ROSANE DE OLIVEIRA

CARACTERIZAÇÃO DE LIPOPROTEÍNAS DE
***Leptospira interrogans* EXPRESSAS**
EM *Escherichia coli*

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

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Área de Concentração: Biotecnologia

Orientador: Dra Ana Lucia Tabet
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CERTIFICADO

Certificamos que o Projeto intitulado “Caracterização de lipoproteínas de *Leptospira interrogans* expressas em *Escherichia coli*” **protocolo nº 471/08**, sob a responsabilidade de Ana Lúcia T. O. Nascimento e Rosane de Oliveira, 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 **14/05/2008**

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Vigência do Projeto: 08/2008 – 07/2012	Nº de animais/espécie	ADITIVO
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São Paulo, 18 de junho de 2010.

Dra. Denise V. Tambourgi
Presidente da CEUAIB

De acordo:

Dr. Otávio Azevedo Mercadante
Diretor do Instituto Butantan

**A Jurema e Edison meus queridos pais,
a minha irmã Emilia e a minha Tia Luci.
Obrigada por tudo...**

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*“O sucesso nasce do querer, da determinação
e persistência em se chegar a um objetivo.
Mesmo não atingindo o alvo, quem busca e
vence obstáculos, no mínimo fará coisas admiráveis”*

José de Alencar

RESUMO

OLIVEIRA, R. **Caracterização de lipoproteínas de *Leptospira interrogans* expressas em *Escherichia coli***. 2013. 217 f. Tese (Doutorado em Biotecnologia) - Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

Leptospirose é uma zoonose altamente disseminada causada por espiroquetas patogênicas do gênero *Leptospira*. Nos centros urbanos, os roedores são os mais importantes reservatórios da doença porque eliminam, continuamente, leptospiras vivas pela urina. Os humanos podem ser infectados pelo contato com solo ou água contaminada com urina contendo leptospiras. Desde que o controle dos roedores e medidas sanitárias não são facilmente implementadas, o desenvolvimento de uma vacina confiável é necessário para o combate da leptospirose. O sequenciamento genômico de *L. interrogans* sorovar Copenhageni e ferramentas de bioinformática permitiram a identificação de novos candidatos vacinais. Desta maneira, os genes LIC10258, LIC12880 e LIC12238 foram selecionados por análises de bioinformática e amplificados do DNA genômico de *L. interrogans* sorovar Copenhageni por metodologia de PCR. Os insertos de DNA foram clonados no vetor de expressão pAE e inseridos em *E. coli* BL21 SI para expressão das proteínas recombinantes. As proteínas rLIC10258, rLIC12880 e rLIC12238 foram purificadas por cromatografia de afinidade. A integridade estrutural das proteínas recombinantes foi avaliada por dicroísmo circular que mostrou que as proteínas estavam estruturadas. Os resultados obtidos com o ensaio de imunofluorescência de fase líquida (L-IFA) e proteólise com proteinase K (PK) sugerem que as proteínas nativas correspondendo a rLIC10258, rLIC12880 e rLIC12238 estão localizadas na membrana das leptospiras. rLIC10258, rLIC12880 e rLIC12238 foram reconhecidas por soro de pacientes diagnosticados com leptospirose, sugerindo que estas proteínas são, provavelmente, expressas durante a infecção. A imunização de hamsters seguido de desafio com dose letal de leptospiras virulentas mostrou que as proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 não são capazes de conferir proteção aos animais contra leptospirose. A avaliação da capacidade de adesão das proteínas recombinantes com laminina, colágeno tipo I, colágeno tipo IV, fibronectina celular e fibronectina plasmática mostrou que rLIC10258, proteína que possui um domínio tipo OmpA, interage com a laminina e fibronectina plasmática. A ligação foi dose dependente e saturável e portanto, esta proteína foi nomeada Lsa66 (*Leptospiral surface adhesin of 66 kDa*). Não foi detectada interação dos componentes da matriz extracelular com rLIC12880 e rLIC12238. Todas as proteínas recombinantes foram capazes de ligar ao plasminogênio e gerar plasmina, mostrando atividade proteolítica específica. Não foi observada interação do fator H e C4BP com as proteínas recombinantes, ao passo que apenas rLIC12238 foi capaz de ligar com o fibrinogênio. Dessa maneira, é possível que estas proteínas possam desempenhar algum papel na patogênese da doença.

Palavras-chave: Leptospirose. *Leptospira interrogans*. Proteínas recombinantes. Vacinas.

ABSTRACT

OLIVEIRA, R. **Characterization of lipoproteins of *Leptospira interrogans* expressed in *Escherichia coli***. 2013. 217 p. Ph. D. thesis (Biotechnology) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2013.

Leptospirosis is worldwide zoonosis caused by pathogenic spirochaetes of the genus *Leptospira*. In the urban settings, rodents are the most important reservoirs of the disease because they continuously shed live leptospires in their urine. Humans can be infected through contact with soil or water contaminated with urine containing leptospires. Since the control of the rodents and sanitation measures are not easily implemented, the development of reliable vaccine is necessary to combat the leptospirosis. The whole-genome sequences of *L. interrogans* serovar Copenhageni and bioinformatics tools allowed the identification of novel vaccine candidates. Thus, the genes sequences of LIC10258, LIC12880 and LIC12238 were selected by bioinformatics analysis and amplified by PCR methodology from genomic DNA of *L. interrogans* serovar Copenhageni. The DNA inserts were cloned into the *E. coli* expression vector pAE and inserted in BL21 *E. coli* strain for recombinant protein expression. The proteins rLIC10258, rLIC12880 and rLIC12238 were purified by using affinity chromatography. Structural integrity of the recombinant proteins was evaluated by circular dichroism that showed well folded proteins. The results obtained with liquid-phase immunofluorescence method (L-IFA) and proteolysis with proteinase K (PK) suggest that native proteins corresponding rLIC10258, rLIC12880 and rLIC12238, are located at the leptospiral membrane. rLIC10258, rLIC12880 and rLIC12238 were recognized by antibodies present in sera from human patients diagnosed with leptospirosis, suggesting that these proteins are probably expressed during infection. The immunization of hamsters followed by challenge with lethal dose of virulent leptospires showed that the recombinant proteins rLIC10258, rLIC12880 and rLIC12238 are unable to confer protection to animals against leptospirosis. Evaluation of the adhesion capacity of the recombinant proteins with laminin, collagen type I, collagen type IV, cellular fibronectin and plasma fibronectin showed that rLIC10258 having the OmpA-like domain interacts to laminin and plasma fibronectin. The binding was dose-dependent and saturable and therefore it was named Lsa66 (Leptospiral surface adhesin of 66 kDa). No specific interaction to the extracellular matrix components was detected with rLIC12880 e rLIC12238. All recombinant proteins were capable to bind plasminogen and to generate plasmin, showing specific proteolytic activity. No interaction to factor H or C4BP was detected with recombinant proteins, whereas that only rLIC12238 was capable to bind fibrinogen. Thus, it is possible that these proteins may play a role in leptospiral pathogenesis.

Keywords: Leptospirosis. *Leptospira interrogans*. Recombinant proteins. Vaccines

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LISTA DE ABREVIATURAS E SIGLAS

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

BSA: albumina de soro bovino

DC: dicroísmo circular

DO: densidade óptica

ELISA: ensaio imunoenzimático – *enzyme linked immuno sorbent assay*

EMJH: Ellinghausen-McCullough / Johnson-Harris

FIOCRUZ: Fundação Oswaldo Cruz

FITC: isotiocinato de fluoresceína

IPTG: isopropil- β -galatosídeo

KD: constante de dissociação

kDa: kiloDalton

L-IFA: imunofluorescência de fase líquida

LPHS: síndrome de hemorragia pulmonar associada à leptospirose

LPS: lipopolissacarídeo

MAT: teste de microaglutinação – *microscopic agglutination test*

MEC: componentes da matriz extracelular

OMP: proteína de membrana externa

OMS: Organização Mundial da Saúde

OPD: o-fenilenodiamina

PBS: solução fosfato salina, pH 7,4

PCR: reação de polimerização em cadeia

PK: proteinase K

PLG: plasminogênio

PMSF: fenilmetilsulfonilflúor

SDS: sulfato dodecil de sódio

TLR: receptor *toll-like*

X-gal: 5-bromo-4-cloro-3-indolil- β -D-galactopiranosídeo

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1 INTRODUÇÃO

Inicialmente conhecida como doença de Weil's, as manifestações clínicas da leptospirose foram descritas, pela primeira vez, em 1886. Em 1915, Inada e Ido isolaram o agente etiológico da leptospirose, uma nova espiroqueta denominada *Spirocheta icterohaenorrhagiae japonica*. Porém, em 1918, devido a características morfológicas diferenciadas, esta espiroqueta passou a integrar um novo gênero, *Leptospira*, pertencente a ordem Spirochaetales (KOBAYASHI, 2001).

1.1 Classificação

As bactérias do gênero *Leptospira* pertencem a família *Leptospiraceae*, ordem *Spirochaetales*, filo *Spirochaetes*, Reino *Eubacteriaceae*.

Até meados de 1989, todas as espécies de leptospiros patogênicas eram denominadas *Leptospira interrogans*, enquanto que *L. biflexa* incluía todas as espécies saprofíticas (LEVETT, 2001; EVANGELISTA; COBURN, 2010). Adicionalmente, baseando-se na expressão diferenciada de lipopolissacarídeos (LPS) de superfície, as leptospiros estão agrupadas em 24 sorogrupos e 250 sorovares. A heterogenicidade dos carboidratos que compõem a cadeia lateral dos LPS determinam a diversidade antigênica entre os sorovares, enquanto que sorovares antigenicamente relacionados estão agrupados em um sorogrupo (VINETZ, 2001; EVANGELISTA; COBURN, 2010).

Atualmente, análises de hibridização de DNA tem classificado as leptospiros em 13 espécies patogênicas (*L. alexanderi*, *L. alstoni*, *L. borgpetersenii*, *L. inadai*, *L. interrogans*, *L. fainei*, *L. kirschneri*, *L. licerasiae*, *L. noguchii*, *L. santarosai*, *L. terpstreae*, *L. weilii* e *L. wolffi*) e 6 espécies saprofíticas (*L. biflexa*, *L. meyeri*, *L. yanagawae*, *L. kmetyi*, *L. vanthiellii* e *L. wolbachii*) (ADLER; DE LA PENA MOCTEZUMA, 2010; EVANGELISTA; COBURN, 2010).

Além disso, análises filogenéticas do gene constitutivo 16S do RNA ribossômico sugerem a classificação das leptospiros em patogênicas (*L. alexanderi*, *L. alstoni*, *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. santarosai* e *L. weilii*), intermediárias (*L. inadai*, *L. fainei*, *L. licerasiae* e *L. wolffi*) e saprofíticas (*L. biflexa*, *L. wolbachii*, *L. kmetyi*, *L. meyeri*, *L. vanthiellii*, *L. terpstreae* e *L. yanagawae*) (KO; GOARANT; PICARDEAU, 2009; EVANGELISTA; COBURN, 2010).

1.2 Morfologia e biologia das leptospiiras

As leptospiiras são bactérias finas e espiraladas com aproximadamente 1 µm de diâmetro por 6-20 µm de comprimento. A presença de dois flagelos no espaço periplasmático dão motilidade a estas bactérias que, diferentemente de outras espiroquetas, possuem formato característico de ponto de interrogação, devido as suas extremidades terminadas em ganchos (PLANK; DEAN, 2000; LEVETT, 2001; ADLER; DE LA PENA MOCTEZUMA, 2010).

As leptospiiras são obrigatoriamente aeróbias e apresentam crescimento lento em uma temperatura ótima entre 28 e 30 °C e pH entre 7,2 e 7,6. Estas espiroquetas crescem em meio de cultura líquido ou sólido enriquecido com vitaminas, sais de amônio e ácidos graxos de cadeia longa que são utilizados como única fonte de carbono e metabolizados por β -oxidação. O meio de cultura mais utilizado é o Ellinghausen-McCullough/Johson-Harris (EMJH) composto por ácido oléico, albumina sérica bovina e polisorbato (Tween) (LEVETT, 2001).

As leptospiiras, assim como outras bactérias Gram-negativas, possuem uma estrutura de membrana dupla e a presença de LPS de superfície, porém também apresentam características semelhantes as das bactérias Gram-positivas como a associação entre a membrana citoplasmática e a camada de peptideoglicano (LEVETT, 2001; EVANGELISTA; COBURN, 2010).

Na membrana externa, o LPS constitui o principal antígeno das leptospiiras, que apesar de ser estruturalmente semelhante ao de outras bactérias Gram-negativas, apresenta uma toxicidade 12 vezes menor para camundongos quando comparado ao LPS de *Escherichia coli*. A menor endotoxicidade do LPS das leptospiiras pode ser atribuído a uma alteração na composição do lipídeo A, que possui uma unidade dissacarídica de glucosamida metilada e fosforilada. Esta característica única encontrada no LPS destas espiroquetas também contribui para uma atividade imune específica, com ativação do receptor *Toll-like 2* (TLR2) ao invés do TLR4, o receptor clássico de LPS (HAAKE; MATSUNAGA, 2010).

Além do LPS estão presentes na superfície das leptospiiras as proteínas de membrana externa (OMP), entre elas, proteínas funcionais e estruturais, hipotéticas, porinas e um grande número de lipoproteínas. Devido a sua localização, especula-

se o envolvimento destas OMPs nas interações patogéno-hospedeiro e na patogenicidade das leptospiros (GAMBERINI et al., 2005; ATZINGEN et al., 2008).

A grande maioria das proteínas de superfície, as lipoproteínas, encontram-se ancoradas a um ácido graxo constituinte da membrana externa por uma ligação covalente com a sua porção amino-terminal (HAAKE et al., 2000; HAAKE; MATSUNAGA, 2010). Malmstrom e colaboradores (2009) utilizando ensaios modificados de cromatografia líquida e espectrometria de massa, determinaram o número de cópias de proteínas por célula de *Leptospira* confirmando que as lipoproteínas são predominantes na membrana externa. Neste ensaio, a lipoproteína de membrana majoritária das leptospiros, LipL32, foi a mais abundante com 38000 cópias por célula (MALMSTROM et al., 2009). Por estarem localizadas na superfície das leptospiros, as lipoproteínas participam da adaptação e interação com o meio ambiente (HAAKE et al., 2000; HAAKE; MATSUNAGA, 2010) e como outras OMPs, provavelmente estão envolvidas na patogênese da doença.

1.3 Leptospirose - Epidemiologia

A leptospirose é uma zoonose causada por espiroquetas patogênicas do gênero *Leptospira*. Trata-se de uma doença de distribuição mundial, ocorrendo tanto em áreas rurais quanto urbanas de regiões tropicais, subtropicais e temperadas (EVANGELISTA; COBURN, 2010).

Nos países desenvolvidos, a leptospirose está geralmente associada com atividades aquáticas de lazer em ambientes contaminados, enquanto que nos países em desenvolvimento, as condições sanitárias precárias e a alta infestação de roedores infectados são fatores determinantes para a alta incidência da doença nestes países. Porém, a leptospirose também pode estar associada com atividades ocupacionais, acometendo indivíduos que trabalham em ambientes úmidos e alagadiços, como plantações de arroz, esgotos e minas (KOBAYASHI, 2001; ADLER; DE LA PENA MOCTEZUMA, 2010).

Nos centros urbanos, os roedores domésticos como *Rattus norvegicus* (rato de esgoto), *Rattus rattus* (rato preto) e *Mus musculus* (camundongo), são os principais reservatórios da doença, pois albergam as leptospiros nos túbulos proximais renais, eliminando-as vivas no ambiente e contaminando água, solo,

alimentos e outros animais. Entretanto, quase todas as espécies de mamíferos podem servir de reservatório de leptospiros (FAINE et al., 1999).

No Brasil, a leptospirose é uma doença endêmica, podendo tornar-se epidêmica nas capitais e regiões metropolitanas durante os períodos chuvosos, devido a fatores como enchentes, redes de saneamento básico deficientes, condições inadequadas de habitação e a presença de grande número de roedores infectados (BRASIL, 2005).

Segundo dados do Ministério da Saúde, entre os anos de 2005 e 2011 foram confirmados 27483 casos, apresentando média anual de 3926 casos (Portal da Saúde. Disponível em: http://portal.saude.gov.br/portal/arquivos/pdf/tab_casos_conf_ser_hist_1997_2012.pdf. Acesso em: 05 de junho de 2013). Neste mesmo período, 2674 pacientes foram a óbito, com média anual de 382 óbitos e letalidade de aproximadamente 10% (Portal da Saúde. Disponível em: http://portal.saude.gov/portal/arquivos/pdf/tab_obitosseriehistorica2012.pdf. Acesso em 05 de junho de 2013).

As Regiões Sudeste e Sul apresentaram as maiores porcentagens de casos confirmados, 37 e 33%, respectivamente, seguidas das Regiões Nordeste, Norte e Centro-Oeste com 19, 10 e 1% do total de casos confirmados no Brasil, respectivamente. O Estado que reportou os maiores números de casos e óbitos foi São Paulo, com 5876 casos confirmados e 688 óbitos, correspondendo a 21,5 e 26% dos casos confirmados e óbitos totais no Brasil, respectivamente.

1.4 Leptospirose humana

A infecção humana ocorre pelo contato direto ou indireto com a urina de animais infectados. As leptospiros penetram ativamente por meio das mucosas (ocular, digestiva, respiratória e genital), da pele lesionada e da pele íntegra em contato por períodos prolongados com água contaminada (BRASIL, 2005). Após a penetração, as leptospiros percorrem as vias linfáticas e sanguíneas, estabelecendo-se nos túbulos renais, fígado, cérebro e olhos onde se multiplicam (FAINE et al., 1999).

O tempo de incubação da doença até o surgimento dos primeiros sintomas é variável, podendo ser de algumas horas até 28 dias (FAINE et al., 1999).

As manifestações clínicas e o grau de severidade da leptospirose são muito variados e dependentes de fatores como: espécie da leptospira infectante e carga bacteriana, bem como idade e condição imunológica do indivíduo infectado (BRASIL, 2005).

Geralmente, os pacientes apresentam um quadro de sintomas amplo e inespecífico, o que dificulta a diferenciação da leptospirose de outras doenças febris agudas. Os sintomas mais comuns incluem: febre, calafrios, cefaleia, mal estar geral e mialgia (FAINE et al., 1999; LEVETT, 2001).

Entre 5 e 15% dos pacientes evoluem para a forma severa da leptospirose, denominada síndrome de Weil's. Os sintomas característicos desta síndrome, que apresenta taxa de mortalidade de 4 a 50%, são: icterícia, falência renal e hemorragias.

Recentemente, a síndrome de hemorragia pulmonar associada à leptospirose (LPHS) tem sido relatada como outra manifestação severa da doença. A LPHS apresenta sintomas inespecíficos como: dor no peito, tosse e dispneia, que geralmente aparecem entre o quarto e sexto dia da doença, podendo levar o paciente a óbito em até 72 horas (FAINE et al., 1999; TOYOKAWA; OHNISHI; KOIZUMI, 2011).

1.5 Diagnóstico e tratamento

Os métodos utilizados para o diagnóstico da leptospirose são usualmente sorológicos. No Brasil, os métodos mais utilizados são o teste ELISA (ensaio imunoenzimático – *enzyme linked immuno sorbent assay*) para pesquisa de anticorpos IgM específicos contra componentes de leptospiros patogênicas e o teste de microaglutinação (MAT – *microscopic agglutination test*), ambos sorológicos (BRASIL, 2005).

O MAT é o método padrão ouro recomendado pela Organização Mundial da Saúde (OMS). Apesar disso, é um método pouco sensível, de difícil padronização e execução, sendo realizado apenas por laboratórios de referência. Neste teste, amostras de soros de pacientes são submetidas à reação com suspensões de diferentes sorovares de leptospira. O MAT apresenta baixa sensibilidade na fase inicial da doença, já que anticorpos contra antígenos de superfície da leptospira não são detectados nos primeiros dias após a infecção. Além disso, por se tratar de um

ensaio sorovar específico, os laboratórios que realizam o MAT necessitam manter culturas vivas de diferentes sorovares de *Leptospira* (FAINE et al., 1999; LEVETT, 2003).

Recentemente, alguns protocolos de PCR tem sido propostos, como uma alternativa ao MAT, para o diagnóstico da leptospirose. Alguns genes, como por exemplo, *secY*, *rrs*, *flaB*, *LigA*, *LigB*, *LipL32*, *rrl* e *LA3521*, tem sido utilizados como alvos e amplificados por reação de polimerase em cadeia (PCR) do liquor e urina de suspeitos de leptospirose (MERIEN et al., 1992; BROWN et al., 1995; KOIZUMI et al., 2003; FONSECA CDE et al., 2006; KOSITANONT et al., 2007).

Atualmente, foi divulgado um novo método de diagnóstico da leptospirose desenvolvido por pesquisadores da Fundação Oswaldo Cruz (FIOCRUZ). Neste método, a proteína recombinante *LigA* imobilizada em um cartão detecta a presença de anticorpos específicos em uma gota de sangue do paciente suspeito (FUNDAÇÃO OSWALDO CRUZ, 2010).

Quando diagnosticada, a leptospirose é tratada com antibióticos como as penicilinas e doxiciclinas. Entretanto, o tratamento é geralmente mais eficiente nos primeiros dias após a infecção. Além disso, procedimentos como diálise e terapia intensiva são necessários quando os pacientes apresentam complicações renais, respiratórias ou hemorrágicas (FAINE et al., 1999; LEVETT, 2001).

1.6 Sequenciamento genômico

Por ser o agente etiológico da maior parte das infecções em humanos, *L. interrogans* foi a primeira espécie a ser sequenciada (REN et al., 2003; NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b). Outra espécie patogênica, *L. borgpetersenii* também teve o seu genoma sequenciado, já que infecta rebanhos de bovinos, suínos e equinos causando grande impacto econômico na pecuária (BULACH et al., 2006). Além das espécies patogênicas, a espécie de vida livre, *L. biflexa* foi sequenciada na tentativa de desvendar os mecanismos da patogenicidade das leptospirosas comparando o genoma de espécies patogênicas e saprofíticas (PICARDEAU et al., 2008).

O genoma das leptospirosas é composto por dois cromossomos circulares, porém a espécie saprofítica *L. biflexa* possui, adicionalmente, um terceiro replicon denominado P74, ausente nas espécies patogênicas. Acredita-se que este replicon

seja essencial a espécie saprofítica, já que possui genes constitutivos do metabolismo basal que são encontrados no cromossomo I das leptospiros patogênicas. Além disso, a comparação dos genomas permitiu a identificação de 1431 genes exclusivos das espécies patogênicas, sem ortólogos em *L. biflexa* (PICARDEAU et al., 2008).

O genoma de *L. interrogans* é maior em tamanho (4,6 Mb) do que os genomas de *L. borgpetersenii* e *L. biflexa* (3,9 Mb). Enquanto, *L. borgpetersenii* não é capaz de sobreviver fora do hospedeiro (BULACH et al., 2006), tem sido relatada a capacidade de *L. interrogans* sobreviver em ambientes úmidos ou água fresca por períodos de até 110 dias (TRUEBA et al., 2004). A comparação destes genomas indica que a perda de genes relacionados com a sobrevivência fora do hospedeiro limitam a transmissão de *L. borgpetersenii* apenas pelo contato direto com indivíduos infectados. Em contrapartida, o maior genoma de *L. interrogans* provavelmente é devido a presença de genes relacionados a sobrevivência desta espiroqueta tanto no meio ambiente quanto em mamíferos hospedeiros.

Recentemente, a espécie patogênica *L. santarosai* (CHOU et al., 2012) e a espécie intermediária *L. licerasiae* (RICALDI et al., 2012) tiveram o seu genoma totalmente sequenciado.

1.7 Mecanismos de patogenicidade e virulência

Os mecanismos moleculares de patogenicidade e virulência das leptospiros são, ainda, pouco conhecidos. Sendo assim, o sequenciamento de cinco espécies de *Leptospira* possibilitaram que análises genômicas identificassem uma grande variedade de proteínas hipotéticas que provavelmente estão envolvidas na patogênese da leptospirose (REN et al., 2003; NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b; PICARDEAU et al., 2008; CHOU et al., 2012; RICALDI et al., 2012).

Loa22, uma lipoproteína com domínio tipo OmpA, foi o primeiro fator de virulência geneticamente identificado em *Leptospira*. Apesar da função de Loa22 permanecer desconhecida, leptospiros mutantes em *loa22* tornaram-se avirulentas em ensaios utilizando hamsters e porquinhos da índia como modelos animais (RISTOW et al., 2007).

Além da Loa22, a motilidade e o LPS das leptospiiras também são considerados fatores de virulência. Em relação a motilidade, ensaios mostraram que leptospiiras mutantes no gene *fliY*, que codifica a proteína FliY do motor flagelar perdem a virulência e a capacidade de infectar porquinhos da Índia (LIAO et al., 2009), enquanto que estudos dirigidos por Murray e colaboradores (2010) relataram a perda da virulência de leptospiiras mutantes em LPS, em modelo de hamster (MURRAY et al., 2010).

Nos últimos anos, o grupo de pesquisa deste trabalho, tem identificado proteínas de membrana hipotéticas capazes de se ligar a componentes da matriz extracelular, auxiliando na adesão das leptospiiras aos tecidos dos hospedeiros. Entre elas: Lsa24 que interage com a laminina (BARBOSA et al., 2006); Lsa21 que se liga a laminina, fibronectina plasmática e colágeno IV (ATZINGEN et al., 2008), Lsa63 ligante de laminina e colágeno IV (VIEIRA et al., 2010b), entre outras. Outros grupos também demonstraram a capacidade de adesão de proteínas de leptospira a componentes da matriz extracelular do hospedeiro, como as proteínas de leptospira semelhantes à imunoglobulina, LigA e LigB (CHOY et al., 2007), e LipL32, a lipoproteína de membrana majoritária das leptospiiras (HAUK et al., 2008; HOKE et al., 2008).

Recentemente, este grupo de pesquisa demonstrou a capacidade das leptospiiras de utilizarem proteases do hospedeiro para a penetração tecidual e invasão (VIEIRA et al., 2009). Desde então, várias proteínas que se ligam ao plasminogênio (PLG) e são capazes de convertê-lo em plasmina tem sido identificadas (VERMA et al., 2010; VIEIRA et al., 2010a; MENDES et al., 2011; DOMINGOS et al., 2012).

Além de proteínas que se ligam aos componentes da matriz extracelular e PLG, tem sido identificadas proteínas de leptospiiras que se ligam aos reguladores do sistema complemento, Fator H e C4BP (VERMA et al., 2006; BARBOSA et al., 2009; CASTIBLANCO-VALENCIA et al., 2012). A ligação destas proteínas ao Fator H e C4BP inibiria o ataque do sistema complemento facilitando a disseminação das leptospiiras e consequente colonização dos órgãos dos hospedeiros.

1.8 Vacinas profiláticas e Vacinologia reversa

As medidas de controle e prevenção da leptospirose são direcionadas ao controle da população de roedores, à melhoria das condições higiênico-sanitárias da população, ações de educação em saúde e alterações do meio ambiente (BRASIL, 2005). Entretanto, como estas são medidas de difícil implementação, o desenvolvimento de uma vacina é crítico para o controle e prevenção da doença.

As vacinas contra a leptospirose disponíveis no mercado são veterinárias e compostas de bactérias inativadas ou preparações de membrana de leptospiros patogênicas (GAMBERINI et al., 2005; KO; GOARANT; PICARDEAU, 2009; ADLER; DE LA PENA MOCTEZUMA, 2010). Apenas alguns países, como Japão, China e Cuba, possuem vacinas contra a leptospirose licenciadas para uso humano (KOIZUMI; WATANABE, 2005; ADLER; DE LA PENA MOCTEZUMA, 2010). Estas vacinas conferem proteção induzindo a produção de anticorpos contra LPS presentes na membrana das leptospiros. Contudo, estas vacinas falham em induzir uma proteção a longo prazo contra a infecção. Além disso, é importante ressaltar que existem variações da composição dos carboidratos do LPS das leptospiros e, baseado na expressão diferenciada destes LPS de superfície, as leptospiros patogênicas estão distribuídas em aproximadamente 250 sorovares (KOIZUMI; WATANABE, 2005; EVANGELISTA; COBURN, 2010). Sendo assim, as vacinas veterinárias e humanas disponíveis conferem imunidade sorovar-específica, não sendo capazes de proteger contra sorovares não incluídos em sua composição (GAMBERINI et al., 2005; KOIZUMI; WATANABE, 2005; ATZINGEN et al., 2010).

Devido as limitações encontradas nas vacinas contra a leptospirose disponíveis atualmente no mercado, tem sido desenvolvidas novas estratégias para a identificação de antígenos que sejam capazes de conferir proteção cruzada contra diferentes sorovares.

O sequenciamento completo do genoma de *Neisseria meningitidis* sorogrupo B (TETTELIN et al., 2000) propiciou o surgimento de uma nova estratégia para identificação de candidatos vacinais, denominada “vacinologia reversa”. Nesta metodologia, os prováveis candidatos vacinais são selecionados após a análise *in silico* dos genomas sequenciados (PIZZA et al., 2000; RAPPUOLI, 2000). Esta mesma estratégia foi empregada satisfatoriamente na busca de candidatos vacinais contra infecção por *Streptococcus pneumoniae* (WIZEMANN et al., 2001),

Porphyromonas gingivalis (ROSS et al., 2001), *S. pyogenes* (REID et al., 2002), entre outros.

Desta maneira, para a realização do presente estudo, os genes LIC10258, LIC12880 e LIC12238 foram selecionados do genoma de *L. interrogans* sorovar Copenhageni para serem avaliados quanto à possibilidade de serem bons candidatos vacinais. Estes genes foram selecionados após análises de bioinformática que identificaram prováveis proteínas de membrana.

2 OBJETIVO

Clonar, expressar e caracterizar proteínas com potencial vacinal e/ou diagnóstico identificadas no genoma da *L. interrogans* sorovar *Copenhageni*

3 MATERIAL E MÉTODOS

3.1 Análise por bioinformática

Os genes foram selecionados do genoma de *L. interrogans* sorovar Copenhageni disponível no servidor público, <http://aeg.lbi.ic.unicamp.br/world/lic/>. Esta seleção teve como base a localização celular das proteínas hipotéticas codificadas por estes genes, que foi feita por meio do programa PSORT (NAKAI e HORTON, 1999) (<http://psort.nibb.ac.jp/>) e CELLO (YU et al., 2006) (<http://cello.life.nctu.edu.tw/>). Os genes LIC12880 e LIC12238 codificam prováveis lipoproteínas, esta identificação foi realizada pelo programa LipoP (JUNCKER et al., 2003) (<http://www.cbs.dtu.dk/services/LipoP/>) que analisa a presença de uma sequência consenso denominada *lipobox*. O programa SMART (SCHULTZ et al., 1998) (<http://smart.embl-heidelberg.de/>) foi utilizado para identificar a presença de peptídeo sinal nos genes selecionados. Os genes ainda foram analisados pelo programa BLAST (ALTSCHUL et al., 1990) (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) e CLUSTAL 2.1 (LARKIN et al., 2007) (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). O BLAST (ALTSCHUL et al., 1990) analisa as similaridades destas sequências com a de outras espécies e sorovares de *Leptospira* e também com a de outros organismos presentes no banco de dados, enquanto que o programa CLUSTAL 2.1 (LARKIN et al., 2007) gera filogramas baseados nas similaridades entre as sequências analisadas pelo BLAST (ALTSCHUL et al., 1990).

3.2 Construção dos oligonucleotídeos e amplificação dos genes e fragmentos de interesse

Os genes LIC10258, LIC12880, LIC12238 e os fragmentos correspondentes as porções N-terminal, intermediária e C-terminal do gene LIC10258 foram obtidos por PCR a partir do DNA genômico de *L. interrogans* sorovar Copenhageni M20, utilizando-se nucleotídeos *forward* (F) e *reverse* (R) desenhados no programa *Generunner* (www.generunner.net/), contendo os sítios de restrição para as enzimas *Bam*HI/*Nco*I; *Xho*II/*Nco*I e *Xho*II/*Hind*III para LIC10258 e seus fragmentos, LIC12880

e LIC12238, respectivamente. A sequência dos oligonucleotídeos construídos para cada um dos genes está descrita no quadro abaixo:

Quadro 01- Sequência de oligonucleotídeos utilizados para amplificação dos genes e fragmentos de interesse

Gene	Oligonucleotídeo
LIC10258	F: 5' <u>GGATCC</u> GAAGCCTTCTCACCCAATTG3' R: 5' <u>CCATGG</u> TTAAGTGAAAGATAAAAATCGATTG3'
LIC10258 N-terminal	F: 5' <u>GGATCC</u> GAAGCCTTCTCACCCAAT3' R: 5' <u>CCATGG</u> TTAATCTGTTCCATCCCAAAT3'
LIC10258 Intermediário	F: 5' <u>GGATCCTCCTCTCGTATCAATATTTCTCAA</u> 3' R: 5' <u>CCATGG</u> TTAATGATCTTCTTCTAACACCAG3'
LIC10258 C-terminal	F: 5' <u>GGATCC</u> ATCAAACTTGGAATGGC3' R: 5' <u>CCATGG</u> TTAAGTGAAAGATAAAAATCGATTG3'
LIC12880	F: 5' <u>CTCGAGGAAGTTGTCCGAGTCTAT</u> 3' R: 5' <u>CCATGG</u> TATTGATTGTTTAATTCAG3'
LIC12238	F: 5' <u>CTCGAGTGTTTTAAACCTACCGGAG</u> 3' R: 5' <u>AAGCTI</u> CTACTTCATCGCTTTTTTCTATATC3'

Notas: LIC: identificação do gene no genoma de *L. interrogans* sorovar Copenhageni

Abreviaturas (em inglês): F: *forward* e R: *reverse*

As sequências que correspondem aos sítios de restrição *Bam*HI/*Nco*I; *Xho*II/*Nco*I e *Xho*II/*Hind*III para LIC10258 e seus fragmentos, LIC12880 e LIC12238, respectivamente, estão sublinhadas.

As temperaturas de fusão utilizadas foram determinadas de acordo com o programa *Generunner* e subtraídas em 5 °C, foram estas 63, 56, 58 e 60 °C para LIC10258, LIC12880, LIC12238 e os fragmentos do gene LIC10258, respectivamente. Os insertos foram amplificados sem a sequência do peptídeo sinal. A amplificação e o tamanho dos insertos de interesse foi verificada por meio de eletroforese em gel de agarose 1% em tampão TBE (450 mM Tris-Borato e 10 mM

EDTA). Os insertos de interesse foram purificados do gel de agarose utilizando o *kit GFxPCR DNA and gel band purification* (GE Healthcare) e os produtos obtidos foram quantificados em espectrofotômetro.

3.3 Clonagem no vetor pGEM-T Easy

Os insertos de DNA obtidos foram clonados no vetor pGEM-T Easy. A reação de ligação foi realizada segundo a reação padrão estabelecida pelo fabricante. Os produtos de ligação foram utilizados para transformar bactérias *E. coli* DH5 α previamente tornadas competentes pelo método descrito por Mandel e Higa (1970) (MANDEL; HIGA, 1970).

Para a transformação foram utilizados 50 ng do produto de ligação para 50 μ L de bactéria competente. O produto da transformação foi semeado em meio de cultura contendo 100 μ g/mL de ampicilina, 100 mM de IPTG (isopropil- β -D-tiogalactopiranosídeo) e 50 mg/mL de X-gal (5-bromo-4-cloro-3-indolil- β -D-galactopiranosídeo). Incubou-se a 37 °C por uma noite. As colônias positivas selecionadas foram inoculadas em 5 mL de meio de cultura com 100 μ g/mL de ampicilina e incubadas a 37 °C, sob agitação, por uma noite. 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. A presença dos insertos de interesse nos plasmídeos obtidos foi confirmada por meio de eletroforese em gel de agarose, após a digestão dos plasmídeos com suas respectivas enzimas de restrição.

3.4 Subclonagem no vetor de expressão pAE

Os insertos correspondentes aos genes LIC10258, LIC12880, LIC12238 e aos fragmentos N-terminal/LIC10258, Intermediário/LIC10258 e C-terminal/LIC10258 foram removidos dos plasmídeos recombinantes (pGEM-T Easy/inserto) por digestão com as enzimas de restrição e tampões apropriados. O vetor de expressão pAE (RAMOS et al., 2004) foi digerido separadamente com as mesmas enzimas de restrição utilizadas para obtenção dos insertos. Após digestão, tanto os insertos quanto o vetor de expressão foram submetidos à eletroforese em gel de agarose e

posteriormente purificados pelo *kit GFX PCR DNA and Gel Band Purification* (GE Healthcare).

A ligação dos insertos ao vetor pAE foi realizada em uma reação contendo inserto:vetor em uma proporção 5:1 em massa de DNA. Bactérias competentes *E. coli* DH5 α foram transformadas com os produtos de ligação e semeadas em placas com meio de cultura e antibiótico apropriados e incubadas a 37 °C por uma noite. As colônias positivas e negativas selecionadas foram inoculadas em 5 mL de meio de cultura com 100 μ g/mL de ampicilina e incubadas a 37 °C, sob agitação, por uma noite. Com estes inóculos foi realizada uma seleção por fenol-clorofórmio, a fim de confirmar a presença do inserto no vetor. A obtenção do DNA plasmidial (pAE/inserto) dos inóculos com clones positivos e a confirmação da presença dos insertos neste DNA plasmidial foi feito da mesma maneira descrita para o vetor pGEM-T Easy.

3.5 Sequenciamento dos clones

Tanto as construções no vetor de clonagem (pGEM-T Easy/inserto) quanto as construções no vetor de expressão (pAE/inserto), foram sequenciadas pelo método da terminação de cadeia (SANGER; NICKLEN; COULSON, 1977) no sequenciador automático *3100 Genetic Analyze* da *Applied Biosystems*.

3.6 Análise da presença dos genes em diferentes cepas de *Leptospira*

A presença dos genes LIC10258, LIC12880 e LIC12238 foi avaliada em espécies patogênicas de *Leptospira* e em outros sorovares patogênicos de *L. interrogans*, utilizando reação de PCR. Foram utilizados os mesmos oligonucleotídeos desenhados para a clonagem dos genes citados acima, de acordo com o genoma de *L. interrogans* sorovar Copenhageni. A reação de PCR foi realizada nas mesmas condições utilizadas para obtenção dos insertos para clonagem.

Os sorovares patogênicos utilizados de *L. interrogans* foram: Icterohaemorrhagiae, Hardjo, Pomona e Canicola. As espécies patogênicas avaliadas foram: *L. borgpetersenii* sorovar Castellonis, *L. santarosai* sorovar

Shermani, *L. noguchii* sorovar Panama e *L. kirschneri* sorovar Grippytyphosa. Foi utilizada também a espécie não patogênica *L. biflexa* sorovar Patoc.

3.7 Extração de RNA total de diferentes espécies e sorovares de *Leptospira*

Culturas de leptospiros (20 mL) com 7 dias de crescimento foram centrifugadas a 6000 rpm, por 30 minutos, a 4 °C. Para extração do RNA do sedimento bacteriano obtido foi utilizado o reagente TRIzol (Invitrogen) segundo as recomendações do fabricante. Foram extraídos o RNA total de culturas de *L. interrogans* sorovares Copenhageni, Icterohaemorrhagiae, Hardjo, Pomona e Canicola e das espécies patogênicas *L. borgpetersenii* sorovar Castellonis, *L. santarosai* sorovar Shermani, *L. noguchii* sorovar Panamá, *L. kirschneri* sorovar Grippytyphosa e da espécie não patogênica *L. biflexa* sorovar Patoc. Também foram extraídos o RNA total de *L. interrogans* sorovar Icterohaemorrhagiae mantidas por 16 horas nas temperaturas de 37 ou 39 °C, ou suplementadas com 120 mM de NaCl e mantidas na temperatura de 28 °C.

3.8 Reação de Transcrição Reversa (RT-PCR)

O RNA total (2 µg) obtido das culturas de leptospiros foi tratado com 1U de DNase I (Invitrogen) por 15 minutos a temperatura ambiente. Para inativação da DNase I, foi adicionado 1 µL de solução de EDTA a 25 mM e, em seguida, incubou-se a amostra a 65 °C por 10 minutos. A reação de transcrição reversa foi realizada com o *kit SuperScript III First-Strand Synthesis System for RT-PCR* (Invitrogen) conforme as recomendações do fabricante. 2 µL do cDNA obtido foi empregado como molde para a reação de PCR. Foram utilizados os mesmos oligonucleotídeos desenhados para a clonagem dos genes LIC10258, LIC12880 e LIC12238, de acordo com o genoma de *L. interrogans* sorovar Copenhageni. Para análise da integridade dos transcritos obtidos foi realizada amplificação utilizando oligonucleotídeos elaborados para o 16S ribossomal (16S-F 5'CAAGTCAAGCGGAGTAGCAATACTCAGC e 16S-R 5'GATGGCAACATAA GGTGAGGGTTGC). Todas as amostras foram testadas na presença e ausência da enzima *transcriptase reversa* para que a possibilidade de contaminação com DNA

fosse descartada. Os produtos de RT-PCR obtidos foram analisados em gel de agarose 1%.

3.9 Expressão das proteínas e fragmentos recombinantes

Bactérias competentes *E. coli* BL21 SI (*Salt Induced*) foram transformadas com o plasmídeo recombinante 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 uma noite. Após este período, as culturas saturadas foram diluídas (1:25) em um novo meio e incubadas até atingir uma densidade óptica (DO) entre 0,6 e 0,8. A indução foi realizada adicionando-se diferentes concentrações de NaCl e incubando as culturas, novamente, a mesma temperatura por um período de 3 horas. A seguir as bactérias foram centrifugadas (5000 rpm/15 minutos/4 °C) e os sedimentos bacterianos 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). As bactérias foram lisadas em banho de gelo por sonicação e centrifugadas novamente. Os sedimentos formados foram ressuspensos em PBS (137 mM NaCl; 2,7 mM KCl; 10 mM Na₂HPO₄; 2 mM KH₂PO₄) e 9 M uréia, e alíquotas do sobrenadante da sonicação e do sedimento ressuspensionado foram analisadas por eletroforese em gel de poliacrilamida/SDS (sulfato dodecil de sódio).

3.10 Solubilização das proteínas e fragmentos recombinantes

As proteínas recombinantes rLIC12880, rLIC10258 e os fragmentos rN-terminal/LIC10258, rIntermediário/LIC10258 e rC-terminal/LIC10258 foram solubilizados em tampão contendo 8 M uréia e 10% glicerol como descrito por Saini e colaboradores (2002) por 2 horas, sob agitação, a 37 °C (SAINI et al., 2002).

3.11 Purificação e diálise das proteínas e fragmentos recombinantes

A purificação das proteínas e fragmentos recombinantes obtidos foi feita em resina de afinidade a metal. Foi utilizada a resina *Chelating Sepharose* (GE Healthcare) carregada com Ni²⁺. Contaminantes foram lavados com tampões

contendo baixas concentrações de imidazol (20, 40 e 60 mM). As proteínas rLIC10258, rLIC12880, rN-terminal/LIC10258, rIntermediário/LIC10258 e rC-terminal/LIC10258 foram renaturadas durante o processo de purificação utilizando-se tampões com concentrações decrescentes de uréia. As proteínas e fragmentos recombinantes foram eluídos com 1 M de imidazol. As frações de todas as etapas da purificação foram coletadas e analisadas em gel de SDS-PAGE. As alíquotas que continham a proteína recombinante rLIC12238 foram dialisadas contra PBS e 0,1 mM PMSF a 4 °C durante uma noite. As alíquotas que continham as proteínas recombinantes rLIC10258, rLIC12880, N-terminal/LIC10258, Intermediário/LIC10258 e C-terminal/LIC10258 foram dialisadas contra PBS; 0,01% glicina e 10% glicerol a 4 °C durante a noite. Após diálise, a concentração das proteínas recombinantes foi avaliada em gel SDS-PAGE, utilizando-se concentrações pré-estabelecidas de albumina de soro bovino (BSA) e o programa *GelQuant*.

3.12 Dicroísmo circular (DC)

As proteínas rLIC10258, rLIC12880 e rLIC12238 foram submetidas à diálise realizada a 4 °C, com tampão contendo Na_2HPO_4 e NaH_2PO_4 em concentração final de 1 M. Foram realizadas 3 trocas da solução para certificar-se que o tampão contendo as proteínas não apresentariam íons Cl^- , os quais interferem na leitura do espectropolarímetro (Jasco J810). Os valores de leitura foram obtidos no intervalo de 184 a 260 nm. Os resultados foram expressos em elipicidade molar, segundo a fórmula:

$$[\theta] \text{ molar} = 100 \times \theta \text{ (degrees)} / C(\text{M}) \times l \text{ (cm)}$$

onde θ é elipicidade, C a concentração da proteína e l o caminho ótico. Os dados obtidos foram comparados com a predição teórica feita pelo servidor público PSIPRED (JONES, 1999) (<http://bioinf.cs.ucl.ac.uk/psipred/>).

3.13 Imunização de camundongos

Foram realizadas 3 imunizações com 15 dias de intervalo entre elas em grupos de 4 camundongos BALB/c, fêmeas, com idade de 6 a 8 semanas. Os animais foram imunizados, no dorso, com 10 μg de proteína ou fragmento recombinante adsorvido em alumínio, por via subcutânea. O grupo controle foi

imunizado com PBS e alúmem. Os animais foram sangrados via plexo retro-orbital 15 dias após cada dose de imunização. O sangue coletado foi incubado a 4 °C durante a noite e foi centrifugado por 5 minutos a 8000 rpm. O soro foi cuidadosamente coletado e armazenado a -20 °C.

3.14 Titulação de anticorpos IgG produzidos em camundongos por ELISA

A detecção de anticorpos IgG anti-rLIC10258, anti-rLIC12880, anti-rLIC12238 e anti-fragmentos da proteína rLIC10258 após as imunizações foi feita por ensaio imunoenzimático ELISA conforme descrito por Sato e colaboradores (1991) (SATO, 1991). As proteínas e fragmentos recombinantes (50 ng/poço) em tampão carbonato 0,05 M pH 9,6 foram adsorvidas em placas de microdiluição (High Binding, Nunc) e incubadas com soros hiperimunes em diluição seriada (previamente incubadas com 25% de extrato de *E. coli* para que ocorresse a adsorção dos anticorpos inespecíficos). A seguir, as placas foram incubadas com anticorpo anti-IgG de camundongo conjugado com peroxidase (Sigma) na diluição 1:5000. A reação enzimática foi revelada pela adição de 1 mg/mL de OPD (δ -phenylenediamina, Sigma) diluído em tampão fosfato-citrato pH 5 contendo 0,03% (vol/vol) de peróxido de hidrogênio. A reação foi interrompida pela adição de solução de H₂SO₄ 8 M. A DO foi determinada em leitor de microplacas usando o comprimento de onda de 492 nm. O título individual foi determinado como sendo a maior diluição do soro com valor da DO₄₉₂ maior do que 0,1.

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

A detecção de anticorpos IgG e IgM anti-proteínas e anti-fragmentos recombinantes no soro destes indivíduos foi feita por ELISA de maneira semelhante ao experimento de detecção de anticorpos de camundongos, introduzindo-se algumas modificações tais como: a diluição única dos soros de 1:100, os anticorpos secundários utilizados foram anti-IgG e anti-IgM humanos conjugados a peroxidase (Sigma) na diluição 1:5000. A revelação da placa ocorreu de forma semelhante, assim como a leitura dos dados. Foram realizadas cinéticas de concentração de extrato bacteriano, 5 e 20%, para adsorção prévia de anticorpos inespecíficos.

Foram consideradas amostras positivas aquelas que apresentaram valores maiores do que o estabelecido pelo *cutoff*. Os valores de *cutoff* foram calculados a partir de valores obtidos para indivíduos normais. Foram utilizadas amostras de soro de 6 e 18 indivíduos saudáveis para as proteínas e fragmentos recombinantes, respectivamente. A fórmula utilizada para o cálculo do *cutoff* foi a média da DO dos indivíduos normais + 3 vezes o desvio padrão desta média.

3.16 Ensaio de Western blotting

O reconhecimento das proteínas e fragmentos recombinantes por anticorpos monoclonais anti-His tag e por anticorpos policlonais homólogos foi analisado por Western blotting. Sendo assim, as proteínas e fragmentos a serem avaliados foram separados por gel de poliacrilamida/SDS e transferidos para membrana de nitrocelulose *Hybond-ECL* (GE Healthcare) em sistema semi-úmido (GE) por 1 hora a 0,8 mA por cm² em tris-glicina acrescido de 1,85% de SDS (tampão de transferência). A eficiência da transferência foi avaliada por coloração de *Ponceau S* (Sigma). As proteínas e fragmentos recombinantes foram descorados com PBS/Tween 0,05% sob agitação e a membrana foi incubada com solução de bloqueio (PBS/Leite desnatado 10%) durante uma noite a temperatura de 4 °C. Lavou-se a membrana por 15 minutos com PBS/Tween 0,05%, e logo após a mesma foi incubada com anticorpo monoclonal de camundongo anti-polihistidina (Sigma) na diluição 1:3000 ou anticorpo policlonal de camundongo anti-rLIC0258, anti-rLIC12880, anti-rLIC12238 ou anti-fragmentos nas diluições 1:800, 1:800; 1:2000 e 1:500, respectivamente, por 2 horas a temperatura ambiente, sob agitação. A membrana foi lavada por 15 minutos com PBS/Tween 0,05%, e incubada com anticorpo anti-IgG de camundongo conjugado com peroxidase (Sigma) na diluição 1:5000, por 1 hora a temperatura ambiente, sob agitação. Lavou-se novamente a membrana por 15 minutos com PBS/Tween 0,05%, e as proteínas e os fragmentos foram detectados utilizando *kit ECL Western Blotting Detection Reagents* (GE Healthcare). As membranas com as proteínas recombinantes foram reveladas após exposição a filmes de raio-X (Kodak) por curtos períodos de tempo (1 a 10 minutos), enquanto que as membranas com os fragmentos recombinantes foram reveladas com a ajuda do programa de imagem molecular *Carestream* (Equilab, Whitestone, Nova York) conectado ao equipamento *Gel Logic 2200PRO*.

3.17 Ensaio de imunofluorescência de fase líquida (L-IFA)

A localização celular das proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 foi analisada por L-IFA de acordo com o protocolo descrito por Oliveira e colaboradores (2008) (OLIVEIRA et al., 2008). Para este ensaio foram utilizadas 2,5 mL de culturas vivas de *L. interrogans* sorovar Copenhageni M20 que foram centrifugadas a 6000 rpm por 15 minutos. O sedimento bacteriano obtido foi lavado, 3 vezes, com PBS contendo 50 mM de NaCl, e após a última lavagem foi ressuspenso em 200 µL do mesmo PBS acrescido de 6 µg/mL de iodeto de propídeo e incubado por 45 minutos a 37 °C. Após a incubação, as leptospiros foram centrifugadas e gentilmente lavadas com PBS e incubadas a 4 °C, por 30 minutos, com anticorpo policlonal obtido em camundongo contra cada uma das proteínas recombinantes deste estudo, na diluição de 1:50. As leptospiros também foram incubadas com anticorpo policlonal anti-LipL32 e anti-Groel, proteínas que foram utilizadas como controle positivo e negativo do ensaio, respectivamente. Após a incubação, as leptospiros foram centrifugadas, lavadas e incubadas com anticorpo anti-mouse IgG conjugado ao FITC (isotiocianato de fluoresceína) (Sigma), por 30 minutos a 4 °C, na diluição de 1:50. Após a incubação com o anticorpo secundário, as leptospiros foram centrifugadas, lavadas e ressuspendidas em PBS contendo solução *antifading* (ProLong Gold; Molecular Probes). As leptospiros marcadas com imunofluorescência foram examinadas em microscópio de imunofluorescência confocal *LSM 510 META* (Zeiss, Alemanha).

3.18 Ensaio de digestão com proteinase K (PK)

Este ensaio foi realizado segundo protocolo descrito por Domingos e colaboradores (2012) (DOMINGOS et al., 2012). Alíquotas de 5 mL de *L. interrogans* sorovar Copenhageni M20, contendo 10^8 bactérias por alíquota, foram centrifugadas a 6000 rpm por 10 minutos a temperatura ambiente e gentilmente lavadas com PBS contendo 50 mM de NaCl. As leptospiros foram ressuspendidas em tampão de proteólise (10 mM Tris-HCl e 5 mM CaCl_2) contendo 25 µg/mL de PK (Sigma) e incubadas sob agitação de 70 rpm, a 37 °C pelos períodos de 0, 30, 60, 120 e 240 minutos. Após o período de incubação, a atividade proteolítica da PK foi interrompida pela adição de 100 mM de PMSF e as leptospiros tratadas foram

centrifugadas por 5 minutos a 6000 rpm, lavadas com PBS (50 mM de NaCl) por duas vezes e ressuspensas em 1 mL de PBS (50 mM de NaCl). Policlonais contra rLIC10258 (1:1000), rLIC12880 (1:1000), rLIC12238 (1:1000), LipL32 (1:4000) e DnaK (1:2000) foram utilizados para detectar a presença destas proteínas na superfície das leptospiros tratadas com PK, em ensaio de ELISA, como já descrito anteriormente. LipL32 e DnaK foram utilizadas como controle positivo e negativo do ensaio, respectivamente. Os resultados foram analisados estatisticamente pelo *Student's two-tailed t test*, comparando-se a presença das proteínas na superfície das leptospiros tratadas e não tratadas com PK. $P < 0,05$ foi considerado estatisticamente significativo.

3.19 Ensaio de adesão das proteínas e fragmentos recombinantes a componentes da matriz extracelular (MEC)

Os ensaios de adesão foram realizados segundo Barbosa e colaboradores (2006) com modificações (BARBOSA et al., 2006). Placas de ELISA (High binding, Nunc) foram adsorvidas com 1 µg de laminina (Sigma), colágeno tipo I (Sigma), colágeno tipo IV (Sigma), fibronectina celular (Sigma), fibroectina plasmática (Sigma) e os controles negativos BSA (proteína não-glicosilada) e fetuína (proteína glicosilada), diluídos em 100 µL de PBS. As placas foram incubadas por 2 horas a 37 °C e, após incubação foram lavadas, três vezes, com PBS-Tween 0,05%. O bloqueio foi feito por 1 hora a 37°C com 200 µL de PBS contendo 10% de leite e após as placas foram incubadas a 4 °C por 16 horas. Após incubação, a solução bloqueio foi descartada e adicionou-se 1 µg de cada proteína e fragmento recombinante diluído em 100 µL de PBS em cada poço. As placas foram incubadas por 90 minutos a 37 °C e, após incubação foram lavadas, seis vezes, com PBS-Tween 0,05%. A adesão das proteínas e fragmentos recombinantes aos componentes da matriz extracelular foi detectada pela adição de anticorpos policlonais anti-proteínas e anti-fragmentos recombinantes, produzidos em camundongos, seguida da incubação com anticorpo anti-IgG de camundongo conjugado a peroxidase. A reação foi revelada com OPD, como já descrito anteriormente e a DO foi determinada em leitor de microplacas usando o comprimento de onda de 492 nm. Para cada proteína recombinante testada, quatro poços foram adsorvidos com cada um dos componentes de matriz extracelular

utilizados neste ensaio. Para controle da especificidade da reação do anticorpo, em um destes poços, a proteína ou fragmento recombinante não foi adicionada. Para análises estatísticas, a ligação das proteínas ou fragmentos recombinantes aos MECs foi comparada a ligação dos controles negativos (BSA e fetuína) aos MECs pelo *Student's two-tailed t test*. O valor de $P < 0,05$ foi considerado estatisticamente significativo. Em outro ensaio, anticorpos monoclonais anti-His tag (Sigma), na diluição 1:1000, foram utilizados para detectar a ligação de rLIC10258 e seus fragmentos a laminina e fibronectina plasmática.

3.20 Ensaio de ligação das proteínas e fragmentos recombinantes ao PLG, fator H, fibrinogênio, componentes do sistema complemento e C4BP

A ligação das proteínas e fragmentos recombinantes aos componentes citados foi avaliada por um ensaio de ELISA modificado. Placas de ELISA (High binding, Nunc) foram adsorvidas com 100 μ L/poço de 10 μ g/mL de PLG (Calbiochem), fator H (Calbiochem), fibrinogênio (Sigma), complemento humano (Sigma) e C4BP (Complements Technology) diluídos em PBS por uma noite a 4 °C. BSA e fetuína foram utilizados como controles negativos. Após incubação, as placas foram lavadas uma vez com PBS-Tween 0,05% e bloqueadas com PBS contendo 10% de leite desnatado por 2 horas a 37 °C. A solução de bloqueio foi descartada e adicionou-se 1 μ g/poço de proteína e fragmento recombinante diluído em 100 μ L de PBS. As placas foram incubadas por 2 horas a 37 °C e, após incubação foram lavadas, quatro vezes, com PBS-Tween 0,05%. A ligação das proteínas e fragmentos recombinantes foi detectada pela adição de anticorpos policlonais anti-proteínas e anti-fragmentos recombinantes (1:1000), produzidos em camundongos, seguida da incubação com anticorpo anti-IgG de camundongo conjugado a peroxidase (Sigma) na diluição 1:5000. Em outro ensaio, anticorpos monoclonais anti-His tag (Sigma), na diluição 1:1000, foram utilizados para detectar a ligação das proteínas e fragmentos recombinantes ao PLG e da proteína rLIC12238 ao fibrinogênio. A reação foi revelada com OPD, como já descrito anteriormente e determinou-se a intensidade de absorção à 492 nm em leitor de microplacas. Para análises estatísticas, a ligação das proteínas ou fragmentos recombinantes ao PLG foi comparada a ligação das mesmas aos controles negativos (BSA e fetuína) pelo

Student's two-tailed t test. O valor de $P < 0,05$ foi considerado estatisticamente significativo.

3.21 Ensaio de atividade enzimática da plasmina

Para este ensaio, placas de ELISA foram adsorvidas com 1 µg de proteína recombinante ou BSA por 16 horas a 4 °C. As placas foram lavadas uma vez com PBS/Tween 0,05% e bloqueadas com PBS/Leite desnatado 10% por 2 horas a 37 °C. A solução bloqueio foi descartada e 100 µL/poço de 10 µg/mL de PLG humano foram adicionados e incubados por 2 horas a 37 °C. Após 3 lavagens com PBS/Tween 0,05% foram adicionados 4 ng/poço de ativador de plasminogênio tipo uroquinase (uPA – Sigma) e 0,04 mM de substrato específico para plasmina. As placas foram incubadas por aproximadamente 16 horas a 37 °C e a degradação do substrato foi mensurada por leitura em espectrofotômetro no comprimento de onda 405 nm. Para análises estatísticas, a atividade enzimática da plasmina foi comparada ao controle negativo (BSA) pelo *Student's two-tailed t test*, sendo o valor de $P < 0,05$ considerado estatisticamente significativo.

3.22 Ensaio de inibição da formação do coágulo de fibrina

Para este ensaio 0,5 mL de solução de PBS contendo 1 mg/mL de fibrinogênio foi incubada com 3500 nM de rLIC12238 por 2 horas a 37 °C. Após incubação, 90 µL da solução contendo rLIC12238 e fibrinogênio foi transferida para placas de ELISA e a formação do coágulo de fibrina foi iniciada pela adição de 10 µL/poço de trombina (10 U/mL – Sigma) e monitorada em leitor de microplacas (Thermo – OD = 595 nm) a cada 1 minuto por 10 minutos e por mais 35 minutos, a cada 5 minutos. As soluções contendo apenas fibrinogênio com ou sem adição de trombina foram utilizadas como controles positivo e negativo do ensaio, respectivamente. Para análises estatísticas, a inibição da formação do coágulo pela proteína recombinante foi comparada com o controle positivo do ensaio pelo *Student's two tailed t test*, o valor de $P < 0,05$ foi considerado estatisticamente significativo.

3.23 Curvas de dose resposta

A especificidade da ligação das proteínas e fragmentos recombinantes com a laminina, fibronectina plasmática, PLG e fibrinogênio foi analisada em ensaio de dose-dependência e saturação por ELISA. Neste ensaio, placas de ELISA foram adsorvidas com concentrações fixas de laminina, fibronectina plasmática, PLG e fibrinogênio (1 µg/poço) por 16 horas a 4 °C. As placas foram lavadas três vezes e bloqueadas com PBS/Leite desnatado 10% por 2 horas a 37 °C. Após o bloqueio as placas foram incubadas com as seguintes concentrações das proteínas recombinantes: laminina e rLIC10258 (0 a 1000 nM); fibronectina plasmática ou PLG e rLIC10258 (0 a 2000 nM); PLG e rLIC12880 (0 a 2000 nM); PLG e rLIC12238 (0 a 2000 nM), fibrinogênio e rLIC12238 (0 a 4000 nM), laminina e fragmentos Intermediário e C-terminal (0 a 2000 nM), fibronectina plasmática e fragmento Intermediário (0 a 3000 nM) e PLG e fragmento Intermediário (0 a 4000 nM). A ligação das proteínas recombinantes foi detectada pela adição de anticorpos policlonais anti-proteínas recombinantes (1:1000), produzidos em camundongos, seguido da incubação com anticorpo anti-IgG de camundongo conjugado a peroxidase (Sigma) na diluição 1:5000. A reação foi revelada com OPD, como já descrito anteriormente e determinou-se a intensidade de absorção à 492 nm em leitor de microplacas. Os dados do ensaio de ELISA foram utilizados para calcular a constante de dissociação (KD) de acordo com método descrito por Pathirana e colaboradores (2006) e Lin et al. (2009) (ZUERNER et al., 2000; PATHIRANA et al., 2006), com base na equação:

$$A = [\text{proteína}] A_{\text{max}} / ([\text{proteína}] K_D + A_{\text{max}})$$

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.

3.24 Interação das proteínas e fragmentos recombinantes desnaturados com MECs, PLG e fibrinogênio

Neste ensaio, placas de ELISA foram adsorvidas com 100 µL/poço de 10 µg/mL de laminina, fibronectina plasmática, PLG e fibrinogênio por 2 horas a 37 °C.

As placas foram lavadas com PBS-T e bloqueadas com 200 μ L de 10% (wt/vol) de leite desnatado acrescido de 1% de BSA, por 16 horas a 4 °C. Após o bloqueio, as placas foram incubadas por 90 minutos a 37 °C com as proteínas e fragmentos recombinantes previamente desnaturados por aquecimento a 96 °C durante 10 minutos. A ligação das proteínas e fragmentos recombinantes desnaturados foi detectada pela adição de anticorpos policlonais anti-proteínas recombinantes (1:1000) e anti-fragmentos recombinantes (1:500), produzidos em camundongos, seguido da incubação com anticorpo anti-IgG de camundongo conjugado a peroxidase (Sigma) na diluição 1:5000. A reação foi revelada com OPD, como já descrito anteriormente e determinou-se a intensidade de absorção à 492 nm em leitor de microplacas. Para análises estatísticas, a ligação das proteínas e fragmentos recombinantes desnaturados aos componentes foi comparada a ligação das proteínas e fragmentos recombinantes não desnaturados pelo *Student's two-tailed t test*, sendo o valor de $P < 0,05$ considerado estatisticamente significativo.

3.25 Interação das proteínas e fragmentos recombinantes bloqueados com soro homólogo com MECs, PLG e fibrinogênio

Para este ensaio, placas de ELISA foram adsorvidas com 100 μ L/poço de 10 μ g/mL de laminina, fibronectina plasmática, PLG e fibrinogênio por 2 horas a 37 °C. As placas foram bloqueadas com 200 μ L de 10% (wt/vol) de leite desnatado acrescido de 1% de BSA, por 16 horas a 4 °C. Após o bloqueio, as placas foram incubadas por 90 minutos a 37 °C com as proteínas e fragmentos recombinantes previamente incubados com policlonais homólogos produzidos em camundongos (1:200) durante 1 hora a 37 °C. As placas foram lavadas seis vezes com PBS-T e a ligação das proteínas e fragmentos recombinantes bloqueados com soro homólogo foi detectada pela adição de anticorpos monoclonais anti-polyhistidine conjugado a peroxidase (Sigma) na diluição 1:2000. A reação foi revelada com OPD, como já descrito anteriormente e determinou-se a intensidade de absorção à 492 nm em leitor de microplacas. Para análises estatísticas, a ligação das proteínas e fragmentos recombinantes bloqueados aos componentes foi comparada a ligação das proteínas e fragmentos recombinantes não bloqueados pelo *Student's two-tailed t test*, o valor de $P < 0,05$ foi considerado estatisticamente significativo.

3.26 Ensaio de inibição da adesão de leptospiros vivos a MECs, PLG e fibrinogênio pelas proteínas recombinantes

Neste ensaio, placas de ELISA foram adsorvidas com 10 µg/mL de laminina, fibronectina plasmática, PLG ou fibrinogênio (100 µL/poço) por 16 horas a 4 °C. As placas foram lavadas uma vez com PBS/Tween 0,05% e bloqueadas com PBS/Leite desnatado 10% por 2 horas a 37 °C. A solução de bloqueio foi descartada e as placas incubadas com concentrações crescentes de proteínas recombinantes (0 a 5 µg) por 90 minutos a 37 °C. Após 3 lavagens, foram adicionadas 4×10^7 de *L. interrogans* sorovar Copenhageni cepa M20 vivas (100 µL/poço) e incubou-se por 90 minutos a 37 °C. As placas foram lavadas para retirada das leptospiros não ligadas e foram adicionados anticorpos anti-LipL32 (1:4000), produzidos em camundongos, para quantificar de forma indireta as leptospiros ligadas, já que a LipL32 é uma proteína majoritária da membrana externa de *Leptospira* (HAAKE et al., 2000). Em seguida, adicionou-se anticorpos anti-mouse IgG conjugado a peroxidase (Sigma) como descrito por Barbosa e colaboradores (2006) (BARBOSA et al., 2006). A reação foi revelada utilizando o substrato OPD como já descrito neste relatório. Os resultados foram analisados estatisticamente pelo *Student's two-tailed t test*, comparando-se a adesão das *Leptospiras* aos MECs, PLG e fibrinogênio na ausência e presença de proteína recombinante. $P < 0,05$ foi considerado estatisticamente significativo.

3.27 Ensaio de competição entre PLG e fibronectina plasmática pelo fragmento Intermediário

Para este ensaio, placas de ELISA foram adsorvidas com 10 µg/ml de fragmento Intermediário (100 µL/poço) por 16 horas a 4 °C. As placas foram lavadas, uma vez, com PBS-T e bloqueadas com PBS/Leite desnatado 10% por 2 horas a 37 °C. As placas foram incubadas com 1 µg de PLG com concentrações crescentes de fibronectina plasmática (0 a 1 µg) por 2 horas a 37 °C. Após quatro lavagens, a ligação da fibronectina plasmática ao fragmento Intermediário foi detectada por anticorpos contra PLG (1:4000, Calbiochem) seguido da incubação com anticorpo anti-IgG de camundongo conjugado a peroxidase (Sigma) na diluição 1:5000. Experimento semelhante foi realizado para avaliar a interferência do PLG na

ligação da fibronectina plasmática ao fragmento Intermediário. Neste ensaio, as placas adsorvidas com o fragmento Intermediário foram incubadas com 1 µg de fibronectina plasmática com concentrações crescentes de PLG (0 a 1 µg) e a ligação foi detectada por anticorpos contra fibronectina plasmática (1:1000, Sigma). A reação foi revelada com OPD, como já descrito anteriormente e determinou-se a intensidade de absorção à 492 nm em leitor de microplacas.

3.28 Ensaio de imunização/desafio com as proteínas recombinantes

Neste ensaio, grupos de doze hamstêrs Golden Syrian machos (6-8 semanas) foram imunizados subcutaneamente no dorso com 50 µg de proteína recombinante rLIC10258, rLIC12880 e rLIC12238, bacterina (10^9 leptospiros inativadas) ou PBS adsorvidos no adjuvante Alhydrogel ($\text{Al}(\text{OH})_3$ – Brenntag Biosector) na concentração de 10%. Foram realizadas duas imunizações com intervalos de 15 dias. Os grupos imunizados com bacterina e PBS foram utilizados como controle positivo e negativo do experimento, respectivamente. A bacterina foi preparada a partir de cultura de *L. interrogans* sorovar Kennwicki (cepa Pomona Fromm) virulenta e recém isolada de animal infectado conforme descrito por Silva e colaboradores (2007) (SILVA et al., 2007). Para a preparação da bacterina, a densidade celular da cultura utilizada foi estimada por meio da contagem do número de células em câmara de Petroff-Hausser em microscópio de campo escuro e aplicação da seguinte fórmula:

$$\text{número de células por mL} = \text{número de células contadas} \times \text{diluição} \times 50000$$

A seguir, a cultura foi centrifugada a 6000 rpm por 20 minutos a temperatura ambiente. O sedimento obtido foi lavado duas vezes com tampão PBS suplementado com 5 mM de MgCl_2 e ressuspensionado em PBS. As bactérias foram inativadas por aquecimento a 56 °C por 20 minutos. A bacterina foi armazenada a – 20 °C. No ensaio de desafio com a proteína rLIC10258, dois animais de cada grupo foram sangrados, via punção cardíaca, 15 dias após a segunda imunização (antes do desafio), enquanto que no ensaio com as proteínas rLIC12880 e rLIC12238, seis animais de cada grupo foram sangrados, via retro orbital, 15 dias após cada imunização. Os soros obtidos foram analisados por ELISA para determinação do título de anticorpos. Duas semanas após a segunda imunização, os hamsters foram inoculados intraperitonealmente com *L. interrogans* sorovar Kennwicki (cepa Pomona Fromm) virulenta e isolada recentemente de hamstêrs Golden Syrian infectados. A

dose de bactérias para o ensaio com a proteínas rLIC10258 foi 2×10^5 leptospiros/animal, enquanto que no ensaio com as proteínas rLIC12880 e rLIC12238 a dose foi de $2,5 \times 10^3$ leptospiros/animal. Os animais desafiados foram monitorados por 21 dias e os sobreviventes foram sangrados, via punção cardíaca, e sacrificados. Para análise da presença de leptospiros, os rins dos animais sobreviventes foram coletados, macerados e ressuspensos em solução salina nas diluições 1:10 e 1:100 e cultivadas em meio EMJH semi-sólido modificado a 28°C. Os ensaios de imunoproteção foram realizados no Laboratório de Zoonoses da Faculdade de Medicina Veterinária da USP.

3.29 Culturas de *Leptospiras* e soros de pacientes diagnosticados com leptospirose

As diferentes cepas e sorovares de *Leptospira* spp utilizadas neste trabalho foram cedidas pela Faculdade de Medicina Veterinária da Universidade de São Paulo. Estas cepas foram cultivadas em meio EMJH modificado (RODRIGUEZ et al., 2002) enriquecido com 10% de soro de coelho a temperatura de 28 °C.

Os soros de pacientes diagnosticados com leptospirose utilizados neste estudo fazem parte de uma soroteca e foram cedidos pela Dra Eliete C. Romero do Instituto Adolfo Lutz.

4 RESULTADOS E DISCUSSÃO

4.1 Análise por bioinformática

Os genes LIC10258, LIC12880 e LIC12238 foram selecionados do genoma de *L. interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004b), a partir da análise *in silico* feita em programas disponíveis em servidores públicos.

De acordo com o programa SMART (SCHULTZ et al., 1998) todos os genes deste estudo codificam proteínas com peptídeo sinal de exportação entre os aminoácidos 1 e 28 e baseado nos programas de localização celular PSORT (NAKAI e HORTON, 1999) e CELLO (YU et al., 2006), os genes LIC10258, LIC12880 e LIC12238 codificam proteínas com provável localização na membrana externa das leptospiros.

Segundo o programa LipoP (JUNCKER et al., 2003), os genes LIC12880 e LIC12238 codificam prováveis lipoproteínas. O programa LipoP (JUNCKER et al., 2003) identifica genes que codificam lipoproteínas baseado na presença de uma sequência consenso denominada *lipobox*, na qual a presença de um resíduo de cisteína indica que a proteína será lipidada. Comparando as sequências do peptídeo sinal de bactérias gram-negativas, foi proposto por Wu, em 1996, o *lipobox* em *E. coli* (WU, 1996). Nas espiroquetas, o *lipobox* está representado segundo a sequência consenso Leu(Ala,Ser)₄-Leu(Val,Phe,Ile)₃-Ile(Val,Gly)₂-Ala(Ser,Gly)₁-Cys₊₁ que foi proposta por Haake (2000) após comparação e análise de 26 sequências conhecidas de lipoproteínas de espécies de *Treponema* spp, *Borrelia* spp e *Leptospira* spp (HAAKE, 2000).

O programa BLAST (ALTSCHUL et al., 1990) analisou a presença destes genes em outros organismos e identificou domínios conservados. A análise revelou que LIC10258 apresenta domínio tipo OmpA em sua porção C-terminal. Este domínio tem sido identificado em proteínas envolvidas na patogenicidade de bactérias gram-negativas (TORRES et al., 2006; RISTOW et al., 2007; ZHANG et al., 2010), como por exemplo: PAL (*peptidoglycan-associated lipoprotein*) de *E. coli* (CASCALES et al., 2002); OprF, uma proteína de membrana externa majoritária de *Pseudomonas aeruginosa* (BRINKMAN; BAINS; HANCOCK, 2000) e a Sal-OmpA de *Salmonella typhimurium*, uma proteína com potencial vacinal contra a salmonelose (SINGH et al., 2003). Nas leptospiros, é importante ressaltar que a primeira proteína

descrita como fator de virulência, Loa22, possui domínio tipo OmpA (RISTOW et al., 2007). Além disso, proteínas identificadas com este domínio tem sido testadas como possíveis candidatos vacinais contra a leptospirose (YAN et al., 2010).

Todos os genes selecionados para este estudo apresentaram identidade com genes presentes em espécies de leptospiros com o genoma totalmente sequenciado. Atualmente, cinco espécies de *Leptospira* já foram sequenciadas: *L. interrogans* sorovar Lai cepas 56601 e IPAV (REN et al., 2003; ZHONG et al., 2011), *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130 (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b), *L. borgpetersenii* sorovar Hardjo-bovis cepas L550 e JB197 (BULACH et al., 2006), *L. biflexa* sorovar Patoc cepa Patoc1 (Paris e Ames) (PICARDEAU et al., 2008), *L. santarosai* sorovar Shermani cepa LT821 (CHOU et al., 2012) e *L. licerasiae* cepas VAR010 e MMD0835 (RICALDI et al., 2012). A similaridade dos genes LIC10258, LIC12880 e LIC12238 com sequências presentes nas espécies de leptospiros citadas acima e em outras espécies que ainda não foram totalmente sequenciadas pode ser visualizada nos filogramas gerados pelo programa CLUSTAL 2.1 (LARKIN et al., 2007). A distância menor ou maior entre os ramos do filograma demonstra maior ou menor identidade entre as sequências. Como pode ser observado na figura 01, todos os genes possuem alta identidade com sequências presentes em *L. interrogans* sorovar Lai. Além disso todos os genes estão presentes em *L. borgpetersenii* sorovar Hardjo-bovis com menor identidade. Os genes LIC12880 e LIC12238 possuem 73 e 82% de similaridade com a espécie patogênica *L. santarosai* sorovar Shermani, enquanto que os genes LIC10258 e LIC12238 possuem pequena similaridade com a espécie de patogenicidade intermediária *L. licerasiae*. Além disso, apenas LIC12238 apresentou 39% de similaridade com uma sequência identificada na espécie saprofítica *L. biflexa* sorovar Patoc. A tabela 01 apresenta um resumo dos dados obtidos após análise *in silico* dos genes LIC10258, LIC12880 e LIC12238.

Figura 01 – Análise *in silico* da similaridade dos genes LIC10258, LIC12880 e LIC12238 em diferentes espécies de *Leptospira* pelos programas BLAST e CLUSTAL 2.1

Tabela 01 - Análise *in silico* dos genes LIC10258, LIC12880 e LIC12238

Gene	Descrição da Função	Conservação (identidade)	Localização Celular (Probabilidade)		Peptídeo Sinal	Sítio de Lipidação
			PSORT	CELLO		
LIC10258	Proteína hipotética com domínio OmpA	99% (Lai) 79% (LBH) 44% (LlicsVM)	40%	70%	sim	não
LIC12880	Lipoproteína hipotética sem domínio conhecido	99% (Lai) 76% (LBH) 73% (LSS)	50%	45%	sim	sim
LIC12238	Proteína hipotética sem domínio conhecido	99% (Lai) 77% (LBH) 82% (LSS) 50% (LlicsVM) 39% (LBP)	50%	40%	sim	sim

Notas: LIC: identificação do gene no genoma de *L. interrogans* sorovar Copenhageni
 Abreviaturas: Lai: *L. interrogans* sorovar Lai; LBH: *L. borgpetersenii* sorovar Hardjo-bovis;
 LBP : *L. biflexa* sorovar Patoc; LlicsVM: *L. licerasiae* serovar Varillal;
 LSS: *L. santarosai* serovar Shermani

4.2 Amplificação dos genes LIC10258, LIC12880 e LIC12238 por PCR

Os genes LIC10258, LIC12880 e LIC12238 foram obtidos por amplificação (PCR) do DNA genômico extraído da cultura de *L. interrogans* sorovar Copenhageni. As bandas de DNA obtidas através da amplificação foram analisadas em gel de agarose e apresentaram o tamanho esperado de 1695, 792 e 444 pb para os clones referentes a LIC10258, LIC12880 e LIC12238, respectivamente. A figura 02 apresenta os produtos obtidos da reação de PCR purificados a partir do gel de agarose.

Figura 02 - Análise do DNA de interesse amplificado por reação de PCR em gel de agarose 1%

As bandas obtidas apresentaram os tamanhos esperados de 1695 pb para LIC10258 (linha 2), 792 pb para LIC12880 (linha 3) e 444 pb para LIC12238 (linha 4). Linha 1: Kb Plus DNA Ladder (marcador molecular).

4.3 Clonagem no vetor pGEM-T Easy

Este vetor foi utilizado para otimizar as reações de digestão com as enzimas específicas de cada clonagem. O pGEM-T Easy (Figura 03) é um vetor linearizado, com uma timidina 3'-terminal em ambas extremidades, o que evita a recircularização do vetor e possibilita a ligação de produtos de PCR pois estes possuem, normalmente, uma adenosina em cada extremidade 3', adicionada pela atividade transferase terminal da *Taq* polimerase. O pGEM-T Easy contém os promotores T7 e SP6 e sítio de múltipla clonagem interrompendo a região codificadora da enzima β -galactosidase, o que permite a identificação dos clones recombinantes positivos e negativos por seleção das colônias bacterianas de cor branca e azul, respectivamente, em placas de meio LB, contendo ampicilina, IPTG e X-Gal. Sendo assim, os insertos de DNA obtidos por meio da reação de PCR foram clonados em vetor pGEM-T Easy e utilizados para transformar bactérias competentes DH5 α . Após extração do DNA plasmidial das bactérias competentes, os plasmídeos contendo os genes LIC10258, LIC12880 e LIC12238 foram digeridos com as enzimas de restrição correspondentes para certificação da presença de insertos (dados não mostrados).

Figura 03 - Mapa do vetor de clonagem pGEM-T Easy

O vetor comercial pGEM-T Easy possui origem de replicação (f1ori), gene de resistência a ampicilina (Amp^r) e o gene β -galactosidase para metabolismo de lactose (lacZ).

Fonte: Promega (2010).

4.4 Subclonagem no vetor de expressão pAE

Este vetor foi desenvolvido para expressar proteínas recombinantes contendo 6 resíduos de histidina na região N-terminal, o que permite a purificação das mesmas em coluna de cromatografia carregada com níquel. Desta maneira, os insertos correspondentes aos genes LIC10258, LIC12880 e LIC12238 foram removidos dos plasmídios recombinantes (pGEM-T Easy/inserto) e subclonados no vetor de expressão pAE (Figura 04) (RAMOS et al., 2004).

Figura 04 - Mapa do vetor de expressão pAE

O vetor de expressão pAE possui o promotor T7 (PT7), sítio de ligação ao ribossomo (RBS), códons para expressão de seis resíduos de histidina (6xHis), códon de início da transcrição (ATG), região de terminação de transcrição (T7term), origem de replicação (F1ori) e gene de resistência à ampicilina (Amp^r).

Os clones positivos foram selecionados por meio da extração rápida por fenol-clorofórmio e após a obtenção do DNA plasmidial (pAE/inserto) os clones foram digeridos com as enzimas de restrição correspondentes para confirmação da presença de inserto (Figura 05).

Figura 05 - Análise dos insertos de DNA obtidos por digestão do DNA plasmidial (pAE/inserto) em gel de agarose 1%

Após seleção por fenol-clorofórmio os clones positivos foram purificados e os plasmídeos obtidos digeridos com as enzimas apropriadas. A) 2-5 (LIC10258), onde 2 e 4 – pAE contendo inserto, 3 e 5 – pAE digerido com as enzimas *BamHI/NcoI*. 6-9 (LIC12880), onde 6 e 8 – pAE contendo inserto, 7 e 9 – pAE digerido com as enzimas *XhoI/NcoI*. B) LIC12238, onde 2 e 4 – pAE contendo inserto, 3 e 5 – pAE digerido com as enzimas *XhoI/HindIII*. 1. marcador molecular (λ hind III).

4.5 Sequenciamento dos clones

O sequenciamento confirmou a presença dos insertos de DNA referentes aos genes LIC10258, LIC12880 e LIC12238, tanto no vetor de clonagem pGEM-T Easy quanto no vetor de expressão pAE. No vetor de expressão pAE, apenas o clone referente a LIC10258 não foi totalmente sequenciado, porém o mesmo já havia sido completamente sequenciado no vetor pGEM-T Easy. Todas as sequências dos clones apresentaram identidade quando comparadas as sequências de bases nitrogenadas do gene original. A sequência codificadora foi confirmada pelo programa BLAST (ALTSCHUL et al., 1990) mostrando 99, 98 e 100% de identidade com as sequências dos clones referentes a LIC10258, LIC12880 e LIC12238, respectivamente, tanto no vetor pGEM-T Easy quanto no vetor pAE. No cromatograma resultante do sequenciamento dos clones no vetor pAE, foi possível identificar a presença do códon de iniciação (ATG), seguido das bases nitrogenadas (CAT/CAC) que codificam os resíduos de histidina e o início da região codificadora dos genes LIC10258, LIC12880 e LIC12238 (Anexo A). Os resultados obtidos

confirmam que as sequências clonadas foram introduzidas na fase correta de leitura do vetor de expressão pAE.

4.6 Análise da presença dos genes LIC10258, LIC12880 e LIC12238 e de seus transcritos em diferentes cepas de *Leptospira*

As leptospiros são classificadas em sorovares devido à diversidade antigênica resultante da composição heterogênea dos carboidratos presentes nos lipopolissacarídeos da sua membrana (FAINE et al., 1999; LEVETT, 2001; BHARTI et al., 2003). Em consequência, um amplo número de espécies de mamíferos serve como hospedeiro reservatório de *Leptospira* e mais de 250 sorovares já foram descritos (HAAKE; MATSUNAGA, 2010). Portanto, a busca por proteínas presentes em diversas espécies e sorovares de *Leptospira* é um fator importante para obtenção de bons antígenos vacinais. Sendo assim, a presença dos genes LIC10258, LIC12880 e LIC12238 foi analisada em sorovares patogênicos de *L. interrogans* e em espécies patogênicas de *Leptospira*. A espécie não patogênica, *L. biflexa* sorovar Patoc também foi avaliada (Figura 06).

Figura 06 - Análise da presença dos genes LIC10258, LIC12880 e LIC12238 em diferentes espécies e sorovares de *Leptospira* em gel de agarose 1%

A expressão do gene 16S rRNA foi utilizado como controle da integridade dos DNAs obtidos. Não foi adicionado DNA no controle negativo (-).

O gene LIC12238 foi detectado em todas as espécies e sorovares testados, incluindo na espécie saprofítica *L. biflexa*. Este resultado está em concordância com análise *in silico* feita através do programa BLAST (ALTSCHUL et al., 1990), que mostra uma sequência presente em *L. biflexa* com 39% de similaridade com o gene LIC12238 de *L. interrogans* sorovar Copenhageni.

Os genes LIC10258 e LIC12880 foram detectados em todos os sorovares de *L. interrogans*. O gene LIC12880, por sua vez, também foi detectado nas espécies patogênicas *L. borgpetersenii* sorovar Castellonis, *L. santarosai* sorovar Shermanii e *L. noguchii* sorovar Panama. A expressão do gene 16S rRNA foi utilizado como controle da integridade das amostras de DNA obtidas.

Com o intuito de analisar se estes genes estão sendo transcritos nos sorovares e espécies de *Leptospira* em que estão presentes, foi realizada a análise da expressão dos genes LIC10258, LIC12880 e LIC12238 nos sorovares e espécies de *Leptospira* citados acima. Para este ensaio foi extraído o RNA total dos sorovares e espécies de *Leptospira* utilizados para análise genômica e a presença dos transcritos nas amostras obtidas foi analisada por RT-PCR, empregando os mesmos oligonucleotídeos utilizados para a amplificação dos genes LIC10258, LIC12880 e LIC12238. A expressão do gene 16S rRNA foi usada como controle da integridade dos transcritos obtidos e a contaminação das amostras com DNA genômico foi descartada, uma vez que não foi observada amplificação nas amostras não tratadas com a enzima *transcriptase reversa*. Apenas o cDNA de LIC12238 foi amplificado em todas as amostras em que este gene foi detectado. Não houve amplificação do transcrito do gene LIC10258 no sorovar Icterohaemorrhagiae de *L. interrogans* e o cDNA do gene LIC12880 não foi detectado nas espécies *L. borgpetersenii*, *L. santarosai* e *L. noguchii* (Figura 07).

Figura 07 - Análise da expressão dos genes LIC10258, LIC12880 e LIC12238 em diferentes espécies e sorovares de *Leptospira* em gel de agarose 1%

A expressão do gene 16S rRNA foi utilizado como controle da integridade dos transcritos obtidos. (+): Enzima *transcriptase reversa* presente e (-): Enzima *transcriptase reversa* ausente.

Muitos patógenos bacterianos adaptam-se ao ambiente em que se encontram regulando a expressão de alguns de seus genes. Tem sido demonstrado que a expressão de alguns genes de *Leptospira* são regulados por mudanças de temperatura, pH ou osmolaridade (NALLY; TIMONEY; STEVENSON, 2001; LO et al., 2006; OLIVEIRA et al., 2010). Portanto, para verificar se a expressão dos genes LIC10258 e LIC12880 estaria sendo regulada por alguma das condições descritas acima foi extraído o RNA total de cultura de *L. interrogans* sorovar Icterohaemorrhagiae, *L. borgpetersenii* sorovar Castellonis, *L. santarosai* sorovar Shermani e *L. noguchii* sorovar Panama suplementada com 120 mM de NaCl. A adição de sal ao meio de cultura das leptospiros mimetiza as condições fisiológicas encontradas por este organismo no hospedeiro. Também foram extraídos o RNA total destas culturas mantidas, por uma noite, a 37 ou 39 °C para simular as condições encontradas pelas leptospiros durante os estágios inicial e febril da infecção.

Não foi detectada a expressão do gene LIC12880 em nenhuma das condições testadas (dados não mostrados). Por outro lado, a figura 08 mostra que, apesar da transcrição do gene LIC10258 não estar sendo regulado pela temperatura, uma vez que não houve amplificação do cDNA obtido das culturas de *L. interrogans* sorovar Icterohaemorrhagiae mantidas a 37 e 39 °C, foi possível

detectar a amplificação do cDNA obtido da cultura suplementada com 120 mM de NaCl, sugerindo que a transcrição deste gene pode estar sendo regulada por condições fisiológicas de osmolaridade encontradas no hospedeiro.

Figura 08 - Análise da regulação da expressão do gene LIC10258 por fatores ambientais em gel de agarose 1%

A expressão do gene 16S rRNA foi utilizado como controle da integridade dos transcritos obtidos. (+): Enzima *transcriptase reversa* presente e (-): Enzima *transcriptase reversa* ausente.

4.7 Expressão das proteínas recombinantes

Para obtenção das proteínas recombinantes desta tese foi realizado um estudo cinético de indução com NaCl em diferentes concentrações. Para este estudo foi utilizada a cepa *E. coli* BL21 SI (*Salt Induced*), que possui o gene T7 RNA polimerase integrado ao genoma sob o controle do promotor *proU* indutível por NaCl (BHANDARI; GOWRISHANKAR, 1997). Sendo assim, bactérias *E. coli* BL21 SI foram transformadas e induzidas em diferentes concentrações de indutor. As concentrações utilizadas foram 3, 30 e 300 mM de NaCl. Após indução, as bactérias foram lisadas e as frações protéicas obtidas (sobrenadante e sedimento) analisadas em gel de SDS-PAGE. A proteína recombinante rLIC12238 foi expressa na forma solúvel enquanto rLIC10258 e rLIC12880 foram expressas na forma insolúvel como corpúsculos de inclusão.

A massa molecular de cada proteína foi previamente analisada *in silico*, a partir da composição de aminoácidos utilizando o programa *Generunner*. A figura 09 apresenta a expressão das proteínas recombinantes na cepa *E. coli* BL21 SI, sendo possível observar rLIC12238 com a massa molecular esperada de 17,6 kDa presente no sobrenadante do lisado bacteriano após indução com 300mM de NaCl. rLIC10258 e rLIC12880 foram observadas com massa molecular esperada de 65,7 e

30,68 kDa, no sedimento bacteriano após indução com 30 e 300 mM de NaCl, respectivamente.

Figura 09 - Análise da expressão das proteínas recombinantes em gel de SDS-PAGE

As bandas de proteínas com os tamanhos esperados de aproximadamente 17,6; 65,7 e 30,6 kDa, correspondendo a rLIC12238 (A), rLIC10258 (B) e rLIC12880 (C), respectivamente, foram visualizadas por Comassie blue. A expressão das proteínas foi analisada em diferentes concentrações de NaCl: 3mM (linhas 3 e 4); 30 mM (linhas 5 e 6) e 300 mM (linhas 7 e 8). rLIC12238 foi expressa na forma solúvel (A – linha 7) enquanto rLIC10258 e rLIC12880 (B e C – linhas 4, 6 e 8) foram expressas na forma insolúvel como corpúsculos de inclusão. Linha 1: massa molecular em kDa. Linha 2: extrato não induzido.

O acúmulo de proteínas sob a forma de corpúsculos de inclusão é uma característica importante do sistema de expressão em *E. coli* que ocorre devido um desequilíbrio entre a agregação e solubilização das proteínas recombinantes *in vivo* durante o processo de dobramento de sua estrutura. As proteínas recombinantes presentes nos corpúsculos de inclusão apresentam a vantagem de estarem protegidas contra a ação de proteases. Em contrapartida, durante o processo de solubilização destes corpúsculos de inclusão podem ocorrer perdas da proteína de interesse. Além disso, é necessário que a conformação estrutural destas proteínas seja recuperada, porém a renaturação da proteína recombinante, através da remoção do agente desnaturante utilizado no processo de solubilização, pode não ser bem sucedida (SORENSEN et al., 2004).

Sendo assim, na tentativa de obter as proteínas recombinantes rLIC10258 e rLIC12880 sob a forma solúvel, repetiu-se o estudo de cinética de indução de proteínas utilizando bactérias competentes *E. coli* BL21 DE3, BL21 Star (DE3) pLys e C43 (DE3). As diferentes cepas de *E. coli* DE3 também possuem o gene da T7

RNA polimerase, porém dirigido pelo promotor *lac UV5*, o qual é induzido por IPTG (STUDIER et al., 1990). A cepa *E. coli* BL21 Star (DE3) pLys, por sua vez, carrega o plasmídeo pLys que produz pequenas quantidades de T7 lisozima, um inibidor natural da T7 RNA polimerase. A redução da expressão basal da T7 RNA polimerase permite a expressão de proteínas recombinantes mais estáveis, particularmente para proteínas tóxicas (STUDIER, 1991). A cepa *E. coli* C43 (DE3) contém mutações genéticas selecionadas fenotipicamente para conferir tolerância a toxicidade. Esta cepa tem sido usada para produzir proteínas que são pouco expressas em *E. coli* BL21 DE3 (MIROUX; WALKER, 1996). As cepas de *E. coli* acima citadas foram transformadas e induzidas com IPTG nas concentrações de 0,01; 0,1 e 1 mM. Porém, como em todas as condições testadas rLIC10258 e rLIC12880 foram expressas sob a forma insolúvel, optou-se então por expressar estas proteínas recombinantes em *E. coli* BL21 SI.

4.8 Solubilização das proteínas rLIC10258 e rLIC12880

Foram testados dois protocolos de solubilização de proteínas recombinantes descritos na literatura (PATRA et al., 2000; SAINI et al., 2002). Inicialmente, testou-se a solubilização das proteínas em condições desnaturantes na presença de 1M de uréia em tampão aquoso com diferentes pHs, como o descrito por Patra e colaboradores (2000) (PATRA et al., 2000). Os diferentes pHs testados para rLIC10258 foram 6,8; 8; 9 e 12, e para rLIC12880 foram 6,8; 9 e 12. Os resultados mostraram que houve pequena solubilização da proteína rLIC10258 no tampão com pH12, enquanto que rLIC12880 não foi solubilizada em nenhuma das condições testadas (dados não mostrados).

Em paralelo, foi testado a solubilização das proteínas rLIC10258 e rLIC12880 em 8 M de uréia na presença de 10% de glicerol (SAINI et al., 2002). Esta metodologia permite a renaturação da proteína durante a etapa de purificação através da remoção gradativa da uréia na presença de glicerol que evita a agregação protéica. Tanto a proteína recombinante rLIC10258, quanto rLIC12880 foram satisfatoriamente solubilizadas (Figura 10).

Figura 10 - Análise das proteínas recombinantes após solubilização com 8 M uréia e 10% glicerol em gel de SDS-PAGE

As setas indicam as bandas correspondentes as proteínas rLIC10258 (A) e rLIC12880 (B). Linha 1: massa molecular. Linha 2: extrato não induzido. Linha 3: sobrenadante da sonicação. Linha 4: sedimento da sonicação. Linha 5: sobrenadante da solubilização e linha 6: sedimento da solubilização.

4.9 Purificação e diálise das proteínas recombinantes

Como o sistema de expressão de proteínas utilizado neste projeto adiciona uma sequência de 6 resíduos de histidina (6xHis) na extremidade N-terminal da proteína recombinante, as proteínas rLIC10258, rLIC12880 e rLIC12238 foram purificadas por cromatografia de afinidade utilizando resina carregada com níquel. Durante o processo de purificação, a proteína recombinante se liga ao níquel via resíduos de histidinas, os contaminantes são lavados com tampão contendo baixa concentração de imidazol e a proteína de interesse é eluída com alta concentração de imidazol. rLIC12238 foi purificada a partir do sobrenadante obtido após lise bacteriana, já que esta proteína foi expressa de maneira solúvel, enquanto que rLIC10258 e rLIC12880, expressas de maneira insolúvel, foram purificadas após a solubilização das mesmas em tampão contendo 10% de glicerol e 8 M uréia.

As alíquotas que continham as proteínas recombinantes foram dialisadas contra tampões apropriados. No caso das proteínas rLIC10258 e rLIC12880 foram realizadas diversas trocas do tampão de diálise para diluir a concentração de glicerol, até que a última troca eliminasse por completo o glicerol da amostra. Porém, não foi possível a retirada do glicerol das amostras contendo as proteínas

recombinantes rLIC10258 e rLIC12880, uma vez que a retirada do mesmo ocasionou a precipitação destas proteínas.

Após diálise, a concentração das proteínas recombinantes foi estimada em gel de SDS-PAGE com concentrações pré-estabelecidas de BSA e análise densitométrica do programa *GelQuant* (Figura 11).

Figura 11 - Estimativa da concentração das proteínas recombinantes em gel de SDS-PAGE

Linhas 1 a 5: BSA nas concentrações 4, 3, 2, 1 e 0,5 µg, respectivamente.
Linha 6: rLIC10258. Linha 7: rLIC12880 e Linha 8: rLIC12238.

4.10 Avaliação da atividade imunogênica das proteínas recombinantes em camundongos

Os animais foram imunizados com 3 doses de proteínas recombinantes e sangrados via plexo retro-orbital após cada dose. Os soros obtidos foram avaliados quanto à presença de anticorpos anti-rLIC10258, anti-rLIC12880 e anti-rLIC12238. Os títulos de anticorpos policlonais presentes no soro dos animais de cada grupo foram avaliados por ELISA.

O grupo imunizado com a proteína rLIC10258 só apresentou altos títulos de anticorpos após a terceira dose, enquanto que os grupos imunizados com rLIC12880 e rLIC12238 já apresentavam altos títulos após a segunda dose. O grupo controle, por sua vez, não apresentou quaisquer títulos. A média dos títulos de anticorpos obtidos por cada grupo após cada imunização está apresentada no quadro 02.

Quadro 02 - Títulos de anticorpos policlonais obtidos após imunização de camundongos com as proteínas recombinantes

Proteína Recombinante	1ª Sangria	2ª Sangria	3ª Sangria
rLIC10258	25	5333	255000
rLIC12880	8200	229400	508000
rLIC12238	6400	179200	1014000

4.11 Análise da estrutura das proteínas recombinantes por DC

O DC é uma técnica para o estudo dos elementos de estrutura secundária das proteínas, tais como, α -hélice, estrutura β e estrutura randômica, as quais contribuem na formação de sua estrutura terciária. Esta técnica utiliza a interação diferencial de moléculas com centros assimétricos (quirais) com a luz circularmente polarizada a esquerda ou direita (elipticidade), que é medida pelo polarímetro. Como as proteínas estão repletas de centros quirais distribuídos e arranjos ao longo da cadeia principal em α -hélices, estruturas β e outras estruturas, a estrutura secundária destas moléculas podem ser caracterizadas por espectroscopia de DC (CANTOR, 1981). Proteínas recombinantes que foram expressas na forma de corpúsculos de inclusão podem perder a estrutura no processo de renaturação, ou durante a estocagem, após purificação. Portanto, para certificar-se da integridade da conformação das proteínas recombinantes obtidas, as mesmas foram analisadas por dicróismo circular. A figura 12 mostra a curva obtida após análise dos dados fornecidos pelo espectropolarímetro. Todas as proteínas apresentaram uma mistura de estruturas secundárias, não sendo possível identificar predominância de nenhuma estrutura. O perfil das curvas apresentadas por estas proteínas, foi condizente com a predição teórica obtida no servidor público PSIPRED (JONES, 1999) que apontou a presença de uma mistura de estruturas secundárias (α -hélice, folha β e randômica) para estas proteínas. Além disso, é importante ressaltar que o perfil das curvas obtidas para todas as proteínas recombinantes não são semelhantes ao de proteínas sem conformação.

Figura 12 - Espectros obtidos das proteínas rLIC10258, rLIC12880 e rLIC12238 por dicroísmo circular

4.12 Presença de anticorpos IgG e IgM humanos contra proteínas recombinantes em soros de pacientes diagnosticados com leptospirose

O reconhecimento imune das proteínas recombinantes deste estudo por anticorpos IgG e IgM de amostras de soros de pacientes diagnosticados com leptospirose foi avaliada por ELISA. Inicialmente estabeleceu-se o valor de *cutoff* a partir de 6 amostras de soros de indivíduos normais que relataram não terem entrado em contato com a doença. Para este ensaio foram feitas incubações prévias dos soros com extrato de *E. coli* nas concentrações de 5 e 20%, evitando com isso a interferência de proteínas contaminantes de *E. coli* presentes na amostra dos recombinantes. Porém, mesmo com a adsorção, os valores individuais de reatividade de anticorpos IgG contra as proteínas rLIC10258 e rLIC12880 foram altos. Os valores de *cutoff* obtidos para estas proteínas foram de 0,367 e 0,343 nm, respectivamente. Em contrapartida, para rLIC12238 a adsorção resultou na diminuição da reatividade e o valor do *cutoff* foi de 0,046 nm. Em relação aos anticorpos IgM, mesmo sem a adsorção, os valores individuais de anticorpos contra as proteínas rLIC12880 e rLIC12238 foram baixos, uma vez que os valores de absorbância foram inferiores a 0,1. Apenas a proteína rLIC10258 apresentou valores maiores que foram satisfatoriamente diminuídos com a adsorção prévia. Portanto, para anticorpos IgM todos os valores de *cutoff* foram abaixo de 0,1 nm.

A próxima etapa foi avaliar a antigenicidade destas proteínas através do reconhecimento por anticorpos IgG e IgM de 20 amostras de pacientes diagnosticados com leptospirose (Anexo B). Das proteínas testadas, rLIC12880 foi a que apresentou menores percentuais de respondedores, 5% e 15% para IgG e IgM, respectivamente. O percentual de respondedores da proteína rLIC12238 foi de 35% tanto para IgG quanto para IgM. rLIC10258 apresentou percentual de respondedores maior para IgG do que para IgM, 35% e 10%, respectivamente (Figura 13). Análises estatísticas revelaram que comparativamente nenhum destes valores foi significativo.

Figura 13 - Análise da presença de anticorpos IgG e IgM contra rLIC10258, rLIC12880 e rLIC12238 em soro de pacientes diagnosticados com leptospirose

Amostras de soros de 20 indivíduos diagnosticados com leptospirose foram analisadas quanto à presença de anticorpos IgG e IgM contra as proteínas recombinantes por ELISA.

Das 20 amostras testadas, 10 amostras eram referentes a fase convalescente da doença, com resultado MAT positivo, e as outras 10 amostras, correspondiam a fase inicial da doença, com resultado MAT negativo. O MAT é o método referência para diagnóstico da leptospirose, sendo a técnica recomendada OMS. Neste teste o soro de pacientes é submetido a reação com suspensões de antígenos de diferentes sorovares de leptospirosas vivas (FAINE et al., 1999; LEVETT, 2003). Porém, como o MAT é uma técnica que apresenta baixa sensibilidade na fase inicial da doença e por se tratar de um ensaio sorovar específico, a identificação de antígenos de leptospirosas que possam ser reconhecidos ainda na fase inicial da leptospirose, como a proteína Lp29 (NEVES et al., 2007) e as proteínas MPL17 e MPL21 (OLIVEIRA et al., 2008), poderão facilitar o diagnóstico e tratamento precoce da doença. Além do mais, testes sorológicos baseados em proteínas recombinantes oferecem algumas vantagens como maior sensibilidade e especificidade, já que maiores concentrações de antígenos podem ser usadas (NEVES et al., 2007).

Devido aos dados apresentados, foi feita a comparação entre a frequência de respondedores que apresentaram anticorpos IgG e IgM na fase inicial e convalescente da doença. A tabela 02 mostra que a proteína rLIC10258 foi a que apresentou maior frequência de respondedores, para anticorpos IgG, na fase inicial da doença (35%). Para a proteína rLIC12238 o percentual de respondedores da fase convalescente, tanto para IgG quanto para IgM, foi significativamente maior que na

fase inicial da doença, enquanto que para rLIC12880 não houve respondedores para fase inicial

Tabela 02 - Distribuição de respondedores para anticorpos IgG e IgM na fase inicial (MAT-) e convalescente (MAT+) da doença

Fases da Doença	IgG			IgM		
	rLIC210258	rLIC12880	rLIC12238	rLIC10258	rLIC12880	rLIC12238
Fase Inicial MAT (-) n=10	3 (30%)	---	1 (10%)	---	---	1 (10%)
Fase Convalescente MAT (+) n=10	4 (40%)	1 (10%)	6 (60%)	2 (20%)	3 (30%)	6 (60%)
Total n=20	7 (35%)	1 (5%)	7 (35%)	2 (10%)	3 (15%)	7 (35%)

4.13 Reconhecimento de rLIC10258, rLIC12880 e rLIC12238 por anticorpos monoclonais anti-His tag e policlonais homólogos por Western blotting

Para confirmar se as proteínas recombinantes estavam sendo expressas com os resíduos de histidina na porção N-terminal e para verificar o reconhecimento das proteínas rLIC10258, rLIC12880 e rLIC12238 por anticorpos policlonais homólogos, foi realizado um ensaio de Western blotting. Portanto, as proteínas recombinantes foram aplicadas em gel de poli-acrilamida/SDS e transferidas para membranas de nitrocelulose. As membranas foram incubadas com anticorpos monoclonais anti-His tag ou com anticorpos policlonais anti-rLIC10258, ou anti-rLIC12880, ou anti-rLIC12238, nas diluições 1:3000, 1:800, 1:800 e 1:2000, respectivamente. A figura 14 mostra que os anticorpos monoclonais anti-His tag reconhecem as proteínas recombinantes deste estudo, da mesma forma que os soros policlonais homólogos foram capazes de reconhecer as proteínas recombinantes para as quais foram produzidos.

Figura 14 - Detecção das proteínas recombinantes por anticorpos contra resíduos de histidina e policlonais homólogos por Western blotting

As proteínas recombinantes foram reconhecidas por anticorpo monoclonal anti-His tag (A) e por policlonais contra as proteínas rLIC10258, rLIC12880 e rLIC12238 produzidos em camundongos (B).

4.14 Análise da localização celular das proteínas codificadas pelos genes LIC10258, LIC12880 e LIC12238 por ensaio de digestão com PK e imunofluorescência de fase líquida (L-IFA)

Devido a sua localização, proteínas associadas à membrana externa são importantes na relação patógeno-hospedeiro. Proteínas expostas à superfície servem como alvos potenciais para o sistema imunológico do hospedeiro, além de estarem envolvidas nos processos de adesão e colonização (BARBOSA et al., 2006; ATZINGEN et al., 2009). A partir do genoma de espécies patogênicas de *Leptospira* sequenciadas (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b; BULACH et al., 2006), análises *in silico* identificaram mais de 200 proteínas com predição de estarem presentes na superfície destas leptospiros (NASCIMENTO et al., 2004a; NASCIMENTO et al., 2004b).

Os genes LIC10258, LIC12880 e LIC12238 codificam proteínas com probabilidade de estarem associadas à membrana externa, porém é necessário que a localização destas proteínas seja confirmada experimentalmente.

Um método, que vem sendo utilizado, para investigar se proteínas de leptospiros estão expostas na membrana externa é o ensaio de digestão com PK (PINNE; HAAKE, 2009; OLIVEIRA et al., 2010; MENDES et al., 2011). 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 LIC10258, LIC12880 e LIC12238 na superfície das leptospiros foi detectada pela adição de anticorpos contra as proteínas recombinantes.

A figura 15 mostra que as proteínas codificadas pelos genes LIC10258, LIC12880 e LIC12238 foram susceptíveis ao tratamento com PK. Após 30 minutos de incubação, foi observada uma redução, estatisticamente significativa, da proteínas codificada pelo gene LIC10258 e também da proteína de membrana externa, LipL32, usada como controle positivo do ensaio ($P < 0,05$). A redução das proteínas codificadas pelo genes LIC12880 e LIC12238 só foi estatisticamente significativa após 1 hora de tratamento com PK ($P < 0,05$). Como esperado, não foi observada alteração na detecção da proteína citoplasmática, DnaK, usada como controle negativo do ensaio. Sendo assim, os resultados obtidos neste ensaio sugerem que as proteínas codificadas pelos genes LIC10258, LIC12880 e LIC12238 estão presentes na superfície de *L. interrogans* sorovar Copenhageni.

Figura 15 - Análise da localização celular por digestão com proteinase K

Leptospiros vivas foram incubadas com 25 µg/mL de PK por diferentes períodos de tempo (0 a 240 minutos). As leptospiros tratadas foram adsorvidas em placas de ELISA e anticorpos contra as proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 foram adicionados para detectar a presença das proteínas nativas correspondentes as recombinantes na superfície das leptospiros. A proteína de membrana externa, LipL32, e a proteína citoplasmática, DnaK, foram incluídas como controle positivo e negativo do ensaio, respectivamente. Os dados são representativos de quatro experimentos independentes. O desvio padrão representa a média de cada ponto realizado em triplicata. Significância estatística em relação as leptospiros não tratadas com PK (tempo zero) pelo *Student's two tailed t test* (* $P < 0,05$).

Outro método utilizado para avaliar se as proteínas deste estudo estavam localizadas na superfície de *L. interrogans* sorovar Copenhageni foi o ensaio de L-IFA utilizando leptospiros vivos. Neste método o DNA genômico das leptospiros pode ser visualizado por marcação com iodeto de propideo (Figura 16 – coluna 1) seguido da detecção com anticorpo policlonal contra cada uma das proteínas recombinantes na presença de anticorpo anti-mouse IgG conjugado ao FITC. Fluorescência verde pode ser observada, na figura 16 (coluna 2) para as proteínas rLIC10258, rLIC12880 e rLIC12238 e para o controle positivo LipL32, mas não para GroEL, uma proteína citoplasmática, utilizada como controle negativo. A localização destas proteínas na superfície de *L. interrogans* sorovar Copenhageni foi observada pela sobreposição das imagens observadas na figura 16 (coluna 3). Portanto, os dados obtidos com este ensaio indicam que rLIC10258, rLIC12880 e rLIC12238 são proteínas expostas na superfície das leptospiros.

Figura 16 - Análise da localização celular das proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 por L-IFA

Leptospiras vivas foram marcadas com iodeto de propídeo (1 – IP) e as proteínas nativas na superfície das leptospiras foram detectadas pela adição de policlonais contra rLIC10258, rLIC12880 e rLIC12238 seguido da incubação com anti-mouse IgG conjugado ao FITC (2 – FITC). A presença das proteínas na superfície das leptospiras pode ser visualizada pela sobreposição das imagens (3 – Sobreposição). A proteína de membrana externa, LipL32 e a proteína citoplasmática, GroEL foram usadas como controle positivo e negativo do experimento, respectivamente.

4.15 Adesão das proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 aos MECs

A adesão e a colonização dos tecidos do hospedeiro por patógenos é um evento crítico no processo de infecção. Já algum tempo, tem sido demonstrado que

a interação de patógenos com componentes da matriz extracelular desempenha um papel primário na colonização dos tecidos do hospedeiro. Além disso, sabe-se que proteínas expostas na superfície dos patógenos provavelmente estariam envolvidas neste processo (BARBOSA et al., 2006; ATZINGEN et al., 2009).

Os mecanismos pelos quais espécies patogênicas de *Leptospira* invadem e colonizam as células hospedeiras são ainda pouco conhecidos. Porém, nos últimos anos, várias adesinas de leptospira tem sido descritas. A primeira adesina de leptospira, Lsa24 (BARBOSA et al., 2006), foi descrita pelo nosso grupo e desde então, novas adesinas tem sido relatadas, tais como Lsa21 (ATZINGEN et al., 2008), LipL32 (HAUK et al., 2008), Lp95 (ATZINGEN et al., 2009), LipL53 (OLIVEIRA et al., 2010) e Lsa63 (VIEIRA et al., 2010b).

Como os genes selecionados para este estudo codificam proteínas com probabilidade de estarem localizadas na membrana externa, especula-se a possibilidade destas proteínas interagirem com componentes da matriz extracelular. Sendo assim, para analisar a capacidade de ligação das proteínas rLIC10258, rLIC12880 e rLIC12238 aos MECs, placas de microdiluição foram adsorvidas com laminina, colágeno tipo I, colágeno tipo IV, fibronectina celular, fibronectina plasmática e os controles negativos BSA e fetuína. A adesão destas proteínas aos ECMs foi avaliada por ELISA.

Como apresentado na figura 17A, não foi observada a ligação da proteína rLIC12880 com nenhum dos MECs testados. A ligação da proteína rLIC12238 com a laminina e fibronectina plasmática não foi estatisticamente significativa e portanto não foi considerada. Apenas rLIC10258 apresentou eficiente ligação a laminina e fibronectina plasmática, e por isso foi nomeada Lsa66 (*Leptospiral surface adhesin of 66* kDa) (OLIVEIRA et al., 2011). A interação desta proteína com estes dois componentes da matriz foi estatisticamente significativa quando comparado aos controles negativos BSA e fetuína ($P < 0,005$). A interação de rLIC10258 com a laminina e a fibronectina plasmática também foi observada quando anticorpos monoclonais anti-His foram empregados (Figura 17B) (* $P < 0,005$; ** $P < 0,05$). Também foi analisada a ligação da proteína rLIC10258 desnaturada e bloqueada com soro homólogo com a laminina e fibronectina plasmática. Como apresentado na figura 17C, tanto a desnaturação quanto o bloqueio de rLIC10258 não foram capazes de inibir totalmente a interação desta proteína com a laminina e fibronectina

plasmática, sugerindo a participação de epítomos não imunogênicos e não conformacionais nesta interação.

Figura 17 - Adesão das proteínas rLIC10258, rLIC12880 e rLIC12238 aos MECs

A interação das proteínas recombinantes aos MECs foi avaliada por ensaio de ELISA. Foram empregados policlonais contra as proteínas recombinantes (A) ou monoclonais anti-His tag (B) para detecção desta interação. Os dados representam uma média de três ensaios independentes. O desvio padrão representa a média de cada ponto realizado em triplicata. Para análises estatísticas, a interação das proteínas recombinantes aos componentes da matriz extracelular foi comparada com a interação dos controles negativos pelo *Student's two-tailed t test* (* $P < 0,005$ e ** $P < 0,05$). (C) Análise do efeito da desnaturação e bloqueio da proteína rLIC10258 na interação com a laminina e fibronectina plasmática. Neste ensaio, rLIC10258 foi desnaturada por aquecimento a 96 °C durante 10 minutos ou bloqueada com policlonais homólogos produzidos por camundongos por 90 minutos a 37 °C. Os dados são representativos de dois experimentos independentes. Significância estatística em relação a rLIC10258 não tratada pelo *Student's two-tailed t test* (* $P < 0,005$ e ** $P < 0,05$).

A especificidade da adesão de rLIC10258 a laminina e a fibronectina plasmática foi avaliada por meio de ensaios de dose-dependência e saturação variando a concentração da proteína recombinante (0 a 2000 nM) na presença fixa dos componentes de matriz (1 µg/poço). A figura 18 mostra que a ligação foi dose-dependente e alcançou a saturação com aproximadamente 500 e 1500 nM de proteína recombinante na ligação com laminina e fibronectina plasmática,

respectivamente. Baseado nos dados de ELISA, a constante de dissociação (KD) calculada para rLIC10258 com a laminina foi de $55,4 \pm 15,9$ nM, enquanto que para rLIC10258 com fibronectina plasmática foi de $290,83 \pm 11,82$ nM.

Figura 18 - Análise da especificidade da ligação de rLIC10258 com a laminina e fibronectina plasmática

A interação da laminina (A) e fibronectina plasmática (B) foi avaliada com concentrações crescentes de rLIC10258 (0 a 2000 nM). O desvio padrão representa a média de cada ponto realizado em triplicata. As proteínas rLIC12880 e rLIC12238 foram incluídas como controle negativo.

4.16 Contribuição dos carboidratos da laminina e fibronectina plasmática na interação com rLIC10258

O metaperiodato de sódio é um reagente que oxida os grupamentos hidroxil dos carboidratos mantendo a estrutura da cadeia polipeptídica intacta (WOODWARD; YOUNG; BLOODGOOD, 1985). Portanto, o envolvimento dos carboidratos da laminina e fibronectina plasmática na interação com a proteína recombinante rLIC10258 foi investigada através do tratamento desses MECs com metaperiodato de sódio. Sendo assim, tanto a laminina quanto a fibronectina plasmática foram oxidadas com concentrações crescentes desse reagente (0 a 100 mM) durante 15 minutos a 4 °C, no escuro.

Como pode ser observado na figura 19, não houve redução da ligação da proteína rLIC10258 com a laminina oxidada. Por outro lado, foi observada diminuição da ligação desta proteína com a fibronectina plasmática oxidada. O efeito da oxidação na interação entre o componente e rLIC10258 foi dose-dependente e estatisticamente significativo quando comparada a fibronectina não tratada (* $P < 0,01$; ** $P < 0,005$ e *** $P < 0,001$). Esses resultados sugerem que os resíduos de açúcar presentes na fibronectina plasmática são importantes na interação com a proteína rLIC10258.

Figura 19 - Contribuição dos resíduos de açúcar na interação de rLIC10258 com a laminina e a fibronectina plasmática

Laminina e fibronectina plasmática foram imobilizadas e tratadas com metaperiodato (0 a 100 mM) por 15 minutos, no escuro, a 4 °C. Barras representam a média dos valores de absorbância a 492 nm $\pm$ o desvio padrão de três replicatas para cada grupo experimental. Os dados são representativos de três experimentos independentes. Significância estatística em relação a fibronectina plasmática não oxidada pelo *Sudent's two-tailed t test* (* $P < 0,01$; ** $P < 0,005$ e *** $P < 0,001$).

4.17 Ligação das proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 ao PLG humano

Tem sido demonstrado que explorar as proteases do hospedeiro é uma estratégia comum dos patógenos para superar as barreiras impostas pelos componentes da matriz extracelular e tecidos epiteliais. Vários patógenos tornam-se organismos proteolíticos após capturar o PLG do hospedeiro e convertê-lo em plasmina, uma serino protease de amplo espectro, capaz de degradar constituintes da matriz extracelular e membrana basal (LAHTEENMAKI; KUKKONEN; KORHONEN, 2001; LAHTEENMAKI; KUUSELA; KORHONEN, 2001).

Já foram identificadas e caracterizadas lipoproteínas de membrana externa de espiroquetas que se ligam ao PLG, incluindo *B. burgdorferi* (BRISSETTE et al., 2009) e *B. hermsii* (ROSSMANN et al., 2007). Recentemente, foi demonstrado por nosso grupo, a capacidade das leptospiros de se ligarem ao PLG e convertê-lo em plasmina utilizando-se de ativadores presentes no plasma do hospedeiro (VIEIRA et al., 2009). Desde então, receptores do PLG tem sido identificados em *L. interrogans* (VERMA et al., 2010; VIEIRA et al., 2010a; MENDES et al., 2011; DOMINGOS et al., 2012).

Portanto, a capacidade de ligação das proteínas recombinantes deste estudo com o PLG foi avaliada por ELISA. O PLG humano e os controles negativos BSA e fetuína imobilizados em placas de microdiluição foram incubados com as proteínas rLIC10258, rLIC12880 e rLIC12238 e a interação foi quantificada utilizando-se anticorpos policlonais homólogos ou monoclonais anti-His tag. Todas as proteínas recombinantes deste estudo foram capazes de se ligar ao PLG humano, como pode ser observado nas figuras 20A e 20B. A interação foi estatisticamente significativa quando comparada aos controles negativos BSA e fetuína (* $P < 0,005$; ** $P < 0,0005$ e *** $P < 0,00005$). Também foi investigado o efeito da desnaturação e bloqueio com anticorpos homólogos das proteínas rLIC10258, rLIC12880 e rLIC12238 na ligação com o PLG. Como apresentado na figura 20C, apenas a ligação de rLIC10258 ao PLG não foi totalmente abolida quando esta proteína foi desnaturada ou bloqueada com policlonais anti-rLIC10258. Esses resultados sugerem o envolvimento de sítios de ligação lineares e não imunogênicos na ligação entre rLIC10258 e PLG.

Figura 20 - Análise da ligação das proteínas rLIC10258, rLIC12880 e rLIC12238 ao PLG humano

A ligação das proteínas recombinantes ao PLG foi avaliada por ensaio de ELISA utilizando anticorpos policlonais anti-proteínas recombinantes (A) ou anticorpos monoclonais anti-His tag (B). Os dados representam uma média de três ensaios independentes e o desvio padrão representa a média de cada ponto realizado em triplicata. Para análises estatísticas, a interação das recombinantes com o PLG foi comparada com a interação dos controles negativos pelo *Student's two-tailed t test*. (A) * $P < 0,005$; ** $P < 0,0005$; *** $P < 0,00005$; (B) * $P < 0,005$. (C) Influência da desnaturação e bloqueio das proteínas recombinantes na interação com o PLG. Neste ensaio, as proteínas recombinantes foram desnaturadas por aquecimento a 96 °C durante 10 minutos ou bloqueadas com anticorpos homólogos produzidos por camundongos por 90 minutos a 37 °C. Os dados são representativos de dois experimentos independentes. Significância estatística em relação às proteínas não tratadas pelo *Student's two-tailed t test*. * $P < 0,005$; ** $P < 0,0005$.

A especificidade da ligação das proteínas rLIC10258, rLIC12880 e rLIC12238 com o PLG foi analisada por meio de ensaios de dose-dependência e saturação. A figura 21 mostra que quando houve variação crescente da concentração das proteínas recombinantes (0 a 2000 nM) na presença de concentração fixa de PLG (1µg/poço) a interação foi saturável e ocorreu de maneira dose-dependente. Baseado nos dados de ELISA, as constantes de dissociação (KD) das proteínas recombinantes com o PLG foram de $68,82 \pm 25,25$; $167,39 \pm 60,14$ e $11,97 \pm 1,06$ nM para rLIC10258, rLIC12880 e rLIC12238, respectivamente.

Figura 21 - Análise da especificidade da ligação de rLIC10258, rLIC12880 e rLIC12238 com o PLG humano

A interação de 1 µg de PLG foi avaliada com concentrações crescentes das proteínas recombinantes (0 a 2000 nM). Os dados são representativos de dois experimentos independentes. O desvio padrão representa a média de cada ponto realizado em triplicata.

4.18 Atividade enzimática da plasmina

Tem sido demonstrado que plasmina pode ser gerada tanto pelo PLG ligado à superfície de *L. interrogans* quanto pelo PLG ligado a proteínas recombinantes na presença de ativador exógeno (VIEIRA et al., 2010a; MENDES et al., 2011; DOMINGOS et al., 2012). Portanto para determinar se o PLG ligado às proteínas recombinantes deste estudo pode adquirir atividade proteolítica, foi realizado um ensaio de ELISA modificado. As proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 foram imobilizadas em placas de microdiluição e, após bloqueio e

incubação com PLG, adicionou-se o ativador de PLG tipo uroquinase (uPA) juntamente com o substrato cromogênico específico para plasmina. A reação ocorreu por 16 horas e a atividade da plasmina foi quantificada através da clivagem do substrato por absorbância a 405 nm. O PLG capturado pelas proteínas recombinantes pode ser convertido a plasmina, como demonstrado indiretamente pela atividade proteolítica da plasmina gerada. Não foi observada atividade proteolítica para o controle negativo BSA, assim como para os controles em que não foi adicionado PLG, uPA ou substrato cromogênico (Figura 22).

Figura 22 - Atividade enzimática da plasmina ligada as proteínas recombinantes

A clivagem de substrato específico para plasmina após ativação do PLG ligado as proteínas recombinantes foi avaliado através de ELISA modificado. As proteínas imobilizadas receberam os seguintes tratamentos: PLG + uPA + substrato para plasmina (PLG + uPA + S), ou controles com a ausência de um dos três componentes (PLG + uPA; PLG + S; uPA + S). BSA foi empregado como controle negativo. As barras representam as médias das absorbâncias a 405 nm como medida da clivagem do substrato $\pm$ desvio padrão de quatro replicatas para cada grupo, sendo representativo de dois experimentos independentes. Diferenças estatisticamente significantes estão evidenciadas por * $P < 0,02$; ** $P < 0,001$ e *** $P < 0,00001$.

4.19 Ligação das proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238 ao fibrinogênio, componentes do sistema complemento humano, fator H e C4BP

O fibrinogênio, uma glicoproteína plasmática de 340 kDa composta de dois pares dos peptídeos A α , B β e Y ligados por meio de pontes de sulfeto, é uma das principais proteínas envolvidas nos processos de coagulação sanguínea, sendo essencial para a cicatrização de feridas e consequente reparação dos tecidos (BAMFORD et al., 2007; CHOY et al., 2011; LIN et al., 2011). A virulência de alguns patógenos, como por exemplo, *Staphylococcus aureus* (MOREILLON et al., 1995),

cepas do grupo A de estreptococos (MEEHAN et al., 2001) e da espiroqueta *T. denticola* (BAMFORD et al., 2007) tem sido relacionada com a interação com o fibrinogênio. Além disso, recentemente, foi relatada a capacidade da proteína LigB de leptospiros de interagir com o fibrinogênio (CHOY et al., 2011; LIN et al., 2011).

O sistema complemento constitui a primeira linha de defesa do sistema imune inato e sua ativação direciona a uma cascata de reações que resulta na formação do complexo lítico de ataque à membrana e opsonização do microrganismo invasor (JANEWAY; FLAVELL, 1999).

Uma estratégia utilizada por diversos patógenos para evitar os efeitos destrutivos causados pela ativação do sistema complemento é sequestrar do hospedeiro, proteínas reguladoras deste sistema, como o Fator H e a C4BP (VERMA et al., 2006; BARBOSA et al., 2009; FRAGA; BARBOSA; ISAAC, 2011; CASTIBLANCO-VALENCIA et al., 2012).

O fator H e a C4BP são as principais proteínas reguladoras da via alternativa e clássica do sistema complemento, respectivamente. O fator H é uma proteína plasmática de 150 kDa composta por 20 domínios nomeados de *short consensus repeat* (SCRs) que inibe a via alternativa do complemento ligando-se ao C3b e deslocando o Bb da C3 convertase, além de atuar como um cofator para o Fator I que cliva o C3b em sua forma inativa, o iC3b (RIPOCHE et al., 1988). A C4BP é uma glicoproteína plasmática de 570 kDa composta de 7 cadeias α e uma única cadeia β ligadas por um núcleo central que inibe a via clássica do complemento acelerando a diminuição e evitando a formação da C4b2a convertase e atuando como cofator para o fator I na inativação do C4b (GIGLI; FUJITA; NUSSENZWEIG, 1979).

Vários patógenos de interesse médico e veterinário, incluindo as espiroquetas *B. burgdorferi* (ALITALO et al., 2002; STEVENSON et al., 2002), *B. hermsii* (HOVIS et al., 2004) e *T. denticola* (MCDOWELL et al., 2005) são capazes de se ligar ao fator H e a proteína similar ao fator H-1 (FHL-1). Além disso, tem sido relatado que *B. recurrentis* e *B. duttonii* são capazes de se ligar a C4BP (MERI et al., 2006).

Há várias décadas tem sido demonstrada a habilidade das leptospiros patogênicas de resistirem ao sistema complemento, apesar de não serem conhecidos os mecanismos que possibilitavam esta resistência (CINCO; BANFI, 1983). Porém, nos últimos anos tem sido descrito que as leptospiros são capazes de interagir com o fator H e C4BP, o que inibiria o ataque do sistema complemento e explicaria a resistência ao soro humano apresentada por este patógeno (VERMA et

al., 2006; BARBOSA et al., 2009). As proteínas LfhA/Lsa24 (VERMA et al., 2006), LenB (STEVENSON et al., 2007), LigA e LigB (CASTIBLANCO-VALENCIA et al., 2012) foram identificadas como ligantes de fator H nas leptospiros, enquanto que as proteínas LcpA (BARBOSA et al., 2010), LigA e LigB (CASTIBLANCO-VALENCIA et al., 2012) são receptores para o C4BP. Recentemente, as proteínas Lsa25, Lsa33, Lsa30 e Lsa23 foram identificadas e caracterizadas por nosso grupo como receptores de C4BP nas leptospiros (DOMINGOS et al., 2012; SOUZA et al., 2012).

Todas as proteínas recombinantes desse estudo são receptores de PLG (VIEIRA et al., 2010a; OLIVEIRA et al., 2011). Além disso, a proteína rLIC10258 interage com a laminina e fibronectina plasmática (OLIVEIRA et al., 2011). Portanto, as proteínas rLIC10258, rLIC12880 e rLIC12238 provavelmente estão envolvidas nos processos de adesão e penetração nos tecidos do hospedeiro. Sendo assim, especula-se a possibilidade destas proteínas participarem de outros mecanismos de patogenicidade e virulência das leptospiros, como por exemplo, evasão imune. Sendo assim, a capacidade de ligação das proteínas recombinantes deste estudo com o fibrinogênio, os componentes do sistema complemento humano, o fator H e C4BP foi avaliada por ELISA. Os componentes e os controles negativos BSA e fetuína imobilizados em placas de microdiluição foram incubados com as proteínas rLIC10258, rLIC12880 e rLIC12238 e a interação foi quantificada utilizando-se anticorpos policlonais anti-proteínas recombinantes.

Como mostra a figura 23A, não foi detectada a ligação das proteínas recombinantes deste estudo com os componentes do complemento humano, fator H e C4BP. Em relação ao fibrinogênio, apenas rLIC12238 foi capaz de se ligar a este componente (Figura 23A). A interação desta proteína com o fibrinogênio foi estatisticamente significativa quando comparado aos controles negativos BSA e fetuína. A ligação de rLIC12238 com o fibrinogênio também foi observada quando anticorpos monoclonais anti-His foram empregados (Figura 23B). Além disso, também foi analisada a influência da desnaturação e bloqueio com soro homólogo da proteína rLIC12238 na ligação com o fibrinogênio. Como apresentado na figura 23C, apenas a desnaturação de rLIC12238 foi capaz de inibir totalmente a interação desta proteína com o fibrinogênio, sugerindo a participação de epítomos não imunogênicos nesta interação.

Figura 23 - Análise da ligação das proteínas rLIC10258, rLIC12880 e rLIC12238 a componentes do soro e plasma humano

A interação das proteínas recombinantes com o fibrinogênio, fator H, complemento humano e C4BP foi analisada por ensaio de ELISA, utilizando anticorpos policlonais anti-proteínas recombinantes (A) ou anticorpos monoclonais anti-His tag (B). Os dados representam uma média de três ensaios independentes. O desvio padrão representa a média de cada ponto realizado em triplicata. Para análises estatísticas, a ligação de rLIC12238 ao fibrinogênio foi comparada com a ligação dos controles negativos pelo *Student's two-tailed t test*. * $P < 0,001$. (C) Análise do efeito da desnaturação e bloqueio da proteína rLIC12238 na interação com o fibrinogênio. Neste ensaio, rLIC12238 foi desnaturada por aquecimento a 96 °C durante 10 minutos e bloqueada com policlonais homólogos produzidos por camundongos por 90 minutos a 37 °C. Os dados são representativos de dois experimentos independentes. Significância estatística em relação a rLIC12238 não tratada pelo *Student's two-tailed t test*. ** $P < 0,0001$.

A especificidade da ligação de rLIC12238 com o fibrinogênio foi analisada por ensaio de dose-dependência e saturação. A figura 24 mostra que quando houve variação crescente da concentração da proteína (0 – 4000 nM) na presença de concentração fixa de fibrinogênio (1 µg/poço) a interação ocorreu de maneira dose-dependente. A saturação desta ligação foi alcançada com aproximadamente 3500 nM de proteína recombinante. Baseado nos dados de ELISA, a constante de dissociação (KD) calculada para a interação entre rLIC12238 e o fibrinogênio foi de $733,27 \pm 276,79$ nM. Como controle negativo desta ligação foi utilizada a proteína rLIC12880 que não interage com o fibrinogênio.

Figura 24 - Análise da especificidade da ligação de rLIC12238 com o fibrinogênio

A interação de 1 µg de fibrinogênio foi avaliada com concentrações crescentes de rLIC12238 (0 a 4000 nM). Os dados são representativos de dois experimentos independentes. O desvio padrão representa a média de cada ponto realizado em triplicata. A proteína rLIC12880 foi incluída como controle negativo.

4.20 Inibição da formação do coágulo de fibrina pela proteína rLIC12238

O fibrinogênio é uma das principais proteínas envolvidas nos processos de coagulação sanguínea (BAMFORD et al., 2007; CHOY et al., 2011; LIN et al., 2011) e a virulência de alguns patógenos (MOREILLON et al., 1995; MEEHAN et al., 2001; BAMFORD et al., 2007) tem sido relacionada com a sua interação com o fibrinogênio.

Recentemente, foi demonstrado por Lin e colaboradores (2011) que a proteína LigB de leptospiros é capaz de ligar ao fibrinogênio e inibir a adesão e agregação plaquetária, além da formação do coágulo de fibrina. Estes resultados sugerem a presença, nas leptospiros, de um mecanismo de regulação da coagulação sanguínea do hospedeiro, o que poderia explicar os quadros hemorrágicos observados em casos clínicos de leptospirose (LIN et al., 2011). Choy et al. (2011) também relataram a inibição da formação do coágulo de fibrina pela interação de LigB com o fibrinogênio e sugerem que esta inibição, possivelmente, prejudicaria a cicatrização de feridas facilitando a entrada das leptospiros na circulação, sua disseminação e consequente estabelecimento da infecção (CHOY et al., 2011).

A proteína rLIC12238 foi capaz de interagir com o fibrinogênio e, portanto, para analisar se esta interação poderia interferir na formação do coágulo de fibrina, foi realizado um ensaio onde o fibrinogênio foi pré-incubado com rLIC12238 e a conversão do fibrinogênio solúvel em fibrina insolúvel foi induzida pela presença de trombina. A formação do coágulo foi detectada pelo aumento da densidade ótica por um período de 45 minutos. Como relatado para a proteína LigB (CHOY et al., 2011; LIN et al., 2011), os dados apresentados na figura 25 mostram que a proteína rLIC12238 foi capaz de inibir a formação do coágulo de fibrina. Esta inibição foi estatisticamente significativa ($*P<0,01$), reduzindo em 33% a formação do coágulo após 45 minutos de ensaio. Os resultados obtidos sugerem que por meio da ligação de rLIC12238 ao fibrinogênio e consequente inibição da conversão deste fibrinogênio em fibrina, esta proteína pode estar envolvida na patogenicidade das leptospiros, interferindo no processo de coagulação do hospedeiro.

Figura 25 - Efeito de rLIC12238 na formação do coágulo de fibrina induzido por trombina

Fibrinogênio (1 mg/mL) foi pré-incubado com a proteína rLIC12238 (3500 nM) por 2 horas a 37 °C. A formação do coágulo foi desencadeada pela adição de trombina (10 U/mL) e foi quantificada pelo aumento da densidade ótica (595 nm) durante 45 minutos. Os dados são representativos de dois experimentos independentes. O desvio padrão representa a média de cada ponto realizado em triplicata. Significância estatística em relação ao controle positivo pelos *Student's two tailed t test*. $*P<0,01$. (+) Fb + Tb: controle positivo. (-) Fb: controle negativo. Fb: fibrinogênio. Tb: trombina.

4.21 Inibição da adesão de leptospiros vivos a MECs, PLG ou Fibrinogênio pelas proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238

Tem sido demonstrado que as proteínas recombinantes Lsa24/Lfha/LenA (BARBOSA et al., 2006), Lsa63 (VIEIRA et al., 2010b), Lsa20 (MENDES et al., 2011), Lsa25 e Lsa33 (DOMINGOS et al., 2012; OLIVEIRA et al., 2013 – manuscrito submetido) promovem um efeito inibitório na adesão das leptospiros a componentes da matriz extracelular, PLG e fibrinogênio. Devido a este fato foi avaliado o efeito de concentrações crescentes das proteínas recombinantes deste estudo (0 a 5 µg) na inibição da ligação de leptospiros a laminina, fibronectina plasmática, PLG e fibrinogênio.

Os resultados mostram que rLIC10258 foi capaz de inibir a adesão de leptospiros a laminina, a fibronectina plasmática e ao PLG (figura 26A). Da mesma forma, rLIC12880 inibiu a ligação das leptospiros ao PLG (figura 26B), enquanto rLIC12238 inibiu a interação das leptospiros ao PLG e fibrinogênio (figura 26C).

A inibição promovida pelas proteínas recombinantes deste estudo foi parcial, porém estatisticamente significativa (* $P < 0,05$). Resultados semelhantes foram obtidos com as proteínas Lsa24/Lfha/LenA (BARBOSA et al., 2006), Lsa63 (VIEIRA et al., 2010b), Lsa20 (MENDES et al., 2011), Lsa25 e Lsa33 (DOMINGOS et al., 2012; OLIVEIRA et al., 2013 – manuscrito submetido) que também inibiram parcialmente a adesão das leptospiros aos MECs, PLG e fibrinogênio. Esta inibição parcial é esperada e pode ser explicada pela presença de outros receptores em *L. interrogans* que se ligam a laminina, fibronectina, PLG e fibrinogênio.

Figura 26 - Inibição da adesão de *L. interrogans* a MECs, PLG e fibrinogênio pelas proteínas rLIC10258, rLIC12880 e rLIC12238

Laminina, fibronectina plasmática e PLG imobilizadas foram incubadas com concentrações crescentes de rLIC10258 (A) ou PLG imobilizado foi incubado com concentrações crescentes de rLIC12880 (B) ou PLG e fibrinogênio foram incubados com concentrações crescentes de rLIC12238 (C), seguido da adição de 4×10^7 de leptospiros vivos. A detecção da adesão das leptospiros ligadas aos MECs e PLG foi realizada indiretamente utilizando-se anticorpos policlonais anti-LipL32. As barras representam a média de absorbâncias a 492 nm $\pm$ desvio padrão de três replicatas de dois experimentos independentes. Diferenças estatisticamente significantes estão evidenciadas: * $P < 0,05$.

4.22 Avaliação da atividade imunoprotetora das proteínas rLIC10258, rLIC12880 e rLIC12238

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 (GAMBERINI et al., 2005; KOIZUMI; WATANABE, 2005; ATZINGEN et al., 2010; CAO et al., 2011).

Vários grupos de pesquisa tem testado a atividade imunoprotetora de proteínas associadas à membrana externa, como por exemplo, OmpL1 e LipL41 (HAAKE et al., 1999), LipL32 (BRANGER et al., 2001; SEIXAS et al., 2007), LigA e LigB (KOIZUMI; WATANABE, 2004; PALANIAPPAN et al., 2006; SILVA et al., 2007; BRISSETTE et al., 2009; YAN et al., 2009; CAO et al., 2011) e proteínas OmpA-like (YAN et al., 2010).

Nosso grupo também tem demonstrado que proteínas, com probabilidade de serem expostas à superfície, produzidas em nosso laboratório (rLIC12730, rLIC10494 e rLIC12922) são capazes de conferir proteção parcial de animais desafiados com dose letal de leptospiros (ATZINGEN et al., 2010).

Portanto para avaliar a atividade imunoprotetora das proteínas rLIC10258, rLIC12880 e rLIC12238, hamsters foram imunizados com duas doses destas proteínas recombinantes e desafiados com dose letal 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 leptospiros, e por exibirem características que mimetizam a infecção severa em humanos (HAAKE, 2006).

Foram realizados quatro ensaios independentes, dois com a proteína rLIC10258 e dois com as proteínas rLIC12880 e rLIC12238. Nestes ensaios foi empregada como controle positivo de proteção uma vacina composta de leptospiros inativadas pelo calor - *L. interrogans* sorovar Kennewicki (cepa Pomona Fromm).

A figura 27 mostra a curva de sobrevivência dos hamsters imunizados com rLIC10258, bacterina e PBS e desafiados com 2×10^5 de *L. interrogans* sorovar Kennewicki (cepa Pomona Fromm) virulenta.

Os resultados obtidos no primeiro experimento, mostram que a proteína rLIC10258 conferiu 20% de proteção aos animais desafiados com dose letal de

leptospiras virulentas. A bacterina foi capaz de conferir 100% de proteção, enquanto que no grupo PBS não houve sobreviventes (Figura 27A e Tabela 03). No segundo experimento, 30% dos animais imunizados com rLIC10258 sobreviveram. A bacterina novamente conferiu 100% de proteção, enquanto que 20% dos animais imunizados com PBS sobreviveram (Figura 27B e Tabela 03). A análise da cinética das curvas de sobrevivência pelo método de Kaplan-Meier (KAPLAN e MEIER, 1958) revelou que apesar da proteção conferida por rLIC10258 ter sido maior no segundo experimento, apenas a proteção obtida por rLIC10258 no primeiro experimento foi estatisticamente significativa ($P < 0,0092$).

Figura 27 - Curva de sobrevivência dos hamsters imunizados com rLIC10258 e desafiados com leptospiras virulentas

Após duas imunizações com rLIC10258, bacterina e PBS, os animais foram desafiados com 2×10^5 de *L. interrogans* sorovar Kennewicki (cepa Pomona Fromm) virulenta. Os animais foram monitorados quanto à morbidade por 21 dias. A) Experimento 1. B) Experimento 2.

Tabela 03 - Atividade imunoprotetora e efeito esterilizante da proteína rLIC10258

Experimento 1				Experimento 2		
Antígenos + Adjuvante	% Proteção	P value	Presença de leptospi- ras nos rins dos sobreviventes (positivo/total)	% Proteção	P value	Presença de leptospi- ras nos rins dos sobreviventes (positivo/total)
Bacterina	100% (10/10)	0,0001	0/10	100% (10/10)	0,0002	0/10
PBS	0% (0/10)	----	----	20% (2/10)	----	0/2
rLIC10258	20% (2/10)	0,0092	1/2	30% (3/10)	0,6768	2/3

Para analisar a resposta humoral dos animais imunizados com a proteína rLIC10258 e PBS, dois hamsters de cada grupo foram sangrados, via punção cardíaca, 15 dias após a segunda imunização (antes do desafio). Também foram analisados os soros obtidos dos animais que sobreviveram ao desafio. A média dos títulos de anticorpos obtidos após imunização com rLIC10258 foram de 200 e 400 no primeiro e segundo experimento, respectivamente. Um aumento dos títulos de anticorpos foi observado nos hamsters que sobreviveram ao desafio com leptospi-*ras virulentas*. No primeiro experimento, a média dos títulos de anticorpos obtidos após o desafio foi de 800, enquanto que no segundo experimento foi de 1600. Não foi detectada a produção de anticorpos nos animais imunizados com PBS em nenhum dos experimentos realizados.

A atividade imunoprotetora das proteínas rLIC12880 e rLIC12238 também foi avaliada. A figura 28 mostra a curva de sobrevivência dos hamsters imunizados com rLIC12880, rLIC12238, bacterina e PBS e desafiados com $2,5 \times 10^3$ de *L. interrogans* sorovar Kennewicki (cepa Pomona Fromm).

Os resultados obtidos mostram que, no primeiro experimento, as proteínas recombinantes rLIC12880 e rLIC12238 foram capazes de proteger 33 e 50% dos hamsters desafiados com dose letal de leptospi-*ras virulentas*, respectivamente. A bacterina conferiu 100% de proteção, enquanto que 8% dos animais imunizados com PBS sobreviveram (Figura 28A e Tabela 04). No segundo experimento, não houve sobreviventes no grupo imunizado com rLIC12880 e apenas 16,5% dos

animais imunizados com rLIC12238 sobreviveram (Figura 28B e Tabela 04). A bactéria, por sua vez, conferiu 100% de proteção, enquanto que 25% dos animais não vacinados sobreviveram. De acordo com a análise da cinética das curvas de sobrevivência pelo método de Kaplan-Meier (KAPLAN e MEIER, 1958), a proteção conferida pelas proteínas rLIC12880 e rLIC12238 não foi estatisticamente significativa em nenhum dos experimentos (Tabela 04).

Figura 28 - Curva de sobrevivência dos hamsters imunizados com rLIC12880 e rLIC12238 e desafiados com leptospiros virulentas

Após duas imunizações com rLIC12880, rLIC12238, bactéria e PBS, os animais foram desafiados com 2×10^3 de *L. interrogans* sorovar Kennewicki (cepa Pomona Fromm) virulenta. Os animais foram monitorados quanto à morbidade por 21 dias. A) Experimento 1. B) Experimento 2.

Tabela 04 - Atividade imunoprotetora e efeito esterilizante das proteínas rLIC12880 e rLIC12238

Experimento 1				Experimento 2		
Antígenos + Adjuvante	% Proteção	P value	Presença de leptospi- ras nos rins dos sobreviventes (positivo/total)	% Proteção	P value	Presença de leptospi- ras nos rins dos sobreviventes (positivo/total)
Bacterina	100% (12/12)	0,0001	0/12	100% (11/11)	0,0003	0/11
PBS	8,3% (1/12)	----	----	25% (3/12)	----	2/3
rLIC12880	33,3% (4/12)	0,3206	4/4	0% (0/12)	0,1648	ND
rLIC12238	50% (6/12)	0,0938	6/6	16,5% (2/12)	0,8258	1/2

Para analisar a resposta humoral dos animais imunizados com as proteínas rLIC12880, rLIC12238 e PBS, seis hamsters de cada grupo foram sangrados, via retro-orbital, 15 dias após cada imunização. Também foram analisados os soros obtidos dos animais que sobreviveram ao desafio. O quadro 03 mostra que, tanto no primeiro quanto no segundo experimento, houve aumento da média dos títulos de anticorpos produzidos pelos hamsters após a segunda imunização (dia 30 – antes do desafio) com as proteínas rLIC12880 e rLIC12238. Nos dois experimentos realizados, também foi observado um aumento dos títulos de anticorpos dos hamsters que sobreviveram ao desafio com leptospi-
ras virulentas. Não foi detectada a produção de anticorpos nos animais imunizados com PBS em nenhum dos desafios realizados (Quadro 03).

Quadro 03 - Resposta humoral dos hamsters imunizados com rLIC12880 e rLIC12238 antes e depois do desafio

Experimento1			Experimento2			
Hamster vacinados com:	Após 1 ^a imunização Dia 15	Após 2 ^a imunização Dia 30	Após desafio Dia 51	Após 1 ^a imunização Dia 15	Após 2 ^a imunização Dia 30	Após desafio Dia 51
rLIC12880	70	1375	1665	150	2500	---
rLIC12238	60	325	550	100	200	600
PBS	---	---	---	---	---	---

Os hamsters que sobreviveram foram sacrificados e tiveram seus rins coletados para cultura e isolamento de leptospiros. As tabelas 03 e 04 mostram que o efeito da bacterina foi esterilizante em todos os experimentos realizados, uma vez que não foi detectada a presença de leptospiros nos rins dos sobreviventes. Em contrapartida, as proteínas rLIC10258, rLIC12880 e rLIC12238 não podem ser consideradas esterilizantes, já que foram detectadas leptospiros nos rins de alguns sobreviventes.

É importante ressaltar que a existência de sobreviventes no grupo de animais não vacinados e a dificuldade de reproduzir os resultados em ensaios de desafio, tem sido descrita pelo nosso grupo e por outros grupos de pesquisa (BRANGER et al., 2001; PALANIAPPAN et al., 2006; ATZINGEN et al., 2010). Em ensaios de desafio realizados por Branger e colaboradores (2001) e Palaniappan et al. (2006) foram observadas taxas de 50% e 80% de sobrevivência do grupo de animais não vacinados, respectivamente. Atzingen e colaboradores (2010) também relataram 20% de sobrevivência do grupo controle e significância estatística de apenas um de dois experimentos realizados.

Sendo assim, os resultados obtidos com estes ensaios sugerem que as proteínas rLIC10258, rLIC12880 e rLIC12238 não possuem atividade imunoprotetora e portanto não podem ser consideradas boas candidatas vacinais contra a leptospirose.

4.23 Amplificação e clonagem dos fragmentos do gene LIC10258

A proteína recombinante rLIC10258 é capaz de interagir com laminina, fibronectina plasmática e PLG (OLIVEIRA et al., 2011). Sendo assim, para analisar qual parte da proteína rLIC10258 é responsável pela ligação aos componentes acima citados, as sequências de DNA correspondentes as porções N-terminal, intermediária e C-terminal do gene LIC10258 foram obtidas por amplificação (PCR) do DNA genômico extraído da cultura de *L. interrogans* sorovar Copenhageni. Os produtos de PCR obtidos foram analisados por eletroforese em gel de agarose 1% e apresentaram o tamanho esperado de 570, 952 e 513 pb para os clones referentes as porções N-terminal, Intermediária e C-terminal do gene LIC10258, respectivamente. A figura 29 esquematiza os fragmentos clonados da proteína codificada pelo gene LIC10258, enquanto que a figura 30 apresenta os fragmentos de DNA obtidos após amplificação por PCR.

Figura 29 - Esquema da clonagem dos fragmentos do gene LIC10258

Foram clonadas as porções N-terminal (aminoácidos 30 ao 219), Intermediária (aminoácidos 170 ao 483) e C-terminal (aminoácidos 420 ao 590) da proteína codificada pelo gene LIC10258. O fragmento N-terminal foi clonado sem o peptídeo sinal (aminoácidos 1 ao 29).

Figura 30 – Análise dos fragmentos do gene LIC10258 amplificados por meio de reação de PCR em gel de agarose 1%

As bandas obtidas apresentaram o tamanho esperado de 570 pb para N-terminal/LIC10258 (linha 2), 952 pb para Intermediária/LIC10258 (linha 3) e 513 pb para C-terminal/LIC10258 (linha 4). Kb Plus DNA Ladder: marcador molecular (linha 1).

Os insertos de DNA obtidos por meio de PCR foram clonados no vetor pGEM-T Easy e utilizados para transformar bactérias competentes DH5 α . Após extração do DNA plasmidial das bactérias competentes, os plasmídeos contendo os fragmentos do gene LIC10258 foram digeridos com as enzimas de restrição *Bam*HI e *Nco*I para certificação da presença dos insertos (figura 31).

Figura 31 – Análise dos insertos de DNA obtidos por digestão do DNA plasmidial (pGEM-T Easy/inserto) em gel de agarose 1%

Os plasmídeos recombinantes obtidos foram digeridos com as enzimas *Bam*HI e *Nco*I para confirmação da presença do inserto de interesse. A) N-terminal/LIC10258; B) Intermediária/LIC10258 e C) C-terminal/LIC10258, onde 1: Kb DNA Ladder (marcador molecular). 2: plasmídeo contendo inserto e 3: plasmídeo digerido + inserto liberado.

Os insertos correspondentes as porções N-terminal, Intermediária e C-terminal do gene LIC10258 foram removidos dos plasmídeos recombinantes (pGEM-

T/inserto) e subclonados no vetor de expressão pAE (RAMOS et al., 2004). Os clones positivos foram selecionados por meio da extração rápida por fenol-clorofórmio e após obtenção do DNA plasmidial (pAE/inserto) os clones foram digeridos com as enzimas *Bam*HI e *Nco*I para confirmação da presença dos insertos (figura 32).

Figura 32 – Análise dos insertos de DNA obtidos por digestão do DNA plasmidial (pAE/inserto) em gel de agarose 1%

Após seleção por fenol-clorofórmio, os clones positivos foram purificados e os plasmídeos obtidos digeridos com as enzimas apropriadas. Linhas 3 a 6: N-terminal/LIC10258, onde linhas 3 e 5: plasmídeo digerido + inserto liberado e linhas 4 e 6: plasmídeo contendo inserto. Linhas 7 a 10: Intermediário/LIC10258, onde linhas 7 e 9: plasmídeo digerido + inserto liberado e linhas 8 e 10: plasmídeo contendo inserto. Linhas 11 a 14: C-terminal/LIC10258, onde linhas 11 e 13: plasmídeo digerido + inserto liberado e linhas 12 e 14: plasmídeo contendo inserto. Linha 1: Kb Plus DNA Ladder e linha 2: λ hind III (Marcadores moleculares).

4.24 Sequenciamento dos clones

O sequenciamento confirmou a presença dos insertos de DNA correspondentes as porções N-terminal, Intermediária e C-terminal do gene LIC10258, tanto no vetor de clonagem pGEM-T Easy quanto no vetor de expressão pAE. Todas as sequências codificadoras foram confirmadas pelo programa BLAST (ALTSCHUL et al., 1990) mostrando 100% de identidade quando comparadas com a sequência de bases nitrogenadas do gene LIC10258, tanto no vetor pGEM-T Easy quanto no vetor pAE (Anexo C).

4.25 Expressão e purificação dos fragmentos recombinantes

Os fragmentos correspondentes as porções N-terminal, Intermediária e C-terminal do gene LIC10258 foram expressos, solubilizados e purificados sob as mesmas condições que a proteína rLIC10258. Sendo assim, bactérias *E. coli* BL21

SI transformadas com o vetor de expressão pAE foram induzidas a expressar os fragmentos recombinantes. Após indução com 30 mM de NaCl, os fragmentos recombinantes foram expressos na forma insolúvel como corpúsculos de inclusão e solubilizados com 8 M uréia e 10% glicerol de acordo com o protocolo descrito por Saini e colaboradores (2002) (SAINI et al., 2002). Após solubilização, os fragmentos recombinantes foram purificados por cromatografia de afinidade utilizando resina carregada com níquel. Os fragmentos purificados foram analisados em gel de SDS-PAGE e apresentaram a massa molecular esperada de 25,5; 36,9 e 23,3 kDa para os fragmentos N-terminal, Intermediário e C-terminal, respectivamente (Figura 33).

Figura 33 - Análise dos fragmentos recombinantes purificados em gel de SDS-PAGE

Os fragmentos recombinantes correspondentes às porções N-terminal, Intermediária e C-terminal da proteína rLIC10258 foram purificados por cromatografia de afinidade a metal. As bandas de proteínas com os tamanhos esperados de aproximadamente 25,5; 36,9 e 23,3 kDa, correspondendo aos fragmentos N-terminal, Intermediário e C-terminal respectivamente, foram visualizadas por Coomassie blue. MM: marcador molecular.

4.26 Reconhecimento dos fragmentos N-terminal, Intermediário e C-terminal por anticorpos monoclonais anti-His tag e policlonais homólogos por Western blotting

Neste ensaio, os fragmentos recombinantes foram aplicados em gel SDS/PAGE e transferidos para membrana de nitrocelulose. As membranas foram incubadas com anticorpos monoclonais anti-His tag (1:2000) e também com anticorpos policlonais contra os fragmentos N-terminal, Intermediário e C-terminal na diluição 1:500. A figura 34A mostra que os soros policlonais homólogos foram

capazes de reconhecer tanto a proteína rLIC10258 quanto os fragmentos recombinantes originados desta proteína. Além disso, os fragmentos recombinantes também foram reconhecidos por anticorpos monoclonais anti-His tag (Figura 34B).

Figura 34 – Reconhecimento dos fragmentos recombinantes por anticorpos contra resíduos de histidina e policlonais homólogos por Western blotting

Os fragmentos recombinantes foram detectados por anticorpo monoclonal anti-His tag (A) e por policlonais contra os fragmentos recombinantes do gene LIC10258 produzidos em camundongos (B).

4.27 Presença de anticorpos IgG e IgM humanos contra os fragmentos recombinantes da proteína rLIC10258 em soros de pacientes diagnosticados com leptospirose

A proteína recombinante rLIC10258 é reconhecida por anticorpos presentes nos soros de pacientes diagnosticados com leptospirose, apresentando 35 e 10% de respondedores para anticorpos IgG e IgM humanos, respectivamente. Além disso, rLIC10258 foi reconhecida por 35% de anticorpos IgG de pacientes na fase inicial da doença, quando o MAT ainda é negativo. Sendo assim, 36 amostras de soros de 18 pacientes diagnosticados com leptospirose foram analisadas quanto a presença de anticorpos IgG e IgM contra os fragmentos recombinantes da proteína rLIC10258 (Anexo D). O valor de *cutoff* foi estabelecido a partir de 18 amostras de soros de indivíduos normais que relataram não terem entrado em contato com a doença. Os valores de *cutoff*, contra anticorpos IgG, obtidos para os fragmentos recombinantes N-terminal, Intermediário e C-terminal foram de 0,105; 0,150 e 0,155 nm, respectivamente, enquanto que contra anticorpos IgM todos os valores de *cutoff* foram abaixo de 0,1 nm.

Como não foram detectados anticorpos IgM humanos contra os fragmentos recombinantes em nenhuma das amostras testadas, a tabela 05 apresenta apenas o percentual de respondedores que apresentaram anticorpos IgG contra os fragmentos recombinantes de rLIC10258. Como pode ser observado, não foram detectados anticorpos IgG contra o fragmento C-terminal em nenhuma das amostras testadas. Por outro lado, tanto o fragmento recombinante N-terminal quanto o Intermediário foram reconhecidos por anticorpos IgG de pacientes diagnosticados com leptospirose. Para ambos fragmentos recombinantes, o percentual de respondedores foi maior na fase convalescente da doença. Porém, é importante ressaltar que estes fragmentos apresentaram 27,7% de respondedores na fase inicial da doença. Este resultado foi semelhante ao da proteína rLIC10258 que foi reconhecida por 35% de amostras de pacientes na fase inicial da doença, quando o MAT ainda é negativo.

Tabela 05 - Distribuição de respondedores para anticorpos IgG na fase inicial (MAT-) e convalescente (MAT+) da doença

Fases da Doença	N-terminal	Intermediário	C-terminal
Fase Inicial MAT (-) n=18	5 (27,7%)	5 (27,7%)	----
Fase Convalescente MAT (+) n=18	7 (38,8%)	12 (66,6%)	----
Total n=36	12 (33,3%)	17 (47,2%)	----

4.28 Ligação dos fragmentos recombinantes de rLIC10258 a laminina, fibronectina plasmática e PLG

Como a proteína recombinante rLIC10258 é capaz de ligar a laminina, fibronectina plasmática e PLG (OLIVEIRA et al., 2011), foram obtidos fragmentos recombinantes correspondentes as porções N-terminal, Intermediária e C-terminal desta proteína com o intuito de identificar qual o fragmento responsável pela interação de rLIC10258 com os componentes acima citados. Sendo assim, placas de microdiluição foram adsorvidas com laminina, fibronectina plasmática, PLG e BSA (controle negativo) e a interação destes componentes com os fragmentos recombinantes N-terminal, Intermediário e C-terminal foi avaliada por ELISA.

Como mostra a figura 35A, não foi observada a ligação do fragmento N-terminal a nenhum dos componentes testados. Em contrapartida, o fragmento Intermediário foi capaz de se ligar a todos os componentes, enquanto o fragmento C-terminal interagiu apenas com a laminina. Resultados semelhantes foram obtidos quando anticorpos monoclonais anti-His foram utilizados (Figura 35B). As interações foram estatisticamente significantes quando comparadas com o controle negativo BSA (* $P < 0,0005$; ** $P < 0,005$).

Também foi analisado o efeito da desnaturação ou bloqueio com soro homólogo dos fragmentos recombinantes na ligação com a laminina, fibronectina

plasmática e PLG. A figura 36A mostra que tanto a desnaturação quanto o bloqueio do fragmento Intermediário não foram capazes de inibir totalmente a ligação deste fragmento a laminina, fibronectina plasmática e PLG. Da mesma forma, a desnaturação e o bloqueio do fragmento C-terminal também não foram capazes de abolir totalmente a adesão do fragmento C-terminal a laminina (Figura 36B). Esses resultados sugerem a participação de sítios de ligação lineares e não imunogênicos na interação entre os fragmentos recombinantes e laminina, fibronectina plasmática e PLG. É importante ressaltar que resultados semelhantes foram obtidos com a proteína rLIC10258 (Figura 17C).

Figura 35 - Ligação dos fragmentos recombinantes com MECs e PLG

A ligação dos fragmentos recombinantes a laminina, fibronectina plasmática e PLG foi avaliada por ensaio de ELISA utilizando anticorpos policlonais anti-fragmentos recombinantes (A) ou anticorpos monoclonais anti-His tag (B). Os dados representam uma média de três ensaios independentes. Para análises estatísticas, a interação dos fragmentos recombinantes aos componentes foi comparada com a interação dos controles negativos pelo *Student's two-tailed t test* (* $P < 0,0005$; ** $P < 0,005$).

Figura 36 - Efeito da desnaturação e bloqueio dos fragmentos recombinantes na interação com MECs e PLG

Laminina, fibronectina plasmática e plasminogênio foram incubados com o fragmento Intermediário desnaturado a 96 °C por 10 minutos ou bloqueado com policlonais homólogos (A). Da mesma maneira, o fragmento C-terminal desnaturado ou bloqueado foi incubado com laminina (B). Os dados são representativos de dois experimentos independentes. Significância estatística em relação aos fragmentos recombinantes não tratados pelo *Student's two-tailed t test* (* $P < 0,005$; ** $P < 0,05$).

A especificidade da adesão dos fragmentos Intermediário e C-terminal a laminina foi avaliada em ensaio de dose-dependência e saturação. A figura 37A mostra que quando houve variação crescente da concentração dos fragmentos recombinantes (0 a 2000 nM) na presença fixa de laminina (1 µg/poço) a interação foi saturável e dose-dependente. A saturação desta ligação foi alcançada com aproximadamente 1000 nM de fragmentos recombinantes. Baseado nos dados de ELISA, as constantes de dissociação (KD) dos fragmentos recombinantes com a laminina foram de $51,10 \pm 5,50$ nM e $75,32 \pm 14,50$ nM para os fragmentos Intermediário e C-terminal, respectivamente. O fragmento N-terminal foi incluído como controle negativo da adesão.

Da mesma maneira, a especificidade da interação do fragmento Intermediário com a fibronectina plasmática e o PLG foi analisada em ensaio de dose-dependência e saturação variando a concentração do fragmento Intermediário (0 a 4000 nM) na presença fixa dos componentes (1 µg/poço). A figura 37B e 37C mostra que a ligação foi dose-dependente e alcançou a saturação com aproximadamente 2000 e 3000 nM de fragmento Intermediário para fibronectina plasmática e PLG, respectivamente. Baseado nos dados de ELISA, a constante de dissociação calculada para o fragmento Intermediário com a fibronectina plasmática foi de $166,03 \pm 41,50$ nM, enquanto que para o fragmento Intermediário com o PLG foi de

744,70 $\pm$ 177,50 nM. Os fragmentos N-terminal e C-terminal foram incluídos como controles negativos da interação.

Figura 37 - Análise da especificidade da ligação dos fragmentos recombinantes com laminina, fibronectina plasmática e PLG

Para avaliar a capacidade dos fragmentos Intermediário e C-terminal aderirem a laminina e do fragmento Intermediário interagir com a fibronectina plasmática e PLG, placas de ELISA foram adsorvidas com laminina (A), fibronectina plasmática (B) e PLG (C) e incubadas com concentrações crescentes dos fragmentos recombinantes (0 a 4000 nM). A ligação foi detectada com policlonais anti-fragmentos recombinantes (1:1000) seguido da incubação com anti-IgG mouse peroxidase (1:5000). Os dados são representativos de três experimentos independentes. O desvio padrão representa a média de cada ponto realizado em triplicata.

4.29 Contribuição dos carboidratos da laminina e fibronectina plasmática na interação com os fragmentos recombinantes de rLIC10258

Para analisar a participação dos carboidratos da laminina na interação com os fragmentos Intermediário e C-terminal de rLIC10258, este componente foi oxidado com concentrações crescentes de metaperiodato de sódio (0 a 100 mM). A figura 38A mostra que não foi observada diminuição da ligação destes fragmentos com a laminina oxidada.

A fibronectina plasmática também foi tratada com metaperiodato de sódio para investigar o envolvimento de seus açúcares na ligação com a porção Intermediária da proteína rLIC10258. Como pode ser observado na figura 38B, houve diminuição da interação deste fragmento com a fibronectina plasmática oxidada. O efeito da oxidação na interação entre este componente e o fragmento Intermediário foi dose-dependente e estatisticamente significativo quando comparado a fibronectina não tratada (* $P < 0,01$). Resultados semelhantes foram obtidos na análise da interação da proteína rLIC10258 com a laminina e fibronectina plasmática oxidada. Sendo assim, esses resultados corroboram que apenas os resíduos de açúcares da fibronectina plasmática estão envolvidos na interação com a proteína rLIC10258.

Figura 38 - Contribuição dos resíduos de açúcar na interação dos fragmentos recombinantes com a laminina e a fibronectina plasmática

Laminina (A) e fibronectina plasmática (B) foram imobilizadas e tratadas com metaperiodato (0 a 100 mM) por 15 minutos, no escuro, a 4 °C. Barras representam a média dos valores de absorbância a 492 nm $\pm$ o desvio padrão de três replicatas para cada grupo experimental. Os dados são representativos de dois experimentos independentes. Significância estatística em relação a fibronectina plasmática não oxidada pelo *Sudent's two-tailed t test* (* $P < 0,01$).

4.30 Interferência da fibronectina plasmática na ligação do PLG ao fragmento Intermediário de rLIC10258

Tanto a fibronectina plasmática quanto o PLG são capazes de interagir com o fragmento Intermediário. Devido a esse fato foi analisado se estes componentes competem pelo mesmo sítio de ligação na porção Intermediária da proteína rLIC10258. Portanto foram realizados ensaios para avaliar a ligação do fragmento Intermediário ao PLG na presença de concentrações crescentes de fibronectina plasmática (0 a 1 μ g) e da ligação do fragmento Intermediário a fibronectina plasmática na presença de concentrações crescentes de PLG (0 a 1 μ g). Os

resultados mostram que a presença de fibronectina plasmática não interfere na ligação do PLG ao fragmento Intermediário (Figura 39A). Da mesma forma, a presença de PLG também não interfere na ligação da fibronectina plasmática a porção Intermediária de rLIC10258 (Figura 39B), sugerindo que estes componentes não competem pelo mesmo sítio de ligação no fragmento Intermediário.

Figura 39 - Fibronectina plasmática e PLG não competem pela ligação ao fragmento Intermediário

(A) O efeito da fibronectina plasmática na ligação do PLG (10 µg/ml) ao fragmento Intermediário imobilizado (10 µg/ml) foi analisado pela adição de concentrações crescentes de fibronectina plasmática (0 to 1 µg). (B) Da mesma forma, o efeito do PLG na ligação da fibronectina plasmática (10 µg/ml) ao fragmento Intermediário imobilizado (10 µg/ml) foi investigado pela adição de concentrações crescentes de PLG (0 to 1 µg). A detecção da ligação do fragmento intermediário com o PLG (A) ou fibronectina plasmática (B) foi realizado utilizando-se anticorpos específicos contra os componentes. Os dados são representativos de dois experimentos independentes. O desvio padrão representa a média de cada ponto realizado em triplicata.

4.31 Região imunodominante da proteína rLIC10258

Para analisar qual a porção imunodominante da proteína rLIC10258 foram obtidos fragmentos recombinantes correspondentes as porções N-terminal, Intermediária e C-terminal desta proteína. O fragmento Intermediário foi construído com regiões de sobreposição tanto no fragmento N-terminal, entre os aminoácidos 170 e 219, quanto no C-terminal, entre os aminoácidos 420 e 483 (Figura 29).

Os resultados mostraram que tanto o fragmento Intermediário quanto o C-terminal foram capazes de interagir com a laminina. O perfil da ligação destes fragmentos com a laminina e os valores de KD obtidos foram semelhantes aos apresentados pela proteína rLIC10258 (Figura 18A). Portanto, como o fragmento Intermediário e C-terminal possuem uma região de sobreposição entre os aminoácidos 420 e 483, os resultados obtidos sugerem que a interação entre estes fragmentos e a laminina ocorra nesta região comum.

Em relação a fibronectina plasmática e o PLG, apenas o fragmento Intermediário foi capaz de interagir com estes componentes, sugerindo que estas interações não ocorrem nas regiões que o fragmento Intermediário possui em comum com os fragmentos N-terminal e C-terminal. O perfil da ligação do fragmento Intermediário com a fibronectina plasmática e o valor de KD obtidos foram semelhantes ao apresentado pela proteína rLIC10258 (Figura 18B), por outro lado a afinidade da ligação entre o fragmento Intermediário e PLG foi menor do que o obtido para rLIC10258 (Figura 21). Além disso, o ensaio de competição revelou que a fibronectina plasmática e o PLG não competem pelo mesmo sítio de ligação no fragmento Intermediário.

Sendo assim, os resultados indicam que a região imunodominante da proteína rLIC10258, responsável pela ligação a laminina, fibronectina plasmática e PLG está localizada entre os aminoácidos 220 e 483. A interação com a laminina possivelmente ocorre entre os aminoácidos 420 e 483, enquanto que entre os aminoácidos 220 e 419 possivelmente estão localizados os sítios de ligação a fibronectina plasmática e PLG (Figura 40). Além disso, os resultados obtidos sugerem que o domínio OmpA-like na porção C-terminal da proteína rLIC10258 não está envolvido na interação desta proteína com a laminina, fibronectina plasmática e PLG.

Figura 40 - Esquema demonstrando o domínio imunodominante da proteína rLIC10258

Proteína rLIC10258

A região imunodominante da proteína rLIC10258 está situada entre os aminoácidos 220 e 483. Entre os aminoácidos 220 e 419 estão localizados os sítios de ligação da fibronectina plasmática e PLG, enquanto que a ligação da laminina ocorre entre os aminoácidos 420 e 483.

5 CONCLUSÃO

Todas as proteínas recombinantes deste estudo ligam-se ao PLG. Além disso, a proteína rLIC10258 é capaz de aderir a laminina e fibronectina plasmática e a proteína rLIC12238 interage com o fibrinogênio. Portanto, as proteínas deste estudo podem auxiliar tanto na adesão ao hospedeiro quanto na penetração dos tecidos por meio da conversão do PLG em plasmina. Além disso, os resultados obtidos sugerem que estas proteínas estão localizadas na superfície de *L. interrogans* sorovar Copenhageni e estão sendo expressas durante a infecção no hospedeiro. Portanto, as proteínas codificadas pelos genes LIC10258, LIC12880 e LIC12238, apesar de não possuírem atividade imunoprotetora, provavelmente estão envolvidas na patogenicidade das leptospiros.

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**APÊNDICES – ARTIGOS CIENTÍFICOS PUBLICADOS EM PERIÓDICOS,
CAPÍTULO DE LIVRO E MANUSCRITOS SUBMETIDOS**

- APÊNDICE A** – ATZINGEN, M. V., et al. Identification and characterization of novel adhesins in *Leptospira*. **Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology**, p. 704-713, 2010.
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- APÊNDICE C** – OLIVEIRA, R., et al. Characterization of novel OmpA-like protein of *Leptospira interrogans* that binds extracellular matrix molecules and plasminogen. **Plos One**, v. 6, p. e21962, 2011.
- APÊNDICE D** – ATZINGEN, M. V., et al. Evaluation of immunoprotective activity of six leptospiral proteins in the hamsters model of leptospirosis. **Open Microbiol J**, v. 6, p. 79-87, 2012.
- APÊNDICE E** – VIEIRA, M. L., et al. Plasminogen binding proteins and plasmin generation on the surface of *Leptospira* spp: the contribution to the bacteria-host interactions. **J Biomed Biotechnol**, p. 758513, 2012.
- APÊNDICE F** – OLIVEIRA, R., et al. Adhesins of *Leptospira interrogans* mediate the interaction to fibrinogen and inhibit fibrina clot formation *in vitro*. Manuscrito submetido.
- APÊNDICE G** – VIEIRA, M. L., et al. Leptospiral extracelular matrix adhesins as mediators of pathogen-host interactions. Manuscrito submetido.

Identification and Characterization of Novel Adhesins in *Leptospira*

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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' CACCGATGAAAAAAGAAAATGAATTGAG 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>GGATCCCT</u> GAAATATACGAA (EcoRI) R: 5' <u>GAATTCCT</u> ACTGTCTCCTC (BamHI)
LIC12906	Lsa24 [26]	YP_002822	Probable Lipoprotein with a domain of unknown function (DUF1554)	F: 5' <u>CGCGCTCG</u> AGAAAAAGGAAGAA (Xho I) R: 5' <u>AAGCTTTT</u> ATTGAAGAATAATTCC (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.

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In Vitro Identification of Novel Plasminogen-Binding Receptors of the Pathogen *Leptospira interrogans*

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Abstract

Background: Leptospirosis is a multisystem disease caused by pathogenic strains of the genus *Leptospira*. We have reported that *Leptospira* are able to bind plasminogen (PLG), to generate active plasmin in the presence of activator, and to degrade purified extracellular matrix fibronectin.

Methodology/Principal Findings: We have now cloned, expressed and purified 14 leptospiral recombinant proteins. The proteins were confirmed to be surface exposed by immunofluorescence microscopy and were evaluated for their ability to bind plasminogen (PLG). We identified eight as PLG-binding proteins, including the major outer membrane protein LipL32, the previously published rLIC12730, rLIC10494, Lp29, Lp49, LipL40 and MPL36, and one novel leptospiral protein, rLIC12238. Bound PLG could be converted to plasmin by the addition of urokinase-type PLG activator (uPA), showing specific proteolytic activity, as assessed by its reaction with the chromogenic plasmin substrate, D-Val-Leu-Lys 4-nitroanilide dihydrochloride. The addition of the lysine analog 6-aminocaproic acid (ACA) inhibited the protein-PLG interaction, thus strongly suggesting the involvement of lysine residues in plasminogen binding. The binding of leptospiral surface proteins to PLG was specific, dose-dependent and saturable. PLG and collagen type IV competed with LipL32 protein for the same binding site, whereas separate binding sites were observed for plasma fibronectin.

Conclusions/Significance: PLG-binding/activation through the proteins/receptors on the surface of *Leptospira* could help the bacteria to specifically overcome tissue barriers, facilitating its spread throughout the host.

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Introduction

Leptospirosis is an emerging infectious disease with worldwide distribution and a zoonosis that is of human and veterinary concern. Pathogenic *Leptospira* are the etiological agents of leptospirosis, a disease with greater incidence in tropical and subtropical regions [1,2,3,4,5]. Leptospirae cause chronic infection in domestic and wild mammalian species that harbor the bacteria in their renal tubules, shedding them through the urine into the environment, thus constituting a source of re-infection in other animals. Humans are accidental and terminal hosts in the transmission process of leptospirosis [3,6]. Leptospirosis represents a great economic burden as it affects the public health system and livestock [3].

The molecular pathogenesis of leptospirosis is poorly understood. To date, few virulence factors contributing to the pathogenesis of the disease have been identified [7,8,9]. It is well documented that the interaction of pathogens with the extracellular matrix (ECM) may play a primary role in the colonization of host tissues, as long-lasting infections may occur if microorganisms

reach the sub-epithelial tissues [10]. Recently, the ability of the leptospirae to adhere to ECM macromolecules has been shown, and some adhesins, ECM-binding proteins, have been identified [9,11,12,13,14,15,16,17,18,19]. However, under normal conditions, ECM is not exposed to bacteria. The proteolytic activity achieved by subversion of host proteases by pathogens, such as plasmin, has been demonstrated to be important in various bacterial infections [20]. Plasmin is a broad-spectrum serine protease component of the fibrinolytic system, which has the zymogen plasminogen (PLG) as its main component.

In our previous work, we found that *Leptospira* species are able to bind PLG and generate plasmin, in the presence of activator, on the outer surface *in vitro* [21], an activity already shown for the pathogenic spirochetes *Borrelia* and *Treponema* [22,23,24,25,26]. We have also revealed that leptospirae express multiple PLG-binding proteins, as reported for other microorganisms. Recently, Verma et al. [27] have shown that the protein LenA of *L. interrogans* [14], formerly LfhA/Lsa24 [12,27], is a surface receptor for human plasminogen.

In the present study, we further evaluated the ability of 14 recombinant proteins of *Leptospira* to mediate binding to PLG *in vitro*. Eight of the 14 assayed were identified as novel PLG-receptor proteins, including the major outer membrane protein LipL32 [28], the previously published rLIC12730, rLIC10494, Lp29, Lp49, LipL40 and MPL36 [29,30,31], and one novel leptospiral protein rLIC12238. Bound PLG could be converted to plasmin by the addition of urokinase-type PLG activator (uPA), showing specific proteolytic activity. We also show that PLG interaction with LipL32, also an ECM adhesin, was affected by collagen type IV but not plasma fibronectin. Our data provide an array of novel leptospiral receptors for human plasminogen, which constitutes an important step for the understanding of the molecular pathogenesis and infection process of these bacteria.

Results

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 PLG. Fourteen proteins available in our laboratory were selected: twelve were previously published, LipL32 [28,32], LipL40, MPL36 [29], rLIC10509 [33], Lp29, Lp49 [30], Lsa27 [11], MPL21, MPL17 [31], rLIC10494, rLIC12730 [12], and Lsa63 [19], and two are novel proteins, rLIC12922 and rLIC12238. Table 1 summarizes the features of the selected proteins and gene conservation within the sequenced genomes [34,35,36,37].

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*. Gene locus, protein reference number, given name, sequences of primers used for PCR amplifications and molecular mass of recombinants are depicted in Table 1. The recombinant proteins were purified by nickel affinity chromatography, and an aliquot of each protein was analyzed by SDS-PAGE (Fig. 1). All purified proteins were represented by major bands, although in some cases such as Lsa27 and MPL21, other less intense protein bands were also observed. Structural integrity of the purified proteins not previously published, LipL40, MPL36, rLIC12922 and rLIC12238, was assessed by 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 the recombinant proteins LipL40, MPL36 and rLIC12922, while rLIC12238 showed a predominant signal of β -strands, with minimum ellipticity around 215 nm (data not shown).

Cellular localization of the recombinant proteins

To determine whether the proteins not previously characterized, namely Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922 and rLIC12238, are surface-exposed proteins, we set out to analyze the corresponding protein location on the bacteria using living *L. interrogans* serovar Copenhageni cells and the liquid-phase immunofluorescence method. Leptospire were visualized by propidium iodide staining followed by protein detection with polyclonal mouse antiserum raised against the protein in the presence of anti-mouse IgG antibodies conjugated to FITC. The localization of the protein-green light lying on the leptospire was achieved by superimposing the two fields, and the results obtained are shown in Fig. 2. For each protein, two fields are depicted where we can see, in general, green fluorescence spots along the bacteria. An intense green fluorescence could be observed for LipL32, a well-known major outer membrane

protein of *Leptospira*, used as a positive control [38], but not with GroEL, a protoplasmic-cylinder marker used as a negative control [39]. The PBS control showed only red fluorescence of leptospire stained with propidium iodide.

Recombinant leptospiral proteins bind human plasminogen

We have reported that *L. interrogans* binds PLG and that several proteins could act as receptors [21]. These data prompted us to investigate whether the selected surface-exposed proteins are capable of binding human PLG *in vitro*. We have employed Western blotting assay as a first screening to identify PLG-binding proteins. The results show that the proteins LipL32, Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730 and rLIC12238 were interacting with PLG, while no binding was seen with Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12730 and rLIC12922 (Fig. 3A). In addition, the data clearly indicate that binding only occur with the expected molecular mass corresponding to the recombinant proteins (Fig. 3A). The binding of the proteins to PLG was also evaluated by ELISA. The fourteen recombinant leptospiral proteins, and BSA, used as negative control, were individually immobilized onto 96-wells plates, incubated with human PLG and the results obtained from three independent experiments are shown in Fig. 3B. Proteins LipL32, Lp29, Lp49, LipL40, MPL36, rLIC10494 and rLIC12238 presented statistically significant binding to PLG ($*P<0.001$ and $**P<0.0001$), while proteins, Lsa63, Lsa27, rLIC10509, MPL21, MPL17, rLIC12730, rLIC12922 and BSA did not show significant binding activity (Fig. 3B). The ELISA data are in agreement with the one obtained with Western blotting except for rLIC12730 and rLIC12238 that show discrepancy in binding with the two methodologies. The rLIC12730 depicted a stronger binding by Western blotting when compared to ELISA data, while rLIC12238 showed an opposite behavior (Fig. 3A, 3B).

Comparison of encoding sequences of the detected leptospiral plasminogen-binding proteins and those of published plasminogen-binding proteins

To investigate whether the identified PLG-binding proteins showed sequence similarity with other published spirochetal PLG-binding proteins [40,41,42,43,44], we carried out sequence alignment analysis using the Clustal X program and a tree-display NJ plot [45,46]. The constructed dendrogram showed that leptospiral PLG-binding proteins do not display sequence similarity or share conserved domains with other reported spirochetal PLG-binding proteins (data not shown).

Role of lysine residues in the plasminogen binding activity

It is known that PLG kringle domains frequently mediate interactions with lysine residues of the bacterial receptors [20]. These domains participate in the binding of PLG with intact live *L. interrogans* serovar Copenhageni strain L1–130, since the derivative and analogue of lysine, ACA, almost totally inhibited binding [21]. Sequence analysis showed that the native PLG binding proteins, LipL32, Lp29, Lp49, LipL40, MPL36, LIC10494, LIC12238 and LIC12730, contain 9.6, 9.3, 10.7, 7.8, 10.6, 9.1, 8.8 and 5.5% lysine residues, respectively. Based on these findings, the participation of lysine residues in the binding of the recombinant proteins was evaluated by the addition of ACA to the assay. As depicted in Fig. 4A, when 2 mM ACA was added to the reaction, the binding of the proteins to PLG was almost completely abolished.

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 [70]
LIC11352	LipL32 ^a	YP_001316	Major outer membrane protein (MOMP), LipL32 lipoprotein	Lai (100%); LBH (98%)	F: 5' CACCGGTGCTTCGGTGGTCTG 3' R: 5' ATTACTTAGTCGCGTCAGAAGC 3'	30.2
LIC10314	Lsa63 ^b	YP_000304	Conserved hypothetical protein with Borrelia_P83 domain	Lai (98%); LBH (87%) LBP (39%)	F: 5' GGATCCTTATTTTCTCAGGAAAG 3' (BamH I) R: 5' GGTACCCTAAGGTTTAATTTTTT 3' (Kpn I)	63.0
LIC10509	rLIC10509 ^c	YP_000493	Putative lipoprotein	Lai (98%)	F: 5' CCGGGATCCAAAAGAGCAAAGAAG 3' (BamH I) R: 5' GGTACCCTACTCGAGACGCCAGGA-CCTTC 3' (Kpn I)	22.0
LIC12892	Lp29 ^d	YP_002808	Putative lipoprotein		F: 5' CTCGAGGCAGTACATTACAATC-TTGCT 3' (Xho I) R: 5' CATGGCTCTTAGGAGCCTGGAAA 3' (Nco I)	29.6
LIC10793	Lp49 ^d	YP_000772	Putative lipoprotein, Surface antigen	Lai (99%); LBH (86%)	F: 5' CTCGAGAGCGGAGACTTTTCTTACTT 3' (Xho I) R: 5' CCATGGTTAAAAACCATCTCTACG-ATAAAC 3' (Nco I)	49.1
LIC12895	Lsa27 ^e	YP_002811	Putative lipoprotein	Lai (79%)	F: 5' GGATCCCTGAAATATACGAA 3' (EcoRI) R: 5' GAATTCCTACTGTTCTCTCTC 3' (BamHI)	27.0
LIC13131	MPL21 ^{a,f}	YP_003039	Hypothetical protein with Ycel domain	Lai (98%); LBP (45%)	F: 5' CACCACGTCTCAAAGTTACGCTTCAG 3' R: 5' TTCTCACCATCCAGCTCGG 3'	21.9
LIC10765	MPL17 ^{a,f}	YP_000745	Conserved hypothetical protein	Lai (100%); LBH (80%) LBP (41%)	F: 5' CACCGAAAGTCCCCTAAGGTTCAAA 3' R: 5' TGCAGGAGTCCACATTTTA 3'	15.4
LIC10091	LipL40 ^a	YP_000088	Putative lipoprotein	Lai (100%); LBH (84%)	F: 5' CCATGGGACTCGAGACGCCTCTCC-TAAAGATCC 3' R: 5' CTCCATGGTCATTTCAAACCTT-CTACGGGGC 3'	39.0
LIC10054	MPL36 ^a	YP_000054	Putative lipoprotein with Rare lipoprotein A (RplA) like domain	Lai (100%); LBH (88%) LBP (50%)	F: 5' CACCACGTCTTGTCGCTCGGTAGAG 3' R: 5' CCAAGTATTCTATTATACGTCCGAG 3'	35.1
LIC10494	rLIC10494 ^g	YP_000478	Putative lipoprotein	Lai (99%)	F: 5' CACCACTGCTAGGGCTGCAGAAA 3' R: 5' ACTTTGAGAGCTTCGTCTCGT 3'	25.8
LIC12730	rLIC12730 ^g	YP_002650	Hypothetical protein with TPR domain and 4 NHL repetition	Lai (100%); LBH (90%) LBP (37%)	F: 5' CACCAGTCTGACGGAATCCCAA 3' R: 5' TCTTGCAGATGAGTTGATCC 3'	77.4
LIC12922	rLIC12922 ^h	YP_002837	Conserved hypothetical protein	Lai (100%); LBH (89%) LBP (48%)	F: 5' CACCGAATCACTCAACAGAGTCATTGC 3' R: 5' ATCAATCTAAATGAAACGTCTCTTC 3'	40.0
LIC12238	rLIC12238 ^h	YP_002173	Putative lipoprotein	Lai (100%); LBH (81%) LBP (39%)	F: 5' CTCGAGTGTTTTAAACCTACCGGAG 3' (Xho I) R: 5' AAGCTTCTACTTCATCGCTTTTCTATATC 3' (Hind III)	17.6

¹<http://aeg.lbi.ic.unicamp.br/world/lic/> [35].²<http://www.ncbi.nlm.nih.gov/protein/>.³Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> [71,72].^aPreviously published by Gamberini et al. [29].^bVieira et al. [19].^cPreviously published by Gómez et al. [33].^dPreviously published by Neves et al. [30].^ePreviously published by Longhi et al. [11].^fPreviously published by Oliveira et al. [31].^gPreviously published by Barbosa et al. [12].^hThis work; Lai: *L. interrogans* serovar Lai [34]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [36]; LBP: *L. biflexa* serovar Patoc [37].

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Figure 1. SDS-PAGE analysis of purified recombinant proteins. Recombinant proteins were expressed in *E. coli* BL21 (DE3) or SI and purified from the bacterial cell lysate using Ni^{2+} -charged chelating Sepharose columns. M: molecular mass protein marker.
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Activation of plasminogen-bound proteins

PLG bound to the surface of *L. interrogans* is converted to enzymatically active plasmin by the addition of activator [21]. To determine if PLG bound to recombinant proteins can acquire proteolytic activity, 96-well plates were coated with the test proteins, blocked, and then incubated with PLG. Unbound PLG was washed away and a plasmin-type PLG activator 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 plasmin-specific chromogenic substrate (absorbance at 405 nm). As shown in Fig. 4B, the PLG captured by the proteins could be converted into plasmin, as demonstrated indirectly by specific proteolytic activity. The negative control BSA did not bind PLG (see Fig. 4) and did not show any proteolytic activity, as with the controls lacking PLG, uPA or the chromogenic substrate.

Characterization of the binding of recombinant proteins to PLG

The interactions between the recombinant proteins and PLG were assessed on a quantitative basis as illustrated in Fig. 5A. Dose-dependent and saturable binding was observed when increasing concentrations (0 to 1,000 nM) of the recombinant proteins MPL36, LipL40, LipL32, rLIC10494 and rLIC12238 were allowed to individually adhere to a fixed PLG amount (1 μg). For the proteins Lp29, Lp49 and rLIC12730, the saturation level was not 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 Fig. 5B; for the ones that reached equilibrium, the highest and the lowest K_D values were for LipL32 (25.87 ± 5.09 nM) and MPL36 (3.52 ± 3.95 nM), respectively.

Effects of salt and heparin on LipL32- and rLIC12238 - plasminogen interactions

As the proteins LipL32 and rLIC12238 showed the highest PLG binding and plasmin activity (Fig. 3B and Fig. 4B), they were chosen for further studies. To verify whether binding of these recombinant proteins to PLG is affected by added Cl^- [47] or negatively charged heparin [48], binding assays were performed in

the presence of different concentrations of NaCl or heparin. As shown in Fig. 6A, the increase in Cl^- anions to physiological conditions did not affect binding, and interference was only detected at a higher salt concentration, 350 mM, for both proteins. Similarly, the addition of heparin (up to 500 IU) did not have any significant effect on the interaction of the proteins with PLG (Fig. 6B).

Collagen type IV, but not plasma fibronectin, competes with LipL32 for binding to PLG

LipL32 has been previously demonstrated to be a leptospiral adhesin capable of binding collagen type IV and plasma fibronectin [15,16]. To see if the extracellular matrix components compete with LipL32 for the binding site of PLG or interfere somehow in the interaction with PLG, the binding assay was performed in the presence of increasing concentrations of collagen type IV or plasma fibronectin. The results show that plasma fibronectin had no effect on the binding of LipL32 to PLG (Fig. 7). On the other hand, the addition of collagen type IV decreased LipL32 binding to PLG in a dose-dependent fashion (Fig. 7), suggesting that both molecules compete for the same binding site.

Discussion

The interaction of the human plasminogen system has been suggested to be a feature that significantly contributes to the virulence of many bacterial pathogens by facilitating the initial anchoring to the endothelium [20]. Through capturing the host PLG on its surface, followed by activation to enzymatically active plasmin, the bacteria are turned into proteolytic organisms, a characteristic that has been shown to contribute to the degradation of ECM components, tissue penetration and invasion. In our previous work, we showed that leptospires are capable of capturing PLG on its outer surface and that the addition of exogenous activator converted it into enzymatically active plasmin capable of degrading ECM fibronectin [21]. In addition, multiple PLG-binding proteins present in the *L. interrogans* serovar Copenhageni whole cell lysates were detected, but remained unidentified [21]. Moreover, as expected, our data suggest the involvement of outer membrane proteins in the binding. Indeed, Verma and colleagues [27] have recently demonstrated that LenA, a surface leptospiral

Figure 2. Cellular localization of proteins in *L. interrogans* by L-IFA. Protein recognition by L-IFA. Proteins in live *L. interrogans* serovar Copenhageni isolates were recognized by FITC-conjugated antibodies against Lp29, Lp49, LipL40, MPL36, LIC10494, LIC12730, LIC12922, LIC12238 and LipL32 (surface-exposed lipoprotein, positive control), but not against GroEL (a protoplasmic cylinder marker) and PBS (negative control) under confocal microscopy. The leptospires were identified using the DNA counterstain propidium iodide. Panels are composite images of propidium iodide- and FITC-stained slides. Magnification, 600 $\times$.
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protein [14] is a bacterial receptor for human plasminogen. The availability of several putative surface-exposed recombinant proteins of *L. interrogans* in our laboratory prompted us to investigate the ability of these proteins to interact with PLG *in vitro*, in order to reveal and extend the array of plasminogen receptors in *Leptospira*.

The fourteen proteins selected for the screening of PLG binding were cloned and expressed in *E. coli* as full-length recombinant proteins. The purified proteins exhibited major bands in SDS-PAGE making them suitable for PLG binding assays. The structural integrity of the unpublished purified proteins was attested by CD spectroscopy revealing a mixture of α -helix and β -strand structures. The other recombinant proteins had their CD

spectra confirmed and were similar to the ones previously reported [11,12,19,30,31,33]. The cellular localization of the proteins Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922 and rLIC12238, was shown to be the cell surface, similar to findings for the previously published LipL32 [28], Lsa63 [19], rLIC10509 [33], Lsa27 [11], MPL21 and MPL17 [31].

Several invasive gram-positive and gram-negative bacteria have been shown to interact with the host PLG system [20,49,50,51,52], a phenomenon that has also been observed with viruses [53,54,55] and parasites [56], either by expression of PLG receptors or stimulation of host activators. In the case of spirochetes, the plasminogen activation system was studied with several species of *Borrelia* with *Treponema denticola* and suggested to have an important role during infection [22,23,24,25,26].

The human plasminogen heavy chain contains five triple-disulfide-linked peptide regions of ~ 80 amino acids, called kringles [57]. The process of binding to bacterial receptors on the cell surface has been shown for several pathogens to be mediated by the PLG kringle domains through the lysine-binding sites present [20]. Similarly, *Borrelia* [24] and *Leptospira* [21] also bind PLG via kringle domains. We have identified eight leptospiral plasminogen-binding proteins: LipL32 [28,32], rLIC10494, rLIC12730 [12], Lp29, Lp49 [30] and LipL40 and MPL36 [29] and one novel protein rLIC12238. The specificity of the binding of these recombinant proteins was attested by Western blotting. Interestingly, two proteins showed disparate binding intensity by Western blotting and ELISA. This could be explained by the fact that ELISA is a quantitative method but Western blotting is not. In addition, due to the denaturing condition of this latter method, it is possible that the exposure of some epitopes led to better binding for rLIC12730, while the inverse situation was seen with rLIC12238 which apparently needs a more native condition for PLG binding.

Plasminogen-binding proteins of *B. burgdorferi*, including the outer-surface lipoprotein OspA, have been identified [41]. Recently, Brisette and coworkers [44] identified three proteins of *B. burgdorferi*, ErpP, ErpA and ErpC, as being PLG-binding proteins. Interestingly, an evaluation of the primary sequence of spirochete PLG-binding proteins show that the proteins do not share similarity, with the exception of the Erp family protein of *B. burgdorferi* [44], BbCRASP-1 of *B. hermsii* [43] and HcpA of *B. recurrentis* [40].

Our data with the PLG-binding proteins point out to a primary role for lysine residues in plasminogen binding, as the lysine analog 6-aminocaproic acid (ACA) significantly inhibited binding [21,27,58]. However, no correlation between the number of lysine residues and PLG binding was detected. Indeed, the highest percentage of lysine residues was identified in Lsa63 [19], a protein that did not bind PLG. Moreover, lysine residue position does not seem to correlate with binding because they were distributed among all the protein sequences studied. This suggests that protein conformation and steric hindrance should be important factors in the binding of the protein/receptor to PLG. Plasminogen-binding proteins were able to generate active plasmin in the presence of urokinase-type PLG activator, as measured by the cleavage of plasmin-specific chromogenic substrate.

The recombinant proteins bind to PLG in a concentration-dependent and saturable fashion, indicating the specificity of the binding, for the proteins LipL32, LipL40, MPL36, rLIC12238 and rLIC10494. The calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG varied from 25.87 ± 5.09 nM for LipL32 to 3.52 ± 3.95 nM for MPL36. The plasminogen interaction/activation has been shown to be suppressed by the presence of Cl anions [47], while there is

Figure 3. Binding of leptospiral recombinant proteins to human plasminogen. **A.** The proteins were electrophoresed in 12% SDS-PAGE gels and transferred to nitrocellulose membranes, followed by overnight incubation with 3 μ g/mL PLG; after washing, the membrane was incubated with mouse anti-human PLG (1:750), followed by incubation with anti-mouse IgG (1:5,000). Protein reactivity was revealed by ECL reagent/exposure to X-ray films. **B.** The recombinant proteins LipL32, Lsa63, rLIC10509, Lp29, Lp49, Lsa27, MPL21, MPL17, LipL40, MPL36, rLIC10494, rLIC12730, rLIC12922 and rLIC12238 were coated onto 96-well ELISA plates (10 μ g/ml) and allowed to interact with purified human PLG (10 μ g/ml). BSA was 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. * $P < 0.001$; ** $P < 0.0001$. doi:10.1371/journal.pone.0011259.g003

Figure 4. Role of lysine residues in recombinant protein-PLG interaction and activation to enzymatically active plasmin. A: Binding of the immobilized proteins LipL32, Lp29, Lp49, LipL40, MPL36, rLIC10494, rLIC12730 and rLIC12238 (10 μ g/ml) to PLG (10 μ g/ml) was carried out in the presence (PLG+ACA) or absence (PLG) of the lysine analogue 6-aminocaproic acid. BSA was used as a negative control for nonspecific binding. The bound PLG was detected and quantified by specific antibodies. Bars represent the mean absorbance at 492 nm $\pm$ the standard deviation of four replicates for each sample and are representative of two independent experiments. Statistically significant differences are shown by * ($P < 0.001$) and ** ($P < 0.0001$). **B:** 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 * ($P < 0.00001$), ** ($P < 0.005$) and *** ($P < 0.05$). doi:10.1371/journal.pone.0011259.g004

evidence that heparin dextran derivatives could contribute to the regulation of plasmin activity, not only by impeding plasmin generation, as a result of their binding to plasminogen but also by directly affecting the catalytic activity of the enzyme [48]. The

addition of Cl^- did not inhibit LipL32 or rLIC12238-PLG interactions, showing in fact, a positive effect with increasing sodium chloride concentration. The presence of heparin had no influence on the binding of either protein to PLG. Similar results

Figure 5. Characterization of recombinant proteins binding to PLG. A. PLG (10 μ 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 and Lp49; 1:500 for rLIC12730), 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. B. 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.
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were obtained by Brissette and coworkers [44] studying the effect of both compounds on the interactions of ErpP recombinant protein of *B. burgdorferi* and plasminogen. Furthermore, Verma et al. [27] showed that heparin had no effect on LenA-PLG interactions but that the addition of salt led to an increase in binding.

The identified PLG-binding proteins Lp29, Lp49, LipL40 and MPL36 have been previously reported to be reactive with sera from leptospirosis patients [29,30] while rLIC12238 and rLIC10494 react with sera from hamsters infected with *Leptospira* (Nascimento, AL et al., unpublished results). These data suggest that the proteins are expressed and exposed during the course of infection. We confirmed here the localization of these four proteins and presented their PLG-binding properties. Taken together, our results implicate that these proteins play a possible role in virulence.

The protein LipL32 is a major outer membrane antigen expressed during mammalian infection [16,28]. It has been shown

that LipL32 is recognized by patients' sera [15,59] and has the ability to bind to ECM macromolecules [15], activities that suggest a role in the pathogenesis of leptospires. Intriguingly, it has also been demonstrated that LipL32 is not essential for either acute or chronic infection with *L. interrogans* [60]. Here, we provide another activity for LipL32, as a PLG-binding protein, giving further support to its importance during infection. Additionally, our results suggest that collagen type IV and PLG interact with the same site of LipL32, a situation that does not occur with plasma fibronectin. The dual activity of LipL32 in binding PLG and ECM macromolecules together with our previous findings of fibronectin degradation by plasmin-coated leptospires [21], may have implications on the mechanisms of the invasion process.

In conclusion, we have identified and characterized eight proteins as being PLG-binding proteins of *Leptospira*. There is evidence that at least five of them (LipL32, Lp29, Lp49, LipL40

A

B

Figure 6. Effects of ionic strength and heparin in PLG binding by LipL32 and rLIC12238. Binding of LipL32 and rLIC12238 (10 μ g/ml) to immobilized PLG (10 μ g/ml) was performed with the addition of increasing concentrations of NaCl (0 to 350 mM) (A) or heparin (0 to 500 IU) (B). The detection of bound-PLG was assayed by specific antibodies. Bars represent the mean absorbance values $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. The data show statistically significant differences in **A**, shown by * ($P<0.05$), but not in **B**.
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and MPL36) are expressed during natural infection [28,29,30]. PLG-binding/activation through the proteins/receptors on the surface of *Leptospira* could help the bacteria to specifically overcome tissue barriers, facilitate invasion and colonize mammalian tissues. We believe that these studies provide new insights into leptospiral pathogenesis and infection.

Materials and Methods

Bioinformatics characterization of the proteins

Predicted coding sequences (CDSs) were analyzed as their cellular localization predictions by PSORT program, <http://psort.nibb.ac.jp> [61,62]. The web servers SMART, [http://smart.](http://smart.embl-heidelberg.de/)

[embl-heidelberg.de/](http://smart.embl-heidelberg.de/) [63,64], PFAM, <http://www.sanger.ac.uk/Software/Pfam/> [65], and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [66] 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 genomic DNA using complementary primer pairs. The gene sequences were amplified without the signal peptide tag, 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,

Figure 7. Effect of extracellular matrix macromolecules on PLG binding by LipL32. Binding of LipL32 (10 μ g/ml) to immobilized PLG (10 μ g/ml) was performed with the addition of increasing concentrations of plasma fibronectin or collagen type IV (0 to 600 mM). The detection of bound-PLG was assayed by specific antibodies. Bars represent the mean absorbance values $\pm$ the standard deviation of four replicates for each condition and are representative of two independent experiments. Results of statistically significant interference with binding in comparison with the positive control (no addition of purified ECM macromolecules) are shown: $P<0.0003$ (*).
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Houston, TX) with appropriate vector-specific T7 (F: TAATAC-GACTCACTATAGGG) and pAE (R:CAGCAGCCAACT-CAGTTCCT) primers. Cloning, expression and purification of the recombinant proteins Lsa63, LIC10509, Lp29, Lp49 and Lsa27 have been previously described [11,19,30,33]. The sequences coding for MPL21, MPL17, MPL36, LipL40, LipL32, LIC10494, LIC12730 and LIC12922 were cloned into the *E. coli* Gateway cloning (pENTR) and expression (pDEST17) system (Invitrogen), according to the protocol described elsewhere [29]. The expression and purification of MPL21 and MPL17 were performed as reported elsewhere [31], and rLIC10494 and rLIC12730, as previously described [12]. The recombinant proteins MPL36 and LipL40 were purified from *E. coli* BL21 (DE3) and *E. coli* BL21-SI, respectively. Bacteria were grown in Luria-Bertani (LB) broth with or without NaCl, at 37°C or 30°C, and at log-phase they were induced by 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) or 3 mM NaCl for 3 h at 37°C or 30°C, respectively. The bacteria cell lysates were cleared by centrifugation at 3,000 $\times$ g for 15 min, and the supernatants were loaded onto Ni²⁺-charged Sepharose beads. The beads were washed with increasing concentrations of imidazole (20 to 60 mM) and the proteins eluted with 0.5 M imidazole. The proteins LipL32 and rLIC12922 were expressed in *E. coli* BL21-SI for 3 h at 30°C with the addition of 150 mM NaCl to the log-phase culture grown in LB broth without NaCl. The proteins were expressed in the soluble form, as the above protocol described for the proteins MPL36 and LipL40. The LIC12238 CDS was cloned into the pGEM-T-Easy vector (Promega), and transferred into the pAE expression vector [67]. The construct was transformed into *E. coli* BL21-SI, and the log-phase culture, grown at 30°C, was induced by incubation in the presence of 300 mM NaCl for 3 h. The protein rLIC12238 was expressed in the soluble form and purified as described above.

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 180 to 260 nm. The molar ellipticity (Φ) is expressed in deg.cm².dmol⁻¹.

Antisera production against recombinant proteins

The recombinant proteins were adsorbed onto 10% (vol/vol) of Alhydrogel (2% Al (OH)₃, Brenntag Biosector, Denmark), used as adjuvant. Each recombinant protein (10 μ g) was subcutaneously injected in female BALB/c mice (4–6 weeks old). Two subsequent booster injections were given at two-week intervals with the same protein preparations. Negative-control mice were injected with PBS/Alhydrogel. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the pooled sera were analyzed by enzyme-linked immunosorbent assay for the determination of antibody titers. All animal studies were approved by the Ethics Committee of Instituto Butantan, under protocol 506/08, São Paulo, SP, Brazil.

Liquid-phase immunofluorescence assay (L-IFA)

The localization of the recombinant proteins by L-IFA was performed as previously described [31]. In brief, 2.5-ml suspensions of live leptospires (*L. interrogans* serovar Copenhageni strain M 20, routinely cultured as described elsewhere [21] were harvested

at 10,000 rpm for 15 min, washed twice with PBS (with 50 mM NaCl), resuspended in 200 μ l of PBS with 6 μ g/ml propidium iodide to stain the nuclei, and incubated for 45 min at 37°C. After incubation, the leptospires were gently washed with PBS and incubated for 30 min at 4°C with polyclonal mouse antiserum against each recombinant protein 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 immunofluorescently labeled leptospires were examined with the use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany).

Plasminogen binding

The binding of the recombinant proteins to PLG 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 recombinant proteins or bovine serum albumin (BSA) as negative control. Plates were washed once with PBS supplemented with 0.05% (vol/vol) Tween 20 (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 human plasminogen 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 plasminogen (Sigma-Aldrich) (1:4,000 in PBS). Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG), diluted 1:5,000 in PBS. After three washings, 100 μ l/well of 1 mg/ml *o*-phenylenediamine (OPD) plus 1 μ l/ml H₂O₂ in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 μ l/well of H₂SO₄ (2 N). Readings were taken at 492 nm.

Effects of salt and heparin on recombinant proteins-plasminogen interactions

To assess the role of ionic interactions or of heparin-binding domains in LipL32 or rLIC12238 binding to plasminogen, increasing concentrations of NaCl (0–350 mM) or heparin (0–500 IU) (Roche) were added to the PBS-based buffer with plasminogen to the recombinant protein-coated wells. In both experiments, the detection of bound-plasminogen was performed as described above.

SDS-PAGE and affinity blotting

The purified recombinant proteins were electrophoresed in 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Hybond-ECL, GE Healthcare) in semi-dry equipment. The membranes were blocked with 5% BSA for 2 h at 37°C, washed three times (10 min for each wash) with PBS-0.5% Tween-20 solution and incubated overnight with 3 μ g/mL PLG 4°C, followed by 2h incubation at room temperature. The membranes were then washed three times and incubated with mouse anti-human PLG (1:750) for 3 h at room temperature, followed by three more washings and 1 h incubation at room temperature with anti-mouse IgG (1:5,000). The membranes were washed and the protein's reactivity was revealed using the ECL reagent (GE Healthcare) with subsequent exposure to X-ray films.

Characterization of the binding

To determine the role of lysines in plasminogen-recombinant protein interactions, the lysine analog 6-aminocaproic acid (ACA)

(Sigma-Aldrich) was added together with plasminogen at a final concentration of 2 mM to the recombinant protein-coated wells. To assess the role of ionic interactions or of heparin-binding domains in LipL32 or rLIC12238 binding to plasminogen, increasing concentrations of NaCl (0–350 mM) or heparin (0–500 IU) (Roche) were added to the PBS-based buffer with plasminogen which was then added to the recombinant protein-coated wells. In both experiments, the detection of bound-plasminogen was performed as described above.

Plasmin enzymatic activity assay

First, 96-well ELISA plates were coated overnight with 10 µg/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) non-fat dry milk for 2 h at 37°C. The blocking solution was discarded and 100 µl/well of 10 µg/ml human plasminogen 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 the plasmin-specific substrate D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (Sigma-Aldrich) 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.

Dose-response curves

First, 96-well ELISA plates were coated overnight in PBS at 4°C with 100 µl of 10 µg/ml plasminogen. Plates were then blocked and increasing concentrations of the purified recombinant proteins (0–1 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 appropriate dilutions (1:4,000 for LipL32; 1:5,000 for rLIC12238, LipL40 and MPL36; 1:1,000 for Lp29 and Lp49; 1:500 for rLIC12730), followed by horseradish peroxidase-conjugated anti-mouse IgG (Sigma) (1:10,000 in PBS). 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. [68] and Lin et al. [69], 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).

Interference of collagen type IV and plasma fibronectin with PLG binding by LipL32

First, 96-well plates were coated overnight at 4°C with 100 µl of 10 µg/ml recombinant LipL32 in PBS, washed once and blocked with 10% (w/v) non-fat dry milk for 2 h at 37°C. The plates were then incubated for 2 h at 37°C with 200 nM PLG together with increasing concentrations of collagen type IV (Sigma-Aldrich) or plasma fibronectin (Sigma-Aldrich) (both from 0 to 600 nM). After four washings, PLG binding was quantified by specific antibodies as described above.

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Author Contributions

Conceived and designed the experiments: MV ALTON. Performed the experiments: MV MA TO RO DA. Analyzed the data: MV MA TO RO DA SV ALTON. Contributed reagents/materials/analysis tools: MV MA TO RO DA SV ALTON. Wrote the paper: MV ALTON.

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Characterization of Novel OmpA-Like Protein of *Leptospira interrogans* That Binds Extracellular Matrix Molecules and Plasminogen

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Abstract

Leptospira interrogans is the etiological agent of leptospirosis, a zoonotic disease of human and veterinary concern. The identification of novel proteins that mediate host-pathogen interactions is important for understanding the bacterial pathogenesis as well as to identify protective antigens that would help fight the disease. We describe in this work the cloning, expression, purification and characterization of three predicted leptospiral membrane proteins, LIC10258, LIC12880 (Lp30) and LIC12238. We have employed *Escherichia coli* BL21 (SI) strain as a host expression system. Recently, we have identified LIC12238 as a plasminogen (PLG)-binding receptor. We show now that Lp30 and rLIC10258 are also PLG-receptors of *Leptospira*, both exhibiting dose-dependent and saturating binding (K_D , 68.8 ± 25.2 nM and 167.39 ± 60.1 nM, for rLIC10258 and rLIC12880, respectively). In addition, LIC10258, which is a novel OmpA-like protein, binds laminin and plasma fibronectin ECM molecules and hence, it was named Lsa66 (Leptospiral surface adhesin of 66 kDa). Binding of Lsa66 to ECM components was determined to be specific, dose-dependent and saturable, with a K_D of 55.4 ± 15.9 nM to laminin and of 290.8 ± 11.8 nM to plasma fibronectin. Binding of the recombinant proteins to PLG or ECM components was assessed by using antibodies against each of the recombinant proteins obtained in mice and confirmed by monoclonal anti-polyhistidine antibodies. Lsa66 caused partial inhibition on leptospiral adherence to immobilized ECM and PLG. Moreover, this adhesin and rLIC12238 are recognized by antibodies in serum samples of confirmed leptospirosis cases. Thus, Lsa66 is a novel OmpA-like protein with dual activity that may promote the attachment of *Leptospira* to host tissues and may contribute to the leptospiral invasion. To our knowledge, this is the first leptospiral protein with ECM and PLG binding properties reported to date.

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Introduction

Leptospirosis is a febrile disease caused by pathogenic spirochaetes of the genus *Leptospira*. The disease is been considered an important re-emerging infectious disease [1]. The transmission of leptospirosis has been associated with exposure of individuals in close proximity to wild or farm animals. Lately, the disease became prevalent in cities with sanitation problems and large population of urban rodent reservoirs, which contaminate the environment through their urine [1,2]. Due to the broad spectrum of symptoms, such as, fever, chills, headache, and myalgias, and similarity with other tropical illness, leptospirosis often remains largely under diagnosed. Progression to multi-organ system complications occurs in 5 – 15% of cases, with mortality rates of 5 – 40% [1,2]. The disease also has a great economic impact in the agricultural industry since it affects livestock inducing abortions, stillbirths, infertility, reduced milk production and death [1,2]. Commercially available vaccines, consisting of heat or chemically inactivated

leptospirins, named bacterins, provide serovar-specific protection against infection. The lack of serovar cross-protection in addition to the need for annual revaccination imposes a major limitation of whole-cell *Leptospira* vaccines [3]. The search for novel protein antigens that could elicit a broad protective and long-term immunity is currently under investigation.

Surface-associated proteins, as a result of their location, are likely to be important in host-pathogen interactions, hence their potential to promote several activities, including adhesion. The interaction of pathogens with the extracellular matrix (ECM) has been well documented [4]. In the case of leptospirins, some adhesion ECM-binding molecules have been characterized thus far. These include the Lsa24/Len protein family [5,6], LigA/LigB [7], Lsa21 [8], LipL32 [9], TlyC [10], Lp95 [11], Lsa63 [12] and OmpL37 [13]. We were the first group to describe that *Leptospira* species are able to bind PLG and generate plasmin, in the presence of activator, on the outer surface *in vitro* [14]. More recently, we have shown that eight out of fourteen recombinant

proteins available in our laboratory are PLG-binding proteins [15]. We believe that understanding the molecular mechanism of pathogenesis of *Leptospira* should help in the identification of novel vaccine candidates.

In the present study, we describe the cloning, expression, purification and characterization of three predicted membrane proteins, LIC10258, LIC12880 and LIC12238, identified in the genome sequences of *L. interrogans* serovar Copenhageni [16]. One of them, LIC12238, was previously described as a plasminogen-binding protein [15]. We now show that rLIC12880, previously named Lp30 [17] and rLIC10258 are also plasminogen-receptors of *Leptospira*. In addition, rLIC10258, which is a novel OmpA-like protein, is also capable to bind laminin and plasma fibronectin ECM molecules and hence it was named Lsa66 (Leptospiral surface adhesin of 66 kDa). The recombinant proteins are recognized by antibodies in serum samples of individuals in convalescent phase of the disease. Furthermore, the coding sequences for LIC10258, LIC12880 and LIC12238 may be expressed on the surface of bacteria because they are detected by immunofluorescence assay with intact living leptospires. Thus, it is possible that Lsa66 and LIC12238 proteins are expressed during infection and may play a role in pathogenesis.

Methods

Leptospira strains and sera

The non-virulent *Leptospira* strains used were: *L. interrogans* serovar Canicola strain Hound Utrecht IV, *L. interrogans* serovar Copenhageni strain M 20, *L. interrogans* serovar Icterohaemorrhagiae strain RGA, *L. interrogans* serovar Pomona strain Pomona, *L. interrogans* serovar Hardjo strain Hardjoprajitno, *L. borgpetersenii* serovar Castellonis strain Castellon 3, *L. kirshneri* serovar Grippotyphosa strain Moskva V, *L. santarosai* serovar Shermani strain 1342 K, *L. noguchii* 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%) [18]. *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.

Characterization of the protein *in silico*

Predicted coding sequence (CDS) LIC10258, LIC12880 and LIC12238 were identified using non redundant protein and *L. interrogans* serovar Copenhageni databases the *L. interrogans* serovar Copenhageni [16]; cellular localization prediction was performed by PSORT, <http://psort.nibb.ac.jp> [19] and CELLO, <http://cello.life.nctu.edu.tw/> [20] programs. The SMART, <http://smart.embl-heidelberg.de/> [21], PFAM, <http://www.sanger.ac.uk/Software/Pfam/> [22], and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> [23] web servers were used to search for predicted functional and structural domains. Sequence analysis was performed by BLAST [24] using Conserved Domain Database [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, 64°C (LIC10258) or 56°C (LIC12880) or 58°C (LIC12238) - 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. One microgram 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 LIC10258-F/LIC10258-R or LIC12880-F/LIC12880-R or LIC12238-F/LIC12238-R as described above. Samples quantity and integrity were verified by amplification of a 1,042 bp 16 S ribosomal cDNA fragment using oligomers:

16F 5'CAAGTCAAGCGGAGTAGCAATACTCAGC 3' and 16S-R 5' GATGGCAACATAAGGTGAGGGTTGC 3'.

The effect of temperature on LIC10258 transcript levels within the *L. interrogans* serovar Icterohaemorrhagiae strain was assessed by culturing leptospires at 37°C and 39°C. Additional cultures grown at 30°C were shifted overnight to 37°C and to 39°C, to simulate conditions encountered by bacteria upon entry into the host and in a febrile stage. Induction of LIC10258 expression by osmolarity was examined by centrifugation of cultures grown at 30°C in EMJH supplemented with 1% rabbit serum followed by resuspension in fresh EMJH medium or in EMJH containing 120 mM NaCl. Cultures were incubated for 24 h before being harvested for RNA isolation. The same procedures were performed with LIC12880 in the *L. borgpetersenii* serovar Castellonis, *L. santarosai* serovar Shermani and *L. noguchii* serovar Panama strains.

DNA recombinant techniques

Predicted CDS LIC10258, LIC12880 and LIC12238, without signal peptides, were amplified by the PCR from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 genomic DNA using the primer pairs listed 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 [26] at the restriction sites depicted in Table 1. 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 with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA).

Expression and purification of recombinant protein

Expression and purification of the recombinant proteins Lp30 and rLIC12238 have been previously described by Neves et al (2007) [17] and Vieira et al (2010) [15], respectively. Protein expression of the Lsa66 was achieved in *E. coli* BL21 (SI) strain by the action of T7 DNA polymerase under control of the osmotically

Table 1. Gene locus, protein name, gene bank 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
LIC10258	rLIC10258	YP_000249	Hypothetical protein with ompA domain	Lai (99%); LBH (79%)	F: 5' GGATCCGAAGCCTTCT-CACCCAATTG 3' (BamH I) R: 5' CCATGGTTAAAGTGAAAG-ATAAAAATCGATTG 3' (Nco I)	65.74 kDa
LIC12880	Lp30	YP_002796	Putative lipoprotein	Lai (99%); LBH (76%)	F: 5' CTCGAGGAAGTTGT-CCGAGTCTAT 3' (Xho I) R: 5' CCATGGTTATTGATT-GTTTAAATTCAG 3' (Nco I)	30.68 kDa
LIC12238	rLIC12238	YP_002173	Hypothetical protein	Lai (99%); LBH (77%); LBP (39%)	F: 5' CTCGAGTGTTTTAA-ACCTACCGGAG 3' (Xho I) R: 5' AAGCTTCTACTTCATCG-CTTTTCTATATC 3' (Hind III)	17.635 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; LBH: *L. borgpetersenii* serovar Hardjo-bovis; LBP: *L. biflexa* serovar Patoc. doi:10.1371/journal.pone.0021962.t001

induced promoter proU. *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 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 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 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 containing 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% (vol/vol) glycerol and 8 M urea. The protein was then purified through metal chelating chromatography in a Sepharose fast flow column (GE Healthcare) and fractions were analyzed in 12% SDS-PAGE. The protein was 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 1000 ml of buffer) containing decreasing gradient of glycerol (10 – 0%), pooled and stored at 4°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 180 to 260 nm. The molar ellipticity (Φ) is expressed in deg.cm.dmol⁻¹. Spectra data was submitted to DICROPROT web server, <http://dicroprot-pbil.ibcp.fr/>, using the method that calculated the secondary structure content from the ellipticity experimental data [27].

Antiserum

Five female BALB/c mice (4 – 6 weeks old) were immunized subcutaneously with 10 µg of the recombinant proteins. The

recombinant protein was 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 under protocol n° 471/08. 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 15% 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-Lsa66 (1:800), anti-Lp30 (1:800), anti-rLIC12238 (1:2,000) mouse serum or anti-his antibody (1:3,000) (GE Healthcare) 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 proteins reactivity was revealed by ECL reagent kit chemiluminescence substrate (GE Healthcare) with subsequent exposition to X-Ray film.

Microscopic agglutination test (MAT)

The microscopic agglutination test was performed according to Faine et al. (1999) [1]. In brief, an array of serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasi-man, Grippotyphosa, 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.

ELISA for detection of human antibodies

Human IgG and IgM antibodies against Lsa66, Lp30 and rLIC12238 were evaluated by ELISA. In brief, samples (negative and positive MAT sera from 10 confirmed leptospirosis patients) were diluted 1:100 and evaluated for total IgG and IgM using peroxidase-conjugated anti-human IgG and IgM antibodies, 1:5,000 (Sigma, USA). Cutoff values were set at three standard deviations above the mean OD₄₉₂ of sera from 6 individuals, unexposed to leptospirosis, from the city of São Paulo, Brazil.

Plasminogen binding assay

The binding of the recombinant proteins to PLG 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 µl of 10 µg/ml of the human plasminogen; fetuin and bovine serum albumin (BSA) were employed, as negative control. Plates were washed once with PBS supplemented with 0.05% (vol/vol) Tween 20 (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 of 10 µg/ml recombinant proteins 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 polyclonal mouse anti-recombinant proteins (1:1,000 in PBS). In another assay, anti-His tag monoclonal antibodies (Sigma) were employed to detect protein binding at 1:1000 dilution. Plates were washed again and incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG), diluted 1:5,000 in PBS. After three washings, 100 µl/well of 1 mg/ml *o*-phenylenediamine (OPD) plus 1 µl/ml H₂O₂ in citrate phosphate buffer (pH 5.0) were added. The reactions were carried out for 5 min and stopped by the addition of 50 µl/well of H₂SO₄ (2 N). Readings were taken at 492 nm.

Plasmin enzymatic activity assay

96-well ELISA plates were coated overnight with 10 µg/ml recombinant proteins (or BSA for negative control) in PBS at 4°C. 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 plasminogen 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) was added. Subsequently, 100 µl/well of plasmin-specific substrate D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride (Sigma-Aldrich) was added at a final concentration of 0.4 mM in PBS. Plates were incubated overnight at 37°C and substrate degradation was measured by taken the readings at 405 nm.

Binding of recombinant proteins to ECM

Protein attachment to individual macromolecules of the extracellular matrix was analyzed according to a previously published protocol [6] with some modifications. Briefly, ELISA plate wells were coated with 1 µg of laminin, collagen type I, collagen type IV, cellular fibronectin, plasmatic 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 containing 0.05% Tween 20 and then blocked with 200 µL of 1% BSA (overnight at 4°C). One microgram of

each protein (Lsa66~152 nM, Lp30~326 nM and rLIC12238~567 nM) was added per well in 100 µL of PBS, and proteins were allowed to attach to the different substrates for 2 h at 37°C. After washing six times with PBS-T, bound proteins were detected by adding an appropriate dilution of mouse antiserum in 100 µL of PBS. Dilutions of mouse antiserum against each recombinant protein were equalized as to give an OD_{492 nm} value of 1.0, as follows: Lsa66 (LIC10258) 1:800; Lp30 (LIC12880) 1:10,000 and rLIC12238 (LIC12238) 1:10,000. In addition, anti-polyhistidine monoclonal antibodies were employed as protein-binding probes at 1:1000 dilution. 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 determination of dose-dependent attachment of Lsa66 to laminin and plasma fibronectin, protein concentrations varying from 0 to 1,500 nM in PBS were used. For statistical analyses, the binding of Lsa66 to ECM macromolecules was compared to its binding to BSA and fetuin by Student's two-tailed *t* test.

Dissociation constant (K_D) for the recombinant proteins binding to ECM and PLG

The ELISA data were used to calculate the dissociation constant (K_D) according to the method previously described [28] 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).

Inhibition of live leptospires binding to laminin, plasma fibronectin and plasminogen by Lsa66 and Lp30

ELISA plates were coated with laminin or plasma fibronectin or plasminogen (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 recombinant proteins (0 to 5.0 µg). After three washings, 100 µL/well of 4×10⁷ live *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), based on the fact that LipL32 is a major outer membrane leptospiral protein [29]; the procedure was followed by horseradish peroxidase-conjugated anti-mouse IgG antibodies, essentially as described in Barbosa et al. (2006) [6]. The detection was performed by OPD as above described.

Liquid-phase immunofluorescence assay (L-IFA)

The localization of LIC10258, LIC12880 and LIC12238 encoded proteins by L-IFA was performed as described Oliveira et al (2010) [30]. In brief, suspensions of 2.5 ml live leptospires (~10⁹ 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 leptospire were washed gently with PBS and incubated for 30 min at 4°C with polyclonal mouse anti-serum against Lsa63, rLipL32 or rGroEL at a 1:50 dilution. The leptospire 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 leptospire were washed and resuspended in PBS-antifading solution (ProLong Gold, Molecular Probes). The immunofluorescence-labeled leptospire were examined by use of a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany).

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.

ECM components and plasminogen

All macromolecules, including the control proteins fetuin and BSA, were purchased from Sigma Chemical Co. (St. Louis, Mo.). Laminin-1 and collagen Type IV were 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. Plasminogen native, purified from human plasma, was purchased from Merck.

Nucleotide sequence accession numbers

Gene bank accession number for protein sequences LIC10258, LIC12880 and LIC12238 is AAS68886, AAS71433 and ASS70810, respectively (see Table 1). The protein can also be accessed by the genome nomenclature for the gene locus, LIC number (*Leptospira interrogans* Copenhagen).

Results

Bioinformatic analysis

The genes encoding LIC10258, LIC12880 and LIC12238 were identified by analysis of the genome sequences of the chromosome I of *L. interrogans* serovar Copenhageni and each one is present as a single copy [16]. The CDSs LIC10258, LIC12880 and LIC12238 have signal peptide ranging from amino acid 1 to 28, according to SMART web server, and are predicted to be outer membrane proteins based PSORT [19] and CELLO [20] web servers. The LIC12280 and LIC12238 are putative lipoproteins, with a

Figure 1. Sequence analysis. (A) Sequence alignment of the OmpA C-like domain with the C-terminal region of CDS LIC10258. (B) Sequence alignments comparison of C-terminal region of CDS LIC10258 with proteins having OmpA-like domain from diverse pathogens: 1. *Escherichia coli* (chain H); 2. PAL of *Haemophilus influenza*; 3. *Helicobacter pylori* (chain K); 4. *Helicobacter pylori* (chain B); 5. *Neisseria meningitidis*; 6. *Vibrio alginolyticus*; 7. *Escherichia coli* (chain A); 8. *Verrucomicrobium spinosum* DSM 4136; 9. *Sorangium cellulosum*; 10. *Acinetobacter* sp. ADP1 (putative antigen).

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Figure 2. Distribution and expression of LIC10258, LIC12880 and LIC12238 genes in saprophytic and pathogenic leptospires. (A) Genomic DNA from *L. biflexa* Patoc and from nine 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. Amplification of 16S DNA shows template integrity. No DNA was added to the negative control reaction (-). (B) RT-PCR analysis of LIC10258, LIC12880 and LIC12238 transcripts in high-passage *Leptospira* strains. Reactions were performed with specific primers designed according to *L. interrogans* serovar Copenhageni. Samples quantity and integrity were

verified by amplification of 16S ribosomal cDNA fragment. +: reverse transcriptase present. -: reverse transcriptase omitted. No cDNA was added to the negative control reaction (-). (C) Transcript analysis of LIC10258 in *L. interrogans* serovar Icterohaemorrhagiae after submission of bacterial culture to temperature upshift from 30 to 37°C, 30 to 39°C and to physiological osmolarity.
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cleavage site for signal peptidase II, according to Lipop program [23]. Blast analysis showed that the CDS LIC10258 has a putative OmpA-like domain at the C-terminal, from amino acid 504 to 583. Sequence alignment of the OmpA C-like domain with the C-terminal region of LIC10258 is depicted in Fig. 1A. Sequence alignments of OmpA-like conserved domain from diverse pathogens [25] are depicted in fig. 1B. The LIC10258 is present in the genome sequences of *L. interrogans* serovar Lai (99% identity) [31], of *L. borgpetersenii* serovar Hardjo-bovis (79% identity) [32], but is absent in *L. biflexa* serovar Patoc [33]. Similar putative coding sequence of LIC12880 was found in *L. interrogans* serovar Lai (99% identity), in *L. borgpetersenii* serovar Hardjo-bovis (76% identity), but is absent in *L. biflexa* serovar Patoc. The CDS LIC12238 was identified in *L. interrogans* serovar Lai (99% identity), in *L. borgpetersenii* serovar Hardjo-bovis (77% identity) and in *L. biflexa* serovar Patoc (39% identity). Table 1 summarizes the main features of the selected proteins and gene conservation within the sequenced genomes [31,32,33].

Distribution and expression of LIC10258, LIC12880 and LIC12238 genes among *Leptospira* strains

The LIC10258 gene is present in the main serovars of *L. interrogans*, Copenhageni, Icterohaemorrhagiae, Hardjo, Pomona and Canicola but no DNA amplification was detected in *L. borgpetersenii* serovar Castellonis, *L. santarosai* serovar Shermani, *L. noguchii* serovar Panama, *L. kirschneri* serovar Grippotyphosa and in *L. biflexa* serovar Patoc (Fig. 2A). Gene LIC12880 was found in all serovars tested of *L. interrogans* and in *L. borgpetersenii* serovar Castellonis, *L. santarosai* serovar Shermani, *L. noguchii* serovar Panama, but absent in *L. kirschneri* serovar Grippotyphosa and in *L. biflexa* serovar Patoc (Fig. 2A). Also shown in this figure is the DNA conservation of LIC12238 that was identified in all tested strains. 16S DNA amplification was performed to attest template integrity (Fig. 2A). The expression of LIC10258, LIC12880 and LIC12238 by *in vitro* cultured leptospires was evaluated by PCR amplification of reversely transcribed total RNA. The results obtained revealed the presence of LIC10258 transcripts in four *L. interrogans* strains mentioned above but no amplification was detected with serovar Icterohaemorrhagiae (Fig. 2B). In the case of LIC12880, transcripts were observed only in serovars of *L. interrogans*, although the gene was found in other pathogenic strains (Fig. 2A e 2B). Transcripts of LIC12238 were identified in all analyzed strains (Fig. 2B). DNA contamination was discarded as no amplification was observed in the absence of reverse transcriptase. The integrity of total RNA used in RT-PCR experiment was assured by the presence of a 1,042-bp 16S ribosomal cDNA fragment in all samples (Figure 2B). We set out to examine whether environmental factors, such as osmolarity and temperature could influence LIC10258 and LIC12880 regulation at the transcriptional level. Induction of LIC10258 and LIC12880 expression by osmolarity was assessed by growing cultures at 30°C in EMJH supplemented with 1% rabbit serum and resuspending them in fresh EMJH medium or in EMJH containing 120 mM NaCl. The addition of 120 mM NaCl to the medium mimics physiological conditions (~300 mosmol per liter) encountered by leptospires upon entry into the host [34]. We also evaluated gene expression of both genes from cultures subjected to temperature upshifts from 30°C to 37°C and from 30°C to

39°C during an overnight period to simulate conditions experienced by leptospires in the early stages of infection and during febrile stage. The results obtained show that cultures of *L. interrogans* serovar Icterohaemorrhagiae submitted to temperature upshift from 37°C to 39°C had no effect on the level of LIC10258 transcript (Fig. 2C). However, LIC10258 transcription (Fig. 2C) could be detected when the same culture strain was shifted to physiological osmolarity, at 30°C. In the case of LIC12880, no transcripts were observed in *L. borgpetersenii* serovar Castellonis, *L. santarosai* serovar Shermani and *L. noguchii* serovar Panama culture strains submitted to the same temperature and osmolarity conditions above described (data not shown).

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*. Gene locus, protein reference number, given name, sequences of primers used for PCR amplifications and molecular mass of recombinants are illustrated in Table 1. The recombinant proteins were purified by nickel affinity chromatography, and an aliquot of each protein was analyzed by SDS-PAGE (Fig. 3A–C). All purified proteins were represented by major bands (Fig. 3D). To further confirm that these proteins are indeed his-tag recombinants we carried out immunoblotting technique and probed them with his-tag antibodies. The data is shown in figure 3E and, as we can see, anti-His tag antibodies recognized the 3 recombinant proteins. However, in the case of rLIC12238 and Lsa66 preparation, other protein bands were also detected, being in the first case probably due to non-specific reaction and in the second, possibly caused by some protein degradation. Similar data were obtained when blotted recombinant proteins were probed with the respective homolog antiserum raised in mice (Fig. 3F).

Structural integrity of the purified proteins was assessed by circular dichroism (CD) spectroscopy

As depicted in Fig. 4, the maximum at 192 nm of α -helix with a broad minimum ellipticity around 215 nm, probably of β -strands, in the CD spectrum show that Lsa66 has both secondary structures; maximum ellipticity of 198 nm of β -strands was detected with rLIC12238, while maximum ellipticity around 200 nm was observed with the protein Lp30. According to DICOPTROT web server, the α -helix percentage of the proteins was higher in Lsa66 followed by rLIC12238, being the lower value for Lp30. These results are consistent with a mixture of α -helices and β -strands and are in agreement with the *in silico* analysis that showed both secondary structures (<http://bioinf.cs.ucl.ac.uk/psipred/>).

Detection of the LIC10258, LIC12880 and LIC12238 coding sequences by immunofluorescence confocal microscopy

To evaluate whether the selected CDSs are located at the bacterial membrane, we set out to analyze the protein position by using living organisms and the liquid-phase immunofluorescence method. Leptospires were visualized by propidium iodide staining (Fig. 5, column A) followed by protein detection with polyclonal

Figure 3. Protein analysis by SDS-PAGE and Western blotting. (A) Lsa66, (B) Lp30 and (C) rLIC12238 expression from NaCl-induced *E. coli* BL21-SI. M: molecular mass protein marker; NI: non-induced total bacterial extract; SL: supernatant after bacterial cell lysis and centrifugation; IL: inclusion body pellet after bacterial lysis and centrifugation; SS: soluble fraction of the induced culture in the presence of 8 M urea; IS: insoluble fraction of the induced culture in the presence of 8 M urea. (D) Coomassie blue stained purified recombinant proteins. (E) and (F) are western blotting analysis of the recombinant proteins probed with anti-His tag antibodies and the respective homolog antiserum, respectively.
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mouse antiserum raised against the protein in the presence of anti-mouse IgG antibodies conjugated to FITC. Green fluorescence could be observed in figure 5 column B, for Lsa66, Lp30,

rLIC12238 and LipL32, an outer membrane protein used as a positive control [29], but not with GroEL, a protoplasmic-cylinder marker, used as a negative control [35]. The localization of the

Figure 4. Circular dichroism spectra of the recombinant proteins. CD spectra of recombinant proteins Lsa66, Lp30 and rLIC12238. Far-UV CD spectra are presented as an average of five scans recorded from 190 to 260 nm. Experimental conditions, as described in M&M.
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Figure 5. Localization of proteins in *L. interrogans* by L-IFA. Confocal microscopy was performed with live *L. interrogans* using antisera specific for LIC10258, LIC12880, LIC12238, LipL32 (surface-exposed lipoprotein) and GroEL (protoplasmic cylinder marker). FITC-conjugated secondary antibodies were used to detect the surface-bound antibodies (B). Leptospires were identified by propidium iodide (A) staining of the DNA. Co-localization is shown in the merged images (C).
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protein-green light lying on the leptospire was achieved by merging both fields and the results obtained are shown in figure 5, column C.

Recombinant leptospiral proteins bind human plasminogen

We have reported that leptospire bind PLG and that several proteins could act as receptors, including the recombinant protein rLIC12238 of this work [14,15]. We then decided to investigate whether the selected surface-exposed proteins, Lp30 and Lsa66, were also capable of binding human PLG *in vitro*. Human purified plasminogen was coated to ELISA plates, allowed to interact with

the recombinant proteins Lsa66 and Lp30 and the results obtained from three independent experiments using antibodies against the recombinant proteins are shown in Fig. 6A. The binding of the proteins were also evaluated with anti-polyHis monoclonal antibodies (Fig. 6B). The interactions between the recombinant proteins and PLG were assessed on a quantitative basis as illustrated in Fig. 6C. Dose-dependent and saturable binding was observed when increasing concentrations (0 to 2,000 nM) of the recombinant proteins Lsa66 and Lp30 were allowed to individually adhere to a fixed PLG amount (1 µg). Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with PLG is 68.8 ± 25.2 nM and 167.39 ± 60.1 nM, for Lsa66 and Lp30, respectively (Fig. 6C).

Figure 6. Recombinant proteins binding to human plasminogen. (A) Binding of leptospiral recombinant proteins to human plasminogen. Human purified plasminogen (10 µg/ml) was coated to ELISA plates and allowed to interact with the recombinant proteins Lsa66 and Lp30 (10 µg/ml). BSA and fetuin were used as a negative control for nonspecific binding. The binding was detected by specific antibodies. Bars represent the mean of absorbance at 492 nm $\pm$ the standard deviation of three replicates for each protein and are representative of three independent experiments. For statistical analyses, the binding of Lsa66 and Lp30 to human PLG was compared to its binding to BSA as well as fetuin by two-tailed *t* test (**P*<0.005 and ***P*<0.0005). (B) Similar as described in (A) but the binding of the recombinant proteins was detected by specific anti-polyhistidine monoclonal antibodies. (C) Ten µg/ml pf PLG was immobilized into 96-wells ELISA plates and 0 to 2,000 nM of each recombinant protein was added for interaction. The binding was detected using antiserum raised in mice against each protein in appropriate dilutions (1:500 for Lsa66 and 1:500 for Lp30) followed by horseradish peroxidase-conjugated anti-mouse IgG. Data represent the mean absorbance values $\pm$ the standard deviation of three replicates for each experimental group. The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that have reached the equilibrium concentration. (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). BSA was employed as negative control. Bars represent mean absorbance at 405 nm, as a measure of relative substrate degradation $\pm$ the standard deviation of four replicates for each experimental group and are representative of two independent experiments. Statistically significant binding in comparison to the negative control (BSA) are show: **P*<0.001 and ***P*<0.02. doi:10.1371/journal.pone.0021962.g006

Plasmin generation from PLG-bound proteins

PLG bound to the surface of *L. interrogans* is converted to enzymatically active plasmin by the addition of activator [14]. To evaluate if PLG bound to recombinant proteins can acquire proteolytic activity, as reported for rLIC12238 [15], 96-well plates were coated with the test proteins, blocked, and then incubated with PLG. Unbound PLG was washed away and the uPA-type PLG activator 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 plasmin-specific chromogenic substrate (absorbance at 405 nm). As shown in Fig. 6D, PLG captured by the proteins could be converted into plasmin, as indirectly demonstrated by specific proteolytic activity. The negative control BSA did not bind PLG (see Fig. 6A) and did not show any proteolytic activity. The same situation occurred with the controls lacking PLG, uPA or the chromogenic substrate.

Adhesion of recombinant proteins to ECM components

The Lsa66, Lp30 and rLIC12238 proteins are suggested to be surface-exposed by immunofluorescence microscopy. We therefore investigated whether these proteins could mediate host colonization by adhering to extracellular matrix proteins. Thus, laminin, collagen Type I, collagen Type IV, cellular fibronectin, plasma fibronectin, and the control protein fetuin were immobilized on microdilution wells and recombinant protein attachment was assessed by an ELISA-based assay using antibodies against the proteins. As shown in Fig. 7A, Lsa66 protein exhibited efficient adhesiveness to laminin and plasma fibronectin. No statistically significant adhesiveness was observed with this protein when wells were coated with collagen Type I and IV, plasma fibronectin or with the highly glycosylated control protein fetuin. No binding was observed with the proteins Lp30 and rLIC12238 with ECM components (Fig. 7A). The Lsa66 interaction with ECM molecules was also observed when anti-polyhistidine monoclonal antibodies were employed (Fig. 7B). The interaction between Lsa66 with laminin and with plasma fibronectin was also assessed on a quantitative basis as depicted in Fig. 6B and 6C, respectively. A dose-dependent and saturable binding was observed when increasing concentrations of the recombinant protein (0 – 1000 nM) were allowed to adhere to a fixed laminin concentration (1 µg) (Fig. 7C) or recombinant protein (0– 2000 nM) to plasma fibronectin (1 µg) (Fig. 7D). Binding saturation level was reached by protein concentration of $\sim$ 500 nM, in the case of laminin, and above 1500 nM for plasma fibronectin (Fig. 7C and 7D), respectively. Also shown in these figures are the calculated dissociation equilibrium constants (K_D) for the recombinant

protein Lsa66 with laminin, 55.4 ± 15.9 nM and with plasma fibronectin, 290.8 ± 11.8 nM.

Inhibition of *L. interrogans* attachment to ECM and plasminogen by Lsa66 and Lp30

The inhibitory effect promoted by Lsa66 on leptospiral adherence to laminin, plasma fibronectin and by Lsa66 and Lp30 to plasminogen was quantified by an ELISA-like assay. ECM- and plasminogen-coated microtiter wells were incubated with increasing concentration of Lsa66 (Fig. 8A) and Lp30 (Fig. 8B) previous to the addition of 4×10^7 *L. interrogans*. Bound leptospires were probed with anti-LipL32 serum, as LipL32 is a major outer membrane leptospiral protein [29]. Lsa66 caused an inhibition of leptospires attachment to laminin (*P*<0.05), to plasma fibronectin (*P*<0.05) and to plasminogen (*P*<0.05) (Fig. 8A). In the case of Lp30, inhibition of leptospiral adherence to plasminogen was achieved with 1.25 µg (*P*<0.05) (Fig. 8B). The experiment was performed in triplicate and repeated three times with similar results.

Reactivity of recombinant proteins with sera from confirmed cases of leptospirosis

To investigate whether LIC10258, LIC12880 and LIC12238 coding sequences are capable to promote an immune response from an infected host, we evaluated the reactivity of the proteins with antibodies present in serum samples of confirmed early (MAT -) and convalescent (MAT +) phases of leptospirosis patients. We performed an ELISA using 20 serum samples, 10 of each phase of the disease. The results depicted in fig. 9 show that although several samples presented IgM- and IgG-antibodies against the recombinant proteins in the convalescent phase, positive MAT serum samples, no or very low reactivity was achieved at the early phase of the disease where MAT was negative. The best performances were obtained with Lsa66 and rLIC12238 proteins (Fig. 9A and 9C). It is worth mentioning that 30% of ELISA IgG antibodies were detected with Lsa66 with MAT negative serum (Fig.9A).

Discussion

A number of studies have established OmpA as a multifaceted molecule with many diverse roles. OmpA outer membrane protein of *Escherichia coli* and other enterobacteria have been reported to act as adhesin/invasin, to participate in biofilm formation and to have role as immune target and evasin [36]. OmpA-like domains (named after the C-terminal domain of *E. coli* OmpA protein) have been shown to non-covalently associate with peptidoglycan [37].

Figure 7. Binding characteristics of Lsa66, Lp30 and rLIC12238 to ECM components. (A) Wells were coated with 1 μ g of laminin, collagen type I, collagen type IV, cellular fibronectin, plasma fibronectin and the control proteins BSA and fetuin. 1 μ g of the recombinant protein was added per well and the binding was measured by an ELISA-based assay. In (A) the protein binding was detected by polyclonal antibodies against each protein, while in (B) protein binding was evaluated by anti-polyhistidine monoclonal antibodies. Data represent the mean $\pm$ the standard deviation from three independent experiments. For statistical analyses, the attachment of recombinant proteins to the ECM components was compared to its binding to BSA as well as fetuin by the two-tailed *t* test (**P*<0.005). (C) and (D) are recombinant proteins dose-dependent binding experiments with laminin (C) and plasma fibronectin (D); in both cases bindings were detected by polyclonal antibodies against each protein; each point was performed in triplicate and expressed as the mean absorbance value at 492 nm $\pm$ standard error for each point. Lp30 and rLIC12238 were included as a negative control. The dissociation constants (*K_D*) are depicted and were calculated based on ELISA data for the recombinant proteins that reached equilibrium up to a concentration of 1,000 nM, in the case of laminin and 2,000 nM, for the plasma fibronectin. doi:10.1371/journal.pone.0021962.g007

Members of this family include the *E. coli* outer membrane protein OmpA [36], the *E. coli* lipoprotein PAL (Peptidoglycan-associated lipoprotein) [38], the *Neisseria meningitidis* RmpM, a putative peptidoglycan binding protein, [39], the *E. coli* motor protein MotB [40] and the flagellar motor proteins PomB and MotY of *Vibrio alginolyticus* [41], which interact with the inner membrane. Moreover, peptidoglycan-associated lipoprotein (Pal) is a potential vaccine candidate against *Haemophilus influenza* [42]. The first protein identified in pathogenic *Leptospira* having a C-terminal OmpA consensus was Loa22 [43], followed by Omp52 identified in *L. santarosai* serovar Shermani, and shown to be reactive with sera of human patients infected with leptospires [44]. Recently, an OmpA70 was identified in *L. interrogans* serovar Copenhageni [45]. The OmpA-like protein Loa22 was reported to be essential for the leptospiral virulence [43] and to promote inflammatory responses in cultured rat renal cells [46]. More recently, Yan and colleagues (2010) [47] have reported OmpA-like proteins as novel vaccine candidates for leptospirosis. Thus, it is possible that OmpA-like proteins have a function in leptospiral pathogenesis.

We have identified one novel putative protein with OmpA-like domain at C-terminus (OmpA C-like) encoded by the LIC10258 gene, we named Lsa66, and two putative lipoproteins, one encoded by the gene LIC12880, previously reported as Lp30 [17], and the other, LIC12238, described as a plasminogen-binding protein [15]. DNA amplification of LIC10258 was present in many serovars of *L. interrogans* but was absent in other pathogenic species tested, including *L. borgpetersenii* serovar Castellonis. Because this gene was identified in *L. borgpetersenii* serovar

Hardjo-bovis genome sequences [32], it is possible that the gene is absent only in the serovar evaluated. Intriguingly, expression of LIC10258 was not observed in serovar Icterohamorrhagiae under normal growth conditions. Likewise, no LIC12880 transcripts were detected in 3 pathogenic species of *Leptospira*. The gene LIC10258 was expressed only under physiological osmolarity in *L. interrogans* serovar Icterohamorrhagiae, a characteristic shared with *ligA* and *B* genes [48] and LIC10368 [8]. The addition of 120 mM NaCl to the culture medium reproduces the hosts serum osmolarity ($\sim$ 300 mosM), thus providing a more physiological environment for leptospiral growth. Indeed, this is not surprising because 6% of the *L. interrogans* genes were revealed to be susceptible to osmoregulation [49]. Osmotic control of gene expression has been reported as an environmental cue associated with virulence in a variety of pathogens [34], including *Tcp pilli* of *V. cholerae* [50] and *invA* of *S. Typhimurium* [51]. Nevertheless, expression of the LIC12880 gene seemed to be restricted only to serovars of *L. interrogans* because bacterial cultures shifted to physiological conditions had no effect on gene expression.

The LIC10258, LIC12880 and LIC12238 coding sequences were cloned, without the signal peptide, and the proteins expressed in *E. coli* with a molecular mass of 66, 30 and 17 kDa, respectively. Recombinant proteins were purified by Ni²⁺-chelating chromatography as a major protein band and were recognized by anti-His tag monoclonal antibodies and by polyclonal antiserum obtained in mice against each of them. Assessment of secondary structure of the recombinant proteins after the purification process has been performed by CD spectroscopy and showed a mixture of α -helices

Figure 8. Inhibition of *L. interrogans* attachment to ECM and plasminogen by Lsa66 and Lp30. ECM- or plasminogen-coated microtiter wells were incubated with increasing concentration (0–5.0 μ g) of Lsa66 (A) or plasminogen-coated microtiter wells and increasing concentration (0–5.0 μ g) of Lp30 (B) for 1 h30 min prior to the addition of 4×10^7 leptospires. Wells were probed with anti-LipL32 serum. Data are expressed as $A_{492 \text{ nm}} \pm$ S.E. 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.pone.0021962.g008

A **B** **C**

Figure 9. Reactivity of the recombinant antigens Lsa66, Lp30 and rLIC12238 with serum samples of individuals diagnosed with leptospirosis. Positive sera (responders) were determined by ELISA with the recombinant proteins and serum samples from patients in both phases of the disease. The reactivity was evaluated as IgM or 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 five healthy individuals. **A**, **B** and **C** show the data for Lsa66, Lp30 and rLIC12238, respectively.
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and β -strands structures, similar to the data predicted by bioinformatics, rendering the recombinant proteins suitable for further studies.

The ability to interact with the host PLG system has been shown for several invasive gram-positive and gram-negative bacteria [52,53]. Moreover, this interaction has been described for some virus and parasites [54,55]. The plasminogen activation system was studied with several species of *Borrelia* and with *Treponema denticola* and suggested to have an important role during infection [56,57]. We have reported that *Leptospira* species were also capable to bind PLG and generating plasmin, in the presence of activator, on the outer surface *in vitro* [14]. Furthermore, we have demonstrated that plasmin-coated virulent *L. interrogans* bacteria were capable of degrading purified extracellular matrix fibronectin, a step that may contribute to leptospiral invasiveness [14]. Verma et al. (2010) [58] have demonstrated that the protein LenA of *L. interrogans* [5], formerly LfhA/Lsa24 [6,59], is a surface receptor for human plasminogen. More recently, we have shown eight novel PLG-receptor proteins of *Leptospira*, including rLIC12238 of this work [15]. We now identified Lsa66 and Lp30, as novel PLG-binding proteins. The highest binding affinity was achieved for rLIC12238 ($K_D = 11.97 \pm 1.06$ nM, in [15]) followed by Lsa66 ($K_D = 68.82 \pm 25.24$ nM), being the lowest value for Lp30 ($K_D = 167.39 \pm 60.14$ nM). The binding affinity value obtained for Lsa66 is of the same order of magnitude of the ones calculated for other recombinant proteins reported from our laboratory [15]. Bound PLG, as demonstrated with rLIC12238, could be converted to plasmin by the addition of urokinase-type PLG activator (uPA), showing specific proteolytic activity. Thus, it is possible that this protein may contribute to leptospiral infectiveness.

Several leptospiral adhesins have been described to date. These include 36-kDa fibronectin-binding protein [60], Lsa24 [6] /LfhA [59], LigA and LigB proteins [7], Len-family proteins [5], Lsa21 [8], LipL32 [9], Lsa27 [61], Lp95 [11], TlyC [10], LipL53 [30], Lsa63 [12] and OmpL37 [13]. Lsa66 exhibits extracellular matrix-binding properties, and it is a laminin and plasma fibronectin-binding protein. Estimated binding affinity of Lsa66 was higher to laminin than with plasma fibronectin. It is thus possible that besides acting as PLG-receptor, this protein may also play a role in the attachment to host tissues.

Lsa66 inhibited adhesion of intact *L. interrogans* to immobilized plasminogen, laminin and plasma fibronectin. Comparable effect was exhibited by Lp30 upon plasminogen. The inhibitory effect promoted by the recombinant proteins was partial and in agreement with the presence of additional binding proteins contributing to the leptospiral adherence to extracellular matrix components and plasminogen. Similar results were obtained with other leptospiral adhesins, Lsa24 [6], LigA and LigB proteins [7], Lsa63 [12] and OmpL37 [13].

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Immunofluorescence of LIC10258, LIC12880 and LIC12238 coding sequences at the surface of leptospire, as a result of antiserum recognition raised against each protein was detected as single spots. This could be explained since quantitative proteomics studies of *L. interrogans* have estimated only 75 and 11 copies per cell of LIC10258 and LIC12238, respectively, while LIC12880 did not fall in protein abundance scale to have its number of copies calculated [62]. On the other hand, 3,800 and 15,000 copies per cell were estimated of LipL32 and GroEL, respectively. These findings could explain the focal distribution and low fluorescence intensity of our coding sequences, contrasting with the fluorescence detected for LipL32. Interestingly, although present in high copy number, no green fluorescence was detected when anti-GroEL was added, attesting the integrity of the bacteria.

Lsa66 and rLIC12238 share serum recognition properties with the previously described adhesins, LipL32 [63], LigA and LigB proteins [64], Lsa27 [61], Lsa63 [12] and OmpL37 [13] that have shown positive reactivity with serum samples from patients diagnosed with leptospirosis. Interestingly, the OmpA-like leptospiral lipoprotein Loa22, although reactive with convalescent mouse sera and essential for virulence of *L. interrogans* in the animal model [43], does not bind ECM molecules [6]. This suggests that the binding of Lsa66 to laminin or plasma fibronectin does not occur through the OmpA-like domain at the C-terminus. Protein recognition by serum of confirmed leptospirosis samples together with the immunofluorescence data suggest that Lsa66 and rLIC12238 may be surface exposed.

In conclusion, we present in these studies one novel OmpA-like protein, Lsa66, and two predicted lipoproteins that are PLG-binding receptors. Lsa66, in addition, is an ECM binding protein that reacts with antibodies present in both phases of the disease. Thus, it is possible that this dual activity of Lsa66 may promote the attachment to host via ECM and may help the leptospire to overcome tissue barriers by plasmin generation. Revealing bacteria–host interactions at a molecular level should elucidate our understanding of the host physiology and facilitate the search for vaccine targets against leptospirosis.

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Author Contributions

Conceived and designed the experiments: RO APG SAV ALTON. Performed the experiments: RO ZMM APG ECR. Analyzed the data: RO ECR SAV ALTON. Contributed reagents/materials/analysis tools: RO ECR SAV ALTON. Wrote the paper: RO ALTON.

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

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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-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].

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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 gua-

nidine-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 [37] 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 purified 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.

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' CACCGATGAAAAAAAAAGAAAATGAATTGAG 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, 42].²<http://www.ncbi.nlm.nih.gov/protein/>.³Protein BLAST - <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi> [43, 44].^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 [45]; LBH: *L. borgpetersenii* serovar Hardjo-bovis [46]; LBP: *L. biflexa* serovar Patoc [47].

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 [40].

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 [41]. 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, 42]. 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*) [43, 44]. 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) [45]. 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) [46]. LIC10368, LIC11030 and LIC10821 CDS were not identified in *L. borgpetersenii* [46]. Only LIC12253 CDS were

identified in the genome sequences of *L. biflexa* serovar Patoc (39 % identity with LEPBI_I0560) [47]. 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-

Fig. (1). Distribution of LIC10821 and LIC10672 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. Amplification of 16S DNA shows template integrity. No DNA was added to the negative control reaction (-).

length protein in *E. coli*. Recombinant proteins were expressed 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

Fig. (2). Analysis of purified recombinant proteins by metal chelating chromatography through SDS-12% PAGE. Purified protein eluted from Ni^{+2} -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.

lent 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 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-adjuvant 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

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)	<i>P</i> value*	Culture (Negative/Total)	% Protection (No. Surviving/Total)	<i>P</i> 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 [41] and Logrank test compared to PBS survival curves.

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

ND: not determined

compared to the PBS-control (Fig. 4A and Table 2). Heat inactivated whole-cell leptospores (bacterin) afforded 100% protection while no survival animal was seen with PBS-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 [41] 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 [48]. Predicted outer membrane proteins have been identified during genome annotation of pathogenic *L. interrogans* serovars Lai [45] and Copenhageni [30] and *L. borgpetersenii* [46]. 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 [49]. 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 [41] 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 [49]. 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 vaccine 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 Kennewick 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.

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Review Article

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

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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'CACCGGTGCTTTTCGGTGGTCTG 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'GGTAGCCCTACTCGAGACAGCCAGGACCTTC 3' (KpnI)	22.0
LIC12892	Lp29 ^{d,h}	YP_002808	Putative lipoprotein	Lai (99%); LBH (86%)	F: 5'CTCGAGGCGATACATTACAACTTGCT 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'CCAAGTATCTATTTATACGTCCGAG 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' GGATCCGGAAGCCTTCTCAGCCCAATTG 3' (BamH I) R: 5' CCATGGTTAAAGTGAAGATAAAATCGATTTC 3' (Nco I)	65.7
LIC12880	Lp30 ⁱ	YP_002796	Putative lipoprotein	Lai (99%); LBH (76%)	F: 5' CTCGAGGGAAGTTGCCGAGTCTAT 3' (Xho I) R: 5' CCAATGGTTATTTGATTTTAAATTCAG 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' CTCGAGTGTACAAACGAAAAGAAAGGT 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' CTCGAGGAGGAGAAAACCGGACGATAC 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 Leptospire. *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 leptospire 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 leptospire 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 leptospire 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 lsPBS 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-passaged 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 leptospires/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 leptospires 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 Leptospire. 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.

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MANUSCRITO SUBMETIDO- Adhesins of *Leptospira interrogans* mediate the interaction to fibrinogen and inhibit fibrin clot formation *in vitro*

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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 (CHOU et al., 2012) 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 were 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 possible mechanisms by which fibrinolysis occur during leptospirosis. Moreover, decreasing fibrin clot would cause an imbalance of the coagulation cascade that may facilitate bleeding and help bacteria dissemination.

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. The control of the disease has been hampered by the lack of prophylactic interventions. 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, previously shown to adhere to extracellular matrix (ECM) components and plasminogen (PLG), as novel Fg-binding proteins. These proteins were able 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 interfere with the coagulation cascade and fibrinolytic system that might lead to bacteria spreading.

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 (FAINE et al., 1999a). The transmission occurs through contact with environmental water contaminated by leptospires shed in the urine of animal carriers (SETTE; RAPPUOLI, 2010). 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 (MALMSTROM et al., 2009). *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 (DALEY, PETERS;LARSEN, 2008). 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* (ALBERTS et al., 2007). Yet, when evaluated, these proteins failed to show hemolytic activity in human erythrocytes (VARKI et al., 2009).

Fg and PLG are key proteins in the coagulation cascade and fibrinolysis, respectively, and critical determinants of bacterial virulence and host defense (FRANTZ, STEWART;WEAVER, 2010). 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 (LODISH et al., 2000).

We have previously shown that PLA-associated *Leptospira* renders the bacteria with proteolytic activity capable of degrading ECM components (LODISH et al., 2000) 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 (MUIZNIEKS, WEISS;KEELEY, 2010). The adhesion of physiological osmotically induced *Leptospira* with Fg was described but their effect on fibrin

formation was not ascertained (HAYES et al., 2011). The leptospiral proteins LigB and OmpL37 were shown to interact with Fg and LigB was reported to reduce fibrin clot formation (LJUNGH; WADSTROM, 1996).

We thus decided to evaluate if Fg-associated *Leptospira* was capable to inhibit the fibrin clot formation and the ability of six recombinant proteins to act as leptospiral Fg-receptors. We report that *Leptospira* strains and the recombinant ECM- and PLG-interacting proteins, rLIC12238 (PARVEEN; LEONG, 2000), Lsa33 (CAMERON et al., 2004), Lsa30 (MILLER, MILLER;WHITE, 1966), OmpL1 (ITO; YANAGAWA, 1987) and rLIC11360 and rLIC11975 (Siqueira et al., submitted to publication), 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 nº 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%) (Turner, 1970). *Leptospira* cultures are maintained in Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil.

Bioinformatics characterization of the proteins. Predicted coding sequences (CDSs) were analyzed for their cellular localization by PSORT program, <http://psort.nibb.ac.jp> (THOMAS; HIGBIE, 1990). The web servers SMART, <http://smart.embl-heidelberg.de/>

(BARBOSA et al., 2006), PFAM, <http://www.sanger.ac.uk/Software/Pfam/> (BREINER et al., 2009), and LipoP, <http://www.cbs.dtu.dk/services/LipoP/> (BARBOSA et al., 2006) 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. 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: TAATACGACTCACTATAGGG) and pAE (R: CAGCAGCCAACTCAGTTCCT) primers. Detailed of cloning, expression and purification of the recombinant proteins Lsa33, rLIC12238, Lsa30, OmpL1, rLIC11360 and rLIC11975 has been previously described (VERMA et al., 2006) (Siqueira et al., manuscript submitted to publication).

Antiserum. Five female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 µg 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.

Binding of soluble human Fg to *Leptospira*. For accessing the leptospiral binding to soluble human Fg, ELISA plates were coated with 10⁸ leptospires per well, allowed to set for 3 h at 37°C and then blocked with 200 µL 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 µL of a Fg solution (Fg-Sigma) (10 µg/mL in low salt PBS - IsPBS-containing 50 mM NaCl) were added per well and incubation proceeded for 2 h. After extensive washing, 100 µL of solution containing goat anti-Fg (1:25,000 in IsPBS) were added per well and incubation was carried out for 1 h at 37°C. After three washings, 100 µL of solution containing

peroxidase (HRP)-conjugated rabbit anti-goat IgG (1:50,000 in IsPBS) 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 µL/mL H₂O₂ was added (100 µL per well). The reaction was allowed to proceed for 10 min and interrupted by the addition of 50 µL of 8 M H₂SO₄. 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. BSA was used as a negative control of binding. For statistical analyses, the binding of Fg to each leptospiral strain was compared to negative control using Student's two-tailed t test.

Human Fg degradation by leptospires coated with PLA. Virulent *L. interrogans* serovar Kennewicki strain Pomona Fromm (10⁸ leptospires/sample) were treated in 200 µL IsPBS with the addition of: (a) 10 µg PLG (b) 3U urokinase (uPA) (c) 10 µg PLG and 3U uPA (PLA) or (d) 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 IsPBS, resuspended in 100 µL IsPBS containing 15 mg/mL human purified Fg (Sigma, USA), and incubated for 16 h at 37°C. As controls, one sample received 1 µg aprotinin (Sigma, USA) to the Fg-leptospires incubation, and one sample containing only Fg (lacking leptospires) was employed. The leptospires were removed by centrifugation, 20 µL 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 anti-human Fg antibodies and secondary antibodies conjugated with HRP, followed by ECL (GE Healthcare) development.

Effect of Fg-bound leptospires on fibrin clot formation. Live leptospires (10⁹, 10⁷, 10⁵ cells/mL) were harvested at 6,000 rpm for 20 min at room temperature, washed once with IsPBS, resuspended in 0.5 mL of IsPBS plus 1 mg/mL of Fg (Sigma), and incubated for 2 h at 37°C. ELISA plates were placed with 90 µL/well of leptospires plus Fg and 10 µL/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 compared with the positive control by Student's two tailed t test.

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 µL of 10 µg/mL of the human Fg (Sigma); gelatin (Difco) was employed as negative 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 µL of 10 µg/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 and 1:800 for OmpL1. 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 µL/well of 1 mg/mL OPD plus 1 µL/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 µL/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 and 1:400 for OmpL1. The reaction was developed with 1 mg/mL OPD plus 1 µL/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 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. 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.

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 (MALMSTROM et al., 2009) 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 (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).

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 (* $P < 0.05$).

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. 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 (* $P < 0.05$).

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. 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%).

Metaperiodate treatment of Fg. Microtitre wells were coated with 1 µg of Fg 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 Fg was treated with sodium metaperiodate (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 metaperiodate - treated Fg was evaluated as described above.

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 (VIEIRA, ATZINGEN et al., 2010); the procedure was followed by the

addition of HRP-conjugated anti-mouse IgG antibodies, essentially as described in Atzingen et al., (2008) (LAHTEENMAKI, KUUSELA; KORHONEN, 2001). The detection was performed by OPD, as above described.

RESULTS

Adhesion of *Leptospira* spp. to Fg 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. As a control, we have employed BSA. Figure 1A depicts one representative experiment showing 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 exposed in Figure 1B. The data show that all the strains studied promoted an inhibition of fibrin formation, although the pathogenic strains seem more competent. The results suggest that *Leptospira* could modulate fibrin clot formation.

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. 1C). The results show that PLA generation on the surface of *Leptospira* cause degradation of human Fg (Fig. 1C, lane 5), which is not observed when one of the reaction components is missing (Fig. 1C, lanes 1, 2, 3 and 4) and completely prevented in the presence of a serine protease inhibitor, aprotinin (Fig. 1C, lane 6). The data suggest that PLA-coated leptospires were capable to employ their proteolytic activity to interfere with one of the coagulation cascade substrate.

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 six proteins, all of them previously shown to be leptospiral adhesins, four were already published, rLIC12238 (LUO et al., 2000), Lsa33 (MATSUNAGA et al., 2005), Lsa30 (CONFER; AYALEW, 2013), OmpL1 (SMITH et al., 2007) and two, rLIC11360 and rLIC11975, are submitted to publication. Table 1 summarizes features of the selected proteins, gene locus, given name, gene conservation within the 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 6XHis tag at the N-terminus and purified by nickel affinity chromatography, as previously described (PARSONS, LIN;ORBAN, 2006).

Interaction of leptospiral recombinant proteins with Fg. Proteins of *Leptospira* have been reported to bind Fg (BARBOSA et al., 2006). We thus decided to investigate whether the selected surface-exposed proteins were capable of binding human Fg *in vitro*. Six recombinant leptospiral proteins, expressed and purified in our laboratory, and gelatin, used as negative control, were individually immobilized onto 96-wells plates and incubated with human Fg. The binding was quantified by ELISA and the results obtained from three independent experiments are shown in Fig. 2. The protein bound to Fg was probed with the respective homolog polyclonal antiserum raised in mice (Fig. 2A) or with the monoclonal antibody anti-histidine tag (Fig. 2B). The percentage of binding for each protein with Fg, measured as a function of time is shown in Fig. 2C. 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. 2C). The binding of recombinant proteins to Fg was also assessed after blocking the proteins with the corresponding antibody (Fig. 2D) and after submitting them to denaturing conditions at 96°C for 10 min (Fig. 2E). The binding was totally inhibited by 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. 2D). 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. 2E), suggesting that with these last proteins epitopes involved in the binding were already exposed or the binding is based on linear epitopes rather than conformational ones.

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. 3A. Dose-dependent and saturable binding was observed when increasing 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 mg/mL). Saturation was reached with all except Lsa30 protein, due to the impossibility to achieve this protein at higher concentrations. Based on the ELISA data, the calculated dissociation equilibrium constants (K_D) for the recombinant proteins with Fg are depicted in Fig. 3B; the highest and the lowest K_D values were for rLIC12238 (733.3 ± 276.8 nM) and Lsa33 (128 ± 89.9 nM), respectively.

Effect of Fg oxidation on recombinant protein binding. In order to address the role of Fg sugar moieties in the interaction with recombinant proteins, Fg was oxidized by sodium metaperiodate, ranging from 5 to 100 mM, at 4°C for 15 min. The effect of periodate concentration on the binding with each protein is shown in Fig. 3C. This mild treatment ensures cleavage of vicinal carbohydrate hydroxyl groups, while polypeptide chain structure remains intact (MALMSTROM et al., 2009). Oxidation effect was dose-dependent and a considerable reduction (83%) in attachment of rLIC11360 to metaperiodate-treated Fg was observed at a 100 mM concentration of periodate, followed by 66% and 43% for rLIC11975 and Lsa30, respectively. Almost no reduction in the interaction of oxidized-Fg was obtained with the proteins Lsa33, OmpL1 and rLIC12238 (Fig. 3C). These results indicate that Fg sugar residues are important for the interaction of rLIC11360, rLIC11975 and Lsa30 proteins.

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. Each recombinant protein was pre-

incubated with 1 mg/mL of Fg at 37°C for 2h. 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. 4A and the percentage of inhibition is illustrated in Fig. 4B. The proteins rLIC11360 and Lsa30 were not employed on these assays because they were kept at pH12 for solubility and thrombin is not active above pH10. The data show that the four proteins tested promoted an inhibition of 40-50% on the fibrin clot formation, suggesting a possible involvement of these proteins in the coagulation process.

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 μ M) of rLIC12238 (A), rLIC11975 (B), Lsa23 (C), rLIC11360 (D), Lsa33 (E) and OmpL1 (F) 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 (PINNE, MATSUNAGA;HAAKE, 2012). The results are depicted in Figure 5 A to F, and show that the proteins caused a modest, but significant reduction in the number of leptospires adhering to Fg ($*P < 0.05$) with 0.5, 1.0, 1.0, 0.1, 0.25 and 1.0 μ M of rLIC12238, rLIC11975, rLIC11360, Lsa30, Lsa33 and OmpL1, respectively. We have performed three independent experiments with comparable results.

DISCUSSION

Fg, a 340 kD glycoprotein, is the major clotting protein present in blood plasma with an important role in coagulation and thrombosis. Activation of prothrombin to thrombin promotes the conversion of soluble Fg into insoluble fibrin to form a clot (BLOM, 2002). Several studies have suggested roles of PLG and Fg in host bacterial interactions (LIMA et al., 2013). The fibrinolytic system that generates PLA from PLG and its activators uPA, decomposes

the fibrin clot by degrading fibrin (MEKALANOS, 1992). This system is negatively regulated by PLG activator inhibitor 1 (PAI-1). A balance between coagulation system and anticoagulation is necessary to avoid pathophysiological conditions, such as, bleeding and thrombosis (ATZINGEN et al., 2008).

Several Fg-binding proteins of important pathogens, such as, *Staphylococcus aureus* (VIEIRA, 2009), group B *Streptococcus* (VIEIRA, DE MORAIS et al., 2010), *Lactobacillus salivarius* (VIEIRA, DE MORAIS et al., 2010) and the spirochetes *Treponema pallidum* (KERR, 1999), *T. denticola* (ATZINGEN et al., 2009) 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 (ATZINGEN et al., 2009).

The adhesion of virulent *L. interrogans* strain Fiocruz L1-130 to Fg has been shown to be induced by physiological osmolarity, but the effect of this binding on fibrin clot formation was not evaluated (PINNE, MATSUNAGA;HAAKE, 2012). The leptospiral proteins, LigA and LigB (CARVALHO et al., 2009) and OmpL37 have been described as Fg-binding proteins. In addition, the interaction of LigB with Fg was shown to inhibit thrombin-catalyzed fibrin formation.

In this work, we have assessed the interaction of three leptospiral strains cultivated under normal conditions with Fg. We have assayed 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 six leptospiral adhesin- and PLG-binding proteins, rLIC12238, Lsa33, Lsa30, OmpL1, rLIC11360 and rLIC11975 (Siqueira et al., manuscript submitted to publication) 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. Attachment of recombinant proteins to Fg was specific, dose-dependent and saturable with all proteins but Lsa30. The interaction of all six 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.

The inhibitory effect observed with leptospires and with the six recombinant proteins on fibrin formation was moderate, similar to the level observed with LigB, but contrasting with the total clotting impediment promoted by the Fg-binding proteins, SdrG of *S. epidermidis* and ClfA of *S. aureus*. 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.

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.

We have described the interaction of *Leptospira* with fibrinolytic system and shown that this association renders the bacteria with proteolytic activity capable of degrading ECM components. 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. Indeed, the increased plasma levels of Fg degradation products detected in leptospirosis has provided evidence for fibrinolysis activity. 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. The activation of coagulation cascade with increased levels of plasma Fg during leptospirosis has been detected. 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. 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, 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.

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Legend to Figures

Figure 1. Adhesion of leptospires to Fg. (A). The binding of soluble human Fg (10 µg/mL) to immobilized strains of leptospires (*L. interrogans* sorovar 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).

Two independent experiments were performed, and the data represent the mean ± the standard deviation from one representative experiment. For statistical analyses, the attachment of Fg to leptospires was compared to its binding to negative control BSA, by the two-tailed *t* test (***P* < 0.001). **(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 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} ± SD for each point. **(C)** Degradation of Fg by PLA-coated leptospires. *L. interrogans* serovar Kennewicki strain Pomona Fromm (LPF) (10⁸/sample) were treated with the addition of 10 µg PLG (3), 3U uPA (4) 10 µg PLG and 3U uPA (PLA) (5, 6) or no additions (untreated) (2). (1) Control lacking leptospires and (6) Addition of 1 µg aprotinin to the Fg-leptospires incubation. The cells were incubated with 15 mg/mL human purified Fg for 16 h at 37°C, and the supernatants were fractioned by SDS-PAGE and transferred to membranes. The Fg was detected by specific antibodies followed by ECL reagent development. 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).

Figure 2. Recombinant proteins binding to human Fg. Human Fg (10 µg/mL) was coated to ELISA plates and allowed to interact with the recombinant proteins (10 µg/mL). Gelatin was used as a negative control for nonspecific binding. The reaction was detected with polyclonal antibodies raised against each proteins (M&M) in (A), (C) and (E) while monoclonal anti-polyhistidine HRP antibodies was employed in (B) and (D). In **(C)** the percentage of binding for each protein with Fg was measured as a function of time up to 120 min. 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 are shown in **(D)** and **(E)**. 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 recombinant proteins to human Fg was compared to its binding to gelatin or untreated recombinant binding, in the case of **(D)** and **(E)** by two-tailed *t* test (**P* < 0.05).

Figure 3. Binding characterization of recombinant proteins to human Fg. (A) Ten mg/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. Data represent the mean absorbance values $\pm$ the standard deviation of three replicates for each experimental group. (B) The dissociation constant (K_D) was calculated based on ELISA data for the recombinant proteins that have reached the equilibrium concentration. (C) Immobilized Fg was treated with sodium metaperiodate (5 to 100 mM) for 15 min at 4°C in the dark, prior to the binding with recombinant proteins (as described in Fig. 2). Mean absorbance values at 492_{nm} ($\pm$ the standard deviations of three independent experiments) were compared to those obtained with untreated Fg (0 mM).

Figure 4. Inhibition of fibrin clot formation by recombinant proteins. ELISA plates were coated with 1 mg/mL of Fg plus recombinant proteins, pre-incubated for 2 h at 37°C, and 10U/mL of thrombin. (A) 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. In (B) the inhibition of fibrin clot by recombinant proteins is shown after 45 min. Bars represent the mean absorbance values $\pm$ standard deviation of three replicates for each condition and are representative of two independent experiments.

Figure 5. Inhibition of *L. interrogans* attachment to human Fg by recombinant proteins. Fg-coated microtiter wells were incubated with increasing concentration (0 – 4.5 μ M) of rLIC12238 (A), rLIC11975 (B), rLIC11360 (C), Lsa30 (D), Lsa33 (E) and OmpL1 (F) for 90 min prior to the addition of 4×10^7 leptospires. Wells were probed with anti-LipL32 serum (1:4,000). Data are expressed as OD492_{nm} $\pm$ 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).

Table 1. Gene locus, protein given name, NCBI 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%) LlicsVM (60%)	F: 5' <u>CTCGAGG</u> ATCTACAAGGTGGGGTTT TTAC3' (XhoI)	33.1 kDa
				LSS (34%) LBP (31%)	R: 5' <u>CCATGGT</u> TACTGAGGTTTACTTGG TCC3' (NcoI)	
LIC11975	Lsa36	YP_0019141	Outer membrane protein	Lai (99%) LlicsVM (54%) LBP (38%)	F: 5' <u>CTCGAGGA</u> ATGTAGCGGTGCGG3' (XhoI) R: 5' <u>AAGCTT</u> TTCATCCCCAAAAGATAA TTCA 3' (HindIII)	36.12 kDa
LIC10973	OmpL1 ^b	YP_000947.1	Outer membrane protein	Lai (94%) LBH (87%) LSS (87%) LlicsVM (51%) LBP (44%)	F: 5' <u>GGATCC</u> AAAAATATGCAATTGTAG G3' (BamHI) R: 5' <u>GGTACCT</u> TAGAGTTCGTGTTTA TAACC3' (KpnI)	32.4 kDa
LIC11360	Lsa23	YP_0013241	Putative lipoprotein	Lai (99%) LBH (69%) LSS (67%) LlicsVM (44%)	F: 5' <u>GGATCC</u> GAACTTCCTTACTTTTC CCCTAAC 3' (BamHI) R: 5' <u>AAGCTT</u> GAATGTTGACTAGAGG CATTACT 3' (HindIII)	22.53 kDa
LIC12238	rLIC12238 ^c	YP_002173	Hypothetical protein	Lai (99%) LBH (77%) LBP (39%) LlicsVM (50%)	F: 5' <u>CTCGAGT</u> GTTTTAAACCTACC GGAG 3' (Xho I) R: 5' <u>AAGCTT</u> CTACTTCATCGCTTTTTC TATATC 3' (Hind III)	17.635 kDa
LIC11087	Lsa30 ^d	YP_0010571	Hypothetical protein	Lai (99%) LSS (81%) LBH (75%) LBP (30%) LlicsVM (29%)	F: 5' <u>CTCGAGG</u> GAGATTCCAGAAAG AAAAC 3' (XhoI) R: 5' <u>AAGCTT</u> ACCTGTGTGATGCGC TTCT 3' (HindIII)	30 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 ; LBH: *L. borgpetersenii* serovar Hardjo-bovis ; LBP: *L. biflexa* serovar Patoc ; LlicsVM: *L. licerasiae* serovar Varillal ; LSS: *L. santarosai* serovar Shermani

^a Previously published by Domingos *et al.*, 2012

^b Previously published by Fernandes *et al.*, 2012

^c Previously published by Vieira *et al.*, 2010 and Oliveira *et al.*, 2011

^d Previously published by Souza *et al.*, 2012

Figure 01

Figure 02

Figure 03

Figure 04

Figure 05

MANUSCRITO SUBMETIDO - Leptospiral extracellular matrix adhesins as mediators of pathogen-host interactions

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Abstract

Leptospirosis is been considered an important re-emerging infectious disease that affects people and animal worldwide. The disease is an important zoonosis caused by pathogenic *Leptospira* spp. Several studies in the last ten years have presented new insights into *L. interrogans* potential virulence strategies. This review summarizes our current knowledge of bacterial attachment to extracellular matrix (ECM) components and discusses the possible role of these interactions in leptospiral pathogenesis. Leptospiral proteins show different binding specificity for extracellular matrix molecules: some are exclusive laminin-binding proteins (Lsa24/LfhA/LenA, Lsa27), while others have a broader spectrum binding profiles (LigB, Lsa21, LipL53). These proteins mediate the interaction of *Leptospira* with ECM and may play a primary role in the colonization of host-tissues. Moreover, there are multifunctional proteins that exhibit binding activities towards a number of target proteins including plasminogen-plasmin and regulators of the complement system, and as such, may also act in bacterial dissemination and immune evasion processes. Many ECM-interacting proteins are recognized by human leptospirosis serum samples, or detected in cellular infiltrates of liver or kidney of hamsters experimentally infected or identified in liver and kidney tissues of fatal human cases of leptospirosis, indicating their expression during infection. This compilation of data should enhance our understanding of the molecular mechanisms of leptospiral pathogenesis.

Introduction

Spirochetes are motile, helically shaped bacteria that include the genera *Leptospira*, *Leptonema*, *Borrelia* and *Treponema*. *Borrelia* and *Treponema* are the causative agents of

Lyme disease, relapsing fever and syphilis. The genus *Leptospira* encompasses both pathogenic and saprophytic species. Pathogenic *Leptospira* are the etiological agent of leptospirosis while saprophytic bacteria are free-living environmental organisms. *Leptospira* are classified according to serovar status, where 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. Some pathogenic species are transmitted through direct contact, whereas the majority is usually transmitted via contact with contaminated soil and water, such as *L. borgpetersenii* and *L. interrogans*, respectively. Leptospirosis is considered an important re-emerging infectious disease that affects populations worldwide. In tropical and subtropical regions, the disease is endemic, while in temperate climates, the disease is associated with occupational and recreational activities. According to available data, it is estimated that more than 500,000 cases of leptospirosis occur annually and some studies indicate that the disease can represent 20 – 40% of febrile illnesses of unknown cause. Leptospirosis also represents a major economic burden, as the disease promotes abortions, stillbirths, infertility, failure to thrive, reduced milk production and death in animals such as cows, pigs, sheep, goats, horses and dogs.

Leptospire enter the host mainly via intact sodden or damaged skin or mucosa. Infection produces a wide spectrum of clinical manifestations, varying from subclinical infection to severe manifestations. The initial phase exhibits wide-ranging and nonspecific symptoms such as fever, chills, headache and myalgia, common to other diseases such as influenza, dengue and some viral diseases.

Leptospirosis can progress to a severe condition known as Weil's syndrome, corresponding to 5 – 15% of the reported cases. This manifestation is generally characterized by hemorrhage, jaundice, kidney failure, meningitis and hypotension. In the last few years, another severe manifestation of leptospirosis, the leptospirosis pulmonary hemorrhage syndrome (LPHS), has been the focus of public health concern. It was first described in North Korea and China, and has been increasingly reported worldwide. LPHS is estimated to account for up to 70% mortality rates due to pulmonary hemorrhage.

Currently available veterinary and human 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. 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 research groups have tested the protective activity of several recombinant proteins as potential vaccines. Nevertheless, only a few proteins are able to confer partial protection against challenge with virulent leptospires and it is anticipated that a multicomponent acellular vaccine will be necessary for complete protection.

In the past ten 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 ECM molecules. This review aims to assemble the current knowledge of leptospiral adhesins focusing on host interactions for this pathogen and pointing out new trends for the characterization of these attachment proteins.

Genomics and proteomics

Currently, there are ten published leptospiral genome sequences: two strains of *L. interrogans* serovar Lai, 56601 and IPAV, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, two strains of *L. borgpetersenii* serovar Hardjo, L550 and JB197, *L. santarosai* serovar Shermani strain LT821, two strains of *L. licerasiae*, VAR010 and MMD0835 and two strains of the saprophyte *L. biflexa*, Paris and Ames. Two

chromosomes characterize the genomes of pathogenic strains, with a size ranging from 3.9 to 4.6 Mb. Comparative genomics between pathogenic and saprophyte strains has identified unique genes present in pathogenic species that could be important for virulence. Additionally, genome annotation has predicted several genes with a potential role in pathogenesis and virulence. Hypothetical and conserved hypothetical genes of unknown function are overrepresented in pathogenic species and their role remains to be determined.

The main mechanism in the pathogenesis of leptospirosis, as in other spirochetal diseases, is the ability of the pathogens to rapidly disseminate within the host during the early stage of infection. Putative outer membrane proteins would be available to the immune response during host infection and hence, constitute important targets for immune protection through mechanisms such as antibody-dependent phagocytosis and complement-mediated killing. In this regard, a major limitation in the field of leptospirosis has been the identification of membrane-associated proteins through conventional biochemical and molecular methods. Accordingly, whole-genome sequencing has made an impressive impact in the microbial field landscape and has helped to overcome these limitations. The available sequences combined with bioinformatics tools and DNA recombinant techniques have allowed the prediction of all proteins *in silico* and their production in laboratory, independently of their abundance and without the need to grow the microorganism *in vitro*. Among these coding sequences are the hypothetical ones, predicted to be located at the surface of *Leptospira*. Our group has been particularly interested in this class of proteins, and thus far we have characterized several of them as possible leptospiral adhesins that we believe may be involved in host-interactions.

Genomic tools are important for revealing general aspects of the organism and were the first strategy for high-throughput identification of proteins. However, it does not indicate which proteins are in fact being expressed under a certain condition. For instance, it has been shown that some leptospiral virulence factors are downregulated after several passages in culture medium (Lig and Lsa21). Additionally, several studies have reported that environmental cues affect leptospiral protein expression, at both, the transcriptional and proteomic level. Yet, most of proteomics data have revealed basically the same proteins, i.e., proteins that are abundantly expressed. One

of the most important contributions to the leptospiral field came from the work of Malmstrom and colleagues . These authors, by using the strategy of combining three mass spectrometry based proteomic methods, provided information on the number of copies of expressed proteins per cell under a particular condition. Using *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, they validated the expression of a significant number of hypothetical proteins and provided a complete profile of leptospiral proteins.

Extracellular matrix

The extracellular matrix (ECM) is an intricate network of macromolecules, whose primary role includes supporting, and connecting cells and tissues. Nonetheless, the role of the ECM surpasses a structural function in relation to tissue components, since its composition and organization influences various physiological processes required for tissue morphogenesis, differentiation and homeostasis, such as cell adhesion, migration, proliferation and cell differentiation . Two main classes of macromolecules constitute the ECM: (i) polysaccharide chains of glycosaminoglycans (GAGs) and (ii) fibrous proteins, such as collagen, elastin, fibronectin, and laminin .

The GAGs are long unbranched polysaccharides constructed of repeating disaccharide units. The disaccharide units contain an amino sugar (N-acetylgalactosamine, GalNAc or N-acetylglucosamine, GlcNAc) and a uronic acid (glucuronate or iduronate) or glucose. GAGs are highly negatively charged molecules that permit high hydration, viscosity and interaction with other molecules . The most important GAGs for the ECM composition are hyaluronic acid, chondroitin sulfate, dermatan sulfate, keratin 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 the scaffold for the attachment of other ECM components, providing tensile strength, regulating cell adhesion and supporting chemotaxis and migration . In addition to the collagens and GAGs, multi adhesive matrix proteins that interact with a variety of collagen types, other matrix proteins, polysaccharides, cell-surface receptors and signaling molecules, compose ECM. Their major function is to attach cells to the ECM . Laminin is a multi-adhesive protein that binds to numerous proteoglycans, type IV collagen, and certain receptors on the cell 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 provides a molecular bridge between the collagen scaffold and the other ECM components, as it contains different domains of high-affinity binding sites for collagen, heparin, gelatin and integrins (via an RGD motif), thereby attaching cells to the ECM . Plasma fibronectin circulating in the blood can bind to fibrin, activate platelets and enhance blood clotting . The elastin results in the assembly of the monomeric precursor tropoelastin . Elastin proportionally provides a degree of elasticity to the ECM, and further permits molecular interaction with ECM components such as proteoglycans, fibulin and fibrillin .

ECM and pathogens

From the pathogen point of view, the ECM represents a physical barrier that must be overcome to establish local infection or reach the circulation and preferred sites of infection. However, as an abundant constituent with multiple adhesive properties, instead of blocking the dissemination of the microorganisms, these barriers can be on the other hand exploited in a way to facilitate the interaction with the host. Bacterial adhesion to the host cells and tissues is the initial and crucial step for colonization and infection, and it also helps the long-term persistence of the pathogen within the host. Pathogens may achieve access to the ECM components after a tissue injury since in the normal state ECM is not exposed to bacteria .

Based on these assertions, it is essential to understand the mechanisms of bacterial adhesion for the purpose of preventing pathogens from initiating first binding and colonization to help fight infections. Bacterial adhesion to host ECM is initially mediated by loose associations, followed by stronger and more intimate interactions through bacterial components. Due to their privileged exposed localization, allowing environmental sensing and interactions, the adherence to ECM molecules is frequently mediated by OMPs .

It is well documented the ECM and cell-binding capacities of several pathogens, including *Pneumococcus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus*, among others . The ability of spirochetes to adhere to eucaryotic cells and ECM molecules has also been reported ; . Several studies have demonstrated that the Lyme disease spirochete *B. burgdorferi* binds to a variety of mammalian cell types *in vitro*

and to ECM components . For example, *B. burgdorferi* has been shown to express a 47-kDa OMP called BBK32 and protein RevA which confer attachment to fibronectin , ErpX which interacts with host laminin and a GAG-binding adhesin termed Bgp . *T. pallidum* also interacts with ECM through the OMPs: Tp0155 and Tp0483 show specific binding to fibronectin and Tp0751 binds to a variety of laminin isoforms .

Leptospiral adhesion to ECM

Miller and colleagues (1966) reported for the first time the adhesion of *Leptospira* to cultured mammalian cells . Later, studies correlated the efficiency of leptospiral attachment to various cell types with bacterial virulence, since virulent organisms were more capable of attachment . Ito and Yanagawa (1987) were the first to describe the binding of *L. interrogans* to mouse fibroblast ECM, further correlating this ability to virulence. Their work corroborates previous findings that highly virulent leptospires attached more efficiently than non-virulent bacteria of the same serovar . These findings suggested that the binding capacity could be decreased or lost during virulence attenuation, and thus, could occur through differentially expressed protein constituents. Additionally, the leptospiral attachment was found to be heat inactivated, indicating the participation of protein-containing moieties in the interaction .

The interaction of *Leptospira* with Vero cells was shown to be inhibited by fibronectin competition, suggesting the leptospiral interaction with this ECM molecule . The direct attachment of *L. interrogans* to the ECM proteins laminin, collagen type I, collagen type IV, cellular and plasma fibronectin were first demonstrated by Barbosa and colleagues (2006), indicating the existence of multiple adhesion molecules . Leptospiral binding to elastin and its precursor, tropoelastin, was reported by Lin and colleagues (2009), further stressing the presence of a broad spectrum binding proteins . Moreover, the interaction of *L. interrogans* to the GAGs chondroitin sulfate and heparan sulfate was demonstrated by Breiner and colleagues .

First 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 . This adhesin was described to be expressed in virulent *L. interrogans*, totally lost during virulence attenuation and absent in the

saprophytic strain *L. biflexa* serovar Patoc I. Evidence of surface-exposed localization of this 36-kDa protein was provided by proteinase K treatment, which abolished its fibronectin-binding activity, as assessed by Western blotting. Moreover, these studies revealed that the gelatin-binding domain of fibronectin was involved in the interaction. Unfortunately, the work did not identify the 36-kDa protein with adhesive properties, and to date, it still remains unknown. Tables 1 and 2 depict features of the published ECM-binding proteins, and Figure 1 illustrates a model of leptospiral 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.

Lsa24/LfhA and related Len proteins

The studies performed by Barbosa and colleagues (2006) reported the first leptospiral protein exhibiting attachment to purified ECM molecules . 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. Laminin sugar moieties appear to be involved in the interaction, since laminin oxidation by sodium metaperiodate reduces the Lsa24 adhesion. 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. The authors suggested that Lsa24 could mediate the adhesion of *Leptospira* to the host via laminin. In the same year, Verma and colleagues (2006) described this protein as a novel binding protein of both factor H- and factor H-related protein 1 of *L. interrogans*, named LfhA (leptospiral factor H-binding protein A) . Thus, it seems that Lsa24/LfhA/LenA is a multifunctional binding protein of *Leptospira*. Subsequently, Stevenson and colleagues (2007) reported that LIC12906 of *L. interrogans* possesses five paralogs, distributed among virulent leptospires and absent in non-pathogenic species . Because of their similarities to mammalian endostatins, these proteins called Len proteins. LenA protein (formerly called LfhA and Lsa24) and the additional homologs were named, LenB, LenC, LenD, LenE and LenF. LenB was found to bind human factor H, and all Len proteins showed laminin-binding capacity. In addition, LenB, LenC, LenD, LenE and LenF exhibited

affinity for fibronectin, as assessed by ligand-blotting experiments.

LipL32

LipL32 is a subsurface lipoprotein found in pathogenic leptospires, expressed during mammalian infection and recognized by leptospirosis serum samples. It is the most abundant protein expressed by *Leptospira*, estimated at 38,000 copies per cell. Furthermore, LipL32 is highly conserved and restricted to pathogenic serovars, thus being absent in saprophytic ones and suggesting its role in pathogenesis. The ECM-binding capacity of LipL32 has been shown by independent studies. Hoke and colleagues (2008) showed that recombinant LipL32 selectively binds to the individual components laminin and collagens I and V, where the carboxy-terminal is designated as the region binding. Hauk and colleagues (2008) observed that LipL32 interacts with collagen IV and plasma fibronectin, and confirmed that the carboxy-terminal portion was responsible for binding.

In addition to attachment to ECM molecules, LipL32 was shown to bind plasminogen, the precursor of the broad-spectrum serine protease plasmin. These studies indicated that LipL32 could interact simultaneously with plasminogen and plasma fibronectin, suggesting a dual binding activity. It has been speculated that one strategy for bacterial pathogenesis may be concomitant plasminogen/plasmin and ECM binding. Plasmin-associated bacteria could promote ECM degradation and consequently facilitate dissemination.

However, despite LipL32 abundance in bacterial cell 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*. Thus, although LipL32 is one of the most studied proteins, there is still controversy over its function in *Leptospira*.

Leptospiral immunoglobulin-like (Lig) proteins

Three proteins containing tandem repeats of the bacterial immunoglobulin-like (Big) domain, named LigA, LigB and LigC (for leptospiral immunoglobulin-like proteins), have been identified. The Big superfamily includes well-characterized virulence determinants that mediate pathogen attachment to host cells and tissues, such as intimin of enteropathogenic *E.*

coli and invasins of *Yersinia pseudotuberculosis*. The *L. interrogans* Lig-proteins are present only in pathogenic species, expressed during infection, absent after attenuation with culture passage, and upregulated under physiologic osmolarity stimuli. 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. Choy and colleagues (2007) firstly described LigA and LigB as leptospiral ECM-binding proteins capable of interacting with fibronectin, laminin and collagen I and IV. The adhesive regions were attributed to the unique regions of each protein, instead of the shared Big repeats. Further studies by Choy and colleagues (2011) have also shown LigB to interact with collagen type III. 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 non-repeated region in the variable C-terminal portion, which also binds laminin, but not collagen. Lin and colleagues, (2009) showed that Lig proteins also interact with elastin and its soluble precursor, tropoelastin. The binding sites are common to both molecules, and were identified as the 4th Big repeat in the conserved domain between LigA and LigB, and to the regions comprising the 7th-8th, 9th and 12th Big repeats in the variable LigB portion. Therefore, the regions responsible for fibronectin and laminin binding seem to differ from that for elastin and tropoelastin, thus demonstrating the specificity and role of Big domains in ECM interactions. It is possible that the leptospiral interaction with elastin and its precursor involves other adhesins, still to be identified, since the LigA and LigB binding regions only partially inhibited *L. interrogans* attachment to elastin. Using the shotgun phage display technique, LigB was shown to further exhibit specific binding to heparin. The LigB portion that participates in the interaction includes the 12th Big domain and the first 112 residues from the non-repetitive carboxy-terminal, the same regions that interact with other ECM molecules already discussed.

Outer membrane protein A (OmpA)-like proteins

Outer membrane protein A (OmpA) is a major heat-modifiable surface-exposed protein in *E. coli* and is one of the best-characterized OMPs. The structure of OmpA and OmpA-like proteins is characterized by an amino-terminal

domain that forms an eight-stranded, anti-parallel β barrel, embedded in the OM, constituting a structural porin function. OmpA protein of *E. coli* and other enterobacteria have been reported to act as adhesin/invasin. OmpA-like proteins have also been described in other pathogens, including the *Neisseria meningitidis* RmpM, the flagellar motor proteins PomB and MotY of *Vibrio alginolyticus* and the peptidoglycan-associated lipoprotein (Pal) of *Haemophilus influenza*. Furthermore, it has been reported that OmpA-like proteins actively participate in pathogenesis process, such as adhesion, serum resistance, invasion and host cell activation.

The first protein identified in pathogenic *Leptospira* was a lipoprotein of 22 kDa with a carboxy-terminal OmpA domain, named Loa22, 22-kDa *Leptospira* lipoprotein with OmpA domain. Subsequently, the proteins Omp52 and OmpA70 were identified in *L. santarosai* serovar Shermani and in *L. interrogans* serovar Copenhageni, respectively. The OmpA-like protein Loa22 was reported to be essential for the leptospiral virulence, since mutants lacking Loa22 expression resulted in attenuated virulence in an animal model of acute infection. Thus, it is possible that OmpA-like proteins have a function in leptospiral pathogenesis. However, work performed in our laboratory showed that Loa22 does not interact with all ECM molecules tested: laminin, collagen I, collagen IV, cellular and plasma fibronectin. Hence, despite being 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 to 583 amino acid residues in the carboxy-terminal region. The recombinant protein, named Lsa66, showed laminin and plasma fibronectin binding properties. The interaction of Lsa66 to ECM components was determined to be specific, dose-dependent and saturable, with a K_D of 55.4 ± 15.9 nM and of 290.8 ± 11.8 nM for laminin and plasma fibronectin, respectively. Moreover, antibodies in serum samples of confirmed leptospirosis cases recognized Lsa66, suggesting its expression during infection. As expected, Lsa66 only partially inhibited adhesion of live *L. interrogans* to immobilized laminin and plasma fibronectin, taking into account the low copy number of this protein in leptospires (75 copies) and the existence of other adhesins. The presence of this protein in *Leptospira*, as a fibronectin-ligand binding, was further corroborated by the work of Pinne and colleagues (2012) using

high-throughput microarray strategies. Lsa66 is also a plasminogen-plasmin-interacting protein. Collectively, these data suggest the involvement of Lsa66 in leptospiral pathogenesis.

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. The majority of these proteins are annotated as hypothetical with no assigned function. By data mining this pool of sequences, our group has identified several novel leptospiral adhesins. Some of these proteins exhibited exclusive laminin-binding property when screened against several individual ECM components. These are: Lsa27, Lsa20, and Lsa25 and Lsa33. All these proteins are most probably surface-exposed, as assessed by indirect liquid phase immunofluorescence or proteinase K accessibility. The participation of the laminin sugar moieties in the interaction with the leptospiral proteins was tested for Lsa25 and Lsa33, and shown to be significant only for Lsa25. In addition to mediating ECM interactions through laminin, Lsa33 and Lsa20 are also plasminogen-interacting proteins and, hence, may be possibly involved in leptospiral invasiveness and dissemination. Moreover, Lsa25 and Lsa33 are able to capture C4bp, a regulator of the classical pathway complement system, and may have a role in leptospiral immune evasion. The putative lipoprotein encoded by LIC12976, identified as an adhesin by shotgun phage-display approach, was also shown to have exclusive laminin-binding activity.

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. 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-passaged strains. It is well known that prolonged culture promotes attenuation of leptospiral virulence, and it has been suggested that expression of virulence factors may be downregulated upon sequential *in vitro* culture passages. Moreover, environmental factors such as osmolarity and temperature

promote upregulation of Lsa21 expression at the transcriptional level, another characteristic this protein shares with Lig proteins. Osmotic control of gene expression and transition of temperatures, from ambient to mammalian body or higher during the febrile stage, have been correlated with changes in the expression of virulence determinants in many pathogens . Moreover, it has been demonstrated that *L. interrogans* also regulates protein synthesis in response to *in vitro* temperature and osmolarity changes . The gene encoding Lsa21 is present in pathogenic strains of *L. interrogans* and absent in the saprophytic *L. biflexa* . Although the gene for LipL53 is present in all pathogenic strains, its transcript was only detected in serovar Icterohaemorrhagiae under regular culture conditions . Furthermore, the adhesin Lsa21 has been shown to be present in liver and kidney tissues of human fatal cases of leptospirosis, and LipL53 is recognized by leptospirosis human serum samples, additionally suggesting the role of these adhesins in leptospirosis pathogenesis . Proteomics studies of *L. interrogans* have identified a protein encoded by the gene LIC10314 that is differentially expressed under virulence conditions . The recombinant protein, named Lsa63, was characterized as an adhesin that binds strongly to laminin and collagen type IV . The protein Lsa63 is highly conserved among pathogenic strains and absent in *L. biflexa*. The protein sequence shows a p83/p100 conserved domain that seems to be largely distributed in spirochetes . Although the function of this domain is uncertain, p83/p100 containing proteins have been shown to elicit an immune response in hosts and are candidates for Lyme borreliosis serodiagnosis . 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. Lsa63 was found to partially inhibit the binding of intact *L. interrogans* to immobilized laminin and collagen IV. The inhibitory effect promoted by the recombinant protein was modest, and is it expected due to the presence of several *L. interrogans* ECM-binding proteins that would contribute to the leptospiral adherence to laminin and collagen IV .

The protein encoded by the gene LIC11087 was genome annotated as hypothetical and predicted to be surface-exposed. We have evaluated the recombinant protein, named Lsa30, and shown that it is also an ECM-interacting protein with the ability to mediate attachment to laminin and plasma fibronectin, . This protein binds to laminin and plasma

fibronectin in a dose-dependent and saturable manner, with dissociation equilibrium constants (K_D) of 292 ± 24 nM and 157 ± 35 nM, respectively. Moreover, Lsa30 is a plasminogen receptor, capable of generating plasmin in the presence of activator. This protein may interfere with the complement cascade by interacting with C4bp regulator. The Lsa30 is probably expressed during infection because antibodies present in serum samples of hamsters experimentally infected can recognize this protein. Thus, Lsa30 is another versatile leptospiral adhesin that may play a role in mediating adhesion to hosts.

OmpL1 is a 31 kDa leptospiral transmembrane OMP, containing 320 amino acid residues, described by Haake and colleagues . Several studies have pointed out the importance of OmpL1 as a serological marker for the diagnosis of human leptospirosis and as a vaccine against the disease . Although the gene is restricted to pathogenic serovars of *Leptospira*, its function is uncertain. Analysis of recombinant OmpL1 immunogenicity in inoculated BALB/c mice showed that the protein has the capacity to promote humoral and cellular immune responses, as indicated by high antibody titers and proliferation of lymphocytes, strengthening its potential as a subunit vaccine. Fernandes and colleagues (2012) showed that OmpL1 has the ability to mediate attachment to laminin and plasma fibronectin, with a K_D value of 2099.93 ± 871.03 nM and 1239.23 ± 506.85 nM, respectively. Like previously described adhesins, OmpL1 inhibited the binding of live *Leptospira* to the same ECM components. In addition, OmpL1 mediates leptospiral binding to plasminogen/plasmin generation, characterizing this protein as multifunctional adhesin that possibly contributes to leptospiral infection and dissemination . Pinne et al. (2010) described the interaction of the protein OmpL37 (LIC12263) with the ECM components laminin, plasma fibronectin and elastin from aorta and human skin. However, intriguingly, recombinant OmpL37 did not impair leptospiral adhesion to elastin, and the presence of rabbit antiserum for OmpL37 acted as an enhancer in this interaction. The authors discuss that this effect may be due to a conformational alteration imposed by antibody binding or a stabilization of the ligand-adhesin complex by the antibodies . OmpL37, a protein present only in pathogenic serovars, could be involved in pathogenesis, facilitating the primary attachment of leptospires to human skin elastin and later promoting leptospiral binding to elastin-rich vascular structures . In the same study, OmpL47 (LIC13050) was

described as binding weakly to laminin, collagen type III, fibronectin and elastin, but these interactions were not further characterized.

Lp95, an adhesin that activates cellular adhesion molecules (CAMs)

CAMs are surface receptors present in eukaryotic cells that mediate cell-cell or cell-ECM interactions. Several cells and extracellular molecules have been reported to use CAMs in host defense against infection. However, several microorganisms, including protozoa, fungi, bacteria and viruses are reported to employ CAMs during their pathogenesis. *Mycobacterium tuberculosis*, *Yersinia* spp., *Shigella* spp., and *Neisseria* spp., among others, are examples of bacteria that employ. Selectins, integrins, ICAM-1 (intercellular adhesion molecule 1) and VCAM (vascular cell adhesion molecule) are stimulated by *S. aureus* and their role suggested to be mitogenic with massive cytokine release. Protein activation of endothelial CAMs has been described for the pathogenic spirochetes *L. interrogans*, *Borrelia* spp. and *Treponema* spp. The leptospiral proteins encoded by the genes LIC10365, LIC10507, LIC10508, LIC10509 have been shown to be present in cellular infiltrate of liver and kidney of hamsters experimentally infected with virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, 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). 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. Furthermore, the same work showed a novel leptospiral protein encoded by LIC12690, named Lp95 (leptospiral protein of 95 kDa) that is capable of specifically activate E-selectin in HUVECs. In addition to CAM induction, Lp95 binds to laminin and fibronectin, where the carboxy-terminal portion is 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.

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. 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: LIC13135, LIC11755, LIC11051, LIC11612, LIC13066, LIC10258, LIC11028, LIC13198, LIC12631, LIC11436, LIC12952, LIC10714, LIC10713, LIC10537 and LIC11893. Among them, only LIC10258, named Lsa66, had been previously characterized as a fibronectin adhesin. Western blotting only confirmed six proteins with fibronectin-binding capacities: LIC11612 (MFn1), LIC10714 (MFn2), LIC11051 (MFn6), LIC11436 (MFn7), LIC10258 (MFn8, Lsa66), and LIC10537 (MFn9). Some proteins previously characterized as fibronectin adhesins were not included in the assay: LIC12236, LIC13143 (TlyC) and LIC10977 (LenB). Previously assayed proteins that did not exhibit binding to plasma fibronectin were further confirmed as non-ligands; these are: LIC11834 (Lsa33), LIC12253 (Lsa25), LIC11469 (Lsa20), LIC12895 (Lsa27) and LIC12906 (Lsa24/LenA).

Conclusions

In recent years, major advances were made in the leptospiral field. Studies aimed at elucidating the pathogenesis of *Leptospira* on the basis of available genome sequences, proteomics and microarrays have been published and, the remarkable number of novel leptospiral ECM-interacting proteins (30) 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 for ECM were described. In addition, there are multifunctional proteins that exhibit binding activities towards a number of target proteins, such as plasminogen-plasmin and regulators of the complement system, and may have several tasks in leptospiral pathogenesis.

However, there is a lack of understanding of the exact function of these ECM-interacting proteins, as most of the studies have been performed *in vitro*. These findings will have to be extended to suitable *in vivo* models to gain information on the precise mechanism of these ECM-binding proteins in leptospiral pathogenesis. The information on the molecular structure of these proteins should contribute to identifying a multicomponent acellular vaccine in addition to potential targets for antimicrobial therapy that may help fight leptospirosis.

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Legend to figure 1

Figure 1. Schematic representation of the *Leptospira* cell membrane. The leptospiral membrane shares characteristics of both gram-negative and gram-positive bacteria. Depicted are outer membrane (OM), inner membrane (IM) and lipopolysaccharide (LPS). In the periplasmic space (PS), the peptidoglycan (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 the published adhesins, identified and characterized by our research group.

Figure 01

ANEXO A – Cromatogramas dos sequenciamentos das construções no vetor pAE contendo os insertos de DNA dos genes selecionados

LIC10258

LIC12880

LIC12238

ANEXO B – Soro de pacientes diagnosticados com leptospirose empregados nos imunoensaios com as proteínas recombinantes rLIC10258, rLIC12880 e rLIC12238

Amostra	Paciente	MAT	Sorovar (Título)
1	1	negativo	-----
2	1	positivo	Copenhageni (1/800)
3	2	negativo	-----
4	2	positivo	Icterohaemorrhagiae (1/3200)
5	3	negativo	-----
6	3	positivo	Autumnalis (1/1600)
7	4	Negativo	-----
8	4	positivo	Icterohaemorrhagiae (1/1600)
9	5	Negativo	-----
10	5	Positivo	Icterohaemorrhagiae (1/800)
11	6	Negativo	-----
12	6	Positivo	Copenhageni (1/3200)
13	7	Negativo	-----
14	7	Positivo	Icterohaemorrhagiae (1/6400)
15	8	Negativo	-----
16	8	Positivo	Copenhageni (1/1600)
17	9	Negativo	-----
18	9	Positivo	Cynopteri (1/400)
19	10	Negativo	-----
20	10	Positivo	Icterohaemorrhagiae (1/6400)

ANEXO C – Cromatogramas dos sequenciamentos das construções no vetor pAE contendo os insertos de DNA dos fragmentos do gene LIC10258

N-terminal

Intermediário

C-terminal

**ANEXO D - Soro de pacientes diagnosticados com leptospirose empregados
nos imunoensaios com os fragmentos recombinantes do gene LIC10258**

Amostra	Paciente	MAT (Sorovar/Título)	Amostra	Paciente	MAT (Sorovar/Título)
1	1	negativo	19	10	negativo
		Positivo			Positivo
2	1	Icterohaemorrhagiae (1/1600)	20	10	Icterohaemorrhagiae (1/1600)
3	2	negativo	21	11	negativo
		Positivo			Positivo
4	2	Icterohaemorrhagiae (1/400)	22	11	Icterohaemorrhagiae (1/25600)
5	3	negativo	23	12	negativo
		Positivo			Positivo
6	3	Icterohaemorrhagiae (1/6400)	24	12	Icterohaemorrhagiae (1/51200)
7	4	negativo	25	13	negativo
		Positivo			Positivo
8	4	Hebdomadis (1/1600)	26	13	Bataviae(1/400)
9	5	negativo	27	14	negativo
		Positivo			Positivo
10	5	Canicola (1/6400)	28	14	Copenhageni(1/6400)
11	6	negativo	29	15	negativo
		Positivo			Positivo
12	6	Australis (1/3200)	30	15	Copenhageni (1/800)
13	7	negativo	31	16	negativo
		Positivo			Positivo
14	7	Icterohaemorrhagiae (1/1600)	32	16	Copenhageni(1/6400)
15	8	negativo	33	17	negativo
		Positivo			
16	8	Icterohaemorrhagiae (1/12800)	34	17	Positivo
					Copenhageni (1/800)
17	9	negativo	35	18	negativo
		Positivo			Positivo
18	9	Icterohaemorrhagiae (1/12800)	36	18	Icterohaemorrhagiae (1/3200)