLIC10672. After challenge (day

Fig. (2). Analysis of purified recombinant proteins by metal chelating chromatography through SDS-12% PAGE. Purified protein eluted from Ni²⁺-charged chelating sepharose column with 1 M imidazole. Lane M, molecular mass protein marker; lane 1, rLIC10821 (37 kDa); lane 2, Lsa66 (65 kDa); lane 3, Lsa25 (24 kDa); lane 4, Lsa21 (21 kDa); lane 5, rLIC11030 (37 kDa); lane 6, rLIC10672 (23 kDa). Protein bands were visualized by Coomassie blue staining. Positions of molecular mass standards are indicated on the left (in kDa). (*) denotes each protein band.

Fig. (3). Induction of humoral immune response in hamsters. The animals were immunized subcutaneously with PBS (negative control), Lsa21, Lsa66, rLIC11030, rLIC10821, Lsa25 or rLIC10672, 50 µg each and boosted after two weeks. Animals were bled on day 30 (prior challenge) and 51 (after the challenge) to determine antigen specific total IgG responses in sera of immunized groups by ELISA method. Microdilution plates were coated with recombinant proteins and incubated with a serial dilution of serum from the immunized hamsters for IgG measurements. The data represent a pooled serum from two hamsters per group (days 30) or survival animals after challenge (day 51). All antibody titers refer to challenge assay 1.

51) the titers measured were 1:3,200 of anti-Lsa21, 1:800 of anti-Lsa66, 1:800 of anti-rLIC11030, 1:400 of anti-rLIC10821, 1:400 of anti-Lsa25 and 1:100 of anti-rLIC10672. No significant level of antibody was detected in control animals immunized with PBS-adjutant at any time point. An increment of antibody titer was observed after the bacterial challenge with all the proteins, except for Lsa25 and rLIC10672 that an opposite effect was detected (Fig. 3). Although the reason for the antibody decay is unknown, we may speculate that a shift in IgG subclass response might have occurred or that because Golden Syrian hamsters are not isogenic animals, thus immune response may be different.

Protection Induced by Hamster Immunization with Recombinant Proteins

Evaluation of recombinant proteins protection in hamster model followed by challenge with virulent leptospires was performed in two independent assays. The surviving plots are presented in Fig. (4) and the data obtained with the statistical analysis are depicted in Table 2. Experiment 1 (Fig. 4A) shows that immunization with the Lsa21 and rLIC11030 proteins conferred partial protection of 30%, followed by 20 % protection for the proteins Lsa66, rLIC10821 and Lsa25, against lethal challenge with $3-5 \times 10^7$ leptospires when compared to the PBS-control (Fig. 4A and Table 2). Heat inactivated whole-cell leptospires (bacterin) afforded 100% protection while no survival animal was seen with PBS-

Fig. (4). Survival curves of hamsters immunized with recombinant proteins and challenged with virulent leptospires. The animals were immunized subcutaneously with PBS (negative control), bacterin (killed whole-leptospires) (positive control), Lsa21, Lsa66, rLIC11030, rLIC10821, Lsa25 or rLIC10672. Two weeks after the second immunization (day 30) with the recombinant proteins, the animals were challenged intraperitoneally with $3-5 \times 10^7$ of *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF) in PBS. The animals were monitored for morbidity until day 21 post-challenge. **A** and **B** depicted the data of two independent experiments.

Table 2. Protective Efficacy of Recombinant Proteins in a Hamster Model

Antigen	Experiment 1			Experiment 2		
	% Protection (No. Surviving/Total)	P value*	Culture (Negative/Total)	% Protection (No. Surviving/Total)	P value*	Culture (Negative/Total)
killed whole-leptospores	100 % (10/10)	<0.0001	10/10	100 % (10/10)	0.0002	10/10
PBS	0 % (0/10)	-----	ND	20 % (2/10)	-----	2/2
Lsa21	30 % (3/10)	0.0470*	1/3	20 % (2/10)	0.9224	2/2
Lsa66	20 % (2/10)	0.0092*	1/2	30 % (3/10)	0.6768	1/3
rLIC11030	30 % (3/10)	0.0144*	2/3	30 % (3/10)	0.5102	2/3
rLIC10821	20 % (2/10)	0.0640	2/2	20 % (2/10)	0.8628	1/2
Lsa25	20 % (2/10)	0.0640	1/2	30 % (3/10)	0.3784	3/3
rLIC10672	0 % (0/10)	0.4180	ND	40 % (4/10)	0.3393	4/4

The statistical analysis was performed by Kaplan-Meier method [40] and Logrank test compared to PBS survival curves.

* P-values (< 0.05) considered to indicate statistical significance.

ND: not determined

control group. In the second experiment (Fig. 4B), 40% of the animals immunized with the proteins rLIC10672 survived, 30% survived with Lsa66, rLIC11030 and Lsa25, 20% survived with Lsa21, rLIC10821 and in PBS-control group. Bacterin afforded 100% protection (Fig. 4B, Table 2). Kaplan-Meier evaluation [40] of the kinetics of the survival curves showed a statistically significant protective effect, for the animals immunized with Lsa21, Lsa66 and rLIC11030 proteins only with the data of experiment 1. The survival animals at day 21 post-infection were sacrificed and their kidneys collected for culture, isolation of leptospores and the results are summarized in Table 2. It is interesting to note that in the experiment 1, a shift showing a delay in animal death was observed with all proteins, except with rLIC10672. Moreover, 70% of survived animals had no leptospores in their kidneys (Table 2). The rLIC10672, however, produced inconsistent results between the experiments.

DISCUSSION

Vaccines are one of the most successful methods of preventing infectious diseases [47]. Predicted outer membrane proteins have been identified during genome annotation of pathogenic *L. interrogans* serovars Lai [44] and Copenhageni [30] and *L. borgpetersenii* [45]. Almost 50% of these proteins were assigned as conserved or hypothetical proteins of unknown function [30]. Several of these proteins are predicted to be located at surface of leptospores and therefore we believe that they may be involved in pathogenesis and protective immunity. Chang and colleagues [19] working with putative outer membrane proteins of *Leptospira* that had no similarity with other organisms deposited in the NCBI database, identified novel antigens that although afforded partial protection against lethal challenge could act as a multicomponent vaccine against leptospirosis. Putative leptospiral outer membrane proteins with an OmpA-like domain have also been shown to elicit protective activity against hamster leptospirosis [20]. Moreover, our group has shown partial protection conferred in hamsters by two recombinant proteins,

rLIC12730 and rLIC10494 [48]. More recently, Felix and colleagues [21] have shown the data obtained in challenge assays with hamsters immunized individually with 27 recombinant proteins, but their end results were not very promising.

In this work, we report the immune response induced by two previously identified adhesins, LIC10368 - Lsa21 [24], LIC10258 - Lsa66 [26], and four hypothetical outer membrane proteins of unknown function, encoded by the genes, LIC11030, LIC10821, LIC12253 and LIC10672. The proteins Lsa21, Lsa66 and LIC11030 have been experimentally shown to be surface exposed in leptospores by live immunofluorescence microscopy [24-26]. DNA amplification was not observed in the non pathogenic *L. biflexa* strain for all studied genes, except for LIC10821 and LIC12253 [27].

The effectiveness of these proteins to elicit protective immunity in hamster model using alum as an adjuvant was evaluated. Our results demonstrated that Lsa21, Lsa66 and rLIC11030 proteins afforded partial protection, as revealed by enhanced survival and reduced leptospiral isolation in kidney. Kaplan-Meier analysis [40] of the kinetics of the survival curves confirmed partial protective, statistically significant effect, for the animals immunized with these proteins in one of the experiments. The number of surviving in control animals in one of the assays (0/10 against 2/10) could be responsible for the inconsistencies obtained. Noteworthy are the displacement of the curves when these proteins were administered. Comparable data were shown when OmpA-like domain proteins [20] and recombinant proteins, rLIC12730 and rLIC10494, were administered in hamsters [48]. The possible synergistic immunoprotection effect of these proteins, in combination or not, with other antigens merits further evaluation. In fact, protective effect of combined proteins have been previously observed with LipL41-OmpL1 [11] and with rLp1454 - Lp1118 - MCEII [19]. Preparations using novel adjuvants can also be used to improve and maybe, modulate the immune response. Delivery systems can also be employed in order to increment the vac-

cine efficacy. Work performed with leptosome-entrapped leptospiral proteins, antigens entrapped with PC-liposomes and *E. coli* lipid liposomes [50] have shown to improve immune response. Moreover, leptospiral antigens delivered by *Mycobacterium smegmatis* lipids (smegmosomes) were shown an enhancement in both Th1 and Th2 immune responses [51].

Survival protection elicited in hamsters immunized with recombinant proteins could be correlated with the antibody responses for the proteins Lsa21, Lsa66, rLIC11030 and rLIC10821, suggesting the participation Th2 immune response, although Th1 contribution cannot be excluded. The recombinant proteins were able to promote sterilizing immunity in 70% of the survived animals, as bacteria could not be recovered from kidneys (see Table 2). Moreover, the fact that some of the animals immunized with these recombinant proteins were clinically normal may suggest that bacterial burden was probably lower when compared to negative PBS control group.

CONCLUSION

We introduced in this work six proteins, including two novel leptospiral proteins. Five of these proteins are potential antigens that induced partial immunoprotection activity against challenge with the virulent Kennewicki strain Pomona Fromm strain. The employment of new generation adjuvants and the combination of one or more proteins should improve the immune protective activity of these proteins and may lead to the development of a multicomponent vaccine that could generate cross-protection against a wide range of pathogenic *Leptospira* serovars.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by FAPESP, CNPq and Fundacao Butantan, Brazil; MVA, MLV, RO, RFD, RSM and ATB have scholarship from FAPESP.

REFERENCES

- [1] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and Leptospirosis. Melbourne, Australia: Medi Sci 1999.
- [2] Bharti AR, Nally JE, Ricaldi JN, et al. Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 2003; 3: 757-71.
- [3] Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD Jr, Riley LW. Urban epidemic of severe leptospirosis in Brazil. *Salvador Leptospirosis Study Group*. *Lancet* 1999; 354: 820-5.
- [4] Plank R, Dean D. Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect* 2000; 2: 1265-76.
- [5] de la Pena-Moctezuma A, Bulach DM, Kalambaheti T, Adler B. Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjovovis. *FEMS Microbiol Lett* 1999; 177: 319-26.
- [6] Adler B, de la Pena Moctezuma A. *Leptospira* and leptospirosis. *Vet Microbiol* 2009; 140: 287-96.
- [7] Martinez R, Perez A, Quinones Mdel C, et al. Efficacy and safety of a vaccine against human leptospirosis in Cuba. *Revista panamericana de salud publica = Pan. Am J Public Health* 2004; 15: 249-55.
- [8] Yan Y, Chen Y, Liou W, et al. An evaluation of the serological and epidemiological effects of the outer envelope vaccine to leptospirosis. *J Chin Med Assoc* 2003; 66: 224-30.
- [9] McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. *Curr Opin Infect Dis* 2005; 18: 376-86.
- [10] Cullen PA, Xu X, Matsunaga J, et al. Surfaceome of *Leptospira* spp. *Infect Immun* 2005; 73: 4853-63.
- [11] Haake DA, Mazel MK, McCoy AM, et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect Immun* 1999; 67: 6572-82.
- [12] Branger C, Sonrier C, Chatrenet B, et al. Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. *Infect Immun* 2001; 69: 6831-8.
- [13] Seixas FK, da Silva EF, Hartwig DD, et al. Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. *Vaccine* 2007; 26: 88-95.
- [14] Seixas FK, Fernandes CH, Hartwig DD, Conceicao FR, Aleixo JA, Dellagostin OA. Evaluation of different ways of presenting LipL32 to the immune system with the aim of developing a recombinant vaccine against leptospirosis. *Can J Microbiol* 2007; 53: 472-9.
- [15] Palaniappan RU, McDonough SP, Divers TJ, et al. Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. *Infect Immun* 2006; 74: 1745-50.
- [16] Koizumi N, Watanabe H. Leptospiral immunoglobulin-like proteins elicit protective immunity. *Vaccine* 2004; 22: 1545-52.
- [17] Silva EF, Medeiros MA, McBride AJ, et al. The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. *Vaccine* 2007; 25: 6277-86.
- [18] Yan W, Faisal SM, McDonough SP, et al. Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. *Microbes Infect* 2009; 11: 230-7.
- [19] Chang YF, Chen CS, Palaniappan RU, et al. Immunogenicity of the recombinant leptospiral putative outer membrane proteins as vaccine candidates. *Vaccine* 2007; 25: 8190-7.
- [20] Yan W, Faisal SM, McDonough SP, et al. Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. *Vaccine* 2010; 28: 2277-83.
- [21] Felix SR, Hartwig DD, Argondizzo AP, et al. Subunit approach to evaluation of the immune protective potential of leptospiral antigens. *Clin Vaccine Immunol* 2011; 18: 2026-30.
- [22] Koizumi N, Watanabe H. Leptospirosis vaccines: past, present, and future. *J Postgrad Med* 2005; 51: 210-4.
- [23] Vernel-Pauillac F, Merien F. Proinflammatory and immunomodulatory cytokine mRNA time course profiles in hamsters infected with a virulent variant of *Leptospira interrogans*. *Infect Immun* 2006; 74: 4172-9.
- [24] Atzingen MV, Barbosa AS, De Brito T, et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol* 2008; 8: 70.
- [25] Mendes RS, Von Atzingen M, de Moraes ZM, et al. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infect Immun* 2011; 79: 4657-67.
- [26] Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. *PLoS ONE* 2011; 6: e21962.
- [27] Domingos RF, Vieira ML, Romero EC, et al. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC Microbiol* 2012; 12: 50.
- [28] Vieira ML, Pimenta DC, de Moraes ZM, Vasconcellos SA, Nascimento AL. Proteome Analysis of *Leptospira interrogans* Virulent Strain. *Open Microbiol J* 2009; 3: 69-74.
- [29] Turner LH. Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires. *Trans R Soc Trop Med Hyg* 1970; 64: 623-46.
- [30] Nascimento AL, Verjovski-Almeida S, Van Sluys MA, et al. Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz J Med Biol Res* 2004; 37:459-77.

- [31] Yu CS, Lin CJ, Hwang JK. Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Sci* 2004; 13: 1402-6.
- [32] Yu CS, Chen YC, Lu CH, Hwang JK. Prediction of protein subcellular localization. *Proteins* 2006; 15: 64: 643-51.
- [33] Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, Bork P. SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res* 2006; 34: D257-60.
- [34] Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci USA* 1998; 95: 5857-64.
- [35] Finn RD, Mistry J, Schuster-Bockler B, *et al.* Pfam: clans, web tools and services. *Nucleic Acids Res* 2006; 34: D247-51.
- [36] Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* 2003; 12: 1652-62.
- [37] Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 1997; 10: 1-6.
- [38] Ramos CR, Abreu PA, Nascimento AL, Ho PL. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res.* 2004; 37: 1103-9.
- [39] Bhandari P, Gowrishankar J. An *Escherichia coli* host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. *J Bacteriol* 1997; 179: 4403-6.
- [40] Vernel-Pauillac F, Goarant C. Differential cytokine gene expression according to outcome in a hamster model of leptospirosis. *PLoS neglected tropical diseases* 2011; 4: e582.
- [41] Kaplan EL, Meier P. Nonparametric estimation from incomplete data. *J Am Stat Assoc* 1958; 53: 457-81.
- [42] Nascimento AL, Ko AI, Martins EA, *et al.* Comparative genomics of two *Leptospira* interrogans serovars reveals novel insights into physiology and pathogenesis. *J Bacteriol* 2004; 186: 2164-72.
- [43] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; 215: 403-10.
- [44] Altschul SF, Madden TL, Schaffer AA, *et al.* Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997; 25: 3389-402.
- [45] Ren SX, Fu G, Jiang XG, *et al.* Unique physiological and pathogenic features of *Leptospira* interrogans revealed by whole-genome sequencing. *Nature* 2003; 422: 888-93.
- [46] Bulach DM, Zuerner RL, Wilson P, *et al.* Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci USA* 2006; 103: 14560-5.
- [47] Picardeau M, Bulach DM, Bouchier C, *et al.* Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS ONE* 2008; 3: e1607.
- [48] Bagnoli F, Baudner B, Mishra RP, *et al.* Designing the next generation of vaccines for global public health. *OMICS* 2011; 545-66.
- [49] Atzingen MV, Goncalves AP, de Moraes ZM, *et al.* Characterization of leptospiral proteins that afford partial protection in hamsters against lethal challenge with *Leptospira interrogans*. *J Med Microbiol* 2010; 59: 1005-15.
- [50] Faisal SM, Yan W, McDonough SP, Chang CF, Pan MJ, Chang YF. Leptosoma-entrapped leptospiral antigens conferred significant higher levels of protection than those entrapped with PC-liposomes in a hamster model. *Vaccine* 2009; 27: 6537-45.
- [51] Faisal SM, Yan W, McDonough SP, Mohammed HO, Divers TJ, Chang YF. Immune response and prophylactic efficacy of smegmosomes in a hamster model of leptospirosis. *Vaccine* 2009; 27: 6129-36.

Received: June 01, 2012

Revised: July 23, 2012

Accepted: July 18, 2012

© Atzingen *et al.*; Licensee Bentham Open.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.

Review Article

Plasminogen Binding Proteins and Plasmin Generation on the Surface of *Leptospira* spp.: The Contribution to the Bacteria-Host Interactions

Monica L. Vieira,^{1,2} Marina V. Atzingen,¹ Rosane Oliveira,^{1,2} Renata S. Mendes,¹
Renan F. Domingos,^{1,2} Silvio A. Vasconcellos,³ and Ana L. T. O. Nascimento^{1,2}

¹ Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil

² Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Avenida Prof. Lineu Prestes, 1730, 05508-900 São Paulo SP, Brazil

³ Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia, USP, Avenida Prof. Dr. Orlando Marques de Paiva, 87, 05508-270 São Paulo, SP, Brazil

Correspondence should be addressed to Ana L. T. O. Nascimento, tabet@butantan.gov.br

Received 13 February 2012; Revised 11 June 2012; Accepted 25 June 2012

Academic Editor: Edward F. Plow

Copyright © 2012 Monica L. Vieira et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Leptospirosis is considered a neglected infectious disease of human and veterinary concern. Although extensive investigations on host-pathogen interactions have been pursued by several research groups, mechanisms of infection, invasion and persistence of pathogenic *Leptospira* spp. remain to be elucidated. We have reported the ability of leptospires to bind human plasminogen (PLG) and to generate enzymatically active plasmin (PLA) on the bacteria surface. PLA-coated *Leptospira* can degrade immobilized ECM molecules, an activity with implications in host tissue penetration. Moreover, we have identified and characterized several proteins that may act as PLG-binding receptors, each of them competent to generate active plasmin. The PLA activity associated to the outer surface of *Leptospira* could hamper the host immune attack by conferring the bacteria some benefit during infection. The PLA-coated leptospires obstruct complement C3b and IgG depositions on the bacterial surface, most probably through degradation. The decrease of leptospiral opsonization might be an important aspect of the immune evasion strategy. We believe that the presence of PLA on the leptospiral surface may (i) facilitate host tissue penetration, (ii) help the bacteria to evade the immune system and, as a consequence, (iii) permit *Leptospira* to reach secondary sites of infection.

1. Introduction

The spirochete *Leptospira interrogans* is a highly invasive pathogen and the causal agent of leptospirosis, one of the most widespread zoonosis of human and veterinary concern. The disease represents a great economic burden because it has an effect on the public health system and the livestock [1–4]. The disease occurs mainly in urban regions lacking adequate sanitary conditions, associated with activities that involve direct contact with contaminated water, soil, or animals [3, 5, 6]. The leptospires chronically infect mammal species, which harbor the bacteria in their renal tubules, shedding them through the urine into the environment,

thus constituting a source of reinfection to other animals. Humans are accidental and terminal hosts in the transmission process of leptospirosis [1, 7]. The leptospires enter the body via abrasions on skin or actively through mucosa, spreading to any tissue, but specially colonizing kidneys and liver [2].

The understanding of molecular aspects of the pathogenesis, the virulence, and invasion processes by which the leptospires infect the hosts and initiate tissue colonization remains to be elucidated, despite the availability of genomic sequencing of five strains of *Leptospira* that have identified more than 200 putative membrane proteins that might be involved in pathogenesis [8–12]. Yet, only few virulence

factors related to the pathogenesis of the disease have been reported [13–16].

The ability of the leptospires to adhere to extracellular matrix (ECM) proteins is assumed to be crucial during the first steps of pathogenesis [17]. Indeed, this ability has been recently and firstly described by our group [18] and a number of adhesins, ECM-binding proteins, have been identified [15, 18–26]. After leptospiral adherence, the next fundamental step must be to overcome the barriers imposed by epithelial tissues and extracellular matrixes, to reach the bloodstream and secondary sites of infection within the hosts. Proteolytic activity has been demonstrated to be important during the penetration of several pathogenic microorganisms [27]. Because the presence of ECM degrading enzymes is limited to few bacterial pathogens, one of the strategies employed to surmount this deficiency is the interaction with protease-mediated systems of the hosts [27].

Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has PLG as a main component. PLG is a circulating single-chain zymogen that is converted to plasmin by cleavage of the peptide bond between Arg-560 and Val-561, mediated by PLG activators-like uPA (urokinase-type PLG activator) and tPA (tissue-type PLG activator). Once activated to plasmin the bacteria are endowed with membrane-associated proteolytic activity, a characteristic that have been demonstrated to contribute to the degradation of ECM components, tissue penetration, and invasion. It has been reported that several pathogens, including the spirochetes *Borrelia* spp. and *Treponema* spp., bind PLG on the surface and converts it to plasmin by host activators [28–30]. The binding of PLG and plasmin generation promotes ECM molecules degradation and it is essential for dissemination of the bacteria through the host tissues, thus suggesting its importance in invasiveness process [27, 31–35].

In this paper, we highlight and compile the recent studies performed by our group describing and characterizing the leptospiral binding to PLG [36], the identification of possible PLG receptors [37–40], and the aspect of the leptospiral immune escape strategy associated to plasmin proteolytic activity [41]. The disclosure of this proteolytic system on the surface of *Leptospira* spp. and the implications associated to bacterial infectivity are discussed.

2. Materials and Methods

2.1. Bacteria Isolates and Culture Conditions. Virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 is routinely cultured at Faculdade de Medicina Veterinária da Universidade de São Paulo by iterative passages in Golden Syrian hamsters for maintenance of virulence [1]. The organs-derived leptospires were cultured at 28°C in semisolid modified Elinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% rabbit sera. Nonvirulent culture-attenuated *L. interrogans* serovar Copenhageni strain M20 are equally routinely cultured by maintenance in liquid-modified EMJH medium supplemented with 10% rabbit sera [42]. For the experiments

of PLG binding, to exclude the PLG interference from the rabbit serum supplementing the culture medium, serum-free leptospires were obtained by three passages in liquid modified EMJH medium supplemented with 10% *Leptospira* enrichment EMJH (BD, Difco), cultured at 28°C.

2.2. Labeling of Leptospires with Plasmin. The leptospires were treated with PLG and uPA based in the protocol described by Coleman et al. [43], with modifications. A total of 7.0×10^9 leptospires were centrifuged at $6,000 \times g$ for 10 min at 25°C, resuspended in 1.4 mL of EMJH culture medium supplemented with 10% *Leptospira* enrichment EMJH, divided into 7 aliquots of 0.2 mL each (1.0×10^9 leptospires) in 2 mL microcentrifuge tubes, and recentrifuged. The 7 tubes containing the leptospires received different treatments: (a) 30% plasma and 3 U uPA (urokinase, Sigma) in 100 μ L low-salt PBS (lsPBS—with 50 mM NaCl), (b) 5 μ g PLG (native PLG purified from human plasma was from Calbiochem) and 3 U uPA in 100 μ L lsPBS, (c) 2 μ g PLG and 3 U uPA in 100 μ L lsPBS, (d) 0.5 μ g PLG and 3 U uPA in 100 μ L lsPBS, (e) 5 μ g PLG in 100 μ L lsPBS, (f) 3 U uPA in 100 μ L lsPBS, and (g) 100 μ L lsPBS. All the preparations were incubated for 1 h at 37°C, under gentle shaking, prior to the addition of the uPA, and followed by 1 h incubation at 37°C. Leptospires were then centrifuged and washed three times with 0.7 mL lsPBS.

2.3. Measurement of Enzymatic Activity of Plasmin-Coated Leptospires. The treated leptospires (1.0×10^9 per sample) were resuspended in 300 μ L lsPBS and divided into 3 aliquots. Each aliquot received 100 μ L of 0.5 mg/mL of the chromogenic substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride (Sigma) in lsPBS, to a final substrate concentration of 0.25 mg/mL. The suspensions were incubated for 1.5 h at 37°C, under gentle shaking, and then centrifuged at $6,000 \times g$ for 10 min at 25°C. The supernatants (150 μ L) were transferred to 96-well microplates and the cleavage of the specific plasmin substrate was quantified with a microplate reader set at a wavelength of 405 nm.

2.4. 6-Aminocaproic Acid (ACA) Binding-Inhibition Assay. Low-passage virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (1.0×10^9 leptospires/sample) was treated with PLG and uPA, as described above, except for the addition of increase concentrations of ACA (Sigma) ranging from 0 to 1,000 mM, added together with the PLG. PLG quantity was set at 5 μ g and uPA at 3 U. Aliquots treated with only PLG, uPA, or ACA, and just lsPBS treated, were used as controls. The enzymatic plasmin activity was measured as described before.

2.5. Liquid-Phase Immunofluorescence Assay (L-IFA). Live bacteria suspensions (2.5×10^9) were harvested at $12,800 \times g$ for 15 min, washed twice with lsPBS, resuspended in 200 μ L lsPBS containing 8 μ g of human PLG, and incubated for 45 min at 37°C. After the incubation, 6 μ g/mL of propidium iodide (Sigma) were added to stain the nuclei, and the suspensions were incubated for more 45 min at 37°C. After

this time, the leptospires were gently washed three times with lsPBS and incubated for 45 min at 37°C with mouse-produced antiserum against human PLG (Sigma) at a 1:50 dilution. The leptospires were washed three times and incubated with goat anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma) at a dilution of 1:50 for 45 min at 37°C. After this incubation, the leptospires were washed twice and resuspended in lsPBS-antifading solution (ProLong Gold; Molecular Probes). The immunofluorescence-labeled leptospires were examined by the use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany). As control for cell integrity, we used antibodies against recombinant *Leptospira* proteins LipL32 or GroEL, produced in mice, following all the mentioned procedures for PLG.

2.6. SDS-PAGE and Affinity Blotting. Total leptospiral protein extracts for SDS-PAGE were prepared from 10 mL of $\sim 10^9$ bacteria in EMJH serum-free cultures. The cells were harvested by centrifugation, washed three times with 5 mM MgCl_2 in lsPBS, and resuspended in 100 μL PBS. The proteins were loaded into 10% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in semidry equipment. The membranes were blocked for 2 h at 37°C with 5% BSA, washed three times (10 min for each wash) with PBS-0.5% Tween-20 solution (PBS-T), and incubated overnight with 3 $\mu\text{g}/\text{mL}$ PLG or 3 $\mu\text{g}/\text{mL}$ PLG + 100 mM ACA at 4°C, followed by a 2 h incubation at room temperature. Then, the membranes were washed three times and incubated with mouse anti-human PLG (1:750, Sigma) for 3 h at room temperature, following by more than three washings and 1 h incubation at room temperature with anti-mouse IgG-peroxidase conjugated (1:5,000, Sigma). The membranes were washed and the protein's reactivity was revealed by ECL reagent (GE Healthcare) with subsequent exposition to X-Ray films (Kodak).

2.7. Assay for the Degradation of Immobilized ECM Macromolecules. 96-well plates were coated overnight at 37°C with 0.5 $\mu\text{g}/\text{well}$ of cellular fibronectin (Sigma) or laminin (Sigma), washed four times with 200 μL per washing with PBS-T, and blocked with 2% BSA in PBS-T for 2 h at 37°C, followed by two washings. The spirochetes (1.0×10^8 leptospires/per sample) were treated as described above with 10 μg PLG and 3 U uPA or lsPBS (untreated). Bacteria were washed, resuspended in 100 μL lsPBS, and transferred to the plate previously coated wells. The plates were centrifuged at $180 \times g$ for 15 min to ensure the contact of the leptospires with the immobilized ECM components. The plates were incubated at 37°C for 20 h and washed 5 times to remove the bacteria. The degradation of the ECM components were detected by reduction in absorbance followed by incubation with antifibronectin or antilaminin IgG antibodies (1:5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma), anti-IgG peroxidase conjugated antibodies (1:5,000 dilution in 100 μL PBS-T for 45 min at 37°C, Sigma), and 100 $\mu\text{L}/\text{well}$ of 1 mg/mL *o*-phenylenediamine—OPD—in citrate phosphate buffer, pH 5.0 plus 1 $\mu\text{L}/\text{mL}$ H_2O_2 . The reaction was stopped

with 50 $\mu\text{L}/\text{well}$ 4N H_2SO_4 , and the absorbance was measured at 492 nm. Percentage degradation was calculated by de formula $(A - B)/A(100)$, where A = the mean PBS samples absorbance (positive control group) and B = the mean experimental group absorbance (untreated or plasmin).

2.8. Bioinformatics Characterization of the Proteins. Predicted coding sequences (CDSs) were analyzed as their cellular localization predictions by PSORT program, <http://psort.hgc.jp/> [44, 45]. The SMART <http://smart.embl-heidelberg.de/> [46, 47], PFAM <http://www.sanger.ac.uk/resources/software/> [48], and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [49] web servers were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs.

2.9. Cloning, Expression, and Purification of Recombinant Proteins. Amplification of the CDSs was performed by PCR from *L. interrogans* serovar Copenhageni genomic DNA using complementary primer pairs. The gene sequences were amplified without the signal peptide tag and predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). The final constructs were verified by DNA sequencing on an ABI Prism 3730.L sequencer (Seq-Wright, Houston, TX, USA) with appropriate vector-specific T7 (F: TAATACGACTCACA-TATAGGG) and pAE (R: CAGCAGCCAACTCAGTTCCT) primers. Cloning, expression, and purification of the recombinant proteins have been previously described as summarized in Table 1.

2.10. Plasminogen Binding to Recombinant Proteins. The binding of the recombinant proteins to PLG was evaluated by a modified ELISA, based in the protocol described by Brissette et al. [50], as follows: 96-well plates (Costar High Binding, Corning) were coated overnight in PBS at 4°C with 100 μL of 10 $\mu\text{g}/\text{mL}$ of the recombinant proteins or bovine serum albumin (BSA, Sigma) as negative control. Plates were washed once with PBS-T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) nonfat dry milk. The blocking solution was discarded and 100 μL of 10 $\mu\text{g}/\text{mL}$ human PLG (Calbiochem) in PBS was incubated for 2 h at 37°C. Wells were washed four times with PBS-T and incubated for 1 h at 37°C with mouse anti-human PLG (1:4,000 in PBS, Sigma). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG, Sigma), diluted 1:5,000 in PBS. After three washings, 100 $\mu\text{L}/\text{well}$ of 1 mg/mL OPD plus 1 $\mu\text{L}/\text{mL}$ H_2O_2 in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 $\mu\text{L}/\text{well}$ of H_2SO_4 (4 N). Readings were taken at 492 nm.

2.11. Enzymatic Activity Assay of Plasmin Bound to Recombinant Proteins. ELISA plates were coated overnight with 10 $\mu\text{g}/\text{mL}$ recombinant proteins or BSA in PBS at 4°C. Plates were then washed once with PBS-T and blocked with PBS with 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The blocking solution was discarded and 100 $\mu\text{L}/\text{well}$ of 10 $\mu\text{g}/\text{mL}$ human PLG (Calbiochem) was added, followed by

TABLE 1: Leptospiral selected proteins for characterization of the binding to PLG. Gene locus, protein name, NCBI reference sequence, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Gene loci ¹	Recombinant protein given name	NCBI reference sequence number ²	Description/Function	Conservation (identity) ³	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC11352	LipL32 ^{a,h}	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100%); LBH (98%)	F: 5'CACCGGTGCTTTCGGTGGTCTG 3' R: 5'ATTACTTAGTCGCGTCAGAAAGC 3'	30.2
LIC10314	Lsa63 ^{b,h}	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98%); LBH (87%); LBP (39%)	F: 5'GGATCCTTATTTTCTCAGGAAAG 3' (BamHI) R: 5'GGTACCCCTAAGGTTTAAATTTTTT 3' (KpnI)	63.0
LIC10509	rLIC10509 ^{c,h}	YP_000493	Putative lipoprotein	Lai (98%)	F: 5'CCGGGATCCAAAAAGAGCAAGAAAG 3' (BamHI) R: 5'GGTAGCCCTACTCGAGACAGCCAGACCTTC 3' (KpnI)	22.0
LIC12892	Lp29 ^{d,h}	YP_002808	Putative lipoprotein	Lai (99%); LBH (86%)	F: 5'CTCGAGGCGATACATTACAACTTGGCT 3' (XhoI) R: 5'CCATGGCTCTTAGGAGCCCTGGAAA 3' (NcoI)	29.6
LIC10793	Lp49 ^{d,h}	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5'CTCGAGAGCGGAGACTTTTCTTACTT 3' (XhoI) R: 5'CCATGGTTAAAAACCAATCTACGATAAAC 3' (NcoI)	49.1
LIC12895	Lsa27 ^{e,h}	YP_002811	Putative lipoprotein	Lai (79%)	F: 5'GGATCCCTGAAATATACGAA 3' (EcoRI) R: 5'GAATCTTACTGTCTCCTTC 3' (BamHI)	27.0
LIC13131	MPL21 ^{a,f,h}	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5'CACCACGTCCTCAAAGTTACGCTTCAG 3' R: 5'TTCTCACCATCCAGCTCGG 3'	21.9
LIC10765	MPL17 ^{a,f,h}	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%); LBP (41%)	F: 5'CACCGAAAGTCCCGTAAGGTTCAAA 3' R: 5'TGCAGGAGTCCACATTITA 3'	15.4
LIC10091	LipL40 ^{a,h}	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5'CCATGGGACTCGAGACGCCCTCCTCTAAAGATCC 3' R: 5'CTCCATGGTCAATTTCAAAAACCTTCTACGGGGC 3'	39.0
LIC10054	MPL36 ^{a,h}	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%); LBP (50%)	F: 5'CACCACGCTTGTGCGTCGGTAGAG 3' R: 5'CCAAGTATCTATTTATACGTCGCGAG 3'	35.1
LIC10494	rLIC10494 ^{g,h}	YP_000478	Putative lipoprotein	Lai (99%)	F: 5'CACCACCTGCTAGGGCTGCAGAAA 3' R: 5'ACTTTGAGAGCTTCGCTCTCGT 3'	25.8
LIC12730	rLIC12730 ^{g,h}	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%); LBP (37%)	F: 5'CACCAGTTCTGACGGACTTCCCAA 3' R: 5'TCTTGCGAATGAGTTGATCC 3'	77.4

TABLE 1: Continued.

Gene loci ¹	Recombinant protein given name	NCBI reference sequence number ²	Description/Function	Conservation (identity) ³	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC12922	rLIC12922 ^h	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%); LBP (48%)	F: 5' CACCGAATCACTCAACAGAGTCATTGC 3' R: 5' ATCAATCTAAATGAAACGCTCTCTTC 3'	40.0
LIC12238	rLIC12238 ^{h,i}	YP_002173	Hypothetical protein	Lai (99%); LBH (77%); LBP (39%)	F: 5' CTCGAGTGTTTTAAACCTACCGGAG 3' (Xho I) R: 5' AAGCTTCTACTTCATCGCTTTTCTATATC 3' (Hind III)	17.6
LIC10258	Lsa66 ⁱ	YP_000249	Hypothetical protein with ompA domain	Lai (99%); LBH (79%)	F: 5' GGATCCGAAGCCTTCTCAGCCCAATTG 3' (BamH I) R: 5' CCATGGTTAAAGTGAAGATAAAATCGATTTC 3' (Nco I)	65.7
LIC12880	Lp30 ⁱ	YP_002796	Putative lipoprotein	Lai (99%); LBH (76%)	F: 5' CTCGAGGAAGTTGTCGAGTCTAT 3' (Xho I) R: 5' CCAATGGTTATTGATTTGTTTAAATTCAG 3' (Nco I)	30.7
LIC11469	Lsa20 ^j	YP_001430	Hypothetical protein	Lai (100%); LBH (82%); LBP (29%)	F: 5' CTCGAGCCCAATTTCTTTTCGATCCAAATC 3' (Xho I) R: 5' AAGCTTTCATCTCTACTGACGCC 3' (Hind III)	20.0
LIC11030	rLIC11030 ^{s,j}	YP_001000	Putative lipoprotein with unknown domain (DUF1565)	Lai (100%)	F: 5' CTCGAGTGTACAAACGAAAAAGAGGT 3' (Xho I) R: 5' AAGCTTTTAGTTGCAAGGATTGGA 3' (Hind III)	37.0
LIC11834	rLIC11834 ^k (Lsa33)	YP_001783	Putative lipoprotein	Lai (99%); LBH (87%); LBP (31%)	F: 5' CTCGAGGATCTACAAGGTGGGGTTTTCAC3' (Xho I) R: 5' CCATGGTTTACTGAGGTTTACTTTGGTCC3' (Nco I)	33.1
LIC12253	rLIC12253 ^k (Lsa25)	YP_002188	Conserved hypothetical protein	Lai (100%); LBH (77%); LBP (39%)	F: 5' CTCGAGGAGGAGAAACCGGACGATAC 3' (Xho I) R: 5' CCATGGTTAGGGAAGACTTCTAACACATC 3' (Nco I)	24.0

¹ <http://aeg.lbi.ic.unicamp.br/world/lic/>.² <http://www.ncbi.nlm.nih.gov/protein/>.³ Protein BLAST — <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>.^a Previously published by Gamberini et al. [78];^b Previously published by Vieira et al. [37];^c Previously published by Gómez et al. [79];^d Previously published by Neves et al. [80];^e Previously published by Longhi et al. [19];^f Previously published by Oliveira et al. [81];^g Previously published by Barbosa et al. [18];^h Previously published by Vieira et al. [37];ⁱ Previously published by Oliveira et al. [38];^j Previously published by Mendes et al. [39];^k Previously published by Domingos et al. [40];Lai: *L. interrogans* serovar Lai [8]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [11]; LBP: *L. biflexa* serovar Patoc [12].

incubation for 2 h at 37°C. Wells were washed three times with PBS-T, and then 4 ng/well of human uPA (Sigma) were added. Subsequently, 100 μ L/well of the plasmin-specific substrate *D*-valyl-leucyl-lysine 4-*p*-nitroanilide dihydrochloride (Sigma) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate was measured by taking readings at 405 nm.

2.12. Dose-Response Curves and Dissociation Constant of the Proteins-PLG Interactions. ELISA plates were coated overnight in PBS at 4°C with 100 μ L of 10 μ g/mL PLG. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0–1 μ M) were added (100 μ L/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at appropriate dilutions, followed by horseradish peroxidase-conjugated anti-mouse IgG (1:10,000 in PBS, Sigma). The binding was evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [51] and Lin et al. [52], based on the equation: $A = A_{\max} [\text{protein}] / (K_D + [\text{protein}])$, where A is the absorbance at a given protein concentration, $A_{\max}$ is the maximum absorbance for the ELISA plate reader (equilibrium), $[\text{protein}]$ is the protein concentration, and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

2.13. Two-Dimensional Gel Electrophoresis (2-DE Gels). Virulent low passage leptospires culture was harvested by centrifugation at 12,800 $\times$ g at 4°C for 10 min. Pellet was washed ($\times$ 5) by resuspension in PBS containing 5 mM MgCl₂. The pellet was resuspended in DeStreak rehydration solution (GE Healthcare, USA) and lysed by vigorous vortexing. The cellular debris was separated by centrifugation at 20,800 $\times$ g for 10 min, and the supernatant was collected. Total protein content was determined according to the Bradford method (Pierce Biotechnology, USA). Samples containing 200 μ g of protein were adjusted to 125 μ L with DeStreak rehydration solution (GE Healthcare, USA), along with 0.8% (v/v) IPG buffer, with a pH range of 3–10 (GE Healthcare).

First-dimension isoelectric focusing was performed using the IPGphor-System (GE Healthcare, USA), and the second dimension was conducted on the Ettan DALTsix system (GE Healthcare). The IPG gel strips (7 cm) with a linear separation of immobilized pH ranging from 3 to 10 were rehydrated directly with the solubilized samples. The focusing protocol was 30 V for 180 Vh, 150 V for 300 Vh, 350 V for 350 Vh, 500 V for 500 Vh, 1000 V for 1,000 Vh, 3,000 V for 3,000 Vh, and 5,000 V for 40,000 Vh, with a 50 μ A/strip maximum-setting at 20°C. The strips were equilibrated twice (reduced and alkylated) for 15 min in 15 mL equilibration solution (0.05 M Tris-HCl, pH 8.8, 6.0 M urea, 30% [v/v] glycerol, and 2% [w/v] SDS), first with the addition of 1% DTT, and finally with 2.5% iodoacetamide. After equilibration, the strips were attached to the 12%

SDS-PAGE. The gels were stained by Coomassie Blue R350 (PhastGel BlueR-GE Healthcare). The gels were analyzed with Image Master-2D Platinum version 6.0 software (GE Healthcare, USA).

2.14. Mass Spectrometry and Protein Identification. The samples were analyzed by MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry, using α -cyano-4-hydroxycinnamic acid as the matrix on an Ettan MALDI-TOF/Pro instrument (Amersham Biosciences, USA). The spots preparation, mass spectrometry, and database search for protein identification were performed according to the protocol already described in Vieira et al. [53]. Prediction for protein localization was performed by LipoP program [49].

2.15. Human Sera and Microscopic Agglutination Test. Confirmed-leptospirosis serum samples were obtained from Instituto Adolfo Lutz collection, Sao Paulo, Brazil, as previously described [41]. In brief, a laboratory-confirmed case of leptospirosis was defined by the demonstration of a four-fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered the titer that was the highest sample dilution with 50% of agglutination. MAT (microscopic agglutination test) was considered negative when the titer was below 100. In addition to the MAT-negative and MAT-positive paired samples of the same individuals with laboratory and clinic leptospirosis confirmations, we also employed sera from normal healthy donors (normal human sera—NHS), without a known history of leptospirosis, with confirmed negative MAT.

2.16. Human IgG and C3b Deposition on Leptospires. *L. interrogans* serovar Pomona strain LPF (2.5×10^7 cells/well) were diluted in 50 μ L lPBS, coated onto microplates, and allowed to stand at 37°C for 1 h, followed by overnight at 4°C. The plates were washed twice with 200 μ L lPBS and blocked with 5% nonfat dry milk and 2.5% BSA for 2 h at 37°C. After addition of 50 μ L of pooled sera/well (NHS, MAT-negative or MAT-positive) as a source of IgG, the plates were allowed to incubate for 30 min on ice, and then washed three times. The coated leptospires were treated with 50 μ L/well of 5 μ g/mL uPA (urokinase, Sigma) and 40 μ g/mL PLG (Calbiochem) for 2 h at 37°C, or only with uPA, only with PLG, or no additions, as negative controls. After three washings, the plates were incubated with anti-human IgG Fc region specific antibodies (Calbiochem) at a dilution of 1:5,000 for 1 h at 37°C, and then with secondary antibodies conjugated with peroxidase at a dilution of 1:10,000 for 1 h at 37°C. The IgG depositions on leptospires were evaluated by the peroxidase substrate OPD and readings were taken at 492 nm. When dilutions of NHS were used, they were performed with lPBS.

The protocol of C3b deposition on leptospires was similar to the IgG deposition described before, with exception of the incubation with NHS, placed at 37°C for 50 min, and use of anti-human C3b primary antibodies (1:5,000)

FIGURE 1: Recognition of PLG binding to *Leptospira* by L-IFA. Live virulent *L. interrogans* serovar Copenhageni isolates were treated with PLG, and the recognition was assessed through polyclonal anti-PLG antibodies under a confocal immunofluorescence microscope (PLG 1 to 3). PLG-treated leptospires stained for the proteins LipL32 (outer surface protein marker) or GroEL (a protoplasmic-cylinder marker) and untreated leptospires (PBS), are shown as controls (a) DNA propidium iodide-stained, (b) FITC-stained and (c) A+B composite images.

(Calbiochem), followed by secondary antibodies conjugated with peroxidase (1 : 5,000).

2.17. Human C3b Degradation by Leptospires. *L. interrogans* serovar Pomona strain LPF (10^8 leptospires/sample) were treated in 200 μ L lsPBS with the addition of 10 μ g PLG and

3 U uPA (plasmin), 10 μ g PLG, or no additions (untreated). The cells were incubated for 1 h at 37°C with the PLG, and for one more hour after the addition of uPA. The cells were washed three times with lsPBS, and then resuspended in 100 μ L lsPBS containing 15 μ g/mL human purified C3b (Calbiochem), being incubated for 20 h at 37°C. The leptospires were removed by centrifugation, 20 μ L of the supernatants

FIGURE 2: Cleavage of the plasmin-specific chromogenic substrate by plasmin-bound leptospires. Live low-passage virulent and high-passage nonvirulent *L. interrogans* serovar Copenhageni cells received the following treatments: PBS only (PBS), uPA alone (uPA), 5 µg PLG alone (PLG), PLG in crescent quantities (0.5, 2 and 5 µg) together with uPA, and 30% human plasma together with uPA (30% plasma). Bars represent mean absorbance as a measure of relative substrate degradation $\pm$ the standard deviation of three replicates for each experimental group and are representative of three independent experiments. *Virulent leptospires experiments: statistically significant ($P < 0.0001$) in comparison to the PBS control; **nonvirulent leptospires experiments: statistically significant ($P < 0.01$) in comparison to the PBS control. The 30% plasma, as PLG source, and all the PLG + uPA samples of the virulent leptospires were statistically significant ($P < 0.001$) in comparison to the same nonvirulent bacteria tested samples.

FIGURE 3: Inhibition of PLG binding to *L. interrogans* by ACA. Low-passage virulent *L. interrogans* serovar Copenhageni cells were treated with the following: PLG together with uPA (PLG + uPA), PLG together with uPA with the addition of crescent concentrations of ACA (0.01 to 1,000 mM ACA), PLG alone (PLG), uPA alone (uPA), ACA alone (ACA), and no additions (PBS). The cleavage of the plasmin-specific substrate D-Val-Leu-Lys 4-nitroanilide dihydrochloride by the treated spirochetes was measured by absorbance readings at 405 nm. Bars represent mean absorbance $\pm$ the standard deviation of three replicates for each experimental group and are representative of three independent experiments. Statistically significant substrate degradation inhibition results in comparison to the positive control (PLG + uPA) are shown: $P < 0.01$ (*) and $P < 0.0001$ (**).

were separated by 10% SDS-PAGE and then transferred to nitrocellulose membranes in semi-dry equipment. The membranes were blocked by incubating overnight at 4°C with 5% nonfat dry milk and 1% BSA. The C3b detection was made by incubations with anti-human C3b antibodies and secondary antibodies conjugated with peroxidase, followed by ECL (GE Healthcare) development.

2.18. Serum Susceptibility Testing for Treated Leptospire. Complement-mediated killing of leptospire was evaluated after incubation with nonimmune NHS, as described by Meri et al. [54], with some modifications. Approximately 5×10^8 *L. interrogans* serovar Pomona strain LPF per sample were treated with 40 µg PLG and 4 U uPA in 200 µL IsPBS for 3 h at 37°C. As a control, one aliquot of *L. interrogans* serovar Pomona strain LPF was incubated only with PBS. Then, the samples were divided into aliquots containing 1.0×10^8 cells, which were added by 80 µL NHS and 80 µL EMJH medium supplemented with 10% *Leptospira* enrichment. The bacteria suspensions were incubated at 37°C for one hour, followed by a incubation of 5 min on ice for stopping complement activation. After, 20 µL of each sample (four replicates) were transferred to microplates filled with 180 µL of EMJH medium/well. The plates were sealed with a sterile adhesive film and incubated at 30°C for 4 days. Bacterial growth was determined by counting leptospire in a Petroff-Hausser chamber under dark-field microscopy.

2.19. Statistics. For the data that were tested for statistical significance, the student's two-tailed test was applied, considering the minimum of $P < 0.05$.

3. Results and Discussion

3.1. Binding of Human Plasminogen by *L. interrogans* Cells. Live-immunofluorescence microscopy (L-IFA) was employed in order to evaluate the ability of *L. interrogans* cells to bind human PLG. L-IFA revealed that leptospire bind PLG in their outer surface along the entire cell, as seen when the bacteria were probed with antibodies anti-PLG (Figure 1). PLG bound to leptospire does not seem to damage the bacterial membrane because no fluorescence of GroEL, a cytoplasmatic heat-shock protein [55], was achieved when serum anti-GroEL was employed (Figure 1). The leptospiral recombinant proteins LipL32, an abundant outer membrane protein of *Leptospira* [56], used as positive control, showed an intense surface fluorescence. We also observed that the spirochete regular movement was conserved, suggesting that the flagella remained undamaged. Taken together, these data suggest that the PLG-binding does not interfere with the membrane structure or dynamics [36].

3.2. Activation and Enzymatic Activity of Plasminogen-Bound *Leptospira*. The binding of PLG to leptospiral surface was further analyzed by plasmin enzymatic activity. Leptospire were incubated with purified PLG, followed by plasmin activation after addition of uPA, conferring the bacteria a surface-associated plasmin activity (Figure 2), as measured

FIGURE 4: Binding of human PLG to *L. interrogans* proteins by affinity blotting. Total protein extracts were resolved by 10% SDS-PAGE and electroblotted into nitrocellulose membranes. The membranes were incubated with PLG (a) or PLG + ACA (b), and the binding was detected by anti-PLG antibodies and peroxidase-conjugated antibodies. The reactivity was revealed by ECL (GE Healthcare) and exposure to X-Ray films. NV: high passage, nonvirulent *L. interrogans* serovar Copenhageni strain M 20; Vir: low-passage, virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. The arrows indicate reactivity protein regions seen in the virulent but absent in the nonvirulent bacterial extract. Positions of protein molecular mass markers are shown on the left.

by the degradation of plasmin's chromogenic specific substrate [36].

The labeling with PLG alone did not trigger proteolytic activity suggesting that *L. interrogans* serovar Copenhageni is dependent on the host PLG activation system, probably not possessing an endogenous mechanism, as already reported for other pathogens [57–64]. In contrast, the ability of PLG activation is shown for a number of pathogenic microorganisms [57–64].

Both the virulent and nonvirulent strains of *L. interrogans* serovar Copenhageni tested were capable of capturing PLG, although the virulent strain seems to be more efficient in the PLG-binding than the nonvirulent bacteria (~2.5 fold) (Figure 2) [36]. PLG is present in plasma at a concentration of approximately 20.8 ± 1.9 mg/100 mL [65]. Our data shows that virulent *L. interrogans*, but not the attenuated strain, have the ability to sequester PLG from human plasma in the conditions assayed. At any rate, once in the host, the infectious leptospires can acquire PLG when reach the blood circulation.

3.3. Inhibition by 6-Aminocaproic Acid (ACA) of Proteolytic Activity Acquired by *L. Interrogans*. It is well known that the

FIGURE 5: Fibronectin and laminin degradation by plasmin-coated leptospires. Spirochetes were incubated in IsPBS with no additions (UT) and with addition of PLG and uPA (plasmin). ELISA plate wells coated with 0.5 μ g/well fibronectin (a) or laminin (b) were incubated for 20 h with the spirochetes from each experimental group to allow substrate digestion. Plates were then incubated with antibodies antilaminin or antifibronectin. A reduction in absorbance was interpreted as substrate digestion, relative to controls lacking bacteria (0.5 μ g/well purified laminin or cellular fibronectin, corresponding to 0% degradation). Percent degradation (a reduction in absorbance value relative to control lacking bacteria) was calculated as described in Section 2. Bars represent mean percent substrate degradation relative to the positive control (0% degradation) $\pm$ the standard deviation of three replicates. The experiment was independently performed three times with similar results. $P < 0.001$ (*) and $P < 0.01$ (**).

lysine-binding sites of PLG kringle domains frequently mediate interactions with lysine residues of the cellular receptors [27]. ACA, a lysine analogue, decreased the proteolytic activity of virulent *L. interrogans* serovar Copenhageni incubated with PLG, in a dose-dependent manner (Figure 3). The total inhibition (100%) of plasmin activity achieved with the higher ACA concentrations employed shows that the PLG interaction with these spirochetes occurs through the lysine-binding sites of the kringle domains [36].

FIGURE 6: Binding of leptospiral recombinant proteins to human plasminogen. The recombinant proteins LipL32, Lsa63, rLIC10509, Lp29, Lp49, Lsa27, MPL21, MPL17, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922, rLIC12238, Lsa66, Lp30, Lsa20, rLIC11030, rLIC11834, and rLIC12253 were coated onto 96-well ELISA plates (10 μ g/mL) and allowed to interact with purified human PLG (10 μ g/mL). BSA and gelatin were used as a negative control for nonspecific binding. Binding was detected and quantified by specific antibodies. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of four replicates for each protein and are representative of three independent experiments. Statistically significant binding in comparison to negative control BSA are shown by * ($P < 0.001$); ** ($P < 0.0001$); \$ ($P < 0.02$); # ($P < 0.0005$). The binding of rLIC11834 and rLIC12253 was compared to its binding to gelatin ^X ($P < 0.005$).

FIGURE 7: Activation of recombinant protein-PLG to enzymatically active plasmin. Cleavage of specific plasmin substrate by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG + uPA + specific plasmin substrate (PLG + uPA + S) or controls lacking one of the three components (PLG + uPA; PLG + S; uPA + S). BSA was employed as a negative control. Bars represent the mean absorbance values at 405 nm, as a measure of relative substrate cleavage, $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. Statistically significant differences are shown by * ($P < 0.00001$), ** ($P < 0.005$), *** ($P < 0.05$), \$ ($P < 0.001$), \$\$ ($P < 0.02$), and \$\$\$ ($P < 0.003$).

3.4. Analysis of Plasminogen-Binding *L. Interrogans* Proteins on Cell Lysates. Assessment of leptospiral proteins binding to PLG was first visualized by affinity blotting using total whole-cell lysates (Figure 4(a)). Our results are indicative that multiple leptospiral proteins are capable of binding PLG, although these affinity blotting experiments do not simulate the protein-PLG interaction *in vivo*. As expected, the data also demonstrate that there are similarities between the PLG-binding proteins of the virulent and nonvirulent strains, but also revealed the presence of some reactive bands on the low-passage virulent strain that are absent in the nonvirulent one, indicating that virulent strains have more PLG binding proteins. Thus, based on the data from enzymatic activity

and affinity blotting presented here we cannot discriminate whether the increased bound activity of the virulent strain is due to only differential expression of a different set of membrane receptors, an increased expression of the same receptors, or both. Comparable to the results obtained with the intact leptospires, the affinity blotting was inhibited by the presence of the lysine analog, ACA, supporting the central role of lysine residues of the proteins in the PLG interactions (Figure 4(b)) [36].

3.5. Plasmin-Coated *Leptospira* Degrades Immobilized ECM Molecules. Plasmin is a broad-spectrum serine protease that

FIGURE 8: Characterization of recombinant proteins binding to PLG. In (a), (b), and (c) PLG ($10 \mu\text{g/mL}$) was immobilized in 96-well ELISA plates, and each recombinant protein at 0 to 1,000 nM was added for interaction. The binding was detected using antiserum raised in mice against each protein at appropriate dilutions (1:4,000 for LipL32; 1:5,000 for rLIC12238, LipL40, and MPL36; 1:1,000 for Lp29, Lp49, Lsa20, and rLIC11030; 1:500 for rLIC12730, Lsa66, and Lp30; 1:750 for rLIC11834 and rLIC12253), followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values $\pm$ the standard deviation of six replicates for each experimental group. The results are representative of two independent experiments. In (d) The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that reached equilibrium up to a concentration of 1,000 nM.

is capable to degrade directly laminin and fibronectin. To evaluate whether the plasmin associated to the leptospire surface can directly break these ECM components, purified soluble human fibronectin and laminin were adhered into 96-well plates, and the reaction was evaluated by exposing the ECM proteins with low-passage virulent *L. interrogans*, pretreated with PLG/uPA. As shown in Figure 5, the plasmin-coated leptospire were able to significantly cleave down the

immobilized ECM macromolecules, when compared to the ones exposed to untreated bacteria [36].

Given that leptospire are highly invasive pathogens that are thought to penetrate the skin or break in the skin to initiate infection, the ability of digesting ECM macromolecules via plasmin activity might be an important step for leptospiral pathogenesis. To date, this is the first proteolytic activity detected in *Leptospira* that can promote

FIGURE 9: Two-dimensional gel analysis of *L. interrogans* PLG binding proteins. Leptospiral total cell lysates were separated by isoelectric point (pH 3–10) and molecular mass on 12% SDS-PAGE. Replicate gels were Coomassie Blue stained (a) or transferred to membranes and submitted to PLG affinity immunoblotting (b). The reactive spots are enumerated on (a). The results are representative of three independent experiments performed in duplicates.

ECM degradation and it is a plausible mechanism that can contribute to leptospiral invasiveness [1, 66].

3.6. Recombinant Leptospiral Proteins Bind Human Plasminogen. In order to investigate PLG binding proteins in *Leptospira* we benefitted from the availability of several recombinant proteins of *L. interrogans* available in our laboratory. We evaluated the ability of these proteins to interact with PLG *in vitro*. Table 1 summarizes the features of these proteins and gene conservation within the sequenced genomes [8–12]. The binding of these proteins to PLG was quantified by ELISA and the results are shown in Figure 6. Proteins LipL32, Lp29, Lp49, LipL40, MLP36, rLIC10494, rLIC12730, rLIC12238, Lsa66, Lp30, Lsa20, and Lsa33 (rLIC11834) presented significant binding to PLG while proteins Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12922, rLIC11030, Lsa25 (rLIC12253), BSA, and gelatin did not show significant binding activity (Figure 6) [37–40].

3.7. Activation of Plasminogen-Bound Proteins. To evaluate whether purified PLG-binding proteins were independently

FIGURE 10: Human IgG and C3b binding to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospores/well) and IgG (a) or C3b (b) were bound by incubating bacteria with 100% NHS. Bacteria were treated with PBS, 40 μ g/mL PLG (PLG), 5 μ g/mL uPA (uPA), or PLG + uPA (PLA). Presence of human IgG or C3b deposited on leptospores was determined by ELISA. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

capable to generate active plasmin in the presence of uPA PLG activator, we measured the degradation of plasmin specific chromogenic substrate in a modified ELISA. The PLG captured by the proteins could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity (Figure 7). The negative control BSA, that showed no PLG binding, produced no significant proteolytic activity, as well as the controls lacking PLG, uPA, or the chromogenic substrate [37–40].

3.8. Characterization of the Binding of Recombinant Proteins to PLG. The binding between each recombinant protein and PLG was assessed on a quantitative basis as illustrated in Figures 8(a), 8(b), and 8(c). Dose-dependent and saturable binding was observed when increasing concentrations (0 to 1,000 nM) of the recombinant proteins MPL36, LipL40, LipL32, rLIC10494, rLIC12238, Lsa66, Lp30, Lsa33 (rLIC11834), and Lsa20 was allowed to individually adhere to a fixed amount of PLG (1 μ g), indicating the specificity of the binding. For the proteins Lp29, Lp49, rLIC12730, Lsa25 (rLIC12253), and rLIC11030, the saturation level was not

TABLE 2: *L. interrogans* PLG-binding proteins identified by MALDI-TOF mass spectrometry. The proteins are identified as the genomic nomenclature (LIC: *Leptospira interrogans* serovar Copenhageni), and are listed and respective NCBI reference sequence, predicted name/function and predicted cellular localization, in theoric molecular mass and isoelectric point (pI).

Gene locus ¹	NCBI reference sequence number ²	Gene/protein function	Localization ³	Theoric molecular mass (kDa)	pI
LIC12816	YP_002733	Hypothetical protein	Membrane	14.7	9.5
LIC10788	YP_000767	flaA-1 (Flagellar filament sheath protein)	Membrane	35.0	7.6
LIC11194	YP_001164	Putative citrate lyase	Cytoplasm	37.2	6.1
LIC12875	YP_002791	tuf (Elongation factor Tu)	Cytoplasm	43.6	5.7
LIC12795	YP_002712	Acetyl-CoA acetyltransferase	Cytoplasm	47.4	6.0
LIC12407	YP_002339	Glutamine synthetase protein	Cytoplasm	55.4	5.9
LIC10524	YP_000508	dnaK (Heat shock protein)	Cytoplasm	69.1	5.1

¹<http://aeg.lbi.ic.unicamp.br/world/lic/>.

²<http://www.ncbi.nlm.nih.gov/protein/>.

³Lipo P: Juncker et al., [49].

reached, even at the highest concentration tested (1,000 nM). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG are depicted in Figure 8(d); for the ones that reached equilibrium, the highest and the lowest K_D values were for Lsa20 (509.13 ± 77.47 nM) and MPL36 (3.52 ± 3.95 nM), respectively, [37–40].

3.9. Proteomic Approach to Leptospiral PLG Binding Protein Identification. In order expand our knowledge on PLG binding proteins, we decided to employ two-dimensional gel electrophoresis followed by affinity immunoblotting, as an alternative strategy to identify novel leptospiral PLG-binding proteins. Total protein extracts of low passage *L. interrogans* were separated by isoelectric point and molecular mass (Figure 9(a)), transferred to membranes, and incubated with PLG and anti-PLG antibodies for detection of reactive spots (Figure 9(b)). The reactive spots were excised and prepared for MALDI-TOF mass spectrometry and protein identification. Under the conditions assayed, we could identify seven proteins capable of binding PLG, as depicted in Table 2. Two were annotated as membrane and five as cytoplasmatic proteins (Table 2). Along with the membrane proteins, one is hypothetical and the other is a putative flagellar filament sheath protein (FlaA-1); the cytoplasmatic proteins are citrate lyase, Tuf (elongation factor), acetyl-CoA acetyltransferase, glutamine synthetase, and the heat shock protein DnaK. While the role of membrane proteins on the PLG sequestering within the host is expected, the meaning of cytoplasmatic proteins remains to be investigated. Intriguingly, none of the PLG-binding recombinant proteins was detected in these experiments. One possible explanation is that the identified proteins, except LIC12816, are expressed in high amounts per cell, while the proteins assayed in our laboratory were below the detection limit, as reported by quantitative proteomics [67]. However, LipL32 that was shown to have the highest expression level per leptospiral cell, was not detected in our proteomics studies. Although the reason for this is unknown, we might speculate that because LipL32 is always presented as isoforms in 2D gels [53, 68, 69], it is possible that these isoforms do not

bind PLG. Several cytoplasmatic proteins of other pathogenic microorganisms have been identified as PLG binding proteins, such as *Bifidobacterium animalis* DnaK [70], *Paracoccidioides brasiliensis*, *Schistosoma bovis* and *Streptococcus pneumoniae* enolases [71] and *Streptococcus pneumoniae* GAPDH [72]. For some of these proteins, there is the possibility that at some moment they could be exposed in the bacterial outer membrane. Indeed, it has been recently reported that *B. burgdorferi* enolase, which is both a cytoplasmic and membrane-associated protein, is also a PLG-binding protein [73]. It is also possible that the PLG binding to these cytoplasmatic proteins are not relevant for the bacteria.

3.10. Human IgG and C3b Deposition on Leptospires. Plasmin-generation on surface of *L. interrogans* may facilitate the bacteria to degrade opsonizing IgG and C3b molecules, and therefore contribute to immune evasion. To test this hypothesis, microtiter plates coated with virulent *L. interrogans* serovar Pomona were incubated with NHS, as a source of C3b and nonspecific IgG molecules. Bacteria were then treated with PLG and uPA, and the resulting surface-associated opsonins were quantified by specific antibodies. The results show that the incubation of bacteria with PLG and uPA resulted in a decrease in IgG Fc region (Figure 10(a)) and C3b depositions (Figure 10(b)) at the leptospiral surface. No effect on IgG or C3b binding to *L. interrogans* was observed with only PLG or uPA [41].

3.11. IgG Binding from Human Leptospirosis Immune Serum to *L. Interrogans*. To evaluate whether PLA activity observed against nonspecific human IgG bound to leptospire could also be functional to immune sera from patients diagnosed with leptospirosis, we performed the same experiment using MAT negative (preimmune phase) and MAT positive (convalescent immune phase) pools of human sera. As expected, MAT positive sera resulted in significant higher IgG depositions on bacterial surface when compared to NHS and MAT negative sera (Figure 11). When PLA was generated on leptospire, a noticeable decrease in the amount of IgG

FIGURE 11: IgG binding from human leptospirosis immune serum to *L. interrogans*. Bacteria were coated onto microtiter plates (25×10^6 leptospires/well) and IgG was bound by incubating bacteria 50% nonimmune human sera (NHS) or confirmed paired leptospirosis patients sera: MAT negative (MAT-) or MAT positive (MAT+). Bacteria were treated with PBS, 40 μ g/mL PLG (PLG), 5 μ g/mL uPA (uPA), or PLG + uPA (Pla). Presence of human IgG deposited on leptospires was determined by ELISA using Fc-specific anti-human IgG antibodies. Bars represent the mean absorbance values at 492 nm $\pm$ the standard deviation of three replicates for each experimental group and are representative of two independent experiments.

deposition from both negative and positive MAT sera was detected [41].

Opsonization obstruction by antibodies or complement system appears to be an interesting approach for the bacteria to evade the first line of human immune defense upon infection. In fact, the expression of proteins on the bacterial surface that sequester host complement regulators FH, FHL-1, or C4BP and therefore the inhibition of the complement cascade by the alternative or classical pathways has been employed by several pathogens [74–77]. Moreover, the proteolytic cleavage of antibodies that prevents the activation of complement classical pathway or Fc receptor-mediated phagocytosis, is another well-known strategy employed by pathogenic bacteria [74–77].

3.12. Human C3b Degradation by Leptospires. Meri and colleagues [54] reported the capability of *Leptospira*-bound factor H (FH) to act as a cofactor for the serum protease factor I in cleaving C3b. Thus, we decided to assess whether the decrease in C3b deposition by *Leptospira*-generated PLA was due to C3b cleavage. *L. interrogans* serovar Pomona were coated with PLA by treatment with PLG and uPA, and then incubated with human purified C3b in fluid-phase. The resulting supernatant together with anti-C3b-specific antibodies was analyzed by Western blotting (Figure 12). The data revealed the appearance of two lower molecular mass bands of estimated 60 and 20 kDa, concurrently with the decrease in the masses of the alpha (105 kDa) and beta (75 kDa) C3b chains that took place only with PLA-coated

FIGURE 12: Detection of human C3b. Leptospires (10^8 /sample) were treated with 10 μ g PLG and 3 U uPA (Plasmin), only with PLG (PLG), or left untreated (UT). The cells were incubated with 15 μ g/mL human purified C3b for 20 h at 37°C, and the supernatants were resolved by SDS-PAGE and transferred to membranes. The C3b was detected by specific antibodies followed by ECL reagent development and exposition to X-Ray films. There are indicated the native human C3b alpha chain (105 KDa) and beta-chain (75 KDa), as well as the degradation products (arrows).

leptospires. Untreated or leptospires only treated with PLG showed no gradation. The results unmistakably show that the human C3b is cleaved by leptospires-coated PLA, indicating that the reduction in deposition is most probably due to degradation [41].

3.13. Serum Susceptibility Testing for Treated Leptospires. It has been described that *L. interrogans* serovar Pomona is partially susceptible to complement killing upon incubation with normal human sera [54]. To evaluate whether the PLG/PLA binding to leptospires confers protection on exposure to NHS, leptospires were treated with PLG+uPA (Pla) or with PBS, as negative control, and incubated for one hour with pooled NHS. The bacteria were harvested and counted in a dark-field microscopy using Petroff-Hausser chamber, at the fourth day of incubation. The number of untreated leptospires was almost four times less when compared to the plasmin-coated leptospires ($0.35 \times 10^8 \pm 0.1 \times 10^8$ versus $1.28 \times 10^8 \pm 0.3 \times 10^8$ leptospires/mL), suggesting that the leptospires endowed with active plasmin are less vulnerable to components present in NHS [41].

4. Conclusions

The interaction of the human PLG system has been suggested to be a feature that significantly contributes to the virulence of many bacterial pathogens by facilitating the initial anchoring to endothelium and penetration [27]. Our group has been studying the interaction of *Leptospira* with PLG/plasmin generation system and the possible implications for pathogenesis. We demonstrated that leptospires interact with PLG and can acquire plasmin activity associated to the surface with no apparent damage to the surface, what occurs through multiple proteins. Although nonvirulent leptospires can interact with PLG, there seems to be a correlation between the efficiency of PLG capturing and virulence, suggesting a role in virulence and infection. Moreover, the generation of enzymatically active PLA on the leptospiral surface decreased C3b and IgG depositions that may constitute a novel mechanism by which leptospires could evade the immune system until reach immunologically safe environments. This latter assertion will also have implication to the bacterial persistence within the host. We believe that the disclosure of this enzymatically system will shed light on the molecular mechanism of leptospiral pathogenesis.

Acknowledgments

The authors are deeply indebted to Alexsander Seixas de Souza (Departamento de Parasitologia, Instituto Butantan, Sao Paulo, Brazil) for the use of the confocal microscope facilities and the helpful discussion. This work was supported by FAPESP, CNPq, and Fundação Butantan, Brazil; M.L.Vieira; M.V.Atzingen; R.Oliveira; R.S.Mendes; R.F.Domingos have scholarships from FAPESP (Brazil).

References

- [1] S. Faine, B. Adler, C. Bolin, and P. Perolat, *Leptospira and Leptospirosis*, MediSci, Melbourne, Australia, 2nd edition, 1999.
- [2] P. N. Levett, "Leptospirosis," *Clinical Microbiology Reviews*, vol. 14, no. 2, pp. 296–326, 2001.
- [3] J. M. Vinetz, "Leptospirosis," *Current Opinion in Infectious Diseases*, vol. 14, no. 5, pp. 527–538, 2001.
- [4] A. R. Bharti, J. E. Nally, J. N. Ricaldi et al., "Leptospirosis: a zoonotic disease of global importance," *Lancet Infectious Diseases*, vol. 3, no. 12, pp. 757–771, 2003.
- [5] A. I. Ko, M. Galvão Reis, C. M. Ribeiro Dourado, W. D. Johnson, and L. W. Riley, "Urban epidemic of severe leptospirosis in Brazil," *Lancet*, vol. 354, no. 9181, pp. 820–825, 1999.
- [6] D. A. Haake, M. Dundoo, R. Cader et al., "Leptospirosis, water sports, and chemoprophylaxis," *Clinical Infectious Diseases*, vol. 34, no. 9, pp. e40–43, 2002.
- [7] R. Plank and D. Dean, "Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans," *Microbes and Infection*, vol. 2, no. 10, pp. 1265–1276, 2000.
- [8] S. X. Ren, G. Fu, X. G. Jiang et al., "Unique physiological and pathogenic features of *Leptospira* interrogans revealed by whole-genome sequencing," *Nature*, vol. 422, no. 6934, pp. 888–893, 2003.
- [9] A. L. T. O. Nascimento, S. Verjovski-Almeida, M. A. Van Sluys et al., "Genome features of *Leptospira* interrogans serovar Copenhageni," *Brazilian Journal of Medical and Biological Research*, vol. 37, no. 4, pp. 459–478, 2004.
- [10] A. L. T. O. Nascimento, A. I. Ko, E. A. L. Martins et al., "Comparative Genomics of two *Leptospira* interrogans serovars reveals novel insights into physiology and pathogenesis," *Journal of Bacteriology*, vol. 186, no. 7, pp. 2164–2172, 2004.
- [11] D. M. Bulach, R. L. Zuercher, P. Wilson et al., "Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 39, pp. 14560–14565, 2006.
- [12] M. Picardeau, D. M. Bulach, C. Bouchier et al., "Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis," *PLoS One*, vol. 3, no. 2, Article ID e1607, 2008.
- [13] J. Matsunaga, M. A. Barocchi, J. Croda et al., "Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily," *Molecular Microbiology*, vol. 49, no. 4, pp. 929–945, 2003.
- [14] P. Ristow, P. Bourhy, F. W. da Cruz McBride et al., "The OmpA-like protein Loa22 is essential for *Leptospira* virulence," *PLoS pathogens*, vol. 3, no. 7, article e97, 2007.
- [15] M. V. Atzingen, A. S. Barbosa, T. De Brito et al., "Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection," *BMC Microbiology*, vol. 8, article no. 70, 2008.
- [16] Y. Luo, Y. Liu, D. Sun et al., "InvA protein is a Nudix hydrolase required for infection by pathogenic *Leptospira* in cell lines and animals," *The Journal of Biological Chemistry*, vol. 286, no. 42, pp. 36852–36863, 2011.
- [17] E. C. Boyle and B. B. Finlay, "Bacterial pathogenesis: exploiting cellular adherence," *Current Opinion in Cell Biology*, vol. 15, no. 5, pp. 633–639, 2003.
- [18] A. S. Barbosa, P. A. E. Abreu, F. O. Neves et al., "A newly identified leptospiral adhesin mediates attachment to laminin," *Infection and Immunity*, vol. 74, no. 11, pp. 6356–6364, 2006.
- [19] M. T. Longhi, T. R. Oliveira, E. C. Romero et al., "A newly identified protein of *Leptospira* interrogans mediates binding to laminin," *Journal of Medical Microbiology*, vol. 58, no. 10, pp. 1275–1282, 2009.
- [20] H. A. Choy, M. M. Kelley, T. L. Chen, A. K. Møller, J. Matsunaga, and D. A. Haake, "Physiological osmotic induction of *Leptospira* interrogans adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen," *Infection and Immunity*, vol. 75, no. 5, pp. 2441–2450, 2007.
- [21] B. Stevenson, H. A. Choy, M. Pinne et al., "Leptospira interrogans endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement," *PLoS One*, vol. 2, no. 11, Article ID e1188, 2007.
- [22] P. Hauk, F. Macedo, E. C. Romero et al., "In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin," *Infection and Immunity*, vol. 76, no. 6, pp. 2642–2650, 2008.
- [23] D. E. Hoke, S. Egan, P. A. Cullen, and B. Adler, "LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*," *Infection and Immunity*, vol. 76, no. 5, pp. 2063–2069, 2008.
- [24] M. V. Atzingen, R. M. Gómez, M. Schattner et al., "Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells," *Journal of Infection*, vol. 59, no. 4, pp. 264–276, 2009.

- [25] E. Carvalho, A. S. Barbosa, R. M. Gómez et al., "Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity," *FEBS Letters*, vol. 583, no. 8, pp. 1381–1385, 2009.
- [26] M. L. Vieira, Z. M. de Moraes, A. P. Gonçalves, E. C. Romero, S. A. Vasconcellos, and A. L. T. O. Nascimento, "Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV," *Journal of Infection*, vol. 60, no. 1, pp. 52–64, 2010.
- [27] K. Lähteenmäki, P. Kuusela, and T. K. Korhonen, "Bacterial plasminogen activators and receptors," *FEMS Microbiology Reviews*, vol. 25, no. 5, pp. 531–552, 2001.
- [28] J. L. Coleman, T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach, "*Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers," *Infection and Immunity*, vol. 63, no. 7, pp. 2478–2484, 1995.
- [29] M. S. Klemperer, R. Noring, M. P. Epstein et al., "Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *Borrelia burgdorferi*," *Journal of Infectious Diseases*, vol. 171, no. 5, pp. 1258–1265, 1995.
- [30] L. T. Hu, G. Perides, R. Noring, and M. S. Klemperer, "Binding of human plasminogen to *Borrelia burgdorferi*," *Infection and Immunity*, vol. 63, no. 9, pp. 3491–3496, 1995.
- [31] J. L. Coleman, J. A. Gebbia, J. Piesman, J. L. Degen, T. H. Bugge, and J. L. Benach, "Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice," *Cell*, vol. 89, no. 7, pp. 1111–1119, 1997.
- [32] J. L. Coleman and J. L. Benach, "Use of the plasminogen activation system by microorganisms," *Journal of Laboratory and Clinical Medicine*, vol. 134, no. 6, pp. 567–576, 1999.
- [33] J. L. Coleman and J. L. Benach, "The generation of enzymatically active plasmin on the surface of spirochetes," *Methods*, vol. 21, no. 2, pp. 133–141, 2000.
- [34] A. Nordstrand, A. Shamaei-Tousi, A. Ny, and S. Bergström, "Delayed invasion of the kidney and brain by *Borrelia crocidurae* in plasminogen-deficient mice," *Infection and Immunity*, vol. 69, no. 9, pp. 5832–5839, 2001.
- [35] W. B. Haile, J. L. Coleman, and J. L. Benach, "Reciprocal upregulation of urokinase plasminogen activator and its inhibitor, PAI-2, by *Borrelia burgdorferi* affects bacterial penetration and host-inflammatory response," *Cellular Microbiology*, vol. 8, no. 8, pp. 1349–1360, 2006.
- [36] M. L. Vieira, S. A. Vasconcellos, A. P. Gonçalves, Z. M. De Moraes, and A. L. T. O. Nascimento, "Plasminogen acquisition and activation at the surface of *Leptospira* species lead to fibronectin degradation," *Infection and Immunity*, vol. 77, no. 9, pp. 4092–4101, 2009.
- [37] M. L. Vieira, M. V. Atzingen, T. R. Oliveira et al., "In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*," *PLoS One*, vol. 5, no. 6, p. e11259, 2010.
- [38] R. Oliveira, Z. M. de Moraes, A. P. Gonçalves, E. C. Romero, S. A. Vasconcellos, and A. L. T. O. Nascimento, "Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen," *PLoS One*, vol. 6, no. 7, Article ID e21962, 2011.
- [39] R. S. Mendes, M. Von Atzingen, Z. M. de Moraes et al., "The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection," *Infection and Immunity*, vol. 79, no. 11, pp. 4657–4667, 2011.
- [40] R. F. Domingos, M. L. Vieira, E. C. Romero et al., "Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions," *BMC Microbiology*, vol. 12, article 50, 2012.
- [41] M. L. Vieira, Z. M. de Moraes, S. A. Vasconcellos, E. C. Romero, and A. L. T. O. Nascimento, "In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*," *Microbial Pathogenesis*, vol. 51, no. 5, pp. 360–365, 2011.
- [42] L. H. Turner, "Leptospirosis III. Maintenance, isolation and demonstration of leptospirae," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 64, no. 4, pp. 623–646, 1970.
- [43] J. L. Coleman, E. J. Roemer, and J. L. Benach, "Plasmin-coated *Borrelia burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix," *Infection and Immunity*, vol. 67, no. 8, pp. 3929–3936, 1999.
- [44] K. Nakai and P. Horton, "PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization," *Trends in Biochemical Sciences*, vol. 24, no. 1, pp. 34–35, 1999.
- [45] K. Nakai and M. Kanehisa, "Expert system for predicting protein localization sites in gram-negative bacteria," *Proteins: Structure, Function and Genetics*, vol. 11, no. 2, pp. 95–110, 1991.
- [46] I. Letunic, R. R. Copley, B. Pils, S. Pinkert, J. Schultz, and P. Bork, "SMART 5: domains in the context of genomes and networks," *Nucleic Acids Research*, vol. 34, pp. D257–260, 2006.
- [47] J. Schultz, F. Milpetz, P. Bork, and C. P. Ponting, "SMART, a simple modular architecture research tool: identification of signaling domains," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 11, pp. 5857–5864, 1998.
- [48] R. D. Finn, J. Mistry, B. Schuster-Böckler et al., "Pfam: clans, web tools and services," *Nucleic Acids Research*, vol. 34, pp. D247–251, 2006.
- [49] A. S. Juncker, H. Willenbrock, G. Von Heijne, S. Brunak, H. Nielsen, and A. Krogh, "Prediction of lipoprotein signal peptides in Gram-negative bacteria," *Protein Science*, vol. 12, no. 8, pp. 1652–1662, 2003.
- [50] C. A. Brissette, K. Haupt, D. Barthel et al., "*Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen," *Infection and Immunity*, vol. 77, no. 1, pp. 300–306, 2009.
- [51] R. D. Pathirana, N. M. O'Brien-Simpson, P. D. Veith, P. F. Riley, and E. C. Reynolds, "Characterization of proteinase-adhesin complexes of *Porphyromonas gingivalis*," *Microbiology*, vol. 152, no. 8, pp. 2381–2394, 2006.
- [52] Y. P. Lin, D. W. Lee, S. P. McDonough, L. K. Nicholson, Y. Sharma, and Y. F. Chang, "Repeated domains of *Leptospira* immunoglobulin-like proteins interact with elastin and tropoelastin," *Journal of Biological Chemistry*, vol. 284, no. 29, pp. 19380–19391, 2009.
- [53] M. L. Vieira, D. C. Pimenta, Z. M. de Moraes, and A. L. Nascimento, "Proteome analysis of *Leptospira interrogans* virulent strain," *The Open Microbiology Journal*, vol. 3, pp. 69–74, 2009.
- [54] T. Meri, R. Murgia, P. Stefanel, S. Meri, and M. Cinco, "Regulation of complement activation at the C3-level by serum resistant leptospirae," *Microbial Pathogenesis*, vol. 39, no. 4, pp. 139–147, 2005.
- [55] D. A. Haake and J. Matsunaga, "Characterization of the leptospiral outer membrane and description of three novel

- leptospirosis membrane proteins," *Infection and Immunity*, vol. 70, no. 9, pp. 4936–4945, 2002.
- [56] D. A. Haake, G. Chao, R. L. Zuerner et al., "The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection," *Infection and Immunity*, vol. 68, no. 4, pp. 2276–2285, 2000.
 - [57] K. C. Young, G. Y. Shi, D. H. Wu et al., "Plasminogen activation by streptokinase via a unique mechanism," *Journal of Biological Chemistry*, vol. 273, no. 5, pp. 3110–3116, 1998.
 - [58] H. E. McCoy, C. C. Broder, and R. Lottenberg, "Streptokinases produced by pathogenic group C streptococci demonstrate species-specific plasminogen activation," *Journal of Infectious Diseases*, vol. 164, no. 3, pp. 515–521, 1991.
 - [59] H. R. Lijnen, F. De Cock, B. Van Hoef, B. Schlott, and D. Collen, "Characterization of the interaction between plasminogen and staphylokinase," *European Journal of Biochemistry*, vol. 224, no. 1, pp. 143–149, 1994.
 - [60] B. Schlott, K. H. Gührs, M. Hartmann, A. Röcker, and D. Collen, "NH₂-terminal structural motifs in staphylokinase required for plasminogen activation," *Journal of Biological Chemistry*, vol. 273, no. 35, pp. 22346–22350, 1998.
 - [61] K. Okada, S. Ueshima, T. Takaishi, H. Yuasa, H. Fukao, and O. Matsuo, "Effects of fibrin and α_2 -Antiplasmin on plasminogen activation by staphylokinase," *American Journal of Hematology*, vol. 53, no. 3, pp. 151–157, 1996.
 - [62] O. A. Sodeinde, Y. V. B. K. Subrahmanyam, K. Stark, T. Quan, Y. Bao, and J. D. Goguen, "A surface protease and the invasive character of plague," *Science*, vol. 258, no. 5084, pp. 1004–1007, 1992.
 - [63] S. P. Leytus, L. K. Bowles, J. Konisky, and W. F. Mangel, "Activation of plasminogen to plasmin by a protease associated with the outer membrane of *Escherichia coli*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 3 I, pp. 1485–1489, 1981.
 - [64] Z. Li, V. A. Ploplis, E. L. French, and M. D. P. Boyle, "Interaction between group A streptococci and the plasmin(ogen) system promotes virulence in a mouse skin infection model," *Journal of Infectious Diseases*, vol. 179, no. 4, pp. 907–914, 1999.
 - [65] D. Collen, G. Tytgat, H. Claeys, M. Verstraete, and P. Wallén, "Metabolism of plasminogen in healthy subjects: effect of tranexamic acid," *Journal of Clinical Investigation*, vol. 51, no. 6, pp. 1310–1318, 1972.
 - [66] M. A. Barocchi, A. I. Ko, M. Galvão Reis, K. L. McDonald, and L. W. Riley, "Rapid translocation of polarized MDCK cell monolayers by *Leptospira* interrogans, an invasive but nonintracellular pathogen," *Infection and Immunity*, vol. 70, no. 12, pp. 6926–6932, 2002.
 - [67] J. Malmström, M. Beck, A. Schmidt, V. Lange, E. W. Deutsch, and R. Aebersold, "Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*," *Nature*, vol. 460, no. 7256, pp. 762–765, 2009.
 - [68] P. A. Cullen, S. J. Cordwell, D. M. Bulach, D. A. Haake, and B. Adler, "Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai," *Infection and Immunity*, vol. 70, no. 5, pp. 2311–2318, 2002.
 - [69] J. E. Nally, J. P. Whitelegge, S. Bassilian, D. R. Blanco, and M. A. Lovett, "Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection," *Infection and Immunity*, vol. 75, no. 2, pp. 766–773, 2007.
 - [70] M. Candela, M. Centanni, J. Fiori et al., "DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts," *Microbiology*, vol. 156, no. 6, pp. 1609–1618, 2010.
 - [71] S. V. Nogueira, F. L. Fonseca, M. L. Rodrigues et al., "Paracoccidioides brasiliensis enolase is a surface protein that binds plasminogen and mediates interaction of yeast forms with host cells," *Infection and Immunity*, vol. 78, no. 9, pp. 4040–4050, 2010.
 - [72] R. Lottenberg, C. C. Broder, M. D. Boyle, S. J. Kain, B. L. Schroeder, and R. 3rd. Curtiss, "Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor," *Journal of Bacteriology*, vol. 174, no. 16, pp. 5204–5210, 1992.
 - [73] A. M. Floden, J. A. Watt, and C. A. Brissette, "*Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein," *PLoS One*, vol. 6, no. 11, Article ID e27502, 2011.
 - [74] A. M. Blom, T. Hallström, and K. Riesbeck, "Complement evasion strategies of pathogens-Acquisition of inhibitors and beyond," *Molecular Immunology*, vol. 46, no. 14, pp. 2808–2817, 2009.
 - [75] J. D. Lambris, D. Ricklin, and B. V. Geisbrecht, "Complement evasion by human pathogens," *Nature Reviews Microbiology*, vol. 6, no. 2, pp. 132–142, 2008.
 - [76] P. F. Zipfel, R. Würzner, and C. Skerka, "Complement evasion of pathogens: common strategies are shared by diverse organisms," *Molecular Immunology*, vol. 44, no. 16, pp. 3850–3857, 2007.
 - [77] S. H. M. Rooijackers and J. A. G. van Strijp, "Bacterial complement evasion," *Molecular Immunology*, vol. 44, no. 1–3, pp. 23–32, 2007.
 - [78] M. Gamberini, R. M. Gómez, M. V. Atzingen et al., "Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis," *FEMS Microbiology Letters*, vol. 244, no. 2, pp. 305–313, 2005.
 - [79] R. M. Gómez, M. L. Vieira, M. Schattner et al., "Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein endothelial cells (HUVECS) and express during infection," *Microbial Pathogenesis*, vol. 45, no. 5–6, pp. 315–322, 2008.
 - [80] F. O. Neves, P. A. E. Abreu, S. A. Vasconcellos, Z. M. De Moraes, E. C. Romero, and A. L. T. O. Nascimento, "Identification of a novel potential antigen for early-phase serodiagnosis of leptospirosis," *Archives of Microbiology*, vol. 188, no. 5, pp. 523–532, 2007.
 - [81] T. R. Oliveira, M. T. Longhi, Z. M. De Moraes et al., "Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays," *Clinical and Vaccine Immunology*, vol. 15, no. 11, pp. 1715–1722, 2008.

RESEARCH ARTICLE

Open Access

"Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions"

Renan F Domingos^{1,2}, Monica L Vieira^{1,2}, Eliete C Romero³, Amãne Paldes Gonçalves⁴, Zenaide M de Moraes⁴, Silvio A Vasconcellos⁴ and Ana L T O Nascimento^{1,2*}

Abstract

Background: Leptospirosis is considered a re-emerging infectious disease caused by pathogenic spirochaetes of the genus *Leptospira*. Pathogenic leptospires have the ability to survive and disseminate to multiple organs after penetrating the host. Leptospires were shown to express surface proteins that interact with the extracellular matrix (ECM) and to plasminogen (PLG). This study examined the interaction of two putative leptospiral proteins with laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, PLG, factor H and C4bp.

Results: We show that two leptospiral proteins encoded by LIC11834 and LIC12253 genes interact with laminin in a dose - dependent and saturable mode, with dissociation equilibrium constants (K_D) of 367.5 and 415.4 nM, respectively. These proteins were named Lsa33 and Lsa25 (Leptospiral surface adhesin) for LIC11834 and LIC12253, respectively. Metaperiodate - treated laminin reduced Lsa25 - laminin interaction, suggesting that sugar moieties of this ligand participate in this interaction. The Lsa33 is also PLG - binding receptor, with a K_D of 23.53 nM, capable of generating plasmin in the presence of an activator. Although in a weak manner, both proteins interact with C4bp, a regulator of complement classical route. *In silico* analysis together with proteinase K and immunofluorescence data suggest that these proteins might be surface exposed. Moreover, the recombinant proteins partially inhibited leptospiral adherence to immobilized laminin and PLG.

Conclusions: We believe that these multifunctional proteins have the potential to participate in the interaction of leptospires to hosts by mediating adhesion and by helping the bacteria to escape the immune system and to overcome tissue barriers. To our knowledge, Lsa33 is the first leptospiral protein described to date with the capability of binding laminin, PLG and C4bp *in vitro*.

Background

Leptospirosis is a zoonosis caused by pathogenic species of the genus *Leptospira*. Greater incidence of human infection occurs in tropical and subtropical countries [1,2]. The transmission of leptospirosis has been correlated with exposure of individuals in close proximity to wild or farm animals [1,3]. Recently, the disease became prevalent in cities with sanitation problems and large urban rodent reservoirs that contaminate the environment through their urine [4].

Pathogenic *Leptospira* spp. have ability to adhere and rapidly disseminate within the host during the early stage

of infection. Surface - associated proteins are potential targets to mediate host - pathogen interactions, and therefore are likely to elicit several activities, including adhesion. The adhesion of leptospires to ECM components of the host was considered to be essential in the initial stage of the infection [5]. Indeed, we have reported that pathogenic leptospires are capable of binding several ECM molecules [6]. To date, several leptospiral ECM binding adhesins have been described [6–18]. After the adhesion, pathogens have to overcome tissue barriers in order to reach blood circulation and organs. We have reported that leptospires have the ability of binding PLG at their surface and that plasmin (PLA) can be generated in the presence of activator [19]. In addition, Verma and colleagues [20] and our group have described several leptospiral proteins as PLG - binding receptors [17,18,21]. More recently, we have reported that PLA generation on

* Correspondence: tabet@butantan.gov.br

²Pós-Graduação Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Avenida Prof. Lineu Prestes, 173005508-900, São Paulo, SP, Brazil
Full list of author information is available at the end of the article

Leptospira decreased opsonization and that it might be an important aspect of the immune escape strategy and survival [22].

L. interrogans serovar Copenhageni genome annotation identified many unknown coding sequences predicted to be surface exposed proteins. Characterization of these proteins, with no previously assigned function, should increase our understanding of this intriguing pathogen's biology. In this work, we present our studies with two leptospiral coding sequences, LIC11834 and LIC12253, named Lsa33 and Lsa25, respectively. The genes were cloned and the proteins expressed using *E. coli*. The recombinant proteins were purified and their ability to bind various ECM and serum components was evaluated. We report that these proteins are novel surface adhesins capable of binding to laminin. In addition, Lsa33 can also interact to PLG and both proteins bind the complement regulator of the classical pathway C4bp. We believe that these proteins are likely to be involved in *Leptospira* - host interactions.

Results

Bioinformatic analysis

The selected coding sequences, LIC11834 and LIC12253, are genome annotated as hypothetical proteins, and one of them, LIC11834, is a putative lipoprotein, having lipoprotein signal peptide (signal peptidase II) and a cleavage site between amino acids 17–18. According to SMART web server, LIC11834 has a signal peptide from 1 to 21 amino acids and a FecR domain from amino acid 60 to 162. PFAM predicts that this domain is involved in regulation of iron dicitrate transport and that FecR is probably a sensor that recognizes iron dicitrate in the periplasm. LIC12253 presents a signal peptide from amino acid 1 to 21 and a DUF1566 (Domain of Unknown Function) from amino acid 58 to 164 [23,24]. The LIC11834 coding sequence can be classified as alpha - beta protein, being the percentage of 36.57 for

alpha-helix and 29.13 for beta strands secondary structure. In the case of coding sequence LIC12253, the protein can be classified as mixed, having a predicted secondary structure composition percent of 11.01, 19.38 and 69.60 for alpha - helix, beta strands and others, respectively. Cellular localization predicts as extra - cellular (non-cytoplasmic branch) for both proteins. The solvent accessibility composition (core/surface ratio) for the CDs LIC11834 and LIC12253 is expected to be 59.87 and 66.52% of amino acid residues exposed with more than 16% of their surface, respectively. All the predictions above were performed with PrecitedProtein web server [25,26]. The presence and identity of both coding sequences among *Leptospira* sequenced genomes are depicted in Table 1.

Distribution and expression of LIC11834 and LIC12253 genes among *Leptospira* strains

The presence of LIC11834 and LIC12253 genes in 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. The gene LIC11834 was amplified by PCR in all strains belonging to the pathogenic species excluding in *L. santarosai* serovar Shermani (Figure 1A). No DNA amplification was detected in the non - pathogenic *L. biflexa* serovar Patoc. In the case of LIC12253 gene, DNA band was amplified in all pathogenic strains and a less intense band was detected in the saprophytic strain (Figure 1A). The expression of LIC11834 and LIC12253 genes was evaluated by PCR amplification of reversely transcribed total RNA. LIC11834 gene product was detected only in *L. interrogans* specie serovars Canicola, Pomona, Copenhageni, Icterohaemorrhagiae and Hardjo. No expression was observed in non-pathogenic strain. LIC12253 gene expression could be identified in all pathogenic strain tested (Figure 1B). Integrity of total RNA used in RT - PCR experiments was assured by the

Table 1 Gene locus, given names, features, gene conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins

Gene locus ¹	Given name ²	Description/ Function	Conservation (identity) ³	Sequence of primers for PCR amplification	Molecular mass
LIC11834	Lsa33	Putative lipoprotein	Lai (99%) LBH (87%) LBP (31%)	F:5'CTCGAGGATCTACAAGGTGGGGTTTTTAC3' XhoI R:5'CCATGGTTACTGAGGTTTACTTGGTCC3' NcoI	33.1 kDa
LIC12253	Lsa25	Conserved hypothetical protein	Lai (100%) LBH (77%) LBP (39%)	F:5'CTCGAGGAGGAGAAACCGGACGATAC 3' XhoI R:5' CCATGGTTAGGGAAGACTTCTAACACATC3' NcoI	24.07 kDa

¹http://aeg.lbi.ic.unicamp.br/world/lic/; LIC: *Leptospira interrogans* Copenhageni

²Lsa: Leptospiral surface adhesin of 33 and 24 kDa; we have named the latter as Lsa25 because Lsa24 has been already described (Barbosa et al., 2006)

³http://blast.ncbi.nlm.nih.gov/Blast.cgi/

Figure 1 Analysis of the LIC11834 and LIC12253 genes and their transcripts among different leptospiral strains. (A) Analysis by PCR of the LIC11834 (2) and LIC12253 (3) genes in pathogenic serovars (*L. interrogans*, *L. borgpetersenii*, *L. kirshnery*, *L. noguchi* and *L. santarosai*) and in the non - pathogenic *L. biflexa* strain. 16 S rRNA gene expression was used as an internal control (1). The negative control contained no DNA, indicated by (-). **(B)** Analysis of the LIC11834 (2) and LIC12253 (3) transcripts by RT - PCR using 2 µg total RNA extracted from different serovars belonging to the pathogenic species and the saprophytic *L. biflexa* serovar Patoc strain Patoc. 16 S rRNA gene expression was used as an internal control (1). Reverse transcriptase present, +; reverse transcriptase omitted, -. The negative control contained no cDNA, indicated by (-).

presence of a 1,042 - bp 16 S ribosomal cDNA fragment in all samples (Figure 1B).

Cloning and characterization of recombinant proteins

The amplified DNA sequences of LIC11834 and LIC12253 were cloned into an *E. coli* pAE vector [27] and the corresponding proteins were expressed as full-length with 6X His sequence tag at their N - terminal. Expression of recombinant proteins was elicited from cultures of *E. coli* BL21 SI after addition of NaCl (300 mM). Recombinant protein Lsa33 is expressed in its soluble form, while Lsa25 is expressed in its insoluble form, as inclusion bodies (data not shown). Protein Lsa25 was recovered from inclusion bodies after solubilization with 8 M urea. The purification was performed by metal chelating chromatography under normal (Lsa33) or denaturing condition, followed by refolding by gradually removal of urea (Lsa25). The proteins were recovered with 1.0 M imidazol. Evaluation of protein purification has shown that most of the

contaminants were washed away and proteins were represented as single major bands. The recombinant protein bands were further confirmed by western blotting probed with monoclonal anti - His tag antibodies and with polyclonal antiserum raised against each protein (data shown). The calculated 33.1 kDa and 24.07 kDa molecular masses of the recombinant proteins comprise the vector fusion plus the encoded amino acids. Structural integrity of the purified proteins was assessed by circular dichroism (CD) spectroscopy. The minima at 208 and 222 nm, and the maximum at 192 nm in the CD spectrum showed the high α - helical secondary structure content of both recombinant proteins (data not shown).

Recognition of the LIC11834 and LIC12253 coding sequences by immunofluorescence confocal microscopy

The assessment of the selected CDSs on the bacterial cell membrane was performed using living organisms and the liquid - phase immunofluorescence method.

Leptospire were visualized by propidium iodide staining (Figure 2, column A) followed by protein detection with polyclonal mouse antiserum raised against each protein in the presence of anti - mouse IgG antibodies conjugated to FITC. Green fluorescence could be observed in Figure 2 column B, for LIC11834, LIC12253 and LipL32, an outer membrane protein used as a positive control [28], but not with GroEL, a protoplasmic - cylinder marker, used as a negative control [29]. The localization of the protein - green light lying on the leptospire was achieved by merging both fields and the results obtained are shown in Figure 2, column C.

Cellular localization of the LIC11834 and LIC12253 coding sequences by protease assay

We have performed proteinase K accessibility assay by using the previously described assay (37, 41) with some modifications. Live leptospire were treated with 25 µg/ml of proteinase K and aliquots of the bacterial suspensions were taken at time 0, 1, 3 and 5 h; the

suspensions were sedimented and the resuspended bacteria were used to coat microplates, followed by incubation with polyclonal antibodies against each protein, including the controls, LipL32 and DnaK, for outer [28] and cytoplasmic [30] protein. The reactions proceeded as described in Methods. The leptospiral coding sequences LIC11834 and LIC12253 were both susceptible to protease treatment after 1 h incubation, similar to the positive control LipL32 (Figure 3). Almost no reduction was observed with DnaK cytoplasmic protein (Figure 3).

Recombinant protein Lsa25 is recognized by antibodies of confirmed cases of leptospirosis

To examine whether LIC11834 and LIC12253 leptospiral coding sequences are able to elicit an immune response from an infected host, we evaluated the reactivity of the recombinant proteins Lsa25 and Lsa33 with antibodies present in serum samples of early (MAT -) and convalescent (MAT +) phases of leptospirosis patients. ELISA was performed using 24 and 33 serum samples of negative MAT and of positive MAT, respectively. The recombinant protein Lsa33 was almost non-reactive with samples from both phases of the disease (Figure 4A), while Lsa25 showed 46 and 48% reactivity for negative and positive MAT, respectively (Figure 4B). When the two proteins were assayed together, a small increment was

Figure 2 Recognition of coding sequences LIC11834 and LIC12253 proteins in *L. interrogans* by their respective antibodies. Liquid - phase Immunofluorescence Assay (L - IFA) was performed with live *L. interrogans* under confocal microscopy using antiserum for LIC11834, LIC12253, LipL32 (surface - exposed lipoprotein) and GroEL (protoplasmic cylinder marker). Leptospire were identified by propidium iodide staining of the DNA (A). FITC - conjugated secondary antibodies were used to detect the surface - bound antibodies (B). Co - localization is shown in the merged images (C).

Figure 3 Protease accessibility assay of LIC11834 and LIC12253 encoded proteins of *L. interrogans*. Viable leptospire were incubated with 25 µg/ml of proteinase K at the indicated times. The suspensions were sedimented, washed, resuspended in PBS and coated in a microplate. Antibodies against recombinant proteins Lsa33, Lsa25, LipL32 and DnaK were added. After incubation, anti-IgG peroxidase conjugated was added and the reaction was developed with OPD peroxidase substrate. Blanks were run in parallels but antibodies against the proteins were omitted. Readings were taken at 492 nm. Bars represent the mean of absorbance ± the standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the signal was compared between 0 hour and hours of treatment with PK by two-tailed t test (* $P < 0.05$).

Figure 4 Reactivity of the recombinant antigens Lsa33 and Lsa25 with serum samples of individuals diagnosed with leptospirosis. Responders were determined by ELISA with the recombinant proteins and serum samples from patients of both phases of the disease. The reactivity was evaluated as IgG antibodies. Serum was considered MAT positive or MAT negative if agglutination was detected when the sera were tested for their reactivity's with isolates of the 22 serovars (see Methods). The cutoff values are depicted as horizontal bars and were defined as the mean plus 3 standard deviations obtained for sera from 12 healthy individuals. (A) shows the data for Lsa33, (B) for Lsa25, and (C) depicts the data when both proteins were employed (Lsa33 plus Lsa25).

observed for positive MAT samples (58%) (Figure 4C). Our data suggest that Lsa25 might be an interesting protein for early diagnose of leptospirosis.

Recombinant proteins adhesion to ECM components

The ability of Lsa33 and Lsa25 proteins to mediate host colonization by adhering to extracellular matrix proteins was examined by ELISA. Laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, and the control proteins fetuin and gelatin were immobilized on microdilution wells and recombinant protein attachment was assessed by ELISA using antibodies

against the proteins. As shown in Figure 5A, both recombinant proteins exhibited adhesiveness to laminin, while no statistically significant binding was observed with these proteins when wells were coated with collagen Type I and IV, cellular and plasma fibronectin, gelatin or with the highly glycosylated control protein fetuin. The interaction of recombinant proteins with laminin was also observed when anti - polyhistidine monoclonal antibodies were employed to probe the ligands (Figure 5B). The binding between Lsa33 and Lsa25 with laminin was also evaluated on a quantitative basis as depicted in Figure 5C. A dose - dependent and saturable binding

was observed when increasing concentrations of the recombinant proteins (0–6 µM) were allowed to adhere to a fixed laminin concentration (1 µg) (Figure 5C). Binding saturation level was achieved by protein concentration of ~4 and 5 µM for Lsa33 and Lsa25, respectively. Based on ELISA data, the calculated dissociation equilibrium constants (*K_D*) for the recombinant protein Lsa33 and Lsa25 with laminin is 367.5 and 415.4 nM, respectively. The role of laminin sugar moieties in the binding with Lsa33 and Lsa25 was assessed with laminin previously oxidized by increasing concentrations of sodium metaperiodate, ranging from 5 to 100 mM. The effect of metaperiodate concentration on the interaction is displayed in Figure 5D. Laminin oxidation had some effect on the interaction with Lsa25, being the reduction of 40% achieved at the highest metaperiodate concentration employed (100 mM). However, the attachment of Lsa33 to metaperiodate - treated laminin had no interference

on the binding. These results indicate that the binding of the proteins to laminin occur in a different manner and that sugar residues contribute to some extent for the interaction of Lsa25 with this ECM glycoprotein.

Interaction of recombinant proteins to serum components

Our group has recently reported that leptospires interact with PLG and that several proteins could act as PLG - receptors [17–19,21]. Protein binding to complement regulators factor H and C4bp have also been shown [31,32]. Therefore, we set out to evaluate whether the recombinant proteins Lsa33 and Lsa25 were capable of binding human PLG, factor H and C4bp *in vitro*. The components, human PLG, factor H and C4bp and the control proteins, gelatin and fetuin, were individually immobilized onto 96 - wells plates followed by incubation with the recombinant leptospiral proteins. The results obtained using polyclonal antibodies against each

protein to probe the reactions showed that Lsa33 and Lsa25 interact with C4bp while only Lsa33 appears to bind to PLG (Figure 6A). No reaction was observed with factor H and the control proteins (Figure 6A). Similar results were achieved when binding was performed using monoclonal anti - his tag antibodies (Figure 6B). Both data show that while Lsa33 protein depicted a statistically significant absorption value for the interaction with PLG, the Lsa25 appears to have only a weak or no adherence to this component. These data were further confirmed when the reaction between the recombinant proteins and PLG were assessed on a quantitative basis as illustrated in Figure 6C. Dose - dependent and saturable binding was observed when increasing concentrations (0 to 1.0 μ M) of the recombinant protein Lsa33 were allowed to adhere to a fixed PLG amount (1 μ g), while very low absorption was detected with Lsa25 (Figure 6C). Based on the ELISA data, the calculated K_D for the recombinant protein Lsa33 with PLG is 23.53 ± 4.66 nM (Figure 6C). This K_D value is in the same order of magnitude with the ones obtained with several recombinant proteins in our laboratory [21].

PLG bound to leptospires and to several recombinant proteins, acting as PLG receptor, can acquire proteolytic activity in the presence of an activator, as we have previously shown [17–19,21]. Therefore, we investigated whether Lsa33 bound to PLG could also generate the enzymatically active plasmin. As a negative control, we have included the recombinant protein Lsa63, previously shown to be non-reactive with PLG [21]. Microplates were coated with the test protein, blocked, and then incubated with PLG. Unbound PLG was washed away and the urokinase - type PLG activator (uPA) was added together with a plasmin - specific chromogenic substrate. The reaction was carried out overnight and the plasmin activity was evaluated by measuring the cleavage of the substrate (absorbance at 405 nm). As shown in Figure 6D, the PLG captured by the Lsa33 protein could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity. The negative controls Lsa63 and BSA did not show any proteolytic activity, similar to the controls lacking PLG, uPA or the chromogenic substrate.

The interaction of recombinant proteins with C4bp was studied in function of protein concentration. We have employed anti -Lsa33 and anti-Lsa25 polyclonal (Figure 6E) and anti-His tag monoclonal antibodies (Figure 6F) to probe the binding. Dose - response curves were obtained with both antibodies but the best response was achieved with anti-His tag monoclonal (Figure 6F), probably because of their homogeneous nature. However, C4bp was not saturated with the protein concentration range employed and therefore the K_D could not be calculated. Lsa63, a His - tag recombinant protein that does

not bind C4bp was also included, as a negative control, showed very low interaction and did not respond to increase protein concentration.

Inhibition of *L. interrogans* attachment to laminin or to PLG by Lsa33 and Lsa25

It has been reported that the several recombinant proteins with adhesin activity revealed an inhibitory effect on the binding of leptospires to ECM macromolecules [6]. We therefore performed experiments to assess whether the recombinant proteins had an effect on the binding of *Leptospira* to laminin or PLG by employing ELISA to detect the interaction in function of protein concentration (0–10 μ g). The results demonstrate that the addition of increasing concentrations of Lsa33 reduced the leptospiral binding to laminin and to PLG molecules in a dose - dependent manner (Figure 7A). Binding decrease in the number of leptospires interacting to laminin and PLG was statistically significant with 1.25 μ g of Lsa33 (*, $P < 0.05$). This interference was also evaluated with the binding of leptospires to laminin in the presence of increasing concentrations of Lsa25 (0–10 μ g), resulting in a similar effect as obtained with Lsa33 (*, $P < 0.05$) (Figure 7B). We have also assessed the competition for the binding of Lsa33 to PLG by increasing laminin concentration (0 to 1.0 μ g). The results revealed that both ligands compete for the binding with Lsa33 as a decrease of 40% in the binding was already detected with 0.25 μ g of laminin (*, $P < 0.05$) (Figure 7C). These experiments were performed in triplicate and Figure 7 shows one representative data of two independent experiments.

Discussion

Complement is a key component of the innate immune system responsible for protection against pathogenic microorganisms [33]. Factor H is a host fluid - phase regulator of the alternative complement pathway. Pathogenic leptospiral complement - resistant strains were found to bind factor H from human serum and this interaction seems to be associated to their serum resistance [31,34]. C4b - binding protein is an inhibitor of complement classical pathway system. This protein controls the complement classical pathway by interfering with the formation and regeneration of C3 convertase and acting as a cofactor to the serine proteinase factor I in the proteolytic inactivation of C4b [33,35]. It has been shown that pathogenic leptospiral strains can obtain C4bp from the host and that this acquisition preserves its cofactor activity [36]. The surface bound C4bp elicits factor I mediated cleavage of C4b, an interaction that appears to contribute to complement resistance by *Leptospira*, through the classical route [37]. Leptospiral binding proteins to C4bp, factor H and factor H - like

Figure 6 Recombinant proteins binding to serum components. (A) Human purified PLG, factor H and C4bp (10 µg/ml) were coated onto ELISA plates and allowed to interact with the recombinant proteins Lsa33 and Lsa25 (10 µg/ml). Gelatin and fetuin were used as negative controls for nonspecific binding. The binding was detected by antibodies raised against each recombinant protein (1:750). Bars represent the mean of absorbance at 492 nm ± the standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the binding of Lsa33 and Lsa25 was compared to its binding to gelatin by two - tailed t test (* $P < 0.05$ and ** $P < 0.005$). (B) Similar as described in (A) but the binding of the recombinant proteins was detected by anti - polyhistidine monoclonal antibodies (1:200). Included is a His - tag recombinant protein Lsa63 that does not bind C4bp. (C) Recombinant proteins dose - dependent binding experiments with PLG. The binding was detected by polyclonal antibodies against each protein; each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control. The dissociation constant (K_D) is depicted and was calculated based on ELISA data for the recombinant protein that reached equilibrium. (D) Plasmin generation by PLG bound to recombinant proteins was assayed by modified ELISA as immobilized proteins received the following treatment: PLG + uPA + specific plasmin substrate (PLG + uPA + S), or controls lacking one of the three components (PLG + uPA; PLG + S; uPA + S). Lsa63 and BSA were employed as negative controls. Bars represent mean absorbance at 405 nm, as a measure of relative substrate degradation ± the standard deviation of four replicates for each experimental group and are representative of three independent experiments. Statistically significant binding in comparison to the negative control (BSA) are shown: * $P < 0.05$. (E) Recombinant proteins dose - dependent binding experiments with C4bp. The binding was detected by polyclonal antibodies raised against each protein (1:750); each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control. (F) Similar to (E) but the protein binding was detected by anti-polyhistidine monoclonal antibodies (1:200); included is a His - tag recombinant protein Lsa63 that does not bind C4bp; each point was performed in triplicate and expressed as the mean absorbance value at 492 nm ± standard error for each point. Gelatin was included as a negative control.

Figure 7 Inhibition of *L. interrogans* attachment to immobilized laminin and PLG by recombinant proteins; The effect of laminin concentration on the binding of PLG to Lsa33. (A) Laminin or PLG (1 µg/well) was adsorbed onto microtiter plates followed by incubation with increasing concentrations of Lsa33 (0 to 10 µg) and in (B) laminin was adsorbed onto microtiter plates followed by incubation with increasing concentrations of Lsa25 (0 to 10 µg). In (A) and (B) the incubations were allowed to proceed for 90 min at 37°C. Live leptospires (100 µl/well of 4×10^7 *L. interrogans* serovar Copenhageni strain M20 leptospires) were added and incubated for another 90 min at 37°C. The unbound leptospires were washed away, and the quantification of bound leptospires was performed indirectly by anti - LipL32 antibodies produced in mice (1: 4,000 dilution) followed by horseradish peroxidase - conjugated antimouse IgG antibodies. Each point represents the mean absorbance value at 492 nm $\pm$ standard deviation of three replicates. Data are representative of two independent experiments (* $P < 0.05$). (C) The effect of laminin on the binding of PLG (10 µg/ml) to immobilized rLIC11834 (10 µg/ml) was assessed with the addition of increasing concentrations of laminin (0 to 1.0 µg). The detection of rLIC11834-bound PLG was performed by use of specific antibodies anti - PLG. Bars represent the mean absorbance values $\pm$ standard deviation of four replicates for each condition and are representative of two independent experiments. Results of statistically significant interference on the binding in comparison with the control (no addition of laminin) are shown: * $P < 0.05$.

have also been identified in *Leptospira* [9,31,32]. Interaction of C4bp and of factor H with other pathogens has been described, including the spirochetes *Borrelia* spp. [33,37–41].

The capacity of the leptospires to adhere to extracellular matrix components has been reported and to date, several leptospiral adhesins have been identified. These include 36 - kDa fibronectin - binding protein [42], LfhA/Lsa24 [6,31], LigA and LigB proteins [7,8], Len-family proteins [9], Lsa21 [10], LipL32 [12,43], Lsa27 [13], Lp95 [11], TlyC [14], LipL53 [44], Lsa63 [15], OmpL37 [45], Lsa66 [17] and Lsa20 [18]. We have reported that *Leptospira* species were also capable to bind PLG and generating plasmin, in the presence of host activator, on the outer surface *in vitro* [19]. In addition, we have described that plasmin - coated virulent *L. interrogans* bacteria were capable to degrade purified extracellular matrix components fibronectin [19] and laminin (Vieira et al., unpublished data), a step that may contribute for dissemination of the bacteria through the host tissues. More recently, we have shown that plasmin generation on the bacterial surface decreases the

deposition of C3b and IgG and hence, opsonization and phagocytosis, a process that could facilitate leptospires to evade the immune system [22]. Several PLG-receptor proteins in *Leptospira* have been identified [17,18,20,21].

By data mining the genome sequences of *L. interrogans*, searching for surface exposed proteins that could mediate host - pathogen interactions, we have identified two proteins annotated as *Leptospira* conserved hypothetical, one of them, predicted to be a novel lipoprotein, LIC11834, and the other, LIC12253, has recently been shown to be non-protective in leptospiral challenge assay [46]. Both selected coding sequences were cloned and the recombinant proteins expressed in *E. coli*. We report that these proteins, Lsa33 and Lsa25, are laminin - binding adhesins and in the case of Lsa33, capable to bind PLG generating enzymatically active plasmin. Although weak, both proteins showed the ability to bind human purified C4bp, suggesting that these proteins have the potential to participate in leptospiral immune evasion by interfering with the complement classical pathway.

Due to the high degree of antigenic variation among leptospires, we examined the gene/protein conservation

among important species of *Leptospira*. The LIC11834 and LIC12253 genes are conserved in five serovars of *L. interrogans* and in other species tested but in the case of *L. santarosai* serovar Shermani the gene LIC11834 is absent. However, LIC11834 transcripts were detected only in serovars of *L. interrogans*, while LIC12253 appears to be expressed in all strains evaluated. None of the proteins seems to be expressed in the saprophytic strain, *L. biflexa* serovar Patoc.

The recombinant proteins Lsa33 and Lsa25 were expressed with molecular mass of 33 - and 24 - kDa, respectively, having a 6XHis tag at N - terminal. The purified proteins exhibited single major bands in SDS - PAGE and were recognized by anti - His tag monoclonal antibodies and by homolog sera from mice immunized with each recombinant protein. Secondary structure of the recombinant proteins after the purification process was evaluated by CD spectroscopy and showed a predominance of alpha helices in both cases, similar to the data predicted by bioinformatics, indicating the suitability of recombinant proteins for further studies. The LIC12253 coding sequence is probably higher immunogenic than LIC11834 because it was recognized by approximately 45% of serum samples of both phases, initial and convalescent, of confirmed leptospirosis's cases. Interestingly, the LIC11834 protein although presented almost no reactivity among these serum samples, showed a slightly augment effect on serum reactivity when was assayed together with LIC12253. Immunofluorescence using live leptospires showed LIC11834 and LIC12253 coding sequences at the surface of bacteria, as a result of antiserum recognition raised against each protein. *In silico* analysis, proteinase K accessibility and immunofluorescence data together suggest that these proteins are likely to be surface exposed. In addition, the recombinant proteins partially inhibited leptospiral adherence to immobilized laminin and PLG.

Merien and colleagues [42] identified a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira*. Our group described the first leptospiral laminin - binding protein, named Lsa24 [6]. These studies were followed by the identification of several extracellular matrix binding proteins [7,9-18]. The recombinant proteins Lsa33 and Lsa25 exhibited extracellular matrix - binding properties, and are laminin - binding proteins. The binding affinity dissociation constants estimated for both proteins to laminin showed similar K_D value of that reported for OmpL 37 (410 ± 81 nM) and the same ECM molecule [16]. Thus, it is possible that these proteins have a role in the adhesion of leptospires to hosts.

The PLG activation system with generation of plasmin was described for virus, parasites and bacteria, including the spirochetes *Borrelia* spp. and with *Treponema denticola* [47-50]. Plasmin is a serine protease with the

capacity to degrade a broad spectrum of substrates, including fibrin clots, connective tissue and components of extracellular matrices [51-53]. We have reported that *Leptospira* spp. bind PLG at their surface generating plasmin, when host activator is available, making the bacteria capable to degrade fibronectin [19] and laminin (Vieira, M.L., unpublished results). Verma et al. [20] have demonstrated that the protein LenA of *L. interrogans* [9] is a surface receptor for human PLG. Moreover, we have reported several novel PLG - receptor proteins of *Leptospira* [17,18,21]. We now describe Lsa33 as a novel PLG - binding protein. Similar to the previously reported proteins, bound PLG could be converted to plasmin by the addition of urokinase - type PLG activator (uPA), showing specific proteolytic activity. It is thus possible that the Lsa33 besides playing a role in the attachment to host and acting as PLG - receptor, may also help leptospires to surmount tissue barriers.

The inhibitory effect exerted on the binding of leptospires to laminin and PLG by the recombinant proteins was statistically significant with both, in the case of Lsa33, and with laminin for the Lsa25. The intensity of the interference upon the binding is expected given the presence of several ECM - or PLG-binding proteins in *Leptospira*. These data are comparable to the ones already reported in the literature [6,7,16-18,21]. Partial inhibitory effect was observed by laminin on the binding of Lsa33 to PLG, suggesting a competition for the same binding site.

Conclusions

We report in these studies a characterization of two leptospiral proteins, genome annotated as proteins of unknown function. The recombinant proteins Lsa33 and Lsa25 are laminin binding proteins that might be involved in the attachment to host. Moreover, both proteins showed the ability to bind C4bp, a feature suggesting their possible involvement in the immune evasion of leptospires. The recombinant Lsa33 is also PLG - binding protein that could help the bacteria during the infection process. Thus, it appears that Lsa33 and to a lesser degree, Lsa25, are multifaceted proteins that might have multiple functions in the leptospiral pathogenesis. To date, Lsa33 is the first described laminin -, PLG - and C4bp - leptospiral binding protein.

Methods

Leptospira strains and sera

The pathogenic *Leptospira* strains used were: *L. interrogans* serovar Canicola strain Hound Utrech IV, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Hardjo strain Hardjoprajitno, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona strain Pomona, *L. borgpetersenii* serovar Whitcombi strain

Whitcomb and serovar Grippothyphosa strain Moskva V, *L. kirshneri* serovar Cynoptery strain 3522 C, *L. santarosai* serovar Shermani strain 1342 K, *L. noguchi* serovar Panama strain CZ 214 and *L. biflexa* serovar Patoc strain Patoc, were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco®) 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%) (Turner LH. *Leptospirosis*. 3. *Maintenance, isolation and demonstration of leptospires*. Trans R Soc Trop Med Hyg 1970; 64: 623–646). *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Confirmed - leptospirosis serum samples were from Instituto Adolfo Lutz collection, São Paulo, Brazil.

Microscopic agglutination test (MAT)

The microscopic agglutination test was performed according to [1]. In brief, an array of 22 serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippothyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolffi. All the strains were maintained in EMJH liquid medium (Difco, USA) at 29°C. A laboratory - confirmed case of leptospirosis was defined by the demonstration of a four - fold microagglutination titer rise between paired serum samples. The probable predominant serovar was considered to be the one with the highest dilution that could cause 50% of agglutination. MAT was considered negative when the titer was below 100.

Characterization of the protein *in silico*

Predicted coding sequence (CDSs) LIC11834 and LIC12253 were identified on *L. interrogans* serovar Copenhageni and selection was based on cellular localization; cellular localization prediction was performed by PSORT, <http://psort.nibb.ac.jp> [54] and PredictProtein web server, <https://www.predictprotein.org/> [25]. The SMART [23] <http://smart.embl-heidelberg.de/> and PFAM [55] <http://www.sanger.ac.uk/Software/Pfam/> web servers were used to search for predicted functional and structural domains. The presence of lipobox putative sequence was evaluated by use of the Lipop program [56] <http://www.cbs.dtu.dk/services/LipoP/>. The predicted sequence of the lipobox was also assessed by use of the SpLip program, as described by Setubal et al. [57]. Secondary structure, solvent accessibility and cellular localization predictions were also performed by using PredictProtein web server, <https://www.predictprotein.org/> [25].

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 by guanidine - detergent lysing method using DNAzol® Reagent (Invitrogen), according to the manufacturer's instructions. Primers were designed according to *L. interrogans* serovar Copenhageni genome sequences (GenBank accession AE016823) and are listed in Table 1. PCR was performed in a reaction volume of 25 µl containing 100 ng of genomic DNA, 1 × PCR buffer (20 mM Tris - HCl, pH 8.4, 50 mM KCl), 2 mM MgCl₂, 20 pmol of each specific primer, 200 µM of each dNTP, and 2.5 U Taq DNA Polymerase (Invitrogen). Cycling conditions were: 94 ° C - 4 min, followed by 40 cycles at 94°C - 50 sec, 57°C (LIC11834) or 56°C (LIC12253) - 50 sec, 72°C - 90 sec, and a final extension cycle of 7 min at 72°C. PCR amplified products were loaded on a 1% agarose gel for electrophoresis and visualization with ethidium bromide.

RNA extraction and RT-PCR analysis

For reverse transcription RT - PCR, total RNA was isolated by the acid guanidinium thiocyanate phenol - chloroform method using TRIzol® Reagent (Invitrogen) according to the manufacturer's recommendations. 1 µg of RNA from each sample was treated with 1 U of DNase I Amplification Grade (Invitrogen) for 15 min at room temperature. DNase I was inactivated by the addition of 1 µl 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 µl reaction mix using oligonucleotides LIC11834 - F/LIC11834 - R or LIC12253 - F/LIC12253 - R, as described above. Samples quantity and integrity were verified by amplification of a 1,042 bp 16 S ribosomal cDNA fragment using oligomers:

16S - F 5'
CAAGTCAAGCGGAGTAGCAATACTCAGC 3' and
16S - R 5'GATGGCAACATAAGGTGAGGGTTGC 3'.

DNA recombinant techniques, protein expression and purification

Predicted CDSs LIC11834 and LIC12253, without signal peptides, were amplified by the PCR from *L. interrogans* serovar Copenhageni strain Fiocruz L1 - 130 genomic DNA using the primer pairs depicted in Table 1. The PCR products obtained for each corresponding gene were cloned into pGEM-T easy vector (Promega) and subcloned into the pAE expression vector [27] at the restriction sites shown in Table 1. The pAE vector allows the expression of recombinant proteins with a minimal

6X His - tag at the N - terminus. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA). Protein expression of rLIC11834 and rLIC12253 was achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotically induced promoter proU [58]. *E. coli* BL21 (SI) containing recombinant plasmids were grown at 30°C in Luria - Bertani broth without NaCl and with 100 µg/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6 to 0.8 was reached. Recombinant protein synthesis was induced by the addition of 300 mM NaCl. After three hours, the cells were harvested by centrifugation and the bacterial pellets resuspended in lysis buffer (200 µg/ml of lysozyme, 1% Triton X - 100, 2 mM phenylmethylsulfonyl fluoride [PMSF]). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). The insoluble fractions were washed with 20 ml of buffer (20 mM Tris - HCl, pH 8.0, 500 mM NaCl, 1 M urea and 1% Triton X-100) and resuspended in a buffer containing 20 mM Tris - HCl, pH 8.0, 500 mM NaCl, 5 mM Imidazole, 1 mM β - mercaptoethanol and 8 M urea. The proteins were then purified through metal chelating chromatography in a Sepharose fast flow column (GE Healthcare) and fractions were analyzed in 12% SDS-PAGE. The rLIC12253 protein was refolded by 500 times dilution with 20 mM Tris - HCl, pH 8.0, and 500 mM NaCl before chromatographic purification. The purified recombinant proteins were extensively dialyzed against phosphate - buffered saline (PBS), pH 7.4, 0.1% (wt/vol) glycine solution (1:100), pooled and stored at -20°C.

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) 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 185 to 260 nm. The molar ellipticity (Φ) is expressed in deg.cm.dmol⁻¹.

Antiserum

Five female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 µg of the recombinant proteins. The recombinant proteins were adsorbed in 10% (vol/vol) of Alhydrogel (2% Al(OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Two subsequent booster injections were given at two - week intervals with the same preparation of 10 µg of the proteins. 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. All animal studies were approved by the Ethics Committee of the Instituto Butantan, São Paulo, SP, Brazil. The Committee in Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation.

Immunoblotting assay

The purified recombinant proteins were loaded into 12% SDS - PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi - dry equipment. Membranes were blocked with 5% non-fat dried milk and 2.5% BSA in PBS containing 0.05% Tween 20 (PBS - T) and then incubated with anti - rLIC11834 (1:500), anti - rLIC12253 (1:500) mouse serum or anti - his antibody (1:1,000) (GE Healthcare) for 2 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase (HRP) - conjugated anti - mouse IgG (1:5,000; Sigma) in PBS - T for 1 h. The protein's reactivity was revealed by ECL reagent kit chemiluminescence substrate (GE Healthcare) with subsequent exposition to X - Ray film.

ELISA for detection of human antibodies

Human IgG antibodies against Lsa33 or Lsa25 were detected by ELISA as previously described [59]. In brief, serum samples of negative (24) and positive (33) MAT from confirmed - leptospirosis patients were diluted 1:400 and evaluated for total IgG using goat HRP - conjugated anti-human IgG antibodies (1:5,000, Sigma). Cutoff values were set at three standard deviations above the mean OD_{492nm} of sera from 11 health individuals, unexposed to leptospirosis, from the city of São Paulo, Brazil and one pool of normal serum samples from USA (Sigma).

Proteinase K accessibility assay

Suspensions of 5 ml live leptospires/per treatment (~10⁸ cells/ml) were harvested at 10,000 rpm for 15 min at room temperature, washed once with PBS (with 50 mM NaCl), resuspended in 5 ml of PBS (with 50 mM NaCl) plus with 25 µg/ml of proteinase K (PK) (Sigma Aldrich). Four similar test tubes were then incubated for 0 to 5 h at 37°C and aliquots were taken at 0, 1, 3 and 5 h before the addition of 100 mM of phenylmethylsulfonyl fluoride (PMSF) to stop PK activity. The suspensions were subsequently pelleted by centrifugation at 10,000 rpm for 5 min, washed twice with PBS (with 50 mM NaCl) and resuspended in 1 ml PBS (with 50 mM NaCl) for ELISA analysis using antibodies against Lsa33, Lsa25, Lip32 and DnaK, as described

below. LipL32 and DnaK are membrane and cytoplasmic leptospiral proteins that were employed in our experiment as positive and negative control, respectively.

ELISA for detection cellular localization of the proteins

Leptospirae were coated onto microplates and allowed to stand at room temperature for 16 h. The plates were washed three times with PBS (with 50 mM NaCl) and blocked with 5% non-fat dry milk and 1% BSA for 2 h at 37°C. After incubated for 2 h at 37°C with polyclonal mouse anti - serum against Lsa33, Lsa25, LipL32 or DnaK (dilution of an OD equal to 1). The leptospirae were washed three times with PBS (with 50 mM NaCl) and incubated with 50 µL of a 1:5,000 dilution of HRP - conjugated goat anti - mouse IgG (Sigma) in PBS (with 50 mM NaCl) for 1 h at 37°C. The wells were washed three times with PBS (with 50 mM NaCl), and o - phenylenediamine (OPD) (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 µL/mL H₂O₂ was added (100 µL per well). The reaction proceeded for 5 min and was interrupted by the addition of 50 µL of 4 N H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (TP - reader, Thermo) against the O.D. of blanks, containing all the reaction mixture but antibodies against the proteins. For statistical analyses, the binding of polyclonal mouse anti - serum against Lsa33, Lsa25, LipL32 or DnaK at 0 h incubation was compared with other incubations by Student's two - tailed *t* test.

Binding of recombinant proteins to ECM and to serum components

Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol [6] with some modifications. Briefly, 96 - well plates (Costar High Binding, Corning) were coated with 1 µg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin, human PLG, factor H, C4bp, or gelatin (negative control) and fetuin (highly glycosylated attachment - negative control protein) in 100 µL of PBS for 3 h at 37°C. The wells were washed three times with PBS - T and then blocked with 200 µL of 10% (wt/vol) non-fat dry milk (overnight at 4°C). One microgram of each recombinant protein was added per well in 100 µL of PBS, and protein was allowed to attach to the different substrates for 2 h at 37°C. After washing six times with PBS - T, bound Lsa33 or Lsa25 was detected by adding mouse anti - recombinant proteins (1:750) in 100 µL of PBS or anti - His tag monoclonal (1:200) in 100 µL of PBS. Incubation proceeded for 1 h at 37°C. After three washings with PBS - T, 100 µL of a 1:5,000 dilution of HRP - conjugated goat anti - mouse IgG (Sigma) in PBS was added per well for 1 h at 37°C. The wells were washed three times, and o - phenylenediamine (OPD) (1 mg/mL) in

citrate phosphate buffer (pH 5.0) plus 1 µL/mL H₂O₂ was added (100 µL per well). The reaction proceeded for 10 min and was interrupted by the addition of 50 µL of 4 N H₂SO₄. The absorbance at 492 nm was determined in a microplate reader (TP - reader, Thermo). For statistical analyses, the binding of recombinant proteins to ECM macromolecules and to serum components were compared to its binding to gelatin and by Student's two - tailed *t* test.

Metaperiodate treatment of laminin

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 the same buffer, and laminin was treated with different sodium metaperiodate concentrations (0–100 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 recombinant proteins (1 µg in PBS per well) to periodate - treated laminin was evaluated as described above.

Dose-response curves

First, 96 - well plates were coated overnight in PBS at 4°C with 100 µL of 10 µg/ml PLG, laminin or C4bp. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0–6 µM) were added (100 µL/well in PBS). The assessment of bound proteins was performed by incubation for 1 h at 37°C with the antiserum raised against each protein at the dilution of 1:750, followed by HRP - conjugated goat anti-mouse IgG (Sigma) (1:5,000 in PBS). The reactions were detected with OPD as describe above. The ELISA data were used to calculate the dissociation constant (K_D) according to the method described by Pathirana et al. [60] and Lin et al. [61], based on the equation: $A = A_{\max}[\text{protein}]/(K_D + [\text{protein}])$, where *A* is the absorbance at a given protein concentration, $A_{\max}$ is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

Plasmin enzymatic activity assay

96 - well ELISA plates were coated overnight with 10 µg/ml recombinant proteins in PBS at 4°C. Lsa63, which does not bind PLG [21] and BSA were employed as negative control. Plates were washed once with PBS - T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) non - fat dry milk. The blocking solution was discarded and 100 µL/well of 10 µg/ml human PLG was added, followed by incubation for 2 h at 37°C. Wells were washed three times with PBS - T, and then 4 ng/

well of human uPA (Sigma - Aldrich) were added. Subsequently, 100 µl/well of plasmin - specific substrate D - valyl - leucyl - lysine - p - nitroanilide dihydrochloride (Sigma - Aldrich) were added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate degradation was measured by readings at 405 nm.

Inhibition of live leptospires binding to laminin or to PLG by recombinant proteins

ELISA plates were coated with laminin or PLG (1 µg/well). The plates were washed and blocked with 10% non - fat dry milk in PBS - T for 2 h at 37°C. The blocking solution was discarded, and the wells were incubated for 90 min at 37°C with increasing concentrations of proteins (0 to 10 µg). After three washings, 50 µL/well of 4×10^7 live low - passage virulent *L. interrogans* serovar Copenhageni strain M20 were added for 90 min at 37°C. The unbound leptospires were washed and the quantification of bound leptospires was performed indirectly by anti - LipL32 antibodies produced in mice (1:4,000), given the fact that LipL32 is a major outer membrane leptospiral protein [28]; the procedure was followed by horseradish peroxidase - conjugated anti - mouse IgG antibodies, essentially as described in Barbosa et al. [6]. The detection was performed by OPD as previously described.

Liquid-phase immunofluorescence assay (L - IFA)

The localization of LIC11834 and LIC12253 encoded proteins by L - IFA was performed as described by Oliveira et al. [15]. In brief, suspensions of 2.5 ml live leptospires ($\sim 10^9$ cells/ml) were harvested at 10,000 rpm for 15 min, washed twice with PBS (with 50 mM NaCl), resuspended in 200 µl of PBS with 6 µg/ml propidium iodide to stain the nuclei, and incubated for 45 min at 37°C. After incubation, the leptospires were washed gently with PBS and incubated for 30 min at 4°C with polyclonal mouse anti - serum against Lsa33, Lsa25, LipL32 or GroEL at a 1:50 dilution. The leptospires were washed and incubated with goat anti - mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC, Sigma) at a dilution 1:50 for 30 min at 4°C. After incubation with secondary antibody, the leptospires were washed and resuspended in PBS - antifading solution (ProLong Gold, Molecular Probes). The immunofluorescence - labeled leptospires were examined by employ of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany).

Nucleotide sequence accession numbers

GenBank accession numbers for protein sequences LIC11834 and LIC12235 are AAS70420 and AAS70825, respectively. The protein can also be accessed by the

genome nomenclature for the gene locus, LIC number (*Leptospira interrogans* serovar Copenhageni).

ECM and biological components

The control proteins fetuin and gelatin, were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Difco[®], respectively. 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. PLG, purified from human plasma, was purchased from Merck. Human Factor H was from Calbiochem. C4bp was from Complement Technology, INC.

Author's contributions

RFD performed the molecular cloning studies, protein expression, ECM assays and animal immunizations. MLV carried out the PLG assays and help with the manuscript. ECR evaluated MAT of the collection serum samples. APG and ZMM were responsible for bacteria growth, identification and virulence strain maintenance. SAV participated in the design of the study and help drafted the manuscript. ALTON conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We are deeply indebted to Alexsander Seixas de Souza (Departamento de Parasitologia, Instituto Butantan, Sao Paulo, Brazil) for use of Confocal facilities and helpful discussion. This work was supported by FAPESP, CNPq and Fundação Butantan, Brazil; RFD and MLV have fellowships from FAPESP.

Author details

¹Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 150005503-900, São Paulo, SP, Brazil. ²Pós-Graduação Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Avenida Prof. Lineu Prestes, 173005508-900, São Paulo, SP, Brazil. ³Centro de Bacteriologia, Instituto Adolfo Lutz, Avenida Dr. Arnaldo, 355CEP 01246-902, Sao Paulo, Brazil. ⁴Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia, USP, Avenida Prof. Dr. Orlando Marques de Paiva, 8705508-270, São Paulo, SP, Brazil.

Received: 18 November 2011 Accepted: 23 March 2012

Published: 30 March 2012

References

1. Faine S, Adler B, Bolin C, Perolat P: *Leptospira and Leptospirosis*. Melbourne: Australia MediSci; 1999.
2. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, Lovett MA, Levett PN, Gilman RH, Willig MR, Gotuzzo E, et al: **Leptospirosis: a zoonotic disease of global importance**. *Lancet Infect. Dis.* 2003, **3**(12):757-771.
3. Levett PN: **Leptospirosis**. *Clin. Microbiol. Rev.* 2001, **14**(2):296-326.
4. Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD Jr, Riley LW: **Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group**. *Lancet* 1999, **354**(9181):820-825.
5. Merien F, Truccolo J, Rougier Y, Baranton G, Perolat P: **In vivo apoptosis of hepatocytes in guinea pigs infected with *Leptospira interrogans* serovar icterohaemorrhagiae**. *FEMS Microbiol. Lett.* 1998, **169**(1):95-102.
6. Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, Morais ZM, Vasconcellos SA, Nascimento AL: **A newly identified leptospiral adhesin mediates attachment to laminin**. *Infect. Immun.* 2006, **74**(11):6356-6364.
7. Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA: **Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen**. *Infect. Immun.* 2007, **75**(5):2441-2450.

8. Lin YP, Chang YF: **A domain of the Leptospira LigB contributes to high affinity binding of fibronectin.** *Biochem. Biophys. Res. Commun.* 2007, **362**(2):443–448.
9. Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, Kraicz P, Cooley AE, Creamer TP, Suchard MA, et al: **Leptospira interrogans endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement.** *PLoS One* 2007, **2**(11):e1188.
10. Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima DM, Abreu PA, Nascimento AL: **Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection.** *BMC Microbiol.* 2008, **8**:70.
11. Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, de Moraes ZM, Vasconcellos SA, Nascimento AL: **Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells.** *J. Infect.* 2009, **59**(4):264–276.
12. Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS, Ho PL: **In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin.** *Infect. Immun.* 2008, **76**(6):2642–2650.
13. Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA, Nascimento AL: **A newly identified protein of Leptospira interrogans mediates binding to laminin.** *J. Med. Microbiol.* 2009, **58**(Pt 10):1275–1282.
14. Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, Abreu PA, Fiorini LC, Oliveira ML, Romero EC, Goncalves AP, et al: **Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity.** *FEBS Lett.* 2009, **583**(8):1381–1385.
15. Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL: **Lsa63, a newly identified surface protein of Leptospira interrogans binds laminin and collagen IV.** *J. Infect.* 2010, **60**(1):52–64.
16. Pinne M, Choy HA, Haake DA: **The OmpL37 surface-exposed protein is expressed by pathogenic Leptospira during infection and binds skin and vascular elastin.** *PLoS Negl. Trop. Dis.* 2010, **4**(9):e815.
17. Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL: **Characterization of novel OmpA-like protein of Leptospira interrogans that binds extracellular matrix molecules and plasminogen.** *PLoS One* 2011, **6**(7):e21962.
18. Mendes RS, Atzingen MV, Moraes ZM, Goncalves AP, Serrano SM, Asega AS, Romero EC, Vasconcellos SA, Nascimento AL: **The Novel Leptospiral Surface Adhesin Lsa20 Binds Laminin and Human Plasminogen and Is Probably Expressed during Infection.** *Infect. Immun.* 2011, **79**(11):4657–4667.
19. Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL: **Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation.** *Infect. Immun.* 2009, **77**(9):4092–4101.
20. Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, Stevenson B: **Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen.** *Infect. Immun.* 2010, **78**(5):2053–2059.
21. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, Nascimento AL: **In vitro identification of novel plasminogen-binding receptors of the pathogen Leptospira interrogans.** *PLoS One* 2010, **5**(6):e11259.
22. Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL: **In vitro evidence for immune evasion activity by human plasmin associated to pathogenic Leptospira interrogans.** *Microb. Pathog.* 2011, **51**(5):360–365.
23. Schultz J, Milpetz F, Bork P, Ponting CP: **SMART, a simple modular architecture research tool: identification of signaling domains.** *Proc. Natl. Acad. Sci. U.S.A.* 1998, **95**(11):5857–5864.
24. Letunic I, Doerks T, Bork P: **SMART 6: recent updates and new developments.** *Nucleic acids research* 2009, **37**(Database issue):D229–D232.
25. Rost B, Yachdav G, Liu J: **The PredictProtein Server.** *Nucleic acids research* 2004, **32**(Web Server issue):W321–W326.
26. Rajesh N, Burkhard R: **Mimicking cellular sorting improves prediction of subcellular localization.** *J. Mol. Biol.* 2005, **348**(1):85–100.
27. Ramos CR, Abreu PA, Nascimento AL, Ho PL: **A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide.** *Braz. J. Med. Biol. Res.* 2004, **37**(8):1103–1109.
28. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN, Bolin CA: **The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection.** *Infect. Immun.* 2000, **68**(4):2276–2285.
29. Haake DA, Matsunaga J: **Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins.** *Infect. Immun.* 2002, **70**(9):4936–4945.
30. Stamm LV, Gherardini FC, Parrish EA, Moomaw CR: **Heat shock response of spirochetes.** *Infect. Immun.* 1991, **59**(4):1572–1575.
31. Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraicz P, Timoney JF, Stevenson B: **LfhA, a novel factor H-binding protein of Leptospira interrogans.** *Infect. Immun.* 2006, **74**(5):2659–2666.
32. Barbosa AS, Monaris D, Silva LB, Moraes ZM, Vasconcellos SA, Cianciarullo AM, Isaac L, Abreu PA: **Functional characterization of LcpA, a surface-exposed protein of Leptospira spp. that binds the human complement regulator C4BP.** *Infect. Immun.* 2010, **78**(7):3207–3216.
33. Blom AM: **Structural and functional studies of complement inhibitor C4b-binding protein.** *Biochem. Soc. Trans.* 2002, **30**(Pt 6):978–982.
34. Meri T, Murgia R, Stefanel P, Meri S, Cinco M: **Regulation of complement activation at the C3-level by serum resistant leptospires.** *Microb. Pathog.* 2005, **39**(4):139–147.
35. Gigli I, Fujita T, Nussenzweig V: **Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator.** *Proc. Natl. Acad. Sci. U.S.A.* 1979, **76**(12):6596–6600.
36. Barbosa AS, Abreu PA, Vasconcellos SA, Moraes ZM, Goncalves AP, Silva AS, Dahi MR, Isaac L: **Immune evasion of leptospira species by acquisition of human complement regulator C4BP.** *Infect. Immun.* 2009, **77**(3):1137–1143.
37. Cinco M: **New insights into the pathogenicity of leptospires: evasion of host defences.** *New Microbiol.* 2010, **33**(4):283–292.
38. Ram S, Mackinnon FG, Gulati S, McQuillen DP, Vogel U, Frosch M, Elkins C, Guttormsen HK, Wetzler LM, Oppermann M, et al: **The contrasting mechanisms of serum resistance of Neisseria gonorrhoeae and group B Neisseria meningitidis.** *Mol. Immunol.* 1999, **36**(13–14):915–928.
39. Kraicz P, Skerka C, Brade V, Zipfel PF: **Further characterization of complement regulator-acquiring surface proteins of Borrelia burgdorferi.** *Infect. Immun.* 2001, **69**(12):7800–7809.
40. Carroll MC: **The complement system in regulation of adaptive immunity.** *Nat. Immunol.* 2004, **5**(10):981–986.
41. Hovis KM, McDowell JV, Griffin L, Marconi RT: **Identification and characterization of a linear-plasmid-encoded factor H-binding protein (FhbA) of the relapsing fever spirochete Borrelia hermsii.** *J. Bacteriol.* 2004, **186**(9):2612–2618.
42. Merien F, Truccolo J, Baranton G, Perolat P: **Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of Leptospira interrogans serovar icterohaemorrhagiae.** *FEMS Microbiol. Lett.* 2000, **185**(1):17–22.
43. Hoke DE, Egan S, Cullen PA, Adler B: **LipL32 is an extracellular matrix-interacting protein of Leptospira spp. and Pseudoalteromonas tunicata.** *Infect. Immun.* 2008, **76**(5):2063–2069.
44. Oliveira TR, Longhi MT, Goncalves AP, de Moraes ZM, Vasconcellos SA, Nascimento AL: **LipL53, a temperature regulated protein from Leptospira interrogans that binds to extracellular matrix molecules.** *Microb Infect Institut Pasteur* 2010, **12**(3):207–217.
45. Pinne M, Choy HA, Haake DA: **The OmpL37 surface-exposed protein is expressed by pathogenic Leptospira during infection and binds skin and vascular elastin.** *PLoS neglected tropical diseases* 2010, **4**(9):e815.
46. Félix SR, Hartwig DD, Argondizzo AP, Silva EF, Seixas FK, Seixas Neto AC, Medeiros MA, Lilienbaum W, Dellagostin OA: **Subunit approach to evaluation of the immune protective potential of leptospiral antigens.** *Clin Vaccine Immunol* 2011, **18**(12):2026–30.
47. Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC: **Identification of a Treponema denticola OppA homologue that binds host proteins present in the subgingival environment.** *Infect. Immun.* 2000, **68**(4):1884–1892.
48. LeBouder F, Morello E, Rimmelzwaan GF, Bosse F, Pechoux C, Delmas B, Riteau B: **Annexin II incorporated into influenza virus particles supports virus replication by converting plasminogen into plasmin.** *J. Virol.* 2008, **82**(14):6820–6828.
49. Rojas M, Labrador I, Concepcion JL, Aldana E, Avilan L: **Characteristics of plasminogen binding to Trypanosoma cruzi epimastigotes.** *Acta Trop.* 2008, **107**(1):54–58.
50. Klempner MS, Noring R, Epstein MP, McCloud B, Rogers RA: **Binding of human urokinase type plasminogen activator and plasminogen to Borrelia species.** *J. Infect. Dis.* 1996, **174**(1):97–104.

51. Ponting CP, Marshall JM, Cederholm-Williams SA: **Plasminogen: a structural review.** *Blood Coagul. Fibrinolysis* 1992, **3**(5):605–614.
52. Angles-Cano E: **Overview on fibrinolysis: plasminogen activation pathways on fibrin and cell surfaces.** *Chem. Phys. Lipids* 1994, **67–68**:353–362.
53. Angles-Cano E, de la Pena Diaz A, Loyau S: **Inhibition of fibrinolysis by lipoprotein(a).** *Annals of the New York Academy of Sciences* 2001, **936**:261–75.
54. Nakai K, Kanehisa M: **Expert system for predicting protein localization sites in gram-negative bacteria.** *Proteins* 1991, **11**(2):95–110.
55. Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R, et al: **Pfam: clans, web tools and services.** *Nucleic acids research* 2006, **34**(Database issue):D247–D251.
56. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A: **Prediction of lipoprotein signal peptides in Gram-negative bacteria.** *Protein Sci.* 2003, **12**(8):1652–1662.
57. Setubal JC, Reis M, Matsunaga J, Haake DA: **Lipoprotein computational prediction in spirochaetal genomes.** *Microbiology (Reading, England)* 2006, **152**(Pt 1):113–121.
58. Bhandari P, Gowrishankar J: **An Escherichia coli host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer.** *J. Bacteriol.* 1997, **179**(13):4403–4406.
59. Oliveira TR, Longhi MT, de Moraes ZM, Romero EC, Blanco RM, Kirchgatter K, Vasconcellos SA, Nascimento AL: **Evaluation of leptospiral recombinant antigens MPL17 and MPL21 for serological diagnosis of leptospirosis by enzyme-linked immunosorbent assays.** *Clin. Vaccine Immunol.* 2008, **15**(11):1715–1722.
60. Pathirana RD, O'Brien-Simpson NM, Veith PD, Riley PF, Reynolds EC: **Characterization of proteinase-adhesin complexes of Porphyromonas gingivalis.** *Microbiology (Reading, England)* 2006, **152**(Pt 8):2381–2394.
61. Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, Chang YF: **Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin.** *J. Biol. Chem.* 2009, **284**(29):19380–19391.

doi:10.1186/1471-2180-12-50

Cite this article as: Domingos et al.: "Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions". *BMC Microbiology* 2012 **12**:50.

Submit your next manuscript to BioMed Central and take full advantage of:

- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at
www.biomedcentral.com/submit

Adhesins of *Leptospira interrogans* Mediate the Interaction to Fibrinogen and Inhibit Fibrin Clot Formation *In Vitro*

Rosane Oliveira^{1,2}, Renan F. Domingos^{1,2}, Gabriela H. Siqueira^{1,2}, Luis G. Fernandes^{1,2}, Natalie M. Souza^{1,2}, Monica L. Vieira¹, Zenaide M. de Moraes³, Silvio A. Vasconcellos³, Ana L. T. O. Nascimento^{1,2*}

1 Centro de Biotecnologia, Instituto Butantan, São Paulo, São Paulo, Brazil, **2** Programa Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil, **3** Laboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil

Abstract

We report in this work that *Leptospira* strains, virulent *L. interrogans* serovar Copenhageni, attenuated *L. interrogans* serovar Copenhageni and saprophytic *L. biflexa* serovar Patoc are capable of binding fibrinogen (Fg). The interaction of leptospires with Fg inhibits thrombin- induced fibrin clot formation that may affect the haemostatic equilibrium. Additionally, we show that plasminogen (PLG)/plasmin (PLA) generation on the surface of *Leptospira* causes degradation of human Fg. The data suggest that PLA-coated leptospires were capable to employ their proteolytic activity to decrease one substrate of the coagulation cascade. We also present six leptospiral adhesins and PLG- interacting proteins, rLIC12238, Lsa33, Lsa30, OmpL1, rLIC11360 and rLIC11975, as novel Fg-binding proteins. The recombinant proteins interact with Fg in a dose-dependent and saturable fashion when increasing protein concentration was set to react to a fix human Fg concentration. The calculated dissociation equilibrium constants (K_D) of these reactions ranged from 733.3 ± 276.8 to 128 ± 89.9 nM for rLIC12238 and Lsa33, respectively. The interaction of recombinant proteins with human Fg resulted in inhibition of fibrin clot by thrombin-catalyzed reaction, suggesting that these versatile proteins could mediate Fg interaction in *Leptospira*. Our data reveal for the first time the inhibition of fibrin clot by *Leptospira* spp. and presents adhesins that could mediate these interactions. Decreasing fibrin clot would cause an imbalance of the coagulation cascade that may facilitate bleeding and help bacteria dissemination

Citation: Oliveira R, Domingos RF, Siqueira GH, Fernandes LG, Souza NM, et al. (2013) Adhesins of *Leptospira interrogans* Mediate the Interaction to Fibrinogen and Inhibit Fibrin Clot Formation *In Vitro*. PLoS Negl Trop Dis 7(8): e2396. doi:10.1371/journal.pntd.0002396

Editor: Joseph M. Vinetz, University of California San Diego School of Medicine, United States of America

Received: March 22, 2013; **Accepted:** July 19, 2013; **Published:** August 29, 2013

Copyright: © 2013 Oliveira et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by FAPESP (www.fapesp.br, grant 10/52579-4), CNPq (www.cnpq.br) and Fundacao Butantan, Brazil (www.butantan.gov.br/home/fundacao_butantan.php). RO, RFD, GHS, NMS, LGF and MLV have scholarships from FAPESP (Brazil). The funders, FAPESP, CNPq and Fundacao Butantan, had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: tabet@butantan.gov.br.

Introduction

The spirochete *Leptospira interrogans* is a highly invasive pathogen and the causal agent of leptospirosis, one of the most widespread zoonosis of human and veterinary concern [1,2]. The transmission occurs through contact with environmental water contaminated by leptospires shed in the urine of animal carriers [1,3]. Humans are accidental and terminal hosts in the transmission process of leptospirosis. The leptospires enter the body via abrasions on skin or actively through mucosa, spreading to any tissue, and colonizing target organs [4,3]. *Leptospira* can cause damage of the endothelium of small blood vessels, leading to hemorrhage and localized ischemia in multiple organs. As a consequence, renal tubular necrosis, hepatocellular damage and development of leptospirosis-associated pulmonary hemorrhage syndrome (LPHS) may occur in the host [1,5,6]. The mechanisms responsible for bleeding in leptospirosis are poorly understood. Hemolysins could play an important role in this toxic response and several genes coding for predicted hemolysins were identified in the genome

sequencing *L. interrogans* [7]. Yet, when evaluated, these proteins failed to show hemolytic activity in human erythrocytes [8,9].

Fg and PLG are key proteins in the coagulation cascade and fibrinolysis, respectively, and critical determinants of bacterial virulence and host defense [10]. Fg is the major clotting protein present in blood plasma with an important role in blood coagulation and thrombosis. PLG under the action of its activators generates PLA, a serine-protease capable of degrading ECM components, fibrin, facilitating the pathogen penetration and invasion [11].

We have previously shown that PLA-associated *Leptospira* renders the bacteria with proteolytic activity capable of degrading ECM components [12] that in turn, may help bacterial penetration and dissemination. Furthermore, PLA-coated leptospires have also shown to degrade IgG and C3b that could facilitate the bacterial immune evasion [13]. The adhesion of physiological osmotically induced *Leptospira* with Fg was described but their effect on fibrin formation was not ascertained [14]. The leptospiral proteins LigB and OmpL37 were shown to interact

Author Summary

Leptospirosis is probably the most widespread zoonosis in the world. Caused by spirochaetes of the genus *Leptospira*, it has greater incidence in tropical and subtropical regions. The disease has become prevalent in cities with sanitation problems and a large population of urban rodent reservoirs, which contaminate the environment through their urine. Understanding the mechanisms involved in pathogenesis of leptospirosis should contribute to new strategies that would help fight the disease. We show in this work that *Leptospira* strains, virulent, attenuated or saprophytic are capable of binding fibrinogen (Fg). The interaction of leptospires with Fg inhibits the formation of fibrin clot that may result of an imbalance in the haemostatic equilibrium. In addition, we show that plasminogen (PLG)/plasmin (PLA) generation on the surface of leptospires can lead to Fg degradation, showing evidence of possible route of fibrinolysis in leptospirosis. We also present six leptospiral proteins, as novel Fg-binding proteins, capable of inhibiting fibrin clot formation by thrombin-catalyzed reaction, suggesting that in *Leptospira* these multifunctional proteins could mediate Fg interaction. Our data suggest possible mechanisms that leptospires could employ to affect the coagulation cascade and fibrinolytic system that might lead to bacteria spreading.

with Fg and LigB was reported to reduce fibrin clot formation [15,16,17].

We thus decided to evaluate if Fg-associated *Leptospira* was capable to inhibit the fibrin clot formation and the ability of seven recombinant proteins to act as leptospiral Fg-receptors. We report that *Leptospira* strains and the recombinant ECM- and PLG-interacting proteins, rLIC12238 [18], Lsa33, Lsa25 [19], Lsa30 [20], OmpL1 [21], rLIC11360 and rLIC11975 [22], are capable to adhere to Fg. We also show that this interaction inhibits fibrin clot formation by thrombin-catalyzed reaction. Moreover, PLA-coated *Leptospira* was capable to degrade Fg. Altogether, the results suggest possible pathways that *Leptospira* may interfere with the coagulation/bleeding process.

Methods

Ethics statement

All animal studies were approved by the Ethical Committee for Animal Research of Instituto Butantan, São Paulo, SP, Brazil under protocol n° 798/11. The Committee in Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation.

Leptospira strains and culture conditions

The non-pathogenic *L. biflexa* serovar Patoc strain Patoc 1, the pathogenic attenuated *L. interrogans* serovar Copenhageni strain M-20 and the virulent strains of *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF) and serovar Copenhageni strain Fiocruz L1-130 were cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco) 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%) [23]. *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Unless otherwise stated, experiments were performed with leptospires resuspended in low salt – lsPBS (50 mM), instead of

PBS that contains 137 mM NaCl, because it is an osmolarity condition closer to cultivation, which is approximately 35 mM.

Immunofluorescence assay

For the immunofluorescence assay (IFA), live *L. interrogans* serovar Copenhageni strain M-20 suspensions ($\sim 10^8$) were harvested at $5,000 \times g$ for 15 min, washed three times with lsPBS, resuspended in 200 μ L lsPBS, and fixed with 200 μ L with 4% paraformaldehyde for 40 min at 30°C. After the fixation, propidium iodide (Sigma) diluted 1:50 in lsPBS was added to stain the nuclei, and the suspensions were incubated for 45 min at 30°C. After this time, the leptospires were gently washed three times with lsPBS and blocked with lsPBS containing 5% BSA for 90 min at 30°C, followed by incubation with 16 μ g Fg in 200 μ L lsPBS for 60 min at 37°C. The leptospires were washed three times with lsPBS plus 1% BSA and incubated with goat-produced antiserum against human Fg at a 1:50 dilution for 45 min at 37°C. Leptospires were washed three times again and incubated with rabbit anti-goat IgG antibodies conjugated to fluorescein isothiocyanate (FITC; Sigma) at a dilution of 1:400 for 45 min at 37°C. After this incubation, the leptospires were washed twice with lsPBS containing 1% BSA and once with distilled water. Finally they were resuspended in lsPBS-antifading solution (ProLong Gold; Molecular Probes). The immunofluorescence-labeled leptospires were examined by use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany). As a control for unspecific binding, Fg was absent from the reaction mixtures.

Binding of soluble human Fg to *Leptospira*

For accessing the leptospiral binding to soluble human Fg, ELISA plates were coated with 10^8 leptospires in lsPBS per well (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Copenhageni strain M-20, and *L. biflexa* serovar Patoc strain Patoc 1), allowed to set for 3 h at 37°C and then blocked with 200 μ L of phosphate-buffered saline containing 0.05% Tween (PBS-T) with 10% non-fat dry milk. After 2 h incubation, plates were washed with PBS-T and then 100 μ L of a Fg solution (Fg- Sigma) (10 μ g/mL in lsPBS) were added per well and incubation proceeded for 2 h. After extensive washing, 100 μ L of solution containing goat anti-Fg (1:25,000 in lsPBS) were added per well and incubation was carried out for 1 h at 37°C. After three washings, 100 μ L of solution containing peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:50,000 in lsPBS) were added per well and the reaction continued for 1 h at 37°C. The wells were washed three times, and *o*-phenylenediamine (OPD) (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 μ L/mL H_2O_2 was added (100 μ L per well). The reaction was allowed to proceed for 10 min and interrupted by the addition of 50 μ L of 8 M H_2SO_4 . Readings were taken at 492 nm in a microplate reader (Multiskan EX; Thermo Fisher Scientific, Helsinki, Finland). The binding of Fg to each leptospiral strain was performed in triplicate and a negative control in which Fg was omitted was included in the experiment. In the no-cell control, BSA replaced leptospires. For statistical analyses, the binding of Fg to different strains and control BSA was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons.

Effect of Fg-bound leptospires on fibrin clot formation

Live leptospires (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Copenhageni strain M-20, and *L. biflexa* serovar Patoc strain Patoc 1), 10^9 , 10^7 , 10^5 cells/mL were harvested at $5,000 \times g$ for 20 min at room temperature, washed once with lsPBS, resuspended in 0.5 mL of lsPBS plus 1 mg/mL of Fg (Sigma), and incubated for 2 h at 37°C. ELISA

plates were placed with 90 μ L/well of leptospires plus Fg and 10 μ L/well of thrombin (10 U/mL - Sigma). The fibrin clot formation was measured every 1 min for 10 min and then every 5 min for 35 min by an ELISA plate reader (Multiskan EX Thermo Fisher Scientific) at OD595_{nm}. The positive control of the reaction employed Fg (1 mg/mL) plus thrombin (10 U/mL) while in the negative control thrombin was omitted. For statistical analyses, inhibition of fibrin clot formation was performed by one-way ANOVA, followed by Tukey post-test for pairwise comparisons. Three independent experiments were performed.

Human Fg degradation by leptospires coated with PLA

Virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm (10⁸ leptospires/sample) were treated in 200 μ L lPBS with the addition of: (a) 10 μ g PLG (b) 3U urokinase (uPA) (c) 10 μ g PLG and 3U uPA (generating PLA-coated leptospires) or (d) only lPBS with no additions (untreated). The bacteria were incubated for 1 h at 37°C with the PLG, and for one more hour after the addition of uPA. The cells were washed three times with lPBS to remove the free PLG, uPA and PLA. Then, the bacteria were resuspended in 100 μ L lPBS containing goat anti-human Fg human purified Fg (Sigma, USA) as substrate, and incubated for 16 h at 37°C. Three additional controls were employed: (a) one sample received 1 μ g of the protease inhibitor aprotinin (Sigma, USA) to the Fg-leptospires incubation, (b) one sample contained only Fg without leptospires, and (c) one sample received the PLG and uPA treatment without the addition of leptospires to rule out the free PLA Fg degradation. The leptospires were removed by centrifugation, 20 μ L of the supernatants were separated by 8% SDS-PAGE and then transferred to nitrocellulose membranes in semi-dry equipment. The membranes were blocked by incubating overnight at 4°C with 5% non-fat dry milk and 1% BSA. The Fg detection was performed by incubations with goat anti-human Fg antibodies and rabbit anti-goat secondary antibodies conjugated with HRP, followed by ECL (GE Healthcare) development.

In another assay, after the incubation for Fg degradation, the pelleted leptospires and the remaining cell-free supernatants were also evaluated for the presence of Fg by ELISA. The cells were resuspended in 100 μ L lPBS and coated onto ELISA plates overnight at 4°C, as well as the cell-free supernatants previously diluted 20 times. The plates were washed three times with PBS-T and blocked with solution containing 5% BSA and 5% non-fat dry milk for 2 h at 37°C, following incubations with anti-Fg polyclonal antibodies (1:25,000) and secondary antibodies conjugated to peroxidase (1:50,000). The reactions were developed by addition of 100 μ L/well of solution containing 1 mg/mL OPD and 1 μ L/mL H₂O₂, and stopped by addition of 50 μ L/well H₂SO₄. The samples in which the leptospires received no treatment (only PBS) were considered as having 100% of Fg for comparative purposes.

Bioinformatics characterization of the proteins

Predicted coding sequences (CDSs) were analyzed for their cellular localization by PSORT program, <http://psort.nibb.ac.jp> [24,25]. The web servers SMART, <http://smart.embl-heidelberg.de/> [26,27], PFAM, <http://www.sanger.ac.uk/Software/Pfam/> [28], and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [29] were used to search for predicted functional and structural domains within the amino acid sequences of the CDSs.

Cloning, expression and purification of recombinant proteins

Amplification of the CDSs was performed by PCR from *L. interrogans* serovar Copenhageni strain M-20 genomic DNA using

complementary primer pairs (Table 1). The gene sequences were amplified without the signal peptide tag and cloned into pAE expression vector [30]. The final constructs were verified by DNA sequencing on an ABI Prism 3730_L sequencer (Seq- Wright, Houston, TX) with appropriate vector-specific T7 (F: TAATAC-GACTCACTATAGGG) and pAE (R:CAGCAGCCAACT-CAGTTCCT) primers. Detailed of cloning, expression and purification of the recombinant proteins Lsa33, Lsa25, rLIC12238, Lsa30, OmpL1, rLIC11360 and rLIC11975 has been previously described [18,19,21,20,22]. The proteins rLIC11360 and Lsa30 were kept at pH 12 required for their solubility.

Antiserum

Five female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μ g of each recombinant protein adsorbed in 10% (vol/vol) of Alhydrogel (2% Al(OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same recombinant proteins preparation. Negative - control mice were injected with PBS plus Alhydrogel. Two weeks after each immunization, the mice were bled from the retro - orbital plexus and the pooled sera were analyzed by ELISA for determination of antibody titers.

Fg binding assay and dose-response curves

The binding of the recombinant proteins to Fg was evaluated by a modified ELISA, as follows: 96-well plates (Costar High Binding, Corning) were coated overnight in PBS at 4°C with 100 μ L of 10 μ g/mL of the human Fg (Sigma); LigB7-12 protein of *L. interrogans*, known as having Fg-binding domain [31,17], generous donation from Dr. Odir Dellagostin, UFPEL, RS, Brazil, was employed as a positive control. PspA, an outer membrane protein of *Streptococcus pneumoniae* [32], kind gift from Dr. Luciana Leite, Instituto Butantan, SP, Brazil, was employed as an unrelated negative control protein. Gelatin (Difco) was also employed as negative protein control. Plates were washed three times with PBS-T and blocked for 2 h at 37°C with PBS with 10% (wt/vol) non-fat dry milk. The blocking solution was discarded and 100 μ L of 10 μ g/mL recombinant proteins in PBS was incubated for 2 h at 37°C. Wells were washed three times with PBS-T and incubated for 1 h at 37°C with polyclonal mouse antiserum produced against each recombinant protein. The serum dilution used was determined by titration curve with the corresponding recombinant protein and the value of 1.0 at OD492_{nm} was employed. These values are: 1:1,000 for rLIC12238; 1:750 for Lsa33; 1:500 for rLIC11975 and rLIC11360; 1:400 for Lsa30, 1:800 for OmpL1, 1:500 for Lsa25 and 1:200 for PspA. After incubation, plates were washed again and incubated with HRP-conjugated anti-mouse immunoglobulin G (IgG), diluted 1:5,000 in PBS. After three washings, 100 μ L/well of 1 mg/mL OPD plus 1 μ L/mL H₂O₂ in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 15 min and stopped by the addition of 50 μ L/well of H₂SO₄ (8 M). Readings were taken at OD492_{nm}. In another assay, the assessment of bound proteins was performed by incubation for 1 h at 37°C with monoclonal anti-polyhistidine-HRP (Sigma) at appropriate dilutions: 1:5,000 for rLIC12238; 1:10,000 for rLIC11975 and rLIC11360; 1:1,000 for Lsa30; 1:500 for Lsa33, 1:400 for OmpL1, 1:200 for Lsa25 and 1:200 for LigB7-12. The reaction was developed with 1 mg/mL OPD plus 1 μ L/mL H₂O₂, as described above. The rate of interaction of recombinant proteins to Fg was determined by measuring the reaction as a function of time. The OD492_{nm} value after 2 h interaction was considered the maximal binding (100%) and was used for statistical analyses, using Student's two-tailed t test. For

Table 1. Gene locus, protein name, gene bank reference sequence, protein conservation, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Gene locus ¹	Given name	NCBI reference sequence number ²	Description/ function	Conservation (identity) ³	Sequence of primers for PCR amplification	Recombinant protein molecular mass
LIC11834	Lsa33 ^a	YP_001783.1	Putative lipoprotein	Lai (99%) LBH (87%) LSS (88%) LlicsVM (60%) LBP (31%)	F: 5' <u>CTCGAGG</u> ATCTACAAGGTGGGGTTT TTAC3' (XhoI) R: 5' <u>CCATGGT</u> TACTGAGGTTTACTTGG TCC3' (NcoI)	33.10 kDa
LIC11975	Lsa36 ^b	YP_0019141	Outer membrane protein	Lai (99%) LBH (0%) LSS (0%) LlicsVM (54%) LBP (38%)	F: 5' <u>CTCGAGGA</u> ATGTAGCGGTGCGG3' (XhoI) R: 5' <u>AAGCTT</u> CATCCCAAAGATAA T TCA3' (HindIII)	36.12 kDa
LIC10973	OmpL1 ^c	YP_000947.1	Outer membrane Protein	Lai (94%) LBH (87%) LSS (87%) LlicsVM (51%) LBP (44%)	F: 5' <u>GGATCC</u> AAAACATATGCAATTGTA GG3' (BamHI) R: 5' <u>GGTACCT</u> TAGAGTTCGTGTTTATA ACC3' (KpnI)	32.40 kDa
LIC11360	Lsa23 ^b	YP_0013241	Putative lipoprotein	Lai (99%) LBH (69%) LSS (67%) LlicsVM (44%) LBP (0%)	F: 5' <u>GGATCC</u> GAACTTCCTTACTTTTCC CCTAAC3' (BamHI) R: 5' <u>AAGCTT</u> GAATGTTGACTAGAGGC ATTACT3' (HindIII)	22.53 kDa
LIC12238	rLIC12238 ^d	YP_002173	Hypothetical protein	Lai (99%) LBH (77%) LSS (0%) LlicsVM (50%) LBP (39%)	F: 5' <u>CTCGAGT</u> GTTTTAAACCTACCGG AG3' (XhoI) R: 5' <u>AAGCTT</u> CTACTTCATCGCTTTTTC TATATC 3' (HindIII)	17.63 kDa
LIC11087	Lsa30 ^e	YP_0010571	Hypothetical protein	Lai (99%) LBH (75%) LSS (81%) LlicsVM (29%) LBP (30%)	F: 5' <u>CTCGAGG</u> GAGATTCCAGAAAGAA AAC3' (XhoI) R: 5' <u>AAGCTT</u> ACTCTGTGTGATGCGCTT C T3' (HindIII)	30.00 kDa
LIC12253	Lsa25 ^a	YP_002188.1	Hypothetical protein	Lai (100%) LBH (77%) LSS (79%) LlicsVM (33%) LBP (39%)	F: 5' <u>CTCGAGG</u> GAGAGAAACCGGACGA TAC 3' (XhoI) R: 5' <u>CCATGGT</u> TAGGGAAGACTTCTAAC ACATC 3' (NcoI)	24.07 kDa

¹<http://aeg.lbi.ic.unicamp.br/world/lic/>;²<http://www.ncbi.nlm.nih.gov/protein/>;³<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi/>. This work, Lai: *L. interrogans* serovar Lai [56]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [57]; LBP: *L. biflexa* serovar Patoc [58]; LlicsVM: *L. licerasiae* serovar Varillal [59]; LSS: *L. santarosai* serovar Shermani [60].^aPreviously published by Domingos *et al.*, 2012 [19].^bPreviously published by Siqueira *et al.*, 2013 [22].^cPreviously published by Fernandes *et al.*, 2012 [21].^dPreviously published by Vieira *et al.*, 2010 and Oliveira *et al.*, 2011 [18,36].^ePreviously published by Souza *et al.*, 2012 [20].

doi:10.1371/journal.pntd.0002396.t001

determination of dose-response curves of the binding of recombinant proteins to human Fg, protein concentrations varying from 0 to 4,000 nM in PBS were used. Binding was detected with polyclonal antibodies against each protein at the dilution described above, except for LigB7-12 where monoclonal anti-polyhistidine-HRP was used (1:200). For statistical analyses, the binding of recombinant proteins to human Fg was compared to its binding to gelatin by Student's two-tailed t test.

Antibody inhibition assay

96-well plates were coated with 1 µg of Fg in 100 µL of PBS and allowed to set overnight at 4°C. The wells were washed three times with PBS-T and then blocked with 200 µL of 10% (wt/vol) nonfat dry milk for 2 h at 37°C. Prior to the next step, the proteins were incubated for 1 h at 37°C with the respective antibodies diluted 1:50 in 100 µL of PBS. After the incubation, each recombinant protein (1 µg) was added per well in 100 µL of PBS, and allowed to attach to Fg for 90 min at 37°C. Polyclonal serum obtained in mice against another leptospiral protein, DnaK (1:50), was employed as a control that is not specific for the studied proteins. After washing six times with PBS-T, bound recombinant proteins were detected by adding monoclonal HRP-conjugated mouse anti-polyhistidine-HRP (Sigma) at dilutions described above. Incubation proceeded for 1 h at 37°C. The detection was performed with OPD, as previously described. BSA or gelatin was used as negative control (data not shown). For statistical analyses, the attachment of blocked recombinant proteins to Fg was compared to the binding with the untreated proteins by the two-tailed t test (* $P < 0.05$).

Effect of recombinant protein denaturing on the interaction with Fg

ELISA plates were coated overnight at 4°C with 100 µL of 10 µg/mL Fg. Plates were washed three times with PBS-T and blocked with 200 µL of 10% (wt/vol) nonfat dry milk for 2 h at 37°C. The recombinant proteins were denatured by incubation at 96°C for 10 min; 1 µg of each was added per well in 100 µL of PBS. The recombinant proteins were allowed to attach to Fg at 37°C for 90 min. After washing six times with PBS-T, bound recombinant proteins were detected by incubation with mouse serum raised against the respective protein (dilutions described above) at 37°C for 1 h. After three washings with PBS-T, 100 µL of a 1:5,000 dilution of HRP-conjugated rabbit anti-mouse IgG (Sigma) in PBS was added per well for 1 h at 37°C. The detection was performed with OPD, as previously described. BSA or gelatin was used as negative control (data not shown). For statistical analyses, the attachment of denatured recombinant proteins to Fg was compared to untreated recombinants binding by the two-tailed t test (* $P < 0.05$).

Dissociation equilibrium constant (K_D) for the binding of recombinant proteins to human Fg

The ELISA data were used to calculate the dissociation equilibrium constant (K_D) according to the method previously described [33] based on the equation: $K_D = (A_{max} \cdot [protein]) / A - [protein]$, where A is the absorbance at a given protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader (when the equilibrium is reached), [protein] is the protein concentration and K_D is the dissociation equilibrium constant for a given absorbance at a given protein concentration (ELISA data point).

Fibrin clot formation inhibition assay in the presence of recombinant proteins

The assay of thrombin-catalyzed fibrin clot inhibition was performed in the presence of recombinant proteins. We have

employed the concentration of recombinant proteins in which there was a binding saturation to Fg. Proteins that do not bind Fg were employed at the same concentration range. Protein concentration employed: Lsa33 – 3,000 nM, OmpL1 – 3,000 nM, rLIC12238 – 3,500 nM, rLIC11975 – 2,000 nM, LigB7-12 – 1,500 nM, Lsa25 – 3,000 nM, PspA – 3,000 nM. Recombinant proteins were resuspended in 0.5 mL of PBS plus 1 mg/mL of Fg and incubated for 2 h at 37°C. ELISA plates were coated with 90 µL/well of recombinant proteins plus Fg and 10 µL/well of thrombin (10 U/mL). The fibrin clot formation was measured as previously described. Reduction of the fibrin clot formation was calculated by comparing the value of the last reading point, at 45 min, with the positive control (100%). Analysis was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons.

Inhibition of live leptospires binding to Fg by recombinant proteins

ELISA plates were coated with Fg (1 µg/well) in PBS and allowed to set overnight at 4°C; the wells were then washed and blocked with 10% non-fat dry milk in PBS-T for 2 h at 37°C. The blocking solution was discarded, and the wells were incubated for 90 min at 37°C with increasing concentrations of recombinant proteins (0 to 4.5 µM). After three washings, 100 µL/well of 4×10^7 live *L. interrogans* serovar Copenhageni strain M-20 were added for 90 min at 37°C. The unbound leptospires were washed and the quantification of bound leptospires was performed indirectly by anti-LipL32 antibodies produced in mice (1:4,000), based on the fact that LipL32 is a major expressed membrane leptospiral protein [34]; the procedure was followed by the addition of HRP-conjugated anti-mouse IgG antibodies, essentially as described in Atzingen et al., (2008) [35]. The detection was performed by OPD, as above described.

Results

Binding of human Fg by *L. interrogans* cells

The ability of *L. interrogans* serovar Copenhageni strain M-20 cells to bind human Fg was performed by immunofluorescence assay (IFA). Leptospires were visualized by propidium iodide staining (Fig. 1, panel A) followed by protein detection with goat anti-human Fg, in the presence of anti-goat IgG antibodies conjugated to FITC. Green fluorescence could be observed for Fg (Fig. 1 -Fg1B, Fg2B). The localization of the protein-green light within the leptospires was achieved by superimposing both fields and the results obtained are shown in Fig. 1 - Fg1C and Fg2C. The FgØ shows the control of the reaction in which Fg was absent.

Interaction of human Fg with *Leptospira* spp. and inhibition of fibrin clot formation

We have evaluated the capability of soluble human Fg (10 µg/mL) to interact with immobilized strains of leptospires by ELISA. We performed the experiments using virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, pathogenic attenuated *L. interrogans* serovar Copenhageni strain M-20 and one saprophytic *L. biflexa* serovar Patoc strain Patoc 1. The binding of Fg to each leptospiral strain was performed in triplicate and the data represent the mean $\pm$ the standard deviation from one representative experiment is depicted in Fig. 2A. The measurements were performed in the presence (Fg+) and absence (Fg-) of human Fg while in the no-cell control, BSA replaced leptospires. For statistical analyses, the attachment of Fg to leptospiral strains was performed by one-way ANOVA followed by Tukey post-test for pairwise comparison, asterisks above the bars refer to

Figure 1. Recognition of Fg binding to *Leptospira* by IFA. Live *L. interrogans* sorovar Copenhageni strain M-20 were fixed with 2% paraformaldehyde and treated with 16 μ g of Fg. The recognition was assessed through polyclonal anti-Fg antibodies under confocal immunofluorescence microscope (Fg1 and Fg2). Control in which Fg was absent from the incubation mixture is shown (Fg Ø). Panels A: DNA propidium iodide-stained, B: FITC-stained and C: A+B composite images.
doi:10.1371/journal.pntd.0002396.g001

comparison to BSA (no cell control) (** $P < 0.01$). The comparison among leptospiral strains is also shown (# $P < 0.05$ and ## $P < 0.01$). The results show that all strains tested were able of binding human Fg, but the virulent strain was more efficient. We have assessed the effect of *Leptospira*-bound to Fg on the inhibition of thrombin-catalyzed fibrin clot formation. The reaction was analyzed with the same strains and readings were taken at OD_{595nm} every 1 min for the first 10 min and then every 5 min for 35 min. The complete reaction, Fg plus thrombin, was used as a positive control while in the negative one, thrombin was missing. The determination was performed in two independent experiments and a representative assay is shown in Fig. 2B. The data show that all the strains studied promoted an inhibition of fibrin formation statistically significant compared to the reaction

positive control. However, the small difference observed between the strains pathogenic and saprophyte was not statistically relevant.

PLA-associated leptospires on human Fg

The effect of PLA-coated virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm on human Fg was evaluated by Western blotting using anti-human Fg antibodies (Fig. 3A). The results show that PLA generation on the surface of *Leptospira* cause degradation of human Fg (Fig. 3A, lane 5), which is not observed when at least one of the reaction components is missing (Fig. 3A, lanes 1, 2, 3 and 4) and completely prevented in the presence of a serine protease inhibitor, aprotinin (Fig. 3A, lane 6). The data suggest that PLA-coated leptospires were capable to employ their

Figure 2. (A). Interaction of leptospires to Fg. The binding of soluble human Fg (10 μ g/mL) to immobilized strains of leptospires (*L. interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Copenhageni strain M-20, and *L. biflexa* serovar Patoc strain Patoc 1) was accessed by ELISA. After 2 h interaction, plates were washed and the detection of bound Fg was verified by incubation with goat anti-Fg (1:25,000) followed by incubation with HRP-conjugated rabbit anti-goat IgG (1:50,000). The binding of Fg to each leptospiral strain was performed in triplicate and the data represent the mean $\pm$ the standard deviation from one representative experiment. Fg+ and Fg- stand for the presence and absence of Fg, respectively, while in the no-cell control, BSA replaced leptospires. For statistical analyses, the attachment of Fg to leptospiral strains was performed by one-way ANOVA followed by Tukey post-test for pairwise comparison, asterisks above the bars refer to comparison to BSA (no cell control) (** $P < 0.01$). The comparison among leptospiral strains is also shown (# $P < 0.05$ and ## $P < 0.01$). **(B)** Inhibition of fibrin clot formation by leptospires. 1 mg/mL of Fg plus live leptospires, pre-incubated for 2 h at 37°C, and 10 U/mL of thrombin were placed into ELISA plates. The positive control of the reaction employed Fg (1 mg/mL) plus thrombin (10 U/mL) while in the negative control thrombin was omitted. BSA was used as a negative control of binding. The fibrin clot formation was measured every 1 min for 10 min and then every 5 min for 35 min. Each point was performed in triplicate and expressed as the mean absorbance value at 595_{nm} $\pm$ SD for each point.
doi:10.1371/journal.pntd.0002396.g002

proteolytic activity to interfere with one of the coagulation cascade substrate.

The Fg degradation by PLA-coated leptospires was also evaluated by ELISA. When leptospires were treated with PLG+uPA, the detection of Fg remaining in cell-free solution is decreased when compared to untreated, only PLG or only uPA-treated or when aprotinin was added to the Fg incubation (Fig. 3B). An additional control lacking leptospires was added in order to rule out the contribution of free PLA to the Fg degradation. As expected, the data showed that the remaining Fg level in this control was comparable with the non-proteolytic controls. Cell-bound Fg was also evaluated. PLA-coated leptospires retain Fg

binding ability, though it seems to be diminished (Fig. 3B), probably due to Fg degradation.

Selection of putative surface proteins from genome sequences

The rationale for protein selection was mostly based on cellular localization, since surface proteins are potential receptors for Fg. We have selected seven proteins, all of them previously shown to be leptospiral adhesins and were already published, rLIC12238 [18,36], Lsa33, Lsa25 [19], Lsa30 [20], OmpL1 [21], rLIC11360 and rLIC11975 [22]. Table 1 summarizes features of the selected proteins, gene locus, given name, gene conservation within the

Figure 3. Degradation of Fg by PLA-coated leptospires. (A) *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF) (10^8 /sample) were treated with the addition of 10 μ g PLG (lane 3), 3U uPA (lane 4) 10 μ g PLG and 3U uPA (PLA) (lanes 5, 6) or no additions (untreated) (lane 2). Control lacking leptospires (lane1) and addition of 1 μ g aprotinin to the Fg-leptospires incubation (lane 6) were employed. The cells were incubated with 15 μ g/mL human purified Fg for 16 h at 37°C, and the supernatants were fractionated by SDS-PAGE and transferred to membranes. The Fg was detected by specific antibodies and the reaction was developed by ECL reagent. Indicated on the left are the molecular mass standards, and on the right, the masses of the intact Fg (340 kDa) and its degradation product (D domains, 90 kDa). (B) After treatments and incubation with Fg, the cell-free (supernatants) and cell-bound Fg were coated onto ELISA plates and detected by Fg polyclonal antibodies. A control lacking leptospires received the PLG+uPA treatment to rule out the contribution of contaminating cell-free PLA to Fg degradation.

doi:10.1371/journal.pntd.0002396.g003

sequenced genomes, the sequences of primers used for cloning techniques and molecular mass.

Expression and purification of recombinant proteins

The amplified coding sequences, excluding the signal peptide tags, were cloned and expressed as full-length proteins in *E. coli*. The recombinant proteins were expressed with 6×His tag at the N-terminus and purified by nickel affinity chromatography, as previously described [35].

Interaction of leptospiral recombinant proteins with Fg

Proteins of *Leptospira* have been reported to bind Fg [15,16,17]. We thus decided to investigate whether the selected surface-exposed proteins were capable of binding human Fg *in vitro*. Seven recombinant leptospiral proteins, expressed and purified in our laboratory, LigB7-12, used as positive control [31,16,17], and the

negative protein controls gelatin and pneumococcal recombinant protein PspA, were individually placed onto 96-wells and incubated with previously immobilized human Fg. The binding was quantified by ELISA and the results obtained from three independent experiments are shown in Fig. 4. The protein bound to Fg was probed with the respective homolog polyclonal antiserum raised in mice (Fig. 4A) or with the monoclonal antibody anti-histidine tag (Fig. 4B). The percentage of binding for each protein with Fg, measured as a function of time is shown in Fig. 4C. The protein rLIC11360 promptly reacted with Fg with 40% of binding achieved after 5 min reaction, contrasting with rLIC11975 that showed very low binding activity at this time (Fig. 4C). The binding of recombinant proteins to Fg was also assessed after blocking the proteins with the corresponding antibody and after submitting them to denaturing conditions at 96°C for 10 min (Fig. 4D). The binding was totally inhibited by

Figure 4. Recombinant proteins binding to human Fg. Human Fg (10 $\mu\text{g/mL}$) was coated to ELISA plates and allowed to interact with the recombinant proteins (10 $\mu\text{g/mL}$). Gelatin was used as a negative control for nonspecific binding. The reaction was detected with polyclonal antibodies raised against each protein, including anti-DnaK, used as non-specific antibodies (M&M), in (A), (C) and (D) while monoclonal anti-polyhistidine HRP antibodies was employed in (B). In (C) the percentage of binding for each protein with Fg was measured as a function of time up to 2 h. The statistical analysis was performed by two-tailed *t* test ($*P<0.05$) compared to the binding after 2 h. The effect of denaturing the recombinant proteins by incubation for 10 min at 96°C or blocking the proteins with the respective antibodies for 1 h at 37°C , previously to interaction with Fg is shown in (D). Bars represent the mean of absorbance at $492_{\text{nm}} \pm$ the standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the binding of recombinant proteins to human Fg was compared to its binding to gelatin or untreated recombinant binding, in the case of (D) by two-tailed *t* test ($*P<0.05$). doi:10.1371/journal.pntd.0002396.g004

antibody-blocked protein in the case Lsa30, Lsa33, rLIC11975, rLIC12238 and rLIC11360, while a very low percentage of the binding remained for OmpL1 (8.4%), suggesting the participation of non- immunogenic epitopes on the interaction

(Fig. 4D).

Anti-DnaK serum, control employed as unrelated antibody, promoted a partial decrease on the binding of rLIC11360 and Lsa30, no effect with the protein Lsa33, while a slightly increase

Figure 5. Binding characterization of recombinant proteins to human Fg. (A) Ten $\mu\text{g/mL}$ of Fg was immobilized into 96-wells ELISA plates and 0 to 4,000 nM of each recombinant protein was added for interaction. The bindings were detected using antiserum raised in mice against each protein followed by HRP-conjugated anti-mouse IgG. Gelatin was employed as negative control for each assayed protein (not shown) and Lsa25, as a leptospiral recombinant protein negative control. Data represent the mean absorbance values $\pm$ the standard deviation of three replicates for each experimental group of three independent experiments. The insert shows the dose response curve obtained with 10 $\mu\text{g/mL}$ immobilized Fg and 0 to 3,000 nM LigB7-12, employed as leptospiral Fg-binding protein. The binding was detected with monoclonal anti-polyhistidine HRP antibodies. (B) The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that have reached the equilibrium concentration. Mean absorbance values at 492 nm ($\pm$ the standard deviations of three independent experiments) were compared to those obtained with untreated Fg (0 mM).

doi:10.1371/journal.pntd.0002396.g005

was detected with the proteins rLIC11975, rLIC12238 and OmpL1. At any rate, these differences were not statistically significant and are probably due to the presence of non-specific antibodies in the polyclonal serum. The adhesion of heat-denatured proteins to Fg was almost totally abolished in the case of rLIC12238 and OmpL1, 24% remained with rLIC11360, while 63–77% of the binding continued with Lsa30, Lsa33 and rLIC11975 (Fig. 4D). The results suggest that for some proteins the binding depends on their conformational structures

while with others it probably relies on their linear primary conformation.

Characterization of the binding of recombinant proteins to Fg

The interactions between the recombinant proteins and Fg was assessed on a quantitative basis, as indicated in Fig. 5A. Dose-dependent and saturable binding was observed when increasing

Figure 6. Inhibition of fibrin clot formation by recombinant proteins. Recombinant proteins were resuspended in 0.5 mL of PBS plus 1 mg/mL of Fg and incubated for 2 h at 37°C. The employed concentration of recombinant protein was the one in which there was a binding saturation to Fg: Lsa33 (3,000 nM), OmpL1 (3,000 nM), rLIC12238 (3,500 nM) and rLIC11975 (2,000 nM). The positive control protein LigB7-12 was employed at 1,500 nM, while the negative control proteins, Lsa25 and PspA were used at 3,000 nM. ELISA plates were coated with 90 µL/well of recombinant proteins plus Fg and 10 µL/well of thrombin (10 U/mL). The positive control of the reaction employed Fg (1 mg/mL) plus thrombin (10 U/mL) while in the negative control thrombin was omitted. The fibrin clot formation was measured every 1 min for 10 min and then every 5 min for 35 min. Each point was performed in triplicate and expressed as the mean absorbance value at 595 nm $\pm$ SD for each point. Bars represent the mean absorbance values $\pm$ standard deviation of three replicates for each condition and are representative of two independent experiments. doi:10.1371/journal.pntd.0002396.g006

concentrations (0 to 4,000 nM) of recombinant proteins rLIC12238, Lsa33, OmpL1, rLIC11975 and rLIC11360, or (0 to 2,000) of Lsa30, were allowed to individually adhere to a fixed human Fg amount (10 µg/mL) for 2 h. Saturation was reached with all except Lsa30 protein, due to the impossibility to achieve this protein at higher concentrations. In the case of LigB7-12, dose-response curve was measured using monoclonal anti-His tag antibodies, and the results depicted in the insert of Fig. 5A shows that saturation was not reached in the protein concentration range employed. Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with Fg are depicted in Fig. 5B; the highest and the lowest K_D values were for rLIC12238 (733.3 ± 276.8 nM) and Lsa33 (128 ± 89.9 nM), respectively.

Inhibition of fibrin formation by recombinant proteins-bound to Fg

Since *Leptospira* bound to Fg inhibited thrombin-catalyzed fibrin formation, we decided to investigate whether the recombinant proteins bound to Fg were capable to mediate this interaction. The concentration of recombinant proteins used was the one in which there was a binding saturation to Fg (Lsa33 – 3,000 nM, OmpL1 – 3,000 nM, rLIC12238 – 3,500 nM and rLIC11975 – 2,000 nM). LigB7-12, previously shown to inhibit thrombin-catalyzed fibrin formation [16,17], was employed as positive control at 1,500 nM. Recombinant proteins Lsa25 and pneumococcal PspA that do not interact with Fg, were employed as negative controls, at 3000 nM. Each recombinant protein was pre-incubated with 1 mg/mL of Fg at 37°C for 2 h. The reaction mixtures were used to coat ELISA plates and 10 U/mL of thrombin was added per well. The fibrin clot formation was measured every 1 min for 10 min and then every 5 min for 35 min. The percentage of inhibition was calculated as a function of time, taking the complete reaction at

the last time-point, in the absence of proteins, as a 100% fibrin formation (positive control). In the negative control, thrombin was omitted from the reaction. The measurements were performed in triplicate and representative curves of two independent experiments are shown in Fig. 6. The proteins rLIC11360 and Lsa30 were not employed on these assays because they were kept at pH 12 for solubility and thrombin is not active above pH 10. The data show that the four proteins tested, Lsa33, rLIC12238, rLIC11975 and OmpL1, elicited an inhibition of 40–50% on the fibrin clot formation, similar to the one elicited by LigB7-12 (Fig. 6). This effect on clot formation by the recombinant proteins was similar with all of them, although a lag time was observed within the rLIC12238 inhibition curve. The inhibition promoted by all proteins was statistically relevant when compared to the positive reaction control. The results are consistent with the K_D of the proteins towards Fg that are in the same order of magnitude. In contrast, Lsa25 and PspA, proteins that do not react with Fg, were not capable to inhibit thrombin-catalyzed fibrin production, resulting in curves similar to the positive control of the reaction (Fig. 6).

Inhibition of live leptospires binding to Fg by recombinant proteins

The inhibitory effect exerted by recombinant proteins on leptospiral adherence to Fg was quantified by ELISA. Fg-coated microtiter wells were incubated with increasing concentration (0–4.5 µM) of rLIC12238 (Fig. 7A), rLIC11975 (Fig. 7B), Lsa30 (Fig. 7C), rLIC11360 (Fig. 7D), Lsa33 (Fig. 7E) and OmpL1 (Fig. 7F) for 90 min prior to the addition of 4×10^7 *L. interrogans* serovar Copenhageni strain M-20. Wells were probed with anti-LipL32 serum, given the fact that LipL32 is a major membrane leptospiral protein [34]. The results are depicted in Figure 7 A to F, and show that the proteins caused a modest, but significant reduction in the number of leptospires adhering to Fg (* $P < 0.05$)

Figure 7. Inhibition of *L. interrogans* attachment to human Fg by recombinant proteins. Fg-coated microtiter wells were incubated with increasing concentration (0–4.5 μM) of rLIC12238 (A), rLIC11975 (B), Lsa30 (C), rLIC11360 (D), Lsa33 (E) and OmpL1 (F) for 90 min prior to the addition of 4×10^7 leptospires (*L. interrogans* serovar Copenhageni strain M20). Wells were probed with anti-LipL32 serum (1:4,000). Data are expressed as $OD_{492\text{ nm}} \pm \text{SD}$ of three independent experiments, each performed in triplicate. Significance was assessed by comparison with the “no protein” wells by Students two-tailed *t* test (**P* < 0.05). doi:10.1371/journal.pntd.0002396.g007

with 0.5, 1.0, 1.0, 0.1, 0.25 and 1.0 μM of rLIC12238, rLIC11975, rLIC11360, Lsa30, Lsa33 and OmpL1, respectively. We have performed three independent experiments with comparable results.

Discussion

Fg is the major clotting protein present in blood plasma with an important role in coagulation and thrombosis. Several studies have suggested roles of Fg and PLG in host bacterial interactions [11]. The fibrinolytic system that generates PLA from PLG and its activators, decomposes the fibrin clot by degrading fibrin [37]. This system is negatively regulated by PLG activator inhibitors. A balance between coagulation system and anticoagulation is

necessary to avoid pathophysiological conditions, such as, bleeding and thrombosis [38].

Several Fg-binding proteins of important pathogens, such as, *Staphylococcus aureus* [39], group B *Streptococcus* [40], *Lactobacillus salivarius* [41] and the spirochetes *Treponema pallidum* [42], *T. denticola* [43,44,45] have been reported and characterized. These Fg-binding proteins are bacterial surface or secreted adhesins that although acting through different mechanisms will ultimately lead to enhance the bacterial survival in the host [46].

The adhesion of virulent *L. interrogans* strain Fiocruz L1-130 to Fg has been shown to be induced by physiological osmolality, but the effect of this binding on fibrin clot formation was not evaluated [14]. The leptospiral proteins, LigA and LigB [16,17] and OmpL37 [15] have been described as Fg-binding proteins. In

addition, the interaction of LigB with Fg was shown to inhibit thrombin-catalyzed fibrin formation [16,17].

In this work, we show that *L. interrogans* serovar Copenhageni are capable of binding human Fg on their surface, as visualized by indirect IFA. Moreover, we have employed ELISA to assess the interaction of human Fg with virulent *L. interrogans* serovar Copenhageni Fiocruz L1-130, attenuated *L. interrogans* serovar Copenhageni M-20 and non-pathogenic saprophytic *L. biflexa* serovar Patoc Patoc 1, and the effect of this binding on the thrombin-catalyzed fibrin formation. Moreover, we studied seven leptospiral adhesin- and PLG-binding proteins, rLIC12238 [18], Lsa33, Lsa25 [19], Lsa30 [20], OmpL1 [21], rLIC11360 and rLIC11975 [22] for their capacity to bind Fg and to inhibit fibrin clot formation. We show that all strains tested bind Fg, including the saprophytic one, and that this interaction inhibits fibrin clot formation. Though the virulent strain appears to be more efficient, the values obtained were not statistically significant. Attachment of recombinant proteins to Fg was specific, dose-dependent and saturable with all proteins but Lsa30. The interaction of the proteins with Fg, similar to the *Leptospira*, promoted an inhibition on thrombin-induced fibrin clot formation. Thus, *Leptospira*, similar to other pathogens, express multiple Fg-binding proteins [38,46].

As expected, recombinant proteins partially inhibited attachment of intact *L. interrogans* to immobilized Fg. The inhibitory effect exerted by the recombinant proteins was moderate, ranging from 0.1 to 1.0 μ M of protein concentration to reach significance, and could be explained by the existence of additional *L. interrogans* binding proteins contributing to the leptospiral adherence to Fg. A correlation between inhibition and affinity was detected with the recombinant protein Lsa33, while no correspondence was seen with the others.

The inhibitory effect on fibrin formation observed with leptospirases and with the six recombinant proteins was partial, similar to the LigB, fragment 7–12, employed in this work as positive control, and previously reported by Choy et al., 2011 [16], using LigB fragment 9–11. Our data differ with the total clotting inhibition promoted by the Fg-binding proteins, SdrG of *S. epidermidis* [47], ClfA of *S. aureus* [48] and LigBCen2R [17]. The data suggest that in *Leptospira* other mechanisms might be involved in fibrin formation and/or the main function of these Fg-binding proteins is not associated with the clotting.

We have described the interaction of *Leptospira* with fibrinolytic system and shown that it occurs with virulent, attenuated and saprophyte strains of *Leptospira*. Moreover, we demonstrated that this association renders the bacteria with proteolytic activity capable of degrading ECM components [12]. Several membrane

proteins were identified as PLG-binding receptors capable of generating PLA in the presence of activator, suggesting that the interaction with the fibrinolytic system might be important during leptospirosis [49,18,50,36,19,21,20]. Indeed, the increased plasma levels of Fg degradation products detected in leptospirosis has provided evidence for fibrinolysis activity [51,52]. We show now that *Leptospira* surface-associated PLA activity is capable to degrade Fg *in vitro*, suggesting one possible pathway to generate Fg metabolites during the disease.

Fg is considerably upregulated during inflammation or under exposure to stress such systemic infections [46]. The activation of coagulation cascade with increased levels of plasma Fg during leptospirosis has been detected [53,51,54]. It has been suggested that these findings are possibly associated to severe tissue damage, vascular endothelial injury or a compensating production by the liver in response to the augmented Fg utilization [55,53,51]. Our data show that *Leptospira* either through their Fg-binding proteins or coated with PLA activity would increase the consumption of Fg molecules by sequestering or degrading them. Under these circumstances, a reduction on fibrin clot formation is expected. In addition, leptospirosis patients with clinical bleeding were reported to have lower platelet counts when compared to other patients [54], a condition that would help decrease thrombosis, facilitate bleeding and help bacterial dissemination.

In conclusion, we have provided molecular evidence of the mechanisms that *Leptospira* could employ to interact with components of the coagulation cascade and the fibrinolytic system. In addition, we have shown that six adhesins could mediate the binding of *Leptospira* to Fg and impair thrombin-induced fibrin clot formation. We believe that our results should contribute to the understanding of the complex coagulopathy observed during leptospirosis.

Acknowledgments

We are deeply in debt to Drs. Odir Dellagostin (UFPEL, RS, Brazil), Yung-Fu Chang (Cornell University, Ithaca, New York, USA), Luciana Leite, (Instituto Butantan, SP, Brazil) for their generous donation of LigB and PspA plasmids, and to Dr. Henrique Krambeck Rofatto (Departamento de Parasitologia, Instituto Butantan, São Paulo, Brazil) for use of the confocal microscope facilities and helpful discussion.

Author Contributions

Conceived and designed the experiments: RO SAV ALTON. Performed the experiments: RO RFD GHS LGF NMS MLV ZMdM. Analyzed the data: RO RFD GHS LGF NMS MLV ZMdM SAV ALTON. Contributed reagents/materials/analysis tools: SAV ALTON. Wrote the paper: RO RFD GHS LGF NMS MLV ZMdM SAV ALTON.

References

- Faine S, Adler B, Bolin C, Perolat P (1999) *Leptospira* and Leptospirosis. Melbourne, Australia: MediSci Press.
- Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, et al. (2003) Leptospirosis: a zoonotic disease of global importance. *Lancet Infect Dis* 3: 757–771.
- Levett PN (2001) Leptospirosis. *Clin Microbiol Rev* 14: 296–326.
- Plank R, Dean D (2000) Overview of the epidemiology, microbiology, and pathogenesis of *Leptospira* spp. in humans. *Microbes Infect* 2: 1265–1276.
- McBride AJ, Athanazio DA, Reis MG, Ko AI (2005) Leptospirosis. *Curr Opin Infect Dis* 18: 376–386.
- Marotto PC, Ko AI, Murta-Nascimento C, Seguro AC, Prado RR, et al. (2010) Early identification of leptospirosis-associated pulmonary hemorrhage syndrome by use of a validated prediction model. *J Infect* 60: 218–223.
- Zhang YX, Geng Y, Bi B, He JY, Wu CF, et al. (2005) Identification and classification of all potential hemolysin encoding genes and their products from *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Lai. *Acta Pharmacol Sin* 26: 453–461.
- Hauk P, Negrotto S, Romero EC, Vasconcellos SA, Genovez ME, et al. (2005) Expression and characterization of HlyX hemolysin from *Leptospira interrogans* serovar Copenhageni: potentiation of hemolytic activity by LipL32. *Biochem Biophys Res Commun* 333: 1341–1347.
- Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, et al. (2009) Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS Lett* 17: 1381–5.
- Degen JL, Bugge TH, Goguen JD (2007) Fibrin and fibrinolysis in infection and host defense. *J Thromb Haemost* 5 Suppl 1: 24–31.
- Lahteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiol Rev* 25: 531–552.
- Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL (2009) Plasminogen acquisition and activation at the surface of *Leptospira* species lead to fibronectin degradation. *Infect Immun* 77: 4092–4101.
- Vieira ML, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL (2011) In vitro evidence for immune evasion activity by human plasmin associated to pathogenic *Leptospira interrogans*. *Microb Pathog* 51: 360–365.

14. Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, et al. (2007) Physiological osmotic induction of *Leptospira* interrogans adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun* 75: 2441–2450.
15. Pinne M, Choy HA, Haake DA (2010) The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. *PLoS Negl Trop Dis* 4: e815.
16. Choy HA, Kelley MM, Croda J, Matsunaga J, Babbitt JT, et al. (2011) The multifunctional LigB adhesin binds homeostatic proteins with potential roles in cutaneous infection by pathogenic *Leptospira* interrogans. *PLoS One* 6: e16879.
17. Lin YP, McDonough SP, Sharma Y, Chang YF (2011) *Leptospira* immunoglobulin-like protein B (LigB) binding to the C-terminal fibrinogen alphaC domain inhibits fibrin clot formation, platelet adhesion and aggregation. *Mol Microbiol* 79: 1063–1076.
18. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, et al. (2010) In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira* interrogans. *PLoS One* 5: e11259.
19. Domingos RF, Vieira ML, Romero EC, Goncalves AP, de Moraes ZM, et al. (2012) Features of two proteins of *Leptospira* interrogans with potential role in host-pathogen interactions. *BMC Microbiol* 12: 50.
20. Souza NM, Vieira ML, Alves IJ, de Moraes ZM, Vasconcellos SA, et al. (2012) Lsa30, a novel adhesin of *Leptospira* interrogans binds human plasminogen and the complement regulator C4bp. *Microb Pathog* 53: 125–134.
21. Fernandes LG, Vieira ML, Kirchgatter K, Alves IJ, de Moraes ZM, et al. (2012) OmpL1 Is an Extracellular Matrix- and Plasminogen-Interacting Protein of *Leptospira* spp. *Infect Immun* 80: 3679–3692.
22. Siqueira GH, Atzingen MV, Alves IJ, Moraes ZM, Vasconcellos SA, Nascimento ALTO (2013) Characterization of three novel adhesins of *Leptospira* interrogans. *Am J Trop Med Hyg*: [In press]
23. Turner LH (1970) Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires. *Trans R Soc Trop Med Hyg* 64: 623–646.
24. Nakai K, Kanehisa M (1991) Expert system for predicting protein localization sites in gram-negative bacteria. *Proteins* 11: 95–110.
25. Nakai K, Horton P (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem Sci* 24: 34–36.
26. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A* 95: 5857–5864.
27. Letunic I, Copley RR, Pils B, Pinkert S, Schultz J, et al. (2006) SMART 5: domains in the context of genomes and networks. *Nucleic Acids Res* 34: D257–260.
28. Finn RD, Mistry J, Schuster-Bockler B, Griffiths-Jones S, Hollich V, et al. (2006) Pfam: clans, web tools and services. *Nucleic Acids Res* 34: D247–251.
29. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, et al. (2003) Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci* 12: 1652–1662.
30. Ramos CR, Abreu PA, Nascimento AL, Ho PL (2004) A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 37: 1103–1109.
31. Forster KM, Hartwig DD, Seixas FK, Bacelo KL, Amaral M, et al. (2013) A conserved region of leptospiral immunoglobulin-like A and B proteins as a DNA vaccine elicits a prophylactic immune response against leptospirosis. *Clin Vaccine Immunol* 20: 725–731.
32. Goulart C, Darrieux M, Rodriguez D, Pimenta FC, Brandileone MC, et al. (2011) Selection of family 1 PspA molecules capable of inducing broad-ranging cross-reactivity by complement deposition and opsonophagocytosis by murine peritoneal cells. *Vaccine* 29: 1634–1642.
33. Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, et al. (2009) Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. *J Biol Chem* 284: 19380–19391.
34. Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW, et al. (2009) Proteome-wide cellular protein concentrations of the human pathogen *Leptospira* interrogans. *Nature* 460: 762–765.
35. Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, et al. (2008) Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol* 8: 70.
36. Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, et al. (2011) Characterization of novel OmpA-like protein of *Leptospira* interrogans that binds extracellular matrix molecules and plasminogen. *PLoS One* 6: e21962.
37. Weisel JW (2005) Fibrinogen and fibrin. *Adv Protein Chem* 70: 247–299.
38. Sun H (2006) The interaction between pathogens and the host coagulation system. *Physiology (Bethesda)* 21: 281–288.
39. Flick MJ, Du X, Prasad JM, Raghu H, Palumbo JS, et al. (2013) Genetic elimination of the binding motif on fibrinogen for the *S. aureus* virulence factor ClfA improves host survival in septicemia. *Blood* 121: 1783–1794.
40. Dramsi S, Morello E, Poyart C, Trieu-Cuot P (2012) Epidemiologically and clinically relevant Group B *Streptococcus* isolates do not bind collagen but display enhanced binding to human fibrinogen. *Microbes Infect* 14: 1044–1048.
41. Collins J, van Pijkeren JP, Svensson L, Claesson MJ, Sturme M, et al. (2012) Fibrinogen-binding and platelet-aggregation activities of a *Lactobacillus salivarius* septicemia isolate are mediated by a novel fibrinogen-binding protein. *Mol Microbiol* 85: 862–877.
42. Houston CV, Hof R, Francescutti T, Hawkes A, Boulanger MJ, et al. (2011) Bifunctional role of the *Treponema pallidum* extracellular matrix binding adhesin Tp0751. *Infect Immun* 79: 1386–1398.
43. Haaapasalo M, Muller KH, Uitto VJ, Leung WK, McBride BC (1992) Characterization, cloning, and binding properties of the major 53-kilodalton *Treponema denticola* surface antigen. *Infect Immun* 60: 2058–2065.
44. Edwards AM, Jenkinson HF, Woodward MJ, Dymock D (2005) Binding properties and adhesion-mediating regions of the major sheath protein of *Treponema denticola* ATCC 35405. *Infect Immun* 73: 2891–2898.
45. Bamford CV, Fenno JC, Jenkinson HF, Dymock D (2007) The chymotrypsin-like protease complex of *Treponema denticola* ATCC 35405 mediates fibrinogen adherence and degradation. *Infect Immun* 75: 4364–4372.
46. Rivera J, Vannakambadi G, Hook M, Speziale P (2007) Fibrinogen-binding proteins of Gram-positive bacteria. *Thromb Haemostasis* 98: 503–511.
47. Davis SL, Gurusiddappa S, McCrea KW, Perkins S, Hook M (2001) SdrG, a fibrinogen-binding bacterial adhesin of the microbial surface components recognizing adhesive matrix molecules subfamily from *Staphylococcus epidermidis*, targets the thrombin cleavage site in the Bbeta chain. *J Biol Chem* 276: 27799–27805.
48. Liu CZ, Shih MH, Tsai PJ (2005) ClfA(221–550), a fibrinogen-binding segment of *Staphylococcus aureus* clumping factor A, disrupts fibrinogen function. *Thromb Haemostasis* 94: 286–294.
49. Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, et al. (2010) Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect Immun* 78: 2053–2059.
50. Mendes RS, Von Atzingen M, de Moraes ZM, Goncalves AP, Serrano SM, et al. (2011) The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infect Immun* 79: 4657–4667.
51. Wagenaar JF, Goris MG, Sakundarno MS, Gasem MH, Mairuhu AT, et al. (2007) What role do coagulation disorders play in the pathogenesis of leptospirosis? *Trop Med Int Health* 12: 111–122.
52. Wagenaar JF, Goris MG, Partiningrum DL, Isbandrio B, Hartskeerl RA, et al. (2010) Coagulation disorders in patients with severe leptospirosis are associated with severe bleeding and mortality. *Trop Med Int Health* 15: 152–159.
53. De Francesco Daher E, Oliveira Neto FH, Ramirez SM (2002) Evaluation of hemostasis disorders and anticardiolipin antibody in patients with severe leptospirosis. *Rev Inst Med Trop Sao Paulo* 44: 85–90.
54. Chierakul W, Tientadakul P, Suputtamongkol Y, Wuthiekanun V, Phimda K, et al. (2008) Activation of the coagulation cascade in patients with leptospirosis. *Clin Infect Dis* 46: 254–260.
55. Higgins R, Cousineau G (1977) The pathogenesis of leptospirosis I. Hemorrhages in experimental leptospirosis in guinea pigs. *Can J Comp Med* 41: 174–181.
56. Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, et al. (2003) Unique physiological and pathogenic features of *Leptospira* interrogans revealed by whole-genome sequencing. *Nature* 422: 888–893.
57. Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, et al. (2006) Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci U S A* 103: 14560–14565.
58. Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, et al. (2008) Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS One* 3: e1607.
59. Ricaldi JN, Fouts DE, Selengut JD, Harkins DM, Patra KP, et al. (2012) Whole genome analysis of *Leptospira licerasiae* provides insight into leptospiral evolution and pathogenicity. *PLoS Negl Trop Dis* 6: e1853.
60. Chou LF, Chen YT, Lu CW, Ko YC, Tang CY, et al. (2012) Sequence of *Leptospira santarosai* serovar Shermani genome and prediction of virulence-associated genes. *Gene* 511: 364–370.

Leptospiral extracellular matrix adhesins as mediators of pathogen–host interactions

Monica L. Vieira¹, Luis G. Fernandes^{1,2}, Renan F. Domingos^{1,2}, Rosane Oliveira^{1,2}, Gabriela H. Siqueira^{1,2}, Natalie M. Souza^{1,2}, Aline R.F. Teixeira^{1,2}, Marina V. Atzingen¹ & Ana L.T.O. Nascimento^{1,2}

¹Centro de Biotecnologia, Instituto Butantan, São Paulo, SP, Brazil; and ²Programa Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, São Paulo, SP, Brazil

Correspondence: Ana L.T.O. Nascimento, Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900 São Paulo, SP, Brazil.
Tel.: 5511 2627 9829;
fax: 5511 3726 9233;
e-mail: ana.nascimento@butantan.gov.br

Received 23 August 2013; revised 19 November 2013; accepted 26 November 2013.

DOI: 10.1111/1574-6968.12349

Editor: Mark Schembri

Keywords

leptospirosis; infectious diseases; bacterial diseases.

Abstract

Leptospirosis is been considered an important infectious disease that affects humans and animals worldwide. This review summarizes our current knowledge of bacterial attachment to extracellular matrix (ECM) components and discusses the possible role of these interactions for leptospiral pathogenesis. Leptospiral proteins show different binding specificity for ECM molecules: some are exclusive laminin-binding proteins (Lsa24/LfhA/LenA, Lsa27), while others have broader spectrum binding profiles (LigB, Lsa21, LipL53). These proteins may play a primary role in the colonization of host tissues. Moreover, there are multifunctional proteins that exhibit binding activities toward a number of target proteins including plasminogen/plasmin and regulators of the complement system, and as such, might also act in bacterial dissemination and immune evasion processes. Many ECM-interacting proteins are recognized by human leptospirosis serum samples indicating their expression during infection. This compilation of data should enhance our understanding of the molecular mechanisms of leptospiral pathogenesis.

Introduction

The genus *Leptospira* encompasses both pathogenic and saprophytic species. Pathogenic *Leptospira* are the etiological agents of leptospirosis, while saprophytic bacteria are environment free-living organisms. *Leptospira* are classified according to serovar status – more than 250 pathogenic serovars have been identified. Structural heterogeneity in lipopolysaccharide moieties seems to be the basis for the great degree of antigenic variation observed among serovars. Humans are accidental hosts that become infected through contact with wild or domestic animals or exposure to contaminated soil or water (Adler & de la Pena Moctezuma, 2010).

Leptospirae enter the host mainly via intact sodden or damaged skin or mucosa. The initial phase exhibits wide-ranging and nonspecific symptoms such as fever, chills, headache, and myalgia. Leptospirosis can progress to a severe condition known as Weil's syndrome, corresponding to 5–15% of the reported cases. Another severe

manifestation of the disease, the leptospirosis pulmonary hemorrhage syndrome, was first described in North Korea and China and has been increasingly reported worldwide (McBride *et al.*, 2005).

Currently available veterinarian and human vaccines are based on inactivated whole cell or membrane preparations of pathogenic leptospirae. However, these vaccines do not induce long-term protection against infection and do not provide cross-protective immunity against leptospiral serovars not included in the vaccine preparation. Several recombinant proteins have been tested as potential vaccines, but only a few are able to confer partial protection against challenge with virulent leptospirae (Silva *et al.*, 2007; Yan *et al.*, 2010; Felix *et al.*, 2011).

In the past 10 years, we have witnessed an increase in our knowledge of *Leptospira* and leptospirosis. Modern molecular approaches, such as genomics, proteomics, and mutagenesis, have produced a great amount of data. Several identified proteins have been described to interact with extracellular matrix (ECM) molecules. This review

aims to assemble the current knowledge of leptospiral adhesins focusing on the host interactions for this pathogen and pointing out new trends for the characterization of these attachment proteins.

Extracellular matrix

The ECM is an intricate network of macromolecules, whose primary role includes supporting and connecting cells and tissues. Two main classes of macromolecules constitute the ECM: (1) polysaccharide chains of glycosaminoglycans (GAGs) (Varki *et al.*, 2009) and (2) fibrous proteins, such as collagen, elastin, fibronectin, and laminin (Alberts *et al.*, 2007).

The GAGs are long unbranched polysaccharides constructed of repeating disaccharide units. They are highly negatively charged molecules that permit high hydration, viscosity, and interaction with other molecules (Varki *et al.*, 2009). The most important GAGs for the ECM composition are hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin.

Collagens are the most abundant proteins found in the animal kingdom and the major protein constituents in the ECM, providing tensile strength, regulating cell adhesion, supporting chemotaxis and migration. Laminin is a multi-adhesive protein that binds to numerous proteoglycans, type IV collagen, and certain receptors on the cells surface, such as integrins, an important class of cell adhesion molecules. Fibronectins are complex glycoproteins present in plasma and in ECM that display important multi-adhesive properties. Fibronectin contains different domains of high-affinity binding sites for collagen, heparin, gelatin, and integrins (via RGD motif), thereby attaching cells to the ECM (Chagnot *et al.*, 2012). Plasma fibronectin circulating in the blood can bind to fibrin, activated platelets and enhance blood clotting (Lodish *et al.*, 2000). The elastin results in the assembly of the monomeric precursor tropoelastin (Muiznieks *et al.*, 2010). Elastin proportionally provides a degree of elasticity to the ECM, and further permits molecular interaction with ECM components such as proteoglycans, fibulin, and fibrillin (Hayes *et al.*, 2011).

Leptospiral adhesion to ECM

The adhesion of *Leptospira* to cultured mammalian cells was reported, and the efficiency of attachment was correlated with bacterial virulence (Thomas & Higbie, 1990). The direct attachment of *Leptospira interrogans* to the ECM proteins laminin, collagen type I, collagen type IV, cellular and plasma fibronectin was reported indicating the existence of multiple adhesion molecules (Barbosa

et al., 2006). Leptospiral binding to elastin and its precursor, tropoelastin, was described further stressing the presence of a broad spectrum of binding proteins (Lin *et al.*, 2009). Moreover, the interaction of *L. interrogans* to the GAGs chondroitin sulfate and heparan sulfate has been reported (Breiner *et al.*, 2009).

The first described leptospiral adhesin

The first leptospiral adhesin was a 36-kDa fibronectin-binding protein isolated from the outer sheath of a virulent variant of pathogenic leptospires (Merien *et al.*, 2000). This adhesin was described to be expressed in virulent *L. interrogans*, totally lost during virulence attenuation and absent in the saprophytic strain *Leptospira biflexa* serovar Patoc I. Evidence of surface-exposed localization of this 36-kDa protein was provided by proteinase K treatment that abolished its fibronectin-binding activity. Table 1 and Supporting Information, Table S1 depict features of the published ECM-binding proteins, and Fig. 1 illustrates a model of membrane architecture featuring the leptospiral adhesins described by our group. The drawing illustrates an outer membrane (OM), a cytoplasmic or inner membrane (IM), and the peptidoglycan (PG) cell wall closely associated with the IM. Protein location was based on previous individually published results (Table S1).

LfhA/Lsa24, and related Len proteins

Studies performed in our laboratory described the first leptospiral protein that exhibited attachment to purified laminin (Barbosa *et al.*, 2006). The protein encoded by the gene LIC12906 was named Lsa24 for leptospiral surface adhesin of 24 kDa. Lsa24 binds strongly to laminin in a specific, dose-dependent, and saturable fashion. Complementary experiments showed that Lsa24 could partially block live leptospires from binding to immobilized laminin, evidencing the participation of this protein in attachment, but also implying the expression of other proteins involved in the interaction. This protein was previously described as a binding protein of both, factor H and factor H-related protein 1 of *L. interrogans*, named LfhA (leptospiral factor H-binding protein A; Verma *et al.*, 2006). Subsequently, Stevenson *et al.* (2007) reported that this gene possesses five paralogs, distributed and limited to virulent leptospires (Stevenson *et al.*, 2007). Because of their similarities to mammalian endostatins, these proteins were called Len proteins, for leptospiral endostatin-like. LenA protein (formerly called LfhA and Lsa24) and the homologs were named LenB, LenC, LenD, LenE, and LenF. LenB was found to bind human factor H, and all Len proteins showed laminin-binding

Table 1. Features of identified ECM-binding proteins of *Leptospira*

LIC*	NCBI accession number [†]	Given name	ECM ligands	Method	K _d (nM)	Inhibition of <i>Leptospira</i> interaction	Genetic confirmation	References	Other known host ligands [‡]	References [§]
ND	ND	36 kDa protein	Fibronectin	Western blot	ND	ND	ND	Merien <i>et al.</i> (2000)	ND	–
LIC12906	YP_002822	Lsa24/LenA	Laminin	ELISA Western blot	ND	Protein: partial	ND	Barbosa <i>et al.</i> (2006), Stevenson <i>et al.</i> (2007)	Plasminogen Factor H FHL-1	Verma <i>et al.</i> (2010, 2006)
LIC10997	YP_000971	LenB	Fibronectin Laminin	ELISA Western blot	118 ± 39 106 ± 8	ND	ND	Stevenson <i>et al.</i> (2007)	ND	–
LIC13006	YP_002919	LenC	Fibronectin Laminin	Western blot	ND ND	ND	ND	Stevenson <i>et al.</i> (2007)	ND	–
LIC12315	YP_002249	LenD	Fibronectin Laminin	Western blot	ND ND	ND	ND	Stevenson <i>et al.</i> (2007)	ND	–
LIC13467	YP_003369	LenE	Fibronectin Laminin	Western blot	ND ND	ND	ND	Stevenson <i>et al.</i> (2007)	ND	–
LIC13248	YP_003155	LenF	Fibronectin Laminin	Western blot	ND ND	ND	ND	Stevenson <i>et al.</i> (2007)	ND	–
LIC11352	YP_001316	LipL32	Laminin Collagen I Collagen V Collagen IV Collagen XX Fibronectin	ELISA Phage display [¶]	ND ND ND 599 ± 12 ND 101 ± 30	Protein: no effect Antisera: no effect	<i>L. interrogans</i> <i>lipL32</i> mutants: no effect	Hoke <i>et al.</i> (2008), Hauk <i>et al.</i> (2008), Murray <i>et al.</i> (2009a, b), Chaemchuen <i>et al.</i> (2011)	Plasminogen	Vieira <i>et al.</i> (2010)
LIC10465	YP_000449	LigA	Collagen I Collagen IV Laminin Fibronectin Tropoelastin	ELISA SFSF** ITC**	** ** ** ** ** **	Protein: partial	<i>L. biflexa</i> transformation: positive effect ^{§§}	Choy <i>et al.</i> (2007), Lin <i>et al.</i> (2009)	Fibrinogen Factor H FHL-1 FHR-1 C4bp	Choy <i>et al.</i> (2007), Castiblanco-Valencia <i>et al.</i> (2012)
LIC10464	YP_000448	LigB	Collagen I Collagen IV Laminin Fibronectin Collagen III Elastin Tropoelastin Heparin	ELISA SFSF** ITC** Phage display ^{¶¶}	** ** ** ** ** ** ** ND	Protein: partial	<i>L. biflexa</i> transformation: positive effect ^{§§}	Choy <i>et al.</i> (2007, 2011), Lin <i>et al.</i> (2009), Ching <i>et al.</i> (2012)	Fibrinogen Factor H FHL-1 FHR-1 C4bp	Choy <i>et al.</i> (2007), Castiblanco-Valencia <i>et al.</i> (2012)
LIC10258	YP_000249	Lsa66	Laminin Fibronectin	ELISA Protein microarray	55 ± 15 290 ± 11	Protein: partial	ND	Oliveira <i>et al.</i> (2011), Pinne <i>et al.</i> (2012)	Plasminogen	Oliveira <i>et al.</i> (2011)

Table 1. Continued

LIC*	NCBI accession number [†]	Given name	ECM ligands	Method	K _d (nM)	Inhibition of <i>Leptospira</i> interaction	Genetic confirmation	References	Other known host ligands [‡]	References [§]
LIC12895	YP_002811	Lsa27	Laminin	ELISA	ND	ND	ND	Longhi et al. (2009)	ND	–
LIC11469	YP_001430	Lsa20	Laminin	ELISA SPR	1988 ± 563	Protein: partial	ND	Mendes et al. (2011)	Plasminogen	Mendes et al. (2011)
LIC12253	YP_002188	Lsa25	Laminin	ELISA	415 ± 203	Protein: partial	ND	Domingos et al. (2012)	C4bp	Domingos et al. (2012)
LIC11834	YP_001783	Lsa33	Laminin	ELISA	367 ± 248	Protein: partial	ND	Domingos et al. (2012)	Plasminogen C4bp	Domingos et al. (2012)
LIC12976	YP_002889	rLIC12976	Laminin	Phage display ELISA	ND	ND	ND	Lima et al. (2013)	ND	–
LIC12690	YP_002611	Lp95	Laminin Fibronectin	Western blot	ND ND	ND	ND	Atzingen et al. (2009)	ND	–
LIC12099	YP_002033	LipL53	Laminin Collagen IV Fibronectin	ELISA	ND ND ND	ND	ND	Oliveira et al. (2010)	ND	–
LIC10368	YP_000355	Lsa21	Laminin Collagen IV Fibronectin	ELISA	ND ND ND	ND	ND	Atzingen et al. (2008)	ND	–
LIC10314	YP_000304	Lsa63	Laminin Collagen IV	ELISA	ND ND	Protein: partial	ND	Vieira et al. (2010)	ND	–
LIC11087	YP_001057	Lsa30	Laminin Fibronectin	ELISA	292 ± 24 157 ± 35	ND	ND	Souza et al. (2012)	Plasminogen C4bp	Souza et al. (2012)
LIC10973	YP_000947	Ompl1	Laminin Fibronectin	ELISA	2099 ± 871 1239 ± 506	Protein: partial	ND	Fernandes et al. (2012)	Plasminogen	Fernandes et al. (2012)
LIC12263	YP_002198	Ompl37	Laminin Fibronectin Elastin	ELISA	410 ± 81 359 ± 68 104 ± 19	Protein: no effect Antisera: enhances interaction	ND	Pinne et al. (2010)	Fibrinogen	Pinne et al. (2010)
LIC13050	YP_002962	Ompl47	Laminin Collagen III Fibronectin Elastin	ELISA	ND ND ND ND	ND	ND	Pinne et al. (2010)	Fibrinogen	Pinne et al. (2010)
LIC11612	YP_001570	MFn1	Fibronectin	Protein microarray Western blot	ND	ND	ND	Pinne et al. (2012)	ND	–
							<i>L. biflexa</i> transformation: positive effect			

Table 1. Continued

LIC*	NCBI accession number [†]	Given name	ECM ligands	Method	K _d (nM)	Inhibition of <i>Leptospira</i> interaction	Genetic confirmation	References	Other known host ligands [‡]	References [§]
LIC10714	YP_000698	MFn2	Fibronectin	Protein microarray Western blot	ND	ND	ND	Pinne <i>et al.</i> (2012)	ND	–
LIC11051	YP_001021	MFn6	Fibronectin	Protein microarray Western blot	ND	ND	ND	Pinne <i>et al.</i> (2012)	ND	–
LIC11436	YP_001398	MFn7	Fibronectin	Protein microarray Western blot	ND	ND	<i>L. biflexa</i> transformation: positive effect	Pinne <i>et al.</i> (2012)	ND	–
LIC10537	YP_000521	MFn9	Fibronectin	Protein microarray Western blot	ND	ND	ND	Pinne <i>et al.</i> (2012)	ND	–
LIC13143	YP_003051	TlyC	Laminin Collagen IV Fibronectin	ELISA	184 ± 13 105 ± 1 89 ± 7	Protein: partial	ND	Cavalho <i>et al.</i> (2009)	ND	–

ECM, extracellular matrix; K_d, Dissociation constant; ND, not determined; SSFS, steady-state fluorescence spectroscopy; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance.[†]*Leptospira interrogans* serovar Copenhageni L1-130 genome annotation (<http://aeg.lbi.ic.unicamp.br/world/lic/>; Nascimento *et al.*, 2004).[‡]<http://www.ncbi.nlm.nih.gov/protein/>.[§]Others ligands.[§]References relative to information depicted in 3.[†]For laminin and collagen type XX.^{**}For elastin and tropoelastin.^{**}For heparin.^{‡‡}Determined only for protein fragments.^{‡‡‡}For fibronectin.

Fig. 1. Schematic representation of the *Leptospira* cell membrane. The leptospiral membrane shares characteristics of both Gram-negative and Gram-positive bacteria. Depicted are OM, IM, and lipopolysaccharide (LPS). In the periplasmic space (PS), the PG is found strongly associated with the (IM). In this diagram, the protoplasmic cylinder (PC) is also shown, containing the nucleoid. The endoflagella (F), responsible for bacterial motility, are located in the (PS). Illustrated are well-characterized published adhesins.

capacity. In addition, LenB, LenC, LenD, LenE, and LenF exhibited affinity for fibronectin. However, *lenB* and *lenE* leptospiral mutant strains did not present an attenuated phenotype, suggesting that the corresponding proteins are not crucial for virulence (Murray *et al.*, 2009a).

LipL32

LipL32 was first described as an OM lipoprotein only found in pathogenic leptospires (Haake *et al.*, 2000). The ECM-binding capacity of LipL32 has been shown by independent studies. One showed that recombinant LipL32 selectively binds to the individual components laminin and collagens I and V, where the carboxy-terminal is designated as the binding region (Hoke *et al.*, 2008), while the other reported that LipL32 interacts with collagen IV and plasma fibronectin and confirmed that the carboxy-terminal portion was responsible for the binding (Hauk *et al.*, 2008). Calcium binding to LipL32 was suggested to be important for the interaction with fibronectin (Tung *et al.*, 2009). However, these results were not confirmed by other authors that showed that calcium binding to LipL32, although important to stabilize the protein structure, is not required to mediate interaction with host ECM proteins (Hauk *et al.*, 2012).

However, despite LipL32 abundance in bacteria cell (Malmstrom *et al.*, 2009) and its presence limited to pathogenic species, mutagenesis experiments with mutants lacking the *lipL32* gene surprisingly showed that this protein is not essential for the bacterial survival during acute or chronic infection of *L. interrogans* (Murray *et al.*, 2009b). In addition, LipL32 cellular position was recently reexamined, and evidence supporting subsurface location was presented in place of surface exposure (Pinne & Haake, 2013). Thus, it appears that more studies are needed to establish the role of LipL32 in mediating *Leptospira*–host interaction.

Leptospiral immunoglobulin-like proteins

Members of the Lig family of genes in *L. interrogans* that encode OM proteins with immunoglobulin-like repeats were identified (Matsunaga *et al.*, 2003). LigA was first cloned and characterized by Palaniappan *et al.* (2002) that showed its expression during infection *in vivo*. The *L. interrogans* Lig proteins are present only in pathogenic species, expressed during infection, absent after attenuation by culture passage (Matsunaga *et al.*, 2003), and upregulated under physiological osmolarity stimuli (Matsunaga *et al.*, 2005). Based on this evidence, the Lig proteins were thought to be important for leptospirosis pathogenesis. The potential of the Lig proteins as vaccine candidates and diagnostic markers has also been proposed (Yan *et al.*, 2009).

Choy *et al.* (2007) first described LigA and LigB as leptospiral ECM-binding proteins capable of interacting with fibronectin, laminin, and collagen IV and I. The adhesive regions were attributed to the unique regions of each protein, instead of the shared bacterial immunoglobulin-like (Big) repeats. Further studies have also shown LigB to interact with collagen type III (Choy *et al.*, 2011). The high-affinity fibronectin-binding region was identified and found to comprise amino acid residues from 1014 to 1165. This portion includes part of the Big domain and a nonrepeated region in the variable C-terminal portion, which also binds laminin, but not collagen (Lin & Chang, 2008).

Although leptospiral strains with mutation of *ligB* does not attenuated the virulence in the hamster model of infection (Croda *et al.*, 2008), heterologous expression of *ligA* and *ligB* genes using the saprophyte *L. biflexa* as a surrogate host improved the adhesion to fibronectin and to cultured cells. Although LigB has been shown to interact with collagen and elastin (Choy *et al.*, 2007; Lin *et al.*, 2009), *L. biflexa lig* mutants were not capable of binding collagens (type I and IV) or elastin more efficient than wild-type cells (Figueira *et al.*, 2011). Thus, it appears

that more investigations are needed to establish the function of Lig proteins in virulence.

Outer membrane protein A-like proteins

Outer membrane protein A (OmpA) is a major heat-modifiable surface-exposed protein in *Escherichia coli* and is one of the best-characterized OMPs (Confer & Ayalew, 2013). OmpA protein of *E. coli* and other enterobacteria has been reported to act as adhesin/invasin (Smith *et al.*, 2007).

The first protein identified in pathogenic *Leptospira* containing a carboxy-terminal OmpA domain was a lipoprotein of 22 kDa, named Loa22 (Koizumi & Watanabe, 2003). The OmpA-like protein Loa22 was reported to be essential for the leptospiral virulence, as mutants lacking Loa22 expression resulted in attenuated virulence in an animal model of acute infection (Ristow *et al.*, 2007). However, work performed in our laboratory showed that Loa22 had a modest interaction with the ECM molecules tested: laminin, collagen I, collagen IV, cellular and plasma fibronectin (Barbosa *et al.*, 2006). Hence, although a potential leptospiral virulence factor, Loa22 is not an ECM-interacting protein.

Oliveira *et al.* (2011) identified a novel leptospiral hypothetical protein, encoded by the gene LIC10258, which has a putative OmpA-like domain comprising 504–583 amino acid residues in the carboxy-terminal region. The recombinant protein, named Lsa66, showed laminin and plasma fibronectin-binding properties. Moreover, antibodies in serum samples of leptospirosis cases recognize Lsa66, suggesting its expression during infection. The presence of this protein in *Leptospira*, as a fibronectin ligand, was further corroborated by other studies using high-throughput microarray strategies (Pinne *et al.*, 2012).

Laminin-binding adhesins

Bioinformatics have predicted 184 coding sequences that appeared to be exported to the leptospiral surface, and thus, with the potential to participate in host–pathogen interactions (Nascimento *et al.*, 2004). Most of these proteins are annotated as hypothetical with no assigned function. By picking coding sequences from this pool of sequences, our group has cloned and expressed various proteins that were identified as novel leptospiral adhesins. Some of these proteins exhibited only laminin-binding property when screened against several individual ECM components. These are as follows: Lsa27 (Longhi *et al.*, 2009), Lsa20 (Mendes *et al.*, 2011), Lsa25 and Lsa33 (Domingos *et al.*, 2012), and Lsa26 (Siqueira *et al.*, 2013). All these proteins are most probably surface-exposed, as

assessed by indirect liquid-phase immunofluorescence or proteinase K accessibility. In addition to mediating ECM interactions through laminin, Lsa33, Lsa20, and Lsa26 are also plasminogen-interacting proteins and, hence, may participate to leptospiral invasiveness and dissemination (Mendes *et al.*, 2011; Domingos *et al.*, 2012). Moreover, Lsa25 and Lsa33 are capable to capture C4BP (Domingos *et al.*, 2012), a regulator of the classical pathway complement system, and may have a role in leptospiral immune evasion.

Adhesins with multiple ECM-binding profile

Several leptospiral adhesins with broader spectrum binding to ECM have been described. LipL53 and Lsa21 were reported to interact with laminin, collagen type IV, cellular and plasma fibronectin and are thought to have roles in pathogenesis and virulence of leptospires (Atzingen *et al.*, 2008; Oliveira *et al.*, 2010). Lsa21 and LipL53 share some similarities with the Lig proteins and hence are virulence factor candidates: their protein expression seems to be restricted to highly virulent low-passage strains (Matsunaga *et al.*, 2003; Atzingen *et al.*, 2008; Oliveira *et al.*, 2010). The gene encoding Lsa21 is restricted to pathogenic strains of *L. interrogans*, and the protein is upregulated at the transcriptional level by physiological osmolarity and temperature (Atzingen *et al.*, 2008).

Proteomics studies of *L. interrogans* have identified a protein encoded by the gene LIC10314 that is differentially expressed under virulence conditions (Vieira *et al.*, 2009). The recombinant protein, named Lsa63, was characterized as an adhesin that binds strongly to laminin and collagen type IV (Vieira *et al.*, 2010). The protein Lsa63 is highly conserved among pathogenic strains and absent in *L. biflexa*. This adhesin is probably expressed during infection because the protein is recognized by human leptospirosis serum samples, mostly at the convalescent phase of the disease.

The proteins encoded by the genes LIC11087, LIC11360, and LIC11975 were genome annotated as hypothetical and predicted to be surface-exposed. We have evaluated the recombinant proteins, named Lsa30, Lsa23, and Lsa36, respectively, and shown that they are also ECM-interacting proteins with the ability to mediate attachment to laminin and plasma fibronectin (Souza *et al.*, 2012; Siqueira *et al.*, 2013). Furthermore, the proteins Lsa23 and Lsa30 may interfere with the complement cascade by interacting with C4BP regulator, and in the case of Lsa23, also with factor H. The proteins Lsa23, Lsa36, and Lsa30 are probably expressed during infection because antibodies present in serum samples of hamsters experimentally infected (Lsa30) or human leptospirosis

sera (Lsa23 and Lsa36) can recognize these proteins. The OmpL1 has been described as a serological marker for the diagnosis of human leptospirosis and as a vaccine against the disease (Haake *et al.*, 1999; Natarajaseenivasan *et al.*, 2008). Although the gene is restricted to pathogenic serovars of *Leptospira*, its function is uncertain. Recently, it has been shown that OmpL1 has the ability to mediate attachment to laminin and plasma fibronectin. Like previously described adhesins, OmpL1 inhibited the binding of live *Leptospira* to the same ECM components (Fernandes *et al.*, 2012). These are versatile leptospiral adhesins that may play a role in mediating adhesion to hosts.

Also included in this category of multiple ECM binding are the adhesins OmpL37 (LIC12263) and OmpL47 (LIC13050; Pinne *et al.*, 2010), and the above discussed, LipL32, LigA/LigB and LenB, LenC, LenD, LenE, and LenF proteins (Table S1).

Lp95, an adhesin that activates cellular adhesion molecules

Cellular adhesion molecules (CAMs) are surface receptors present in eukaryotic cells that mediate cell–cell or cell–ECM interactions, and several microorganisms are reported to employ CAMs during their pathogenesis (Boyle & Finlay, 2003). The leptospiral proteins encoded by the genes LIC10365, LIC10507, LIC10508, and LIC10509 have been shown to be present in cellular infiltrate of liver and kidney of hamsters experimentally infected with virulent *L. interrogans*, suggesting that these proteins are expressed during infection. Most importantly, the recombinant proteins were capable of promoting the upregulation of ICAM-1 and E-selectin in human umbilical vein endothelial cells (HUVECs; Vieira *et al.*, 2007; Gomez *et al.*, 2008). Subsequently, we demonstrated for the first time that virulent *L. interrogans*, but also the saprophyte *L. biflexa*, induced an upregulation of CAMs in HUVECs, although the virulent one was more effective on activation (Atzingen *et al.*, 2009). Furthermore, the same work showed a novel leptospiral protein encoded by LIC12690, named Lp95 (leptospiral protein of 95 kDa), that is capable of specifically activating E-selectin in HUVECs. In addition to CAM induction, Lp95 binds to laminin and fibronectin, being the carboxy-terminal portion responsible for the interaction. Thus, Lp95 with its dual activities could act as a protein that contributes to leptospiral attachment to ECM and cell receptors during *Leptospira* infection (Atzingen *et al.*, 2009).

High-throughput screening microarrays

In addition to *in silico* identification of putative surface proteins and their production, such as recombinant

proteins for the characterization of leptospiral ECM-binding proteins, recent work has employed high-throughput microarray strategy to identify novel host ligand-binding proteins (Pinne *et al.*, 2012). These studies have employed OMP microarray containing 401 leptospiral proteins expressed in a cell-free expression system. These cited authors identified 15 candidates as fibronectin-binding proteins, but only six of them were confirmed (Table S1). Among them, only LIC10258, called Lsa66, had been previously characterized as a fibronectin adhesin (Oliveira *et al.*, 2011). However, discrepancies between the array data and the published with purified recombinant proteins are evident. Several proteins with reported fibronectin adhesion properties were identified below the threshold, including the vastly studied LigA, LigB, and LipL32 and therefore admitted as nonbinding proteins (Pinne *et al.*, 2012). LigA and LigB showed an intermediate binding value, while LipL32 a low one. Interestingly, Lsa33, which was shown to bind uniquely to laminin (Domingos *et al.*, 2012), showed a binding value for fibronectin (*c.* 9.3) near the admitted threshold (10) (Pinne *et al.*, 2012). LIC13050 (OmpL47), detected by the same group as fibronectin-interacting protein, had a binding value below 0. The other proteins included in the same group are as follows: LipL53 (Oliveira *et al.*, 2010), Lsa21 (Atzingen *et al.*, 2008), Lp95 (Atzingen *et al.*, 2009), OmpL1 (Fernandes *et al.*, 2012), and all Len proteins (Stevenson *et al.*, 2007). Thus, although the array can rapidly identify ECM-binding proteins, the data will have to be further confirmed with the isolated proteins.

Concluding remarks

In recent years, major advances were made in the leptospiral field. Studies aiming to elucidate the pathogenesis of *Leptospira* based on available genome sequences, proteomics, and microarrays have been published, and the remarkable number of novel leptospiral ECM-interacting proteins recognized demonstrates this progress. This work assembles the current knowledge of leptospiral adhesins and describes their characterizations. These ECM-binding proteins are capable of mediating the attachment of *Leptospira* to mammalian hosts and of starting the process of invasion/colonization. Several laminin- and plasma fibronectin-binding proteins were identified indicating that leptospires have a redundant repertoire of adhesion molecules that are probably part of their invasion strategies. Proteins with exclusive or larger ligand capacity to ECM were described. In addition, there are multifunctional proteins that exhibit binding activities toward several target proteins, such as plasminogen/plasmin and regulators of the complement system, and may have several tasks in leptospiral pathogenesis. The presence of

multiple adherence proteins in pathogens such as *Yersinia* and *Neisseria* has been discussed in terms of compensation mechanisms or the need for cooperative action in adhesin–ligand interactions (Kline *et al.*, 2009; Mikula *et al.*, 2013). This redundancy may explain why mutants lacking virulence factors do not change their phenotypes and point out the heterologous expression of virulent proteins in the saprophyte, *L. biflexa* serovar Patoc, as an attractive model. *In vitro* studies using leptospiral recombinant proteins represent only a model to evaluate possible tasks of these novel proteins that no function could be assigned during genome annotation. However, it is possible that some of these proteins might be expressed only in small amounts in *Leptospira* and therefore could not perform these interactions. In any event, these ECM-binding proteins will have to be further investigated to gain information on their precise role in leptospiral pathogenesis. These studies should contribute to identify a multicomponent acellular vaccine in addition to potential targets for antimicrobial therapy that may help fight leptospirosis.

Acknowledgements

This work was supported by FAPESP, CNPq, and Fundacao Butantan, Brazil; MLV, MVA, RO, RFD, GHS, NMS, ARFT, and LGF have scholarships from FAPESP and CAPES (Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Dr. A. Leyva for helpful discussion and with English editing of the manuscript. The authors report that they have no conflict of interest.

References

- Adler B & de la Pena Moctezuma A (2010) Leptospira and leptospirosis. *Vet Microbiol* **140**: 287–296.
- Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P (2007) *The Extracellular Matrix of Animals*. Garland Science, New York, NY.
- Atzingen MV, Barbosa AS, De Brito T *et al.* (2008) Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol* **8**: 70.
- Atzingen MV, Gomez RM, Schattner M *et al.* (2009) Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *J Infect* **59**: 264–276.
- Barbosa AS, Abreu PA, Neves FO *et al.* (2006) A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun* **74**: 6356–6364.
- Boyle EC & Finlay BB (2003) Bacterial pathogenesis: exploiting cellular adherence. *Curr Opin Cell Biol* **15**: 633–639.
- Breiner DD, Fahey M, Salvador R, Novakova J & Coburn J (2009) *Leptospira interrogans* binds to human cell surface receptors including proteoglycans. *Infect Immun* **77**: 5528–5536.
- Carvalho E, Barbosa AS, Gomez RM *et al.* (2009) Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS Lett* **583**: 1381–1385.
- Castiblanco-Valencia MM, Fraga TR, Silva LB *et al.* (2012) Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *J Infect Dis* **205**: 995–1004.
- Chaemchuen S, Rungpragayphan S, Poovorawan Y & Patarakul K (2011) Identification of candidate host proteins that interact with LipL32, the major outer membrane protein of pathogenic *Leptospira*, by random phage display peptide library. *Vet Microbiol* **153**: 178–185.
- Chagnot C, Listrat A, Astruc T & Desvaux M (2012) Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components. *Cell Microbiol* **14**: 1687–1696.
- Ching AT, Favaro RD, Lima SS *et al.* (2012) *Leptospira interrogans* shotgun phage display identified LigB as a heparin-binding protein. *Biochem Biophys Res Commun* **427**: 774–779.
- Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J & Haake DA (2007) Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun* **75**: 2441–2450.
- Choy HA, Kelley MM, Croda J *et al.* (2011) The multifunctional LigB adhesin binds homeostatic proteins with potential roles in cutaneous infection by pathogenic *Leptospira interrogans*. *PLoS ONE* **6**: e16879.
- Confer AW & Ayalew S (2013) The OmpA family of proteins: roles in bacterial pathogenesis and immunity. *Vet Microbiol* **163**: 207–222.
- Croda J, Figueira CP, Wunder EA Jr, Santos CS, Reis MG, Ko AI & Picardeau M (2008) Targeted mutagenesis in pathogenic *Leptospira* species: disruption of the LigB gene does not affect virulence in animal models of leptospirosis. *Infect Immun* **76**: 5826–5833.
- Domingos RF, Vieira ML, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA & Nascimento AL (2012) Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC Microbiol* **12**: 50.
- Felix SR, Hartwig DD, Argondizzo AP *et al.* (2011) Subunit approach to evaluation of the immune protective potential of leptospiral antigens. *Clin Vaccine Immunol* **18**: 2026–2030.
- Fernandes LG, Vieira ML, Kirchgatter K *et al.* (2012) OmpL1 is an extracellular matrix- and plasminogen-interacting protein of *Leptospira* spp. *Infect Immun* **80**: 3679–3692.
- Figueira CP, Croda J, Choy HA, Haake DA, Reis MG, Ko AI & Picardeau M (2011) Heterologous expression of pathogen-specific genes ligA and ligB in the saprophyte

- Leptospira biflexa* confers enhanced adhesion to cultured cells and fibronectin. *BMC Microbiol* **11**: 129.
- Gomez RM, Vieira ML, Schattner M *et al.* (2008) Putative outer membrane proteins of *Leptospira interrogans* stimulate human umbilical vein endothelial cells (HUVECS) and express during infection. *Microb Pathog* **45**: 315–322.
- Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, Matsunaga J & Wagar EA (1999) Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect Immun* **67**: 6572–6582.
- Haake DA, Chao G, Zuerner RL *et al.* (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect Immun* **68**: 2276–2285.
- Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS & Ho PL (2008) In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun* **76**: 2642–2650.
- Hauk P, Barbosa AS, Ho PL & Farah CS (2012) Calcium binding to *Leptospira* outer membrane antigen LipL32 is not necessary for its interaction with plasma fibronectin, collagen type IV, and plasminogen. *J Biol Chem* **287**: 4826–4834.
- Hayes AJ, Lord MS, Smith SM, Smith MM, Whitelock JM, Weiss AS & Melrose J (2011) Colocalization *in vivo* and association *in vitro* of perlecan and elastin. *Histochem Cell Biol* **136**: 437–454.
- Hoke DE, Egan S, Cullen PA & Adler B (2008) LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. *Infect Immun* **76**: 2063–2069.
- Kline KA, Falker S, Dahlberg S, Normark S & Henriques-Normark B (2009) Bacterial adhesins in host-microbe interactions. *Cell Host Microbe* **5**: 580–592.
- Koizumi N & Watanabe H (2003) Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. *FEMS Microbiol Lett* **226**: 215–219.
- Lima SS, Ching AT, Favaro RD *et al.* (2013) Adhesin activity of *Leptospira interrogans* lipoprotein identified by *in vivo* and *in vitro* shotgun phage display. *Biochem Biophys Res Commun* **431**: 342–347.
- Lin YP & Chang YF (2008) The C-terminal variable domain of LigB from *Leptospira* mediates binding to fibronectin. *J Vet Sci* **9**: 133–144.
- Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y & Chang YF (2009) Repeated domains of *Leptospira* immunoglobulin-like proteins interact with elastin and tropoelastin. *J Biol Chem* **284**: 19380–19391.
- Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D & Darnell J (2000) *Molecular Cell Biology*. W. H. Freeman, New York, NY.
- Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA & Nascimento AL (2009) A newly identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol* **58**: 1275–1282.
- Malmstrom J, Beck M, Schmidt A, Lange V, Deutsch EW & Aebersold R (2009) Proteome-wide cellular protein concentrations of the human pathogen *Leptospira interrogans*. *Nature* **460**: 762–765.
- Matsunaga J, Barocchi MA, Croda J *et al.* (2003) Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Mol Microbiol* **49**: 929–945.
- Matsunaga J, Sanchez Y, Xu X & Haake DA (2005) Osmolarity, a key environmental signal controlling expression of leptospiral proteins LigA and LigB and the extracellular release of LigA. *Infect Immun* **73**: 70–78.
- McBride AJ, Athanazio DA, Reis MG & Ko AI (2005) Leptospirosis. *Curr Opin Infect Dis* **18**: 376–386.
- Mendes RS, Von Atzingen M, de Moraes ZM *et al.* (2011) The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infect Immun* **79**: 4657–4667.
- Merien F, Truccolo J, Baranton G & Perolat P (2000) Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar Icterohaemorrhagiae. *FEMS Microbiol Lett* **185**: 17–22.
- Mikula KM, Kolodziejczyk R & Goldman A (2013) Yersinia infection tools-characterization of structure and function of adhesins. *Front Cell Infect Microbiol* **2**: 169.
- Muiznieks LD, Weiss AS & Keeley FW (2010) Structural disorder and dynamics of elastin. *Biochem Cell Biol* **88**: 239–250.
- Murray GL, Morel V, Cerqueira GM *et al.* (2009a) Genome-wide transposon mutagenesis in pathogenic *Leptospira* species. *Infect Immun* **77**: 810–816.
- Murray GL, Srikrum A, Hoke DE *et al.* (2009b) Major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. *Infect Immun* **77**: 952–958.
- Nascimento AL, Verjovski-Almeida S, Van Sluys MA *et al.* (2004) Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz J Med Biol Res* **37**: 459–477.
- Natarajaseenivasan K, Vijayachari P, Sharma S, Sugunan AP, Selvin J & Sehgal SC (2008) Serodiagnosis of severe leptospirosis: evaluation of ELISA based on the recombinant OmpL1 or LipL41 antigens of *Leptospira interrogans* serovar Autumnalis. *Ann Trop Med Parasitol* **102**: 699–708.
- Oliveira TR, Longhi MT, Goncalves AP, de Moraes ZM, Vasconcellos SA & Nascimento AL (2010) LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. *Microbes Infect* **12**: 207–217.
- Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA & Nascimento AL (2011) Characterization of novel OmpA-like protein of *Leptospira interrogans* that

- binds extracellular matrix molecules and plasminogen. *PLoS ONE* **6**: e21962.
- Palaniappan RU, Chang YF, Jusuf SS *et al.* (2002) Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*. *Infect Immun* **70**: 5924–5930.
- Pinne M & Haake DA (2013) LipL32 is a subsurface lipoprotein of *Leptospira interrogans*: presentation of new data and reevaluation of previous studies. *PLoS ONE* **8**: e51025.
- Pinne M, Choy HA & Haake DA (2010) The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. *PLoS Negl Trop Dis* **4**: e815.
- Pinne M, Matsunaga J & Haake DA (2012) Leptospiral outer membrane protein microarray, a novel approach to identification of host ligand-binding proteins. *J Bacteriol* **194**: 6074–6087.
- Ristow P, Bourhy P, da Cruz McBride FW *et al.* (2007) The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog* **3**: e97.
- Silva EF, Medeiros MA, McBride AJ *et al.* (2007) The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. *Vaccine* **25**: 6277–6286.
- Siqueira GH, Atzingen MV, Alves IJ, de Moraes ZM, Vasconcellos SA & Nascimento AL (2013) Characterization of three novel adhesins of *Leptospira interrogans*. *Am J Trop Med Hyg* **89**: 1103–1116.
- Smith SG, Mahon V, Lambert MA & Fagan RP (2007) A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol Lett* **273**: 1–11.
- Souza NM, Vieira ML, Alves IJ, de Moraes ZM, Vasconcellos SA & Nascimento AL (2012) Lsa30, a novel adhesin of *Leptospira interrogans* binds human plasminogen and the complement regulator C4bp. *Microb Pathog* **53**: 125–134.
- Stevenson B, Choy HA, Pinne M *et al.* (2007) *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE* **2**: e1188.
- Thomas DD & Higbie LM (1990) *In vitro* association of leptospires with host cells. *Infect Immun* **58**: 581–585.
- Tung JY, Yang CW, Chou SW, Lin CC & Sun YJ (2009) Calcium binds to LipL32, a lipoprotein from pathogenic *Leptospira*, and modulates fibronectin binding. *J Biol Chem* **285**: 3245–3252.
- Varki A, Cummings RD, Esko JD *et al.* (2009) *Essentials of Glycobiology*. Cold Spring Harbor, New York, NY.
- Verma A, Hellwage J, Artiushin S, Zipfel PF, Kraiczky P, Timoney JF & Stevenson B (2006) LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect Immun* **74**: 2659–2666.
- Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF & Stevenson B (2010) Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect Immun* **78**: 2053–2059.
- Vieira ML, D'Atri LP, Schattner M *et al.* (2007) A novel leptospiral protein increases ICAM-1 and E-selectin expression in human umbilical vein endothelial cells. *FEMS Microbiol Lett* **276**: 172–180.
- Vieira ML, Pimenta DC, de Moraes ZM, Vasconcellos SA & Nascimento AL (2009) Proteome analysis of *Leptospira interrogans* virulent strain. *Open Microbiol J* **3**: 69–74.
- Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA & Nascimento AL (2010) Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect* **60**: 52–64.
- Yan W, Faisal SM, McDonough SP *et al.* (2009) Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. *Microbes Infect* **11**: 230–237.
- Yan W, Faisal SM, McDonough SP, Chang CF, Pan MJ, Akey B & Chang YF (2010) Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. *Vaccine* **28**: 2277–2283.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Protein conservation, protein copy number per *Leptospira*, and differential expression in response to temperature, physiologic osmolarity and exposure to serum.

Mapeamento de genomas bacterianos: sequenciamento e funcional de leptospiras

Renan F. Domingos^{1,2} (doutorando), Gabriela H. Siqueira^{1,2} (doutorando), Luis G. Fernandes^{1,2} (doutorando), Aline R. F. Teixeira^{1,2} (doutorando), Lucas P. Silva¹ (mestrando), Maria R. Cosate¹(pós-doc), Monica L. Vieira¹ (pós-doc) and Ana L. T. O. Nascimento^{1,2,*}

¹Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São Paulo, SP, Brazil; ²Programa Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Avenida Prof. Lineu Prestes, 1730, 05508-900, São Paulo, SP, Brazil;

*Autor de correspondência, e-mail: ana.nascimento@butantan.gov.br

Telefone: (5511) 2627 9829;

Resumo

Nos últimos anos, vimos assistindo avanços no desenvolvimento de novas vacinas devido a progressos na tecnologia de DNA recombinante e sequenciamento de DNA. O mapeamento de genomas completos, juntamente com o desenvolvimento da bioinformática, permitindo a mineração dos dados, impulsionou a genômica funcional. A revolução genômica, auxiliada pelas metodologias de vacinologia reversa (VR), microarranjos (transcriptômica) e proteômica, teve grande impacto no campo da microbiologia, sobretudo a voltada à área da Saúde. O uso dessas metodologias facilitou a identificação de novos candidatos vacinais de patógenos bacterianos importantes e, nesta busca, fatores de virulência e proteínas associadas à patogenicidade foram encontrados, que auxiliaram no entendimento dos patógenos em estudo. A VR aplicada ao genoma da *Neisseria meningitidis* sorogrupo B, bactéria responsável pela meningite meningocócica e sepsis, representa um marco na aplicação destas metodologias de ponta, e resultou na obtenção de uma vacina, em fase de registro final. A aplicação da VR no genoma da *Leptospira interrogans*, agente etiológico da leptospirose, encontra-se em andamento, e as principais metodologias e estratégias empregadas na busca de antígenos vacinais e fatores de virulência estão apresentadas neste capítulo.

Introdução

Doenças infecciosas representam um grande problema para a saúde pública, sobretudo em países em desenvolvimento. A prevenção destas doenças por meio de medidas profiláticas representa a melhor estratégia de intervenção. Entre os avanços nas condições de saneamento básico, educação, higiene e o uso de antimicrobianos, o maior impacto na melhoria das condições da saúde humana deve-se à vacinação. Doenças infecciosas que não dispõem de vacinas, doenças emergentes causadas por cepas resistentes a antimicrobianos, ou surgimento de novos patógenos demandam o desenvolvimento de novas estratégias vacinais de custo-efetivo.

Com os avanços das técnicas de DNA recombinante e sequenciamento, o antigo Instituto de Pesquisas Genômicas, TIGR-Institute for Genomic Research, em 1995, sequenciou o primeiro genoma completo de um organismo de vida livre, *Haemophilus influenza* [1]. A este, seguiram-se outros sequenciamentos completos de genoma, como o do patógeno causador da úlcera péptica, *Helicobacter pylori* [2] e o da espiroqueta causadora da doença de Lyme, *Borrelia burgdorferi* [3]. A era genômica havia iniciado, e com ela, o desenvolvimento de ferramentas de bioinformática necessárias para auxiliar no entendimento do grande montante de dados gerados nestes projetos. A evolução na tecnologia promoveu uma grande melhora e rapidez dos sequenciadores de DNA, diminuindo drasticamente o seu custo. Este fato propiciou o início do sequenciamento em larga escala ou pan-genômica. Atualmente, temos um total de 10.991 organismos sequenciados, com genoma completo ou não, entre procariotos, eucariotos e vírus, cujas sequências encontram-se disponíveis em bancos de dados públicos (<http://www.ncbi.nlm.nih.gov/genome/browse/>).

A revolução genômica teve um grande impacto no campo da microbiologia. O genoma completo, com suas sequências codificadoras de proteínas anotadas, através de servidores públicos na *web*, representa o potencial “proteômico” do organismo, fundamental para o seu entendimento. A investigação da regulação gênica, expressão de proteína e sua função, deu início à era pós-genômica, ou era “*omics*”, onde a genômica funcional, que inclui a transcriptômica, a proteômica e a vacinologia reversa (VR), tiveram papel fundamental (Figura 1).

Figura 1. Esquema das abordagens experimentais utilizando sequências genômicas. **Proteoma** é o conjunto de proteínas expressas em uma célula ou tecido em determinadas situações. **Transcriptoma** é o conjunto completo de RNAs presente em uma célula ou tecido em determinadas situações. **Vacinologia Reversa** é uma tecnologia moderna aplicada à produção de vacinas, cujo objetivo principal é a identificação de alvos vacinais a partir das sequências genômicas de dado organismo.

Genomas, Patogênese e Vacinas

O sequenciamento completo do genoma da *N. meningitidis* sorogrupo B, principal agente etiológico da septicemia bacteriana e da meningite meningocócica [4], seguido da publicação que identificava candidatos vacinais a partir das sequências genômicas do meningococo sorogrupo B [5], representou um marco da genômica funcional aplicada ao desenvolvimento de vacinas. Outros patógenos importantes tais como *Bacillus anthracis* [6], *Streptococcus pneumoniae* [7], *Staphylococcus aureus* [8, 9], *Chlamydia pneumoniae* [10], *Porphyromonas gingivalis* [11], *Edwardsiella tarda* [12], *Mycobacterium tuberculosis* [13], *L. interrogans* [14] e *Escherichia coli* uropatogênica [15] seguiram esta abordagem de explorar sequências genômicas na busca de candidatos vacinais. Esta metodologia, denominada de “Vacinologia Reversa” – VR – por Rappuoli [16, 17] teve grande impacto no desenvolvimento de vacinas e na identificação de proteínas envolvidas na patogênese de microrganismos importantes. O primeiro patógeno onde a VR foi aplicada foi a *N. meningitidis* sorogrupo B – MenB –

[5], para a qual não existe uma vacina protetora de amplo espectro. Este fato deve-se a presença de um polissacarídeo capsular muito semelhante a um antígeno de humanos (*self-antigen*) e a grande variabilidade de seus antígenos proteicos de superfície. A VR aplicada a MenB identificou três componentes proteicos empregados na formulação de uma vacina. São eles: antígeno de *Neisseria* que se liga a heparina (NHBA, do inglês, *Neisserial heparin-binding antigen*), uma proteína que se liga ao fator H (fHbp, do inglês, *factor H-binding protein*) e a adesina A de *Neisseria* (NadA, do inglês, *Neisserial adhesin A*) [18]. Estes três componentes parecem ter papel importante na virulência da MenB [19-22]. Analisando as características/funções destes antígenos, fica claro que a VR associada à bioinformática, enquanto seleciona antígenos candidatos vacinais, auxilia no entendimento da patogênese do organismo.

Semelhante ao ocorrido com a *N. meningitidis*, onde diversos fatores de virulência foram identificados, outros organismos importantes tiveram proteínas envolvidas na patogenicidade encontradas durante a busca de alvos vacinais [18]. Com *Streptococcus* grupo A e B, formado por bactérias causadoras de sepsis e pneumonia, foram identificados antígenos com papel na adesão, colonização e formação de biofilme [23-25]. No caso da *E. coli*, responsável por infecção intestinal e extra intestinal (trato urinário, sepsis), antígenos de membrana externa, também com função na adesão e colonização, foram caracterizados [26, 27]. Patógenos como *S. aureus*, causador de pneumonia e endocardite [28], *Clostridium difficile*, responsável pela diarreia nosocomial [29], e *Chlamydia trachomatis*, causador da gravidez ectópica e infertilidade [30], tiveram processo similar, com diversos antígenos/fatores de virulência envolvidos na motilidade, esporulação e interação patógeno-hospedeiro identificados.

Vacinologia Reversa versus estratégia convencional no desenvolvimento de vacinas bacterianas

A VR aplicada às sequências genômicas da *N. meningitidis* selecionou, através de ferramentas de bioinformática, novas proteínas que seriam potencialmente localizadas na superfície da bactéria ou exportadas à membrana. Seguiu-se uma clonagem em larga escala onde os “genes candidatos” foram amplificados pela reação em cadeia da polimerase (do inglês, *Polymerase Chain Reaction*, PCR), clonados e as proteínas expressas. As proteínas obtidas foram empregadas para imunizar animais e os antissoros obtidos utilizados para diversos testes, incluindo teste bactericida [5]. Esta

abordagem mostrou importantes vantagens sobre a metodologia convencional clássica, que requer o cultivo do microrganismo, obtenção do sedimento bacteriano, lise, purificação de proteínas ou inativação do organismo, testes de imunogenicidade em modelo animal, e ensaios de desafio ou bactericida, antes da seleção do antígeno candidato vacinal e do início dos testes clínicos. Um esquema comparativo entre as duas abordagens está apresentado na Figura 2.

Na VR, a bioinformática permite a identificação de proteínas, incluindo aquelas que são expressas em baixa quantidade ou mesmo mascarada por outro antígeno imunodominante. Esta metodologia também permite selecionar antígenos de patógenos de difícil cultivo em laboratório. O que se busca é a identificação de genes que potencialmente codificam fatores de virulência, proteínas secretadas ou associadas à membrana. Programas públicos disponíveis em servidores da *web* utilizam algoritmos específicos para a identificação de novas proteínas de superfície, que devido à localização, são potencialmente capazes de mediar resposta imune no hospedeiro.

Figura 2. Representação esquemática comparando as metodologias convencional e vacinologia reversa (VR) para a obtenção de vacinas bacterianas. Na metodologia convencional o patógeno de interesse é semeado em meio de cultura apropriado, e as células recuperadas através de centrifugação. O sedimento celular é inativado, lisado, e o sobrenadante, onde se encontram as proteínas, submetido a purificação por diversos processos de cromatografia para obtenção de proteínas purificadas. O processo de cromatografia selecionado vai depender das características da proteína em questão. Na vacinologia reversa o organismo é obtido de modo semelhante ao da metodologia convencional, e após a lise bacteriana o DNA genômico é extraído e submetido a técnica de sequenciamento. Na sequência de DNA os genes são identificados e selecionados de acordo com sua localização na célula por programas de bioinformática; Genes que codificam proteínas de membrana e de superfície são selecionados para clonagem e expressão da proteína. Proteínas obtidas por ambas metodologias são analisadas quanto a imunogenicidade *in vitro* ou *in vivo*, em modelo animal. Vantagens da VR em relação a convencional: (i) identificação de proteínas que são expressas em quantidades e muito pequenas, e, portanto de difícil obtenção; (ii) não há necessidade de cultivar o organismo para investigação de novas proteínas;

Microarray ou microarranjo

Microarray ou técnica de microarranjo busca medir os níveis de expressão de transcritos em larga escala. O microarranjo de DNA consiste na imobilização de moléculas de DNA, geralmente obtidas por PCR, a uma superfície sólida (*chip*). Os *chips* de DNA são utilizados para definir quais dos genes imobilizados estão transcricionalmente ativos em uma população celular em determinado momento. Para isso, o RNA dessas células é extraído e é sintetizado o cDNA (fita de DNA complementar à fita de RNA). Esse último é marcado com fluoróforos e, finalmente, hibridizado com os fragmentos de DNA previamente imobilizados na superfície do *chip*. A partir de um detector de fluorescência, os sinais emitidos por cada ponto quando há excitação por laser são medidos para quantificar a transcrição dos genes alvo imobilizados. A metodologia de microarranjos está esquematizada na Figura 3.

Figura 3. Esquema da metodologia de microarranjo com cDNA: (1) Isolamento do RNA de células- alvo do estudo; (2) A partir das amostras de RNA extraídas de diferentes tecidos é realizada a técnica de transcriptase reversa na presença de um fluoróforo produzindo as sondas de cDNA marcadas com fluorescência; (3) Hibridização com chips de DNA e medição da fluorescência; (4) Avaliação da atividade transcrricional celular mediante realização de processamento e análises de imagem.

Chips de DNA carregando genomas bacterianos completos podem ser preparados, permitindo a análise da expressão de todo o repertório gênico. É uma ferramenta poderosa para a avaliação de genes diferencialmente expressos em resposta a estímulos e condições diversas. Grifantini e colaboradores [31], em 2002, foram os pioneiros na aplicação da transcriptômica para a identificação de candidatos vacinais, usando como modelo a *N. meningitidis*. Após a interação da bactéria com células humanas epiteliais, os RNAs isolados serviram como base para a identificação de genes bacterianos com expressão diferencial durante esta interação. Foram capazes de reconhecer 347 genes com expressão diferencial, sendo que 189 sofreram regulação positiva. Desses, 40% dos genes codificam para proteínas de membrana, sugerindo que o contato com as células epiteliais promoveu uma substancial reorganização da membrana. Dentre as proteínas de membrana estimuladas por adesão, o grupo identificou 5 proteínas que foram capazes de induzir anticorpos bactericidas em camundongos, sendo promissores candidatos vacinais. O trabalho

mostrou que o Microarranjo é uma técnica válida para a identificação de candidatos vacinais. No entanto, a técnica tem algumas limitações já que não há, na maioria dos casos, correlação direta entre mRNA e o nível de expressão proteica, e esta metodologia não considera eventos regulatórios pós-transcricionais.

Proteômica

A proteômica é um campo de estudo que se propõe a análise do conjunto de proteínas expressas numa célula ou tecido, isto é, o proteoma. A elucidação da sequência genômica de um organismo é apenas o primeiro passo para entender sua biologia. A genômica prediz os quadros de leitura ou proteínas codificadas pelas sequências gênicas, denotando o estado estático de informações herdadas. O genoma não é suficiente para identificar quais proteínas estão sendo realmente expressas na célula em um dado momento e condição. Deste modo, a genômica alavancou a proteômica e facilitou a identificação das proteínas devido à criação de bancos de dados com as sequências codificadoras. Diferentemente do genoma, o proteoma é extremamente dinâmico, podendo ser afetado por condições ambientais, temporais, nutricionais, entre outras [32].

Na proteômica, a técnica de eletroforese bidimensional (2DE) [33, 34] tem importante papel. Nesta técnica as proteínas são submetidas a duas etapas de separação: a primeira consiste em uma focalização isoelétrica na qual as proteínas são separadas pela sua carga elétrica; as proteínas migram até atingirem o pH em que possuam carga líquida igual à zero – o seu ponto isoelétrico (pI). A segunda etapa consiste na separação das proteínas de acordo com suas massas moleculares em eletroforese em gel de poliacrilamida (do inglês, *sodium dodecyl sulfate polyacrylamide gel electrophoresis*, SDS-PAGE). A separação 2DE atinge alto nível de resolução e permite a análise e visualização concomitante de centenas ou milhares de proteínas. A figura 4A ilustra a técnica da focalização isoelétrica (primeira dimensão) seguida da separação pela massa molecular (segunda dimensão); a figura 4B mostra uma eletroforese bidimensional (2D) obtida experimentalmente, após coloração das manchas (do inglês, “spots”) proteicos com prata.

Figura 4: Representação esquemática da técnica de eletroforese bidimensional (2DE). Após a solubilização das proteínas expressas na célula ou tecidos, essa amostra complexa é submetida a duas etapas de separação. **(A)** A primeira separação consiste em uma focalização isoelétrica, onde as proteínas são separadas de acordo com seu ponto isoelétrico. A segunda etapa separa as proteínas de acordo com suas massas moleculares em SDS-PAGE. **(B)** Após coloração do gel com um corante adequado, um mapa de pontos (spots) é visualizado. A análise desses spots é realizada por programas de computador.

Associada à técnica de 2DE, várias aplicações da espectrometria de massas (do inglês, *mass spectroscopy*, MS) desenvolveram-se extensivamente e emergiram como técnicas analíticas suficientemente sensíveis e compatíveis com as exigências da análise proteica em larga escala. A proteômica aplicada à microbiologia médica propiciou a caracterização de proteomas microbianos, estudo de mecanismos patogênicos de doenças infecciosas, identificação de novos alvos terapêuticos, análises de resistência a drogas, busca de novos marcadores moleculares e desenvolvimento vacinal. Em adição, a combinação da proteômica com análises sorológicas é uma técnica útil para a

identificação de candidatos vacinais. O “imunoma” permite a identificação em larga escala dos antígenos expostos na membrana, por exemplo, durante a infecção, que são reconhecidos por anticorpos dos hospedeiros. Essa abordagem foi utilizada para a identificação de candidatos vacinais contra *S. aureus*, quando proteínas de membrana foram resolvidas por 2DE, transferidas para membranas e incubadas com soros de pacientes infectados com a bactéria, identificando uma série de proteínas altamente imunorreativas e 15 candidatos vacinais em potencial [9]. Análises sorológicas do proteoma foram utilizadas para identificar proteínas imunorreativas de *B. anthracis* [35], *S. pneumoniae* [36], *S. iniae* [37], *M. tuberculosis* [38], *Haemophilus parasuis* [39], *Vibrio parahaemolyticus* [40], *Rickettsia heilongjiangensis* [41] entre outros.

Na era pós-genômica, as três principais estratégias descritas foram empregadas nos estudos com diversos patógenos importantes, buscando antígenos candidatos para desenvolvimento de vacinas [42]. Estudos com a *N. meningitidis* sorogrupo B utilizaram a genômica, microarranjos e proteômica [4, 5, 31, 43-45], enquanto que para a bactéria *S. pneumoniae*, a VR comparativa, proteômica e a metodologia clássica foram empregadas [7, 46-48]. Para outros organismos como *B. anthracis*, *S. aureus*, *M. tuberculosis*, *H. pylori*, foram empregadas a VR, imuno-proteômica e proteoma de proteínas de superfície, para a identificação de antígenos vacinais [6, 9, 13, 49-58]. O desenvolvimento de vacinas com estes organismos encontra-se em diferentes fases de desenvolvimento indo desde a fase de identificação, testes pré-clínicos, clínicos à obtenção de registro [59].

Sequenciamento do genoma completo da bactéria *L. interrogans*

A leptospirose é uma zoonose de importância global, que nos últimos dez anos vem sendo considerada uma das principais doenças infecciosas emergentes. É causada por um grupo de bactérias patogênicas pertencentes à família *Leptospiraceae*, gênero *Leptospira*.

A incidência de infecções humanas ocorre, principalmente em países tropicais e subtropicais onde as condições para transmissão são particularmente favoráveis. A contaminação ocorre por meio da exposição direta ou indireta via solo ou água contaminados com urina de mamíferos infectados cronicamente. Em áreas urbanas, os roedores domésticos, em particular o *Rattus norvegicus*, desempenham o papel de principal reservatório da doença, por permitirem a colonização das leptospiros nos

túbulos renais proximais e as excretarem vivas através da urina. Em países desenvolvidos, é tida como doença recreacional e ocupacional, pois está associada a atividades de lazer e de profissionais que trabalham em serviços de água e esgoto, canaviais, arrozais, lixeiros, tratadores de animais entre outras.

A leptospirose também tem grande importância econômica devido às alterações causadas em animais infectados, como gado, ovelha, etc., ocasionando diminuição drástica da produção de leite, alta incidência de abortos e morte precoce [60].

Os sintomas mais frequentes associados à doença variam desde uma infecção subclínica caracterizada por febre, calafrio, dores de cabeça e muscular, podendo até evoluir para formas mais severas como a síndrome de Weil [61] e a síndrome hemorrágica pulmonar (do inglês, *severe pulmonary hemorrhagic syndrome*, SPHS) [62]. Estas formas severas são responsáveis por cerca de 10% a 50% dos casos de mortalidade por leptospirose, principalmente em adultos entre 30-40 anos.

O tratamento com antibiótico é diferenciado dependendo da severidade da doença e duração dos sintomas, tornando-se efetivo quando iniciado na primeira semana de infecção. Devido à ausência de um diagnóstico precoce da doença, grande parte dos pacientes, quando diagnosticados, já apresentam as manifestações severas da mesma e o tratamento, nesta fase, atua apenas na redução da carga bacteriana, não revertendo as alterações patológicas estabelecidas. Dentre os antibióticos recomendados estão: ampicilina e amoxicilina, em casos onde não há relatos de complicações severas, penicilina G, para casos graves, e doxiciclina, tanto para profilaxia como doença branda, além da tetraciclina [60].

Na maioria dos casos, o diagnóstico laboratorial para leptospirose é feito por sorologia através do teste de micro-aglutinação (MAT) [60]. Embora este método apresente inúmeras desvantagens, tais como, baixa sensibilidade na detecção da fase inicial da doença, cultivo em laboratório de diferentes sorovares, e difícil interpretação de resultados devido à reatividade cruzada, o teste é considerado "padrão ouro" para detecção de anticorpos nas amostras de soros de pacientes. É também considerado, até o presente momento, o teste mais apropriado para levantamentos epidemiológicos, além de ser o mais sensível e específico quando comparado com testes alternativos de diagnóstico sorológico [60, 63].

Acredita-se que a melhor estratégia para combater a doença é a adoção de medidas preventivas. O desenvolvimento de uma vacina é extremamente importante, dada a dificuldade de controlar roedores que são os principais disseminadores urbanos

da doença. As primeiras vacinas testadas em animais e humanos, denominadas de bacterinas, são constituídas basicamente por uma suspensão de leptospiros inativadas por uma variedade de métodos, tais como aquecimento, formalina, fenol, irradiação, entre outros e, geralmente causam efeitos adversos [60]. Na tentativa de minimizar essas reações, novas preparações incluindo meio de cultura livre de proteínas, frações subcelulares ou preparações de membrana, demonstraram ser capazes de conferir uma resposta protetora através da indução de anticorpos contra lipopolissacarídeos (LPS) de leptospira [64]. Entretanto, essas vacinas apresentam algumas desvantagens, tais como: (i) proteção de curta duração, (ii) não indução de imunidade cruzada com outros sorovares não incluídos na preparação e (iii) a possível indução de autoimunidade [65].

Apesar das leptospiros terem sido identificadas há 100 anos [60], pouco se sabe sobre os mecanismos de invasão, patogenicidade e infecção deste organismo. Atualmente, temos dez genomas completos de leptospiros sequenciados: duas cepas de *L. interrogans* sorovar Lai, isolados 56601 e IPAV [66, 67], *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130 [68, 69], duas cepas de *L. borgpetersenii* sorovar Hardjo, L550 e JB197 [70], *L. santarosai* sorovar Shermani cepa LT821 [71], duas cepas de *L. licerasiae*, VAR010 e MMD0835 [72] e duas cepas saprófitas, *L. biflexa*, Paris e Ames [73]. Dois cromossomos circulares caracterizam o genoma das leptospiros patogênicas, com tamanho variando de 3,9 a 4,6 Mb. O genoma da *L. interrogans* sorovar Copenhageni forneceu novas perspectivas sobre a fisiologia relacionada ao metabolismo energético, tolerância a oxigênio, sistemas de transdução de sinal e mecanismos de patogenicidade. Adicionalmente, mais de 200 genes que codificam para lipoproteínas e proteínas de membrana externa foram identificados, considerados candidatos potenciais para o desenvolvimento de vacina contra a leptospirose.

A genômica comparativa entre as espécies patogênicas e saprófitas identificou genes únicos que podem ter papel na virulência. Em adição, genes hipotéticos ou hipotéticos conservados de função desconhecida parecem ser abundantes nas espécies patogênicas. O sequenciamento de genomas de leptospiros levou a um aumento significativo de publicações com diversos focos, aumentando o entendimento da biologia desta bactéria.

Vacinologia reversa aplicada ao genoma da *L. interrogans*

Com a disponibilidade das sequências genômicas de *Leptospira* deu-se o início de um projeto Piloto Funcional em larga escala. Este piloto empregou a estratégia da VR, selecionando as sequências codificadoras através do programa PSORT (<http://psort.hgc.jp/form.html>) [74] em proteínas expressas na membrana externa da bactéria e as não expostas. O programa PSORT identificou 2.000 sequências codificadoras como proteínas de superfície. O piloto funcional, com base em outros algoritmos, selecionou 437 proteínas, clonou 357 genes e 151 proteínas recombinantes foram expressas no sistema hospedeiro *E. coli*. Foi possível sondar 100 destas proteínas com soro de pacientes positivos para leptospirose, sendo que 23 foram imunorreativas [14]. O esquema resumindo a estratégia para a seleção das proteínas de membrana da bactéria *L. interrogans* sorovar Copenhageni durante o Piloto Funcional está mostrado na Figura 5.

Figura 5. Desenho esquemático do Piloto Genoma Funcional de *L. interrogans* para a identificação de candidatos vacinais por vacinologia reversa.

O piloto funcional mostrou que a estratégia da VR aplicada a *Leptospira* era viável e nos incentivou a prosseguir. Nosso grupo focou em proteínas de membrana externa conservadas nas cepas patogênicas. São estas proteínas que, provavelmente, estão envolvidas na interação das leptospiros com o ambiente externo, podendo também servir de alvo para o sistema imune do hospedeiro. Dentre estas proteínas, a classe das lipoproteínas é uma das mais importantes, sendo de grande interesse para o desenvolvimento de novas vacinas e diagnóstico [75]. Foram estabelecidos alguns critérios para a seleção das sequências genômicas: ser de membrana externa, podendo ser ou não lipoproteína, ter peptídeo sinal de exportação à membrana, possuir ou não domínios conservados em fatores de virulência de outros patógenos, ser hipotética. O esquema da Figura 6 mostra os critérios de seleção adotados por nosso grupo de pesquisa.

O primeiro critério de seleção é a localização celular. Programas como PSORT [74] e CELLO (<http://cello.life.nctu.edu.tw>) [76, 77] fazem a predição da localização com base na sequência de aminoácidos da proteína. Estas predições têm como base: (i) inferência com base na homologia/similaridade da sequência em banco de dados, nos quais a localização das proteínas depositadas tenha sido experimentalmente investigada; (ii) ferramentas baseadas em sequências-sinal, cuja localização celular é conhecida. Selecionamos as proteínas com predição de localização na membrana externa, incluindo as lipoproteínas. As proteínas com predição de localização no citoplasma são descartadas. Em seguida, utilizamos a ferramenta de BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) [78, 79] que verifica a presença ou não das proteínas no GenBank. No caso de haver similaridade com as sequências depositadas podemos verificar o quão conservadas são estas sequências em leptospiros e em outros organismos. Caso a proteína não tenha sido identificada ela é denominada hipotética, ou seja, é uma proteína nova. A presença de um peptídeo sinal de exportação ou exportação/lipidação, no caso de lipoproteínas, é analisada pelos programas SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) [80] e LipoP (<http://www.cbs.dtu.dk/services/LipoP/>) [81], respectivamente. O primeiro algoritmo faz predição da presença de um peptídeo sinal de clivagem e exportação SpI (peptidase sinal I), enquanto o segundo prediz em adição ao SpI, o SpII (peptidase sinal II), que possui um sítio de clivagem e lipidação (do inglês, *lipobox*). A presença de domínios conservados é analisada via programa PFAM (<http://pfam.sanger.ac.uk>) [82], também por similaridade de sequência; a presença de alguns domínios conservados pode

sinalizar se a proteína selecionada estaria envolvida na patogênese, caso este domínio esteja presente em proteínas caracterizadas com este papel.

Figura 6. Critérios e programas de bioinformática adotados para a seleção de genes a serem clonados e proteínas recombinantes a serem caracterizadas. GenBank: <http://www.ncbi.nlm.nih.gov>; 1- PSORT: <http://psort.hgc.jp/form.html>; 2 - CELLO: <http://cello.life.nctu.edu.tw>; 3 -LipoP: <http://www.cbs.dtu.dk/services/LipoP/>; 4 - BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>; 5 - PFAM: <http://pfam.sanger.ac.uk>

Clonagem dos genes selecionados e expressão das proteínas recombinantes em *E. coli*

As sequências correspondentes aos genes selecionados são amplificadas a partir do DNA genômico de *L. interrogans* sorovar Copenhageni, utilizando oligonucleotídeos sintéticos complementares às sequências e sítios de restrição na fita *forward* e outro na fita *reverse*. Os fragmentos amplificados pela PCR são isolados do gel de agarose e purificados utilizando-se a metodologia de mini-preparação ou mini-prep [83]. Os insertos de DNA obtidos na PCR são clonados no vetor pGEM-Teasy (*Promega*[®]), para clonagem no sistema hospedeiro de *E. coli* e obtenção da proteína

recombinante, ou em um vetor ponte (do inglês, *shuttle vector*) *E. coli*-mamífero, como o pTARGET, para obtenção de vacina de DNA.

O pGEM-T easy é um vetor linearizado, com uma timidina 3'-terminal em ambas as extremidades, o que permite a ligação dos produtos de PCR, onde uma sequência de poli-A é adicionada pela polimerase. A entrada do inserto de DNA no vetor interrompe a região codificadora da enzima β -galactosidase. Essa inativação permite a identificação dos clones recombinantes por seleção azul/branco em placas de meio de cultura/ágar, contendo ampicilina, IPTG e X-Gal, o qual é um substrato cromogênico que, quando degradado, torna-se azul; assim sendo, as bactérias que são incapazes de degradar esse substrato ficam brancas e são, presumivelmente, positivas para a presença do inserto de interesse.

A mistura de ligação dos produtos de PCR e pGEM-T easy é usada na transformação da linhagem *E. coli* DH5 α [83], previamente tornadas competentes pelo método do CaCl₂ (Figura 7A). As colônias recombinantes serão selecionadas em meio contendo ampicilina. Para verificar a presença de inserto, são feitas preparações dos plasmídeos a partir das colônias positivas de *E. coli*. O DNA obtido é digerido com enzimas de restrição apropriadas e analisado em gel de agarose para observar a liberação do inserto de DNA do plasmídeo. Os clones positivos, resistentes a ampicilina, são sequenciados e os insertos contendo as sequências corretas de DNA são removidos por digestão com as enzimas de restrição específicas e subclonados no vetor de expressão pAE [84], nos mesmos sítios de restrição. O vetor pAE possui origem de replicação em *E. coli*, gene que confere resistência a ampicilina, um sítio de ligação ribossomal (do inglês, *ribosome binding site*, RBS), um códon de iniciação (ATG) seguido de sequência de 6 resíduos de histidinas e um sítio de múltipla clonagem. É um sistema de alta expressão, controlado pelo promotor do fago T7 e, portanto a expressão da proteína recombinante só ocorre na presença da T7 RNA polimerase (Figura 7B).

O vetor pTARGET (*Promega*[®]) é um vetor ponte, *E. coli*-mamífero, derivado do vetor pCI (*Promega*[®]). Contém origem de replicação em *E. coli*, gene que confere resistência a ampicilina, gene da β -galactosidase permitindo a seleção visual de colônias (azul e branca), promotor e sequência amplificadora (*enhancer*) de citomegalovírus (CMV) de mamífero para expressão constitutiva da proteína heteróloga, um íntron, um sítio de múltipla clonagem seguido de sequência de poliadenilação, sequência

amplificadora, promotor de SV40 e um gene que confere resistência à neomicina seguido de sequência de poli (A) (Figura 7C) .

Figura 7. (A) Clonagem do gene selecionado por bioinformática em vetor de clonagem e expressão da proteína recombinante (pAE) (B) para desenvolvimento de vacina proteica ou em vetor ponte *E. coli*-mamífero pTARGET (C) para vacina de DNA.

Expressão das proteínas recombinantes no hospedeiro *E. coli*

As cepas de *E. coli*, comumente empregadas para ensaios de expressão, são BL21 SI (*Salt Induced*), que possui o gene da T7 RNA polimerase integrado ao genoma sob controle do promotor *proU* induzível por NaCl [85] e BL21 DE3, que também possui o gene da T7 RNA polimerase, mas dirigida pelo promotor *lacUV5*, o qual é induzido por IPTG (isopropil-B-D-tio-galactopiranosídeo) [86]. Após transformação com clones positivos e a indução da expressão das proteínas recombinantes, em ambas as bactérias, é realizada a análise por eletroforese em SDS-PAGE [87] na presença de

proteínas de massa molecular conhecida (marcador). Os clones que apresentarem bandas com a massa molecular esperada são selecionados para cultivo e indução em volume maior de meio de cultura para estudo da solubilidade das proteínas recombinantes. Caso as proteínas sejam expressas na forma solúvel, a purificação é feita a partir do sobrenadante da cultura, após lise celular e clarificação; se as proteínas forem expressas na forma de corpúsculo de inclusão, a purificação será feita a partir dos corpúsculos solubilizados na presença de algum agente desnaturante, como ureia ou guanidina. A Figura 8 sumariza as etapas necessárias para a obtenção da proteína recombinante purificada.

Figura 8. Expressão e purificação da proteína recombinante para desenvolvimento de vacina proteica.

Caracterização das proteínas recombinantes

As proteínas recombinantes são avaliadas quanto à sua imunogenicidade em modelo animal, com o objetivo de avaliar a capacidade de indução de resposta imunológica, tanto humoral (produção de anticorpos), como celular (produção de citocinas). A capacidade das proteínas em reagir com soros humanos positivos para leptospirose nos dá ideia da expressão da mesma durante o processo de infecção por leptospiras e do potencial da mesma para compor um kit diagnóstico. Nosso grupo já identificou proteínas reativas com estes soros, inclusive na fase inicial da doença, na qual outros métodos são pouco eficazes.

A ligação de proteínas bacterianas a componentes da matriz extracelular (MEC) é a primeira etapa no processo de invasão/colonização. Todas as proteínas recombinantes de *Leptospira* expressas em *E. coli* são avaliadas quanto a sua atividade de adesão. Componentes como laminina, fibronectina e colágeno são utilizados nestes ensaios. Até o momento, descrevemos 17 novas proteínas com atividade de adesão [88-101]. Reatividade a componentes de soro, como plasminogênio e reguladores do sistema complemento (como fator H e C4BP) também são avaliados. A ligação das proteínas recombinantes ao plasminogênio, um zimógeno circulante, nos dá uma ideia da participação da proteína no sistema fibrinolítico, com a geração da plasmina. A plasmina gerada na superfície das leptospiras é capaz de degradar laminina e fibronectina, podendo facilitar a invasão da bactéria no hospedeiro (FIGURA 9) [94-99, 102-104].

Figura 9. Representação esquemática básica da ativação da fibrinólise e inibição da coagulação seguido da ligação de plasminogênio (PLG) e fibrinogênio por *Leptospira*. As leptospiras capturam plasminogênio na sua superfície, que é convertido à plasmina

por ativadores presentes no hospedeiro (uPA e tPA). A plasmina degrada diretamente componentes da matriz extracelular (laminina e fibronectina), facilitando a invasão bacteriana. A plasmina atua também na fibrinólise, degradando fibrina, o que por sua vez inibe a coagulação. As leptospiplas capturam fibrinogênio livre através de seus receptores, resultando na sua depleção. Isso inibe a cascata de geração de coágulos de fibrina, catalisada pela trombina. Os triângulos verdes indicam os inibidores de enzimas e pró-enzimas: $\alpha 2$ -antiplasmina e $\alpha 2$ -macroglobulina atuando sobre a plasmina, inibidores de ativadores de plasminogênio (PAIs, do inglês *Plasminogen Activators Inhibitor*) inibindo a ação de uPA e tPA e antitrombina modulando a ação da trombina.

Identificamos uma proteína recombinante que se liga ao fator H e quatro ao C4BP [96, 98, 99]. Leptospiras patogênicas são capazes de evadir o sistema complemento, enquanto as não patogênicas ou saprófitas são eliminadas pelo hospedeiro [105]. Desse modo, a busca de proteínas que se ligam aos reguladores do sistema complemento é importante, pois as mesmas devem estar envolvidas na patogênese da bactéria e podem ser antígenos vacinais. Em patógenos importantes, como a *N. meningitidis*, proteínas que se ligam ao fator H do sistema complemento mostraram atividade bactericida e fazem parte da vacina contra meningite meningocócica, atualmente aguardando liberação por órgãos competentes na Europa [106, 107]. O estudo da reatividade das proteínas recombinantes com componentes do soro identificou também, seis adesinas que se ligam ao fibrinogênio e inibem a formação de coágulo catalisada pela trombina (ver Figura 9) [108]. A leptospirose severa é acompanhada por múltiplos focos hemorrágicos cujo mecanismo é desconhecido. Nossos dados sugerem que estas proteínas podem mediar a interação de leptospiras com o fibrinogênio e podem auxiliar no entendimento da coagulopatia que acompanha a doença. Um sumário da caracterização das proteínas recombinantes está apresentado no esquema da Figura 10.

Figura 10. Esquema resumindo os principais estudos feitos para a caracterização das proteínas recombinantes de *Leptospira*.

Avaliação da atividade imunoprotetora das proteínas recombinantes - Ensaio de desafio em modelo animal

A atividade imunoprotetora das proteínas recombinantes é avaliada em ensaio de desafio em modelo animal. Hamsters são roedores susceptíveis às leptospiros e empregados nestes experimentos. Estes são imunizados por inoculação subcutânea de 50 µg de proteína recombinante purificada ou vacina de DNA, vacina comercial (*Farrow Sure B*, Pfizer,) ou PBS (do inglês, *phosphate buffered saline*) solução salina, estes últimos utilizados como controle positivo e negativo, respectivamente. As suspensões para inoculação contêm hidróxido de alumínio 10%, empregado como adjuvante. São realizadas duas imunizações com intervalos de aproximadamente 15 dias. Antes de cada imunização, os animais são sangrados via plexo retro-orbital para termos controle do nível de anticorpos induzidos pós-inoculação das proteínas recombinantes. O título de anticorpos é analisado pela técnica de ELISA (do inglês, *Enzyme-Linked Immunosorbent Assay*).

No 28º dia após a 1ª imunização, os animais são infectados por inoculação intraperitoneal com 200 µl de cultura de leptospiros virulentas e um grupo controle

recebe apenas meio de cultura. O esquema de imunização está apresentado na Figura 11. Após 21 dias de observação são contados os animais mortos e os sobreviventes são sangrados via plexo retro-orbital para avaliação do soro quanto à presença de anticorpos anti-leptospira pelo ensaio de MAT. Os rins dos animais sobreviventes são coletados, macerados, suspensos em solução salina nas diluições 10^{-1} , 10^{-2} e 10^{-3} e inoculados em meio semi-sólido para avaliação da presença de leptospiras, com o intuito de analisar atividade esterilizante do antígeno. Estes órgãos são também preservados para cortes e posterior análise histológica.

Figura 11. Esquema de imunização de *hamsters* (não está correto: hamster é hamster, porquinho-da-Índia é Guinea-pig) com as proteínas recombinantes ou vacina de DNA e avaliação da resposta imunológica (anticorpos), imunoproteção (sobreviventes) e atividade esterilizante (presença de leptospiras em meio de cultura).

Identificamos algumas proteínas de leptospiras que induziram proteção parcial contra o desafio com leptospiras virulentas [109, 110]. É possível que a combinação de antígenos proteicos seja necessária para a obtenção de uma maior proteção, semelhante à vacina contra *N. meningitidis*, que emprega 4 proteínas antigênicas [111].

Conclusões

O rápido desenvolvimento nas técnicas de sequenciamento de DNA propiciou o início da era genômica, com o grande acúmulo de sequências de procariotos, vírus e eucariotos. A mineração destes dados, ou *data mining*, impulsionou o desenvolvimento da bioinformática, com seus algoritmos específicos para a predição das sequências

genômicas, quanto a sua localização celular e possível função no organismo. Este processo deu início à era pós-genômica, ou “*omics*”, onde as metodologias de transcriptômica e da proteômica tem importante papel. A genômica funcional, que engloba estas duas metodologias mais a VR, permitiu a identificação de proteínas envolvidas na patogenicidade de patógenos importantes, durante a busca e caracterização de alvos vacinais. No caso da *N. meningitidis* sorogrupo B, uma vacina encontra-se em fase de licenciamento e vários patógenos encontram-se em fase de ensaios pré-clínicos e clínicos. A VR aplicada ao genoma da *L. interrogans* permitiu a identificação de diversas proteínas com possível papel na patogênese, contribuindo para o conhecimento da leptospirose. Embora grandes desafios permaneçam, a evolução da genômica comparativa e da genômica sintética e estrutural como novas ferramentas, deve contribuir para a genômica funcional no desenvolvimento de futuras vacinas.

Agradecimentos. O laboratório de Biotecnologia Molecular II, do Centro de Biotecnologia, Instituto Butantan, tem apoio financeiro da FAPESP, CNPq e Fundação Butantan.

Referências Bibliográficas

1. Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, et al.: **Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd.** *Science* 1995, **269**:496-512.
2. Tomb JF, White O, Kerlavage AR, Clayton RA, Sutton GG, Fleischmann RD, Ketchum KA, Klenk HP, Gill S, Dougherty BA, Nelson K, Quackenbush J, Zhou L, Kirkness EF, Peterson S, Loftus B, Richardson D, Dodson R, Khalak HG, Glodek A, McKenney K, Fitzgerald LM, Lee N, Adams MD, Hickey EK, Berg DE, Gocayne JD, Utterback TR, Peterson JD, Kelley JM, Cotton MD, Weidman JM, Fujii C, Bowman C, Watthey L, Wallin E, Hayes WS, Borodovsky M, Karp PD, Smith HO, Fraser CM, Venter JC: **The complete genome sequence of the gastric pathogen *Helicobacter pylori*.** *Nature* 1997, **388**:539-47.
3. Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb JF, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, van Vugt R, Palmer N, Adams MD, Gocayne J, Weidman J, Utterback T, Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fuji C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter JC: **Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*.** *Nature* 1997, **390**:580-6.
4. Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, Eisen JA, Ketchum KA, Hood DW, Peden JF, Dodson RJ, Nelson WC, Gwinn ML, DeBoy R, Peterson JD, Hickey EK, Haft DH, Salzberg SL, White O, Fleischmann RD, Dougherty BA, Mason T, Ciecko A, Parksey DS, Blair E, Citti H, Clark EB, Cotton MD, Utterback TR, Khouri H, Qin H, Vamathevan J, Gill J, Scarlato V, Masignani V, Pizza M, Grandi G, Sun L, Smith HO, Fraser CM, Moxon ER, Rappuoli R, Venter JC: **Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58.** *Science* 2000, **287**:1809-15.
5. Pizza M, Scarlato V, Masignani V, Giuliani MM, Arico B, Comanducci M, Jennings GT, Baldi L, Bartolini E, Capecchi B, Galeotti CL, Luzzi E, Manetti R, Marchetti E, Mora M, Nuti S, Ratti G, Santini L, Savino S, Scarselli M, Storni E, Zuo P, Broeker M, Hundt E, Knapp B, Blair E, Mason T, Tettelin H, Hood DW, Jeffries AC, Saunders NJ, Granoff DM, Venter JC, Moxon ER, Grandi G, Rappuoli R: **Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing.** *Science* 2000, **287**:1816-20.
6. Ariel N, Zvi A, Grosfeld H, Gat O, Inbar Y, Velan B, Cohen S, Shafferman A: **Search for potential vaccine candidate open reading frames in the *Bacillus anthracis* virulence plasmid pXO1: in silico and in vitro screening.** *Infect Immun* 2002, **70**:6817-27.
7. Wizemann TM, Heinrichs JH, Adamou JE, Erwin AL, Kunsch C, Choi GH, Barash SC, Rosen CA, Masure HR, Tuomanen E, Gayle A, Brewah YA, Walsh W, Barren P, Lathigra R, Hanson M, Langermann S, Johnson S, Koenig S: **Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection.** *Infect Immun* 2001, **69**:1593-8.
8. Etz H, Minh DB, Henics T, Dryla A, Winkler B, Triska C, Boyd AP, Sollner J, Schmidt W, von Ahsen U, Buschle M, Gill SR, Kolonay J, Khalak H, Fraser CM, von Gabain A, Nagy E, Meinke A: **Identification of in vivo expressed vaccine candidate antigens from *Staphylococcus aureus*.** *Proc Natl Acad Sci U S A* 2002, **99**:6573-8.

9. Vytvytska O, Nagy E, Bluggel M, Meyer HE, Kurzbauer R, Huber LA, Klade CS: **Identification of vaccine candidate antigens of *Staphylococcus aureus* by serological proteome analysis.** *Proteomics* 2002, **2**:580-90.
10. Montigiani S, Falugi F, Scarselli M, Finco O, Petracca R, Galli G, Mariani M, Manetti R, Agnusdei M, Cevenini R, Donati M, Nogarotto R, Norais N, Garaguso I, Nuti S, Saletti G, Rosa D, Ratti G, Grandi G: **Genomic approach for analysis of surface proteins in *Chlamydia pneumoniae*.** *Infect Immun* 2002, **70**:368-79.
11. Ross BC, Czajkowski L, Hocking D, Margetts M, Webb E, Rothel L, Patterson M, Agius C, Camuglia S, Reynolds E, Littlejohn T, Gaeta B, Ng A, Kuczek ES, Mattick JS, Gearing D, Barr IG: **Identification of vaccine candidate antigens from a genomic analysis of *Porphyromonas gingivalis*.** *Vaccine* 2001, **19**:4135-42.
12. Srinivasa Rao PS, Lim TM, Leung KY: **Functional genomics approach to the identification of virulence genes involved in *Edwardsiella tarda* pathogenesis.** *Infect Immun* 2003, **71**:1343-51.
13. Betts JC: **Transcriptomics and proteomics: tools for the identification of novel drug targets and vaccine candidates for tuberculosis.** *IUBMB Life* 2002, **53**:239-42.
14. Gamberini M, Gomez RM, Atzingen MV, Martins EA, Vasconcellos SA, Romero EC, Leite LC, Ho PL, Nascimento AL: **Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis.** *FEMS Microbiol Lett* 2005, **244**:305-13.
15. Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HL: **Mucosal immunization with iron receptor antigens protects against urinary tract infection.** *PLoS Pathog* 2009, **5**:e1000586.
16. Rappuoli R: **Reverse vaccinology.** *Curr Opin Microbiol* 2000, **3**:445-50.
17. Rappuoli R: **Reverse vaccinology, a genome-based approach to vaccine development.** *Vaccine* 2001, **19**:2688-91.
18. Delany I, Rappuoli R, Seib KL: **Vaccines, reverse vaccinology, and bacterial pathogenesis.** *Cold Spring Harb Perspect Med* 2013, **3**:a012476.
19. Capecchi B, Adu-Bobie J, Di Marcello F, Ciocchi L, Massignani V, Taddei A, Rappuoli R, Pizza M, Arico B: ***Neisseria meningitidis* NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells.** *Mol Microbiol* 2005, **55**:687-98.
20. Madico G, Welsch JA, Lewis LA, McNaughton A, Perlman DH, Costello CE, Ngampasutadol J, Vogel U, Granoff DM, Ram S: **The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance.** *J Immunol* 2006, **177**:501-10.
21. Schneider MC, Exley RM, Chan H, Feavers I, Kang YH, Sim RB, Tang CM: **Functional significance of factor H binding to *Neisseria meningitidis*.** *J Immunol* 2006, **176**:7566-75.
22. Serruto D, Spadafina T, Ciocchi L, Lewis LA, Ram S, Tontini M, Santini L, Biolchi A, Seib KL, Giuliani MM, Donnelly JJ, Berti F, Savino S, Scarselli M, Costantino P, Kroll JS, O'Dwyer C, Qiu J, Plaut AG, Moxon R, Rappuoli R, Pizza M, Arico B: ***Neisseria meningitidis* GNA2132, a heparin-binding protein that induces protective immunity in humans.** *Proc Natl Acad Sci U S A* 2010, **107**:3770-5.
23. Manetti AG, Zingaretti C, Falugi F, Capo S, Bombaci M, Bagnoli F, Gambellini G, Bensi G, Mora M, Edwards AM, Musser JM, Graviss EA, Telford JL, Grandi G, Margarit I: ***Streptococcus pyogenes* pili promote pharyngeal cell adhesion and biofilm formation.** *Mol Microbiol* 2007, **64**:968-83.

24. Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, Muzzi A, Giusti F, Emolo C, Sinisi A, Hilleringmann M, Pansegrau W, Censini S, Rappuoli R, Covacci A, Masignani V, Barocchi MA: **A second pilus type in *Streptococcus pneumoniae* is prevalent in emerging serotypes and mediates adhesion to host cells.** *J Bacteriol* 2008, **190**:5480-92.
25. Kadioglu A, Weiser JN, Paton JC, Andrew PW: **The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease.** *Nat Rev Microbiol* 2008, **6**:288-301.
26. Moriel DG, Bertoldi I, Spagnuolo A, Marchi S, Rosini R, Nesta B, Pastorello I, Corea VA, Torricelli G, Cartocci E, Savino S, Scarselli M, Dobrindt U, Hacker J, Tettelin H, Tallon LJ, Sullivan S, Wieler LH, Ewers C, Pickard D, Dougan G, Fontana MR, Rappuoli R, Pizza M, Serino L: **Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic *Escherichia coli*.** *Proc Natl Acad Sci U S A* 2010, **107**:9072-7.
27. Nesta B, Spraggon G, Alteri C, Moriel DG, Rosini R, Veggi D, Smith S, Bertoldi I, Pastorello I, Ferlenghi I, Fontana MR, Frankel G, Mobley HL, Rappuoli R, Pizza M, Serino L, Soriani M: **FdeC, a novel broadly conserved *Escherichia coli* adhesin eliciting protection against urinary tract infections.** *MBio* 2012, **3**:
28. McCarthy AJ, Lindsay JA: **Genetic variation in *Staphylococcus aureus* surface and immune evasion genes is lineage associated: implications for vaccine design and host-pathogen interactions.** *BMC Microbiol* 2010, **10**:173.
29. Lawley TD, Croucher NJ, Yu L, Clare S, Sebaihia M, Goulding D, Pickard DJ, Parkhill J, Choudhary J, Dougan G: **Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores.** *J Bacteriol* 2009, **191**:5377-86.
30. Heinz E, Tischler P, Rattei T, Myers G, Wagner M, Horn M: **Comprehensive in silico prediction and analysis of chlamydial outer membrane proteins reflects evolution and life style of the Chlamydiae.** *BMC Genomics* 2009, **10**:634.
31. Grifantini R, Bartolini E, Muzzi A, Draghi M, Frigimelica E, Berger J, Ratti G, Petracca R, Galli G, Agnusdei M, Giuliani MM, Santini L, Brunelli B, Tettelin H, Rappuoli R, Randazzo F, Grandi G: **Previously unrecognized vaccine candidates against group B meningococcus identified by DNA microarrays.** *Nat Biotechnol* 2002, **20**:914-21.
32. Bradshaw RA, Burlingame AL: **From proteins to proteomics.** *IUBMB Life* 2005, **57**:267-72.
33. O'Farrell PH: **High resolution two-dimensional electrophoresis of proteins.** *J Biol Chem* 1975, **250**:4007-21.
34. Klose J, Spielmann H: **Gel isoelectric focusing of mouse lactate dehydrogenase: heterogeneity of the isoenzymes A4 and X4.** *Biochem Genet* 1975, **13**:707-20.
35. Ariel N, Zvi A, Makarova KS, Chitlaru T, Elhanany E, Velan B, Cohen S, Friedlander AM, Shafferman A: **Genome-based bioinformatic selection of chromosomal *Bacillus anthracis* putative vaccine candidates coupled with proteomic identification of surface-associated antigens.** *Infect Immun* 2003, **71**:4563-79.
36. Ling E, Feldman G, Portnoi M, Dagan R, Overweg K, Mulholland F, Chalifa-Caspi V, Wells J, Mizrahi-Nebenzahl Y: **Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse.** *Clin Exp Immunol* 2004, **138**:290-8.
37. Shin GW, Palaksha KJ, Kim YR, Nho SW, Kim S, Heo GJ, Park SC, Jung TS: **Application of immunoproteomics in developing a *Streptococcus iniae* vaccine for**

- olive flounder (*Paralichthys olivaceus*).** *J Chromatogr B Analyt Technol Biomed Life Sci* 2007, **849**:315-22.
38. Malen H, Softeland T, Wiker HG: **Antigen analysis of *Mycobacterium tuberculosis* H37Rv culture filtrate proteins.** *Scand J Immunol* 2008, **67**:245-52.
 39. Zhou M, Zhang A, Guo Y, Liao Y, Chen H, Jin M: **A comprehensive proteome map of the *Haemophilus parasuis* serovar 5.** *Proteomics* 2009, **9**:2722-39.
 40. Li H, Ye MZ, Peng B, Wu HK, Xu CX, Xiong XP, Wang C, Wang SY, Peng XX: **Immunoproteomic identification of polyvalent vaccine candidates from *Vibrio parahaemolyticus* outer membrane proteins.** *J Proteome Res* 2010, **9**:2573-83.
 41. Qi Y, Xiong X, Wang X, Duan C, Jia Y, Jiao J, Gong W, Wen B: **Proteome analysis and serological characterization of surface-exposed proteins of *Rickettsia heilongjiangensis*.** *PLoS One* 2013, **8**:e70440.
 42. Bambini S, Rappuoli R: **The use of genomics in microbial vaccine development.** *Drug Discov Today* 2009, **14**:252-60.
 43. Bernardini G, Braconi D, Lusini P, Santucci A: **Post-genomics of *Neisseria meningitidis*: an update.** *Expert Rev Proteomics* 2011, **8**:803-11.
 44. Giuliani MM, Adu-Bobie J, Comanducci M, Arico B, Savino S, Santini L, Brunelli B, Bambini S, Biolchi A, Capecchi B, Cartocci E, Ciucchi L, Di Marcello F, Ferlicca F, Galli B, Luzzi E, Masignani V, Serruto D, Veggi D, Contorni M, Morandi M, Bartalesi A, Cinotti V, Mannucci D, Titta F, Ovidi E, Welsch JA, Granoff D, Rappuoli R, Pizza M: **A universal vaccine for serogroup B meningococcus.** *Proc Natl Acad Sci U S A* 2006, **103**:10834-9.
 45. Dietrich G, Kurz S, Hubner C, Aepinus C, Theiss S, Guckenberger M, Panzner U, Weber J, Frosch M: **Transcriptome analysis of *Neisseria meningitidis* during infection.** *J Bacteriol* 2003, **185**:155-64.
 46. Seib KL, Zhao X, Rappuoli R: **Developing vaccines in the era of genomics: a decade of reverse vaccinology.** *Clin Microbiol Infect* 2012, **18 Suppl 5**:109-16.
 47. Hiller NL, Janto B, Hogg JS, Boissy R, Yu S, Powell E, Keefe R, Ehrlich NE, Shen K, Hayes J, Barbadora K, Klimke W, Dernovoy D, Tatusova T, Parkhill J, Bentley SD, Post JC, Ehrlich GD, Hu FZ: **Comparative genomic analyses of seventeen *Streptococcus pneumoniae* strains: insights into the pneumococcal supragenome.** *J Bacteriol* 2007, **189**:8186-95.
 48. Morsceck C, Prokhorova T, Sigh J, Pfeiffer M, Bille-Nielsen M, Petersen J, Boysen A, Kofoed T, Frimodt-Moller N, Nyborg-Nielsen P, Schrotz-King P: ***Streptococcus pneumoniae*: proteomics of surface proteins for vaccine development.** *Clin Microbiol Infect* 2008, **14**:74-81.
 49. Jagusztyn-Krynicka EK, Godlewska R: **New approaches for *Helicobacter* vaccine development--difficulties and progress.** *Pol J Microbiol* 2008, **57**:3-9.
 50. Glowalla E, Tosetti B, Kronke M, Krut O: **Proteomics-based identification of anchorless cell wall proteins as vaccine candidates against *Staphylococcus aureus*.** *Infect Immun* 2009, **77**:2719-29.
 51. DelVecchio VG, Sabato MA, Trichilo J, Dake C, Grewal P, Alefantis T: **Proteomics for the development of vaccines and therapeutics.** *Crit Rev Immunol* 2010, **30**:239-54.
 52. Giri PK, Kruh NA, Dobos KM, Schorey JS: **Proteomic analysis identifies highly antigenic proteins in exosomes from *M. tuberculosis*-infected and culture filtrate protein-treated macrophages.** *Proteomics* 2010, **10**:3190-202.
 53. Read TD, Peterson SN, Tourasse N, Baillie LW, Paulsen IT, Nelson KE, Tettelin H, Fouts DE, Eisen JA, Gill SR, Holtzapple EK, Okstad OA, Helgason E, Rilstone J, Wu M, Kolonay JF, Beanan MJ, Dodson RJ, Brinkac LM, Gwinn M, DeBoy

- RT, Madpu R, Daugherty SC, Durkin AS, Haft DH, Nelson WC, Peterson JD, Pop M, Khouri HM, Radune D, Benton JL, Mahamoud Y, Jiang L, Hance IR, Weidman JF, Berry KJ, Plaut RD, Wolf AM, Watkins KL, Nierman WC, Hazen A, Cline R, Redmond C, Thwaite JE, White O, Salzberg SL, Thomason B, Friedlander AM, Koehler TM, Hanna PC, Kolsto AB, Fraser CM: **The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria.** *Nature* 2003, **423**:81-6.
54. Bergman NH, Anderson EC, Swenson EE, Janes BK, Fisher N, Niemeyer MM, Miyoshi AD, Hanna PC: **Transcriptional profiling of *Bacillus anthracis* during infection of host macrophages.** *Infect Immun* 2007, **75**:3434-44.
55. Chitlaru T, Gat O, Grosfeld H, Inbar I, Gozlan Y, Shafferman A: **Identification of in vivo-expressed immunogenic proteins by serological proteome analysis of the *Bacillus anthracis* secretome.** *Infect Immun* 2007, **75**:2841-52.
56. Jaing C, Gardner S, McLoughlin K, Mulakken N, Alegria-Hartman M, Banda P, Williams P, Gu P, Wagner M, Manohar C, Slezak T: **A functional gene array for detection of bacterial virulence elements.** *PLoS One* 2008, **3**:e2163.
57. Chakravarti DN, Fiske MJ, Fletcher LD, Zagursky RJ: **Application of genomics and proteomics for identification of bacterial gene products as potential vaccine candidates.** *Vaccine* 2000, **19**:601-12.
58. Utt M, Nilsson I, Ljungh A, Wadstrom T: **Identification of novel immunogenic proteins of *Helicobacter pylori* by proteome technology.** *J Immunol Methods* 2002, **259**:1-10.
59. Snape MD, Philip J, John TM, Robinson H, Kelly S, Gossger N, Yu LM, Kittel C, Toneatto D, Dull PM, Pollard AJ: **Bactericidal antibody persistence 2 years after immunization with 2 investigational serogroup B meningococcal vaccines at 6, 8 and 12 months and immunogenicity of preschool booster doses: a follow-on study to a randomized clinical trial.** *Pediatr Infect Dis J* 2013, **32**:1116-21.
60. Faine S, Adler B, Bolin C, Perolat P: ***Leptospira* and Leptospirosis.** 1999,
61. Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC: **Sporadic urban leptospirosis.** *Ann Intern Med* 1996, **125**:794-8.
62. McBride AJ, Athanazio DA, Reis MG, Ko AI: **Leptospirosis.** *Curr Opin Infect Dis* 2005, **18**:376-86.
63. Levett PN: **Leptospirosis.** *Clin Microbiol Rev* 2001, **14**:296-326.
64. de la Pena-Moctezuma A, Bulach DM, Kalambaheti T, Adler B: **Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis.** *FEMS Microbiol Lett* 1999, **177**:319-26.
65. Rathinam SR, Rathnam S, Selvaraj S, Dean D, Nozik RA, Namperumalsamy P: **Uveitis associated with an epidemic outbreak of leptospirosis.** *Am J Ophthalmol* 1997, **124**:71-9.
66. Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, Zhang YX, Xiong H, Lu G, Lu LF, Jiang HQ, Jia J, Tu YF, Jiang JX, Gu WY, Zhang YQ, Cai Z, Sheng HH, Yin HF, Zhang Y, Zhu GF, Wan M, Huang HL, Qian Z, Wang SY, Ma W, Yao ZJ, Shen Y, Qiang BQ, Xia QC, Guo XK, Danchin A, Saint Girons I, Somerville RL, Wen YM, Shi MH, Chen Z, Xu JG, Zhao GP: **Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing.** *Nature* 2003, **422**:888-93.
67. Zhong Y, Chang X, Cao XJ, Zhang Y, Zheng H, Zhu Y, Cai C, Cui Z, Zhang Y, Li YY, Jiang XG, Zhao GP, Wang S, Li Y, Zeng R, Li X, Guo XK: **Comparative proteogenomic analysis of the *Leptospira interrogans* virulence-attenuated strain IPAV against the pathogenic strain 56601.** *Cell Res* 2011, **21**:1210-29.

68. Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, Digiampietri LA, Harstkeerl RA, Ho PL, Marques MV, Oliveira MC, Setubal JC, Haake DA, Martins EA: **Genome features of *Leptospira interrogans* serovar Copenhageni**. *Braz J Med Biol Res* 2004, **37**:459-77.
69. Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, Verjovski-Almeida S, Hartskeerl RA, Marques MV, Oliveira MC, Menck CF, Leite LC, Carrer H, Coutinho LL, Degraive WM, Dellagostin OA, El-Dorry H, Ferro ES, Ferro MI, Furlan LR, Gamberini M, Giglioti EA, Goes-Neto A, Goldman GH, Goldman MH, Harakava R, Jeronimo SM, Junqueira-de-Azevedo IL, Kimura ET, Kuramae EE, Lemos EG, Lemos MV, Marino CL, Nunes LR, de Oliveira RC, Pereira GG, Reis MS, Schrieffer A, Siqueira WJ, Sommer P, Tsai SM, Simpson AJ, Ferro JA, Camargo LE, Kitajima JP, Setubal JC, Van Sluys MA: **Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis**. *J Bacteriol* 2004, **186**:2164-72.
70. Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, Cullen PA, Davis J, Johnson M, Kuczek E, Alt DP, Peterson-Burch B, Coppel RL, Rood JI, Davies JK, Adler B: **Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential**. *Proc Natl Acad Sci U S A* 2006, **103**:14560-5.
71. Chou LF, Chen YT, Lu CW, Ko YC, Tang CY, Pan MJ, Tian YC, Chiu CH, Hung CC, Yang CW: **Sequence of *Leptospira santarosai* serovar Shermani genome and prediction of virulence-associated genes**. *Gene* 2012, **511**:364-70.
72. Ricaldi JN, Fouts DE, Selengut JD, Harkins DM, Patra KP, Moreno A, Lehmann JS, Purushe J, Sanka R, Torres M, Webster NJ, Vinetz JM, Matthias MA: **Whole genome analysis of *Leptospira licerasiae* provides insight into leptospiral evolution and pathogenicity**. *PLoS Negl Trop Dis* 2012, **6**:e1853.
73. Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, Creno S, Kuczek ES, Bommezzadri S, Davis JC, McGrath A, Johnson MJ, Boursaux-Eude C, Seemann T, Rouy Z, Coppel RL, Rood JI, Lajus A, Davies JK, Medigue C, Adler B: **Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis**. *PLoS One* 2008, **3**:e1607.
74. Nakai K, Horton P: **PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization**. *Trends Biochem Sci* 1999, **24**:34-6.
75. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, Mazel M, Matsunaga J, Levett PN, Bolin CA: **The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection**. *Infect Immun* 2000, **68**:2276-85.
76. Yu CS, Lin CJ, Hwang JK: **Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions**. *Protein Sci* 2004, **13**:1402-6.
77. Yu CS, Chen YC, Lu CH, Hwang JK: **Prediction of protein subcellular localization**. *Proteins* 2006, **64**:643-51.
78. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: **Basic local alignment search tool**. *J Mol Biol* 1990, **215**:403-10.
79. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ: **Gapped BLAST and PSI-BLAST: a new generation of protein database search programs**. *Nucleic Acids Res* 1997, **25**:3389-402.
80. Petersen TN, Brunak S, von Heijne G, Nielsen H: **SignalP 4.0: discriminating signal peptides from transmembrane regions**. *Nat Methods* 2011, **8**:785-6.

81. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A: **Prediction of lipoprotein signal peptides in Gram-negative bacteria.** *Protein Sci* 2003, **12**:1652-62.
82. Punta M, Coghill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer EL, Eddy SR, Bateman A, Finn RD: **The Pfam protein families database.** *Nucleic Acids Res* 2012, **40**:D290-301.
83. Sambrook J, Gething MJ: **Protein structure. Chaperones, paperones.** *Nature* 1989, **342**:224-5.
84. Ramos CR, Abreu PA, Nascimento AL, Ho PL: **A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide.** *Braz J Med Biol Res* 2004, **37**:1103-9.
85. Bhandari P, Gowrishankar J: **An Escherichia coli host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer.** *J Bacteriol* 1997, **179**:4403-6.
86. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW: **Use of T7 RNA polymerase to direct expression of cloned genes.** *Methods Enzymol* 1990, **185**:60-89.
87. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227**:680-5.
88. Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, Morais ZM, Vasconcellos SA, Nascimento AL: **A newly identified leptospiral adhesin mediates attachment to laminin.** *Infect Immun* 2006, **74**:6356-64.
89. Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Morais ZM, Lima DM, Abreu PA, Nascimento AL: **Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection.** *BMC Microbiol* 2008, **8**:70.
90. Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, de Morais ZM, Vasconcellos SA, Nascimento AL: **Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells.** *J Infect* 2009, **59**:264-76.
91. Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Morais ZM, Vasconcellos SA, Nascimento AL: **A newly identified protein of Leptospira interrogans mediates binding to laminin.** *J Med Microbiol* 2009, **58**:1275-82.
92. Oliveira TR, Longhi MT, Goncalves AP, de Morais ZM, Vasconcellos SA, Nascimento AL: **LipL53, a temperature regulated protein from Leptospira interrogans that binds to extracellular matrix molecules.** *Microbes Infect* 2010, **12**:207-17.
93. Vieira ML, de Morais ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL: **Lsa63, a newly identified surface protein of Leptospira interrogans binds laminin and collagen IV.** *J Infect* 2010, **60**:52-64.
94. Oliveira R, de Morais ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL: **Characterization of novel OmpA-like protein of Leptospira interrogans that binds extracellular matrix molecules and plasminogen.** *PLoS One* 2011, **6**:e21962.
95. Mendes RS, Von Atzingen M, de Morais ZM, Goncalves AP, Serrano SM, Asega AF, Romero EC, Vasconcellos SA, Nascimento AL: **The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection.** *Infect Immun* 2011, **79**:4657-67.
96. Domingos RF, Vieira ML, Romero EC, Goncalves AP, de Morais ZM, Vasconcellos SA, Nascimento AL: **Features of two proteins of Leptospira**

- interrogans with potential role in host-pathogen interactions.** *BMC Microbiol* 2012, **12**:50.
97. Fernandes LG, Vieira ML, Kirchgatter K, Alves IJ, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL: **OmpL1 is an extracellular matrix- and plasminogen-interacting protein of *Leptospira* spp.** *Infect Immun* 2012, **80**:3679-92.
 98. Souza NM, Vieira ML, Alves IJ, de Moraes ZM, Vasconcellos SA, Nascimento AL: **Lsa30, a novel adhesin of *Leptospira* interrogans binds human plasminogen and the complement regulator C4bp.** *Microb Pathog* 2012, **53**:125-34.
 99. Siqueira GH, Atzingen MV, Alves IJ, de Moraes ZM, Vasconcellos SA, Nascimento AL: **Characterization of Three Novel Adhesins of *Leptospira* interrogans.** *Am J Trop Med Hyg* 2013. **In press.**
 100. Fernandes LG, Vieira ML, Alves IJ, de Moraes ZM, Vasconcellos SA, Romero EC, Nascimento AL: **Functional and immunological evaluation of two novel proteins of *Leptospira* spp.** *Microbiology* 2013. **In press.**
 101. Vieira ML, Fernandes LG, Domingos RF, Oliveira R, Siqueira GH, Souza NM, Teixeira AF, Atzingen MV, Nascimento AL: **Leptospiral extracellular matrix adhesins as mediators of pathogen-host interactions.** *FEMS Microbiol Lett* 2013. **In press.**
 102. Vieira ML, Vasconcellos SA, Goncalves AP, de Moraes ZM, Nascimento AL: **Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation.** *Infect Immun* 2009, **77**:4092-101.
 103. Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, Nascimento AL: **In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira* interrogans.** *PLoS One* 2010, **5**:e11259.
 104. Vieira ML, Atzingen MV, Oliveira R, Mendes RS, Domingos RF, Vasconcellos SA, Nascimento AL: **Plasminogen binding proteins and plasmin generation on the surface of *Leptospira* spp.: the contribution to the bacteria-host interactions.** *J Biomed Biotechnol* 2012, **2012**:758513.
 105. Meri T, Murgia R, Stefanel P, Meri S, Cinco M: **Regulation of complement activation at the C3-level by serum resistant leptospires.** *Microb Pathog* 2005, **39**:139-47.
 106. Pizza M, Donnelly J, Rappuoli R: **Factor H-binding protein, a unique meningococcal vaccine antigen.** *Vaccine* 2008, **26 Suppl 8**:I46-8.
 107. Seib KL, Serruto D, Oriente F, Delany I, Adu-Bobie J, Veggi D, Arico B, Rappuoli R, Pizza M: **Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37.** *Infect Immun* 2009, **77**:292-9.
 108. Oliveira R, Domingos RF, Siqueira GH, Fernandes LG, Souza NM, Vieira ML, de Moraes ZM, Vasconcellos SA, Nascimento AL: **Adhesins of *Leptospira* interrogans mediate the interaction to fibrinogen and inhibit fibrin clot formation in vitro.** *PLoS Negl Trop Dis* 2013, **7**:e2396.
 109. Atzingen MV, Goncalves AP, de Moraes ZM, Araujo ER, De Brito T, Vasconcellos SA, Nascimento AL: **Characterization of leptospiral proteins that afford partial protection in hamsters against lethal challenge with *Leptospira* interrogans.** *J Med Microbiol* 2010, **59**:1005-15.
 110. Atzingen MV, Vieira ML, Oliveira R, Domingos RF, Mendes RS, Barros AT, Goncalves AP, de Moraes ZM, Vasconcellos SA, Nascimento AL: **Evaluation of immunoprotective activity of six leptospiral proteins in the hamster model of leptospirosis.** *Open Microbiol J* 2012, **6**:79-87.

111. Vogel U, Taha MK, Vazquez JA, Findlow J, Claus H, Stefanelli P, Caugant DA, Kriz P, Abad R, Bambini S, Carannante A, Deghmane AE, Fazio C, Frosch M, Frosi G, Gilchrist S, Giuliani MM, Hong E, Ledroit M, Lovaglio PG, Lucidarme J, Musilek M, Muzzi A, Oksnes J, Rigat F, Orlandi L, Stella M, Thompson D, Pizza M, Rappuoli R, Serruto D, Comanducci M, Boccadifuoco G, Donnelly JJ, Medini D, Borrow R: **Predicted strain coverage of a meningococcal multicomponent vaccine (4CMenB) in Europe: a qualitative and quantitative assessment.** *Lancet Infect Dis* 2013, **13**:416-25.

Manuscript Number:

Title: The intermediate and C-terminus regions of leptospiral adhesin Lsa66 are responsible for the binding with plasminogen and extracellular matrix components

Article Type: Original Research Paper

Keywords: Leptospirosis; adhesin; plasminogen

Corresponding Author: Dr. Ana Lucia Nascimento, Ph.D

Corresponding Author's Institution: Instituto Butantan

First Author: Rosane Oliveira

Order of Authors: Rosane Oliveira; Renan Domingos; Zenaide Morais; Silvio Vasconcellos; Ivy Alves; Eliete Romero; Ana Lucia Nascimento, Ph.D

Abstract: Leptospirosis, a worldwide zoonotic infection, is an important human and veterinary health problem. We have previously identified a leptospiral multipurpose adhesin, Lsa66, capable of binding extracellular matrix (ECM) components and plasminogen (PLG). In this work, we report the cloning, expression, purification and characterization of three fragments derived from the full-length Lsa66: N-terminus, intermediate and C-terminus regions. We have employed *E. coli* BL21-SI, as expression cells. The recombinant fragments tagged with N-terminal hexahistidine were purified by metal-charged chromatography to major protein bands that are recognized by monoclonal anti-His tag antibodies. The recombinant fragments were evaluated for their capacity to attach to extracellular matrix (ECM) components and to PLG. The intermediate region binds to laminin, plasma fibronectin and to PLG. Laminin also binds to C-terminus. Antibodies in confirmed-leptospirosis serum samples recognized Lsa66, being the immune epitopes located at N-terminus and intermediate fragments. Our data strengthen that Lsa66 is expressed during infection and that this proteins might have a role in bacterial infection.

November 12, 2013

To Dr Jean-Pierre Gorvel
Editor-in-Chief of Microbial Pathogenesis

Dear Sir,

On behalf of the authors, I am submitting the manuscript entitled “**The intermediate and C-terminus regions of leptospiral adhesin Lsa66 are responsible for the binding with plasminogen and extracellular matrix components**”, to be considered for publication, as research paper, *in Microbial Pathogenesis*.

We have previously identified a leptospiral versatile adhesin, Lsa66, capable of binding extracellular matrix (ECM) components and plasminogen (PLG). We have mapped now the regions involved in the binding with ECM, PLG and serum reactivity. We show that the intermediate region binds laminin, plasma fibronectin and PLG. Laminin also binds to C-terminus, most probably via the overlapping region with the intermediate. Antibodies in confirmed-leptospirosis serum samples recognized Lsa66, being the immune epitopes located at N-terminus and intermediate fragments. Our data strengthen that Lsa66 is expressed during infection and that this proteins might have a role in bacterial infection.

I state that all authors have contributed significantly to the work and have seen and approved the content of this manuscript. This work has not been previously published in full or in part and is not being considered for publication elsewhere. Our submission includes a text with 1 table and 7 figures.

We very much look forward to hearing from you as to the suitability of our manuscript for publication.

Yours faithfully,

Ana Lucia T. Oller Nascimento, Ph.D.
Instituto Butantan, Sao Paulo, SP, Brazil
ana.nascimento@butantan.gov.br

**1 The intermediate and C-terminus regions of leptospiral adhesin Lsa66 are responsible
2 for the binding with plasminogen and extracellular matrix components**

3
4
5
6
7
8
9
10
11
12
13
14 Rosane Oliveira^{a,b}, Renan F. Domingos^{a,b}, Zenaide M. de Moraes^c; Silvio A. Vasconcellos^c;
15
16 Ivy J. Alves^d; Eliete C. Romero^e and Ana L. T. O. Nascimento^{a,b,*}
17
18
19
20

21
22 ^aCentro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503-900, São
23 Paulo, SP, Brazil
24

25 ^bInterunidades em Biotecnologia, Instituto de Ciências Biomédicas, USP, Avenida Prof.
26 Lineu Prestes, 1730, 05508-900, São Paulo, SP, Brazil
27

28 ^cLaboratório de Zoonoses Bacterianas do VPS, Faculdade de Medicina Veterinária e
29 Zootecnia, USP, Avenida Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, São Paulo,
30 SP, Brazil
31

32 ^dInstituto Adolfo Lutz, Laboratório Regional de Santos, Núcleo de Ciências Biomédicas,
33 Rua Silva Jardim, 90, 11015-020, Santos, SP, Brazil
34

35 ^eCentro de Bacteriologia, Instituto Adolfo Lutz, Av. Dr. Arnaldo, 355, 01246-902, São
36 Paulo, SP, Brazil
37

38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

*Corresponding author. Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil,
1500, 05503-900, São Paulo, SP, Brazil. Phone: (5511) 26279829; e-mail:
ana.nascimento@butantan.gov.br (A.L.T.O Nascimento).

Running title: Reactive fragments of Lsa66

1
2
3
4 **28 Abstract**
5
6

7 **29**
8

9 **30** Leptospirosis, a worldwide zoonotic infection, is an important human and veterinary health
10
11 **31** problem. We have previously identified a leptospiral multipurpose adhesin, Lsa66, capable
12
13 **32** of binding extracellular matrix (ECM) components and plasminogen (PLG). In this work,
14
15 **33** we report the cloning, expression, purification and characterization of three fragments
16
17 **34** derived from the full-length Lsa66: N-terminus, intermediate and C-terminus regions. We
18
19 **35** have employed *E. coli* BL21-SI, as expression cells. The recombinant fragments tagged
20
21 **36** with N-terminal hexahistidine were purified by metal-charged chromatography to major
22
23 **37** protein bands that are recognized by monoclonal anti-His tag antibodies. The recombinant
24
25 **38** fragments were evaluated for their capacity to attach to extracellular matrix (ECM)
26
27 **39** components and to PLG. The intermediate region binds to laminin, plasma fibronectin and
28
29 **40** to PLG. Laminin also binds to C-terminus. Antibodies in confirmed-leptospirosis serum
30
31 **41** samples recognized Lsa66, being the immune epitopes located at N-terminus and
32
33 **42** intermediate fragments. Our data strengthen that Lsa66 is expressed during infection and
34
35 **43** that this proteins might have a role in bacterial infection.
36
37
38
39
40
41
42
43
44

45 **45** Keywords: Leptospirosis, Lsa66, adhesin, plasminogen, reactive fragments
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1. Introduction

Leptospirosis is a widespread infectious disease caused by pathogenic species of *Leptospira*. The transmission of leptospirosis is associated with exposure of individuals near to wild or farm animals. Lately, the disease has been prevalent in cities with sanitation problems and a large population of rodent reservoirs that contaminate the environment through their urine [1]. Symptoms vary from general flu-like to most severe form of leptospirosis, known as Weil's syndrome, with hepatic, renal and pulmonary involvement and a mortality rate of 5 to 40% [1]. Leptospirosis has also a great economic impact in the agricultural industry because the disease affects livestock inducing abortions, stillbirths, infertility, reduced milk production and death [1].

The genome sequencing of different leptospiral species and serovars is now available [2-8]. Genome annotation together with bioinformatics tools has facilitated the identification of many unknown, putative surface exposed proteins, opening possibilities for studies on protein function. In addition, it is possible that a number of these membrane proteins mediate the initial adhesion to host cells [9-11]. Indeed, several adhesins of *Leptospira* have been identified [12-30]. In addition, some adhesins are also PLG-binding proteins, capable of generating plasmin, which could facilitate the bacteria to overcome tissue barriers and dissemination [17, 19, 20, 26, 27, 31, 32]. Lsa66, is one of those multipurpose adhesins that binds laminin, plasma fibronectin, PLG and is recognized by antibodies in positive leptospirosis serum samples. In addition, this protein was identified in leptospiral outer membrane microarray as fibronectin binding protein [29].

1
2
3
4 69 In this work, we report the cloning, protein expression and purification of three
5
6 70 fragments generated from the full length Lsa66. The purified recombinant fragments were
7
8
9 71 evaluated for their ability to bind ECM components, PLG and leptospirosis positive serum
10
11 72 samples. Our data show that antibodies present in leptospirosis sera recognize the N-
12
13
14 73 terminus and intermediate regions, the central fragment binds plasma fibronectin and
15
16 74 laminin, and C-terminus is reactive only with laminin. The characterization of these
17
18
19 75 interactions is presented.
20
21 76
22
23
24 77
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2. Materials and Methods

2.1. ECM and biological components

Macromolecules (laminin and plasma fibronectin), including the control protein BSA, were purchased from Sigma-Aldrich (St. Louis, MO). Laminin-1 was derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; plasma fibronectin was isolated from human plasma and native PLG, which was purified from human plasma, was purchased from EMD Chemicals, Inc. (San Diego, CA).

2.2. *Leptospira* strains and sera

The non-virulent *Leptospira interrogans* serovar Copenhageni strain M 20 was cultured at 28°C under aerobic conditions in liquid EMJH medium (Difco®) 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%) [33]. *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Confirmed-leptospirosis serum samples were from Instituto Adolfo Lutz collection, São Paulo, Brazil.

2.3. 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 by guanidine-detergent lysing method using DNAzol® Reagent (Invitrogen), according to the manufacturer's instructions. Primers were designed according to *L. interrogans* serovar

Copenhageni genome sequences (GenBank accession [AE016823](#)) and are listed in Table 01. 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 MgCl₂, 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 - 4 min, followed by 40 cycles at 94°C - 50 sec, 60°C - 50 sec, 72°C - 90 sec, and a final extension cycle of 7 min at 72°C. The PCR products obtained for each corresponding fragment were cloned into pGEM-T easy vector (Promega) and subcloned into the pAE expression vector [34] at the restriction sites depicted in Table 01. The pAE vector allows the expression of recombinant proteins with a minimal 6 x His-tag at the N-terminus. All cloned sequences were confirmed by DNA sequencing in an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA).

2.4. Expression and purification of recombinant fragments

Recombinant fragments expression were achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotically induced promoter proU. *E. coli* BL21 (SI) containing recombinant plasmids were grown at 30°C in 2YT broth without NaCl and with 100 μ g/ml ampicillin with continuous shaking until an optical density at 600 nm of 0.6 to 0.8 was reached. Recombinant fragments synthesis was induced by the addition of 30 mM NaCl. After three hours, the cells were harvested by centrifugation, the bacterial pellets resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 μ g/ml of lysozyme, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride [PMSF]). The bacterial cell pellets were lysed on ice with the aid of a sonicator (Ultrasonic Processor; GE Healthcare). The insoluble fraction was washed with 20 ml of buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 M urea and 0,1% Triton X-100) and resuspended in a buffer

1
2
3
4 126 containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% (vol/vol) glycerol and 8 M urea.
5
6
7 127 The recombinant fragments were then purified through metal chelating chromatography in
8
9 128 a Sepharose fast flow column (GE Healthcare) and fractions were analyzed in 15% SDS-
10
11 129 PAGE. The fragments were extensively dialyzed against phosphate-buffered saline (PBS),
12
13 130 pH 7.4, 0.1% (wt/vol) glycine solution (at the proportion of 10 ml of protein per 1000 ml of
14
15 131 buffer) containing decreasing gradient of glycerol (10 – 0%), pooled and stored at 4°C.
16
17
18
19 132

20 21 133 2.5. Antiserum

22
23
24 134 Five female BALB/c mice (4 – 6 weeks old) were immunized subcutaneously with
25
26 135 10 µg of the recombinant fragments. The recombinant fragments were adsorbed in 10%
27
28 136 (vol/vol) of Alhydrogel (2% Al(OH)₃, Brenntag *Biosector*, Denmark), used as adjuvant.
29
30
31 137 Two subsequent booster injections were given at two-week intervals with the same
32
33 138 preparation of 10 µg of the fragments. Negative-control mice were injected with PBS.
34
35
36 139 Twice week after each immunization, the mice were bled from the retro-orbital plexus and
37
38 140 the pooled sera were analysed by enzyme-linked immunosorbent assay (ELISA) for
39
40
41 141 determination of antibody titer.
42

43 142

44 45 143 2.6. Ethics Statement

46
47
48 144 All animal studies were approved by the Ethical Committee for Animal Research of
49
50 145 Instituto Butantan, São Paulo, SP, Brazil under protocol nº 798/11. The Committee in
51
52 146 Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of
53
54
55 147 Animal Experimentation.
56

57
58 148

59 60 149 2.7. Immunoblotting assay

The purified recombinant fragments were loaded into 15% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi-dry equipment. Membranes were blocked with 10% non-fat dried milk and 1% BSA in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-N-terminus, anti-Intermediate and anti-C-terminus mouse serum (1:500) or anti-his antibody (1:2,000) (Sigma) for 2 h at room temperature. After washing, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5,000; Sigma) in PBS-T for 1 h. The fragments reactivity was revealed by use of the ECL reagent kit chemiluminescence substrate (GE Healthcare). The luminescence generated by the reaction was detected with the aid of a Carestream molecular imaging instrument (Equilab, Whitestone, NY) connected to Gel Logic 2200PRO.

161

2.8. Microscopic agglutination test (MAT)

The microscopic agglutination test was performed according to [1] and was described in [17].

165

2.9. ELISA for detection of human antibodies

Human IgG and IgM antibodies against N-terminus, Intermediate and C-terminus were evaluated by ELISA. In brief, samples (negative and positive MAT sera from 18 confirmed leptospirosis patients) were diluted 1:200 and evaluated for total IgG using peroxidase-conjugated anti-human IgG and IgM antibodies, 1:5,000 (Sigma, USA). Cut-off values were set at three standard deviations above the mean OD₄₉₂ of normal human serum obtained from Sigma-Aldrich (St. Louis, MO).

173

2.10. Binding of recombinant fragments to laminin, plasma fibronectin and PLG

The binding of the recombinant fragments to laminin, plasma fibronectin and PLG was evaluated according to a previously published protocol [10] with some modifications as follows: ELISA plate wells were coated with 1 µg of laminin, plasma fibronectin, PLG and BSA (negative control) in 100 µL of PBS for 2 h at 37°C. The wells were washed three times with PBS-T and then blocked with 200 µL of 10% (wt/vol) non-fat dry milk plus 1% BSA (overnight at 4°C). One microgram of each recombinant fragment (N-terminus, Intermediate and C-terminus) was added per well in 100 µL of PBS, and fragments were allowed to attach to the different substrates for 2 h at 37°C. After washing six times with PBS-T, bound fragments were detected by adding an appropriate dilution of mouse antiserum in 100 µL of PBS (1:1,000). Dilutions of mouse antiserum against each recombinant protein were equalized as to give an OD_{492 nm} value of 1.0. In addition, anti-polyhistidine monoclonal antibodies were employed as protein-binding probes at 1:2000 dilutions. Incubation proceeded for 1 h at 37°C, and after three washings with PBS-T, 100 µL of a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG in PBS was added per well for 1 h at 37°C. The wells were washed three times, and *o*-phenylenediamine (1 mg/mL) in citrate phosphate buffer (pH 5.0) plus 1 µL/mL H₂O₂ was added (100 µL per well). The reaction was allowed to proceed for 10 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 (Multiskan EX; Labsystems Uniscience). For statistical analyses, the binding of recombinant fragments to ECM macromolecules and PLG was compared to its binding to BSA by Student's two-tailed *t* test.

196

2.11. Dose-response curves

1
2
3
4 198 First, 96-wells plates were coated with 1 µg of laminin, plasma fibronectin and PLG
5
6 199 in 100 µL of PBS for 2 h at 37°C. Plates were then blocked, and increasing concentrations
7
8 200 of the purified recombinant fragments (0 to 4000 nM) were added (100 µL/well in PBS).
9
10 201 The assessment of bound fragments was performed by incubation for 1 h at 37°C with the
11
12 202 antiserum raised against each fragment at the dilution of 1:1,000 followed by HRP-
13
14 203 conjugated goat anti-mouse IgG (Sigma) (1:5,000 in PBS). The reaction was detected with
15
16 204 OPD as described above.
17
18
19
20
21
22

23 205

24 206 2.12. Dissociation constant (K_D) for the recombinant fragments binding to ECM and PLG

25
26 207 The ELISA data of the dose-response curves were used to calculate the equilibrium
27
28 208 dissociation constant (K_D), according to a method described previously [16], following the
29
30 209 equation $K_D = (A_{max} \cdot [Protein]) / A - [Protein]$, where A is the absorbance at a given
31
32 210 protein concentration, A_{max} is the maximum absorbance for the ELISA plate reader
33
34 211 (equilibrium), [Protein] is the protein concentration, and K_D is the equilibrium dissociation
35
36 212 constant for a given protein concentration (ELISA data point).
37
38
39
40

41 213

42 214 2.13. Interaction of denatured recombinant fragments with ECM and PLG

43
44 215 First, ELISA plates were coated with 100 µL of 10 µg/ml laminin, plasma
45
46 216 fibronectin or PLG, which were allowed to adhere for 2 h at 37°C. Plates were washed
47
48 217 three times with PBS-T and blocked with 200 µL of 10% (wt/vol) non-fat dry milk plus 1%
49
50 218 BSA (overnight at 4°C). The recombinant fragments were denatured by incubation at 96°C
51
52 219 for 10 min; 1 µg of each was added per well in 100 µL of PBS. The recombinant fragments
53
54 220 were allowed to attach to ECM and PLG at 37°C for 90 min. After washing six times with
55
56 221 PBS-T, bound recombinant fragments were detected by incubation with mouse serum
57
58
59
60
61
62
63
64
65

222 raised against the respective fragments (dilutions described above) at 37°C for 1 h. After
223 three washings with PBS-T, 100 µL of a 1:5,000 dilution of HRP-conjugated rabbit anti-
224 mouse IgG (Sigma) in PBS was added per well for 1 h at 37°C. The detection was
225 performed with OPD, as previously described. BSA was used as negative control (data not
226 shown). For statistical analyses, the attachment of denatured recombinant fragments to
227 laminin, plasma fibronectin and PLG was compared to untreated fragments binding by the
228 Student two-tailed *t* test (* *P* < 0.005; ** *P* < 0.05).

229

230 2.14. Antibody inhibition assay

231 Ninety-six well plates were coated with 100 µL of 10 µg/ml laminin, plasma
232 fibronectin and PLG, which were allowed to adhere for 2 h at 37°C. Plates were then
233 blocked overnight with 200 µL of 10% (wt/vol) nonfat dry milk plus 1% BSA. Prior to the
234 next step, the recombinant fragments were incubated for 1 h at 37°C with the respective
235 antibodies diluted 1:200 in 100 µL of PBS. After the incubation, each recombinant protein
236 (1 µg) was added per well in 100 µL of PBS, and allowed to attach to laminin, plasma
237 fibronectin and PLG for 90 min at 37°C. After washing six times with PBS-T, bound
238 recombinant fragments were detected by adding monoclonal HRP-conjugated mouse anti-
239 polyhistidine-HRP (Sigma) diluted 1:2,000. Incubation proceeded for 1 h at 37°C. The
240 detection was performed with OPD, as previously described. BSA was used as negative
241 control (data not shown). For statistical analyses, the attachment of blocked recombinant
242 fragments to ECM and PLG was compared to the binding with the untreated proteins by the
243 Student two-tailed *t* test (* *P* < 0.005; ** *P* < 0.05).

244

245 2.15. Metaperiodate treatment of laminin and plasma fibronectin

1
2
3
4 246 Microtitre wells were coated with 1 µg of laminin or plasma fibronectin in 50 mM
5
6 247 sodium acetate buffer, pH 5.0, and incubated for 16 h at 4°C. Wells were washed three
7
8 248 times with the same buffer, and ECMs were treated with sodium metaperiodate (0–100
9
10 249 mM), in the same buffer for 15 min at 4°C in the dark. After three washes with 50 mM
11
12 250 sodium acetate buffer, wells were blocked with 200 µL of 1% BSA for 1 h at 37°C.
13
14 251 Binding of recombinant fragments (1 µg/100 µL in PBS per well) to metaperiodate-treated
15
16 252 ECMs was evaluated as previously described.
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3. Results

255

3.1. Expression and purification of Lsa66 fragments

257 N-terminus, intermediate and C-terminus portions of the Lsa66 protein (Fig. 1A)
258 were amplified by PCR from genomic *Leptospira* DNA. The amplified fragments were
259 cloned into the pAE vector and proteins were expressed with 6XHis tag at the N-terminus,
260 using *E. coli* BL21 SI as host. The three fragments were expressed in their insoluble form,
261 as inclusion bodies. Fragments were recovered from inclusion bodies and the purification
262 was performed by metal-chelating chromatography under denaturing conditions followed
263 by refolding by gradual removal of urea. The recombinant fragments were purified by
264 nickel affinity chromatography, and an aliquot of each protein was analysed by SDS-PAGE
265 (Fig. 1B). The stained gel shows protein bands of approximately 25, 37 and 23 kDa,
266 corresponding to N-terminus, intermediate and C-terminus regions, respectively (Fig. 1B).
267 The recombinant fragments were confirmed by Western blotting with anti-His tag
268 monoclonal antibodies and with polyclonal antibodies raised in mice against each fragment.
269 The data are depicted in Figure 1C and 1D, respectively, and show that anti-His tag
270 antibodies recognized all recombinant fragments (Fig. 1C), while blotted recombinant
271 fragments were recognized by the respective homolog antiserum (Fig. 1D).

272

3.2. Reactivity of recombinant fragments with sera from confirmed cases of leptospirosis

274 Because antibodies present in confirmed leptospirosis cases recognize Lsa66, we
275 decided to evaluate which portion of the protein was responsible for this reactivity. Thus,
276 N-terminus, intermediate and C-terminus fragments were set to react with antibodies
277 present in serum samples of early (MAT-) and convalescent (MAT+) phases of

leptospirosis. We performed ELISA and employed 18-paired samples from both phases of the disease. N-terminus and intermediate fragments exhibited reactivity for negative and positive MAT (Fig. 2A and 2B), while C-terminus showed no reactivity with samples of both phases of the disease (Fig. 2C). At the early phase of the disease, 28% responders presented IgG antibodies against N-terminus, while at the convalescent phase the number increases to 39%. On the other hand, intermediate fragment showed 28 and 66.5% reactivity for negative and positive MAT, respectively.

285

3.3. Evaluation of Lsa66 recombinant fragments on the interaction to laminin, plasma fibronectin and PLG

Lsa66 is OmpA-like protein with ECM and PLG binding properties. Thus, we set out to investigate which portion of Lsa66 is involved in these interactions. Immobilized laminin, plasma fibronectin, PLG and the control protein BSA were incubated with the recombinant fragments – N-terminus, intermediate and C-terminus. The reaction was probed with polyclonal antibodies against each fragment and showed that the intermediate fragment interacts with laminin, plasma fibronectin and PLG while C-terminus fragment only binds to laminin (Fig. 3A). No binding was observed with N-terminus with any tested component. Similar results were achieved when binding was performed using monoclonal anti-his tag antibodies (Fig. 3B). The binding of Lsa66, intermediate and C-terminus fragments to laminin and the interaction of Lsa66 and intermediate fragment to plasma fibronectin and to PLG was also assessed after submitting them to denaturing conditions at 96°C for 10 min and after blocking the proteins with the corresponding antibody. The adhesion of heat-denatured proteins to laminin was almost totally abolished in the case of Lsa66, intermediate and C-terminus fragment (Fig. 4A), while in the case of antibody

blocking, adhesion to laminin remained 30, 60 and 18% for intermediate, C-terminus and Lsa66, respectively (Fig. 4A). This suggests that non-immune epitopes is involved in the binding, particularly for the binding with C-terminus fragment. For plasma fibronectin, 25 and 50% of the interaction remained after intermediate fragment and Lsa66 were denatured, respectively (Fig. 4B), whereas very low percentage of the interaction to plasma fibronectin has been observed after Lsa66 and intermediate fragment were antibody-blocked (Fig. 4B). Binding of Lsa66 and intermediate fragment to PLG persisted 20 and 40%, respectively, after the proteins were antibody-blocked or heat denatured (Fig. 4C).

3.4. Characterization of recombinant fragments interaction with laminin, plasma fibronectin and PLG

The binding between intermediate and C-terminus fragments with laminin was evaluated on a quantitative basis as depicted in Figure 5A. A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant fragments (0–2000 nM) were allowed to adhere to a fixed laminin concentration (1 μ g). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant fragments with laminin are 51.10 ± 5.50 nM and 75.32 ± 14.50 nM for intermediate and C-terminus, respectively. Likewise, the interaction of intermediate fragment with plasma fibronectin and with PLG was also assessed by keeping the plasma fibronectin and PLG concentration constant (1 μ g) and changing the Intermediate fragment concentration (0–4000 nM), as shown in Figure 5B and 5C, respectively. The calculated dissociation equilibrium constants (K_D) for the intermediate fragment with plasma fibronectin and PLG is 166.03 ± 41.50 nM and 744.70 ± 177.50 nM, respectively.

3.5. Influence of carbohydrates moieties on the binding of Lsa66 and recombinant fragments to laminin and plasma fibronectin

To analyse the involvement of the carbohydrate moieties in the interaction of laminin with Lsa66, intermediate and C-terminus fragment, as well as, in the interaction of plasma fibronectin with Lsa66 and intermediate fragment, laminin and plasma fibronectin were oxidized by increasing concentrations of sodium metaperiodate (5–100 mM) for 15 min at 4 °C. The oxidation effect is shown in Figure 6. No reduction in the interaction of oxidized-laminin was observed with Lsa66, intermediate and C-terminus (Fig. 6A), whereas the reduction in the interaction of oxidized-plasma fibronectin with Lsa66 and intermediate fragment was dose-dependent and statistically significant when compared to non-treated wells – 0 nM (Fig. 6B) (* $P < 0.01$; ** $P < 0.001$). These results indicate the importance of sugar residues in the interaction of plasma fibronectin with Lsa66 and intermediate fragment.

3.6. Reactive regions of Lsa66 protein

Intermediate fragment was generated with an overlap region from 170 to 219 amino acids of N-terminus and with an intersection from 420 to 483 amino acids of C-terminus region (Fig. 1A). The results with leptospirosis serum samples show major reactivity with the intermediate region, minor with the N-terminus and none with C-terminus. Moreover, our data show that both intermediate and C-terminus fragments are capable of binding laminin (Fig. 4A and 5A), while only intermediate fragment has the ability to interact with plasma fibronectin and PLG (Fig. 4B and 4C; Fig. 5B and 5C). Based on these results, we propose the scheme depicted in Fig. 7.

4. Discussion

We have identified a protein with OmpA-like domain at C- terminus (OmpA C-like) in *Leptospira* spp., encoded by the LIC10258 gene, we called Lsa66 [17]. We have reported that this protein is expressed at the surface of bacteria, and capable of interacting with laminin, plasma fibronectin and PLG. Moreover, Lsa66 was reactive with leptospirosis serum samples at the convalescent phase (MAT+). Recently, this protein has been identified as human plasma fibronectin – ligand in leptospiral outer membrane protein microarray studies [29]. OmpA outer membrane protein of *Escherichia coli* and other enterobacteria have been shown to have adhesin/invasin activities [35].

Loa22 was the first protein identified in pathogenic *Leptospira* having a C-terminal OmpA consensus [36]. This protein was further reported to be essential for the leptospiral virulence [37] and to promote inflammatory responses in cultured rat renal cells [38].

OmpA-like proteins of leptospires have been described as vaccine candidates for leptospirosis [39]. Thus, it is possible that OmpA-like proteins have a function in leptospiral pathogenesis.

In this work, we report cloning, expression and purification of the Lsa66 fragments intended to evaluate which portion of the protein was reactive with positive serum samples of leptospirosis and involved in the binding with ECM and PLG. We have generated three fragments from Lsa66 full length protein, originally cloned from 30 to 590 amino acid: the N-terminus portion, from 30 to 219, the intermediate or central region from 170 to 483 and the C-terminus fragment from 420 to 590, containing the OmpA-like domain, from 504 to 583 amino acid. The three fragments were produced in *E. coli*, in their insoluble form, and

1
2
3
4 374 were purified from metal chelating chromatography under denaturation condition, and
5
6 375 refolded after urea removal by dialysis.
7
8

9 376 Leptospire present a vast repertoire of ECM- binding proteins, which most
10
11 377 probably mediate the adhesion of the bacteria to host (Vieira *et al.*, 2013, submitted to
12
13 378 publication). It has been reported that the interaction of *Leptospira* spp. with PLG with the
14
15 379 generation of plasmin on the bacterial surface lead to laminin and fibronectin degradation, a
16
17 380 mechanism that could help bacteria penetration [40, 41]. Indeed, this proteolytic system has
18
19 381 been described for several invasive pathogens [42, 43], including the spirochetes *Borrelia*
20
21 382 and *Treponema* [44, 45]. Several leptospiral proteins have been described with ECM- and
22
23 383 PLG- binding capacities and Lsa66 was one of them [17, 19, 20, 26, 27, 31, 32].
24
25
26
27

28 384 The intermediate and C-terminus fragments of Lsa66 are involved in the binding
29
30 385 with laminin, whereas plasma fibronectin and PLG interact only with the intermediate
31
32 386 region. The K_D values for the binding of laminin with intermediate and C-terminus are in
33
34 387 the same order of magnitude of the one obtained with Lsa66, 55.4 ± 15.9 nM and the same
35
36 388 ligand [17]. The C-terminus reactive site most probably does not encompass the OmpA-like
37
38 389 domain because Loa22 protein does not bind laminin [10]. Moreover, it is possible that the
39
40 390 binding of laminin with C-terminus occurs at the region where there is an overlapping
41
42 391 between the intermediate and C-terminus (420-483). The calculated K_D for the binding of
43
44 392 plasma fibronectin and the intermediate fragment is also similar to the one obtained with
45
46 393 the full-length protein (290.8 ± 11.8 nM). However, the K_D for the binding of PLG and
47
48 394 central region is one order of magnitude higher than the one calculated with Lsa66 ($744.7 \pm$
49
50 395 177.5 versus 68.8 ± 25.2 nM) [17]. These results suggest that in the case of reaction with
51
52 396 PLG, the protein conformation is important, while with laminin and plasma fibronectin
53
54 397 only linear epitopes are crucial for the binding.
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4 398 Lsa66 has shown positive reactivity with serum samples from patients diagnosed with
5
6 399 leptospirosis [17]. In the present study, we show that N-terminus and intermediate
7
8 400 fragments are involved in antibody recognition, with 39 and 67% of responders with MAT
9
10 401 positive, respectively. In addition, the N-terminus region that interacts with antibodies
11
12 402 might belong to the region where there is an intersecting with the intermediate segment.
13
14 403 The fact that there is no contribution of the C-terminus region on antibody recognition
15
16 404 suggests that this region, which comprises the OmpA-like domain, has no immune
17
18 405 epitopes. This may explain why 60% of the binding remained when laminin was set to react
19
20 406 with C-terminus fragment previously blocked with antiserum (see Fig. 4A).
21
22
23
24
25
26
27
28 407
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

408 5. Conclusions

409

410 We have identified ECM, PLG and antibody reactive regions of the adhesin Lsa66.

411 Mapping these binding sites should enlighten our understanding of *Leptospira*-host

412 interaction at molecular level and help identify immune targets needed to fight

413 leptospirosis.

414

415

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgements

This work was supported by FAPESP, CNPq and Fundacao Butantan, Brazil; RO and RFD have scholarships from FAPESP (Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

420

421

Table 1. Lsa66 fragments, sequence of the primers employed for DNA amplification, and molecular mass of expressed recombinant proteins.

Lsa66 fragments	Sequence of primers for PCR amplification	Recombinant fragment molecular mass
N-terminus	F: 5' <u>GGATCC</u> GAAGCCTTCTCACCCAAT 3' (BamHI) R: 5' <u>CCATGG</u> TTAATCTGTTCCATCCCAAAT 3' (NcoI)	25.5 kDa
Intermediate	F: 5' <u>GGATCC</u> TCCTCTCGTATCAATATTTCTCAA 3' (BamHI) R: 5' <u>CCATGG</u> TTAATGATCTTCTTCTAACACCAG 3' (NcoI)	36.9 kDa
C-terminus	F: 5' <u>GGATCC</u> ATCAAACTTGGAATGGC 3' (BamHI) R: 5' <u>CCATGG</u> TTAAAGTGAAAGATAAAAATCGATT 3' (NcoI)	23.3 kDa

References

- [1] Faine S, Adler B, Bolin C, Perolat P. *Leptospira* and Leptospirosis. Melbourne, Australia: MediSci; 1999.
- [2] Ren SX, Fu G, Jiang XG, Zeng R, Miao YG, Xu H, et al. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* 2003; 422:888-93.
- [3] Bulach DM, Zuerner RL, Wilson P, Seemann T, McGrath A, Cullen PA, et al. Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc Natl Acad Sci U S A* 2006; 103:14560-5.
- [4] Picardeau M, Bulach DM, Bouchier C, Zuerner RL, Zidane N, Wilson PJ, et al. Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS One* 2008; 3:e1607.
- [5] Ricaldi JN, Fouts DE, Selengut JD, Harkins DM, Patra KP, Moreno A, et al. Whole genome analysis of *Leptospira licerasiae* provides insight into leptospiral evolution and pathogenicity. *PLoS Negl Trop Dis* 2012; 6:e1853.
- [6] Chou LF, Chen YT, Lu CW, Ko YC, Tang CY, Pan MJ, et al. Sequence of *Leptospira santarosai* serovar Shermani genome and prediction of virulence-associated genes. *Gene* 2012; 511:364-70.
- [7] Nascimento AL, Verjovski-Almeida S, Van Sluys MA, Monteiro-Vitorello CB, Camargo LE, Digiampietri LA, et al. Genome features of *Leptospira interrogans* serovar Copenhageni. *Braz J Med Biol Res* 2004; 37:459-77.
- [8] Nascimento AL, Ko AI, Martins EA, Monteiro-Vitorello CB, Ho PL, Haake DA, et al. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J Bacteriol* 2004; 186:2164-72.
- [9] Merien F, Truccolo J, Baranton G, Perolat P. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar icterohaemorrhagiae. *FEMS Microbiol Lett* 2000; 185:17-22.
- [10] Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM, Vieira ML, et al. A newly identified leptospiral adhesin mediates attachment to laminin. *Infect Immun* 2006; 74:6356-64.
- [11] Choy HA, Kelley MM, Chen TL, Moller AK, Matsunaga J, Haake DA. Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect Immun* 2007; 75:2441-50.
- [12] Stevenson B, Choy HA, Pinne M, Rotondi ML, Miller MC, Demoll E, et al. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS One* 2007; 2:e1188.
- [13] Hoke DE, Egan S, Cullen PA, Adler B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudoalteromonas tunicata*. *Infect Immun* 2008; 76:2063-9.
- [14] Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Moraes ZM, Barbosa AS, et al. In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect Immun* 2008; 76:2642-50.
- [15] Lin YP, Greenwood A, Yan W, Nicholson LK, Sharma Y, McDonough SP, et al. A novel fibronectin type III module binding motif identified on C-terminus of *Leptospira* immunoglobulin-like protein, LigB. *Biochem Biophys Res Commun* 2009; 389:57-62.

- [16] Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, Chang YF. Repeated domains of leptospira immunoglobulin-like proteins interact with elastin and tropoelastin. *J Biol Chem* 2009; 284:19380-91.
- [17] Oliveira R, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. *PLoS One* 2011; 6:e21962.
- [18] Longhi MT, Oliveira TR, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA, et al. A newly identified protein of *Leptospira interrogans* mediates binding to laminin. *J Med Microbiol* 2009; 58:1275-82.
- [19] Mendes RS, Von Atzingen M, de Moraes ZM, Goncalves AP, Serrano SM, Asega AF, et al. The novel leptospiral surface adhesin Lsa20 binds laminin and human plasminogen and is probably expressed during infection. *Infect Immun* 2011; 79:4657-67.
- [20] Domingos RF, Vieira ML, Romero EC, Goncalves AP, de Moraes ZM, Vasconcellos SA, et al. Features of two proteins of *Leptospira interrogans* with potential role in host-pathogen interactions. *BMC Microbiol* 2012; 12:50.
- [21] Lima SS, Ching AT, Favaro RD, Da Silva JB, Oliveira ML, Carvalho E, et al. Adhesin activity of *Leptospira interrogans* lipoprotein identified by in vivo and in vitro shotgun phage display. *Biochem Biophys Res Commun* 2013; 431:342-7.
- [22] Atzingen MV, Gomez RM, Schattner M, Pretre G, Goncalves AP, de Moraes ZM, et al. Lp95, a novel leptospiral protein that binds extracellular matrix components and activates e-selectin on endothelial cells. *J Infect* 2009; 59:264-76.
- [23] Oliveira TR, Longhi MT, Goncalves AP, de Moraes ZM, Vasconcellos SA, Nascimento AL. LipL53, a temperature regulated protein from *Leptospira interrogans* that binds to extracellular matrix molecules. *Microbes Infect* 2010; 12:207-17.
- [24] Atzingen MV, Barbosa AS, De Brito T, Vasconcellos SA, de Moraes ZM, Lima DM, et al. Lsa21, a novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol* 2008; 8:70.
- [25] Vieira ML, de Moraes ZM, Goncalves AP, Romero EC, Vasconcellos SA, Nascimento AL. Lsa63, a newly identified surface protein of *Leptospira interrogans* binds laminin and collagen IV. *J Infect* 2010; 60:52-64.
- [26] Souza NM, Vieira ML, Alves IJ, de Moraes ZM, Vasconcellos SA, Nascimento AL. Lsa30, a novel adhesin of *Leptospira interrogans* binds human plasminogen and the complement regulator C4bp. *Microb Pathog* 2012; 53:125-34.
- [27] Fernandes LG, Vieira ML, Kirchgatter K, Alves IJ, de Moraes ZM, Vasconcellos SA, et al. OmpL1 is an extracellular matrix- and plasminogen-interacting protein of *Leptospira* spp. *Infect Immun* 2012; 80:3679-92.
- [28] Pinne M, Choy HA, Haake DA. The OmpL37 surface-exposed protein is expressed by pathogenic *Leptospira* during infection and binds skin and vascular elastin. *PLoS Negl Trop Dis* 2010; 4:e815.
- [29] Pinne M, Matsunaga J, Haake DA. Leptospiral outer membrane protein microarray, a novel approach to identification of host ligand-binding proteins. *J Bacteriol* 2012; 194:6074-87.
- [30] Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, Abreu PA, et al. Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. *FEBS Lett* 2009; 583:1381-5.
- [31] Vieira ML, Atzingen MV, Oliveira TR, Oliveira R, Andrade DM, Vasconcellos SA, et al. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PLoS One* 2010; 5:e11259.

- [32] Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, Stevenson B. Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infect Immun* 2010; 78:2053-9.
- [33] Turner LH. Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires. *Trans R Soc Trop Med Hyg* 1970; 64:623-46.
- [34] Ramos CR, Abreu PA, Nascimento AL, Ho PL. A high-copy T7 *Escherichia coli* expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. *Braz J Med Biol Res* 2004; 37:1103-9.
- [35] Smith SG, Mahon V, Lambert MA, Fagan RP. A molecular Swiss army knife: OmpA structure, function and expression. *FEMS Microbiol Lett* 2007; 273:1-11.
- [36] Koizumi N, Watanabe H. Molecular cloning and characterization of a novel leptospiral lipoprotein with OmpA domain. *FEMS Microbiol Lett* 2003; 226:215-9.
- [37] Ristow P, Bourhy P, da Cruz McBride FW, Figueira CP, Huerre M, Ave P, et al. The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog* 2007; 3:e97.
- [38] Zhang Y, Bao L, Zhu H, Huang B, Zhang H. OmpA-like protein Loa22 from *Leptospira interrogans* serovar Lai is cytotoxic to cultured rat renal cells and promotes inflammatory responses. *Acta Biochim Biophys Sin (Shanghai)* 2010; 42:70-9.
- [39] Yan W, Faisal SM, McDonough SP, Chang CF, Pan MJ, Akey B, et al. Identification and characterization of OmpA-like proteins as novel vaccine candidates for Leptospirosis. *Vaccine* 2010; 28:2277-83.
- [40] Vieira ML, Vasconcellos SA, Goncales AP, de Moraes ZM, Nascimento AL. Plasminogen acquisition and activation at the surface of leptospira species lead to fibronectin degradation. *Infect Immun* 2009; 77:4092-101.
- [41] Vieira ML, Atzingen MV, Oliveira R, Mendes RS, Domingos RF, Vasconcellos SA, et al. Plasminogen binding proteins and plasmin generation on the surface of *Leptospira* spp.: the contribution to the bacteria-host interactions. *J Biomed Biotechnol* 2012; 2012:758513.
- [42] Siemens N, Patenge N, Otto J, Fiedler T, Kreikemeyer B. *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing. *J Biol Chem* 2011; 286:21612-22.
- [43] Stie J, Bruni G, Fox D. Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PLoS One* 2009; 4:e5780.
- [44] Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, Benach JL. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect Immun* 1995; 63:2478-84.
- [45] Fenno JC, Tamura M, Hannam PM, Wong GW, Chan RA, McBride BC. Identification of a *Treponema denticola* OppA homologue that binds host proteins present in the subgingival environment. *Infect Immun* 2000; 68:1884-92.

Legend to Figures

Figure 1. (A) Schematic representation of Lsa66. The drawing shows, in amino acids, the signal peptide (1 to 29), the OmpA-like domain (504 to 583), N-terminus (30 to 219), intermediate (170 to 483) and C-terminus fragment (420 to 590). **(B) SDS-PAGE.** Comassie blue-stained gel depicting the purified recombinant fragments. M, represents molecular mass standard proteins. **(C) and (D) Western blotting analysis.** The recombinant fragments were membrane blotted and proteins were probed with **(C)** monoclonal anti-His tag antibodies and **(D)** the corresponding homolog antiserum raised in mice.

Figure 2. Recognition of Lsa66 fragments by IgG antibodies in leptospirosis serum samples. Positive sera responders were detected by ELISA with each recombinant fragment and serum samples from leptospirosis in both the early (MAT-) and convalescent (MAT+) phases of the disease. The reactivity was evaluated as total IgG antibodies. The cut-off values are depicted as horizontal bars and were defined as the mean plus 3 standard deviations obtained for commercial sera from healthy individuals. **A, B** and **C** show the data for N-terminus, intermediate and C-terminus fragments, respectively.

Figure 3. Binding of recombinant fragments to ECM components and to PLG. Microtiter plates were coated with 1 µg of laminin, plasma fibronectin and PLG. BSA was included as control. 1 µg of the N-terminus, intermediate and C-terminus fragments were added per well and the binding was measured by ELISA. **(A)** Fragment binding was detected by polyclonal antibodies against each recombinant fragment, while in **(B)** fragment binding was evaluated by anti-polyhistidine monoclonal antibodies. Data represent the mean ± the standard deviation from three independent experiments. For

1
2
3
4 583 statistical analyses, the attachment of recombinant fragments to the ECM macromolecules
5
6 584 and PLG was compared to its binding to BSA by the Student two-tailed *t* test (**P* < 0.0005;
7
8
9 585 ***P* < 0.005).

10
11
12 586 **Figure 4. Effect of denaturing or antibody-blocking the recombinant fragments on the**
13
14 587 **interaction with ECM and PLG.** Wells were coated with 1 µg laminin (A), plasma
15
16 588 fibronectin (B) or PLG (C), and allowed to interact with denatured or antibody-blocked
17
18 589 recombinant fragments. The fragments were denatured by incubation for 10 min at 96°C or
19
20 590 blocked with polyclonal antibodies. Data are expressed as A₄₉₂ nm ± the standard deviation
21
22 591 of three independent experiments, each performed in triplicate. Significance was assessed
23
24 592 by comparison with the “untreated fragments” by Students two-tailed *t* test (**P* < 0.005;
25
26 593 ***P* < 0.05).

27
28
29 594 **Figure 5. Characterization of recombinant fragments binding to ECM components**
30
31 595 **and to PLG.** To evaluate the specificity of interaction of intermediate and C-terminus
32
33 596 fragments to ECM and to PLG, plates were coated with 1 µg laminin (A), plasma
34
35 597 fibronectin (B) and PLG (C), and each recombinant fragment at 0 to 4,000 nM was added
36
37 598 for interaction. The binding was detected using antiserum raised in mice against each
38
39 599 fragment (1:1,000), followed by horseradish peroxidase-conjugated anti-mouse IgG
40
41 600 (1:5,000). Data represent the mean absorbance values ± the standard deviation of three
42
43 601 replicates for each experimental group. The results are representative of two independent
44
45 602 experiments. The dissociation constant (*K_D*) was calculated based on ELISA data for the
46
47 603 recombinant fragments that have reached the equilibrium concentration.
48
49
50
51
52
53
54
55
56
57
58
59 604

Figure 6. Sugar moiety contribution on the interaction of Lsa66/recombinant fragments with ECM. Immobilized laminin (A) or plasma fibronectin (B), 1 µg, was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark, before the reaction was set with the reactive fragments (1 µg). Each bar represents the mean ± standard deviation from triplicate experiments. Significance was assessed by comparison with untreated wells (0 mM) (* P<0,01 and ** P<0,001).

Figure 7. Schematic representation of Lsa66 and its binding regions. The drawing depicts the full length of Lsa66 recombinant protein demarcated by amino acid sequence number: signal peptide region (1 to 29), OmpA-like domain (504 to 583), N-terminus (30-219), intermediate (170-483), immune reactive region (30 to 483), laminin-, plasma fibronectin- and PLG-binding region (170 to 483) and C-terminus with the OmpA-like domain (420-590) and laminin-binding site (420 to 483).

Figure 1
[Click here to download high resolution image](#)

Figure 1

Figure 2

[Click here to download high resolution image](#)

Figure 2

Figure 3
[Click here to download high resolution image](#)

Figure 3

Figure 4

[Click here to download high resolution image](#)

Figure 4

Figure 5
[Click here to download high resolution image](#)

Figure 5

Figure 6
[Click here to download high resolution image](#)

Figure 6

Figure 7
[Click here to download high resolution image](#)

Figure 7

O gene LIC11089 também foi explorado durante o período deste Doutorado e os resultados obtidos pela caracterização da proteína recombinante rLIC11089 farão parte de um artigo que está em fase de confecção para publicação:

Analizou-se a prevalência genômica e dos transcritos do gene LIC11089 em espécies e sorovares patogênicos e em um não patogênico de leptospiros. Verificou-se alta conservação para estes sorovares.

Amplificou-se o gene a partir do genoma de *L. interrogans* sorovar Copenhageni utilizando oligonucleotídeos específicos, pode-se clonar os insertos no vetor pGEMT-Easy e depois clonar no vetor pAE (o sequenciamento confirmou a presença do gene de forma íntegra no vetor).

A expressão da proteína recombinante ocorreu a partir deste clone (pAE/gene) utilizando a cepa *E. coli* BL21 SI. A proteína correspondente ao gene LIC11089 foi obtida sob a forma insolúvel. Esta proteína, então, foi solubilizada utilizando tampão contendo uréia e renaturada. A proteína recombinante obtida apresentou mistura de estruturas secundárias quando avaliada por dicroísmo circular.

Para obtenção de anticorpos policlonais, camundongos foram imunizados com a proteína recombinante obtida. Os soros obtidos após as imunizações foram titulados por ELISA e reconhecidos contra rLIC11089 por *Immunoblotting*.

No intuito de identificar se a proteína é expressa durante o processo de infecção, investigamos a reatividade com soros de pacientes correspondentes à fase inicial da doença, com resultado MAT negativo, e soros de pacientes correspondentes à fase convalescente da doença, com resultado MAT positivo através de ELISA. A proteína recombinante rLIC11089 mostrou cerca de 25% de reatividade tanto para MAT positivo, quanto para negativo.

Pela técnica de acessibilidade pela proteinase K, pôde-se verificar que a proteína em estudo possivelmente é expressa na superfície de leptospiros.

Verificou-se que há adesão à laminina, e que esta adesão ocorre de forma dose-dependente.

Verificou-se também que há ligação ao PLG de forma dose-dependente e saturante. Além disso, houve redução significativa na interação da rLIC11089 com o PLG pela adição de 2 mM AAC (um derivado e análogo de lisina), o que sugere participação dos resíduos de lisina da proteína recombinante nesta interação. Na presença de um ativador, foi possível verificar que o PLG ligado à rLIC11089 é convertido a plasmina, uma serino-protease de amplo espectro, o que pode auxiliar a bactéria a se tornar um ser proteolítico.

Com objetivo de mapear qual o sítio (ou os sítios) de ligação da proteína com os componentes que mostraram adesão, clonou-se 3 regiões do gene e obteve-se 3 frações da proteína recombinante correspondentes às frações N-terminal, intermediária e C-terminal, como descrito acima, porém utilizando oligonucleotídeos específicos para as regiões e a cepa *E. coli* BL21 Star pLys para expressão das proteínas.

Todas as frações foram reconhecidas pelo antissoro produzido contra a proteína íntegra e se ligaram fortemente ao PLG, enquanto que somente a região C-terminal mostrou ligação à laminina.