FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISA GONÇALO MONIZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

PATOGÊNESE DA LEPTOSPIROSE: ESTUDO SOBRE OS FATORES ENVOLVIDOS NA VIRULÊNCIA E DISSEMINAÇÃO DO AGENTE DURANTE A INFECÇÃO NO MODELO ANIMAL DE HAMSTER

ELSIO AUGUSTO WUNDER JÚNIOR

Salvador - Bahia
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“Trate as pessoas como são e elas permanecerão assim. Trate-as como se já fossem o que podem vir a ser e isto as ajudará a se tornarem no que são capazes.”

GOETHE

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À minha família, que mesmo distante sempre me apoiou, me deu forças e me mostrou que independente do caminho que escolho, eles estarão lá, ao meu lado.

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RESUMO

A leptospirose é uma zoonose de importância global e um importante problema de saúde pública principalmente em países em desenvolvimento. É causada por bactérias do gênero Leptospira, uma espiroqueta móvel e de alta morbidade capaz de se disseminar nos tecidos e causar doença crônica em animais hospedeiros. Uma barreira importante para o controle e prevenção da doença tem sido o pouco conhecimento da patogênese do agente, em parte pela falta de ferramentas disponíveis e eficazes de manipulação genética. Um dos objetivos desse estudo foi caracterizar duas cepas mutantes de *Leptospira interrogans*. A interrupção do gene *lipl32*, que codifica para a proteína LipL32, a mais abundante proteína no gênero *Leptospira* e expressa somente na superfície das leptospiras patogênicas, foi realizada através da inserção do transposon Himar1 no sorovar Manilae. A cepa mutante não apresentou nenhuma diferença de crescimento ou de aderência em componentes da matriz celular, comparada com a cepa parental. O mutante foi capaz de produzir doença aguda no modelo animal de hamster e causar colonização crônica no modelo animal de rato, mostrando que LipL32 não possui um papel nestes modelos de infecção. A interrupção do gene *ligB* foi realizada com a utilização, pela primeira vez em leptospiras patogênicas, da técnica de recombinação homóloga por mutagênese dirigida, onde o gene que codifica para a proteína LigB teve uma parte substituída por um cassete de resistência de espectinomicina (Spcr). Essa proteína, identificada como um possível fator de virulência, reconhecida pelo soro de pacientes infectados e importante para a aderência em componentes da matriz celular, mostrou não ser importante para a infecção aguda ou crônica, quando testada frente aos modelos animais, além de não ser necessária para a aderência em cultura de células. Outro objetivo desse trabalho foi estudar a cinética de disseminação da *Leptospira interrogans* no modelo animal de hamster, utilizando uma dose alta (10^8 leptospiras) e baixa (250 leptospiras) de inóculo, além de diferentes rotas de infecção. Nossos resultados demonstraram que leptospiras se disseminam rapidamente em todos os tecidos 01 hora após a infecção com uma alta dose de inóculo e que possivelmente a carga do agente nos tecidos é mais importante para a patogênese do que a sua habilidade para a disseminação. Também demonstramos que a motilidade não é essencial para disseminação, mas pode ser essencial para a carga nos tecidos e letalidade.

Palavras-chave: Leptospira, leptospirose, mutagênese por transposição, LipL32, recombinação homóloga, LigB, Real Time, disseminação.

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ABSTRACT

Leptospirosis is a worldwide zoonosis and a major public health problem especially important in developing countries. Caused by bacteria of *Leptospira* genus, a motile life-threatening spirochete which is able to disseminate to tissues and causes chronic carriage in animal hosts. A significant barrier to the control and prevention of leptospirosis has been the limited understanding of its pathogenesis, due in part to the lack of tools available for the genetic manipulation of this pathogen. One of the purposes of this study was to characterize two mutant strains of *Leptospira interrogans*. Interruption of *lipL32* gene, encoding LipL32, the most abundant protein of pathogenic leptospires and its major outermembrane lipoprotein, was achieved in serovar Manilae using transposon mutagenesis with Himar1. The mutant had normal morphology and growth rate compared to the wild type and was equally adherent to extracellular matrix. The mutant was able to cause acute severe disease manifestations in the hamster model and chronic colonization in the rat model, showing that LipL32 doesn't play a role in neither of those models of infection. Interruption of *ligB* gene was achieved using the homologous recombination by target mutagenesis for the first time in pathogenic leptospires and a spectinomycin resistance (Spcr) gene replaced a portion of the *ligB* coding sequence. This Lig protein, previously identified as a putative virulence factor, recognized by the sera of infected patients and important for the adherence in extracellular matrix components, showed not to be important for acute or chronic infection when tested with animal models and it's not required to mediate bacterial adherence to cultured cells. Another purpose of this study was to determine and analyze the kinetics of dissemination of *Leptospira interrogans* in the hamster model, using a high (10^8 leptospires) and low (250 leptospires) dose of inoculum and different routes of infection. Our results demonstrated that leptospires can rapidly disseminate through all tissues after 1 hour post-challenge with a high inoculum dose and that perhaps the load of the agent in target tissues is more important for pathogenesis than its ability for dissemination. We also demonstrate that motility is not essential for dissemination but can be essential for tissue load and lethality.

Key-words: Leptospira, leptospirosis, transposon mutagenesis, LipL32, homologous recombination, LigB, Real Time, dissemination.

LISTA DE ABREVIATURAS

BSA	Albumina bovina sérica
ELISA	Ensaio imunoenzimático
EMJH	Meio de Ellinghausen e McCullough modificado por Johnson e Harris
FRET	Transferência ressonante de energia de fluorescência
LCR	Líquido céfalorraquidiano
Hap1	Proteína 1 associada à hemólise
Lens	Proteínas de leptospiras do tipo endostatina
Ligs	Proteínas do tipo imunoglobulinas
LipL32	Lipoproteína L32
LPS	Lipopolissacarídeo
MAT	Teste de microaglutinação sorológica
Omp	Proteína de membrana externa
PCR	Reação da polimerase em cadeia
SPHS	Síndrome de hemorragia pulmonar grave
Sph	Esfingomielinase
TLR	Receptor do tipo Toll (2 ou 4)
TNF-α	Fator alfa de necrose tumoral

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1. INTRODUÇÃO

1.1 Aspectos gerais

A leptospirose é considerada a zoonose de maior distribuição mundial (WHO, 1999; LEVETT, 2001). Ela é causada por espiroquetas do gênero *Leptospira*, que inclui 08 espécies patogênicas com mais de 200 sorovares identificados, as quais possuem a habilidade de induzir colonização crônica nos túbulos renais de um grande número de animais selvagens e domésticos (BARTHI, 2003; KO et al., 1999; FAINE, 1999). Em humanos, a doença é adquirida através do contato com reservatórios animais ou com ambiente contaminado pela urina destes e se caracteriza por um amplo espectro de manifestações clínicas: febre, dor de cabeça e mialgia, nos casos leves, e insuficiência renal, icterícia e hemorragia pulmonar nos casos graves (KO, 1999; LEVETT, 2001). A leptospirose é um sério problema também na área de saúde veterinária, principalmente em bovinos, suínos e caninos, e é responsável por importante perda econômica na pecuária (FAINE, 1999; LEVETT, 2001). Embora a leptospirose seja amplamente distribuída e ocorra de forma endêmica em várias partes do globo, como na América do Sul e no Sudeste Asiático (KO et al., 1999, McBRIDE et al., 2005), os conhecimentos acerca da biologia e patologia das leptospiras ainda são limitados (KO et al., 2009).

1.2 Histórico

Em 1886, o médico alemão Adolf Weil descreveu pela primeira vez uma doença infecciosa que causava esplenomegalia, nefrite e icterícia, razão pela qual a forma grave da leptospirose em humanos é até hoje denominada como doença de Weil (FAINE et al., 2000). Em 1907, Stimson observou pela primeira vez o agente em cortes histológicos de rim, corados pela prata, de um paciente com diagnóstico de febre amarela. As bactérias se encontravam em agregados e apresentavam uma forma semelhante ao ponto de interrogação, sendo assim denominadas como *Spirochaeta interrogans* (FAINE et al., 2000). O primeiro isolamento de leptospiras patogênicas foi realizado por Inada e colaboradores, em 1916, no Japão. Os autores mostraram ainda a distribuição da bactéria nos tecidos, as características morfológicas do agente, a proteção passiva em cobaios com o uso de bacterina, bem como o papel do rato como reservatório (INADA et al., 1917).

1.3 Agente

As leptospiras pertencem à ordem *Spirochaetales*, a qual faz parte de um filo bacteriano único, *Spirochaetes*, junto com o *Treponema pallidum* e a *Borrelia burgdorferi*, agentes da sífilis e da doença de Lyme, respectivamente, entre outros. A família *Leptospiraceae* compreende o gênero *Leptospira*, o qual é composto por bactérias helicoidais de 6 a 20µm de comprimento e 0,1µm de diâmetro, aeróbias e móveis, com gancho característico terminal em uma ou ambas as extremidades. Sob condições adversas podem ser alongadas; em alta concentração de sal, em tecidos ou culturas recentes podem ter uma forma cocóide. Sua divisão é por fissão binária. Podem ser encontradas numa grande variedade de espécies animais (cepas patogênicas) e na água (cepas saprófitas) (ELLIS, 1994; FAINE et al., 2000).

As leptospiras possuem uma arquitetura distinta de dupla-membrana, tendo similaridades com bactérias Gram-negativas e Gram-positivas. Assim como em bactérias Gram-positivas, a membrana interna (citoplasmática) das espiroquetas é associada com a parede celular de peptideoglicanos. E como as bactérias Gram-negativas, possuem uma membrana externa que serve como proteção para antígenos (HAAKE, 2000a). As leptospiras são visualizadas apenas através da microscopia de campo escuro, contraste de fase ou em preparações impregnadas pela prata e são extremamente móveis, possuindo dois endoflagelos polares, um em cada extremidade, localizados no espaço periplasmático. Realizam movimentos de rotação ao longo do seu eixo e de translação, movendo-se rapidamente em linha reta ou arcos (FAINE et al., 2000).

As leptospiras são bactérias obrigatoriamente aeróbias e crescem numa temperatura de 28º a 30°C e pH 7,2 a 7,6. Essas bactérias são fastidiosas e necessitam de meio especial para crescimento, enriquecido com albumina bovina sérica (BSA), soro de coelho, vitaminas B1 e B12, amônia e ferro. A sua principal fonte de energia são os ácidos graxos de cadeia longa. O meio de cultura mais utilizado para o seu cultivo é o meio Ellinghausen-McCullough-Johnson-Harris (EMJH) (Ellinghausen et al., 1965; Johnson et al., 1967), que contém ácido oléico, BSA como detoxificante e Tween 80 como fonte de carbono. As leptospiras possuem tempo de geração longo, variando de três horas para espécies saprófitas e 8-18 horas para espécies patogênicas, e o crescimento em meio de cultura pode variar de dois a 30 dias (FAINE et al., 2000).

As leptospiras são bastante sensíveis ao ressecamento, desinfetantes, extremos de temperatura e pH inferior a 6,8 ou superior a 8,0 (FAINE et al. 2000). No entanto, sobrevivem na água e em cultura por longos períodos (TRUEBA et al., 2004), bem como em solos, lama,

coleções de água doce e rios (HENRY et al. 1978). As espécies saprófitas sobrevivem e se multiplicam na água e solo (PICARDEAU et al., 2008), já as patogênicas podem sobreviver no ambiente, mas preferencialmente encontram-se no hospedeiro, onde se multiplicam (FAINE et al., 2000).

1.4 Taxonomia

Na década de 80, o gênero *Leptospira* foi dividido em duas espécies, *L. interrogans*, que compreendia todas as cepas patogênicas, e *L. biflexa* que agrupava as cepas saprófitas isoladas do ambiente, podendo ser diferenciadas pelos requerimentos de crescimento e por reações bioquímicas (LEVETT, 2001). Para propósitos taxonômicos e como auxiliar nos estudos epidemiológicos, as leptospiras foram subdivididas em sorogrupos com base na relação antigênica, determinada por reações de aglutinação cruzada e posteriormente subdivididas em sorovares de acordo com padrões de aglutinação – absorção (ELLIS, 1994). Existem mais de 250 sorovares agrupados em 24 sorogrupos (LEVETT, 2001).

A classificação fenotípica vem sendo substituída pela genotípica, baseada em hibridização DNA-DNA, a qual agrupou as leptospiras em diversas espécies genômicas, que correspondem a grupos de cepas com similaridades no DNA. Até o momento, foram identificadas 14 espécies e 04 genomospécies (YASUDA et al., 1987; PEROLAT et al., 1998; BRENNER et al., 1999; LEVETT et al., 2005; MATTHIAS et al., 2008). As leptospiras patogênicas compreendem Leptospira alexanderi, L. borgpetersenii, L. interrogans, *L. kirschneri, L. noguchii, L. santarosai, L. weilii* e *L.* genomospecies 1. O grupo das leptospiras intermediárias compreende *L. broomii, L. fainei, L. inadai* e *L. licerasiae* (MATTHIAS et al., 2008) e o das saprófitas compreende *L. biflexa, L. meyeri, L. wolbachii, L.* genomospecies 3, 4 e 5 (BRENNER et al., 1999; LEVETT et al., 2005; MATTHIAS et al., 2008). Apesar de ser atualmente aceita e amplamente utilizada, providenciando um protocolo de tipificação rápido e reproduzível, a tipificação genética tornou a taxonomia do gênero confusa (ELLIS, 1994). Um problema dessa classificação é que um mesmo sorovar pode representar mais de uma espécie genômica (BRENNER et al., 1999), sendo assim, a classificação sorológica ainda é aceita na microbiologia clínica e epidemiologia (BHARTI et al., 2003).

Alguns sorovares de leptospira são comumente associados com reservatórios animais em particular, como apresentado na **Tabela 1** (BHARTI et al., 2003).

Tabela 1 – Reservatórios típicos de manutenção para sorovares comuns de *Leptospira* spp.

Hospedeiro de manutenção	Sorovar (es)
Suínos	Pomona, Tarassovi, Bratislava
Bovinos	Hardjobovis, Pomona
Eqüinos	Bratislava
Caninos	Canicola
Ovinos	Hardjobovis
Mão-pelada	Grippotyphosa
Ratos	Icterohaemorrhagiae, Copenhageni
Camundongos	Ballum, Arborea, Bim
Marsupiais	Grippotyphosa
Morcegos	Cynopteri, wolffi

1.5 Epidemiologia

A leptospirose é uma doença infecciosa distribuída em todos os continentes, sendo comum e economicamente importante em todo o mundo (THIERMANN, 1984).

Animais, incluindo humanos, podem ser divididos em hospedeiros de manutenção (reservatórios) e hospedeiros acidentais (incidentais). A doença é mantida na natureza pela infecção crônica dos túbulos renais nos hospedeiros de manutenção, sendo os organismos eliminados pela urina (leptospirúria) (LEVETT, 2001). Um hospedeiro de manutenção é definido como uma espécie na qual a infecção é endêmica e usualmente transmitida de um animal para outro por contato direto. Animais podem ser hospedeiros de manutenção de alguns sorovares e hospedeiros acidentais de outros, sendo que neste último caso a infecção pode ser severa ou fatal (LEVETT, 2001).

Uma vez infectados, os reservatórios apresentam colonização persistente dos túbulos proximais renais e disseminam de forma assintomática o organismo para o ambiente através da urina ou secreções, contaminando coleções de água e solo, sendo a água o principal veículo de transmissão da doença (McBRIDE et al., 2005; LEVETT 2001; BHARTI et al., 2003). O sorovar que predomina no ambiente urbano é o Copenhageni e o seu reservatório principal é o *Rattus norvegicus* (rato marrom ou rato de esgoto) (PEREIRA et al., 2000; ROMERO et al., 2003, TUCUNDUVA et al., 2007).

As leptospiras dependem de condições especiais no meio ambiente para que se mantenham vivas, como umidade e pH neutro à levemente alcalino, podendo, porém

sobreviver por um curto período em pH mais ácido e por até 03 meses em urina diluída com águas da chuva (AMATREDJO et al., 1975). Onde condições como estas são encontradas, a prevalência de infecção acidental é maior. Ambientes favoráveis para a sobrevivência de leptospiras são menos importantes na epidemiologia dos hospedeiros de manutenção (ELLIS, 1994).

Em humanos, a infecção por leptospiras pode ser causada por qualquer um dos sorovares patogênicos, o que torna complexo o estudo epidemiológico da doença. Felizmente, apenas um pequeno número de sorovares é endêmico numa região em particular ou país (ELLIS, 1994). Diversas espécies de mamíferos servem de reservatórios para o agente e mantém a transmissão da leptospirose na natureza. Como determinadas espécies de reservatórios costumam estar associadas a alguns sorovares, o conhecimento sobre quais são os reservatórios e os sorovares circulantes em uma região é essencial para o entendimento da epidemiologia da leptospirose no local (LEVETT 2001; BHARTI et al. 2003).

O período de incubação médio após a infecção de um hospedeiro humano por leptospiras patogênicas é de 7 a 14 dias. A infecção é capaz de produzir uma grande variedade de manifestações clínicas, como uma infecção subclínica seguida de soroconversão, uma doença febril aguda autolimitada e a de uma doença grave e potencialmente letal que pode se apresentar por qualquer combinação entre insuficiência renal aguda, icterícia, sangramentos e pneumonite (LEVETT, 2001; BHARTI et al., 2003; McBRIDE et al., 2005). A forma grave, que se manifesta por icterícia, insuficiência renal aguda e sangramento (síndrome de Weil) tem letalidade >10%. A forma grave associada a sangramento pulmonar maciço é conhecida como síndrome de hemorragia pulmonar grave (SHPS) e apresenta uma letalidade >50% (McBRIDE et al., 2005; GOUVEIA et al., 2008).

O início da doença, tanto nas formas leves e autolimitadas quanto nas formas graves, costuma ser súbito. O paciente apresenta febre alta, algumas vezes acompanhada de calafrios, cefaléia, mialgia, anorexia, prostração, náuseas e vômitos (BHARTI et al., 2003). Este quadro inicial é de difícil diagnóstico e se confunde com doenças como dengue, influenza, gastrenterite e outras viroses (LEVETT, 2001). Este quadro se resolve espontaneamente em poucos dias em mais de 90% dos pacientes e não deixa seqüelas. Entretanto, 5-10% dos pacientes pode evoluir para formas graves da doença, o que em geral ocorre ainda na primeira semana de sintomas (KO et al, 1996; LEVETT, 2001; BHARTI et al., 2003; McBRIDE et al., 2005). Os mecanismos patogênicos que determinam a progressão para formas graves da doença ou para infecções subclínicas permanecem desconhecidos, mas devem estar relacionados a características de virulência do organismo, a dose de inóculo durante a

infecção, a características da resposta imune do hospedeiro ou uma interação entre estes fatores (BHARTI et al. 2003).

1.6 Diagnóstico

Leptospiras são organismos fastidiosos e de crescimento lento, portanto as práticas de diagnóstico concentram-se nos métodos sorológicos. Entretanto, isolamento do agente e exame histopatológico de tecidos são de grande importância. A escolha dos testes a serem realizados, vai depender das condições disponíveis (THIERMANN, 1984).

1.6.1 Técnica de Microaglutinação Sorológica (MAT)

O MAT é o procedimento laboratorial mais utilizado para o diagnóstico de Leptospirose. O teste necessita de cepas representativas dos principais sorogrupos conhecidos mais aqueles que são mantidos pelos animais reservatórios da região. É considerado o teste de referência e baseia-se na identificação, por microscopia de campo escuro, da aglutinação do soro do paciente com antígenos vivos. O teste é considerado específico e sensível na fase imune da doença, permitindo a identificação do sorogrupo infectante, sendo uma importante ferramenta clínico-epidemiológica. Porém, o teste possui baixa sensibilidade na fase inicial da doença (CRODA et al., 2007), bem como em casos crônicos, onde os títulos de anticorpos podem ser muito baixos (ELLIS et al., 1981). A MAT é uma técnica de difícil execução sendo realizada apenas em laboratórios especializados e freqüentemente são necessárias amostras pareadas para a confirmação e diagnóstico da doença. O MAT falha em diferenciar anticorpos vacinais daqueles produzidos pela infecção natural, mas títulos altos são indicativos desta última. Poucas reações falso-positivas ocorrem, já que os antígenos de superfície não são compartilhados com outros organismos. Porém, reações cruzadas causadas por exposição à leptospiras do mesmo sorogrupo podem ocorrer. É um teste trabalhoso, demorado e que utiliza antígenos vivos, um risco para aqueles que realizam o teste (SMITH et al., 1994).

A interpretação do MAT é complicada pela já citada reação cruzada entre sorovares, principalmente na fase inicial da doença, ocorrendo muitas vezes títulos similares para todos os sorovares de um mesmo sorogrupo, além da possibilidade de ocorrerem reações paradoxais, onde os maiores títulos são detectados para um sorogrupo não relacionado com a infecção. As falhas no MAT ocorrem principalmente por detectar tanto IgM quanto IgG, assim como pela diversidade comum de antígenos entre leptospiras (LEVETT, 2001).

1.6.2 Ensaio Imunoenzimático (ELISA)

As deficiências no MAT levaram alguns pesquisadores a desenvolver métodos de diagnóstico baseado na técnica de ensaio imunoenzimático. O maior benefício desta técnica é a possibilidade de ser específico para anticorpos IgM ou IgG (SMITH et al., 1994)

O ELISA é uma técnica simples, segura, facilmente automatizada e, portanto, a mais adequada para o exame de um grande número de amostras, e a incorporação do sistema biotina/avidina eleva seus níveis de detecção (CHAMPAGNE et al., 1991)

Segundo Adler et al., (1982), apesar dos anticorpos detectados pelo ELISA e pelo MAT aparecerem juntos, não houve correlação do tempo pós-infecção onde os testes detectaram os picos da resposta imune, sugerindo que ambos detectam diferentes sistemas de ligação antígeno-anticorpo. Ainda para estes autores, uma reação positiva no ELISA IgM específico indica que a infecção ocorreu no mês prévio e a reação negativa exclui uma infecção ativa.

Diversos ensaios imunoenzimáticos são considerados mais sensíveis que o MAT. As diferenças no desempenho destes testes se referem à sua composição, particularmente variações na preparação do antígeno (SMITH et al., 1994).

Apesar da maior sensibilidade do ELISA, que eleva sua habilidade em detectar infecções recentes e o fato de que antígenos vivos não são requeridos para este teste, possui baixa especificidade quando comparado com o MAT (SMITH et al., 1994; LEVETT, 2001).

1.6.3 Reação da Polimerase em Cadeia (PCR) e PCR em tempo real

O diagnóstico baseado no PCR tem sido desenvolvido efetivamente para um grande número de microorganismos. Devido à sua alta sensibilidade, especificidade e rapidez de amplificação, o PCR tem demonstrado ser de grande utilidade onde as técnicas de cultura existentes falharam ou são inadequadas (AHMED et al., 2009).

Em 1989, Van Eys e colaboradores desenvolveram o primeiro PCR para diagnóstico de leptospirose, utilizando *primers* para amplificação do DNA e detecção de *L. borgpetersenii* sorovar Hardjobovis em urina de bovinos. Estes autores afirmaram que o PCR é uma técnica que ultrapassa a sensibilidade de outras até então exploradas, por detectar um pequeno número de leptospiras, sendo pelo menos tão sensível quanto à cultura e a sorologia, e muito mais sensível que as técnicas baseadas em DNA antes descritas. Para os autores, a especificidade do PCR é determinada pela eficiência de anelamento dos *primers*, que depende da sua identidade seqüencial com o DNA alvo e a temperatura de anelamento. Um aumento nesta temperatura permite poucos erros de ligação entre os *primers* e o DNA, aumentando a

especificidade do anelamento. Além disso, com um maior número de ciclos de amplificação, as chances de síntese de produtos indesejados aumentam.

Gerritsen e colaboradores (1991) avaliaram a técnica de PCR para diagnóstico de leptospirose em urina de bovinos e demonstraram que o congelamento e armazenamento podem afetar a qualidade do DNA, mas não afetam a amplificação. Mérien e colaboradores (1992), utilizando PCR em amostras clínicas de urina, líquido cefalorraquidiano (LCR) e sangue de humanos, afirmaram que este pode ser superior à cultura em sensibilidade e que a posterior hibridização aumenta em dez vezes a sua sensibilidade. Bal e colaboradores (1994) também confirmaram a especificidade da ampliação com a hibridização.

A sensibilidade da técnica de PCR comparada com outras técnicas, como MAT e ELISA, é de 90%, portanto é indicado o uso concomitante de duas técnicas para o diagnóstico preciso de leptospirose (WAGENAAR et al., 2000). Porém, uma das vantagens da técnica de PCR é seu uso prático para o diagnóstico na fase inicial logo após os primeiros sintomas, facilitando o tratamento (MÉRIEN et al., 1995).

Nas últimas duas décadas, diversos testes de PCR convencional foram descritos para o diagnóstico de leptospirose utilizando uma variedade de genes alvo, incluindo *rrs* (MÉRIEN et al., 1992), *flaB* (KAWATABA et al., 2001) e *ompL1* (REITSTETTER, 2006), porém somente dois foram avaliados em estudos clínicos (BROWN et al., 1995; MÉRIEN et al., 1995) e utilizados em larga escala para o diagnóstico. Apesar do seu intenso uso, estes testes possuem limitações. O teste descrito por Mérien et al. (1995) é um teste gênero-específico que amplifica tanto sorovares de leptospiras patogênicas quanto de leptospiras saprófitas, enquanto que o teste descrito por Gravekamp et al. (1993) e avaliado por Brown et al. (1995), necessita dois grupos de primers, os primers G1/G2 (gene *secY*) para detectar todas as leptospiras patogênicas, com exceção da *L. kirschneri* detectada pelos primers B64I/B64II.

Recentemente, diversos métodos baseados na técnica de PCR em tempo real (Real time PCR) foram introduzidos como uma ferramenta rápida e sensível para a detecção de leptospiras, reduzindo o risco de resultados falso-positivos ocorridos por amplificação inespecífica (AHMED et al., 2009).

A técnica de PCR em tempo real envolve o uso de marcadores que intercalam a fita dupla de DNA, como SYBR Green I, ou o uso de sondas fluorescentes como Taqman ou Transferência ressonante de energia de fluorescência (FRET) para emitir um comprimento de onda de tamanho específico para detecção (SLACK et al., 2007). Além disso, o uso de PCR em tempo real permite determinar a quantidade do gene alvo e com isso, a quantificação da carga do agente (SMYTHE et al., 2002).

Existe um número limitado de técnicas de PCR em tempo real para a detecção de leptospiras patogênicas, sendo que nenhum foi clinicamente validado (LEVETT et al., 2005; MÉRIEN et al., 2005; PALANIAPPAN et al., 2005; ROCZEK et al., 2008; SLACK et al., 2006; SMYTHE et al., 2002; STODDARD et al., 2009). Dois desses testes possuem como alvo genes que são universalmente presente em bactérias, o gene *rrs* (MÉRIEN et al., 2005; SMYTHE et al., 2002) e o gene *gyrb* (SLACK et al., 2006). Outros possuem alvos como o gene *lipl32* (LEVETT et al., 2005; ROCZEK et al., 2008; STODDARD et al., 2009), e os genes *ligA* e *ligB* (PALANIAPPAN et al., 2005), considerados genes restritos à leptospiras patogênicas.

Os testes de PCR em tempo real são baseados mais comumente na tecnologia do SYBR Green (LEVETT et al., 2005; MÉRIEN et al., 2005; SLACK et al., 2006) ou Taqman (PALANIAPPAN et al., 2005; SMYTHE et al., 2002; STODDARD et al., 2009). A tecnologia de SYBR Green é popular devido ao seu baixo custo, porém possui a desvantagem de menor especificidade quando comparado com técnicas como Taqman e a necessidade de uma etapa adicional de *melting curve* para verificar o produto amplificado (ESPY et al., 2006).

A utilização de técnicas de PCR em tempo real tornou-se comum no estudo da leptospirose, como o uso do teste desenvolvido por Smythe et al. (2002) para determinar a carga do agente no sangue de pacientes com comprometimento pulmonar (SEGURA et al., 2005) ou para determinar o risco de leptospirose pela determinação da carga do agente em amostra de água de áreas urbanas e rurais no Peru (GANOZA et al., 2006). Porém, avaliações mais consistentes ainda são necessárias e, com isso, o uso desta técnica permanece incerto (AHMED et al., 2009).

Recentemente, um teste de PCR em tempo real baseado na técnica de SYBR Green e utilizando como alvo o gene *secY*, foi desenvolvido e validado clinicamente mostrando uma sensibilidade de diagnóstico de 100% e uma especificidade de diagnóstico de 93%, utilizando a cultura como método padrão (AHMED et al., 2009).

1.6.4 Isolamento

A cultura de leptospiras pode ser um exercício prolongado, pois os meios devem ser examinados por campo escuro a cada 15 dias nos primeiros 03 meses da cultura, o procedimento é difícil, trabalhoso e requer experiência e habilidade e a identificação dos isolados, assim como o próprio procedimento de isolamento, deve ser realizado por laboratórios especializados, razão esta pela qual a cultura não é usada rotineiramente em

laboratório de diagnóstico (SMITH et al., 1994). Sangue, urina, líquor e outras amostras biológicas podem ser utilizadas para isolamento do organismo por cultura. Porém a cultura de leptospiras é difícil e pouco sensível. Além disso, por ser uma bactéria fastidiosa, pode ser necessário até 16 semanas de incubação com altos índices de contaminação (BHARTI et al., 2003).

1.6.5 Outros métodos

A investigação microbiológica deve ser baseada na demonstração de leptospiras nos órgãos internos, como fígado, pulmão, cérebro, rim e glândulas adrenais e fluídos corporais, como sangue, LCR, líquido torácico e peritoneal. Porém, este método possui baixa especificidade e não é sensível o suficiente para detectar leptospiras degradadas (ELLIS, 1994; SMITH et al., 1994).

O teste de imunofluorescência tem sido largamente utilizado. Tem a vantagem de prover um melhor contraste entre as leptospiras e o tecido, comparado com outras técnicas. Porém, necessita de um antisoro policlonal de boa qualidade para reconhecer uma variedade de epitopos que serão expostos devido à degradação das leptospiras causada pela autólise do tecido, e a produção deste requer um regime de inoculações repetidas em coelhos (ELLIS, 1994).

As leptospiras não se coram satisfatoriamente com corantes comuns. A visualização direta do microorganismo em tecidos é tradicionalmente realizada pela coloração com prata ou imunohistoquímica, sendo a última mais sensível e específica (ELLIS, 1994; SMITH et al. 1994).

O exame de fluídos com microscópio de campo escuro tem sido muito utilizado e pode ser uma boa ferramenta nas mãos de um laboratorista experiente, porém muitos artefatos teciduais podem ser confundidos com leptospiras (ELLIS, 1994).

O desenvolvimento de um novo método diagnóstico para a leptospirose, que seja simples e capaz de identificar as fases iniciais da doença é uma das prioridades na pesquisa em leptospiras (CRODA et al., 2007).

1.7 Saúde Pública

O papel da Leptospirose como zoonose vem sendo descrito desde que Weil, em 1881, relatou casos humanos de infecção por *Leptospira spp*. em açougueiros, e do primeiro isolamento do sorovar *pomona* de um fazendeiro (AMATREDJO, 1975).

A leptospirose é um problema grave de saúde pública no mundo, ocorrendo com maior freqüência em localidades tropicais e ambientes rurais dos países em desenvolvimento. Com o aumento da população urbana, principalmente pela migração rural, tornou-se um problema urbano nos países em desenvolvimento. No Brasil, epidemias ocorrem todo o ano nas comunidades pobres durante o período de chuvas, época em que os alagamentos constantes associado às condições precárias de saneamentos destes locais, favorecem o contato com ambiente e água contaminada (KO et al., 1999). Mais de 10.000 casos de leptospirose grave são relatados anualmente no país, sendo mais de 300 em Salvador, onde aproximadamente 15% destes vão a óbito (KO et al., 1999). Nos países desenvolvidos, sua ocorrência é rara e normalmente está associada com atividades recreacionais (KO et al., 1999; McBRIDE et al., 2005). Porém, a situação da leptospirose urbana tende a piorar muito nos próximos 25 anos, quando se acredita que a população das favelas tende a dobrar (WHO, 1999). No Brasil, a leptospirose humana é uma doença de notificação compulsória, muito embora apenas 3.000 casos da doença, um número provavelmente subestimado, sejam declarados por ano (TASSINARI et al., 2004).

Ainda persiste o dogma de que a leptospirose é uma doença ocupacional e esporádica, associada a profissões masculinas como agricultura, pecuária, mineração, manutenção de esgotos e serviços militares (LEVETT 2001; BHARTI et al. 2003). Somente na última década a leptospirose ganhou atenção como um importante problema de saúde pública global. Este reconhecimento deveu-se à emergência da SHPS em todo o mundo (TREVEJO et al. 1998; GOUVEIA et al. 2008), à identificação de surtos de leptospirose durante desastres (CAMPANELLA 1999; SANDERS et al. 1999) e atividades de recreação e turismo (CDC 1998; CDC 2001; MORGAN et al., 2002; SEJVAR et al., 2003), e às grandes epidemias (TANGKANAKUL et al., 2005).

Embora a população de maior risco seja tradicionalmente representada por agricultores de subsistência da zona rural (FAINE et al. 1999; LEVETT 2001; BHARTI et al. 2003), atualmente a leptospirose emerge como uma doença urbana que acomete os moradores pobres de favelas de países em desenvolvimento (McBRIDE et al. 2005; RILEY et al. 2007).

1.8 Tratamento

O tratamento da leptospirose é baseado no uso de antibióticos e de terapias de suporte (FAINE et al., 2000). Embora haja controvérsia do benefício da antibioticoterapia na redução da letalidade associada à doença grave (GUIDUGLI et al., 2000), o emprego de antibióticos parece reduzir a duração da febre, o tempo para normalização da função renal e o tempo de

hospitalização (McCLAIN et al., 1984; WATT et al., 1988). A penicilina cristalina ou ceftriaxone e doxiciclina são os antibióticos recomendados para os pacientes hospitalizados e ambulatoriais, respectivamente (LEVETT 2001; BHARTI et al. 2003; McBRIDE et al. 2005). Seu uso deve ser iniciado tão cedo quanto possível após o diagnóstico clínico e epidemiológico. Atualmente, as medidas de prevenção têm sido totalmente ineficazes no controle da disseminação desta doença (McBRIDE et al. 2005).

Vacinas podem ser alternativas efetivas e com uma relação custo/benefício positiva na prevenção da leptospirose (MAROTTO et al., 1999). Elas devem prevenir a doença em humanos através da imunização de populações de risco ou impedir a transmissão através da imunização de reservatórios animais (LEVETT 2001). A leptospirose é um sério problema também na área de saúde veterinária, principalmente em bovinos, suínos e caninos (FAINE et al., 1999; LEVETT 2001) e existe uma grande variedade de vacinas comerciais disponíveis para uso veterinário. As bacterinas disponíveis consistem de leptospiras inativadas quimicamente ou por calor e são capazes de proporcionar proteção contra a infecção letal, mas não de prevenir a colonização renal (SEGURA et al., 2005; GOUVEIA et al., 2008), mantendo a leptospirose como uma doença de alta prevalência em animais domésticos (HOTEZ et al., 2006).

A prevenção da leptospirose humana através da vacinação é relatada em alguns países como Cuba e China (BHARTI et al., 2003; McBRIDE et al., 2005). Porém, assim como outras bacterinas, possui a desvantagem de ter uma imunidade curta, necessitando de doses repetidas, induzir possíveis reações adversas através dos componentes do meio e dos lipopolisacarideos presentes nas leptospiras (LPS), além de ser necessária a composição com diversos sorovares, já que a resposta induzida é contra os LPS, específicos para cada sorogrupo (ADLER et al., 1980). Tais preocupações fizeram com que estas vacinas não fossem licenciadas fora de seus países (McBRIDE et al. 2005). Esforços vêm sendo feitos na pesquisa e desenvolvimento de subunidades de vacinas contra a leptospirose. Proteínas da membrana externa, conservadas entre as espécies e sorovares patogênicos de leptospiras, são alternativas viáveis (FREUDENSTEIN et al., 1981). Essas proteínas são expressas durante a infecção em mamíferos e estão expostas na membrana de todas as espécies de leptospiras patogênicas, servindo de alvo para a resposta imune do hospedeiro, induzindo assim uma proteção cruzada para todos os sorogrupos de leptospira (FREUDENSTEIN et al., 1981; THIERMANN, 1984). Estudos de imunoproteção mostraram que algumas destas proteínas são eficazes na indução de proteção, como OmpL1 e LipL41 (BRANGER et al., 2001), LipL32 (ADLER et al., 1980; HAAKE et al., 2004) e Ligs (SILVA et al., 2007; KOIZUMI et

al., 2004; PALANIAPPAN et al., 2006). Porém, nenhuma vacina foi capaz de impedir a colonização renal nos modelos estudados.

Medidas individuais de proteção incluem evitar ou reduzir a exposição a águas e solos potencialmente contaminados por leptospiras, assim com evitar o contato com animais potencialmente contaminados. Evidências sugerem que o uso de quimioprofilaxia com doxiciclina antes ou após exposição é capaz de reduzir o risco de doença clínica, mas não impede a ocorrência da infecção como demonstrado pelos testes sorológicos (TAKAFUJI et al., 1984; SEHGAL et al., 2000). Medidas de prevenção coletivas, como desratização, são utilizadas com frequência, entretanto, são custosas e precisam ser repetidas rotineiramente para ter benefício (BARTHI et al., 2003).

1.9 Genética

O isolamento dos fagos LE de leptospiras não patogênicas dos esgotos de Paris (SAINT GIRONS et al., 1990) foi o primeiro avanço na manipulação genética de leptospiras. Porém, estes fagos infectam apenas leptospiras saprófitas, não se replicando em cepas patogênicas. A partir desta descoberta, desenvolveu-se um plasmídeo replicativo contendo a origem de replicação do profago LE1, resultando no primeiro relato de transferência genética *E.coli - L. biflexa* (SAINT GIRONS et al., 2000).

Em 2001, Picardeau e colaboradores realizaram a primeira recombinação homóloga através do uso de um plasmídeo suicida contendo o gene *flaB* de leptospiras, originando um mutante deficiente de endoflagelo e motilidade. Essa mesma técnica foi posteriormente utilizada para estudar outros genes como *recA*, *trpE*, *metY*, *metX* e *metW* (TCHAMEDEU KAMENI et al., 2002; BAUBY et al., 2003; PICARDEAU et al., 2003).

Em 1999, Rubin et al. demonstraram que transposons de eucariotos da família mariner realizam transposição com baixa especificidade em uma ampla gama de bactérias. O Himar1 é um elemento móvel da família dos transposons mariner isolado da mosca do chifre *Haematobia irritans* (LAMPE et al., 1996), que não precisa de fatores associados ao hospedeiro, realiza transposição tanto em eucariotos quanto em procariotos e, com exceção do dinucleotídio TA, não possui requerimentos específicos para inserção, características essas que o tornaram um excelente candidato para a inserção aleatória em leptospiras (BOURHY et al., 2005; LOUVEL et al., 2005). Utilizando um plasmídeo suicida carreando o Himar1, o transposon se insere de maneira aleatória no genoma bacteriano, interrompendo um determinado gene. Com o uso desta técnica, Louvel e colaboradores (2005) criaram uma

biblioteca de mutantes para a pesquisa de genes de metabolismo em leptospiras, como a caracterização da aquisição de ferro em *L. biflexa*.

Bourhy e colaboradores (2005) utilizaram a transposição aleatória de Himar1 para realizar a primeira transferência genética em leptospiras patogênicas, utilizando *L. interrogans* sorovar Lai. Porém, para cada grama de DNA plasmidial se obtém, em média, 10.000 colônias quando se utiliza *L. biflexa* e apenas 100 colônias quando se trabalha com *L. interrogans*. Essa baixa eficiência de transformação pode ser resultado da competência celular e dos mecanismos de recombinação e/ou restrição do DNA, diferentes entre as leptospiras saprofitas e patogênicas (BOURHY et al., 2005).

O primeiro sequenciamento genético completo de uma cepa de *Leptospira* foi realizado em 2003, da *L. interrogans* sorovar Lai (REN et al., 2003). Atualmente, além do sorovar Lai, três outras cepas patogênicas foram sequenciadas, *L. interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004) e duas cepas de *L. borgpetersenii* sorovar Hardjo (BULACH et al., 2006) e uma cepa saprófita, *L. biflexa* sorovar Patoc (PICARDEAU et al., 2008). O genoma da leptospira possui dois cromossomos circulares, sendo que a cepa saprófita possui, além disso, um plasmídeo circular. O sequenciamento proporcionou uma gama de novas perspectivas. A genômica comparada de leptospiras patogênicas e saprofitas pode revelar conhecimentos fundamentais sobre a virulência e biologia. O genoma do sorovar Hardjo, adaptada aos bovinos e tendo o contato direto como principal forma de contágio e, portanto, menos adaptada à sobrevivência no ambiente, mostrou ter uma perda genômica de 700 Kb em relação às cepas *L. interrogans*, sabidamente adaptadas ao ambiente (BULACH et al., 2006). Os estudos de microarranjos também foram beneficiados, permitindo demonstrar diferenças na expressão de genes relacionados com quimiotaxia, motilidade e proteínas de membrana externa, quando comparadas diferentes temperaturas de cultivo (LO et al., 2006; QIN et al., 2006) ou osmolaridade fisiológica (MATSUNAGA et al., 2007) e na triagem de genes de proteínas de membrana externa, que podem ser candidatos vacinais (YANG et al., 2006b).

A técnica do Himar1 e o conhecimento do genoma da Leptospira permitiram a identificação da lipoproteína de superfície Loa22 como o primeiro fator de virulência associado à *Leptospira* (RISTOW et al. 2007). Em 2008, Picardeau utilizou pela primeira vez o método de conjugação para transferência genética em leptospiras, através de uma cepa de *E. coli* doadora e um plasmídeo que possui uma ampla gama de hospedeiros, transferindo o transposon Himar1 para *L. biflexa* e *L. interrogans*. Recentemente, MURRAY et al. (2009) criaram uma biblioteca de mutantes aleatórios com a técnica do Himar1 em *L. interrogans*,

descobrindo que mutantes com genes de função aleatória interrompidos tornaram-se atenuados, validando o uso do transposon para identificação de novos fatores de virulência e para o estudo de função gênica.

Nos últimos anos, temos presenciado um grande progresso no estudo da biologia molecular de Leptospiras, mas comparado com outros grupos de bactérias e mesmo de outras espiroquetas, o conhecimento da genética deste importante grupo ainda é pequeno (BULACH et al., 2000; MÉRIEN et al., 2005). Os avanços são dificultados principalmente pela ausência de ferramentas genéticas adequadas e eficientes (LOUVEL et al., 2005; KO et al, 2009).

1.10 Modelos animais

O uso de modelos animais experimentais é indispensável para compreender a biologia, transmissão, colonização e patogênese da Leptospira. Hamster e cobaios (Guinea Pig) são os modelos experimentais padrão para leptospirose aguda (RANDALL et al., 1944; FAINE et al., 1999). A infecção com baixas doses (<100 leptospiras) resulta numa cinética de infecção e gravidade similar àquela observada em humanos (SILVA et al., 2008). Camundongos e gerbilos têm sido usados para estudar a genética da resposta imune e como modelo para estudos de imunidade mediada por vacinação, porém camundongos são resistentes à infecção e necessitam de altas doses de inóculo para produzir doença, o que não mimetiza a infecção natural (KO et al., 2009). Os ratos são utilizados como modelo para estudar a colonização persistente, mas também exigem altas doses de inóculo (ATHANAZIO et al., 2008; NALLY et al., 2005). O uso de modelos com maior semelhança à fisiologia humana, como cães nos modelos renais (FAINE, 1957) e macacos no modelo pulmonar (PEREIRA et al., 2005) também são válidos. Mais recentemente, DAVIS et al. (2009) descreveram o uso do peixe-zebra como modelo para a infecção e disseminação em leptospirose, porém, para a compreensão dos vários aspectos da patogênese da doença, é essencial o uso de modelos animais bem definidos e estudados, no qual o peixe-sempre não se encaixa (EVANGELISTA et al., 2010).

A inoculação experimental nos estudos da leptospirose é realizada principalmente pela via intraperitoneal (HAAKE, 2006). Este método, apesar de permitir que uma quantidade reproduzível de leptospiras seja introduzida no modelo animal, não reflete a transmissão natural do patógeno. Poucos estudos utilizam métodos que mimetizem a infecção natural, como através da pele (TRUCCOLO et al., 2002) ou pela mucosa ocular (BOLN et al., 2001; LOURDAULT et al., 2009).

1.11 Patogenia

O mecanismo molecular da patogênese da leptospirose ainda não é claro (EVANGELISTA et al., 2010). As leptospiras patogênicas podem penetrar pele e mucosa, se disseminando rapidamente para outros tecidos após a infecção, através da via hematógena assim como da sua capacidade de translocação celular (FAINE et al., 1999; BAROCCHI et al., 2002; LI et al., 2007). As leptospiras possuem mais de 80 genes envolvidos em motilidade e quimiotaxia, sendo a grande maioria destes conservados entre saprófitas e patogênicas. A quimiotaxia pode ter um importante papel na patogenicidade, como sugerido pelo comportamento de motilidade em resposta à hemoglobina (YURI et al., 1993). Estudos sugerem que as leptospiras utilizam a entrada na célula hospedeira e a rápida translocação sem dano ao tecido, como mecanismo para disseminarem aos órgãos alvos e evadir o sistema imune (KO et al., 2009).

A infecção causa uma prolongada leptospiremia até que o hospedeiro possa montar uma resposta imune efetiva, que ocorre uma ou duas semanas apos a exposição (FAINE, 1957). Quando a infecção é instalada, pode ocorrer a evolução para uma doença aguda, o desenvolvimento de imunidade protetora e eliminação do agente ou o desenvolvimento do estado de portador crônico. Neste último caso, estudos indicam que o lúmen dos túbulos renais, local onde a concentração de anticorpos é baixa, é um local de colonização ideal para as leptospiras, sendo provavelmente uma forma de escape do sistema imune (FAINE et al., 2000; ATHANAZIO et al., 2008).

Os rins colonizados por leptospiras apresentam nefrite túbulo-intersticial, focos inflamatórios, necrose tubular e hemorragias. As leptospiras são encontradas em grande número nos túbulos contorcidos proximais, glomérulos e interstício (VAN DEN INGH et al., 1986; NALLY et al., 2004; COX et al., 1981). No fígado, as leptospiras causam dano hepatocelular, com perda da arquitetura tecidual, focos de necrose hepatocitária, focos de inflamação, presença de células de Kupffer aumentadas e eventualmente presença de células apoptóticas (MÉRIEN et al., 1998; NALLY et al., 2004, VAN DEN INGH et al., 1986). O dano pulmonar principal na leptospirose ocorre devido às intensas hemorragias intra-alveolares, levando à insuficiência respiratória (NALLY et al., 2004; PEREIRA et al., 2005; GOUVEIA et al., 2008). Raras leptospiras são visualizadas nos pulmões, o que sugere um mecanismo diferente de patogenia que não a ação direta do microrganismo, assim como a presença do agente em tecidos renais e hepáticos não estão relacionados com lesão (CRODA et al., 2010; NALLY et al., 2004; VAN DEN INGH et al., 1986).

O inicio dos sintomas da doença está relacionado com o surgimento dos anticorpos e a eliminação das leptospiras através da opsonização e lise mediada pelos mesmos. A resposta humoral é específica para o serovar infectante. O dano vascular endotelial é a principal lesão na leptospirose grave e causa extravasamento capilar, hemorragias e vasculites (LEVETT, 2001; KO et al., 2009; NICODEMO et al., 1989). Proteínas com provável atividade de hemolisinas podem estar implicadas na lesão endotelial, como a proteína 1 associada à hemólise (Hap1), também chamada de lipoproteína L32 (LipL32) (LEE et al., 2000; HAAKE et al., 2000b), Esfingomielinase (Sph) (LEE et al., 2002) e Sph2 (ARTIUSHIN et al., 2004). A hemólise apresenta vantagens para a bactéria, disponibilizando ferro e ácidos graxos que são essenciais para o seu crescimento. A fonte de ferro mais abundante no hospedeiro é o heme, e leptospiras são capazes de usar heme e hemoglobina *in vitro* (LOUVEL et al., 2006).

Acredita-se que a patogênese da leptospirose está relacionada com a resposta imune do hospedeiro para componentes de membrana, como os lipopolissacarídeos (LPS), lipoproteínas e peptideoglicanos (CULLEN et al., 2005). Estudos mostram que o LPS das leptospiras ativa os receptores TLR2 nas células humanas ao contrário de outras Gram-negativas que ativam o receptor do tipo Toll 4 (TLR4) e as lipoproteínas induzem resposta imune inata pela ativação de TLR2 (WERTS et al., 2001, YANG et al., 2006a). Entretanto, LPS ativa ambos TLR2 e TLR4 em células de camundongos, indicando que existem diferenças espécie-específica com respeito à ativação dos TLR. Os peptideoglicanos das leptospiras induzem liberação de fator alfa de necrose tumoral (TNF-α) de monócitos através de um mecanismo independente dos efeitos endotóxicos (CINCO et al., 1996). Proteínas de membrana externa (OMP) e glicoproteínas também estão envolvidas no estímulo da resposta celular (YANG et al. 2001; DIAMENT et al., 2002). Recentemente, Murray et al. (2010), identificaram dois mutantes com interrupção de proteínas pertencentes ao LPS da *L. interrogans* sorovar Manilae, mas com função desconhecida. Ambos mutantes mostraram serem atenuados na virulência e eliminados do hospedeiro após 03 dias da infecção, demonstrando que o LPS tem um papel importante na patogênese das leptospiras.

As leptospiras podem ser encontradas em íntima relação com membranas celulares (BAROCCHI et al., 2002; NALLY et al., 2004), sugerindo possuírem mecanismos específicos de adesão. Porém, pouco se sabe sobre adesinas em leptospiras. Estudos descritivos mostram uma correlação entre adesão e virulência. Comparada com leptospiras atenuadas em cultura, as leptospiras virulentas aderem em maior numero à fibronectina, colágeno e laminina (IYO et al., 1987) e aderem às células em cultura e invadem células *Vero* mais rapidamente (MÉRIEN et al., 1997). Algumas prováveis proteínas de adesão foram

descritas, como uma proteína de adesão à fibronectina (MÉRIEN et al., 2000), as Ligs (MATSUNAGA et al., 2003) e as Lens (BARBOSA et al., 2006; STEVENSON et al., 2007).

As lipoproteínas são componentes fundamentais das membranas de eubactérias. Como em outras espiroquetas, o genoma da *Leptospira* spp. codifica para mais lipoproteínas que outras bactérias, contendo aproximadamente 145 genes que codificam para prováveis lipoproteínas e OMPs (SETUBAL et al., 2006; VIRATYOIN et al., 2008; YANG et al., 2006b). Existem aproximadamente 12 proteínas, presentes na superfície das leptospiras, que tiveram sua localização comprovada. Dentre essas, se destacam: as Ligs, Loa22, LipL32, OmpL1, LenA, LenD (KO et al., 2009) e mais recentemente OmpL36, OmpL 37, OmpL47 e OmpL54 (LO et al., 2009).

Com relação às Ligs, foram descritas duas proteínas, LigA e LigB, expressas em todas as espécies patogênicas de leptospira e LigC, um pseudogene presente em algumas espécies (MATSUNAGA et al., 2003). A expressão de LigA e LigB é aumentada quando as espécies patogênicas de leptospiras são expostas às concentrações de sal semelhantes aquelas encontradas no tecido dos hospedeiros, indicando um importante papel na patogênese da leptospirose (MATSUNAGA et al., 2005). Possuem a capacidade de se aderirem a fibronectina, colágeno e laminina (CHOY et al., 2007), estando possivelmente envolvidas na colonização dos tecidos nos hospedeiros, servindo como adesinas.

O único gene, até o momento, que preenche todos os postulados moleculares de Koch para um fator de virulência em leptospiras, é o gene *loa*22 (RISTOW et al., 2007). A lipoproteína Loa22 é exposta na superfície bacteriana (RISTOW et al., 2007), reconhecida pelo soro de pacientes com leptospirose (GAMBERINI et al., 2005) e possui maior expressão no modelo de infecção aguda (NALLY et al., 2007), apesar de que os resultados *in vitro* de aderência à componentes celulares ser fraco (BARBOSA et al., 2006) e existir um ortólogo do gene no genoma de *L. biflexa* (PICARDEAU et al., 2008).

A LipL32 é exposta na superfície e representa 75% das proteínas da membrana externa (HAAKE et al., 2000b; LEE et al., 2000, CULLEN et al., 2002), sendo altamente conservada entre as leptospiras patogênicas e sem presença de ortólogos na *L. biflexa* (HAAKE et al., 2004, PICARDEAU et al., 2008). A LipL32 é altamente expressa durante a infecção aguda letal, comparada com a cultura *in vitro* (NALLY et al., 2007). A porção C terminal da proteína se adere *in vitro* à laminina, fibronectina e colágeno I, IV e V (HAUK et al., 2008; HOKE et al., 2008). O estudo de sua estrutura cristalizada demonstra que essa proteína assemelha-se a proteínas como colagenase, que se ligam aos componentes da matriz extracelular (VIVIAN et al., 2009).

Recentemente, houve um grande progresso no conhecimento dos aspectos básicos da biologia e patogênese da *Leptospira* spp., porém a identificação de fatores de virulência de leptospiras ainda permanece especulativa devido à dificuldade em manipular geneticamente o agente e a falta de ferramentas genéticas para as espécies patogênicas (PICARDEAU et al., 2001; KO et al., 2009). Os estudos para esclarecimento do mecanismo molecular da patogênese das leptospiras e a realização de pesquisa para melhor compreensão de sua biologia e virulência, irão contribuir para o desenvolvimento de novas estratégias de tratamento e prevenção, importantes para combater essa importante e emergente doença infecciosa e seu grande impacto nas populações menos favorecidas.

2. OBJETIVOS

2.1 Objetivo Geral

Caracterizar a patogênese da leptospirose através da identificação de fatores de virulência e o mecanismo de disseminação do patógeno no hospedeiro.

2.2 Objetivos Específicos:

2.2.1 Determinar se a perda de genes que codificam para proteínas associadas à superfície das leptospiras, influencia na capacidade do agente em induzir colonização persistente e doença na infecção experimental:

2.2.1.1 Determinar se LigB, membro da superfamília *Bacterial immunoglobulin-like* (Big), tem papel na virulência da leptospirose experimental;

2.2.1.2 Identificar se LipL32, a maior lipoproteína de membrana (OMP), está envolvida no processo de colonização renal persistente;

2.2.2 Estudo da cinética da disseminação e tropismo das leptospiras para os tecidos do hospedeiro, usando um ensaio de PCR quantitativo durante a infecção experimental:

2.2.2.1 Determinar a cinética da infecção com alta e baixa dose, através do estudo de disseminação da *Leptospira interrogans* sorovar Copenhageni nos tecidos, utilizando a rota intraperitoneal e conjuntival;

2.2.2.2 Estabelecer se existe tropismo específico para o rim ou algum outro tecido em especial e se a carga do patógeno está relacionada com alterações histopatológicas nos diferentes tecidos;

2.2.2.3 Determinar se a cinética de distribuição nos diferentes tecidos é similar, quando comparado com clones virulentos e clones com atenuação da virulência.

3. JUSTIFICATIVA

A leptospirose é uma doença que causa risco de vida numa grande variedade de situações epidemiológicas, sendo considerada a zoonose mais difundida no mundo, devido ao grande número de mamíferos selvagens e domésticos considerados reservatórios do agente (FAINE et al., 2000). A mortalidade devido à leptospirose grave é alta, variando de 10-50%. O diagnóstico conveniente necessita um teste laboratorial, já que os primeiros sintomas da doença não são específicos. Entretanto, o diagnóstico atual depende de um método antiquado, o teste de microaglutinação (MAT), que requer amostras pareadas de soro e é realizada apenas em poucos laboratórios de referência (FAINE et al., 2000; GOUVEIA et al., 2008; SMITH et al., 1994). Além disso, não existe medidas de controle efetivas para a doença que possam ser implementadas, principalmente em áreas mais afetadas, como comunidades carentes de países em desenvolvimento (KO et al., 1999). A falta de diagnóstico e medidas de controle adequadas impede intervenções baseadas na redução da alta mortalidade da doença, além de contribuir para a subnotificação de casos e seu status de doença negligenciada.

O maior impedimento para identificar novas abordagens de intervenção tem sido o conhecimento limitado dos determinantes da doença. Apenas recentemente foi realizado o seqüenciamento do genoma de duas espécies patogênicas e uma saprófita de *Leptospira* (NASCIMENTO et al., 2004; REN et al., 2003; BULACH et al., 2006; PICARDEAU et al., 2008), que levaram à identificação de genes que podem ter algum papel na virulência da leptospirose. Porém, até o momento, apenas o gene que codifica para a lipoproteína de superfície Loa22 foi descrito e reconhecido como um fator de virulência para essa doença (RISTOW et al., 2007). Um dos motivos que previnem os esforços na identificação de novos fatores de virulência é a inabilidade de manipular geneticamente as leptospiras.

Sendo assim, são necessários maiores esforços e estudos na área da biologia e patogênese da doença, que permitam uma melhor compreensão dos mecanismos de infecção e virulência do agente, e no desenvolvimento de protocolos para mutagênese randômica ou dirigida. Esses esforços e seus resultados podem favorecer e facilitar a identificação de fatores de virulência, que serão uteis na elaboração e implementação de melhores e mais adequadas técnicas de diagnóstico e prevenção.

4. MANUSCRITO 1

Título:

Targeted Mutagenesis in Pathogenic *Leptospira* Species: Disruption of the LigB Gene Does Not Affect Virulence in Animal Models of Leptospirosis.

[Mutagênese dirigida em espécies patogênicas de *Leptospira*: interrupção do gene de LigB não afeta a virulência no modelo animal de leptospirose].

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Objetivo específico 1

1. Determinar se a perda de genes que codificam para proteínas associadas à superfície das leptospiras, influencia na capacidade do agente em induzir colonização persistente e doença na infecção experimental:

1.1 Determinar se LigB, membro da superfamília *Bacterial immunoglobulin-like* (Big), tem papel na virulência da leptospirose experimental;

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Targeted Mutagenesis in Pathogenic *Leptospira* Species: Disruption of the LigB Gene Does Not Affect Virulence in Animal Models of Leptospirosis[▽]

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The pathogenic mechanisms of *Leptospira interrogans*, the causal agent of leptospirosis, remain largely unknown. This is mainly due to the lack of tools for genetically manipulating pathogenic *Leptospira* species. Thus, homologous recombination between introduced DNA and the corresponding chromosomal locus has never been demonstrated for this pathogen. Leptospiral immunoglobulin-like repeat (Lig) proteins were previously identified as putative *Leptospira* virulence factors. In this study, a *ligB* mutant was constructed by allelic exchange in *L. interrogans*; in this mutant a spectinomycin resistance (Spc^r) gene replaced a portion of the *ligB* coding sequence. Gene disruption was confirmed by PCR, immunoblot analysis, and immunofluorescence studies. The *ligB* mutant did not show decrease virulence compared to the wild-type strain in the hamster model of leptospirosis. In addition, inoculation of rats with the *ligB* mutant induced persistent colonization of the kidneys. Finally, LigB was not required to mediate bacterial adherence to cultured cells. Taken together, our data provide the first evidence of site-directed homologous recombination in pathogenic *Leptospira* species. Furthermore, our data suggest that LigB does not play a major role in dissemination of the pathogen in the host and in the development of acute disease manifestations or persistent renal colonization.

Leptospirosis is a widespread zoonosis that has emerged as a major public health problem in developing countries in Southeast Asia and South America (6, 22, 29). This increasingly common disease occurs in poor urban centers subject to frequent flooding (20). Rodents are the main reservoir of the disease, excreting the bacteria in their urine (14, 22). Humans are usually infected through contaminated water. More than 500,000 cases of severe leptospirosis are estimated to occur worldwide each year (46), and the fatality rate is 5 to 20% (29).

The control methods for leptospirosis implemented to date have been ineffective (29). A significant barrier to control and prevention of leptospirosis has been our limited understanding of the pathogenesis of the disease, due in part to the lack of genome sequences and tools to genetically manipulate the pathogens. Most of the barriers have now been overcome. The genomes of two pathogenic species and one saprophytic species have been sequenced (8, 32, 39, 40). Furthermore, we developed a transposon-mediated mutagenesis system for pathogenic *Leptospira* species (7). This advance allowed characterization of the first genetically defined virulence factor in pathogenic *Leptospira* spp. (41). However, the generation of targeted mutants of pathogenic species was not feasible until now.

High-molecular-weight leptospiral immunoglobulin-like repeat (Lig) proteins were previously identified as putative virulence factors in pathogenic *Leptospira* spp. (21, 26, 34). This family of three proteins, LigA, LigB, and LigC, belongs to the superfamily of bacterial immunoglobulin-like (Big) repeat domain proteins, which includes virulence determinants such as intimin from enteropathogenic *Escherichia coli*, invasin from *Yersinia pseudotuberculosis*, and BipA from *Bordetella* spp. (26). This superfamily appears to mediate pathogen-host cell interactions, such as invasion and host cell attachment, during infection. Choy et al. and Lin and Chang recently showed that recombinant Lig proteins can mediate in vitro interactions with host extracellular matrix proteins, including fibronectin, fibrinogen, collagen, and laminin (9, 23). In addition, *lig* genes are upregulated at physiological osmolarity (27) and encode surface-exposed proteins that are strongly recognized by sera from human leptospirosis patients (10, 26, 43). Finally, several studies have shown that Lig proteins are protective antigens in animal models of leptospirosis (21, 35, 42).

In this study, we produced a *ligB* mutant of *L. interrogans* by allelic exchange and evaluated the effect of the deletion in this mutant using both cell adhesion assays and animal models. The results provided the first demonstration of targeted mutagenesis of *Leptospira* pathogenic strains.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Leptospires were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (13, 19) or on 1% agar plates at 30°C and were counted in a Petroff-Hausser counting chamber (Fisher Scientific). *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, a virulent clinical isolate from Brazil (20, 32), was used in all experiments. *E. coli* was grown in Luria-Bertani medium. When necessary, spectinomycin or kanamycin was added to culture media at a concentration of 50 μg/ml.

Polyclonal and monoclonal antibodies. We prepared immune sera against previously described recombinant fragments of LigA (LigANI; amino acid positions 625 to 1225) and LigB (LigBNI; amino acid positions 625 to 1257) (42). These fragments contain the 6th to 13th and 6th to 12th Big repeat domains of LigA and LigB, respectively, and do not include the portions of these molecules which have identical amino acid sequences (26). New Zealand White rabbits were immunized intravenously on days 0, 7, and 14 with three doses of 80 μg of recombinant protein fragments, using aluminum hydroxide as an adjuvant. Rabbits were bled on day 28 to obtain immune sera. For quality control, the reactivities of immune sera with recombinant and native Lig proteins were evaluated using enzyme-linked immunosorbent and immunoblot assays as previously described. Prebleed sera, as well as sera from rabbits immunized with phosphate-buffered saline (PBS) and alhydrogel, were used as control samples. Ascites fluid containing monoclonal antibodies (MAb) against a recombinant LigB protein fragment, LigBrep (26, 42), were provided by José Aleixo, Federal University of Pelotas. The LigBrep fragment corresponds to the six N-terminal Big repeat domains of the LigB molecule (amino acid positions 131 to 649).

Targeted mutagenesis. For allelic exchange, the gene fragment which encodes the LigB nonidentical region (nucleotides 1891 to 5450), also called the B2 region, was amplified from the genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 using primers LigBU1F-SmaI (5′ TCCCCCGGG GCTGAAATTAAAAATACCAGTGGAAG 3′) and P2R-SmaI (5′ TCCCCCG GGCCTATGTAGAGATAAGATCCACTTGC 3′). The PCR product was digested with SmaI and cloned into a PvuII-digested pGKm plasmid vector. The resulting plasmid was then digested with EcoRI, which removed a 699-bp sequence from the *ligB* B2 domain. The Spc^r cassette was amplified from plasmid pGSpc DNA using primers Spc-EcorI3′ (5′ ACGGAATTCAACGTAAAG TAAG 3′) and Spc-EcorI5′ (5′ TCGGAATTCAACGCGTCCCGAGC 3′). The PCR product was digested with EcoRI and subsequently inserted into the plasmid from which the 699-bp *ligB* sequence had been removed to obtain plasmid pB2SK (Fig. 1B). For gene inactivation by plasmid insertion, an internal DNA fragment of *ligB* (nucleotides 1891 to 3771), also called the B1 region, was amplified from the genomic DNA of *L. interrogans* Copenhageni strain Fiocruz L1-130 using primers LigBU1F-SmaI (5′ TCCCCCGGGGCTGAAATTAAAA ATACCAGTGGAAG 3′) and LigBU2R-SmaI (5′ TCCCCCGGGCACTTGG TTTAAGGAATTACAAACT 3′). The PCR product was digested with SmaI and cloned into a PvuII-digested pGSpc plasmid vector, resulting in plasmid pB1S. Plasmids pGKm and pGSpc (complete sequences are available on request) were derived from the cloning vector pGEM-7Zf(−) (Promega Corporation, Madison, WI) as previously described (15). We used the *Enterococcus faecalis* kanamycin and *Staphylococcus aureus* Spc^r cassettes as previously described (5).

The plasmid constructs, which are not replicative in *Leptospira* spp., were used to deliver the inactivated allele into *L. interrogans* by electroporation. Cells were grown to exponential phase (optical density at 420 nm, 0.10 to 0.20) and then centrifuged at 4,000 × *g* and concentrated to obtain 10^{10} bacteria/ml in sterile water. Suicide plasmids containing the inactivated allele were subjected to 5 to 30 s of UV treatment (254 nm, 400 μW/cm^2) using a UV chamber (GS Gene linker; Bio-Rad). Two hundred microliters of cells was electroporated (1.8-kV, 200-Ω, 25-μF electric pulse in a prechilled 0.2-cm-diameter cuvette) in the presence of 100 to 500 ng of plasmid DNA and then transferred to 1 ml of EMJH liquid medium, in which the cells were incubated for 24 h at 30°C. The bacteria were then plated on EMJH medium supplemented with spectinomycin (50 μg/ml). After 4 to 6 weeks of incubation, Spc^r colonies were picked and examined for allelic exchange in the target gene by PCR, Western blotting, and immunofluorescence analysis.

Genomic DNA analysis. Genomic DNA was prepared from liquid cultures by use of a cell DNA purification kit (Maxwell, Promega, Madison, WI). To check for double homologous recombination, primers B2EF (5′ CATACACACTTGT AGTCAACAAACAAG 3′) and B2ER (5′ CGTAACGTAATTCGGAACCG 3′) and primers LBN (5′ GGGAATTCCATATGAAGAAAATATTTTGTAT TTCG 3′) and P2R (5′ TATGTAGAGATAAGATCCACTTGC 3′) were used for amplification of the *ligB* locus (Fig. 1A). Gene inactivation by plasmid insertion was confirmed by using primers B1F (5′ ACCTGGAATTCCTCTAA TACGGATATT 3′) and B1R (5′ GAATATAAAGGTTTGGAAAAAGA AACG 3′) for PCR amplification.

Immunoblotting. Mutant and wild-type *L. interrogans* Fiocruz L1-130 strains were grown in EMJH medium until the optical density at 420 nm was 0.2. *Leptospira biflexa* was also used as a control in these experiments. Bacteria were washed in PBS. After the concentration was adjusted to 2×10^8 bacteria/per well (20 μl), the cells were solubilized in 62.5 mM Tris hydrochloride (pH 6.8)–10% glycerol–5% 2-mercaptoethanol–2% sodium dodecyl sulfate. Crude protein extracts were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a discontinuous buffer system. After transfer to nitrocellulose membranes, immunoblots were blocked in 0.05 M Tris-buffered saline (pH 7.4)–0.05% (vol/vol) Tween 20 with 5% (wt/vol) nonfat dry milk. The blots were washed, incubated for 1 h at room temperature with a 1,000-fold dilution of mouse ascites containing MAb to the LigB identical repeat region (LigA/B) or with a 10,000-fold dilution of hyperimmune rabbit antisera to LipL41, and probed with goat anti-mouse and anti-rabbit immunoglobulin G (IgG) antibodies conjugated to alkaline phosphatase (Sigma). Immunoblots were developed in a nitroblue tetrazolium—5-bromo-4-chloro-3-indolylphosphate (BCIP) solution (Bio-Rad).

Immunofluorescence assays. Immunofluorescence labeling was performed using a modified protocol of Cullen et al. (12). Suspensions containing 10^7 live leptospires in 10 μl of PBS were placed on poly-L-lysine-coated (Sigma) slides and incubated for 1 h in a humidified chamber. The slides were washed twice with PBS, blocked with PBS containing 1% bovine serum albumin (BSA) (Sigma) (PBS-BSA), and incubated for 1 h with hyperimmune rabbit antisera to the LigB nonidentical region (LigBNI) and the LigA nonidentical region (LigANI) (diluted 1:100 in PBS-BSA) and with control rabbit antisera to a leishmanial antigen. The slides were washed gently with PBS-BSA and incubated with donkey anti-mouse IgG antibodies conjugated to Alexa dye (Molecular Probes) or with goat anti-rat IgG antibodies conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories) for 1 h at 37°C. The slides were washed twice with PBS-BSA and incubated with 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) (Molecular Probes) for 1 h at room temperature. The slides were washed and then mounted in antifading solution (Prolong; Molecular Probes) and visualized by fluorescence microscopy (Olympus BX51).

Hamster model of acute infection. Groups of four Golden Syrian male hamsters that were 5 to 8 weeks old were inoculated intraperitoneally with 10, 10^2, 10^4, and 10^6 cells of the wild-type and *ligB* mutant *L. interrogans* Fiocruz L1-130 strains. Negative control animals were inoculated intraperitoneally with 1 ml of EMJH medium. Animals were monitored daily for clinical signs of leptospirosis (prostration and jaundice) and survival. Surviving animals were killed after a 21-day postchallenge follow-up period. The 50% lethal dose (LD_{50}) of *L. interrogans* strain Fiocruz L1-130 in 5- to 8-week-old hamsters was approximately 10^1 leptospires. Culture isolation and immunofluorescence studies were performed using kidney and liver samples (42) to determine whether surviving animals had a persistent infection. The protocols used for animal experiments followed the guidelines of the Animal Care and Use Committee of Fundação Oswaldo Cruz.

Rat model of chronic infection. Groups of four or eight Wistar rats (Fiocruz, Rio de Janeiro, Brazil) that were 4 to 5 weeks old were inoculated intraperitoneally with 10^8 cells of the wild-type and *ligB* mutant strains in 1 ml EMJH medium. Control animals were inoculated intraperitoneally with 1 ml of sterile EMJH medium. Animals were sacrificed 15 days after infection. Necropsies were performed immediately after sacrifice. Kidney and liver samples were fixed in 4% formalin, embedded in paraffin, and cut into 4- to 5-μm sections for conventional histology analysis. Renal tissue samples were homogenized in 5 ml of EMJH liquid medium for 10 min. After separation of the supernatant from the tissues, 500 μl of the supernatant was used to inoculate 5 ml EMJH liquid medium, which was subsequently incubated at 29°C. The cultures were examined weekly for growth by dark-field microscopy for up to 6 weeks.

Histopathology studies. Groups of three hamsters were inoculated with 10^6 cells of the wild-type and *ligB* mutant *L. interrogans* Fiocruz L1-130 strains and euthanized on day 9 postchallenge. Tissues (liver, kidneys, and lungs) were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned using routine histological procedures to obtain 4- to 5-μm sections that were then stained with hematoxylin and eosin. For immunohistochemistry analysis, the paraffin was removed from the sections with xylene and ethanol. The tissues were blocked by incubation of sections with 1.0% BSA at room temperature for 20 min. The tissues were incubated with a 1,000-fold dilution of antiserum to LipL32 (17) at room temperature for 1 h. Samples were treated with 0.3% hydrogen peroxide for 15 min at room temperature and then incubated at room temperature for 30 min with goat anti-mouse or anti-rabbit antibodies conjugated to peroxidase (Histostain-Plus kit; Invitrogen). Enzyme reactions were developed using 3,3′-diaminobenzidine (Sigma).

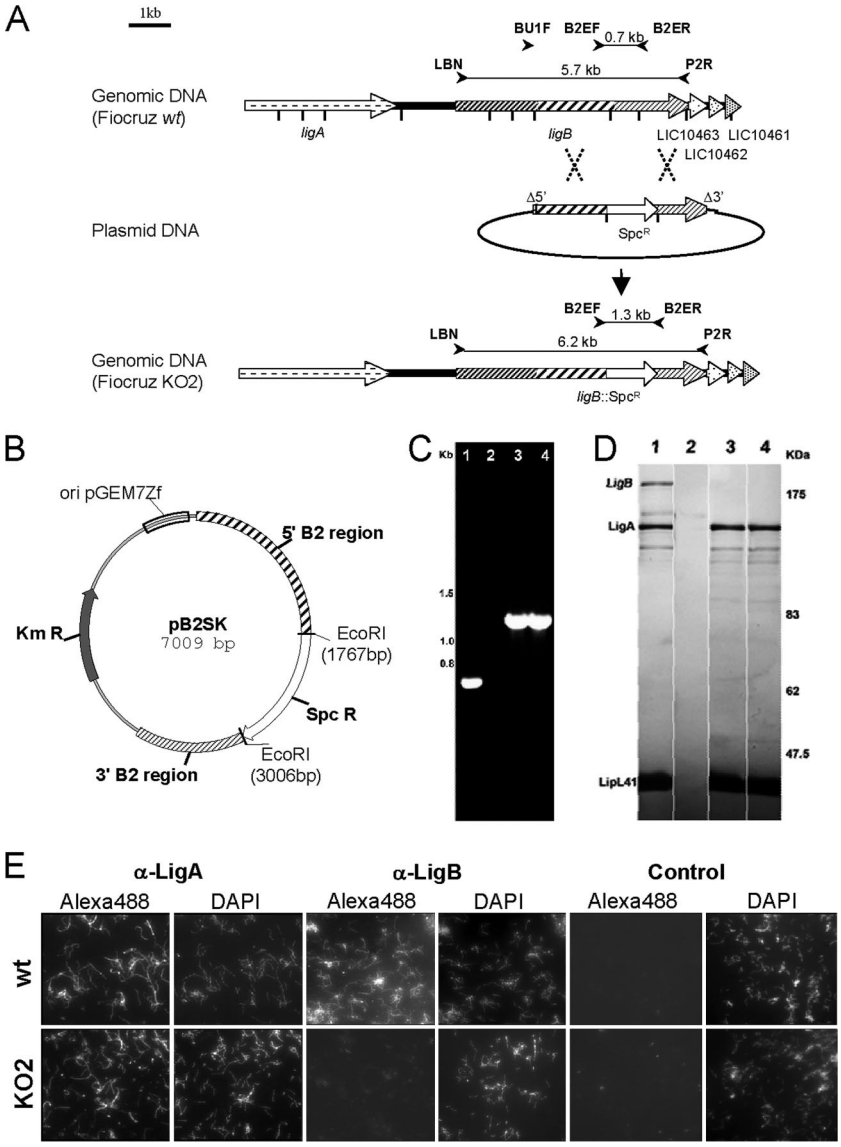

FIG. 1. Disruption of *ligB* in *L. interrogans* strain Fiocruz L1-130. (A) Schematic representation of the genotype of the parental (Fiocruz *wt*) and *ligB* (Fiocruz KO2) mutant strains. The LigB protein has a tripartite structure which includes an N-terminal identical repeat region, a nonidentical repeat region, and the C-terminal region. The vertical bars indicate EcoRI restriction sites. The locations of primers used to check for allelic exchange, as well as the expected sizes of amplified products, are indicated. (B) Map of the pB2SK plasmid, in which the Spc^r cassette was inserted between EcoRI sites in the B2 region of *ligB*. (C) PCR amplification of chromosomal DNA from the *L. interrogans* wild-type strain (lane 1), *L. biflexa* strain Patoc1 (lane 2), the *L. interrogans ligB* KO2 mutant (lane 3), and the *L. interrogans ligB* KO2 mutant reisolated from hamsters after infection (lane 4) with primers B2EF and B2ER, as shown in panel A. (D) Western blot of LigA and LigB expression in the *L. interrogans* wild-type strain (lane 1), *L. biflexa* strain Patoc1 (lane 2), the *L. interrogans ligB* mutant (lane 3), and the *L. interrogans ligB* mutant which was reisolated from hamsters (lane 4). Blots were also probed with LipL41 antiserum as a reference. (E) Immunofluorescence assays were performed with *L. interrogans* wild-type (wt) and *ligB* mutant (KO2) strains. Strains were labeled with antibodies against LigANI (α-LigA0), LigBNI (α-LigB), and a control. Alexa- and fluorescein isothiocyanate-conjugated secondary antibodies were used to detect surface-bound antibodies to LigANI and LigBNI, respectively. A DAPI counterstain was used to document the presence of leptospires. The photomicrograph show the results of one of three representative experiments.

Host cell adhesion assay. Madin-Darby canine kidney (MDCK) cells were harvested by treating cell cultures with 0.05% trypsin and 0.02% EDTA in PBS and then plated on 24-well plates in Dulbecco's modified Eagle's medium (Cultilab) without antibiotics. Cell viability was determined by trypan blue exclusion, and 500-μl portions of cell suspensions containing 2×10^5 cells per ml were layered on round glass coverslips in 24-well tissue culture plates. The plates were incubated for 24 h and washed twice with PBS to remove nonadherent cells. Bacteria were suspended in warm (37°C) cell culture medium at a concentration of 2×10^7 cells per ml. Wild-type and *ligB* mutant strains were used at the same time in each experiment. A 500-μl aliquot of each bacterial suspension was then added to the wells at a bacterium/cell ratio of 100:1. The plates were incubated under static conditions for 1 h at 37°C (30). Experiments were performed in triplicate. Coverslips were washed three times in PBS to remove nonadherent bacteria. An immunofluorescence analysis was performed as described above. The first antibody was anti-LipL32 MAb or anti-Omp Salmonella MAb, and the second antibody was anti-mouse antibody conjugated with Alexa 488 (Molecular Probes). DAPI and Alcian Blue were used to stain the nucleus and the cytoplasm, respectively. The numbers of leptospires and MDCK cells were deter-

mined by examining 10 high-power fields during fluorescence microscopy. Student's *t* test was used to evaluate the significance of differences between the numbers of associated leptospires per host cell in incubations with wild-type and *ligB* mutant strains.

RESULTS

Allelic exchange mutagenesis of *L. interrogans ligB*. Pathogenic *Leptospira* species possess between one and three *lig* genes. *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 contains two *lig* genes, *ligA* (3,675 bp) and *ligB* (5,673 bp), which encode polypeptides with molecular masses of 128 and 201 kDa, respectively. The third gene, *ligC*, was identified as a pseudogene in this *L. interrogans* strain (26). The *ligB* locus was used as a target for mutagenesis by allelic exchange in *L. interrogans* strain Fiocruz L1-130. A gene replacement construct was generated by cloning *ligB* into a suicide vector, which deleted a portion of the *ligB* open reading frame, which was replaced by an Spc^r cassette. The amounts of homologous *L. interrogans* DNA present on the two sides of the spectinomycin marker were 1.8 and 1 kb (Fig. 1A).

The origin of replication used in the plasmid construct was that from pGEM7Zf, which is nonfunctional in *Leptospira* spp. Thus, any Spc^r colonies arising after electroporation of this plasmid into *L. interrogans* should have resulted from recombination of the plasmid with the host genome. Strain Fiocruz L1-130 was electroporated with the UV-irradiated plasmid construct as previously described (38) and plated on solid medium containing spectinomycin. A total of six transformation experiments were performed, one of which yielded two transformants. The two mutant clones were obtained on the same plate and may have been mutants of siblings.

To confirm that homologous recombination events occurred at the origin of the Spc^r phenotype, the *ligB* locus was analyzed by PCR mapping of genomic DNA obtained from *L. interrogans* transformants. PCR amplification with primers B2EF and B2ER, which normally produce a 0.7-kb product with wild-type *L. interrogans*, generated a 1.3-kb DNA fragment with the two Spc^r recombinants analyzed (Fig. 1B). The amplified product was the expected size if a *ligB* mutant resulted from integration of the Spc^r cassette by double-crossover recombination in the *ligB* chromosomal locus (Fig. 1A and B). Furthermore, PCR amplification with primers B2N and P2R yielded a product which had a size that was consistent with a gene replacement event in *ligB* loci. Immunoblotting with an anti-LigA/B MAb confirmed that LigB-reactive polypeptides were not present in the mutant (Fig. 1C). Moreover, by evaluating the reactivity with antibodies raised against LigA, we were able to detect LigA, which is encoded by the *lig* gene located upstream of *ligB* (26). This finding indicates that *ligB* inactivation does not modify *ligA* expression. Immunofluorescence studies also demonstrated that the *ligB* mutant did not express LigB, whereas the wild-type strain did express this protein (Fig. 1D). In contrast, antisera to LigA labeled live *ligB* mutant and wild-type leptospiral strains similarly.

By using another approach, we cloned an internal fragment of *ligB* lacking the 5′ and 3′ ends of the open reading frame in a suicide vector. Five transformants were recovered after electroporation in *L. interrogans* strain Fiocruz L1-130 in one of four transformation experiments. With all clones tested we obtained integration of the plasmid via a single crossover event, which generated two copies of the targeted gene, one with a deletion at the 5′ end of the gene and the other with a deletion at the 3′ end, thereby rendering it inactive, as confirmed by immunoblotting (data not shown).

TABLE 1. Virulence of the wild-type and *ligB* mutant *L. interrogans* Fiocruz L1-130 strains in the hamster model of leptospirosis

Strain	No. deaths (% of total) with a challenge dose of[a]:			
	10^6 Bacteria	10^4 Bacteria	10^2 Bacteria	10^1 Bacteria
Expt 1				
Wild type	4 (100)	4 (100)	4 (100)	ND[b]
ligB KO2 mutant	4 (100)	4 (100)	4 (100)	ND
Expt 2				
Wild type	4 (100)	4 (100)	4 (100)	ND
ligB KO2 mutant	4 (100)	4 (100)	4 (100)	ND
Expt 3				
Wild type	ND	3 (75)	4 (100)	3 (75)
ligB KO2 mutant	ND	4 (100)	4 (100)	4 (100)

[a] Groups of four hamsters were inoculated with each challenge dose.
[b] ND, not determined.

In addition to the differences in genotype mentioned above, the transformants resulting from allelic exchange and plasmid insertion did not produce LigB; hence, the term *ligB* mutant refers to the double-crossover recombinant KO2 mutant below unless indicated otherwise.

Loss of the *ligB* gene does not affect virulence and persistence in animal models. The *ligB* mutant and wild-type strains had similar cell growth kinetics in liquid EMJH medium (the generation time for the parent and mutant strains was approximately 20 h). Inactivation of *ligB* did not affect cell morphology and motility.

In order to determine whether LigB may have a role in virulence in vivo, we evaluated *ligB* mutants and the parental wild-type strain using the standard hamster model for acute leptospirosis. Different numbers of organisms (log increases in the challenge dose) were inoculated intraperitoneally to produce infection. The proportion of hamsters which died and the proportion which survived for each bacterial concentration were used to calculate the LD_{50}. Three independent experiments in which groups of four animals were infected with each challenge dose were performed (Table 1). The LD_{50} was less than 100 bacteria for both the wild-type and mutant strains. Thus, the lack of LigB expression did not result in loss of virulence as measured by the LD_{50}. In addition, no significant differences in the time to death were observed with the *ligB* and wild-type strains (data not shown). The general health status of the hamsters infected with the *ligB* and wild-type strains was also assessed. Infections with the *ligB* and wild-type strains produced similar pathological findings (jaundice, pulmonary hemorrhage, dissociation of hepatic trabecula, and acute damage of renal tubular epithelia with cell swelling in proximal segments). The immunohistochemistry results showed the same distribution of *Leptospira* in the renal parenchyma (Fig. 2).

The virulence of the *ligB* mutant was evaluated using the rat model for renal colonization. In three separate experiments, groups of four or eight rats were infected intraperi-

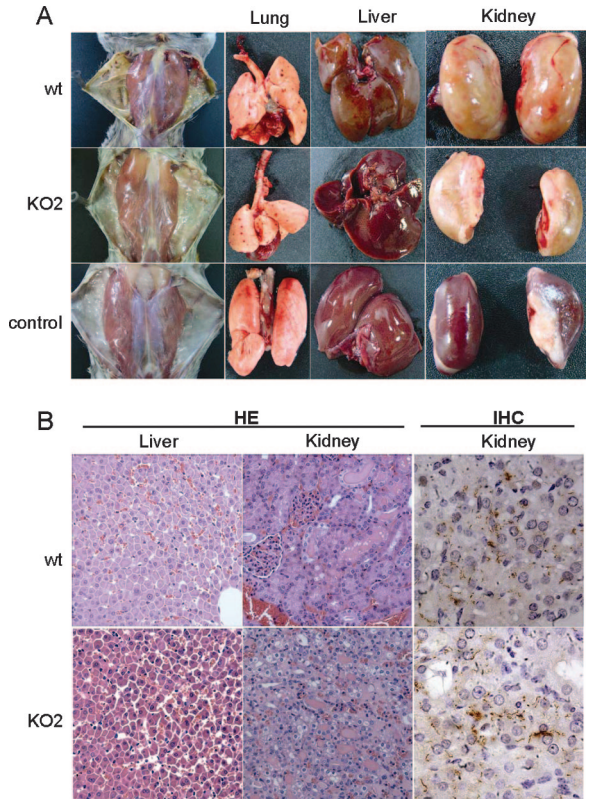

FIG. 2. Pathology in hamsters infected with the *ligB* mutant. (A) Gross appearance of hamsters infected with the wild-type (wt) and mutant *ligB* mutant (KO2) strains and a representative uninfected control hamster. (B) Livers and kidneys from hamsters infected with the wild-type and *ligB* mutant strains of *L. interrogans*. Tissues were stained with hematoxylin and eosin (HE) (magnification, ×400), and the immunohistochemistry analysis was performed with antiserum specific for LipL32 (IHC) (magnification, ×1,000).

toneally with 10^8 cells of either the *ligB* mutant or wild-type strain as previously described (1). Rats were sacrificed 15 days after infection. The *ligB* mutant behaved like the wild type. High levels of both strains were recovered in rat kidneys (Table 2). In hamster and rat experiments, the double-crossover disruptant KO2 was recovered from animals at the time of sacrifice and 2 weeks postchallenge, respectively, and the genotype was confirmed by PCR and immunoblot analysis (Fig. 1). This suggests that the *ligB* disruption was stable in the absence of selection.

In vitro adherence of the *ligB* mutant to MDCK cells. Interactions of *L. interrogans* wild-type and mutant strains with cultured epithelial cells were assayed by examining the adherence of leptospires to epithelial monolayers of MDCK cells. Cell monolayers were incubated using a multiplicity of infection of 100 bacteria per MDCK cell, and subsequent binding was quantified by microscopic analysis. There was not any statistically significant difference between the *L. interrogans ligB* mutant and the wild-type strain in the number of bacteria associated with MDCK cells (Fig. 3). These findings suggest that the *ligB* genotype does not influence in vitro host cell association.

DISCUSSION

Compared to other bacterial species, the genetic data for leptospires and determination of the molecular basis of the pathogenesis of these organisms are in their infancy. Analysis of the complete genome sequences of pathogenic *Leptospira* species revealed that more than 50% of the predicted open reading frames did not exhibit similarity to genes encoding proteins with known functions (8, 32, 39, 40). Until a method for constructing pathogenic leptospire mutants is developed, any function of leptospire proteins, including virulence factors, remains speculative. Previous attempts to inactivate genes in pathogenic *Leptospira* species have been unsuccessful. The putative role of LigB in virulence (21, 26, 34) prompted us to generate an *L. interrogans ligB* mutant.

We used approaches used previously for saprophytic *Leptospira* species (5, 16, 24, 25, 28, 37, 38, 45) to carry out gene targeting by homologous recombination in the pathogen *L. interrogans*. Although the efficiency of the transformations was low, our results show the feasibility of performing allelic exchange in pathogenic *Leptospira* spp. by homologous recombination. Our previous attempts to generate homologous recombination in *L. interrogans* were not successful (unpublished data), presumably due to the target gene chosen. The use of a large region of homologous DNA (more than 1 kb) may have increased the probability of homologous recombination. The *ligAB* locus appears to be the target of fragment rearrangements and recombination events. It has been suggested that *ligA* was created from *ligB* by gene duplication, since the fragments which encode the first six Big domains are identical in the two genes (26). Furthermore, sequence analysis of *lig* genes from collections of pathogenic *Leptospira* species resulted in evidence of recombination between *Leptospira* species at this locus (unpublished data). Finally, in addition to allelic exchange derived from a double homologous recombination event, we showed that targeted integration of a suicide plasmid which contains a 5′- and 3′-truncated fragment of the gene of interest can facilitate targeted mutagenesis in *L. interrogans*.

For members of the superfamily containing the Big proteins, previous studies have demonstrated that compared to the wild-

TABLE 2. Renal colonization of *Rattus norvegicus* with the wild-type and *ligB* mutant strains of *L. interrogans* Fiocruz L1-130 after experimental challenge

Strain	No. animals with evidence of *Leptospira* renal colonization (% of total) based on[a]:	
	Culture isolation	Immunofluorescence
Expt 1		
Wild type	6 (75)	8 (100)
ligB KO2 mutant	8 (100)	8 (100)
Expt 2		
Wild type	4 (100)	4 (100)
ligB KO2 mutant	4 (100)	4 (100)
Expt 3		
Wild type	8 (100)	8 (100)
ligB KO2 mutant	5 (63)	5 (63)

[a] Groups of eight rats and groups of four rats were inoculated with 10^8 leptospires in experiments 1 and 3 and in experiment 2, respectively.

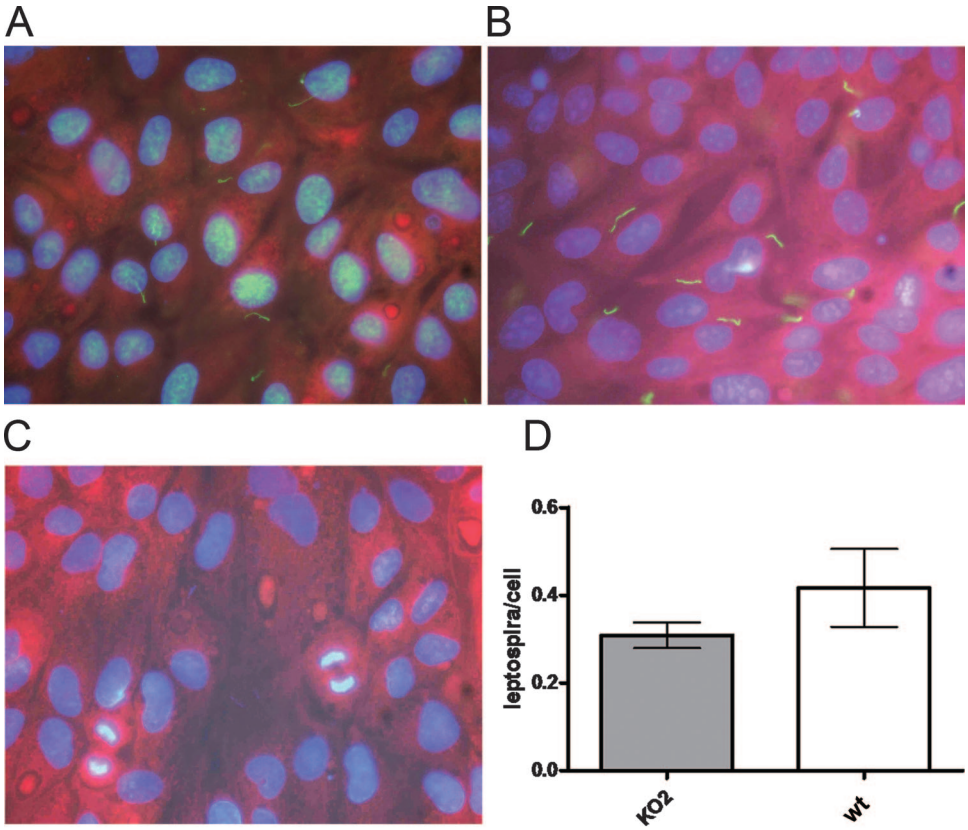

FIG. 3. Adherence of the *L. interrogans ligB* mutant to MDCK epithelial cells. The adherence of the *L. interrogans* wild-type (A) and *ligB* mutant KO2 (B) strains to MDCK epithelial cells was examined. Anti-*Salmonella* OmpA MAb was used as a control (C). Representative micrographs obtained by fluorescence microscopy are shown. (D) Attachment ratios (means ± standard deviations) determined using 10 random fields. wt, wild type.

type strain, an intimin-deficient enteropathogenic *E. coli* strain is defective for adherence to cultured cells and for intestinal colonization (33). Similarly, analysis of a *Y. enterocolitica inv* mutant suggested that invasin is necessary for efficient translocation of the bacteria across the intestinal epithelium (36). *ligB* is conserved among all pathogenic *Leptospira* species and is upregulated when bacteria confront the host environment (26, 34). Furthermore, expression of *ligB* is correlated with the virulence status of *Leptospira* strains (26). Therefore, the working hypothesis has been that LigB is essential for the bacteria to survive, disseminate, and/or colonize in the host.

Yet we found that loss of *ligB* was not associated with a loss of virulence phenotypes. Inoculation of *ligB* mutants produced the same acute disease manifestations and lethal outcomes that were observed in hamsters infected with wild-type strains. In addition, inoculation of *ligB* mutants resulted in efficient renal colonization in experimental rats similar to that observed with wild-type strains.

Moreover, we found that the *L. interrogans ligB* mutant was able to adhere to epithelial cells in vitro. The interaction of *L. interrogans* with host cells is critical for dissemination in the host (4). Although the LigB protein has been shown to bind in vitro to host extracellular matrix moieties (9, 23), our findings suggest that there may be other modes of leptospiral attachment to host epithelial cells. As a caveat, we did not examine whether *ligB* mutants bind to extracellular matrix components, including fibronectin, and further studies are required to evaluate this possibility. Furthermore, the mechanism of association of the pathogen with the host cell in vivo may be quite different than what is observed in vitro; therefore, we cannot exclude the possibility that Lig proteins mediate host cell interactions based on observations made with in vitro assays alone.

Because of the location of the Spc^r cassette in the 3′ end of *ligB*, a truncated LigB protein could have been expressed. However, immunoblot analysis using polyclonal and monoclonal antibodies against recombinant fragments located upstream of the disruption site did not allow identification of any fragments in the mutant strains. Furthermore, whereas these antibodies stained strongly with the wild-type strain in immunofluorescence studies, no signal was associated with the mutant strains. It is therefore unlikely that a truncated *ligB* fragment was expressed in the mutants.

In the present study, the data obtained with the *ligB* mutant suggest that an absence of LigB does not lead to a loss of virulence and a loss of colonization in the acutely and chronically infected animal models, respectively. In our challenge experiments, hamsters and rats were infected by intraperitoneal inoculation of leptospires. We cannot exclude the possibility that LigB may play a role in penetration of the host or other early events during infection. Alternatively, the fact that the *ligB* mutant remained virulent may have been due to func-

tional redundancy in the bacteria. The numerous lipoproteins which are present in leptospires (11) in addition to the LigB protein may compensate for the loss of LigB expression. Several surface-associated *Leptospira* proteins, including LigA, have been shown to interact in vitro with extracellular matrix components (2, 3, 18, 31, 44). Thus, the function of LigB may be replaced to various extents by other lipoproteins which may play a role in host-cell interactions. LigA and LigB proteins contain Big domains that may have redundant functions (9, 27, 34). Choy et al. demonstrated that domains within LigA and LigB proteins bind specifically to fibronectin in vitro (9). A phenotype distinct from that of the parental strain may occur only when both genes are disrupted. Therefore, further studies should include generation of *ligA* and *ligAB* mutants.

In conclusion, we demonstrated for the first time that site-directed homologous recombination can be successfully achieved in pathogenic *Leptospira*. The approaches used in this study, therefore, make it feasible to produce knockout mutations in putative virulence-associated genes in *Leptospira* and evaluate the roles that these genes may play in leptospiral pathogenesis.

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4. MANUSCRITO 2

Título:

Major Surface Protein LipL32 Is Not Required for Either Acute or Chronic Infection with *Leptospira interrogans*.

[LipL32, a maior proteína de superfície não é necessária para a infecção aguda ou crônica causada por *Leptospira interrogans*].

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Objetivo específico 1

1. Determinar se a perda de genes que codificam para proteínas associadas à superfície das leptospiras, influencia na capacidade do agente em induzir colonização persistente e doença na infecção experimental:

1.2 Identificar se LipL32, a maior lipoproteína de membrana (OMP), está envolvida no processo de colonização renal persistente;

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Major Surface Protein LipL32 Is Not Required for Either Acute or Chronic Infection with *Leptospira interrogans*▽†

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***Leptospira interrogans* is responsible for leptospirosis, a zoonosis of worldwide distribution. LipL32 is the major outer membrane protein of pathogenic leptospires, accounting for up to 75% of total outer membrane protein. In recent times LipL32 has become the focus of intense study because of its surface location, dominance in the host immune response, and conservation among pathogenic species. In this study, an *lipL32* mutant was constructed in *L. interrogans* using transposon mutagenesis. The *lipL32* mutant had normal morphology and growth rate compared to the wild type and was equally adherent to extracellular matrix. Protein composition of the cell membranes was found to be largely unaffected by the loss of LipL32, with no obvious compensatory increase in other proteins. Microarray studies found no obvious stress response or upregulation of genes that may compensate for the loss of LipL32 but did suggest an association between LipL32 and the synthesis of heme and vitamin B_{12}. When hamsters were inoculated by systemic and mucosal routes, the mutant caused acute severe disease manifestations that were indistinguishable from wild-type *L. interrogans* infection. In the rat model of chronic infection, the LipL32 mutant colonized the renal tubules as efficiently as the wild-type strain. In conclusion, this study showed that LipL32 does not play a role in either the acute or chronic models of infection. Considering the abundance and conservation of LipL32 among all pathogenic *Leptospira* spp. and its absence in saprophytic *Leptospira*, this finding is remarkable. The role of this protein in leptospiral biology and pathogenesis thus remains elusive.**

Leptospira interrogans is a zoonotic spirochete with a worldwide distribution. In the chronic carrier state, host animals such as rats do not exhibit overt disease but are colonized by *Leptospira* in their renal tubules and shed bacteria in their urine. Humans are incidental hosts that become infected through exposure to contaminated water, soil, or urine. In the acute form of leptospirosis, disease severity ranges from asymptomatic infection to multiple organ failure, pulmonary hemorrhage, and death (13).

The cellular and molecular mechanisms of leptospiral pathogenesis remain unclear. One of the major sites of interaction with the host is the bacterial outer membrane (OM). Analysis of the *L. interrogans* OM has identified a number of proteins (11, 12), the most abundant of which is LipL32, a 32-kDa lipoprotein estimated to account for a remarkable 75% of the OM proteome (11). LipL32 is also the most abundant surface-exposed protein (12).

LipL32 is found in all pathogenic species tested to date and is highly conserved, with average amino acid identity over 98%, but it is not found in saprophytic species (16, 17). LipL32 is expressed during both chronic and acute infection and is highly immunogenic (15, 16, 27). These features have generated interest in LipL32 as a potential diagnostic reagent in both PCRs (21) and enzyme-linked immunosorbent assays (14). There has also been much interest in the potential of LipL32 to generate heterologous immunity, overcoming the limitations of serovar-specific immunity. However, to date LipL32-based vaccines have met with limited success (5, 6).

The abundance, conservation, unique presence in pathogenic species, and immunogenicity of LipL32 are consistent with an important role in pathogenesis. Available microarray data provide little insight into the role of LipL32 since gene expression is unchanged under conditions of different temperatures (22). Recent studies have shown that LipL32 may act as an adhesin binding to collagen, laminin, and fibronectin (18, 19) while LipL32 has also been associated with hemolysis (20). However, the precise role of LipL32 in pathogenesis remains unknown.

In this study an *lipL32* mutant was constructed by transposon mutagenesis. To our surprise, analysis of this mutant in the hamster model of acute infection and rat model of chronic infection showed that LipL32 is not required for causing either acute leptospirosis or renal colonization.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *L. interrogans* serovar Manilae strain L495 was obtained from N. Koizumi, National Institute of Infectious Diseases,

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Tokyo, Japan. Mutant M777 is a Tn*SC189* mutant constructed from strain L495 with an insertion after base 3979065 (*L. interrogans* serovar Lai genome) and retains virulence (25). Bacteria were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton Dickinson) at 30°C without aeration. Plates were made by solidification of medium with 1.5% agar. Kanamycin (25 μg/ml) was added where appropriate. To examine survival in water, mid-log-phase leptospires were centrifuged to remove culture medium, resuspended in sterile deionized water, and then inoculated into 5 ml of sterile deionized water in triplicate at a concentration of 10^8 leptospires/ml. Samples were removed at regular intervals for enumeration using a Petroff-Hausser chamber.

Transposon mutagenesis. Transposon mutagenesis was conducted as described previously (4). In brief, leptospires were grown to late log phase and made electrocompetent by repeated washing in ultrapure water. Plasmid pSC189 (8) containing transposon Tn*SC189* was introduced into cells by electroporation, and transformants were selected on EMJH plates containing kanamycin. The location of transposon insertion was determined by sequencing directly from the chromosome (24). PCR primers used to confirm the mutant genotype were 3453 (5′-CGTCATGGTCTTTGTAGTCTATGG-3′), annealing to the 5′ end of the transposon; 4777 (5′-AAAAGATCTTTAACCAACAGATGCAACGAAAGA-3′), annealing to the 3′ end of *lipL32*; and 4956 (5′-TTTTGATATCTAAGAA TTTCTGATGTTTCAATCGT-3′), annealing to the intergenic region proximal to *lipL32*.

Protein analysis techniques and leptospiral adhesion assays. Preparation of total membranes was carried out as described previously (19). Western blotting and one-dimensional and two-dimensional gel electrophoresis were carried out by standard methods (11). Spots from two-dimensional gels were excised and analyzed by mass spectrometry or identified through Western blotting with anti-LipL32 serum kindly provided by D. Haake (16). Adhesion of leptospires to Matrigel (Becton Dickinson), laminin, and collagen (Sigma) and the laminin binding blot were assayed as described previously (19).

Microarray studies. Microarray analysis comparing M933 and intergenic mutant M777 was performed using three biological replicates, each with a dye swap, resulting in six arrays. RNA purification, probe preparation, hybridizations, and analysis were carried out as described previously (22).

Hamster infection model. Golden hamsters were infected by one of two routes. For systemic infection, groups of eight hamsters were injected intraperitoneally with 10^3 leptospires in 100 μl of EMJH medium and monitored for 14 days. Tissue was fixed in 10% formalin for histopathology analysis. For the mucosal route of infection, groups of 10 hamsters were inoculated with 10^6 leptospires instilled onto the eye in a volume of 10 μl in EMJH medium. In accordance with animal ethics requirements, moribund animals were euthanized.

Rat infection model. Groups of six-week-old Wistar rats (eight per group) were inoculated intraperitoneally with 10^8 leptospires in 1 ml of EMJH medium as described previously (1). Control animals were injected with 1 ml of sterile EMJH medium. We have shown previously that colonization is well established by 15 days and correlates with long-term carriage (1). After 15 days animals were euthanized. Kidney tissue was passed through a sterile syringe, homogenized in 5 ml of EMJH liquid medium, and reinoculated into 5 ml of liquid EMJH medium. Cultures were maintained at 30°C and examined weekly for growth by dark-field microscopy for up to 6 weeks postinoculation.

Touch preparations of sectioned kidney were used for the direct visualization of leptospires. Kidney fragments were spotted onto poly-L-lysine (Sigma-Aldrich)-coated glass slides, which were then dried and fixed in acetone for 3 min. Leptospires were visualized by immunofluorescence as described below. Additional kidney fragments were fixed in 4% formalin, embedded in paraffin, and cut into 4- to 5-μm sections for conventional histology and immunohistochemical studies. Immunofluorescence labeling was performed using a protocol modified from Cullen et al. (12). Slides were washed twice with phosphate-buffered saline (PBS) after fixation, blocked twice with 2% bovine serum albumin (Sigma) (PBS-BSA) for 20 min each at 30°C, and incubated for 1 h with rabbit antiserum to whole *L. interrogans* serovar Icterohaemorrhagiae (diluted 1:200 in PBS-BSA) or control normal rabbit serum at 30°C. Slides were washed with PBS-BSA and incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories) for 1 h at 30°C. Slides were washed, mounted in antifading solution (Prolong-Molecular Probes), and visualized by fluorescence microscopy (Olympus BX51).

Kidney tissue sections were treated according to routine histological procedures, and sections were then stained with hematoxylin and eosin. For immunohistochemical analyses, paraffin was removed from tissue sections with xylene and ethanol. Slides were blocked with 10% powdered milk at room temperature for 20 min and then incubated with a 1:1,000 dilution of rabbit antiserum against LipL32 or whole leptospires at room temperature for 1 h. Slides were treated with 0.3% hydrogen peroxide for 15 min at room temperature and then incubated at room temperature for 30 min with peroxidase-conjugated anti-rabbit immunoglobulin (Histostain-Bulk Kit; Invitrogen). Enzyme reactions were developed using 3,3′-diaminobenzidine (Sigma). Slides were examined in a blinded manner to prevent bias in the interpretation of the results.

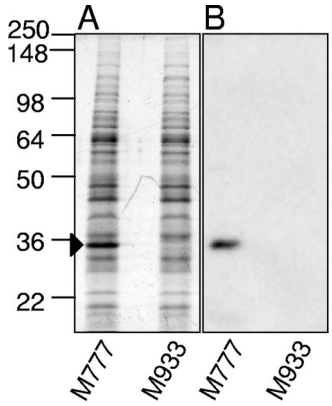

FIG. 1. Confirmation of the *lipL32* mutant phenotype. Whole-cell lysates of M933 (*lipL32* mutant) and M777 (control) were analyzed by SDS-PAGE with Coomassie blue staining (A) and by Western blotting with anti-LipL32 serum (B). The band corresponding to LipL32 is indicated by an arrow. Numbers on the left correspond to approximate molecular sizes (kDa).

RESULTS

Construction of an *L. interrogans lipL32* mutant. Random transposon mutagenesis was performed on *L. interrogans* serovar Manilae as described previously (4), and the location of transposon insertion was determined by direct sequencing from the chromosome (24). In mutant M933 the transposon was determined to have inserted after base 2620245 of the Lai genome, 177 bp into the 816-bp gene encoding LipL32 (also known as LA2637 or LIC11352). An intergenic mutant (M777) constructed from the same parent strain with Tn*SC189* was also used as a control in some experiments (25). The growth rate of M933 in EMJH medium with kanamycin was slightly slower than that of wild-type *L. interrogans* or M777 (data not shown). By dark-field microscopy, M933 had a slightly less uniform cell size, but otherwise bacteria were motile and appeared normal (data not shown). The mutation was confirmed by PCR.

Characterization of the OM of the *lipL32* mutant. LipL32 is the major protein of the OM, accounting for an estimated 75% of OM protein (11). Immunoblotting with specific LipL32 antiserum showed that a band of approximately 32 kDa was absent in the mutant (Fig. 1). Two-dimensional gel electrophoresis analysis of membrane preparations showed no difference in protein profiles between *L. interrogans* serovar Manilae wild type and strain M933 except for the absence of LipL32 (data not shown).

As LipL32 is the major OM protein, surface biotinylation was performed to determine the major surface-exposed proteins in the absence of LipL32. Biotinylated proteins were purified on a streptavidin column and examined on a large-format 7.5 to 15% gradient Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel with Coomassie blue staining or by streptavidin blotting. LipL41 was found to be the major OM protein in mutant M933 (Fig. 2).

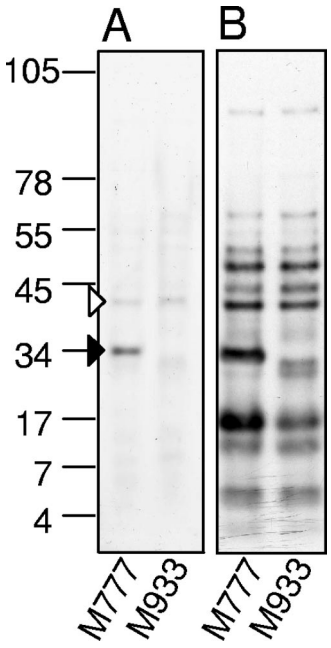

FIG. 2. Detection of surface-exposed proteins in the *lipL32* mutant. M933 (*lipL32* mutant) or M777 (control) cells were surface labeled with biotin, and then surface-exposed proteins were purified on a streptavidin column and separated on a gel. Detection was performed by Coomassie blue staining or streptavidin-horseradish peroxidase detection. The dominant bands correspond to LipL32 (solid arrow head) and LipL41 (open arrow head). Numbers on the left correspond to approximate molecular sizes (kDa).

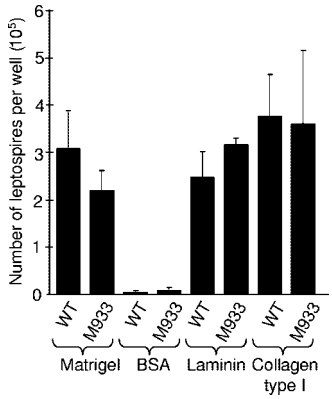

FIG. 3. Leptospiral cell adhesion assay. Leptospires were incubated in microtiter tray wells coated with the indicated substrate for 1 h. After a washing step, adherent leptospires were released by trypsin for direct counting by microscopy. A subset of these data appeared in a previous study (19). Error bars correspond to 1 standard deviation. WT, wild type.

Microarray analysis of the *lipL32* mutant. Loss of the major membrane protein may have potential consequences for the expression of genes encoding other membrane components, potentially providing an indication of function for LipL32. Microarray analysis was conducted to compare gene expression of M933 and the control strain M777. The same samples were used for both two-dimensional gel analysis and microarray analysis. A total of 34 genes (0.96% of total) were upregulated in M933 compared to M777, while 12 genes (0.34%) were downregulated (1.5-fold cut off; $P < 0.01$) (see Table S1 in the supplemental material).

Of the 34 upregulated genes, 18 (53%) had no known function. There was an overrepresentation of genes from cluster of orthologous groups functional category H (coenzyme transport and metabolism), accounting for 21% of upregulated genes, compared to a 3% total in the genome. These genes were from the heme and vitamin B_{12} (cobalamin) synthesis operons. The strongest upregulated protein was LA3881, a hypothetical lipoprotein with alpha integrin-like repeat domains (2.6-fold).

Of the 12 downregulated genes, 7 (58%) had no function attributed. Expression of *lipL32* was very low and likely to be a background signal (0.035-fold). The most strongly downregulated gene was that encoding a putative TonB-dependent receptor, LA2641 (0.072-fold). LA2641 is located on the large chromosome and unlinked to the genes for B_{12} and heme synthesis that are found on the small chromosome.

Adherence to extracellular matrix. It has previously been shown that LipL32 is an adhesin that binds to extracellular matrix (18, 19). A leptospiral adhesion assay (19) was used to determine the adhesion of M933 to a commercially available extracellular matrix preparation, Matrigel. M933 and M777 bound equally to Matrigel-coated microtiter tray wells (Fig. 3). There was likewise no difference in binding to trays coated with collagen type I or laminin.

To investigate the presence in the mutant of other molecules that adhere to extracellular matrix, blots of total membrane were probed with biotinylated laminin. Several bands were found to bind to laminin in both the mutant and the wild type, except for the band at approximately 32 kDa corresponding to LipL32 (Fig. 4). This supports the above data indicating loss of LipL32 in mutant M933 and also demonstrates the presence of additional laminin-binding proteins in M933. An additional band between the 36- and 50-kDa markers was observed in the wild-type sample but not in the mutant. It is possible that this corresponds to a dimer of LipL32.

LipL32 is not required for acute infection in hamsters. To analyze the role of LipL32 in the acute model of infection, hamsters were injected intraperitoneally with 10^3 leptospires. The courses of infection for wild-type serovar Manilae and M933 were indistinguishable, with all hamsters succumbing to infection between days 7 and 9 (Fig. 5 A). Infection with a lower dose of 10^2 leptospires also resulted in no survival for either the wild-type strain ($n = 8$ hamsters) or M933 ($n = 4$), indicating a 100% lethal dose of $<10^2$ leptospires for both strains and showing that there was no subtle attenuation of the *lipL32* mutant.

Histopathological examination of tissues found no difference between the mutant and the wild type. The lungs showed mild to moderate multifocal alveolar hemorrhage while the livers showed diffuse dissociation and single-cell necrosis of hepatocytes with abundant leptospires in the sinusoids. Kidneys from both wild-type- and M933-infected hamsters showed multifocal tubular necrosis, mild tubular proteinuria, and rare hemoglobin casts with numerous leptospires in affected areas. The genotype of M933 reisolated from hamster kidneys was confirmed by PCR using transposon- and gene-specific primers.

Although the loss of LipL32 did not affect the ability of leptospires to cause acute systemic disease after intraperito-

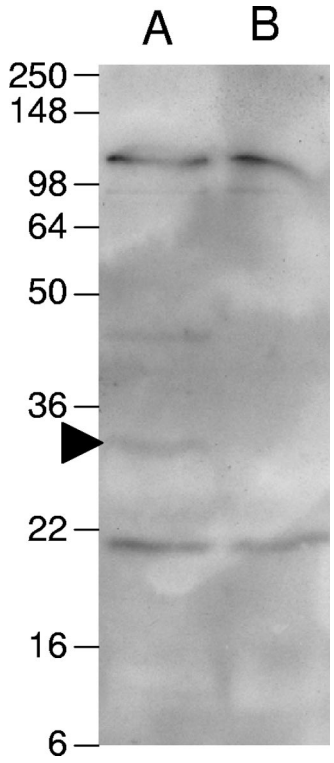

FIG. 4. Laminin binding blot. Bacterial membrane preparations were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with biotinylated laminin and then detected with streptavidin-horseradish peroxidase. The arrowhead indicates the band corresponding to LipL32. The positions of molecular mass markers (kDa) are indicated on the left.

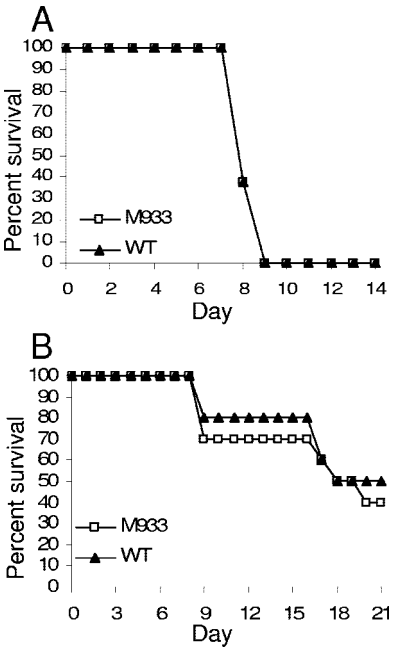

FIG. 5. Survival of hamsters inoculated with M933 (*lipL32* mutant) or wild-type (WT) Manilae. (A) Groups of eight hamsters were inoculated with 10^3 leptospires intraperitoneally and monitored for 14 days. (B) Groups of 10 hamsters were inoculated with 10^6 leptospires via the ocular route and monitored for 21 days.

neal inoculation, LipL32 could play a role early during natural infection when the pathogen penetrates host skin or mucosa prior to dissemination. To evaluate this possibility, hamsters were inoculated with 10^6 leptospires via the conjunctiva. The survival curve for M933 again closely resembled that for wild-type serovar Manilae, indicating no difference in virulence (Fig. 5B).

LipL32 is not required for chronic infection in the rat. In carrier hosts such as rats, *L. interrogans* can establish chronic infections where bacteria colonize the proximal renal tubules and are subsequently shed in the urine. To examine the role of LipL32 in this process, M933, M777, and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were injected into groups of eight rats at 10^8 leptospires per animal. After 14 days the kidneys were removed and examined for colonization by culture isolation and immunofluorescence detection of leptospires in kidney touch preparations. In two experiments all eight animals infected with M933 were colonized (Table 1). This was equal to or greater than the colonization rate observed in rats inoculated with control strains. Histopathological analysis was also conducted on rat kidneys to visualize colonization. Immunohistochemical staining using antiserum to whole bacteria detected leptospires in the lumen of tubules from rats infected with both M933 and M777 (Fig. 6, top row). When anti-LipL32 serum was used, leptospires were detected only in the kidneys of animals infected with the M777 control (bottom row). All reisolated bacteria were shown by PCR to retain the transposon in the original chromosomal location (data not shown).

LipL32 is not required for survival in water. Since LipL32 did not appear to play a role in infection, it was hypothesized that the protein may be required for survival in the environment. To examine survival in water, the mutant M933, M777 (control), and Manilae wild-type strains were centrifuged to remove culture medium and inoculated into sterile deionized water. There was no difference in survival curves over 7 days of counting. After this time all strains lost structural integrity, making identification of live organisms for counting impossible. After 2 weeks of incubation, 100-μl samples were inoculated into 5 ml of EMJH medium; motile leptospires were observed in these cultures after 1 week of incubation, indicating that viability was retained after 2 weeks in water.

TABLE 1. Colonization of rat kidneys in the chronic infection model

Strain	Colonization of rat kidneys (no. of positive animals/total no. of animals tested)			
	Expt 1		Expt 2	
	IFA (%)[a]	Culture (%)[b]	IFA (%)[a]	Culture (%)[b]
M933	8/8 (100)	8/8 (100)	8/8 (100)	8/8 (100)
M777	8/8 (100)	5/6 (83)	6/8 (75)	6/7 (86)
Fiocruz L1-130			8/8 (100)	8/8 (100)

[a] IFA, immunofluorescence assay on kidney touch preparations.
[b] Not all kidney cultures could be interpreted due to contamination.

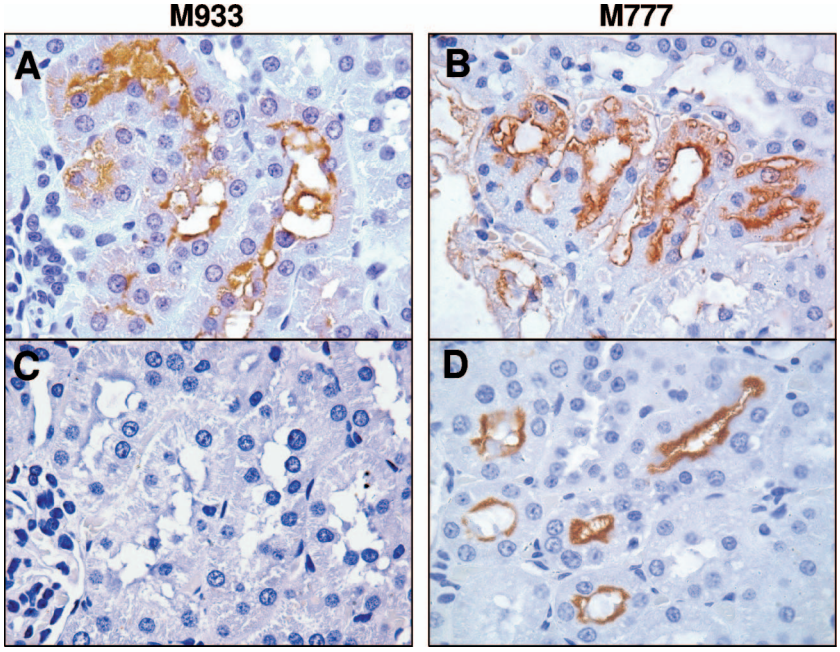

FIG. 6. Rat colonization by M933 (*lipL32* mutant) and M777 (control). Representative photomicrographs of immunohistochemically stained sectioned kidneys from rats infected with the indicated strain. Detection was performed with antiserum to whole *Leptospira* organisms (A and B) or to LipL32 (C and D). Photomicrographs were taken at a magnification of ×1,000.

DISCUSSION

This study describes the construction and characterization of a *lipL32* mutant of *L. interrogans*. A large body of evidence suggests that LipL32 is an important protein in *Leptospira*; LipL32 is by far the most abundant protein in the OM and on the cell surface of pathogenic leptospires, and LipL32 is expressed in vivo and in vitro and stimulates a strong immune response. LipL32 is also absent in saprophytic *Leptospira* spp. while being highly conserved among different pathogenic isolates. It was therefore surprising that the protein is not essential for the survival of *L. interrogans*: a LipL32 mutant exhibits only a slightly delayed growth rate and relatively normal morphology. The slower growth and change in uniformity of cell size might suggest a degree of stress in the mutant; however, this was not supported by the microarray data, which failed to show a general stress response. Taken together, these data suggest that the integrity of the OM was not compromised by the loss of LipL32.

Animal infection experiments showed unequivocally that LipL32 is not required for acute infection in hamsters; mortality in M933-infected hamsters and the pathology of lesions in target organs were found to be the same as those observed in wild-type-infected hamsters. When the mutant strain was administered through a more natural route of infection, the conjunctiva, it was still virulent, indicating that LipL32 is not required for infection by the mucosal route. *L. interrogans* can cause chronic infection in carrier hosts such as rats and dogs, where organisms colonize the renal tubules of the kidneys and are shed in the urine. Further animal studies found that LipL32 is also not required for chronic infection of rats since animal kidneys were colonized when infected with either M933 or the control mutant M777. It is possible that LipL32 is required for virulence in host species not tested in this study or in mechanisms of pathogenesis that were not stringently tested in our model systems. Alternatively, the role of LipL32 may be compensated for by other proteins in the OM although we observed no significant change in the profile of the OM subproteome. To date, only two potential *L. interrogans* virulence factors have been characterized by transposon mutagenesis, the OmpA-like protein Loa22 (29) and the heme oxygenase enzyme, HemO (26).

Recent studies have shown that LipL32 binds to extracellular matrix proteins (18, 19). In this study the *lipL32* mutant showed the same level of adhesion to extracellular matrix as wild-type *L. interrogans* serovar Manilae. A number of other potential leptospiral adhesins were highlighted in an extracellular matrix binding blot, indicating that the adhesin function of LipL32 may be redundant. Additionally, the Lig and Len families of proteins and Lsa21 have all been assigned an adhesin role (2, 3, 9, 32). This study adds to a growing body of evidence that *L. interrogans* has a high degree of redundancy in virulence mechanisms. Recently, several other potential virulence factors with putative virulence roles (such as adhesion or factor H binding) were determined not to be essential for virulence including *ligB* (10) and *ligC*, *lenB*, and *lenE* (25). Redundancy is common in bacterial systems. For example, *Haemophilus influenzae* isolates have up to four *hgp* hemoglobin binding proteins, each of which is sufficient alone for normal hemoglobin-haptoglobin complex utilization (23).

It is a reasonable conclusion that the absence of LipL32 in the mutant would be compensated for by an increase in other OM proteins. Interestingly, the OM protein profile of the LipL32 mutant was largely the same as that for a control strain. LipL41 was found to be the most abundant surface protein in

the absence of LipL32. In *Borrelia burdorferi* OspC is a major OM protein that is required for certain phases of virulence. Mutation of *ospC* can be largely compensated for by the overexpression of other lipoproteins (33). There was no evidence for compensation for the loss of LipL32 in *L. interrogans*. It is possible that existing proteins in the OM can assume the function of LipL32 without a noticeable increase in expression.

Although LipL32 is unlikely to have a transcriptional role in *L. interrogans*, microarray analysis was conducted to identify transcriptional changes induced by the loss of such an abundant lipoprotein. Results did not indicate a general stress response in the mutant. There were few upregulated lipoproteins that may compensate for the loss of LipL32. In general, results indicate a lack of obvious compensation for loss of LipL32. However, there was an upregulation of heme and vitamin B_{12} synthesis pathways, suggesting an association between LipL32 in the uptake or metabolism of these cofactors. Heme and vitamin B_{12} synthesis share common pathways in bacteria (31). The change in expression of these genes may be a response to the strong downregulation of TonB-dependent receptor, LA2641, which may have a role in cofactor uptake. Alternatively, general disruption of the OM proteome may otherwise prevent transport of B_{12} across the OM. The possibility cannot be excluded that secondary suppressor mutations may mask obvious phenotypes in this strain and lead to unexpected transcriptional changes.

Since our findings indicate that LipL32 is not required for normal in vitro growth or pathogenesis in different infection models, the role of this protein remains enigmatic. The aspects of the environmental phase of transmission of *Leptospira* are not well characterized. Although the LipL32 mutant did not show decreased ability to survive in water, it is possible that LipL32 may be important in the normal transmission or environmental phase of the life cycle. We recently showed that an ortholog of LipL32 is found in *Pseudoalteromonas tunicata*, a marine bacterium that is associated with tunicates (19). However, LipL32 is retained in *Leptospira borgpetersenii*, which does not persist well outside the host and has significantly reduced its genome to reflect this (7). Furthermore, LipL32 is absent in the saprophytic species *Leptospira biflexa*, which has only an environmental existence (28). *L. interrogans* has been shown to form biofilms (30), but the role of LipL32 in this process is unknown.

In conclusion, it is intriguing that *L. interrogans* produces LipL32 at very high levels at great metabolic cost when it does not appear to be essential for survival or virulence. Considering the many aspects of LipL32 expression and conservation that indicate it is an important protein, LipL32 remains a paradox.

ACKNOWLEDGMENTS

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6. MANUSCRITO 3

Título:

Infection kinetics of high and low dose of *Leptospira interrogans* in the hamster model determined by a quantitative real time PCR assay

[Cinética da infecção de alta e baixa dose de *Leptospira interrogans* no modelo animal de hamster determinada por um PCR quantitativo em tempo real].

A ser submetido.

Objetivo específico 2

2. Estudo da cinética da disseminação e tropismo das leptospiras para os tecidos do hospedeiro, usando um ensaio de PCR quantitativo durante a infecção experimental:

2.1 Determinar a cinética da infecção com alta e baixa dose, através do estudo de disseminação da *Leptospira interrogans* sorovar Copenhageni nos tecidos, utilizando a rota intraperitoneal e conjuntival;

2.2 Estabelecer se existe tropismo específico para o rim ou algum outro tecido em especial e se a carga do patógeno está relacionada com alterações histopatológicas nos diferentes tecidos;

2.3 Determinar se a cinética de distribuição nos diferentes tecidos é similar, quando comparado com clones virulentos e clones com atenuação da virulência.

Infection kinetics of high and low dose of *Leptospira interrogans* in the hamster model determined by a quantitative real time PCR assay

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ABSTRACT

Leptospira is a motile life-threatening spirochete which is able to disseminate to tissues and causes chronic carriage in animal hosts. However, its ability to spread and the importance of its spread to the pathogenesis of the disease are not well understood. We evaluate the use of a quantitative real time PCR assay (TaqMan) using the *lipl32* gene as a target to determine the kinetics of the leptospiral infection in perfused Golden Syrian Hamster. The analysis after 1 hour of infection using a high intraperitoneal inoculum dose of strain Fiocruz L1130 (10^8 leptospires) demonstrated that leptospires were present in all tissues reaching levels of 10^2-10^5 leptospires/g including the eye and brain with no tropism for any specific tissue. With a low intraperitoneal inoculum dose (250 leptospires), we were able to detect leptospires only after 5 days of infection (10^2-10^4 leptospires/g), and, after 8 days, levels were at 10^5-10^8 leptospires/g in all tissues with macroscopic lesions. Infection with a high inoculum dose of a motile attenuated clone of Fiocruz L1130 (40 sequenced *in vitro* passages) showed that this strain was able to disseminate but not to cause death of the animals with a significant statistical difference when the levels of leptospires detected in tissues after 4 days of infection were compared with the virulent (10^6-10^9 leptospires/g) and avirulent strains (10^4-10^6 leptospires/g). Same result was observed when we tested a non-motile strain that was shown to be avirulent but capable of infecting tissues and detectable after 4 days of infection. However, this strain was not able to cause chronic carriage after 21 days post-challenge. Our results demonstrated that leptospires can rapidly disseminate through all tissues after 1 hour post-challenge with a high inoculum dose and that probably the load of the agent in target tissues is more important for pathogenesis than its ability for dissemination. We also demonstrate that determination of infection kinetics by ocular route can be useful to study and understand the role of genes in the penetration phase of leptospiral infection.

Keywords

Leptospira, Real Time PCR, dissemination kinetics, hamster, tissue load

INTRODUCTION

Leptospirosis is a life-threatening disease which can occur in a diverse range of epidemiological situations (1-3). The spirochetal agent is a unique and genetically and antigenically diverse group of bacteria divided into eight pathogenic *Leptospira* species and >200 serovars (1, 4). The disease is considered the most widespread zoonosis in the world (1) due to the pathogen's ability to induce a carrier state in a wide range of wild and domestic animals. Leptospires can establish chronic carriage in the kidney tubules of reservoir hosts and persist for weeks to months in the environment after excretion (1, 4). Transmission to humans occurs during contact with reservoirs or an environment contaminated with their urine. Infection produces a broad spectrum of clinical manifestations, from a self-limiting febrile illness to liver dysfunction, bleeding, kidney failure and pulmonary hemorrhage (5). In endemic areas, acute leptospirosis accounts for over 10% of hospitalizations for acute febrile illness (3), and leptospirosis epidemics occur predictably after periods of heavy rain and flooding (6). The major impact of leptospirosis is caused by its severe clinical manifestations, with mortality rates varying from 10-50% (1-4).

Leptospires are highly motile bacteria which penetrate abraded skin and mucous membranes, causing a systemic infection in a short period of time by crossing tissue barriers and by haematogenous dissemination (1-3,7). Infection causes prolonged leptospiraemia until the beginning of the immune response from the host, which occurs within two weeks after exposure (8). Leptospires can be detected in the bloodstream minutes after intraperitoneal inoculation (1) and are present in several tissues after two days of infection (8-11, 15), reaching 10^6-10^7 organisms per ml of blood or per g of tissue of patients and animals (10-12). In 1957 and 1964, Faine (9) and Green et al. (15), respectively, were able to determine the kinetics of the leptospiral infection using dark field microscopy or culture. The advent of molecular biology techniques, such as real-time PCR, brought a new perspective for those studies.

Real-time PCR is a rapid and sensitive tool for leptospiral detection, and different target genes and methodologies were reported (10, 16-22). The most common real-time PCR assays for the diagnosis of leptospirosis use the SYBR green technology (16, 17, 19, 22) and target conserved genes among bacteria, such as those of 16S rRNA (17, 20) and the *gyr*B (19). The SYBR green assays, although having a lower cost, has the disadvantage of a lower specificity compared with technologies that use fluorescent probes, as TaqMan (23). Recently, a TaqMan

assay targeting the *lipL*32 gene was described (24), showing high sensitivity and specificity in blood, serum and urine samples. Although the assay was evaluated as a diagnostic tool, it can be a potential tool for pathogenic studies in animal models.

The use of animal models is indispensable to understand the biology, transmission, colonization and pathogenesis of *Leptospira*. The hamster is commonly used as a model for acute leptospirosis due to its high susceptibility to leptospire infection with the clinical features that mimic that of severe human infection (25). Experimental leptospire inoculation is primarily performed through intraperitoneal injection (38), but it does not reflect the natural transmission of the pathogen, and there are only a few experiments using methods to mimic the natural entry of leptospires into hosts (10, 26, 27, 38). In this study, we evaluated the use of a quantitative real time PCR assay (TaqMan) using the *lip*L32 gene as a target to determine the kinetics of the leptospiral infection in perfused Golden Syrian Hamster by the intraperitoneal and ocular routes.

MATERIALS AND METHODS

Bacterial strains and clones. In total, one strain and four clones of *Leptospira interrogans* were used in the experiments. For the challenge experiments, leptospires were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (13,14) at 30°C and bacteria were counted in a Petroff-Hausser counting chamber (Fisher Scientific).

For the spiking and kinetics experiments, *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1 130, a virulent clinical isolate from Brazil (6), was used.

The motile and non-motile clones were obtained from *L. interrogans* serovar Copenhageni strain 2756, a virulent clinical isolate from a patient with severe pulmonary hemorrhagic syndrome (SPHS) enrolled in our surveillance study in Salvador, Brazil (6). This strain was cultivated on 1% EMJH agar plates at 30°C. After 24 days, 3 colonies were identified and selected. The plates were kept at 30°C for 8 more days (32 days in total), and 4 more colonies were identified. Cultivation in liquid EMJH and observation under dark-field microscopy showed that all of the first 3 clones had the same morphological and motility characteristics as the parental strain. However, the last 4 clones showed differences for both morphological and motility characteristics. When tested for virulence in the hamster model, the

chosen motile clone had a LD50 < 10 leptospires, while the chosen non-motile clone was avirulent after infection with 10^8 leptospires (data not shown).

The attenuated Fiocruz L1 130 strain with 42 *in vitro* passages was also plated in EMJH plate, as was the virulent Fiocruz L1 130 strain, to obtain the clones that were used in this study. Both the attenuated and the virulent clones were tested in the hamster model for confirmation of the attenuation and virulence, respectively. Serougrouping, serotyping and VNTR analysis demonstrate that all the clones were *L. interrogans* serovar Copenhageni.

Standard curve and spiking experiments. The DNA from 20ml *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1 130 was extracted using the QIAamp DNA minikit (QIAGEN, Valencia, CA), eluting with 200µl water. DNA was quantified using the Nanodrop instrument (Nanodrop Technologies, Wilmington, DE). A genome size of 4,6Mb was used to determine the genomic equivalent (GE) per microliter of the purified DNA (28). For the standard curve, serial dilutions of the DNA were made starting at 1 x 10^7 GE/5µl to 1 x 10^0 GE/5µl. All the assays for the standard curve were performed in duplicate.

Blood, kidney and liver used for spiking experiments were acquired from non-infected hamsters. The appropriate amount of leptospires was spiked into 1ml of water and whole blood in EDTA to achieve a final concentration of 1 x 10^7 leptospires/ml. After the spiking, serial 10-fold dilutions of 1 x 10^6 down to 1 x 10^0 leptospires/ml were made, using blood and water as diluent. For the spiking in tissues, a serial 10-fold dilution of leptospires was made from 5 x 10^6 to 5 x 10^{-1} leptospires/ml. Then, 50µl of each dilution was spiked into 25mg of kidney and liver reaching concentrations of 1 x 10^7 to 1 leptospires/g. Non-spiked blood and water and kidney and liver spiked with water were used as negative control.

Hamster infection and necropsy. All the experiments were performed using 5-8 week-old Golden Syrian male hamsters. For the experiments of kinetics with the Fiocruz L1 130 strain, two groups of fifteen animals were inoculated intraperitoneally (IP) with a high-dose inoculum (10^8 leptospires) and a low-dose inoculum (250 leptospires) in 1ml of EMJH medium each. Groups of 3 animals infected with a high-dose inoculum were euthanized at 1 hour, 1, 3 and 4 days after infection. For the animals infected with a low-dose inoculum, 03 animals were euthanized at day 3, 5, 8 and 11 after infection. Two groups of three animals were injected

intraperitoneally with 1 ml of EMJH medium and euthanized in the last time point for each experiment as negative control group.

For the experiments with the clones, one group of six animals for each strain was inoculated intraperitoneally with 10^8 leptospires in 1ml of EMJH medium. After 1 hour and 4 days post-infection, sub-groups of two animals were euthanized.

The ocular infection was performed by centrifugation of 30ml culture of leptospires for 10 minutes at 1000rcf and using an inoculum of 10^8 leptospires in 10µl of EMJH medium instilled in the left eye conjunctiva using a micropipette. Groups of four animals were infected and two were euthanized after 7 days of infection for each strain tested.

For each experiment, a group of two animals were left as positive controls. Animals were monitored twice daily for signs of disease and death, up to 30 days post-infection. Moribund animals were immediately sacrificed by inhalation of CO_2.

The necropsy of the animals was made as follows. Animals were sacrificed by inhalation of CO_2 and placed on their backs slightly inclined in the dissecting tray. After sterilization of the abdomen with alcohol 70% and using sets of sterile instruments, the internal organs were exposed, including the heart and lungs. All blood was collected directly from the heart in a Vacutainer® K2 EDTA Tubes (BD Diagnostics) and Glass Serum Tubes, using a 5ml syringe with a 21G needle. A 21G butterfly needle affixed to a 60ml syringe containing sterile saline 0.85% was then inserted into the left ventricle. The right atrium was snipped to allow the residual blood and normal saline to leave the body during the perfusion. Each hamster was perfused with 100ml of saline solution. After perfusion, the heart, right pulmonary lobe, right dorsocaudal hepatic lobe, spleen, right kidney and muscle of the right thigh were carefully removed. The brain was exposed by accessing the cranium and the right eye was also removed. A touch-prep of each tissue was made, as described previously (33) for detection of leptospires by immunofluorescence assay. All the tissues were collected into cryotubes and immediately placed into liquid nitrogen before being stored at -80ºC until extraction. Sera were obtained by centrifugation of the clotted blood at 1000g for 15min at room temperature and kept frozen (-20ºC) until analysis for the presence of antibodies against leptospires by the microagglutination test (MAT), as described previously (4, 40). For the clone as for the different routes of infection experiments, only blood, kidney, liver, lung, spleen and eye were analyzed.

Protocols for animal experiments were approved by the Committee for the Use of Experimental Animals of the Gonçalo Moniz Research Center, Fiocruz.

DNA extraction. Using scissors and scalpels, 25mg of heart, lung, liver, kidney cortex, muscle, brain and eye and 10mg of the spleen were aseptically collected. DNA was extracted using the Dneasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions but using only 100µl of elution volume,

Previous results showed that there was inhibition of the amplification of the DNA from the blood of the hamster obtained with the Qiagen kit. For that reason, DNA was extracted from 200µl of blood using the Maxwell 16 Tissue DNA purification Kit (Promega Corporation, Madison, WI).

Real Time PCR analysis. The concentration of leptospires in all the samples tested was quantified by an ABI 7500 (Applied Biosystems, Foster City, CA) using Platinum Quantitative PCR SuperMix-UDG (Invitrogen Corporation, Carlsbad, CA). The *lipL32* gene was amplified using the set of primers previously described (24), LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3') and LipL32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3') that amplify a fragment of 242bp, which was detected by the probe, LipL32-189P (FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1).

As a control for PCR inhibitors and to monitor nucleic acid extraction efficiency, spiked and experimental specimens were tested for the presence of a hamster housekeeping gene glyceraldehyde-3-phophate dehydrogenase (GAPDH). The primer pair and probe were designed using Primer Express version 1.3 (Applied Biosystems). The forward primer of GAPDH_F (5'-GGT GGA GCC AAG AGG GTC AT-3') and GAPDH_R (5'-GGT TCA CAC CCA TCA CAA ACA T-3') were selected to amplify a fragment that was detected by the probe, GAPDH_P (FAM-5'- ATC TCC GCA CCT TCT GCT GAT GCC-3'-BHQ1). A sample with a threshold cycle (Ct) value between 16 and 21 was considered as positive and further analyzed by real-time PCR targeting *liL32*. In case of a negative sample, a new DNA extraction was performed.

The PCR reaction contained 10µM of each primer, 5µM of the specific probe and 5µl of DNA in a total volume of 25µl. There was no addition of $MgCl_2$ or passive reference dye. The amplification protocol consisted of 2min at 50ºC and 10min at 95ºC, followed by 45 cycles of amplification (95ºC for 15s and 60ºC for 1min). A negative result was assigned where no

amplification occurred or if the Ct was greater than 40 (24). A no template control (NTC) that contained all the reagents described above was also included to detect the presence of contaminating DNA.

For each organ, the DNA was extracted from one sample and the Real Time PCR was performed in duplicate. Considering the amount of tissue that was used for DNA extraction, an equation was applied to express the results as the number of leptospires per gram of tissue or per ml of blood/water.

All the graphics and statistical analyses were performed using the GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Histopathology studies. Kidney, liver and lung tissues sections were treated according to routine histological procedures, and sections of 3 to 5μm thickness were then stained with haematoxylin and eosin. The slides were examined in an optical microscopy (Zeiss Axioscop), using previous standardized protocols for each organ. A semi-quantitative analysis was made, ranking the presence of each parameter as negative, discrete, mild or intense, in a blinded manner to prevent bias in the interpretation of the results.

For immunohistochemical analyses, paraffin was removed from tissue sections with xylene and ethanol. Slides were blocked by incubation of sections with 10% powder milk at room temperature for 20 min, and then incubated with 1,000-fold dilution of rabbit immune monoclonal anti-sera to LipL32 and whole-*L. interrogans* serovar Icterohaemorrhagiae strain RGA and negative control rabbit antisera at room temperature for 1 hour. Slides were treated with 0.3% hydrogen peroxide for 15 min at room temperature and then incubated at room temperature for 30 min with anti-rabbit antibodies conjugated to peroxidase (Histostain-Bulk Kit, Invitrogen). Enzyme reactions were developed using DAB (3.3'- Diaminobenzidine-Sigma). Slides were examined in an optical microscopy (Zeiss Axioscop).

Immunofluorescence labeling was performed as previously described (33). Briefly, slides of touch preps of tissues were washed twice with PBS after fixation, blocked twice with 2% bovine serum albumin (Sigma) (PBS-BSA) for 20 minutes each at 30ºC in a humidified chamber, and incubated for 1 h with hyperimmune rabbit antisera to whole-*L. interrogans* serovar Icterohaemorrhagiae strain RGA (diluted 1:200 in PBS-BSA) and negative control rabbit antisera at 30ºC. The slides were washed gently with PBS-BSA and incubated with goat anti-

rabbit IgG antibodies conjugated to fluorescein isothiocyanate (FITC, Jackson ImmunoResearch Laboratories) for 1 h at 30 °C. Slides were washed, then mounted in anti-fading solution (Prolong-Molecular Probes) and visualized by fluorescence microscopy (Olympus BX51).

Statistical analysis. GraphPad Prism 5.0c (GraphPad Software, San Diego, CA) was used to perform all the statistical analysis. A *P* value of <0.05 was considered to indicate statistical significance.

RESULTS

Standard curve and spiking experiments. Real-time PCR were run in three independent experiments, on duplicate serial dilutions of genomic DNA isolated from *L. interrogans* serovar Copenhageni strain Fiocruz L1 130. The results were similar to those described previously (24), and all the experiments showed positive amplification at 01 GE per reaction (data not shown), with a linear correlation (R^2) of 0.998. The same batch of DNA was used for the subsequent analysis, since a standard curve was run side by side in each plate analyzed. During all the experiments, amplification of 10 GE was 100% positive and more than 50% of the standard curves were positive at 01 GE (data not shown).

The control animals that were used as donors for the spiking experiments tested negative on *lipL32* real-time PCR in tissues and blood. The *gapdh* real-time PCR results showed amplification in all samples tested and all the NTC samples were negative,

For all the spiked tissues and water, the results showed a linear curve with R^2=0.978, but when compared with the standard curve, with exception of the spiked water, there was a difference of one log in the detected amount of leptospires, indicating that there's some potential inhibition in the clinical samples (Figure 1).

This inhibition is more critical for the spiking results in solid tissues. Water and blood spiked with leptospires showed a detection level of 1 x 10^2 leptospires/ml the same level as the theoretical limit of the test for both. However, kidney and liver had a detection level of 1 x 10^4 leptospires/g, one log less than the theoretical limit of the test (Figure 1).

Real Time PCR analysis in hamster infected IP with a low and a high dose inoculum of the Fiocruz L1 130 strain. As described previously (41), the mean LD_{50} for Fiocruz L1 130 strain is 45,9 leptospires in hamster. Doses as high as 10^8 leptospires are normally used for experimental infection and the low dose used here was chosen to be approximately 5x the LD50. Previous pilot experiments were performed to determine the time points for each infection dose, considering the day of death of the animals after infection and the first time point to achieve a detectable number of leptospires per gram of tissue. We tried to use skin from cheek pouch to determine quantification, but since the results for *lipL32* amplification were negative they were excluded from the experiments.

To check for the possibility of discrepant results of quantification when using different pieces of a same tissue, we performed a test doing extraction from three different sites in the same tissue (kidney, liver and lung) from animals infected with high and low doses. There was no significant difference between the extractions in any tissue (Table 1). Those results showed that leptospires are spread equally in the whole tissue. There are no differences between the right and the left kidney also (data not shown). This indicates that using a single DNA extraction, the quantification gives a result applicable to the whole organ.

Hamsters infected IP with 10^8 leptospires (high dose) were euthanized 1 hour, 1, 3 and 4 days after infection. Control infected animals died between the 5 and 6 day post-infection. All the tissues analyzed were positive after 1 hour for the presence of the agent, with a range of 1.2 x 10^2-7.6 x 10^2 leptospires/g for brain, heart, eye, muscle, lung and 5.8 x 10^3 – 2.4 x 10^5 leptospires/g for blood, liver and kidney. Reaching 3.5 x 10^5 leptospires/g, the spleen was the organ that showed the higher number of leptospires per gram after 1 h of infection (Figure 2).

Statistical significance was evidenced only between the first and the third day post-infection ($p<0.005$) in the high infection dose experiments. After 4 days, kidney, liver and spleen reached 1.65 x 10^8, 8.5 x 10^7 and 7.72 x 10^7 leptospires/g respectively, with a difference of 4 logs between the first and the last time point. Eye had the lowest tissue load, 2 x 10^4 leptospires/g and also the lowest difference among time points. Blood had a burden that reached 1.6 x 10^7 leptospires/ml and for the other tissues, the burden for the last day was between 1.4 x 10^5 and 9.3 x 10^5 leptospires/g.

Hamster infected IP with 250 leptospires (low dose) were euthanized at time points 3, 5, 8 and 11 days after infection and the control infected animals died between the 9-11th day post-

infection. Only in blood bacteria were detected after 3 days of infection (1,87 x 10^0 leptospires/ml), achieving 1.3 x 10^2 leptospires/ml 5 days post-infection, when there was also detection of leptospires in the kidney, liver and spleen (2.74 x 10^2, 1.94 x 10^3 and 1.36 x 10^4 leptospires/g, respectively; Figure 2). After 8 days of infection, all the tissues were positive, with a burden ranging from 5.4 x 10^4 leptospires/g for muscle to 6.7 x 10^7 leptospires/g in the kidney, with a statistical difference between time points ($p<0.0001$). With exception of lung and brain, all other analyzed tissues had a decreased in the number of detected leptospires from day 8 to day 11, with no statistically significant difference ($p=0.084$) but with a difference of 2 logs for blood, liver and spleen (Figure 2). This event could be associated with the first appearance of antibodies against leptospirosis measured by MAT, but only 50% of the animals had serological titers ranging from 1:25 to 1:100. No animals infected with a high dose of leptospires had antibodies detected by MAT.

To analyze the distribution of leptospires among the different tissues for the animals infected with 10^8 leptospires, we calculated the percentage of bacterial burden at each time point for kidney, liver, spleen, blood and lung (Table 2). For the first hour and first and third day, spleen had a slightly higher percentage, but in the fourth day all the percentages are very similar. The other analyzed tissues had a percentage of distribution similar to lung (data not shown). The same results were observed when this analysis was performed with tissues from animals infected with 250 leptospires (data not shown).

Necropsy and histopathology analysis. At necropsy, animals infected with the high and low dose of inoculum showed clinical signs and macroscopic lesions after 3 days and 8 days post-infection, respectively, culminating with death of the animals between 2 and 3 days after those time points. For the clinical signs, the most common were anorexia, depression and hemorrhages just before the onset of death. Regarding to gross lesions observed at the necropsy, animals showed slightly signs of jaundice, enlargement of the spleen and hemorrhage of the kidneys. But the most striking feature was hemorrhage of the lungs (Figure 3), that was not caused by CO_2 since negative control animals didn't have the same lesions (data not shown).

Analysis of haematoxylin and eosin staining of sectioned lung, liver and kidney also showed no differences between control animals and those infected with high and low dose for the first two time points. For the last two time points of both inocula, the tissues had the

characteristic histopathologic features of leptospirosis (Figure 3). Regarding the kidney, discrete hemorrhage, inflammation, and mild hyaline degeneration were observed in animals at the last time points for both doses. In the liver, animals infected with a high dose showed discrete hydropic degeneration and infiltrating mononuclear cells. In animals infected with low dosage, we observed discrete hemorrhage and necrosis. Mild steatosis and mild to intense loss of parenchymal architecture were observed in animals inoculated with both high and low dose. In the lungs, mild to intense alveolar hemorrhage and discrete bronchial hemorrhage were observed in all animals (Figure 3).

The localization of leptospires in tissues, by immunohistochemistry, was basically intercellular in the liver, in the kidney interstitium and tubules and in the alveolar septa of the lung (Figure 3), but 70% of the lung tissues were negative.

Conjunctival infection. The kinetics in animals infected by a conjunctival route (CJ) was compared with the regular IP infection, both with 10^8 leptospires (Figure 4). For the animals infected by the IP route, tissues were analyzed after 1 and 4 days of infection, with control animals dying after 5-6 days. Animals infected by CJ were analyzed after 1, 4 and 7 days. Control animals died after 8-9 days.

Only blood, kidney, liver, spleen, lung and eye were analyzed. Both infection routes caused death of the animals, but kinetics on animal infected by CJ were longer. Tissues of animals infected IP were all positive after 1 day post-infection with a burden of 3×10^1 to 3.5×10^5 leptospires/g and this number grew exponentially for all tissues after 4 days of infection, reaching 4.2×10^5 to 3.3×10^7 leptospires/g (Figure 4). In contrast, all tissues from animals infected by CJ were negative for quantification in the first day, and after 4 and 7 days they reached a burden of leptospires comparable with the first and fourth day of animals infected by IP (2.95×10^1 to 1.36×10^4 leptospires/g and 4.9×10^4 to 1.05×10^7 leptospires/g, respectively). The burden of the eye of animals infected by the CJ route was much lower than the IP animals, and the detection was only possible after seven days of infection, reaching only 8.3×10^2 leptospires/g (Figure 4).

Real Time PCR analysis in hamster infected with an attenuated strain and a non-motile clone. Two different clones that had lost its virulence phenotype were also used for the studies of

kinetics. One was a clone of an attenuated Fiocruz strain with 42 *in vitro* cultures and the other was a clone obtained from a virulent strain of serovar Copenhageni that showed markedly deficiency in motility. The dose of infection for those experiments was 10^8 leptospires, and the time point for this experiment was 1 and 4 days post-infection.

The bacterial burden of tissues after one day post-infection for the attenuated clone is similar in comparison with a virulent clone of the Fiocruz strain with low *in vitro* passage (Figure 5A). However, after 4 days of infection, the distribution of leptospires are statistically different between hamsters infected with the two clones ($p=0.014$), with the attenuated strain showing lower burden in all analyzed tissues (Figure 5B). For the non-motile clone, there was no detection of leptospires after 1 day of infection (Figure 5C), and the burden of leptospires in all tissues after 4 days was significantly lower in comparison with the motile clone (Figure 5D). All control animals infected with the virulent and the motile clones died after 5-6 days of infection while the animals infected with the attenuated and the non-motile clones survived. Analysis after 30 days of tissues from animals that survived, showed detection of leptospires only in the kidneys of animals infected with the attenuated clone, with a bacterial burden of 10^4 leptospires/g (data not shown). For both non-virulent clones, there was no detection of leptospires in the eye of infected animals at any time point.

When those same four clones were used to infect hamsters with 10^8 leptospires by the conjunctival route, none of the analyzed tissues infected with the two non-virulent clones were positive for the presence of leptospires after seven days post-infection (data not shown). For the motile clone and the virulent Fiocruz clone, the burden in the tissues was similar to those found in previous experiments using the conjunctival route (Figure 6), with the control animals dying after 8-9 days post-infection. All the controls animals infected with the non-motile clone and the Fiocruz attenuated clone survived and the tissues were still negative after 30 days.

DISCUSSION

Leptospira is a motile life-threatening spirochete which is able to disseminate to tissues. However, its ability to spread and the importance of its spread to the pathogenesis of the disease are not well understood. In this study we described the kinetics of leptospirosis in the hamster model for acute infection, using a previously described (24) real-time PCR assay based on

primers and TaqMan probe to detect the *lipL32* gene, which is only present in pathogenic *Leptospira* spp. In the standard curve using genomic DNA isolated from *L. interrogans* serovar Copenhageni strain Fiocruz L1 130, we showed that this assay was able to detect 10 GE per reaction reaching detection levels as low as 1 GE in more than 50% of the reactions. This result differs from what was described before (24), where the lower limit of detection, for which 95% of the tested samples were positive, was 50 GE/µl. That difference can be related to the combination of the Applied Biosystems instrument with Invitrogen SuperMix and a standardized amount of 5 µl of template DNA for each reaction, increasing the sensibility of the assay.

Since serum was described as not being a good specimen for detection of leptospires using real-time PCR compared with whole blood (24), we decided to work only with the latter. However, our real-time PCR assay was inhibited when using the DNA extracted from the blood of hamsters using the Qiagen kit. Inhibitors in whole blood DNA are well known and described, especially in human whole blood (29, 30). To avoid the confounding factor of blood in the quantification of leptospires in tissues and the possible inhibition of the assay, all the animals used in this study were perfused with saline. The Maxwell tissue kit was used only to do the extraction from the whole blood samples and the Qiagen kit was used with other tissues since no differences were found when comparing both kits (data not shown). The use of the GAPDH amplification as a control for the DNA extraction was also an excellent indicator for the presence of inhibitors.

Analysis of hamster tissues spiked with leptospires demonstrated that there is an important inhibition in clinical specimens, as shown before (24). In the spiked blood, the lower detection value was in accordance to that expected value for the test, as seen in the spiked water, 1 x 10^2 leptospires/ml, but in kidney and liver the lower detection level was 10^4 leptospires/g, and we expected it to be one log lower (Figure 1). In our experiments, since we used the standard curve instead the of threshold cycle (Ct) values as comparative, we were also able to notice an inhibition related to the number of leptospires that were spiked and the number that were detected. Despite the fact that the curves were linear (Figure 1), there is a difference of one log between the two values in every measurement for kidney and liver, noticed only after the counting 10^4 leptospires/ml for blood (Figure 1).

High dose (10^8 leptospires) injected intraperitoneally showed leptospires in all tissues, including eye and brain, after 1 hour of infection, with levels ranging from 10^2-10^5 leptospires/g.

A recent study with guinea pigs (10) found that after an infection of 10^7 leptospires, only after 4 days, it was possible to detect 10^4 leptospires/ml. In our study, after one hour the burden for the blood was already over 10^3 leptospires/ml, reaching over 10^7 leptospires/ml after 4 days of infection (Figure 2). Low dose (250 leptospires) infection showed that detection of leptospires in the blood was possible after 3 days of infection, while in the tissues only after 5 days (Figure 2). Analysis after 4 days showed levels of 10^4-10^8 leptospires/g and macroscopic lesions culminating with death of the animals between 5-6 days after infection.

The relationship between the burden of leptospires in tissues, lesions and death was described previously, where the first appearance of hemorrhages in the animals coincided with a defined number of leptospires (8) or the moment of death coincides with the higher burden of the agent in the tissues (36). In our study, animals infected with a lower dose of inoculum took longer to have detectable leptospires in the tissues and to become ill and die. Despite that, the number of leptospires in every tissue was comparable, when macroscopic lesions, especially hemorrhagic lungs, were visible and animals were near death, reaching numbers over 10^7 leptospires/g in tissues as kidney, liver, spleen and blood (Figure 2) and macroscopic and histopathological changes found were very similar, as hemorrhage lungs and kidneys and hepatic steatosis (Figure 3).

The fact that usually a low number of leptospires are detected in the lung (10, 36) but also that this organ is highly affected by leptospiral infection is well know. A toxin-mediated mechanism and/or the immune responses of the host are suggested as one of the main mechanism of leptospirosis and lung lesions (38, 43, 44). Although the analyses of the lung in our study showed signals of hemorrhage and a considerable number of leptospires were quantified (Figure 2) reaching numbers as high as 10^6 leptospires per gram, fewer leptospires were detected by immunohistochemistry and the most common observation was traces of degraded leptospires, probably related to the action of immune system and release of toxins (35). Although we were able to isolate leptospires from lung of infected animals (data not shown) this discrepancy of results could be explained by one of the downsides of PCR-based assays that don’t discriminate between viable and dead organisms (18).

Spleen had a slightly higher percentage of leptospires distribution (Table 2), but there was no statistical difference in that distribution, showing in our experiments that there was no tropism for any specific organ during the dissemination and multiplication phase of leptospirosis, in

accordance with what was described before (34), that leptospires multiply and migrate randomly through all body tissues. Same observation was made in rats where leptospires also disseminate extensively through all tissues and that the chronic renal colonization is probably related to some immune privilege aspect of the kidney (42). Virulent leptospires rapidly invade the blood and are subsequent distributed to all tissues and that half of total final burden of leptospires in each tissue was already achieved after 1 hour post-infection (data not shown). The presence of higher levels of leptospires in vascular tissues is probably due to haematogenous dissemination and blood flow (15, 37). Leptospires in brain and eye of animals infected with low and high dose were detected at the first hour and eighth day post-infection, respectively, (Figure 2), showing that leptospires can cross the brain-blood barrier, as described in previous studies in animals and humans (15, 31, 32).

In our experiments with hamster model, individual variability was observed, but it was not enough to interfere in the results even when fewer animals were used per group, as showed by the SD for each experiment.

As described before (10), the same high dose inoculated by the conjunctival route showed that leptospires were able to cause a systematic infection and can be detected after 4 days post-challenge and that the bacterial load is similar but with a time difference from animals infected IP. These results showed that, besides the fact that both routes leads to active infection and death of the animals with the same high dose of infection, leptospires inoculated by CJ route need more days to reach the bloodstream, multiply and spread (Figure 4).

Infection with a high inoculum dose of a motile attenuated clone of Fiocruz L1 130 (40 sequenced *in vitro* passages) showed that this strain was able to disseminate but not to multiply in tissues or to cause death of the animals (Figure 5A/B). It seems that there is no difference between the abilities of a virulent and avirulent organism either to penetrate or to spread from the peritoneal cavity after IP injection, but the sole difference appears to be in the ability to survive in the infected animal, as described before (8). This difference was also observed for the infection with the non-motile strain that was shown to be avirulent but capable of infecting tissues and to be detectable after 4 days of infection (Figure 5C/D). However, this strain was not able to cause chronic carriage after 21 days post-challenge, while the attenuated strain was present in the kidney and was able to be re-isolated and keep the avirulent status (data not shown). Both strains were not detected in the eye, and there was a significant statistical

difference when the levels of leptospires detected in tissues after 4 days of infection were compared with the virulent (10^6-10^9 leptospires/g) and avirulent strains (10^4-10^6 leptospires/g). This evidence reinforces the fact that a determined number of leptospires in the tissues are necessary to cause lesions and death of the animals.

The use of a conjunctival route for infection in leptospirosis is not common, despite the fact that this reproduces one natural mode of infection of the agent (38). When we used the attenuated strain and the non-motile strain with the ocular route, there was no detection of leptospire in the tissues after 7 days of infection, while the virulent strain and the motile strain were detected in every tissue (Figure 6), which means that motility is important for penetration and that the attenuation that occurs with high passage *in vitro*, despite keeping the motility phenotype affects the ability for penetration. This observed difference of burden in the tissues of the two non-virulent clones between the infection by IP and ocular route showed that the use of a conjunctival route in the routine of leptospiral experiments with animal models are extremely important, since virulence factors or vaccine mechanisms of protection could be related to the active form of penetration of this agent. It's important to emphasize that the dose necessary to cause disease by natural infection is unknown, but probably is lower than the 10^8 leptospires used in this study. It's clear that a higher number of leptospires are needed to cause disease by ocular inoculation, and since environmental factors are also not included in experimental infection with leptospires, this approach has a potential application.

In summary, our results demonstrated that leptospires can rapidly disseminate through all tissues after 1 hour post-challenge with a high inoculum dose and that the load of the agent in target tissues is more important for pathogenesis than its ability for dissemination. We also demonstrated that motility is not essential for dissemination but can be essential for tissue load and lethality. The assay here described can be a useful tool to verify the importance of target genes in mutant clones for dissemination and penetration and also to verify the effectiveness of vaccines in preventing dissemination and colonization.

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Table 1: Burden of leptospires in kidney, liver and lung of an animal infected with 10^8 leptospires, 1 hour post-infection (A) and an animal infected with 250 leptospires, 8 day post-infection (B). For each organ, three assays of extraction from different sites were performed to determine if the distribution of leptospires are localized or spread equally.

Animal	**Quantification results for three extractions of the same tissue Mean (SD)**		
	Kidney	**Liver**	**Lung**
A	1.07E x 10^4 (0.11)	2.57 x 10^4 (0.14)	2.29 x 10^3 (0.11)
B	9.55 x 10^7 (0.06)	1.05 x 10^7 (0.37)	3.16 x 10^5 (0.23)

Table 2: Percentage analysis of leptospiral dissemination in kidney, liver, spleen, blood and lung along time in hamster infected IP with 10^8 leptospires of the Fiocruz L1 130 strain. The percentage represents the mean result of three independent experiments with the standard deviation.

Tissue	**Time point**			
	1 hour	**1 day**	**3 days**	**4 days**
Kidney	14,04 (1,37)*	13,80 (0,72)	15,19 (0,62)	14,31 (0,34)
Liver	13,81 (0,26)	14,75 (0,46)	13,91 (0,92)	13,79 (1,19)
Spleen	17,78 (0,71)	16,01 (0,71)	14,19 (0,85)	13,74 (0,27)
Blood	12,08 (0,53)	12,45 (0,35)	12,40 (0,36)	12,55 (0,38)
Lung	9,34 (2,63)	8,83 (0,55)	9,80 (0,58)	10,42 (0,73)
Heart	8,91 (2,17)	8,20 (1,52)	8,77 (0,55)	8,99 (0,59)
Muscle	9,28 (2,11)	9,94 (1,51)	8,72 (0,12)	9,67 (0,23)
Brain	6,71 (0,40)	8,80 (0,71)	8,53 (0,91)	8,98 (0,71)
Eye	8,05 (3,98)	7,21 (1,62)	8,49 (0,52)	7,56 (1,71)

* % (SD)

Figure legends

Figure 1. Results of the spiking experiments with the Fiocruz L1 130 strain. Each point represents the mean result (logarithmic scale) of three independent experiment performed in water, kidney, liver and blood. Error bars represent the standard deviation.

Figure 2. Kinetics of leptospirosis dissemination in tissues taken from hamsters infected IP with 10^8 and 250 leptospires of the Fiocruz L1 130 strain. Bacterial load for each tissue was calculated based on the mean result of three perfused hamsters for each time point. Each line represents the mean result of bacterial load (logarithmic scale) of three independent experiments. Error bars represents the standard deviation. Animals infected with 10^8 leptospires (straight line) were analyzed at 1 hour, 1, 3 and 4 days post-infection. Animals infected with 250 leptospires (dotted line) were analyzed at 3, 5, 8 and 11 days post-infection.

Figure 3. Pathology of hamsters infected intraperitoneally with the L1 130 Fiocruz strain. Gross examination of infected hamsters and pathology analysis of liver, kidney and lung infected with 10^8 leptospires at day 4 (A) and 250 leptospires at day 11 (B) post-challenge. First and third columns, tissues were stained by haematoxylin and eosin (HE, x400). Kidney had mild hyaline degeneration, liver had mild loss of parenchymal architecture and steatosis and lungs had hemorrhage. Second and fourth columns, immunohistochemistry with monoclonal antiserum specific to LipL32 (IHC, x1000), showing whole leptospires and degraded cells in kidney tubules, interstitium of liver and alveolar septa of lung.

Figure 4. Kinetics dissemination of leptospires in tissues from hamster infected with 10^8 leptospires of the Fiocruz L1 130 strain comparing the intraperitoneal (IP) and conjunctival (CJ) routes of infection. The bacterial load for each tissue was calculated based on the mean result of two perfused hamsters for each time point. Each line represents the mean (logarithmic scale) of two independent experiments. Error bars represent the standard deviation. Animals infected by the IP route (straight line) were analyzed after 1 and 4 days post-infection. Animals infected by the CJ route of infection (dotted line) were analyzed after 1, 4 and 7 days post-infection).

Figure 5. Kinetics dissemination of leptospires in tissues from hamsters infected intraperitoneally with 10^8 leptospires using two different avirulent clones of serovar Copenhageni. Animals were infected with an attenuated clone of the Fiocruz L1 130 strain with 42 *in vitro* passages and compared with a virulent clone of the same strain (A and B) and also infected with a non-motile clone of a virulent Copenhageni which lost its virulence phenotype, side by side with a motile and virulent clone of the same strain (C and D). The analysis of the tissues were performed 1 day post-infection (A and C) and four days post-infection (B and D) by the mean result of two perfused hamster for all the clones. Each column represents the mean (logarithmic scale) of to independent experiments. Error bars represent the standard deviation.

Figure 6. Kinetics dissemination of leptospires in tissues from hamster infected by conjunctival route, with 10^8 leptospires of a virulent clone of the Fiocruz L1 130 strain and a motile and virulent clone of a Copenhageni strain. Bacterial load for each tissue was calculated based on the mean result of two perfused hamsters after 7 days of infection. Each bar represents the mean (logarithmic scale) of two independent experiments. Error bars represent the standard deviation.

Figure 1.

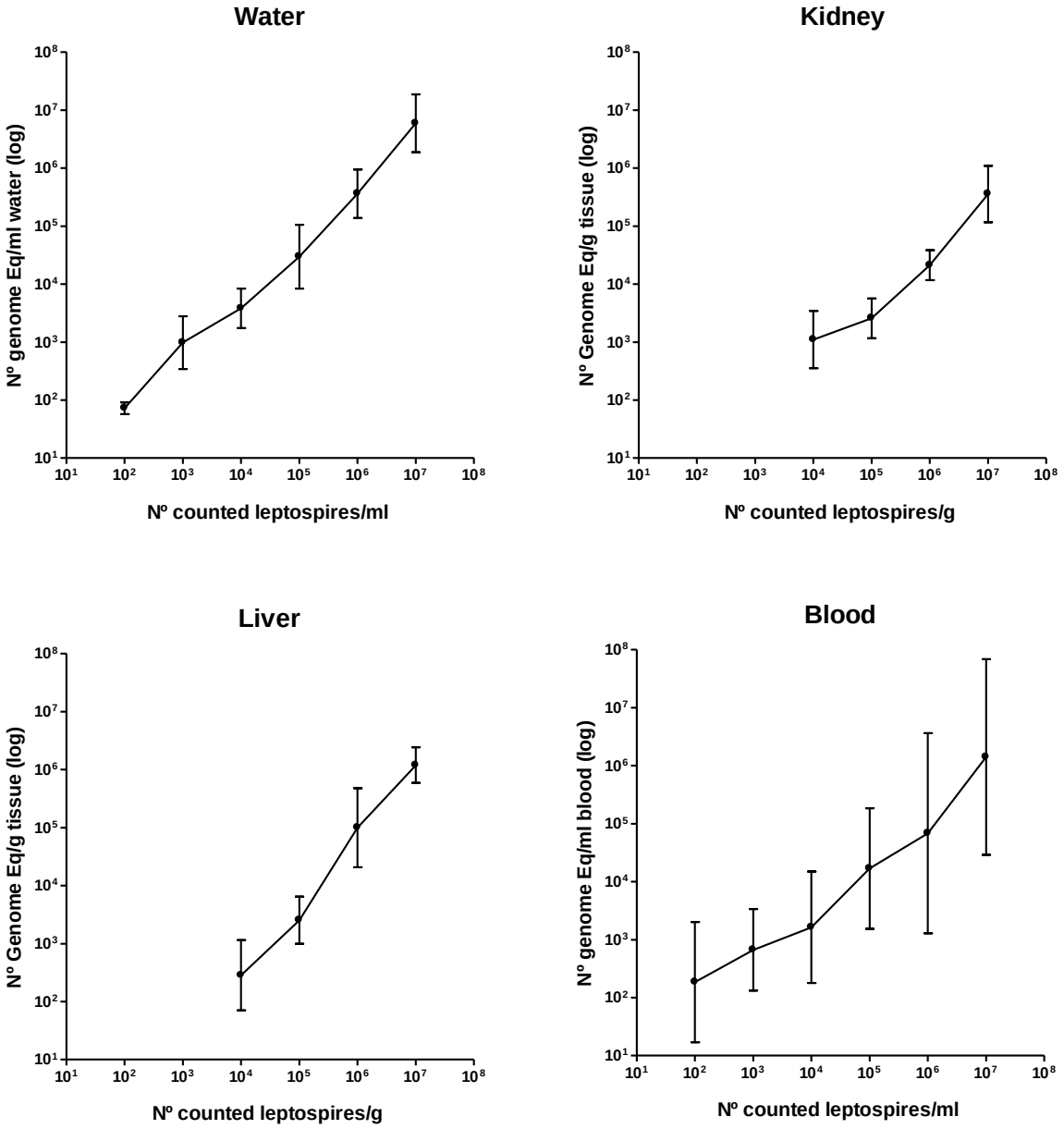

Water
Nº genome Eq/ml water (log)
Nº counted leptospires/ml
Kidney
Nº Genome Eq/g tissue (log)
Nº counted leptospires/g
Liver
Nº Genome Eq/g tissue (log)
Nº counted leptospires/g
Blood
Nº genome Eq/ml blood (log)
Nº counted leptospires/ml

Figure 2.

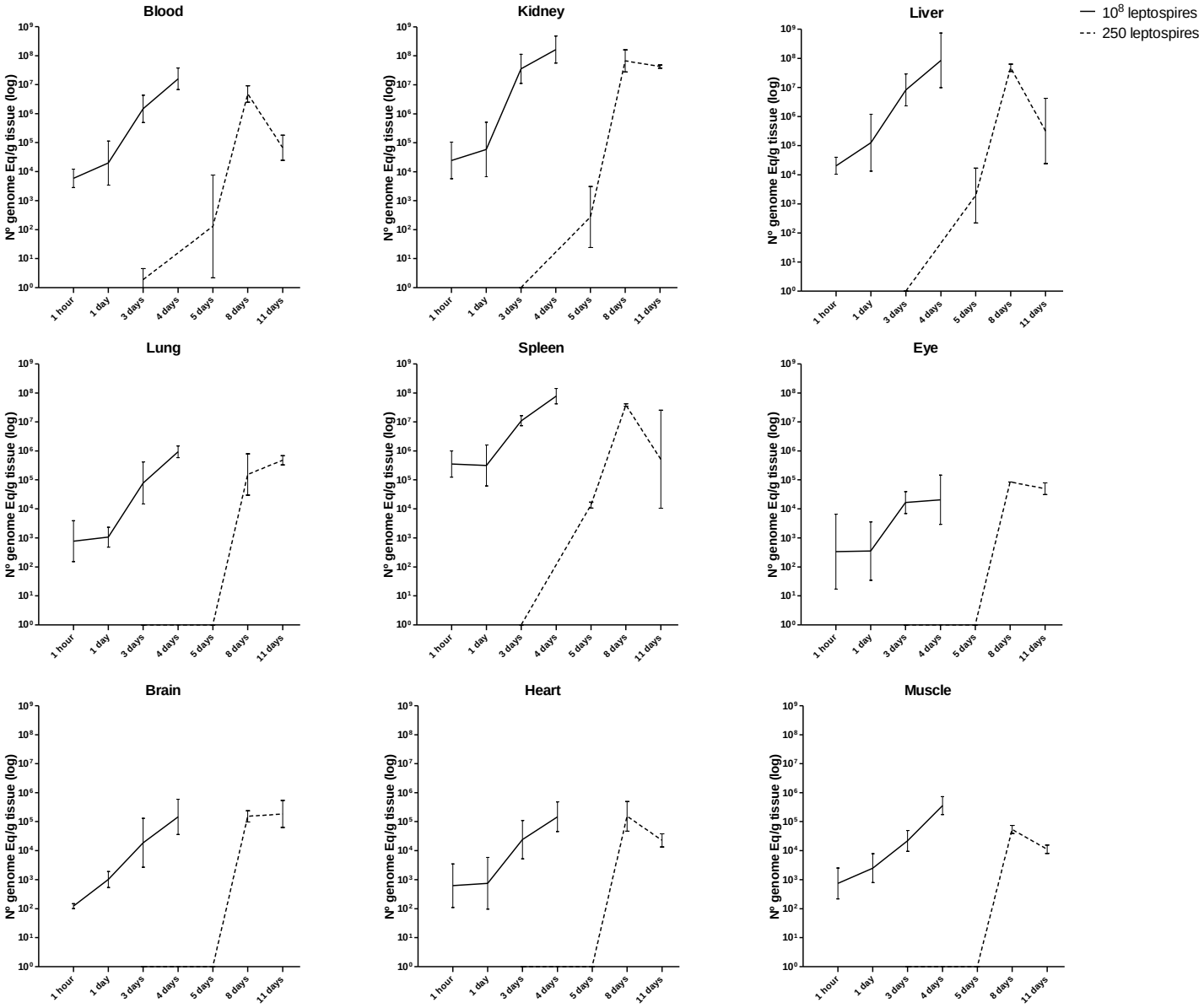

Blood
Kidney
Liver
Lung
Spleen
Eye
Brain
Heart
Muscle
Nº genome Eq/g tissue (log)
1 hour
1 day
3 days
4 days
5 days
8 days
11 days
10^8 leptospires
250 leptospires

Figure 3

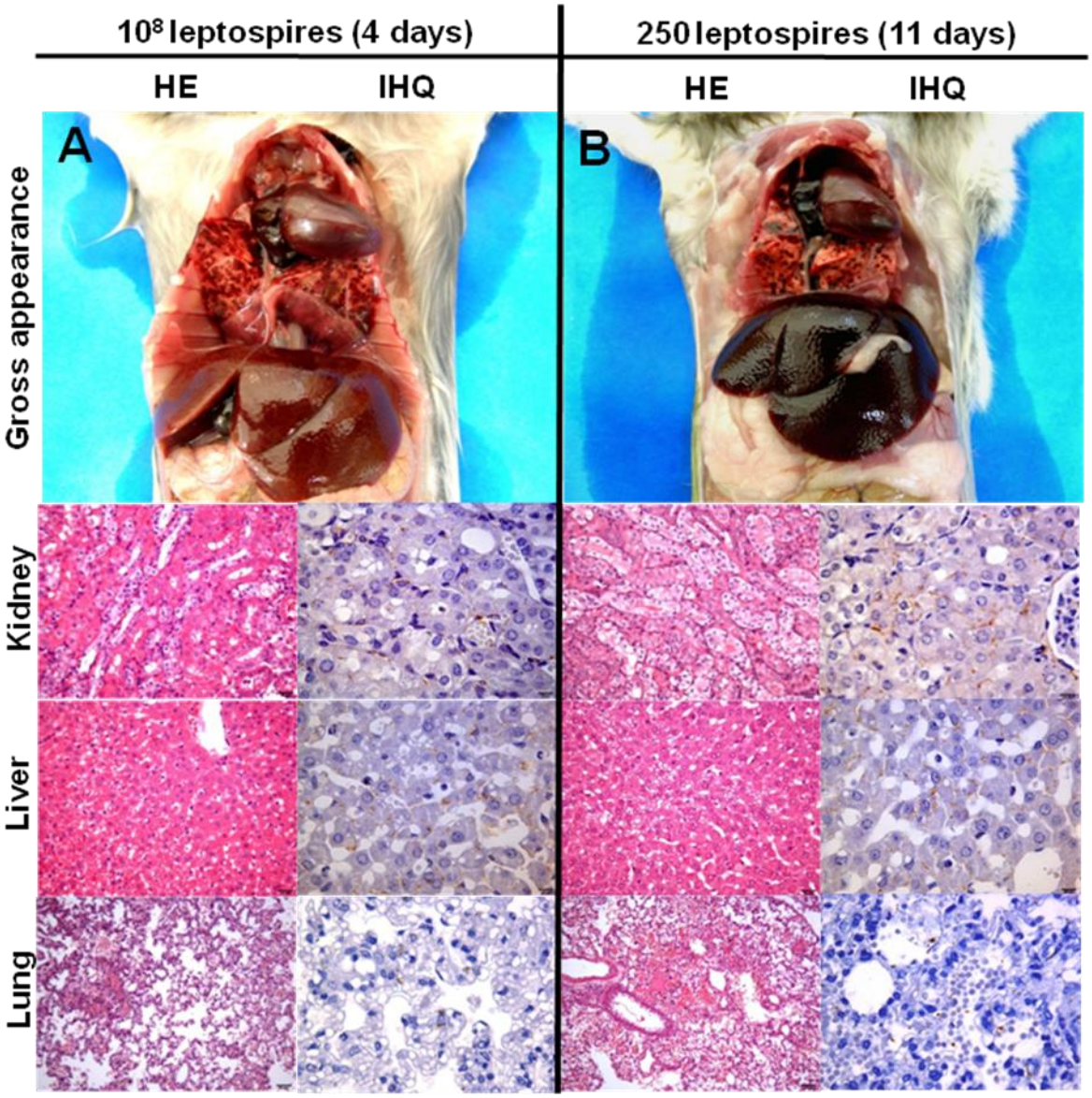

10⁸ leptospires (4 days)
250 leptospires (11 days)
HE
IHQ
HE
IHQ
A
B
Gross appearance
Kidney
Liver
Lung

Figure 4.

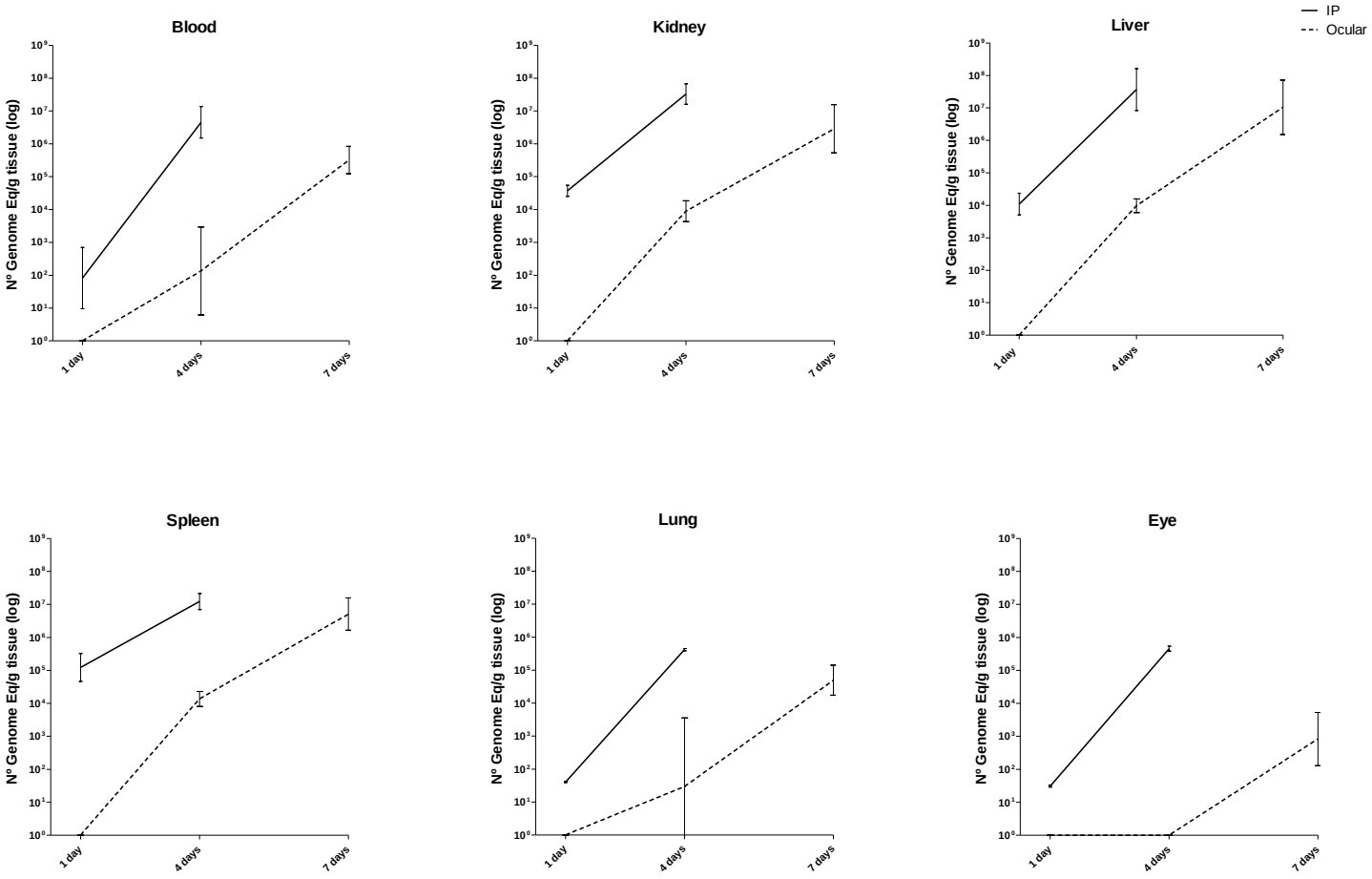

Blood
Kidney
Liver
Spleen
Lung
Eye
IP
Ocular
Nº Genome Eq/g tissue (log)
1 day
4 days
7 days

Figure 5.

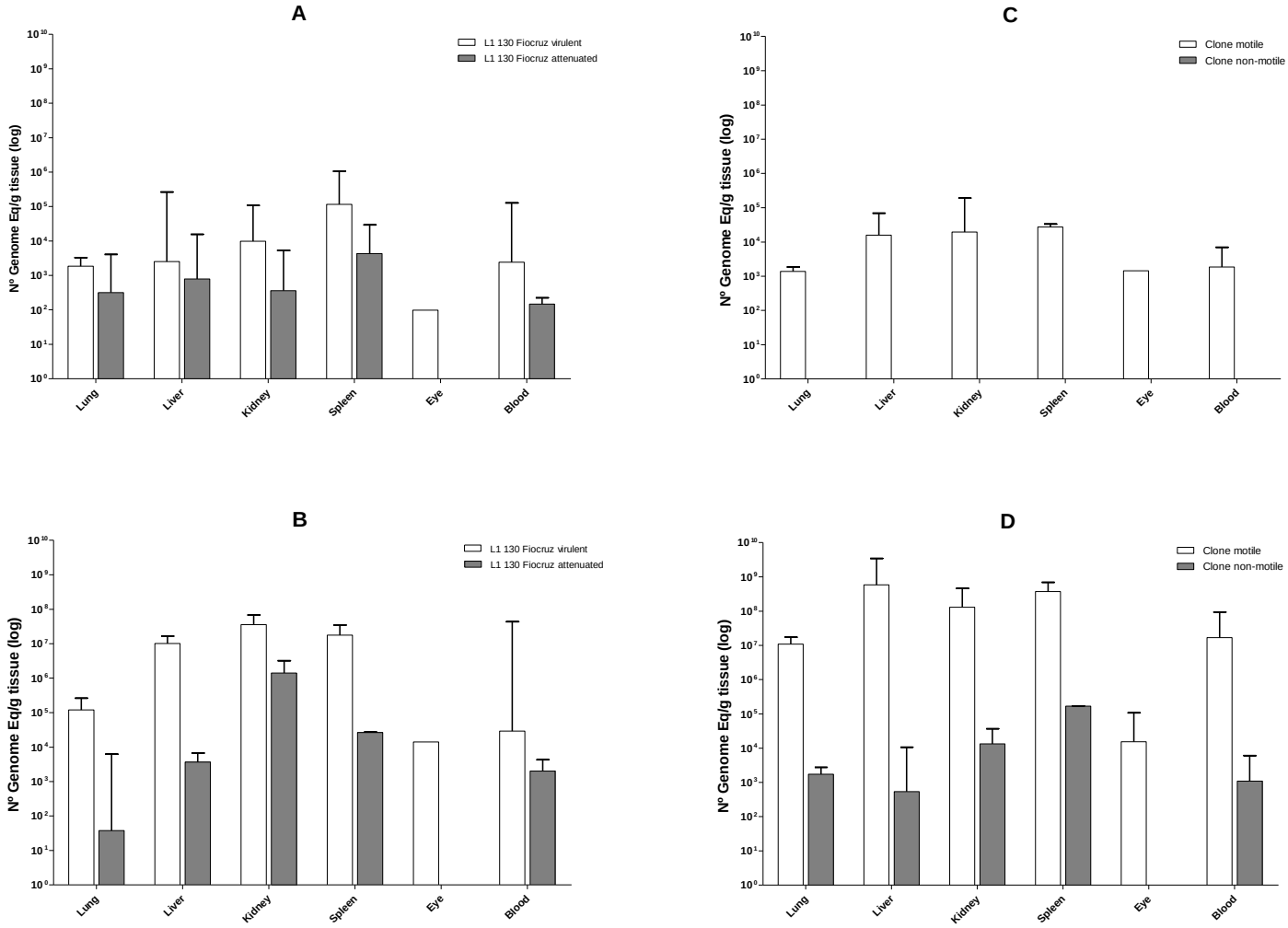
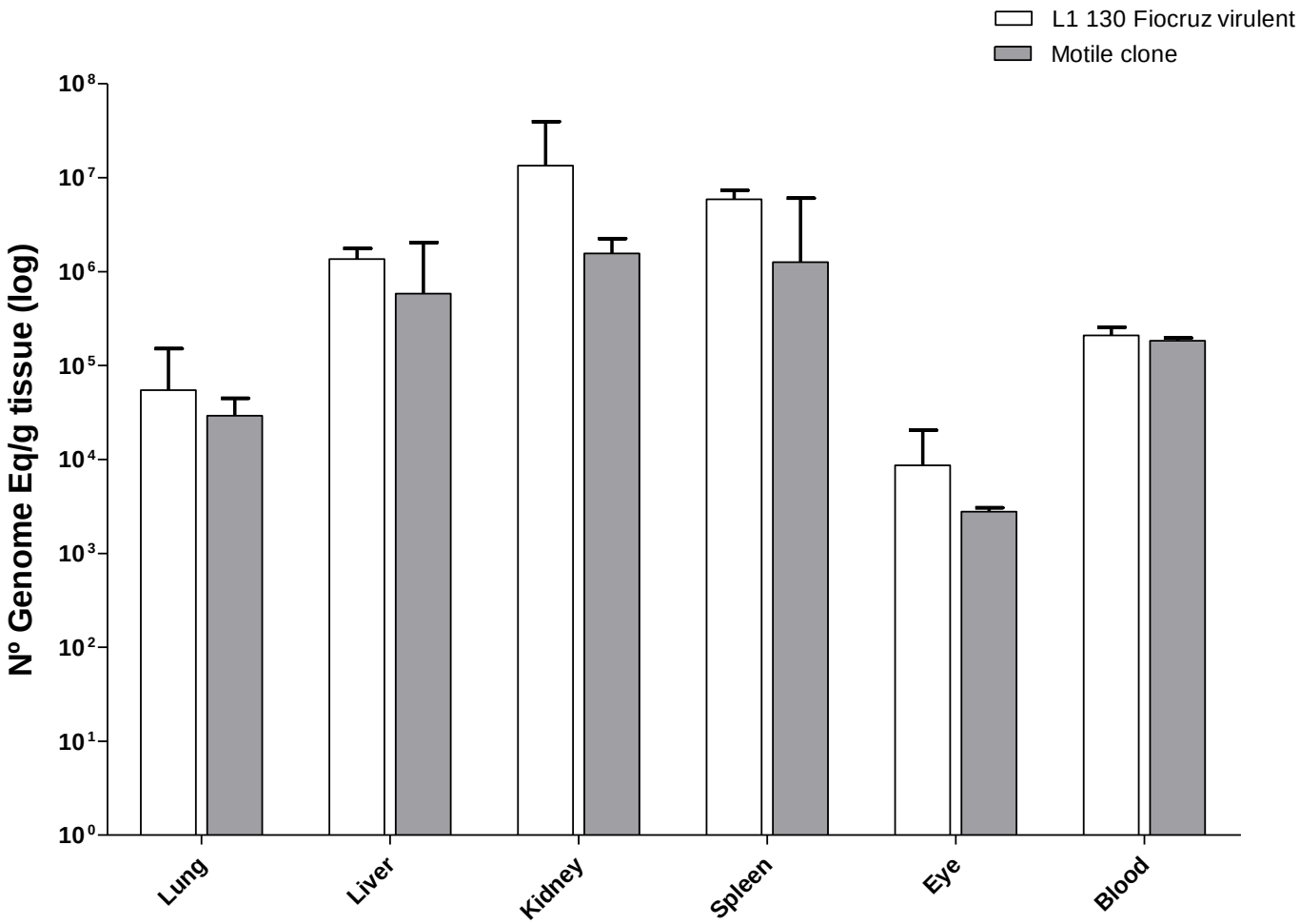

A
L1 130 Fiocruz virulent
L1 130 Fiocruz attenuated
Nº Genome Eq/g tissue (log)
Lung
Liver
Kidney
Spleen
Eye
Blood
C
Clone motile
Clone non-motile
Nº Genome Eq/g tissue (log)
Lung
Liver
Kidney
Spleen
Eye
Blood
B
L1 130 Fiocruz virulent
L1 130 Fiocruz attenuated
Nº Genome Eq/g tissue (log)
Lung
Liver
Kidney
Spleen
Eye
Blood
D
Clone motile
Clone non-motile
Nº Genome Eq/g tissue (log)
Lung
Liver
Kidney
Spleen
Eye
Blood

Figure 6.


L1 130 Fiocruz virulent
Motile clone
Nº Genome Eq/g tissue (log)
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
Lung
Liver
Kidney
Spleen
Eye
Blood

7. DISCUSSÃO

Enquanto a leptospirose emergiu nas últimas décadas como um importante problema de saúde pública no mundo todo, o conhecimento da biologia do agente ainda é limitado, criando uma grande barreira para o eficaz controle e prevenção da doença. Essa limitação é, em parte, devido à falta de ferramentas adequadas de manipulação genética que impossibilita os estudos, principalmente relacionados com a patogênese da doença. A recente publicação do genoma completo de três sorovares patogênicos e um sorovar saprófita (NASCIMENTO *et al*., 2004; REN *et al*., 2003; BULACH *et al*., 2006; PICARDEAU et al., 2008), promoveu um grande avanço no estudo funcional e genético do agente, possibilitando, pela primeira vez em anos de estudos, a descoberta do único fator de virulência descrito em leptospiras, até o momento (RISTOW et al., 2007) e a criação de uma biblioteca de mutantes em leptospiras patogênicas, com o objetivo de estudar a função de genes específicos (MURRAY et al., 2009).

Estudos recentes em outros microorganismos mostraram que bactérias com deficiência em membros da superfamília contendo as proteínas Big possuíam menor aderência em células e translocação celular prejudicada (NOUGAYRÈDE et al., 2003; PEPE et al., 1993). O gene que expressa a proteína LigB, a qual pertence à superfamília das proteínas Big, é conservado em todas as leptospiras patogênicas e tem sua expressão aumentada quando o agente entra em contato com o ambiente idêntico ao encontrado no hospedeiro (MATSUNAGA et al., 2003; PALANIAPPAN et al., 2002). No caso de *lipL32*, diversos estudos indicam a importância da proteína expressa por esse gene em leptospiras. A proteína LipL32 é a mais abundante na membrana externa e na superfície da célula, está presente somente em leptospiras patogênicas, tem característica de adesina em componentes da matriz celular e estimula uma forte resposta imune (HAUK et al., 2008; HOKE et al., 2008).

Essas observações levam a acreditar que ambas as proteínas teriam um papel importante na disseminação e/ou colonização do agente e até mesmo na sua sobrevivência. Ambas as proteínas foram, por longo tempo, consideradas como fatores de virulência devido a sua localização, distribuição, o reconhecimento por soro de pacientes infectados e sua função e/ou atividade descrita. Portanto, foi surpreendente observar que, ao contrário do esperado, as cepas apresentando deficiência na expressão destas proteínas, não apresentaram nenhuma diferença na sua virulência, capacidade de aderência, sobrevivência ou multiplicação.

Nos dois primeiros artigos aqui expostos, relacionados com o primeiro objetivo da tese, descrevemos o uso de duas metodologias distintas para obtenção de cepas mutantes em sorovares patogênicos de *Leptospira* sp. A mutação aleatória por transposon foi utilizada para gerar um mutante de *L. interrogans* sorovar Manilae, deficiente na expressão de LipL32 e a recombinação alélica por mutação dirigida foi utilizada para obtenção de uma cepa mutante de *L. interrogans* sorovar Copenhageni, deficiente na expressão de LigB. Porém, nossos estudos mostraram que ambas as cepas mutadas para os genes correspondentes não apresentaram atenuação de virulência quando testadas frente ao modelo animal de hamster e rato, padrões para a infecção aguda e crônica da doença, respectivamente.

As leptospiras possuem uma grande quantidade de lipoproteínas (CULLEN et al., 2005) em sua estrutura e a redundância de mecanismos de virulência em bactérias é bem conhecida (MORTON et al., 1999). Dessa maneira, é bem provável existir uma redundância de fatores de virulência em espécies de *Leptospira*. No estudo com LipL32, foi demonstrado que mesmo sendo a proteína de maior abundância na superfície das leptospiras, sua deficiência não causou problemas estruturais ou de sobrevivência, nem mesmo super expressão de outras proteínas como método de compensação. Infelizmente, um estudo similar não foi realizado no mutante *ligB*$^{-}$. A proteína LigA possui domínios Big que podem ser redundantes com os domínios similares presentes na LigB (CHOY et al., 2007; MATSUNAGA et al., 2003). Um projeto para o futuro seria a produção de mutantes deficientes para as duas proteínas ou a geração de mutantes deficientes de LigB em cepas que não possuem o gene para LigA.

O papel de possíveis fatores de virulência em leptospirose não havia sido estudado até o momento devido às dificuldades inerentes à manipulação genética em leptospiras, bem como à impossibilidade de se realizar mutagênese sítio-dirigida em leptospiras patogênicas. Recentemente, foi demonstrado que o transposon *mariner Himar 1* permite a mutação aleatória no patógeno *L. interrogans* (BOURHY *et al*., 2005). Apesar de nossos resultados ainda manter dúvidas sobre o verdadeiro papel de LigB e LipL32 nas leptospiras, a descrição e utilização de ambas as técnicas de mutagênese é de extrema importância para o estudo da biologia das leptospiras.

Uma observação importante é que os estudos *in vitro* com o mutante *ligB*$^{-}$ foram conduzidos com culturas de célula, enquanto que os ensaios com o mutante *lipL32*$^{-}$ foram realizados com componentes da matriz celular. Estudos *in vitro* nem sempre refletem a realidade

das interações que acontecem *in vivo*, portanto não pode ser totalmente descartada uma possível atividade de interação dessas proteínas com o tecido do hospedeiro. Porém, é possível dizer que a simples deficiência dessas proteínas não altera a capacidade do agente em termos de multiplicação, colonização e virulência.

O terceiro artigo aqui exposto, e que se refere ao segundo objetivo da tese, descreve o uso de um PCR quantitativo em tempo real baseado na tecnologia TaqMan e que utiliza o gene *lipL32* como alvo. As leptospiras são bactérias móveis, capazes de penetrar em tecidos e de disseminar rapidamente (FAINE, 1957). Porém, a quantificação do agente nos diferentes tecidos, assim com a cinética da infecção com diferentes doses, ainda não foi explorada intensamente.

Com o crescente desenvolvimento de técnicas de Real Time PCR, principalmente para o diagnóstico da doença (SMYTHE et al., 2002; STODDARD et al., 2009), a utilização dessa metodologia para os estudos de cinética e compreensão da patogênese foram favorecidos e começam a ser mais bem exploradas. Num estudo recente onde utilizaram cobaios infectados com *Leptospira* sp., Lourdault e colaboradores (2009) mostraram que a detecção de leptospiras nos tecidos acontece após dois dias da infecção, chegando a alcançar 10^6-10^7 leptospiras por grama de tecido após seis dias de infecção. A quantificação mais apurada de leptospiras nos tecidos oferece uma ferramenta importante não só para os estudos de cinética e disseminação, mas também para a melhor compreensão da patogênese da doença. Em nosso estudo, determinamos a cinética da infecção de leptospirose no modelo animal de hamster, utilizando diferentes doses e rotas de infecção.

A infecção com *L. interrogans* produz infecção letal em hamster e reproduz a apresentação clínica da forma grave da leptospirose humana, incluindo hemorragia pulmonar (LEVETT, 2001; McBRIDE et al., 2005). Sendo o hamster um dos principais modelos de animal para o estudo da infecção aguda por leptospirose, assim como estudos de vacina e de tratamento (HAAKE, 2006), este modelo foi o escolhido para nossos estudos. Nossos resultados demonstraram que as leptospiras se distribuem rapidamente por todos os tecidos sem um tropismo especifico, indicando que possivelmente a distribuição é feita via hematógena. A detecção de leptospiras nos tecidos de animais infectados com alta dose (10^8 leptospiras) foi realizada após uma hora da infecção, em todos os tecidos, sendo que o óbito dos animais foi observado cinco dias pós-infecção. A infecção com baixa dosagem (250 leptospiras) mostrou um retardo na primeira detecção em tecidos, três dias no sangue, sendo que apenas no oitavo dia

todos os tecidos apresentavam detecção, com o óbito dos animais sendo observado após onze dias. Porém, ao compararmos a quantificação de leptospiras nos diferentes tecidos no último ponto de análise antes da morte dos animais infectados, os números entre os animais infectados com alta e baixa dosagem são semelhantes, indicando que a carga do agente nos tecidos possui uma grande relevância para o desfecho da doença. Essa observação foi evidenciada quando realizada a análise da cinética em cepas com atenuação da virulência. Neste caso, apesar de ser possível detectar leptospiras nos tecidos mesmo em cepas que não levaram os animais ao óbito, a carga do agente nos tecidos momentos antes da morte dos animais foi significantemente diferente, sendo bem maior nos animais infectados com a cepa que causou infecção letal.

A via intraperitoneal é a via de inoculação utilizada freqüentemente para a infecção experimental em hamster, porém esta via pode não refletir as condições encontradas durante a infecção natural (ATHANAZIO et al., 2008). O uso da conjuntiva ocular como rota de infecção, apesar de pouco utilizada, é a que mais se assemelha ao modo de infecção que ocorre na natureza. Nossos resultados mostraram que essa via de inoculação proporcionou resultados reproduzíveis e que permitiram observar a cinética da infecção considerando um fator importante da epidemiologia das leptospiras, que é a penetração. Na análise da cinética de infecção das cepas não-virulentas, a infecção via intraperitoneal não causou a morte dos animais, porém houve disseminação nos tecidos a ponto de serem detectadas e quantificadas, apesar da baixa carga, como descrito acima. Porém, quando realizada a infecção dos animais com as cepas não-virulentas utilizando a via ocular, não houve detecção do agente em nenhum tecido analisado. Sabendo-se que o processo de infecção do hospedeiro por um patógeno é complexo e multifatorial, e que diferentes proteínas podem influenciar a patogenia da leptospirose em vários momentos da infecção, a utilização da via ocular em estudos de determinação de fatores de virulência é uma ferramenta de extrema importância.

Em sumário, os resultados aqui apresentados descrevem a evolução das técnicas de manipulação genética em leptospiras patogênicas, incluindo o uso pela primeira vez da troca alélica por mutação dirigida. Com o uso de diferentes técnicas, em diferentes sorovares de *Leptospira* sp., constatamos que duas importantes proteínas, antes descritas como prováveis fatores de virulência, não são relevantes para a infecção aguda e crônica no modelo animal. A descrição de um PCR em tempo real quantitativo para os estudos de cinética de disseminação no modelo animal, além de proporcionar uma melhor compreensão do processo de disseminação,

multiplicação e carga nos diferentes tecidos, nos oferece uma ferramenta importante, principalmente nos estudos de determinantes de virulência e em estudos vacinais. O papel específico dos possíveis fatores de virulência aqui estudados, nas várias etapas da infecção, como a sobrevivência da bactéria *in vivo*, o estímulo ao sistema imune, a adesão durante a colonização ou a capacidade de invasão dos tecidos, permanece especulativo e necessita melhores estudos no futuro. Ainda é preciso uma melhor compreensão dos mecanismos moleculares da patogênese da leptospirose, mas as técnicas aqui descritas serão fundamentais para esse processo, tendo como objetivo comum o desenvolvimento de novas e diferentes estratégias de prevenção para uma doença que possui um importante impacto na saúde, principalmente de populações carentes de países em desenvolvimento.

8. CONCLUSÃO

- Utilizando pela primeira vez o método de mutagênese sítio-dirigida, foi obtido um mutante na cepa *L. interrogans* sorovar Copenhageni com interrupção no gene *ligB*. A interrupção e a expressão da proteína foram testadas através de PCR e Western Blot, respectivamente. Foi comprovada a inserção do cassete de resistência para espectinomicina no gene e a falta de expressão da proteína, mostrando que a recombinação homóloga dirigida pode ser obtida em leptospiras patogênicas;

- Estudos *in vivo* através da infecção intraperitoneal e utilizando o modelo animal padrão de infecção aguda e crônica de leptospirose, respectivamente, foi demonstrado que a falta da proteína LigB não leva à uma atenuação da virulência e colonização;

- Na análise *in vitro* de aderência celular, não foram observadas diferenças entre o mutante *ligB*$^{-}$ e a cepa parental;

- Utilizando a técnica de mutação aleatória, foi obtido um mutante na cepa *L. interrogans* sorovar Manilae com interrupção no gene *lipL32*. Foi comprovada a ausência de expressão da proteína LipL32 através de Western Blot, mostrando que esse método de seleção de mutantes randômicos pode ser útil no estudo de possíveis fatores de virulência em leptospirose;

- Análises *in vitro* demonstraram que não houve diferenças entre o mutante *lipL32*$^{-}$ e a cepa parental com relação à aderência aos componentes da matriz celular e nem alterações na expressão de genes como forma de compensação da perda da proteína LipL32;

- Os estudos *in vivo* com hamster, utilizando a infecção intraperitoneal e ocular, demonstraram que a proteína LipL32 não tem papel na virulência da leptospirose. O mesmo resultado foi observado no modelo de rato, não havendo diferenças na colonização renal destes animais;

- O verdadeiro papel da proteína LigB e LipL32 na biologia das leptospiras ainda se mantém inconclusivo;

- Através da técnica de Real Time PCR, tendo como alvo o gene *lipL32*, comprovamos a alta capacidade de disseminação das leptospiras após a penetração, sendo estas detectadas após uma hora da infecção intraperitoneal com alta dose do patógeno (10^8 leptospiras) em todos os tecidos;

- Durante o processo de penetração e disseminação inicial do agente, não ocorre tropismo específico para nenhum tecido em especial;

- A carga do agente nos tecidos é importante para o desfecho clínico da doença, assim como para as alterações histopatológicas identificadas;

- A técnica de Real Time PCR aqui descrita é uma importante ferramenta para identificação e estudo de possíveis fatores de virulência em leptospirose;

- O uso da conjuntiva ocular como via de inoculação do agente na infecção experimental mostrou ser um método válido para os estudos de patogênese, sendo reproduzível e mais próximo do processo que ocorre na natureza, pois considera o processo de penetração do agente, essencial para o estabelecimento da infecção.

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