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Novas abordagens para estudos em leptospirose: contribuindo para o desenvolvimento de vacinas

André Alex Grassmann

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Dedico.

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“Isn't it sad to go to your grave without ever wondering why you were born? Who, with such a thought, would not spring from bed, eager to resume discovering the world and rejoicing to be part of it?”

Richard Dawkins

Resumo

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A leptospirose é uma doença tropical negligenciada, de caráter zoonótico, responsável por cerca de 873 mil casos humanos e 49 mil mortes em todo o mundo a cada ano. O agente causador pertence ao gênero *Leptospira*, um grupo antigenicamente e geneticamente diverso de espiroquetas, dividido em nove espécies patogênicas, 24 sorogrupos e mais de 250 sorovares. As leptospiras podem infectar praticamente qualquer espécie de mamífero. Roedores podem carrear leptospiras nos túbulos renais, eliminando-as em grande número através da urina, sendo esta a fonte mais importante para novas infecções. Em hospedeiros suscetíveis, as leptospiras patogênicas se espalham no organismo resultando em uma doença febril com icterícia, seguida de falência renal, hepática e cardíaca, que com frequência leva à morte. A vacinação é a abordagem profilática mais efetiva contra a leptospirose. A bacterina é a única vacina licenciada, sendo usada em todo o mundo para algumas espécies animais, enquanto o uso em humanos é permitido em apenas alguns poucos países. A razão para isso é a resposta imune de curta duração e sorovar-específica induzida por essa vacina, que apresenta ainda, efeitos colaterais adversos. Vários esforços para desenvolver uma vacina recombinante protetora contra diferentes sorovares e com resposta de longa duração, falharam. O pouco conhecimento sobre os fatores de virulência e a patogênese de *Leptospira* spp. são as principais razões para o lento avanço na descoberta de antígenos protetores. Esta tese apresenta várias abordagens diferentes de estudos em leptospirose na tentativa de acrescentar ao conhecimento acerca da doença e seu agente etiológico: O cenário atual de desenvolvimento de novas vacinas contra a leptospirose é devidamente revisado. Uma nova cepa virulenta de *L. interrogans* isolada de um cão com leptospirose aguda foi caracterizada e pode ser utilizada em experimentos de infecção em modelo animal, ou, ainda, para o entendimento de mecanismos de virulência. Foi desenvolvido um protocolo para obtenção de leptospiras adaptadas ao hospedeiro, através do cultivo destes organismos dentro de Câmaras de Membrana de Diálise (DMC) implantadas na cavidade peritoneal de ratos. Este protocolo foi utilizado para identificar, a partir do sequenciamento de RNA total (RNA-seq), genes relacionados com as mudanças sofridas pela *Leptospira* spp. para se adaptar ao hospedeiro durante a infecção, novos fatores de virulência e seleção de alvos para mutagênese. Finalmente, uma via alternativa para infecção de hamsters por *L. interrogans* virulenta foi descrita, mimetizando a entrada natural pela via transcutânea de leptospiras no hospedeiro. Esta metodologia pode substituir a injeção intraperitoneal de leptospiras. Juntos, estes achados representam um progresso substancial no campo de estudo da leptospirose e possivelmente irão contribuir para a descoberta futura de antígenos protetores para utilização no desenvolvimento de vacinas aperfeiçoadas contra leptospirose.

Palavras-chave: *Leptospira*, Leptospirose, vacinas, DMC, via de infecção transcutânea.

Abstract

GRASSMANN, André Alex. **New approaches for leptospirosis studies: contributing to vaccine development**. 2015. 125f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is a widespread neglected tropical zoonotic disease responsible for at least 873,000 human cases and 49,000 deaths each year globally. The causative agent belongs to the genus *Leptospira*, a unique and genetically and antigenically diverse group of spirochetes divided into nine pathogenic species, 24 serogroups and more than 250 serovars. Leptospires can infect virtually any mammalian species. Rodents can carry spirochetes in their renal tubules and shed large numbers in their urine, the main source of leptospires for new infections. In susceptible hosts, pathogenic leptospires spread throughout the body, resulting in a febrile icteric illness, followed by renal, hepatic and cardiac failure that can lead to death. Vaccination is the most effective approach for leptospirosis prophylaxis. Bacterins are the only licensed vaccines, and are used worldwide in certain animals, however, human vaccination is approved in only a few countries. The reason for this is that the vaccine induces a serovar specific, short-term immune response that has several adverse side effects. Efforts to develop a new recombinant vaccine with long-term, cross-protective immunity have failed. The lack of knowledge of *Leptospira* spp. virulence factors and pathogenesis is the main reason for the slow progress in the discovery of protective antigens. This thesis describes several different approaches in leptospirosis studies in an attempt to improve understanding of the disease and its causative agent: The current scenario of leptospirosis vaccine development is comprehensively reviewed. A new *L. interrogans* virulent strain isolated from a dog presenting with acute leptospirosis was characterized and can be used for experimental infections in animal models, or for understanding virulence mechanisms. A protocol to obtain host-adapted leptospires cultivated within Dialysis Membrane Chambers (DMC) implanted in rat peritoneum was developed. This protocol was applied to the identification, by total RNA sequencing (RNA-seq), of several genes related to the changes that leptospires undergo during adaptation to infection, new virulence determinants and selection of targets for mutagenesis. Finally, an alternative route of infection of hamsters by virulent *L. interrogans* is described, mimicking the natural transcutaneous entry of leptospires into the host. This methodology could replace the intraperitoneal injection of leptospires. Together, these findings represent substantial progress in the field of leptospirosis, possibly contributing to the future discovery of protective antigens for the development of improved vaccines against leptospirosis.

Keywords: *Leptospira*, leptospirosis, vaccines, DMC, transcutaneous infection route.

Lista de Abreviaturas

EMJH – Ellinghausen-McCullough-Johnson-Harris *medium*

PBS – Tampão Salina de Fosfato (*Phosphate Buffer Saline*)

DMC – Câmara de Membrana de Diálise (*Dialysis Membrane Chamber*)

DMSO – Dimetil sulfóxido

SDS-PAGE – Eletroforese em gel de acrilaminda – dodecil sulfato de sódio

ST – Tipo de sequência (*Sequence type*)

MAT – Teste de microaglutinação

MLST – *Multilocus Sequence Typing*

IM – Método de *Imprint*

HE – Hematoxilina e eosina

TC – Transcutâneo

TLR4 – Receptor do tipo *Toll* 4

TNF – Fator de necrose tumoral

CONCEA – Conselho Nacional de Controle da Experimentação Animal

IGR – Região intergênica

IL – interleucina

IP – Intraperitoneal

IV – *in vitro*

BSA – Albumina sérica bovina

SC – Subcutâneo

CJ – Conjuntival

CDS – Sequencia codificadora

LE – Lesão provocada com agulha

LS – Lesão provocada por escarificação da pele

SL – Sem lesão provocada

WW – Exposição do animal à agua morna

LD50/DL50 – Dose letal à 50% dos animais

LPS – Lipopolissacarídeo

ORF – *Open reading frame*

Sumário

1 INTRODUÇÃO GERAL

A leptospirose é uma zoonose negligenciada de distribuição global, de caráter ocupacional, associada a períodos de chuva intensa, enchentes e saneamento básico precário (McBride et al., 2005). Atualmente, estima-se a ocorrência de 873 mil novos casos de leptospirose humana, levando 49 mil pessoas à morte no mundo (Picardeau, 2013, WHO, 2011). Os sinais clínicos variam desde uma manifestação febril auto-limitante, até uma doença ictérica crônica, com falhas renais e hepáticas (Doença de Weil), ou ainda, podendo progredir para a síndrome hemorrágica pulmonar associada à leptospirose, uma manifestação grave, com taxas de mortalidade superiores a 50% (McBride et al., 2005, Ko et al., 2009).

A leptospirose é uma doença infecciosa causada por espiroquetas patogênicas do gênero *Leptospira.* Atualmente estão descritas 9 espécies patogênicas e pelo menos 250 sorovares, distribuídos em 24 sorogrupos (Bourhy et al., 2014, Cameron, 2015). Esta grande variação sorológica é consequência, principalmente, da variação antigênica no lipopolissacarídeo (LPS), presente na membrana externa da *Leptospira* (Cameron, 2015, McBride et al., 2005). Esta espiroqueta possui duas membranas e entre elas o espaço periplasmático, contendo uma camada de peptideoglicano e dois flagelos ancorados em suas extremidades. Na membrana externa, além do LPS, estão presentes grande quantidade de proteínas transmembrana (Haake e Zuckert, 2015, Ko et al., 2009).

A vacinação é a medida profilática mais promissora para controle da leptospirose (Dellagostin et al., 2011). Seu uso é documentado desde o início do século passado e envolve preparações de células inteiras mortas por métodos físicos ou químicos (Noguchi, 1918). Estas vacinas, entretanto, são reatogênicas, causando inflamação local. Seu uso é comum apenas em poucos países (China, Cuba, Japão, Vietnã e França) em períodos de enchentes (Dellagostin et al., 2011). Bacterinas contra leptospirose estão globalmente disponíveis para cães, bovinos e suínos, porém são necessárias revacinações anuais (Ellis, 2015, Dellagostin et al., 2011). Além disto, estas vacinas protegem apenas contra sorovares presentes nas suas formulações e normalmente a proteção não é esterilizante (Dellagostin et al., 2011, Levett, 2001).

Na última década, um número crescente de estudos investigou a proteção induzida por vacinas recombinantes contra leptospirose utilizando como antígeno proteínas de membrana externa da *Leptospira*. Diversos antígenos leptospirais, até então considerados os mais promissores para compor uma vacina, já foram testados através de diferentes estratégias e com diferentes adjuvantes – LipL32 (Grassmann et al., 2012), LipL41 (Haake et al., 1999), LigANI (Silva et al., 2007), LigB (Yan et al., 2009, Cao et al., 2011), Loa22 (Zhang et al., 2010) – porém nenhum trabalho obteve, simultaneamente, proteção heteróloga total, estatisticamente significativa, esterilizante e sem efeitos adversos (Dellagostin et al., 2011). O desenvolvimento racional de vacinas recombinantes contra leptospirose ainda é limitado pela escassez de estudos focados em elucidar a resposta imune protetora contra esta doença e em identificar fatores de virulência que contribuam para a patogênese e que possam ser utilizados como alvos vacinais (Adler, 2014b).

O desafio em experimentos para avaliação de novas vacinas é realizado pela injeção intraperitoneal (IP) de leptospiras virulentas nos animais vacinados (Haake, 2006). Apesar de o mecanismo de entrada de leptospiras no hospedeiro não estar completamente esclarecido, a injeção IP não simula corretamente a infecção natural que ocorre quando um hospedeiro entra em contato com as leptospiras na natureza, frequentemente através do contato da pele apresentando microlesões ou da mucosa com locais úmidos, contaminados com urina de animais portadores (solo, poças d'água, esgoto a céu aberto) (McBride et al., 2005, Ko et al., 2009). Uma nova metodologia que mimetize o que ocorre na transmissão natural de leptospiras, para indução de leptospirose letal em modelo animal é necessário. Isso proporcionaria a correta avaliação de antígenos vacinais, inclusive aqueles importantes em estágios iniciais da invasão ao hospedeiro.

Nesta tese, são apresentados trabalhos descrevendo a padronização e utilização do cultivo de *L. interrogans* dentro de Câmara de Membrana de Diálise (DMC, do inglês *Dialysis Membrane Chamber*). Esta abordagem permitiu a identificação de genes relacionados com a adaptação de leptospiras ao hospedeiro, que são agora potenciais novos alvos para o desenvolvimento de vacinas recombinantes. Além disso, foi desenvolvida uma metodologia para indução de infecção letal em hamsters baseada na entrada natural de leptospiras através de

microlesões provocadas na pele dos animais. Esta metodologia é confiável, reproduzível e pode sem empregada não apenas na indução de leptospirose para estudos focados no entendimento da patologia da doença, como desafio em estudos de vacinas. Finalmente, é apresentado o isolamento e caracterização molecular de um isolado virulento de *L. interrogans* sorogrupo Icterohaemorrhagiae, identificado com cepa UFPEL-RCA.

2 REVISÃO BIBLIOGRÁFICA

2.1 História da *Leptospira* e da leptospirose

A história moderna da leptospirose inicia em 1886 quando o médico alemão Adolf Weil publicou seus estudos descrevendo um tipo de doença ictérica, acompanhada de disfunção renal, esplenomegalia, conjuntivite e erupções cutâneas (Adler, 2015). Hoje é sabido que a doença descrita é a leptospirose, cuja forma grave é chamada doença de Weil desde aqueles primórdios.

Acredita-se que as leptospiras sejam a causa de vários surtos de doenças febris ictéricas, com registros históricos datando de até mil anos atrás, a maioria deles na Ásia. E foi na Ásia que o pesquisador japonês R. Inada, praticamente concomitante com Stimson na Europa, descreveu pela primeira vez a existência de leptospiras em material de autópsia realizada em pacientes com leptospirose (Inada et al., 1916, Adler, 2015). Já naquele momento, eles descreveram rotas de infecção, patologia, distribuição nos tecidos, liberação de leptospiras na urina, morfologia e motilidade. Em estudos com *guinea pigs*, foi mostrado os sinais da doença, e a presença de espiroquetas em vários tecidos. Inada nomeou o organismo recém descoberto como *Spirochaeta icterohaemorrhagiae*, e o organismo isolado por ele, presente até os dias atuais foi renomeado na década de 1990 como *L. interrogans* Ictero No. 1 (agora já descrita como sorogrupo Icterohaemorrhagiae). Outros grupos na Europa, também de forma simultânea tiveram achados semelhantes ao do japonês, inclusive publicando ensaios sobre modelos animais e novos isolados (Adler, 2015, Faine et al., 1999).

Em sequência aos trabalhos de Inada, Ido e colaboradores, demonstraram o status de carreador assintomático do rato e a liberação de espiroquetas na urina. O grupo japonês descreveu ainda interessantes achados epidemiológicos, como o caráter sazonal da leptospirose, relação com períodos quentes e de chuvas, bem como o caráter ocupacional, com uma prevalência maior em trabalhadores de minas de carvão, por exemplo (Ido et al., 1917b, Ido et al., 1917a).

A partir daí, diversos grupo ao redor do mundo passaram a descrever síndromes causadas por leptospiras em diferentes locais e espécies animais. O nome *Leptospira* foi proposto por Noguchi em 1918 a fim de diferenciar a leptospirose das doenças causadas por outras espiroquetas conhecidas na época (Noguchi, 1918).

Cada novo isolado recebia um novo nome dentro do gênero, até que em 1982 o gênero foi dividido em dois, *L. interrogans* para as patogênicas e *L. biflexa* para as saprófitas, e todas as demais subclassificações se tornaram sorovares e sorogrupos de ambas (Adler, 2015). Com o surgimento de estudos de parentesco de DNA as leptospiras foram subsequentemente classificadas em diferentes espécies (Brenner et al., 1999).

2.2 Sistemática de Leptospiraceae

As leptospiras pertencem ao Filo Spirochaetes, Classe Spirochaetes, Ordem Spirochaetales, Família Leptospiraceae. Outras duas espécies da Ordem Spirochaetales têm grande importância médica, a *Borrelia burgdorferi*, causadora da Doença de Lyme e a *Treponema pallidum* causadora da Sífilis. Além das espécies do gênero *Leptospira*, as espiroquetas *Turneriella parva* e *Leptonema illini* também pertencem a família Leptospiraceae (Levett, 2015).

Atualmente estão descritas 21 espécies dentro do gênero *Leptospira*. Estas espécies são diferenciadas pela hibridização de DNA (atualmente realizada *in silico*) (Bourhy et al., 2014). Nove delas são patogênicas, *L. interrogans*, *L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. alexanderi, L. kmetyi*, e *L. alstonii*, podendo infectar e causar doença em humanos e outros animais. Cinco espécies são de patogenicidade intermediária, *L. inadai, L. broomii, L. fainei, L. wolffii* e *L. licerasiae*, e foram isoladas de humanos e animais e podem causar manifestações clínicas leves. Finalmente, sete espécies são saprófitas e não desenvolvem doença nem em humanos nem animais e são exclusivamente ambientais: *L. biflexa, L. wolbachii, L. meyeri, L. vanthielii, L. terpstrae, L. idonii,* e *L. yanagawae* (Levett, 2015, Bourhy et al., 2014).

Além da classificação taxonômica as leptospiras são classificadas sorológicamente de acordo a sua antigenicidade, determinada majoritariamente pela composição química do lipopolissacarídeo (LPS) presente na membrana externa. Existem pelo menos 250 sorovares de leptospiras, distribuídos em 24 sorogrupos (Cameron, 2015). Alguns sorovares estão representados em diferentes espécies e até o momento não há uma metodologia precisa para identificação a nível de sorovar que não o uso de anticorpos monoclonais capazes de reconhecer especificamente

um dentre as centenas de sorovares existentes. Recentemente, um esquema para *Multilocus Sequence Typing* (MLST) foi desenvolvido e, a partir do sequenciamento de 7 loci e comparação com o banco de dados de sequencias, permite a identificação molecular de sete espécies patogênicas. Essa metodologia é, ainda, confiável para identificação de diversos sorogrupos e sorovares, ainda que não seja completa (Boonsilp et al., 2013).

2.3 Estrutura, fisiologia e metabolismo de leptospiras

Apesar da variedade de espécies e sorovares, as leptospiras mantém um padrão estrutural, sendo todas espiraladas, finas (0,1 µm × 6-20 µm) e altamente móveis. Leptospiras recém isoladas normalmente são mais curtas e móveis do que as que já sofreram diversas passagens *in vitro* (Ellis et al., 1983b), que podem se tornar extremamente alongadas em condições limitantes de nutrientes, e até esféricas em condições ainda mais críticas (Cameron, 2015).

O envelope celular de leptospiras é muito semelhante às Gram-negativas. Possuem uma membrana interna, um espaço periplasmático contendo peptideoglicano, membrana externa onde grande quantidade de LPS fica ancorado, compondo o folheto externo da mesma (Faine et al., 1999, Cameron, 2015). O LPS é relativamente diferente daquele encontrado em Gram-negativas, sendo menos tóxico ao hospedeiro, em função da constituição do lipídeo A (Werts et al., 2001, Que et al., 2002). No espaço periplasmático, a camada de peptideoglicanos está mais próxima a membrana interna do que à externa (Nauman et al., 1969, Cameron, 2015). Neste local há ainda dois flagelos, ancorados um em cada uma das extremidades da bactéria. A motilidade é essencial a virulência de leptospiras (Lambert et al., 2012), que conseguem se locomover a uma velocidade de 20 µm a cada 2-3 segundos em meio líquido (Faine et al., 1999).

O genoma de leptospiras patogênicas codifica para vias completas de biossíntese de aminoácidos e ácidos nucleicos (Faine et al., 1999, Ren et al., 2003), ao contrário de outras espiroquetas, como *B. burgdorferi*, *T. pallidum* e a *L. biflexa* (Cameron, 2015). A fonte de carbono e energia para leptospiras são ácidos graxos de cadeia longa (Henneberry e Cox, 1970). A incapacidade de utilização de glicose para esse fim não se deve à ausência de uma via de utilização de glicose, uma vez

que os genes estão presentes (Nascimento et al., 2004a) e sim a um limitado sistema de transporte deste açúcar. Leptospiras são aeróbias obrigatórias, portanto apresentam um conjunto completo de genes para o ciclo do ácido carboxílico e para cadeia de transporte de elétrons (Nascimento et al., 2004a, Ren et al., 2003). O crescimento de leptospiras normalmente requer uma fonte de nitrogênio, fornecida na forma de amônia (Faine et al., 1999).

2.4 Requerimentos nutricionais, crescimento e cultivo

As leptospiras necessitam de suplementos de fontes de carbono e nitrogênio e presença de vitaminas para crescimento *in vitro*. Ácidos graxos de cadeia longa são essenciais, mas também tóxicos, necessitando a adição de albumina sérica ou ácidos graxos complexados a sorbitol (Tween) para a absorção destes lipídeos tóxicos e sua liberação no meio a níveis não tóxicos (Cameron, 2015, Faine et al., 1999). Como citado anteriormente, suplemento de amônio é necessário como fonte de nitrogênio. Outros suplementos nutricionais necessários são tiamina, biotina, fosfato, cálcio, magnésio, ferro, manganês, cobre e sulfatos (Faine et al., 1999). Vitamina B12 também é normalmente adicionado a meios de cultura, apesar recentemente demonstrado que as leptospiras são capazes de produzir esta vitamina (Nascimento et al., 2004a). O meio de cultura desenvolvido por Ellinghausen e McCullough (Ellinghausen e McCullough, 1965a, Ellinghausen e McCullough, 1965b) e modificado por Johnson e Harris (Johnson e Harris, 1967), o EMJH, inclui todos estes sais e suplementos, sendo o mais comumente utilizado.

O crescimento ideal de leptospiras patogênicas ocorre em 28-30 °C, mas as mesmas crescem normalmente a 37 °C. O pH ótimo é 7.2-7.6. O tempo de duplicação de leptospiras recentemente isoladas de hospedeiros é em torno de 14-18h em EMJH, mas após várias passagens o crescimento *in vitro* é acelerado, com tempo de duplicação de 6-8h (Cameron, 2015, Faine et al., 1999).

2.5 Genômica e proteômica de *Leptospira*

A primeira espécie de *Leptospira* a ter o genoma sequenciado foi *L. interrogans*. Dois sorovares, Lai e Copenhageni, ambos do sorogrupo Icterohaemorrhagiae foram sequenciados quase concomitantemente (Nascimento et al., 2004b, Ren et al., 2003). Assim como as demais espécies de leptospiras, *L.*

interrogans apresenta dois cromossomos. Ambos com 35% de CG, um com 4277 kb e outro, menor, com 350 kb. Outras quatro espécies já tiveram o genoma sequenciado e publicado, a saprófita *L. biflexa* (Picardeau et al., 2008), as patogênicas *L. borgpetersenii* (Bulach et al., 2006) e *L. santarosai* (Chou et al., 2012), e a intermediária *L. licerasiae* (Ricaldi et al., 2012). Recentemente, um esforço internacional, com apoio do J. Craig Venter *Institute*, está sequenciando centenas de cepas de leptospiras, de todas as espécies, a fim de obter um quadro representativo do genoma deste gênero.

O genoma de *L. interrogans* sorovar Lai apresenta 3718 sequencias codificadoras (CDS) (Ren et al., 2003) enquanto *L. borgpetersenii* aparenta uma redução no número de CDS, com apenas 2800 (Bulach et al., 2006). Todos estes genomas sequenciados e publicados até o momento apresentam cerca de 35% de CDS sem função conhecida (Ren et al., 2003, Nascimento et al., 2004a, Nascimento et al., 2004b, Bulach et al., 2006, Chou et al., 2012, Ricaldi et al., 2012). Existem 1547 genes em comum, presentes em *L. interrogans*, *L. biflexa, L. borgpetersenii, L. santarosai* e *L. licerasiae*, a maioria destes genes são *housekeeping*, relacionados com funções vitais (Picardeau, 2015). A análise comparativa do genoma de *L. interrogans, L. borgpetersenii* e *L. biflexa* mostrou a presença de 893 genes específicos para patógenos, dos quais 78% tem função desconhecida, sugerindo a existência de mecanismos de virulência específicos para leptospiras (Picardeau, 2015, Picardeau et al., 2008, Adler, 2014b).

O número máximo de proteínas detectadas em um único estudo foi de 2673 em *L. interrogans* Lai (Cao et al., 2010). Algumas proteínas, com o as proteínas Lig (*Leptospiral immunoglobulin like proteins*) não são expressas em condições normais de cultivo (Matsunaga et al., 2005), sendo reguladas por alterações osmóticas. Diversos estudos de proteômica focaram na determinação da localização subcelular de proteínas de leptospiras (Haake e Matsunaga, 2010), porém até o momento nenhuma técnica parece ser reproduzível (Cullen et al., 2003, Hauk et al., 2009, Vivian et al., 2009, Eshghi et al., 2011, Vieira et al., 2012, Pinne e Haake, 2013). Uma nova abordagem, utilizando crio-eletrotomografia identificou e quantificou ~1800 proteínas expressas em *L. interrogans* (Malmstrom et al., 2009, Beck et al., 2009). Este estudo mostrou, por exemplo, que LipL32 possui aproximadamente 38 mil cópias numa única célula de *Leptospira*. Alguns estudos já demonstraram a ocorrência de

modificações pós-traducionais em leptospiras, como metilação, fosforilação e acetilação (Ricaldi et al., 2013, Cao et al., 2010, Eshghi et al., 2011).

2.6 Leptospirose em humanos

A leptospirose é uma zoonose distribuída em todo o mundo, potencialmente fatal e endêmica em muitas regiões tropicais. É a causa de grandes epidemias pós chuvas fortes e alagamentos (McBride et al., 2005). A infecção resulta de um contato direto ou indireto com animais reservatórios que carregam leptospiras nos túbulos renais e as eliminam no ambiente através da urina. Apesar de muitos animais domésticos e selvagens servirem como reservatório, o rato (*Rattus norvegicus*) é a principal fonte de leptospiras patogênicas na transmissão a humanos. Indivíduos vivendo em zonas urbanas pobres, caracterizadas por condições sanitárias inadequadas e moradias impróprias estão em alto risco de exposição a estes ratos e, por consequência às leptospiras (Haake e Levett, 2015). A leptospirose também é uma doença de caráter ocupacional e recreativo, onde atividades que proporcionam contato com animais potencialmente infeccionados (veterinários, trabalhadores rurais *etc.*) ou água contaminada (praticantes de esportes aquáticos) aumentam o risco de adquirir a doença (Sejvar et al., 2005, Sejvar et al., 2003, Haake et al., 2002).

Um aumento do impacto da leptospirose no mundo é esperado com as mudanças demográficas que favorecem a maior acumulação de população pobre urbana em regiões tropicais sujeitas a tempestades e alagamentos urbanos devido às mudanças climáticas (WHO, 2011). Atualmente são estimados cerca de 873 mil casos de leptospirose humana, com 49 mil mortes a cada ano (Picardeau, 2013, WHO, 2011). Dados de estudos de vigilância prospectiva sugerem que a maioria das infecções por leptospiras em humanos de áreas endêmicas são leves ou assintomáticas. O desenvolvimento de quadro clínico mais severo depende, majoritariamente de três fatores: condições epidemiológicas, suscetibilidade do hospedeiro e virulência da *Leptospira* (Haake e Levett, 2015). A doença ocorre predominantemente em homens, provavelmente devido a uma maior exposição (Katz et al., 2011, Guerra, 2013), enquanto a mortalidade aumenta com a idade, principalmente com pacientes acima dos 60 anos de idade (Lopes et al., 2004). Altos níveis de bacteremia estão associados com prognóstico negativo (Segura et al., 2005, Truccolo et al., 2001) e possivelmente deve-se a choques sépticos causados pelo

LPS, que é ineficientemente reconhecido pelo receptor do tipo Toll 4 (TLR4) (Werts et al., 2001, Nahori et al., 2005). Pacientes com leptospirose severa apresentam altos níveis de interleucina (IL) 6, fator de necrose tumoral (TNF) alfa e IL-10 (Goris et al., 2011, Reis et al., 2013). Pacientes com o alelo HLA DQ6 possuem alto risco de desenvolvimento da doença (Lingappa et al., 2004).

A leptospirose em humanos normalmente se apresenta como uma doença febril aguda, caracterizada por febre, mialgia e dores de cabeça, sendo não específica, e facilmente confundida com outras doenças como a gripe sazonal e dengue (McBride et al., 2005). Novos testes de diagnóstico, em adição ao teste de microaglutinação (MAT), como IgM-ELISA e PCR facilitam o diagnóstico precoce e o tratamento (Picardeau, 2013). Pacientes que progridem para falha de vários órgãos sempre apresentam leptospiremia (Haake e Levett, 2015). Disfunções renais leves normalmente são resolvidas com reposição de líquidos e eletrólitos, mas as mais graves necessitam de diálise e aumentam o risco de morte (Haake e Levett, 2015). É comum um elevado nível de bilirrubina no sangue, devido ao dano hepático e a ruptura de junções intercelulares de hepatócitos (Merien et al., 1998), resultando no vazamento de bilirrubina para fora do ducto biliar. Complicações hemorrágicas são comuns e associadas com anomalias de coagulação (Haake e Levett, 2015). A síndrome hemorrágica pulmonar severa é consequência de hemorragia alveolar, ainda não tem as causas esclarecidas, mas apresenta uma taxa de mortalidade acima dos 50% dos casos (Trevejo et al., 1998, Gouveia et al., 2008).

2.7 Leptospirose em animais

Leptospirose já foi descria em todas as regiões do mundo, com exceção das regiões polares, e em virtualmente todas as espécies animais investigadas para este fim (Ellis, 2015). Entre os animais domésticos parece haver diferentes graus de susceptibilidade, com equinos sendo suscetíveis a diversos sorovares de leptospiras, enquanto a infecção em gatos é muito rara (Levett, 2015). Em teoria, qualquer *Leptospira* patogênica pode infectar qualquer espécie animal, ainda que cada sorovar tenha uma tendência a ser mantido na natureza por uma espécie animal em particular, mesmo que esta possa desenvolver a doença a partir desta infecção (Ellis, 2015). A maior exceção é o roedor (ratos e camundongos) que não desenvolve doença por nenhuma espécie de *Leptospira* (McBride et al., 2005). Os ratos são os hospedeiros

de manutenção do sorogrupo Icterohaemorrhagiae; bovinos e caprinos do sorogrupo Hardjo; suínos e cães dos sorogrupos Canicola e Bratislava (Ellis, 2015).

Bovinos mantém o sorovar Hardjo na natureza, tanto da espécie *L. borgpetersenii* (Hardjobovis) quanto *L. interrogans* (Hardjoprajitno). Infecções por estes sorovares em bovinos normalmente são subclínicas, com exceção de vacas lactantes que podem interromper a produção de leite. Doença severa é muito rara e normalmente está relacionada com infecção pelos sorogrupos Pomona, Icterohaemorrhagiae e Grippotyphosa. Os sinais clínicos normalmente incluem febre, icterícia, anemia hemolítica, ocasionalmente meningite e morte. Abortos, natimortos, nascimentos prematuros e nascimento de bezerros fracos e com baixo peso são os maiores problemas econômicos relacionados com a leptospirose clínica em bovinos (Ellis, 2015).

Da mesma forma que em bovinos, infecções pelo sorovar Hardjo em ovelhas são subclínicas, e a apresentação de complicações surge da infecção pelos sorogrupos Pomona (Vermunt et al., 1994), Grippotyphosa (Ayral et al., 2014), Icterohaemorrhagiae (Leon-Vizcaino et al., 1987), Australis e Sejroe (Ellis et al., 1983a, McKeown e Ellis, 1986). A doença aguda normalmente resulta em abortos, ou icterícia, hematúria e morte ocasional, normalmente em indivíduos jovens (Ellis et al., 1983a, Leon-Vizcaino et al., 1987).

A leptospirose é uma doença comum em suínos ao redor do mundo e é considerada uma importante causa de perdas reprodutivas (Ellis, 2015). As infecções em suínos normalmente estão associadas com os sorogrupos Pomona, Australis e Tarassovi. Apresentações clínicas são mais comuns em animais jovens, enquanto adultos não gestantes normalmente são assintomáticos e podem eliminar leptospiras na urina, especialmente quando infectados com Pomona (Ellis, 2015).

A importância de leptospirose em equinos foi evidenciada apenas recentemente, quando demonstrado a participação desta doença na uveíte recorrente, responsável por grandes perdas econômicas na forma de término prematuro da carreira de cavalos de alto valor comercial ou esportivo (Verma et al., 2013a). Equinos são suscetíveis à uma grande quantidade de sorogrupos, e os mais frequentemente relacionados com a doença nestes animais são Pomona, Grippotyphosa, Icterohaemorrhagiae, Autumnalis, Sejroe, Canicola e Ballum (Ellis, 2015). Apesar da maioria das infecções serem subclínicas, alguns meses após a infecção inicial os

equinos normalmente apresentam uveíte recorrente, uma apresentação clínica autoimune, envolvendo as proteínas leptospirais LruA e LruB (Verma et al., 2010b).

O sorovar Canicola é mantido na natureza apenas por hospedeiros canídeos, mas a soroprevalência deste sorovar tem caído consideravelmente em todo mundo, possivelmente devido à vacinação contra o mesmo (Ellis, 2010). O sorogrupo Icterohaemorrhagiae é o principal causador de infecção acidental em cães (Ellis, 2010). Grippotyphosa, Bratislava e Canicola (Oliveira Lavinsky et al., 2012, Mayer-Scholl et al., 2013, Renaud et al., 2013) também se destacam neste cenário. Assim como em outras espécies, muitas infecções são subclínicas. A leptospirose se apresenta de forma semelhante à doença em humanos, com uma fase anictérica inicial, normalmente auto-limitante, acompanhada de febre, anorexia, náuseas, vômitos e prostração (Ellis, 2015), e uma fase ictérica. Esta segunda fase normalmente é grave, podendo ter em adição aos sinais da fase anictérica, falhas renais, hepáticas e pulmonares, icterícia e progressão para colapso de vários órgãos, o que pode levar a morte (Ellis, 2015).

2.7.1 Modelos animais

Os roedores mais utilizados como modelos experimentais em geral são ratos e camundongos, ambos resistentes a leptospirose, capazes de eliminar grande quantidade de leptospiras na urina (>10^7/ml) sem desenvolver qualquer sinal de doença (Athanazio et al., 2008). Estes animais não são úteis quando é necessário a indução de sinais clínicos e leptospirose aguda, mas podem ser utilizados para desenvolvimento de colonização crônica, especialmente quando os objetivos da pesquisa envolvem fatores de virulência relacionados com a colonização do rim em hospedeiro carreador natural e a eliminação de leptospiras na urina. Ratos podem também servir para manter cepas de leptospiras virulentas ou limpar culturas de leptospiras contaminadas com outros microrganismos a partir do reisolamento por cultivo de rim (Tucunduva de Faria et al., 2007, Athanazio et al., 2008, Monahan et al., 2008, Nally et al., 2011). Camundongos com genes nocauteados, como a linhagem C3H/HeJ podem ser utilizados como modelos suscetíveis por apresentarem a forma aguda da doença, mas ainda são pouco utilizados (Zuerner, 2015).

Animais jovens e suscetíveis, como *guinea pigs, gerbils* e especialmente hamsters são utilizados quando objetiva-se a indução de doença aguda, normalmente

letal (Ellis, 2015, Haake, 2006). Estes animais são utilizados em testes de potência de vacinas, pesquisas de novos alvos vacinais, teste de virulência de cepas, dentre outras aplicações (Dellagostin et al., 2011, Haake, 2006). Hamster é o modelo mais bem caracterizado e utilizado, especialmente para leptospirose aguda, apresentando leptospirose letal e sinais clínicos muito semelhantes aos observados em humanos, inclusive hemorragia pulmonar severa (Dellagostin et al., 2011, Haake, 2006, Silva et al., 2008, da Silva et al., 2010). A forma padrão de inoculação de leptospiras neste modelo é a injeção intraperitoneal, que apesar de fácil execução e repetitividade nos resultados, ignora as defesas naturais da pele e mucosa que as leptospiras encontram durante a entrada natural ao hospedeiro (Zhang et al., 2012b, Lourdault et al., 2009).

2.8 Bases moleculares da patogênese da leptospirose

As bases moleculares da patogênese de *Leptospira* são pouco conhecidas. Como comentado anteriormente, 78% dos genes únicos às espécies patogênicas tem função desconhecida (40% do genoma de *L. interrogans*). As leptospiras não possuem fatores de virulência clássicos comuns a outros patógenos, como sistemas de secreção do tipo III, tipo IV e tipo VI (Nascimento et al., 2004a). Alguns poucos fatores de virulência foram identificados por bioinformática, como colagenase, catalase, heme oxigenasse, hemolisinas e MceI (Adler, 2014b).

Os principais avanços na identificação de fatores de virulência em leptospiras foram alcançados após a obtenção de uma vasta biblioteca de mutantes seja pela inserção aleatória de transposon no genoma (Bourhy et al., 2005), ou pelos poucos mutantes por silenciamento sítio dirigido por troca de alelos (Croda et al., 2008). Vários foram os genes desligados que resultaram na perda da virulência do mutante, a citar: a lipoproteína LruA (Zhang et al., 2013), a provável chaperona HtpG (King et al., 2013, Marcsisin et al., 2013), genes relacionados à síntese do LPS (Marcsisin et al., 2013, Murray et al., 2010), colagenase ColA (Kassegne et al., 2014), a proteína relacionada a adesão celular Mce (Zhang et al., 2012a), heme oxigenasse HemO (Murray et al., 2009a), a lipoproteína de função desconhecida Loa22 (Ristow et al., 2007), o flagelo e consequente movimento celular (Liao et al., 2009, Lambert et al., 2012), a chaperona ClpB (Lourdault et al., 2011), catalase KatE (Eshghi et al., 2012), a proteína relacionada a quimiotaxia LB139 (Eshghi et al., 2014), um componente do

sistema TonB e as proteínas hipotéticas LB194, LA2786 e LA0589 (Marcsisin et al., 2013). É notável destacar que as proteínas extensivamente estudadas LipL32 e LigB, quando silenciadas por mutação a nível gênico não tiveram a virulência atenuada (Murray et al., 2009b, Croda et al., 2008). O problema da abordagem de avaliação da virulência de mutantes para um único gene é a presença de função redundante, onde o silenciamento da expressão de uma proteína relacionada com uma determinada função é compensado por outra proteína, responsável pela mesma função. E isso parece ocorrer em grande quantidade em *Leptospira* spp. Dezenas de proteínas foram descritas como capazes de se ligar a componentes da matriz extracelular (Murray, 2015, Adler, 2014b), ainda que não demonstrada a localização na superfície destas proteínas (indispensável nesse tipo de função). Ainda assim, a adesão parece ser uma característica compartilhada entre dezenas de proteínas na *Leptospira* spp.

Algumas proteínas parecem ligar vários componentes extracelulares, como LipL32, LigA e LigB e as proteínas LenA à LenE (Murray, 2015, Adler, 2014b). Vários fatores levam a crer que as proteínas Lig sejam fatores de virulência de leptospiras. Estas proteínas são induzidas com o aumento da osmolaridade, simulando o que ocorre na transmissão do ambiente para o hospedeiro (Choy et al., 2007). A perda de expressão de Ligs está relacionada à perda de virulência e a longos períodos de cultivo e várias passagens *in vitro* (Matsunaga et al., 2003). Também se ligam a componentes da matriz extracelular, e às proteínas reguladoras do sistema completo proteína ligadora de C4 e Fator H (Fraga et al., 2011). Aliás, a evasão ao ataque do sistema imune parece ser um mecanismo de sobrevivência no hospedeiro usado por leptospiras. Várias evidencias já mostraram a capacidade de diversas proteínas leptospirais de recrutar proteínas que inativam o sistema complemento, protegendo-as do ataque do mesmo (Murray, 2015). Alguns trabalhos mostraram ainda a capacidade de leptospiras permanecerem temporariamente no interior de células fagocíticas (Barocchi et al., 2002) e persistir em macrófagos (Toma et al., 2011).

2.9 Vacinas contra leptospirose

Os primeiros estudos avaliando vacinas para profilaxia da leptospirose datam de apenas um ano após o primeiro isolamento de *Leptospira* (Ido et al., 1917b) , onde células inteiras mortas induziram proteção contra um desafio no modelo de *guinea*

pig. Desde então, estas bacterinas têm sido usadas em humanos, bovinos, suínos e cães e permanecem como as únicas vacinas disponíveis.

Uma revisão detalhada sobre as vacinas contra leptospirose e outros aspectos pertinentes da doença (como a resposta do hospedeiro à infecção) estão apresentados no **Artigo 1** desta Tese. Apesar de diversos antígenos terem sido testados, visando o desenvolvimento de uma nova vacina, na esperança de resolver os problemas encontrados na utilização da bacterina, até o momento não foi descrito um antígeno completamente satisfatório. Isso evidencia a necessidade de investigações focadas no melhor entendimento da leptospirose, dos mecanismos de patogenicidades e a identificação de proteínas expostas na superfície que possam ser utilizadas em testes para desenvolvimento de uma vacina eficiente.

3 OBJETIVOS

3.1 Objetivo Geral

Investigar diferentes aspectos de *L. interrogans* e da leptospirose a fim de contribuir para avanços na área e facilitar a descoberta de novos antígenos para utilização em desenvolvimento racional de vacinas contra leptospirose.

3.2 Objetivos Específicos

- Avaliar a situação atual da leptospirose no mundo e a necessidade de novas vacinas, bem como revisar as vacinas em desenvolvimento para auxiliar na tomada de decisões críticas para desenvolvimento racional de novas vacinas;
- Padronizar a metodologia de cultivo de *L. interrogans* em DMC implantadas no peritônio de ratos;
- Avaliar o transcriptoma por RNASeq de *L. interrogans* cultivada dentro de DMC e em *in vitro* visando identificar genes relacionados com a adaptação deste organismo ao hospedeiro;
- Desenvolver e validar uma nova metodologia para indução de leptospirose letal em hamsters mimetizando a infecção que normalmente ocorre na natureza;
- Isolar e caracterizar leptospiras causadoras de infecção aguda em cães;

4 CAPÍTULOS

4.1 Artigo 1

Recombinant vaccines against leptospirosis

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Recombinant vaccines against leptospirosis

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Key words: leptospira, leptospirosis, bacterin, vaccines, subunit vaccines

Abbreviations: Alum, aluminium mineral salts; ECM, extracellular matrix; IM, inner membrane; LPHS, leptospirosis-associated pulmonary haemorrhage syndrome; LPS, lipopolysaccharide; MAT, microscopic agglutination test; OM, outer membrane; TLR, toll-like receptor

Leptospirosis is an important neglected infectious disease that occurs in urban environments, as well as in rural regions worldwide. Rodents, the principal reservoir hosts of pathogenic Leptospira spp, and other infected animals shed the bacteria in their urine. During occupational or even recreational activities, humans that come into direct contact with infected animals or with a contaminated environment, particularly water, are at risk of infection. Prevention of urban leptospirosis is largely dependent on sanitation measures that are often difficult to implement, especially in developing countries. Vaccination with inactivated whole-cell preparations (bacterins) has limited efficacy due to the wide antigenic variation of the pathogen. Intensive efforts toward developing improved recombinant vaccines are ongoing. During the last decade, many reports on the evaluation of recombinant vaccines have been published. Partial success has been obtained with some surface-exposed protein antigens. The combination of protective antigens and new adjuvants or delivery systems may result in the much-needed effective vaccine.

Introduction

Leptospirosis is a major public health concern, particularly in tropical and sub-tropical regions, with an estimated global burden of 500,000 cases per year,[1] yet it remains a neglected disease.[2] A wide range of mammals are known to be carriers of pathogenic Leptospira spp, with the rat the most common source of transmission to humans in urban settings.[3] The transmission cycle of leptospirosis begins with an infected reservoir host (e.g., rodents) passing leptospires in their urine to the environment (soil or water), and human infection results through the direct or indirect contact with contaminated urine.[3,4] The infection route is most likely mucosal and skin lesions (scratches, cuts or abrasions) are positively associated with infection.[5] Humans are accidental hosts and are not chronic carriers of leptospires. Indeed, there are so few reports of human-to-human transmission that it is not considered a risk, hence the disease is classified as a zoonosis.[3,6] Since its discovery, leptospirosis was considered an occupational hazard,[7] until an outbreak of severe leptospirosis following heavy flooding in Nicaragua[8] and in athletes that participated in aquatic sporting events.[9,10] Furthermore, there are reports of cases among the homeless in the inner cities of the USA.[11,12] In developing or underdeveloped countries, leptospirosis is associated with extreme poverty in urban slums or in subsistence farming communities.[4,7,13,14] Estimates suggest that the worlds slum population will double to reach two billion by 2030,[15] thereby further increasing the impact of leptospirosis. The residents of urban slums and individuals exposed to occupational risk are the main target populations for vaccination programs with the so-called antipoverty vaccines for neglected tropical diseases.[16]

While treatment is recommended for the control of leptospirosis, the disease is often misdiagnosed, and there are differing views as to its efficacy.[17] Laboratory diagnosis remains difficult and is of little direct benefit to the patient, usually the result only becomes available after the critical phase of the disease is over. Prevention strategies using inactivated bacterin-type vaccines have been used with varying degrees of success and reports of severe side effects.[3,17] Over the last ten years a number of recombinant vaccine candidates have been evaluated. This review will give a brief outline of leptospirosis, the causative agent and then focus on the development of a new vaccine against leptospirosis.

Leptospira spp

Leptospirosis is caused by pathogenic spirochaetes belonging to the Leptospira genus. Originally, the genus was divided into *L. biflexa*, which included all the non-pathogenic (saprophytic) strains and *L. interrogans* that comprised the pathogens.[3] Leptospira strains are classified into serogroups that contain antigenically related serovars, there are over 60 saprophytic and 260 pathogenic serovars, and this system remains in use today.[7] Serovars are based on variations in the lipopolysaccharide (LPS) component of the leptospiral cell wall and their definition remains important in epidemiological studies. Serovars tend to infect specific hosts, e.g., serovar Icterohaemorrhagiae is associated with rats and serovar Canicola with dogs, thereby aiding identification of potential sources of transmission in outbreak investigations.[3] With the application of genotyping methods, six

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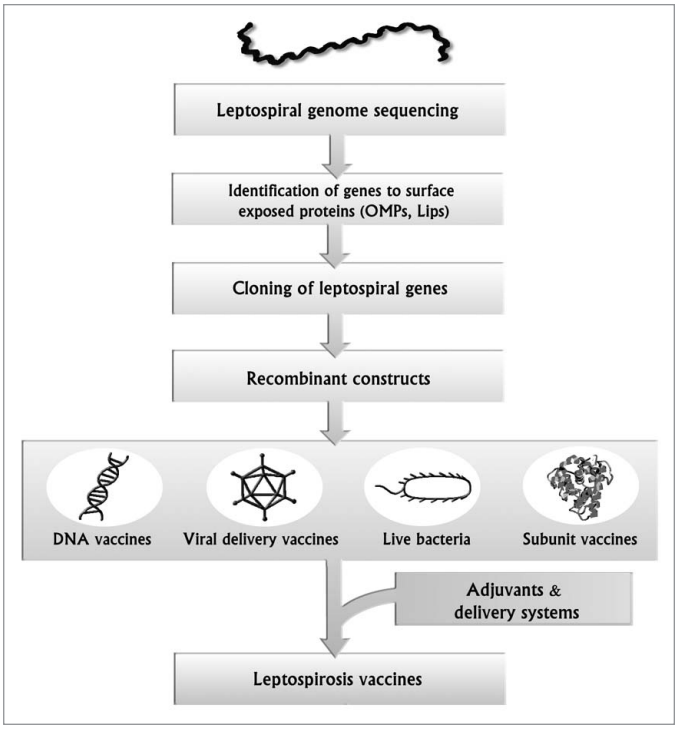

Figure 1. Schematic of different approaches for recombinant leptospiral vaccine development.

saprophytic species and 13 pathogenic species have been identified.[7,18,19] However, the species do not correspond to the serology-based system as a species can include both pathogenic and saprophytic serovars.

Leptospira is derived from the Greek *leptos* for fine or thin and the Latin *spira* for coil and this describes perfectly the morphology of the species. Leptospires are distinguishable from other spirochaetes as one or both ends are usually hooked, forming the typical question mark shape, see **Figure 1**. The bacteria are Gram-negative, including an outer (OM) and inner (or cytoplasmic) membrane (IM) and a peptidoglycan cell wall that is associated with the IM. The principal antigenic component of the OM is LPS and although similar to that of other Gram-negative bacteria, it is not as endotoxic. Leptospires are highly motile and this is achieved by two polar flagella, located at each end and within the periplasmic space. As leptospires are so fine, they are most easily observed by darkfield or immunofluorescent microscopy.[3,20] Leptospira spp are obligate aerobes with optimal growth at 28–30°C, the main energy source is long-chain fatty acids and they require iron. Leptospires, including the pathogenic serovars, are unique among the spirochaetes as they can survive for extended periods outside the host, potentially participating in biofilms and increasing transmission of leptospirosis.[21,22]

Genome sequences are available for *L. biflexa*, *L. interrogans* (serovars Lai and Copenhageni) and *L. borgpetersenii* (two strains of serovar Hardjo).[23-26] The Leptospira genomes are composed of one large and one small chromosome, approximately 2,000 genes are conserved among the different species, while there are over 1,000 pathogen specific genes. The pathogen genomes show evidence of frequent rearrangements by insertion sequences (absent in *L. biflexa*) and there is a significant reduction (~700 kb) in the genome size of *L. borgpetersenii* compared with *L. interrogans*. This reduction has reduced the ability of *L. borgpetersenii* to survive outside the host, suggesting that transmission is evolving toward a direct host-to-host route.

Leptospirosis

In the majority of individuals (>90%), leptospirosis presents as a self-limiting febrile illness, while the more serious Weil's disease (jaundice, renal failure and haemorrhaging) has a case-fatality rate of 5–15%.[17] However, of major concern is the recent appearance of a more serious condition, leptospirosis-associated pulmonary haemorrhage syndrome (LPHS), with mortality rates of 50–70%.[27,28] Leptospirosis presents as two phases: the acute phase where the leptospires replicate and rapidly disseminate throughout the organs of the infected individual (leptospiraemia), followed by the immune phase and the production of antibodies and clearance of the leptospires from the bloodstream.[3] Those patients with severe leptospirosis can suffer acute renal and hepatic failure, pulmonary distress and eventually death, the classic symptoms of Weil's disease. LPHS presents with massive pulmonary haemorrhaging and is associated with lesions in the vascular endothelium, with evidence of autoimmune involvement in this process. Killing of leptospires stimulates the production of proinflammatory cytokines such as tumor necrosis factor α and high levels have been associated with poor outcomes.[29] These pro-inflammatory cytokines stimulate the production of nitric oxide (NO) and high NO concentrations were reported in patients with severe leptospirosis.[30] For a more complete description of the clinical presentations of leptospirosis in humans, the reader is referred to references 3, 6, 7 and 17.

The severity of the disease depends on host susceptibility factors, the infecting species or serovar and the infective dose. Host susceptibility factors, including age, sex, race and income, have been identified as risk factors.[13,14] In the first study of its kind, Lingappa and colleagues found an association between the human leukocyte antigen (HLA)-DQ6 genotype and the risk of leptospirosis following ingestion of contaminated water.[31] More recently, single nucleotide polymorphisms (SNPs) were identified in individuals with a history of leptospirosis. The SNPs associated with susceptibility were found in the HLA loci and the interleukin (IL)-4 gene and its receptor.[32] The infecting serovar is dependent on the range of reservoir hosts in the surrounding environment. Severe urban leptospirosis outbreaks tend to be associated with serovars carried by rats and other rodents while rural outbreaks can involve different serovars because of the greater diversity of the reservoir hosts.[4,7,33] Severe leptospirosis has also been associated with high numbers of leptospires ($\geq 10^5$ leptospires/g) in patient tissue, including lung, liver, kidney and muscle.[28] Furthermore, the concentration of free-living leptospires was reported to be higher in urban water sources and was associated with severe urban leptospirosis.[33]

Domestic animals, chiefly dogs, cattle and pigs, have a greater variety of symptoms and complications.[19] A host-specific

serovar infection is usually asymptomatic, e.g., Canicola in dogs, Bratislava in horses and pigs and Hardjo in cattle. However, other serovars are more serious with a major impact on reproduction, causing abortion, miscarriage and stillbirth. The infections tend to be chronic and result in the constant shedding of leptospires into the environment and hence transmission to other animals as well as humans, reviewed in reference 19.

Laboratory diagnosis is either by culture isolation or through the microscopic agglutination test (MAT).[34] The MAT is considered the standard method for diagnosis, however, paired samples are required and as the second sample should be collected during the second week of the illness,[35] the result has little impact on patient outcome. Treatment of the disease varies according to its severity. Mild forms may resolve through managing the symptoms, while severe leptospirosis requires antibiotics, traditionally penicillin or doxycycline and more recently ceftriaxone.[36] Doxycycline is also used as a prophylactic measure for people traveling to high risk settings, and a recent cost-benefit analysis found that doxycycline was the most efficient treatment strategy.[37]

The Immune Response

Several studies have explored the interactions between Leptospira spp and its hosts, but there is no thorough understanding of these complex relations. Understanding the pathology and immune response involved in leptospirosis is critical for the discovery of correlates of immunity and in developing new vaccines against leptospirosis. Classically, the immune response against leptospirosis is humoral in humans and most animals.[3,19] This is based on evidence that protection can be passively transferred by convalescent sera from human patients or animals, or by anti-Leptospira monoclonal antibodies directed against LPS.[3,38-40] Furthermore, the level of protection correlated with the titer of LPS-specific agglutinating antibodies in the transferred sera.[38] The anti-Leptospira immunoglobulins develop during the first 10 d following infection, the IgM peak is quickly followed by increasing IgG levels.[3,7] The antibodies persist in patient serum for up to five years[41,42] and even low antibody levels are protective.[3,40] Conversely, a humoral immune response with a high titer of agglutinating antibodies does not protect cattle, rather a cellular immune response is required for protection against leptospirosis. High agglutinating antibody titers against LPS in serum from vaccinated cattle were not sufficient for protection.[43,44] Cattle vaccines need to induce a strong Th1 response, with IFNγ secretion by $CD4^+$ and γδ T cells.[45-47] Currently, there are no accepted in vitro assays that correlate with immunity against leptospirosis and this is a major setback for the identification of novel vaccine candidates using high-throughput screening of large numbers of leptospiral antigens.[6]

Leptospiral LPS is atypical, less toxic than LPS from Gram-negative organisms, and has an unusual lipid A moiety.[48] This structure is recognized by Toll-like receptor (TLR) 2 in human macrophages but not by TLR4, the classical LPS recognition receptor.[49] In murine cells leptospiral LPS signals through both TLR2 and TLR4,[50] and this was confirmed with double knockout TLR2 and TLR4-deficient mice, which are susceptible to infection by *L. interrogans*.[51,52] Despite the absence of Toll stimulation in the double knockout, a strong pro-inflammatory response was observed but it did not promote leptospiral clearance. However, a role for TLR4 in susceptible hosts during leptospirosis cannot be ruled out, since it can be mediated by still unidentified ligand(s), other than LPS.[53] A recent study with human blood cells claimed the involvement of not only TLR2 but also TLR4 and TLR5 in the immune response against leptospiraemia.[54] This observation may be justified by the presence of whole leptospires, rather than just LPS, which could signal TLR5 through their flagellin, and TLR4 by danger-associated molecular patterns (DAMPs) released by host cells in response to infection or another unknown mechanism.

Current Vaccines

Vaccines against the human form of leptospirosis were introduced shortly after the description of the disease in Japan, and have been available for nearly a century.[3] The heat-killed whole-cell vaccine preparations (bacterins) were very effective in endemic settings as far back as 1933.[55] Since then there have been numerous descriptions of problems associated with bacterin-type vaccines, including: severe side effects (pain, nausea, fever), short-term immunity and serovar-restricted protection.[3] Bacterins have been used in Japan,[56,57] Cuba,[58,59] France,[60,61] and a purified outer envelope vaccine has been used in China.[62] There are reports of the use of vaccines in other countries in Asia,[63] but a lack of international reports makes it difficult to evaluate these vaccines. When available, these vaccines are mainly for high-risk populations, with reports of efficacy >70%, little or no side effects and protection lasting for up to seven years.[17] However, short-term, serovar-specific protection remains a major issue and regular yearly booster immunizations are recommended for maintenance of protective antibody levels.[3] Furthermore, once a population is vaccinated, serovars not included in the original vaccine preparation may cause new outbreaks. Such a case in Cuba resulted in the inclusion of another serogroup in the original trivalent vaccine, and revaccination of the population.[64] While bacterins are effective in at risk populations, a universal vaccine using this approach is unlikely, therefore research has focused on developing recombinant vaccines.

Potential Targets and Next Generation Vaccines

The drawbacks of bacterin vaccines highlight the need for new vaccine strategies for the prevention of leptospirosis. Consequently, research has focused on leptospiral recombinant antigens capable of eliciting protective immunity. The first successful recombinant leptospirosis vaccine candidate was reported in 1999.[65] The surface-exposed lipoprotein LipL41 and the transmembrane porin OmpL1 were cloned and expressed in *E. coli*. Hamsters immunized with purified recombinant *E. coli* membrane fractions containing LipL41 and OmpL1 induced a significant, synergistic, protective response (71% survival). Although this vaccine was not commercialized, it provided experimental

evidence for the efficacy of recombinant vaccines in preventing leptospirosis. Research has therefore concentrated on identifying virulence determinants such as the surface-exposed antigens recognized by sera collected from leptospirosis patients,[66-68] and bioinformatics analysis of the Leptospira genome sequences has identified homologs to vaccine candidates in other pathogens, **Figure 1**.[69,70] Many leptospiral proteins have since been tested as potential vaccine candidates and the most promising antigens used in vaccine preparations (subunit, DNA, Adenovirus and BCG vaccine constructs), to date are described, see **Table 1**.

The Immunodominant Protein LipL32

This surface-exposed lipoprotein, also known as Hap1, is the major leptospiral outer membrane protein (OMP) and is specific to, and highly conserved among the pathogenic Leptospira spp.[23,71-73] A protein extract containing LipL32 protected all gerbils against challenge.[74] Furthermore, when *lipL32* was cloned into an Adenovirus construct for in vivo expression, 87% of immunized gerbils survived a heterologous challenge, although 51% of the control group also survived.[75] In a follow-up study, recombinant LipL32, in vaccine preparations containing Freund's adjuvant or aluminum hydroxide and saponin, failed to protect gerbils. However, when *lipL32* was cloned into a DNA vaccine vector and used to vaccinate gerbils, 60% survived, compared with 35% of the control group.[76] A *Mycobacterium bovis* BCG vector delivery system expressing LipL32 induced a protective immune response in 56% of immunized hamsters, while only 12% of the control group survived.[77] Of note, the BCG (LipL32) construct induced sterilizing immunity in the surviving hamsters. There was no evidence of disease and the lungs and kidneys did not contain leptospires. Vaccine efficacy of LipL32 in DNA, BCG or Adenovirus constructs is low (38–72%) and the subunit vaccines did not elicit any significant protection in animal models.

The Leptospiral Immunoglobulin-like (Lig) Proteins

These three proteins, LigA, LigB and LigC, belong to the superfamily of bacterial immunoglobulin-like proteins.[66] They are present only in pathogenic Leptospira spp and are highly conserved (63–99% identity at the amino acid level). Furthermore, LigB has been found in every pathogenic Leptospira spp studied to date.[78,79] The Lig proteins are virulence determinants, their expression is increased on entry into the host[80] and they can bind to host tissues including fibrinogen and fibronectin.[81-84] The first evidence of their potential as vaccine candidates was presented in 2004, purified recombinant peptides of LigA and LigB protected mice against lethal challenge (90–100% compared with 20% of the control group).[85] Numerous recombinant peptides of LigA, including the identical region shared with LigB (amino acids 102–630) and the non-identical carboxy-terminus (amino acids 631–1,224), have been expressed in heterologous systems, including yeast,[86] and evaluated in experimental models of leptospirosis.[87,88] The non-identical region of LigA contains the epitope(s) responsible for induction of the protective immune response, see **Table 1**. However, the challenge dose used in the various models varied considerably (10^3–10^8 leptospires) and this appeared to impact on the efficacy of the various LigA vaccine candidates, ranging from 50 to 100%. Even at the higher challenge doses (>10^6 leptospires) there were survivors in the unvaccinated control groups (20–87%), suggesting that virulence was a limiting factor and this reduced vaccine efficacy following statistical analysis of the data.

Due to the ubiquitous and highly conserved nature of LigB among the pathogenic Leptospira spp, it has the greatest potential as a universal vaccine. However, in practice it has proved difficult to produce a subunit vaccine capable of inducing a protective immune response, see **Table 1**. The first report of significant protection (90%) was in a mouse model and with a recombinant LigB peptide in a formulation containing Freund's adjuvant.[85] A more recent study found that hamsters immunized with LigB peptides that corresponded to the identical and non-identical regions of LigA were significantly protected against lethal challenge (50–75%).[89] Furthermore, protection was improved (75–87%) when both peptides were included in the vaccine formulation. Toward identifying the domains within LigB that contain protective epitopes, Cao and colleagues produced a series of recombinant LigB peptides of regions known to interact with the host extracellular matrix (ECM), including fibronectin, laminin, fibrinogen and collagen.[90] However, the protection afforded was not significant, suggesting that the ECM binding domains do not include epitopes capable of stimulating a protective immune response, **Table 1**.

While subunit vaccines stimulate fewer side effects than bacterin vaccines, they are associated with reduced immunogenicity.[91,92] The inclusion of adjuvants in the vaccine formulation can help to avoid this problem, yet they can also cause severe side effects due to their toxicity. The most commonly used adjuvants in vaccine formulations are aluminum mineral salts (alum). Although considered a weak adjuvant, alum induces a Th2 (IgG1 antibody) response.[91] The greatest impact was seen with the LigA subunit formulations containing particulate antigen adjuvants based on liposomes and PLGA microspheres compared with alum (87, 75 and 50% protection, respectively).[93] Furthermore, recombinant LigB peptides formulated with either Freund's or alum adjuvant induced protective immune responses that included both humoral and cell mediated immunity.[85,89]

Other Potential Vaccine Candidates

In *E. coli* and other Gram-negative bacteria, OmpA has several functions, including roles as an adhesin, an invasin and induction of a cellular immune response. Therefore, it is likely that leptospiral proteins containing an OmpA domain will be surface-exposed and hence potential vaccine candidates. Chang and colleagues screened 12 OMPs using the hamster model of leptospirosis. Recombinant Lp1454, Lp1118 and Lp0607, protected hamsters against a lethal challenge (71, 75 and 100%, respectively) and when combined, 87% of the immunized hamsters survived, see **Table 1**.[94] However, in both experiments there were survivors in the unvaccinated control group (43 and 50%, respectively). Toward improving the efficacy of the subunit vaccine based on

Table 1. Leptospirosis vaccine candidates

Antigen (aa)[a]	Conserved[b]	Type[c]	Serovar[d]	Adjuvant	Immunization	Challenge	% Survival		
					Dose/Via[e]	Serovar/ Dose[f]	Vaccinated	Control	Ref.
LigA (68–1224)							90		
LigB (68–1191)	Lb/Li/Lk/Ln/Lw	His	Man (Li)	Freund's	3 x 10 μg/SC/M	Man/10^6	90		85
LigA + LigB							100		
LigA (32–626) + LigA (631–1225)	Lb/Li/Lk/Ln/Lw	GST	Pom (Li)	Alum	2 x 50 μg/SC	Pom/10^8	100	57–87	87
LigA (625–1224)	Lb/Li/Lk/Ln/Lw	His	Cop (Li)	Freund's	80 + 40 μg/SC	Cop/10^3	67–100	0	108
LigA (32–626) + LigA (631–1225)	Lb/Li/Lk/Ln/Lw	DNA	Pom (Li)	DNA	3 x 100 μg/IM	Pom/10^8	100	50–75	88
LigA (631–1225)	Lb/Li/Lk/Ln/Lw	GST	Pom (Li)	Alum	2 x 10 μg/SC	Pom/10^5	50	0	93
				PLGA	1 x 20 μg/SC		75		
				Liposomes	2 x 10 μg/SC		87		
LigB (31–630)	Lb/Li/Lk/Ln/Lw	GST	Pom (Li)	Alum	2 x 50 μg/SC	Pom/10^8	62–75	12–25	89
LigB (630–1418)		His					50–62		
LigB (1418–1890)		GST					25–37		
LigB + LigB + LigB							75–87		
LigB (307–630)::(1014–1165)	Lb/Li/Lk/Ln/Lw	GST	Pom (Li)	Oil	2 x 50 μg/SC	Pom/2.5x10^2	50	0	90
LigB (307–403)::(1014–1165)							50		
LigB (307–630)::(1014–1165)::LipL32 (185–272)							50		
LigB (307–403)::(1014–1165)::LipL32 (185–272)							50		
LigB (47–630)							34		
LipL32 (265–271)	Lb/Li/Lk/Ln/Ls/Lw	Av	Aut (Li)	-	2 x 10^9 pfu/ IM/G	Can/10^4	87	51	75
LipL32 (265–271)	Lb/Li/Lk/Ln/Ls/Lw	DNA	Aut (Li)	-	2 x 100 μg/ IM/G	Can/10^7	60	35	76
			Gri (Lk)				60		
LipL32 (23–273)	Lb/Li/Lk/Ln/Ls/Lw	BCG	Cop (Li)	-	2 x 10^6 cfu/IP	Cop/10^2	12–56 [g]	0–20	77
LipL41 (53–408)	Lb/Li/Lk/Ln/Ls/Lw	OMP	Gri (Li)	-	3 x 50 μg/IP	Grip/10^2	17–29	0–33	65
OmpL1 (1–321)	-						0–100		
LipL41 + OmpL1	All						50–100		
Lp0607 (20–267)	Lb/Li	GST	Pom (Li)	Alum	2 x 50 μg/SC	Pom/10^8	66–100	0–50	94
Lp1118 (26–317)	Li						66–75		
Lp1454 (32–359)	Li						66–71		
Lp0607 + Lp1118 + Lp1454	-						87		
Lp0607 + Lp1118 + Lp1454	-	GST	Pom (Li)	Leptosomes	2 x 10 μg/SC	Pom/10^8	75	0	95
Lp0607 + Lp1118 + Lp1454	-	GST	Pom (Li)	Smegmosomes	2 x 10 μg/SC	Pom/10^8	75	0	96

[a]aa: amino acid coordinates of the polypeptide used in the vaccine preparation. [b]Conserved: >60% identity at the protein level in the Leptospira spp listed: La, *L. alstoni*; Lb, *L. borgpetersenii*; Li, *L. interrogans*; Lk, *L. kirschneri*; Ln, *L. noguchii*; Ls, *L. santarosai*; Lw, *L. weilii*. [c]Type: Recombinant protein expressed in *E. coli* (unless otherwise specified) with a GST, glutathione-S-transferase-tag or His, His-tag; Av, Adenovirus-mediated; BCG, *M. bovis* bacillus Calmette-Guerin; DNA, naked DNA vaccine vector; OMP, recombinant *E. coli* outer membrane preparation; PLGA, poly-lactide-co-glycolic acid. [d]Serovar: Origin of DNA used in cloning; Aut, Autumnalis; Cop, Copenhageni; Gri, Grippotyphosa; Man, Manilae; Pom, Pomona; (Leptospira spp). [e]Dose/Via: cfu, colony forming units; pfu, plaque forming units; Route of immunization in the hamster model (unless otherwise stated); IM, intramuscular; IP, intraperitoneal; SC, subcutaneous; M, mouse model; G, Gerbil model. [f]Challende dose: Number of leptospires used in the challenge dose. [g]Sterilizing immunity in surviving animals.

Table 1. Leptospirosis vaccine candidates (continued)

Antigen (aa)[a]	Conserved[b]	Type[c]	Serovar[d]	Adjuvant	Immunization Dose/Via[e]	Challenge Serovar/ Dose[f]	% Survival Vaccinated	% Survival Control	Ref.
LIC10494 (28–100)	Li						29–50		
LIC12730 (19–126)	Li	His	Cop (Li)	Alum	3 x 50 μg/SC	Cop/2 x 10^5	38–50	0–20	100
LIC12922 (47–166)	Lb/Li						29–30		
Lp4337 (2–429)	Li						67–83		
Lp3685 (7–661)	Li	GST	Pom (Li)	Oil	2 x 50 μg/SC	Pom/10^8	50–67	17	99
Lp0222 (7–196) [or Loa22]	Lb/Li						33–50		

[a]aa: amino acid coordinates of the polypeptide used in the vaccine preparation. [b]Conserved: >60% identity at the protein level in the Leptospira spp listed: La, *L. alstoni*; Lb, *L. borgpetersenii*; Li, *L. interrogans*; Lk, *L. kirschneri*; Ln, *L. noguchii*; Ls, *L. santarosai*; Lw, *L. weilii*. [c]Type: Recombinant protein expressed in *E. coli* (unless otherwise specified) with a GST, glutathione-S-transferase-tag or His, His-tag; Av, Adenovirus-mediated; BCG, *M. bovis* bacillus Calmette-Guerin; DNA, naked DNA vaccine vector; OMP, recombinant *E. coli* outer membrane preparation; PLGA, poly-lactide-co-glycolic acid. [d]Serovar: Origin of DNA used in cloning; Aut, Autumnalis; Cop, Copenhageni; Gri, Grippotyphosa; Man, Manilae; Pom, Pomona; (Leptospira spp). [e]Dose/Via: cfu, colony forming units; pfu, plaque forming units; Route of immunization in the hamster model (unless otherwise stated); IM, intramuscular; IP, intraperitoneal; SC, subcutaneous; M, mouse model; G, Gerbil model. [f]Challende dose: Number of leptospires used in the challenge dose. [g]Sterilizing immunity in surviving animals.

these OMPs, several formulations based on liposomes were evaluated. Liposomes produced from *L. biflexa* (leptosomes) and *E. coli* (escheriosomes) and containing the OMPs, induced significant protection in hamsters (75%) and there were no survivors in the unvaccinated control group.[95] In addition, liposomes produced from *Mycobacterium smegmatis* (smegmosomes) together with the OMPs, protected 75% of immunized hamsters and there were no survivors in the control group.[96] However, none of these liposome-based formulations induced sterilizing immunity among the survivors.

The first leptospiral virulence determinant to fulfil Koch's molecular postulates was Loa22. Inactivation of *loa22* in the *L. interrogans* genome generated a mutant that could not express Loa22 and was no longer virulent.[97] Although the *L. biflexa* genome contains *loa22*, it does not appear to be expressed.[6,98] Loa22 was the first surface-exposed lipoprotein described that contains an OmpA domain.[98] Yan and colleagues evaluated the ability of six putative leptospiral OMPs containing an OmpA domain, including Loa22, to protect hamsters against lethal challenge.[99] Recombinant Lp3685 and Lp4337 induced significant protection in hamsters, 50–67% and 67%, respectively. However, protection following immunization with recombinant Loa22 was not significant (33–50%), see **Table 1**.

Three putative OMPs identified using bioinformatics analysis of the *L. interrogans* serovar Copenhageni genome were evaluated as vaccine candidates.[100] Recombinant formulations of two of the OMPs, LIC10494 and LIC12730, partially protected hamsters against challenge and significantly improved survival in the immunized hamsters, see **Table 1**. The majority of surviving animals (80–100%) were positive for the presence of leptospires.

Animal Vaccines

The development of effective leptospirosis vaccines, with wide ranging protection and few side effects is a basic requirement for any new human vaccine. Indeed, such characteristics are just as important in an animal vaccine. Due to looser restrictions by the regulating authorities, a novel vaccine will likely be approved for use in animals years before a human vaccine. Current veterinary vaccines are commercially available and are widely used in cattle, pigs and dogs. They are bacterin vaccines, made by combining suspensions of different serovars, formulated according to local needs.[19] The vaccine preparation should always include local serovars due to the serovar-specific nature of the bacterins.

Canine vaccines generally include serogroup Canicola, the most important cause of canine leptospirosis worldwide together with serogroup Icterohaemorrhagiae.[101] This vaccine is applied with viral vaccines during routine veterinary checkups. However, others serovars such as Pomona, Grippotyphosa and Bratislava are becoming increasingly important as causes of canine leptospirosis in North America and elsewhere.[102] Cattle and swine are usually vaccinated with serogroups Hardjo and Pomona respectively, as well as Icterohaemorrhagiae to decrease the chances of human infection from contact with these animals.[19] As described previously, protection in cattle is different to that reported in other animals, agglutinating antibodies are not protective, rather a Th1 response is required. Although current commercial vaccines reduce symptoms, renal colonization and leptospiruria still occurs in immunized animals. However, vaccination markedly reduced abortions and stillbirths, the main economic impact of leptospirosis in breeding herds.[46,103]

Animal Models of Leptospirosis

Animals have been used to study leptospirosis since the description of the disease.[104] The golden Syrian hamster (*Mesocricetus auratus)* is regarded as the standard model for leptospirosis vaccine development and it is used by regulating bodies such as the FDA to test commercial vaccines.[105] Hamsters are highly susceptible to leptospirosis, the evolution of the disease is very similar to that of humans and the model is well characterized.[106]

Furthermore, hamsters develop most forms of the acute disease, including LPHS.[107-109] The hamster model has been thoroughly described, with diverse publications on the use of this species in leptospirosis vaccine studies.[3,106,110,111]

Rats belonging to the Rattus genus were recognized as a reservoir host of the pathogenic Leptospira spp in the transmission cycle to humans.[112] Rats remain the most important source of transmission in urban settings.[4] Rats are resistant to the clinical disease and rarely develop severe leptospirosis.[3] The rat model has been used in studies of pathogenesis comparing chronic colonisation in the rat with acute lethal leptospirosis as seen in hamsters, guinea pigs and gerbils.[113-115] As rats are naturally resistant, they are not used as experimental models for vaccine testing.

Mice (*Mus musculus)* are reservoir hosts and are generally resistant to leptospirosis. An advantage of the mouse model is the vast array of laboratory tools and knockout strains available for research of the immune response to leptospirosis.[116,117] However, mice have been used in the initial stages of vaccine development, particularly when screening large numbers of vaccine candidates.[69] Knockout mice (C3H/HeJ) susceptible to leptospirosis have been used in immune protection studies,[85] but the mouse is not recognized as an experimental model for vaccine development.[19]

The guinea pig (*Cavia porcellus*) was the first animal model used in the study of leptospirosis,[104] and the original treatments and vaccines were developed using this model.[118] The guinea pig model offers similar advantages to that of the hamster in terms of susceptibility to acute leptospirosis, which is similar to that seen in humans.[3] The use of guinea pigs to evaluate vaccine candidates has declined somewhat, while the use of hamsters has increased. This probably has more to do with logistics than any other reason, as the guinea pig requires more space in animal facilities than the hamster. However, the hamster model is known to be oversensitive to leptospirosis compared with the guinea pig, therefore the guinea pig may be more suited to the study of virulence determinants.[119]

Gerbils (*Muriones unguiculatus*) are susceptible to acute leptospirosis and this model has been used in several vaccine studies.[74-76] While not as thoroughly described as the hamster, the gerbil represents an alternative to the hamster model, if one is needed. In addition, several primate models, including rhesus monkeys, were used to evaluate a bacterin vaccine.[120] A recent study used marmosets (*Callithrix jacchus*) as an experimental model of leptospirosis.[121] The marmoset develops lesions similar to those seen in the human disease, with the greatest alterations seen in the lungs and kidneys, including LPHS. Primates represent another alternative for the evaluation of vaccine candidates. Furthermore, marmosets have been used in biomedical research for over 50 y and there is a wide range of reagents available for research purposes, a major advantage compared with the hamster and guinea pig models.

Conclusions

Although bacterin vaccines are used in several countries, they have several drawbacks that justify the search for an improved vaccine. Research into recombinant vaccines has identified surface-exposed antigens that can protect against lethal leptospirosis in experimental models. However, various groups have presented divergent results as to vaccine efficacy for the same antigen, particularly when using a subunit formulation. Structural differences in the recombinant proteins are probably the simplest explanation for these observations. This is a recognized problem in recombinant protein expression, however, it should be possible to overcome using alternate expression systems and accurate mapping of the protective epitopes. Various adjuvants and delivery systems have been evaluated, yet it is difficult to recommend any one in particular, although liposomes did improve efficacy when used with LigA. Given that one of the major goals of a recombinant vaccine is to provide heterologous protection, there are very few published reports that address the subject. Of the next-generation vaccine candidates described in this review, none have advanced to clinical trials. This cannot be justified by a lack of infrastructure as several groups have well established community-based cohorts ideally situated for recruiting individuals for clinical trials.[13,28,122] Arguably, there is sufficient data available to justify significant investment in developing a recombinant vaccine against leptospirosis, however, is there the political will for such an investment? The target population for such a vaccine includes those living in extreme poverty in urban slums and they are all too easily ignored.

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4.2 Artigo 2

Host adapted *Leptospira* spp. by Dialysis Membrane Chamber Implantation in Rats

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Host adapted *Leptospira* spp. by Dialysis Membrane Chamber Implantation in Rats

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[Abstract] *Leptospira interrogans* can infect a myriad of mammalian hosts, including humans. Infected hosts shed large number of spirochetes in their urine and the leptospires can survive in different environmental conditions before transmission to another host. Differential gene expression by *Leptospira* spp. permits adaption to these new conditions. Here we describe a protocol for the cultivation of *Leptospira interrogans* within a Dialysis Membrane Chamber (DMC) implanted in a rat peritoneum. This technique was originally described to obtain mammalian adapted *Borrelia burgdorferi*, the Lyme disease spirochete (Akins, et al, 1998; Caimano, 2005). The environment within the DMC is similar to that of the renal proximal convoluted tubules during colonization by *L. interrogans*. The small pore size of dialysis tubing, like the renal tubules walls, excludes large molecules but allows exchange of small nutrients, and is immune privileged. In a 20 to 40 min procedure, dialysis tubing containing spirochetes are surgically implanted into the rat peritoneal cavity and after 9 days are explanted, providing a large number (up to 10^9) of host-adapted leptospires. We have recently shown that *L. interrogans* changes its transcript profile from that under *in vitro* culture when growing inside DMCs, allowing us to identify several genes related to the infection process (Caimano, et al. 2013).

Materials and Reagents:

Adult Female Sprague-Dawley rats (>210 g)

EMJH medium prepared as previously described (Zuerner, 2005) supplemented with an additional 10 mg/ml Fraction V bovine serum albumin (BSA) (Sigma-Aldrich cat# A9647-100G) – final BSA concentration 20 mg/ml

Leptospira interrogans culture (5-7 days, 28 °C)

1 mM EDTA, pH 8.0

Ultrapure water

Regenerated cellulose dialysis membrane tubing (Spectra/Por 6, 8000 MWCO, 32 mm width; Spectrum Labs cat#132582)

Isoflurane

Ketamine/xylazine anesthetic cocktail (see recipe)

Ophthalmic ointment, sterile

0.3 mg/ml buprenorphine HCl

Individually wrapped, sterile surgical gloves

Sterile surgical drape

Equipment:

Dark field microscope

Three 2 L beakers

Hot plate/magnetic stirrer

Sterile extra-long blunt end forceps

0.22 µm Stericup® Filter Unit (Millipore)

Surgical instruments sterilized and kept within sterile package:

- Scalpel blade and holder, no. 10
- 4 in. (~10 cm) iris scissors
- Tissue forceps
- 5 ½ in. (~14 cm) needle holder with built-in scissors
- Ethicon 4-0, SH-1, 27 in. (~68 cm) coated Vicryl, violet-braided suture
- Auto-clip 9 mm stainless steel wound closure clips and applicator

Biosafety cabinet (level 2)

Circulating warm water blanket and pump

Electric hair/fur clippers

Glass bead sterilizer (e.g. Braintree Scientific, Germinator 500)

Animal facilities

Procedures:

A. Preparation of sterile dialysis membrane tubing

1. Cut a strip of sterile dialysis membrane tubing 18-20 cm in length using sterile. Tie off one end of the tubing, cutting off any excess tubing from the tied end.
2. Place tied-off tubing in a previously autoclaved 2 L beaker containing 1 L ultrapure water, a magnetic stir bar and covered with aluminum foil.
3. Place beaker on a hot plate/magnetic stirrer and bring to a rolling boil with constant stirring for 20 min.
4. Transfer the tubing, using extra-long forceps, to an autoclaved 2 L beaker, containing 1 L 1 mM EDTA, stir bar and covered with aluminum foil, already boiling and boil for 20 min.
5. Repeat step 2 and boil for 20 min.

   Note: Closely monitor steps 3 – 5 to ensure that the tubing remains submerged within the boiling solution at all times.
6. In a sterile biosafety cabinet, transfer the tubing to a 0.22 µm Stericup® Filter Unit (Millipore) bottom using sterile blunt tip forceps. Filter sterile water into the same container. Keep in the cabinet until ready to use. Tubing may be prepared several days in advance (store at 4°C) but do not open more than once before use.

B. Preparation of the DMC

1. Just before starting the surgical procedure, dilute the *L. interrogans* culture to a density of 10^4 organisms/ml in 10 ml of EMJH media in a 50 ml conical tube. Prepare one dilution per bag. Do not make one large dilution and then try to aliquot.

   Note: it is important that the final concentration of the BSA in the EMJH medium is 20 mg/ml. This maintains the correct osmotic pressure inside the DMC, and prevents desiccation of the DMC following implantation.
2. Fill dialysis tubing with 9-10 ml of diluted organisms. Tie off the tubing removing as much air as possible and cut off any excess tubing. Place the DMC containing the leptospires into the same 50 ml conical tube and keep in the hood until implantation.

C. Preoperative preparation

1. Place the rat inside an anesthetic chamber previously prepared with isoflurane. As soon as the rat appears drowsy, remove it from the chamber, let it recover for a few seconds, and anesthetize the rat by intramuscular injection with the anesthetic cocktail.

   Note: Ensure complete anesthesia before surgery.

2. Administer preoperative analgesic medication (buprenorphine HCl – 0.05 to 0.1 mg/kg body weight) by subcutaneous injection.
3. Apply a small amount of eye ointment to each eye to avoid dehydration. Check that rat's tongue is not obstructing its airway.
4. Perform abdominal trichotomy. Wipe shaved abdomen with ethanol-wet gauze. Transfer the animal on a sterile surgical drape to the biosafety cabinet.

D. DMC implantation

1. Using a sterile scalpel blade, make a 5 cm incision through the skin only, starting 2.5 cm below the ribcage (using the xiphoid process as a guide). Use tissue forceps to pull up the skin and use the scalpel to separate the skin from the abdominal wall.
2. Use the same scalpel, make a small incision in the abdominal muscle by pulling up the tissue with the tissue forceps and then extend the opening to approximately 4 cm using scissors. Use the white vertical line along the abdomen (the linea alba) as a guide to keep the opening straight and clean.

   Note: A smaller opening requires fewer stitches, causing less trauma to the animal.

3. Using tissue forceps raise one side of the abdominal incision and place the DMC inside rat peritoneal cavity. Gently push the DMC inwards just enough to keep it clear of the suturing. Try not to entangle the intestine around the DMC and place it slightly to the side of the abdomen to minimize interference with the bladder.
4. Suture the abdominal incision using square knots, close both of the ends of the incision (double knotted) and then work up the incision, suturing with double knots approximately 3 mm apart.

5. The skin is closed using surgical staples placed very close together, resulting in one continuous line (Figure 1).

   Note: If more than one implant is planned, the surgical material should be held inside a high temperature glass bead sterilizer.

6. Return the rat to a cage containing fresh bedding, overlaid with a sterile surgical drape. To help maintain body temperature, apply gentle heating to cage bottom using a circulating water pad until the animal is alert and responsive.

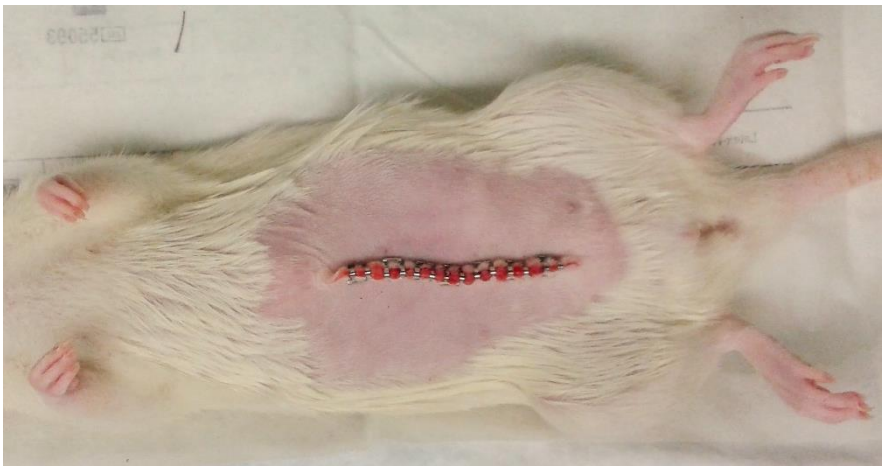

Figure 1. Rat abdominal surgery showing stapled incision post implantation of the DMC containing *L. interrogans*.

E. Postoperative recovery

1. Administer analgesic medication by subcutaneous injection for two days after implant as described in C.2.
2. Monitor animals twice daily for signs of distress, stich abscesses, peritoneal infection, signs of leptospirosis, or other complications for the duration of the experimental protocol.
3. If animals show any sign of distress or discomfort, consult institutional veterinary staff immediately.

F. Explant of the DMC

1. At 8 to 10 days after implantation, euthanize animals by CO_2 asphyxiation.
2. Place animal in a supine position, using sterile surgical scissors to cut just under and along the line of staples to expose the abdominal wall.

3. Use tissue forceps to secure the sutured inner incision and using scissors reopen the sutured incision.
4. Locate and remove DMC using blunt end forceps. Transfer to a sterile petri dish.
5. Remove the contents of the DMC using an 18G needle attached to a sterile 10 ml syringe. Gently transfer the contents to a 15 ml conical tube and keep on ice.

G. DMC content processing

1. Take a few microliters of the DMC contents and analyze under dark field microscopy (200×).
2. Pellet the leptospires in a bench top centrifuge at 4°C, 8000 × g for 15 min. Separate the supernatant and discard or process separately as needed.
3. Wash the pellet three times with PBS.
4. Analyze the organisms as required, e.g. SDS-PAGE, immunoblotting, qRT-PCR, microarrays, RNASeq *etc*.

Recipes

1. **Ketamine/xylazine anesthetic cocktail**

   3.75 mL of 100 mg/ml ketamine HCl

   1.25 mL of 20 mg/ml xylazine HCl

2. **EMJH medium supplemented with an additional 10 mg/ml Fraction V BSA**
   a) Weight 10 mg of Fraction V BSA using analytical balance
   b) Add to 1 L EMJH prepared as previously described (Zuerner, 2005)
   c) Slowly stir in a magnetic stirrer until dissolve BSA, avoiding bubbles formation
   d) Sterilize by filtration using 0.22 µm Stericup® Filter Unit (Millipore)
   e) Store at 4 °C and prewarm before use

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4.3 Artigo 3

A Model System for Studying the Transcriptomic and Physiological Changes Associated with Mammalian Host-Adaptation by *Leptospira interrogans* Serovar Copenhageni

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A Model System for Studying the Transcriptomic and Physiological Changes Associated with Mammalian Host-Adaptation by *Leptospira interrogans* Serovar Copenhageni

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Abstract

Leptospirosis, an emerging zoonotic disease with worldwide distribution, is caused by spirochetes belonging to the genus *Leptospira*. More than 500,000 cases of severe leptospirosis are reported annually, with >10% of these being fatal. Leptospires can survive for weeks in suitably moist conditions before encountering a new host. Reservoir hosts, typically rodents, exhibit little to no signs of disease but shed large numbers of organisms in their urine. Transmission occurs when mucosal surfaces or abraded skin come into contact with infected urine or urine-contaminated water or soil. In humans, leptospires can cause a variety of clinical manifestations, ranging from asymptomatic or mild fever to severe icteric (Weil's) disease and pulmonary haemorrhage. Currently, little is known about how *Leptospira* persist within a reservoir host. Prior *in vitro* studies have suggested that leptospires alter their transcriptomic and proteomic profiles in response to environmental signals encountered during mammalian infection. However, no study has examined gene expression by leptospires within a mammalian host-adapted state. To obtain a more faithful representation of how leptospires respond to host-derived signals, we used RNA-Seq to compare the transcriptome of *L. interrogans* cultivated within dialysis membrane chambers (DMCs) implanted into the peritoneal cavities of rats with that of organisms grown *in vitro*. In addition to determining the relative expression levels of "core" housekeeping genes under both growth conditions, we identified 166 genes that are differentially-expressed by *L. interrogans in vivo*. Our analyses highlight physiological aspects of host adaptation by leptospires relating to heme uptake and utilization. We also identified 11 novel non-coding transcripts that are candidate small regulatory RNAs. The DMC model provides a facile system for studying the transcriptional and antigenic changes associated with mammalian host-adaption, selection of targets for mutagenesis, and the identification of previously unrecognized virulence determinants.

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Introduction

Leptospirosis is a neglected disease of global significance [1,2]. Pathogenic leptospires, shed in animal urine or free-living within contaminated water, enter the host through small abrasions in the skin or contact with mucous membranes of the eyes, nose or throat. Organisms disseminate almost immediately following acquisition, travelling *via* the bloodstream to multiple tissues [3]. *L. interrogans*, an extracellular pathogen, is thought to penetrate host tissues by intercellular migration [4]. In immunocompetent hosts, the majority of leptospires are thought to be cleared by opsonophagocytosis following the appearance of specific antibodies [5]. However, organisms that reach the kidneys, an immunoprivileged site [1], adhere to and colonize the proximal convoluted renal tubules, where they replicate exponentially. The majority of human disease is caused by *Leptospira interrogans* serovar

Author Summary

Leptospirosis, a global disease caused by the unusual bacterium *Leptospira*, is transmitted from animals to humans. Pathogenic species of *Leptospira* are excreted in urine from infected animals and can continue to survive in suitable environments before coming into contact with a new reservoir or accidental host. Leptospires have an inherent ability to survive a wide range of conditions encountered in nature during transmission and within mammals. However, we know very little about the regulatory pathways and gene products that promote mammalian host adaptation and enable leptospires to establish infection. In this study, we used a novel system whereby leptospires are cultivated in dialysis membrane chambers implanted into the peritoneal cavities of rats to compare the gene expression profiles of mammalian host-adapted and *in vitro*-cultivated organisms. In addition to providing a facile system for studying the transcriptional and physiologic changes leptospires undergo during mammalian infection, our data provide a rational basis for selecting new targets for mutagenesis.

(sv.) Copenhageni for which *Rattus norvegicus* serves as a reservoir host [3,6,7]. Experimentally-infected rats can excrete up to 10^7 leptospires/ml of urine for months without clinical signs of infection, thus exemplifying the unique biological equilibrium that can exist between pathogen and reservoir host [8,9,10].

The genome sequences of several pathogenic and saprophytic *Leptospira* spp., including *L. interrogans* sv. Copenhageni, are now complete [6,11,12,13,14,15,16]. *L. interrogans* sv. Copenhageni Fiocruz L1-130 harbors 3728 protein-encoding genes [11,12]. By comparative genomics, Picardeau *et al.* [14] identified 1431 "pathogen-specific" genes that are present within either or both of the pathogenic species, *L. interrogans* and *L. borgpetersenii*, but are absent from the free-living saprophyte *L. biflexa*. Although the majority (62%) of these pathogen-specific genes encode proteins of unknown function, it is possible that some are required by *Leptospira* to respond to unique environmental cues encountered within the mammalian host. Along these lines, the genome of *L. interrogans* contains >200 protein-coding sequences potentially involved in gene regulation, including gene products associated with two component signal transduction systems, alternate sigma factors, anti-sigma factors, and anti-sigma factor antagonists [11,12]. Not surprisingly, the pathogen-specific group also includes numerous gene products whose annotated functions or cellular location suggest a potential role in virulence-related processes such as adherence, digestion of host tissues and extracellular matrix, and evading the host's innate and adaptive immune responses [14,17].

To identify novel leptospiral virulence determinants, investigators have manipulated *in vitro* growth conditions to simulate those encountered within the mammalian host, including increased temperature and/or osmolarity, iron starvation, and the presence of serum [18,19,20,21,22]. However, the extent to which these *in vitro* conditions faithfully reproduce those encountered by *Leptospira in vivo* is unclear. In an effort to characterize leptospires in a truly mammalian host-adapted state, we cultivated virulent low-passage *L. interrogans* sv. Copenhageni within the peritoneal cavities of rats using a modification of our dialysis membrane chamber (DMC) model [23,24]. Given that rats are a natural reservoir host for this species of *Leptospira* [2,25,26], we reasoned that this model would be ideal for this purpose. Originally developed to study host adaption by Lyme disease spirochetes (*Borrelia burgdorferi*) [23,24], this technique, which uses dialysis membrane tubing with an 8000 Da molecular weight cut-off, provides bacteria with access to host nutrients while protecting them from the host's cellular immune response. The DMC model has been instrumental in studying the contribution of mammalian host-specific signals to differential gene expression in *B. burgdorferi* on a genome-wide scale as well as enabling us to characterize the transcriptional and physiological changes integral to the mammalian host-adaptation process [23,24,27,28,29].

In recent years, high-throughput RNA sequencing (RNA-Seq) has replaced microarrays as the method of choice for genome-wide transcriptional profiling in bacteria [30,31]. Unlike microarrays, RNA-Seq allows transcription to be understood at the single-nucleotide level. Here, we used an RNA-Seq approach to compare the transcriptome of virulent low passage *Leptospira interrogans* sv. Copenhageni cultivated within DMCs with that of leptospires grown under standard *in vitro* conditions (30°C in EMJH). Using this approach, we determined the relative expression levels of "core" housekeeping genes under both growth conditions, and, more importantly, we identified 166 genes that are differentially-expressed by leptospires within the mammalian host, the majority of which are pathogen-specific (*i.e.*, not present within saprophytic *Leptospira*). Most notably, our analyses highlight novel physiological aspects of mammalian-host adaptation by leptospires with respect to heme uptake and utilization. Moreover, we identified 11 novel non-coding (ncRNAs) transcripts which represent candidate small regulatory RNAs. In addition to providing a facile system for studying the transcriptional and physiologic changes leptospires undergo during mammalian infection, our data provide a rational basis for selecting new targets for mutagenesis.

Results

Virulent leptospires become mammalian host-adapted during growth within dialysis membrane chambers

Our extensive experience with cultivation of Lyme disease spirochetes in DMCs implanted into rats [23,24,27,32], a natural reservoir for *L. interrogans*, led us to ask whether the DMC model could be used to generate mammalian host-adapted *Leptospira*. In preliminary experiments, we determined that virulent low-passage *L. interrogans* sv. Copenhageni strain Fiocruz L1-130, diluted to low density (1×10^4 leptospires/ml) in EMJH medium, undergoes exponential replication within DMCs, reaching a maximal density of $\sim7\times10^7$ leptospires/ml within 8 days post-implantation (data not shown). Importantly, leptospires recovered from DMCs explanted daily between 8 and 12 days post-implantation were vigorously motile by dark-field microscopy. The polypeptide profiles of leptospires in DMCs explanted between 9 and 12 days were highly similar (data not shown). On the basis of these studies, we chose 10 days as our standard period for intraperitoneal implantation. As shown in Figure 1A, under these conditions, we noted numerous polypeptides whose expression was either increased or decreased in response to mammalian host-derived signals compared to *in vitro*-grown bacteria. The polypeptide differences between *in vitro*- and DMC-cultivated organisms were even more apparent by two-dimensional SDS-PAGE (Figure S1). While a comprehensive quantitative analysis of these differentially-expressed polypeptides is necessary to identify the corresponding leptospiral proteins, these data support our contention that virulent leptospires substantially alter their proteome in response to mammalian host-specific signals.

With *B. burgdorferi*, successful mammalian host-adaptation within DMCs is determined by the reciprocal expression of the outer surface lipoprotein (Osp) A and OspC lipoproteins, which are

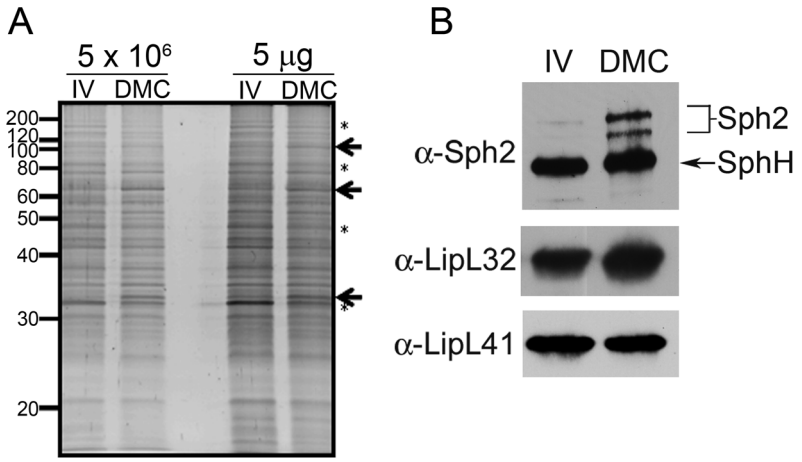

Figure 1. Virulent leptospires become mammalian host-adapted during growth within dialysis membrane chambers. Representative whole cell lysates of leptospires cultivated to late-logarithmic phase in EMJH medium at 30°C *in vitro* (IV) and within dialysis membrane chambers (DMC) implanted into the peritoneal cavities of female Sprague-Dawley rats. (A) Lysates were loaded according to the numbers of leptospires (5×10^6 per lane) or total protein (5 μg per lane) and stained with SYPRO Ruby gel stain. Arrows and asterisks are used to highlight examples of polypeptides whose expression appears to be increased or decreased, respectively, within DMCs compared to *in vitro*. Molecular mass markers are indicated on the left. (B) Immunoblot analyses using rabbit polyclonal antisera directed against Sph2 [34], LipL32 [38] and LipL41 [39]. An arrow is used to indicate a band of the predicted molecular mass for SphH, a second, closely-related sphingomyelinase in *L. interrogans* recognized by antiserum directed against Sph2 [34,37].
doi:10.1371/journal.ppat.1004004.g001

OFF and ON, respectively, within the mammal [23]. However, no expression profile associated with host-adapted *L. interrogans* has been reported and only a handful of leptospiral genes/proteins have been shown to be reproducibly upregulated during mammalian infection. Among these is Sph2, one of four sphingomyelinase-like proteins encoded by *L. interrogans* sv. Copenhageni [12]. Although most strains of *L. interrogans* encodes at least 3 distinct sphingomyelinase-like proteins (Sph1, Sph2 and Sph3), only Sph2 is thought to be a "true" (*i.e.*, enzymatically active) sphingomyelinase [33]. Expression of Sph2 is upregulated *in vitro* in response to serum [21] and/or increased osmolarity [34] and during mammalian infection [35]. On the other hand, SphH, a closely-related pore-forming protein without sphingomyelinase activity [33,36], is expressed constitutively *in vitro* [34,37] and by leptospires colonizing the renal tubules of infected hamsters [37]. Consistent with these previous studies, the level of Sph2 was substantially higher in DMC-cultivated leptospires compared to *in vitro*-grown organisms, whereas SphH was expressed at similar levels under both conditions (Figure 1B). Immunoblots using antisera against LipL32 and LipL41, two leptospiral lipoproteins expressed constitutively *in vitro* and during mammalian infection [38,39,40], were performed as loading controls (Figure 1B). We considered these data as strong indication that DMC-cultivated leptospires are in a mammalian host-adapted state.

RNA-Seq analysis of *Leptospira* cultivated *in vitro* and within DMCs

Having established the feasibility of using DMCs to generate mammalian host-adapted *L. interrogans*, we compared the transcriptional profiles of DMC- and *in vitro*-cultivated leptospires by RNA-Seq. To ensure that our data would be robust and reproducible, we generated Illumina TruSeq libraries from three biologically-independent samples for each growth condition. The sequence statistics and numbers of mapped reads for each biological replicate are summarized in Table 1 and displayed graphically in Figure 2. The total number of reads ranged from ~8–14 million per library, of which 79–94% of reads mapped to the *L. interrogans* sv. Copenhageni Fiocruz L1-130 reference genome [11,12]; only those reads that mapped to a single location on either Chromosome 1 or 2 were used to assess gene expression. The majority (43–55%) of unique sequence reads mapped to protein-coding mRNAs annotated on Chromosome 1, while ~3–5% mapped to predicted ORFs on Chromosome 2; this 12:1 ratio is consistent with the relative coding capacities of the two chromosomes [11,12]. As discussed below, a considerable number of reads (13–20%) in both chromosomes mapped to non-coding regions that represent candidate small regulatory RNAs (sRNAs) (Table 1).

RNA-Seq provides comprehensive coverage of the leptospiral transcriptome under *in vivo* and *in vitro* growth conditions

The genome of *L. interrogans* sv. Copenhageni Fiocruz L1-130 harbors 3728 protein-encoding genes [11,12]. The vast majority (~94%) of these (3489 and 3499 in DMC- and *in vitro*-cultivated leptospires, respectively), were represented in our RNA-Seq data by a mean expression value of ≥1 (Table S2). We observed average mean expression values of 67.2 and 60.5 per gene in DMC- and *in vitro*-cultivated organisms, respectively (data not shown).

By comparative genomics, Picardeau *et al.* [14] identified 2052 "core" protein-coding genes that are shared between pathogenic (*L. interrogans* and *L. borgpetersenii*) and saprophytic (*L. biflexa*) *Leptospira* species. Not surprisingly, many of these core gene products are associated with housekeeping functions, such as motility, energetics and intermediary metabolism, DNA and RNA metabolism, and cell division [14]. Analysis of the protein-coding sequences for the 100 most highly-expressed genes (*i.e.*, Top 100) in DMC-cultivated leptospires revealed that 66 are conserved (*i.e.*, ≥40% amino acid identity over ≥80% of the coding region) between pathogenic and saprophytic *Leptospira* spp. and, therefore, part of the core group (Table S3); of note, the percentage (66%) of core genes within our Top100 is similar to the overall percentage (55%) of core genes within the entire *L. interrogans* sv. Copenhageni

Table 1. Summary of RNA-Seq mapping data.

Library	Total # of Reads	Total # of mapped reads[1]	Uniquely mapped Reads[2]	Chromosome 1[3]				Chromosome 2[3]		
				mRNA	ncRNA	rRNA & tRNA	Intergenic	mRNA	ncRNA	Intergenic
DMC_1	9,150,966	8,327,861 (91.01%)	598,674 (6.54%)	279,333 (46.66%)	125,095 (20.90%)	155,452 (25.97%)	13,828 (2.31%)	22,797 (3.81%)	374 (0.06%)	1,795 (0.30%)
DMC_2	7,902,948	6,543,759 (82.80%)	574,766 (7.27%)	247,398 (43.04%)	161,761 (28.14%)	133,152 (23.17%)	9,997 (1.74%)	20,822 (3.62%)	382 (0.07%)	1,254 (0.22%)
DMC_3	14,089,133	11,125,795 (78.97%)	1,151,053 (8.17%)	589,345 (51.20%)	240,121 (20.86%)	235,839 (20.49%)	33,601 (2.92%)	46,718 (4.06%)	1,136 (0.10%)	4,293 (0.37%)
30°C_1	9,562,316	8,997,777 (94.10%)	870,275 (9.10%)	482,828 (55.48%)	129,770 (14.91%)	192,490 (22.12%)	20,249 (2.33%)	41,187 (4.73%)	770 (0.09%)	2,981 (0.34%)
30°C_2	11,642,652	10,702,058 (91.92%)	842,265 (7.23%)	456,665 (54.22%)	113,539 (13.48%)	201,691 (23.95%)	20,245 (2.40%)	45,486 (5.40%)	918 (0.11%)	3,721 (0.44%)
30°C_3	10,175,557	9,462,697 (92.99%)	805,288 (7.91%)	441,078 (54.77%)	109,817 (13.46%)	186,689 (23.18%)	21,165 (2.63%)	42,184 (5.24%)	809 (0.10%)	3,546 (0.44%)

[1]Total number and percentage (in parenthesis) of reads that mapped to the reference genome with 100% accuracy.
[2]Total number and percentage (in parenthesis) of reads that mapped to a single location within the reference genome [11,12].
[3]Based on the total of uniquely mapped reads for the corresponding sample.
doi:10.1371/journal.ppat.1004004.t001

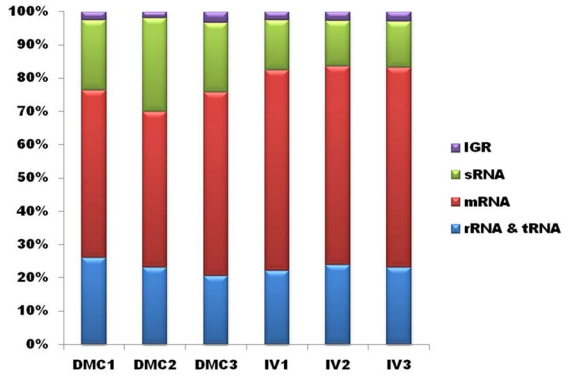

Figure 2. Mapping of RNA-Seq reads. Percentage of uniquely mapping reads from each biological replicate of leptospires cultivated in DMCs or under standard *in vitro* growth conditions (30°C in EMJH).
doi:10.1371/journal.ppat.1004004.g002

genome [14]. Consistent with their proposed housekeeping functions, 62 (94%) of the 66 core genes within the Top 100 were expressed at similar levels *in vitro* and within DMCs (Table S3). Thirty-four of the Top 100 genes are pathogen-specific (*i.e.*, no orthologous gene identified in *L. biflexa*), two of which (*LIC10465/ligA* and *LIC12653*) are found only in *L. interrogans* (*i.e.*, absent in *L. borgpetersensii*, *L. licerasiae* and *L. santarosai*). Eight of the 34 pathogen-specific genes within the Top 100 were upregulated by *L. interrogans* sv. Copenhageni within DMCs (see Table S3 and below).

We also surveyed both DMC- and *in vitro*-derived datasets for genes associated with key metabolic pathways. One unusual metabolic feature of pathogenic leptospires, compared to other spirochetes, is that they are unable to utilize glucose despite encoding a seemingly complete glycolytic pathway, relying instead on β-oxidation of long-chain fatty acids as sources of both carbon and energy [11,41]. By RNA-Seq, we detected uniquely mapped reads for all of the genes thought to be involved in glucose uptake and utilization (KEGG pathway lic00010), each of which was expressed at similar levels in DMCs and *in vitro* (Table S4). However, two genes, *LIC13358* and *LIC20119*, both encoding putative phosphoglucomutases, and *LIC12908*, encoding the only glucose transporter identified in *L. interrogans* [11,12,42], were expressed at extremely low levels, both in DMCs and *in vitro* (Table S2). These data support the findings of Zhang *et al.* [42], who proposed that the inability of pathogenic leptospires to utilize glucose stems from insufficient glucose uptake and/or catalysis rather than an incomplete glycolytic pathway. As one might predict, we detected significant numbers of sequence reads for genes involved in the uptake and β-oxidation of medium and long-chain fatty acids (KEGG pathway lic00071), the citric acid cycle (KEGG lic00020), generation of NAD/NADP (KEGG lic00760), and oxidative phosphorylation (KEGG lic00190). All of the individual genes involved in these energetic pathways were expressed at similar levels under both growth conditions (Table S4).

Genes whose expression was significantly upregulated by leptospires in DMCs compared to *in vitro*-grown bacteria

Using DESeq [43], we identified 166 genes whose expression was either positively- or negatively-regulated by ≥2-fold (adjusted p-value≤0.05) within the mammal (Tables 2 and 3). Although some variance was observed between biological replicates (Table S2), a heat map representing the expression data for all 166

Table 2. *L. interrogans* sv. Copenhageni genes upregulated in DMCs compared to *in vitro*.

Gene ID[1]		Product[1]	Fold (DMC vs IV)	P value (adjusted)
Hypothetical Proteins				
LIC10822		Hypothetical protein	ND @ 30°C	4.00E-02
LIC12077		Hypothetical protein	22.78	1.19E-18
LIC13005		Hypothetical protein	21.12	2.75E-04
LIC13445		Hypothetical protein	17.46	6.13E-06
LIC11059		Hypothetical protein	16.76	2.65E-13
LIC10456		Hypothetical protein	10.59	4.12E-02
LIC10965		Hypothetical protein	9.73	8.94E-08
LIC12340		Hypothetical protein	7.14	3.40E-10
LIC13390		Hypothetical protein	6.60	2.74E-04
LIC10455		Hypothetical protein	6.54	1.04E-02
LIC12120		Hypothetical protein	6.20	1.93E-02
LIC10376		Hypothetical protein	6.14	2.07E-02
LIC11888		Hypothetical protein	5.71	1.33E-08
LIC10535		Hypothetical protein	5.61	2.65E-02
LIC12555		Hypothetical protein	4.76	5.29E-04
LIC10971		Hypothetical protein	4.35	8.70E-03
LIC10775		Hypothetical protein	4.27	8.69E-07
LIC10790		Hypothetical protein	4.15	8.98E-03
LIC11695		Hypothetical protein	4.06	9.65E-03
LIC11492		Hypothetical protein	4.03	4.56E-02
LIC12986		Hypothetical protein	4.03	2.53E-02
LIC10415		Hypothetical protein	3.93	5.73E-06
LIC13354		Hypothetical protein	3.77	4.53E-03
LIC12653		Hypothetical protein	3.63	5.83E-03
LIC12993		Hypothetical protein	3.53	1.19E-03
LIC10593		Hypothetical protein	3.32	3.07E-03
LIC11705		Hypothetical protein	3.14	3.55E-02
LIC10080		Hypothetical protein	3.14	5.99E-03
LIC10374		Hypothetical protein	3.09	2.12E-02
LIC10729		Hypothetical protein	2.97	2.12E-02
LIC10450		Hypothetical protein	2.94	1.21E-02
LIC10454		Hypothetical protein	2.86	4.53E-02
LIC11783		Hypothetical protein	2.82	4.12E-02
LIC10053		Hypothetical protein	2.81	1.23E-02
LIC10460		Hypothetical protein	2.74	5.31E-03
LIC13084		Hypothetical protein	2.72	2.03E-02
LIC12263	*ompL37*	Hypothetical protein	2.67	8.81E-03
LIC20245		Hypothetical protein	2.66	4.04E-02
LIC12719		Hypothetical protein	2.66	2.24E-02
LIC11275		Hypothetical protein	2.66	3.36E-02
LIC13212		Hypothetical protein	2.61	3.75E-02
LIC12353		Hypothetical protein	2.53	4.28E-02
LIC11565		Hypothetical protein	2.52	2.11E-02
LIC10050		Hypothetical protein with OmpA-like domain	2.46	1.54E-02
LIC11177		Hypothetical protein	2.43	2.67E-02
LIC11447		Hypothetical protein	2.42	3.36E-02
LIC10302		Hypothetical protein	2.23	2.28E-02
LIC12071		Hypothetical protein	2.08	3.02E-02

Table 2. Cont.

Gene ID[1]		Product[1]	Fold (DMC vs IV)	P value (adjusted)
Putative or Probable Lipoproteins (unknown function)				
LIC12099	*lipL53*	Lipoprotein	19.50	1.03E-26
LIC11030		Lipoprotein	11.18	1.78E-04
LIC12209		Lipoprotein	7.76	2.75E-15
LIC11058	*lemA*	Lipoprotein	6.27	8.69E-07
LIC10373		Lipoprotein	5.37	4.61E-11
LIC13066		Lipoprotein	5.34	3.09E-09
LIC10462		Lipoprotein[2]	5.08	3.17E-07
LIC10371		Lipoprotein	4.61	9.64E-08
LIC10054	*rlpA*	Lipoprotein	3.84	1.44E-05
LIC13355		Lipoprotein	3.72	1.25E-02
LIC12208		Lipoprotein	3.64	2.07E-04
LIC10461		Lipoprotein	3.54	1.84E-04
LIC10968		Lipoprotein[2]	3.44	2.81E-03
LIC10463		Lipoprotein	3.31	4.81E-04
LIC11082		Lipoprotein	3.15	2.03E-03
LIC11167		Lipoprotein	2.24	2.03E-02
Pathogenicity and/or Virulence				
LIC12760	*colA*	Collagenase precursor	49.03	2.63E-51
LIC12631	*sph2*	Hemolysin/sphingomyelinase-like protein, Sph2	13.92	5.13E-26
LIC11219	*ahpC*	Peroxiredoxin	5.96	7.29E-07
LIC10465	*ligA*	*Leptospira* Ig-like protein LigA[3]	4.57	2.03E-02
LIC12632	*sph1*	Hemolysin/sphingomyelinase-like protein, Sph1	4.02	1.52E-05
LIC12927		Cytochrome c peroxidase[2]	4.23	1.04E-05
LIC13198	*sph3*	Hemolysin/sphingomyelinase-like protein, Sph3	3.57	1.99E-02
LIC12659	*vapB*	Virulence-associated protein	2.90	2.66E-02
Chemotaxis and Motility				
LIC10299	*flgB*	Flagellar basal body rod protein FlgB	7.89	4.89E-16
LIC11889	*flaB*	Flagellin protein	3.95	1.50E-02
LIC10298	*flgC*	Flagellar basal body rod protein FlgC	3.91	2.12E-05
LIC11328	*flgJ*	Flagellum-specific muramidase	3.89	7.28E-03
LIC10297	*fliE*	Flagellar hook-basal body protein FliE	3.16	1.87E-03
LIC11326	*flgH*	Flagellar L-ring protein precursor	2.66	3.03E-02
LIC11186	*flbC*	Flagellar protein	2.37	3.72E-02
Regulatory Functions				
LIC12798		TetR family transcriptional regulator	5.64	3.65E-05
LIC11146		DeoR family transcriptional regulator	4.42	3.08E-03
LIC12034	*fur*	Fur family transcriptional regulator	3.83	7.84E-04
LIC11440		Histidine kinase response regulator hybrid protein	3.36	1.04E-03
LIC10996		EAL-type diguanylate phosphodiesterase	3.23	2.11E-03
LIC11617		ArsR family transcriptional regulator	3.11	7.39E-03
LIC20025		Cyclic nucleotide binding protein	2.41	4.73E-02
Intermediary Metabolism and Biosynthesis of Small Molecules				
LIC13053	*desA*	Fatty acid desaturase	6.72	2.98E-11
LIC12981		Glutathione S-transferase	4.00	2.59E-02
LIC13397	*phoD*	Alkaline phosphatase	3.21	6.13E-04
LIC13085	*coaE*	Dephospho-CoA kinase	3.20	1.79E-02
LIC20148	*hol*	Heme oxygenase	3.06	3.35E-03
LIC12322		Glutaconate CoA transferase-like protein	2.32	4.80E-02
LIC13031		Aminotransferase	2.25	1.46E-02

Table 2. Cont.

Gene ID[1]		Product[1]	Fold (DMC vs IV)	P value (adjusted)
LIC13465		Gly tRNA	4.69	1.69E-02
Cell Processes/Transport				
LIC11694		TonB-dependent outer membrane receptor	14.85	2.26E-13
LIC10964	*phuR*	TonB-dependent outer membrane hemin receptor	3.46	2.23E-02
LIC20149		Multidrug-efflux transporter	2.82	4.22E-02
LIC12992	*sulP*	Sulfate permease	2.81	4.05E-03
DNA Metabolism and Cell Division				
LIC11467	*rcc1*	Regulator of chromosome condensation	7.80	1.92E-03
LIC12737		Site-specific modification DNA-methyltransferase	5.12	6.37E-11
LIC12297		DNA repair protein	4.55	3.80E-02
LIC10131	*mesJ*	Cell cycle protein	3.41	3.62E-04
LIC10252		Exonuclease	3.31	5.86E-03
LIC13389	*mutS*	DNA mismatch repair protein	2.56	3.80E-02
LIC11479	*xerD*	Integrase/recombinase protein	2.91	3.02E-03
Macromolecular Metabolism and Cell Structure				
LIC10537		OmpA-like peptidoglycan-associated periplasmic protein	3.79	1.00E-03
LIC10528	*pbpB*	Penicillin-binding protein 3	2.46	1.99E-02
Miscellaneous				
LIC10149	*frnE*	Polyketide synthase	4.89	1.08E-02
LIC10251		Rad50-like protein	3.22	4.05E-03
LIC11850	*rmsE*	16S ribosomal RNA methyltransferase RsmE	2.41	8.14E-03

[1]Gene designations and protein product descriptions are based on those of [11,12] and the *L. interrogans* sv. Copenhageni Genome Project database (http://aeg.lbi.ic.unicamp.br/world/lic/), except where indicated.
[2]nnotation based on Setubal *et al.* [47].
[3]Revised annotation based on bioinformatics.
doi:10.1371/journal.ppat.1004004.t002

differentially-expressed genes confirmed that each biological replicate clustered with its respective sample source (Figure S2). Of the 110 genes upregulated by *L. interrogans* within DMCs, 106 are on Chromosome 1 while only 4 are on Chromosome 2 (Table 2). All but 3 of the upregulated genes appear to be pathogen-specific (*i.e.*, a paralogous gene/protein could not be identified in *L. biflexa*; 54 of these are unique to *L. interrogans* and an additional 7 are unique to serovar Copenhageni (Figure 3). Almost half (49/110) of the genes upregulated in DMCs encode hypothetical proteins (Figure 4 and Table 3), which is consistent with the overall percentage (40%) of hypothetical genes annotated within *L. interrogans* [6,11]. Based on searches performed using the Conserved Domain Database [44,45], none of the hypothetical proteins encoded by these genes contained readily identifiable functional domains (data not shown). However, one gene (*LIC12986*) recently was shown to be required for leptospires to survive within hamsters and to colonize the renal tubules of mice [46].

Of the remaining upregulated genes, 16 encode putative lipoproteins of unknown function [47] (Figure 4 and Table 3). Surface-exposed spirochetal lipoproteins have been implicated in a wide range of pathogenesis-related functions, including adherence to extracellular matrix components and nutrient acquisition [48]. However, because the mechanism(s) responsible for sorting individual spirochetal lipoproteins remain poorly understood, it is not possible to predict based on amino acid sequence alone which, if any, might function at the pathogen-host interface.

Table 3. Leptospiral genes differentially-expressed within DMCs compared to *in vitro*.

Genes	No of genes in each category		
	Upregulated (%)[1]	Downregulated (%)[1]	Total (%)[2]
Known or predicted function	45 (41%)	20 (36%)	66 (40%)
Unknown or poorly characterized function[3]	65 (59%)	36 (64%)	101 (60%)
Total	110	56	166

[1]Percentage of genes based on the total number of genes in upregulated or downregulated category.
[2]Percentage of genes based on the total number of differentially-expressed genes.
[3]Hypothetical proteins and uncharacterized lipoproteins.
doi:10.1371/journal.ppat.1004004.t003

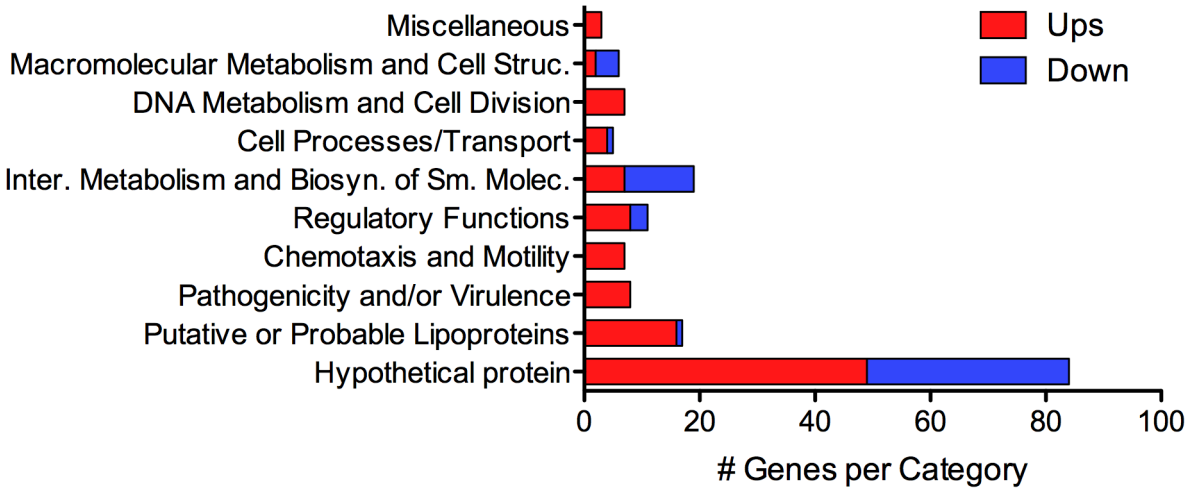

Figure 3. Conservation of *L. interrogans* sv. Copenhageni Fiocruz L1-130 differentially-expressed genes among virulent and saprophytic *Leptospira* spp. Protein sequence similarities were determined using GLSEARCH (v. 34.05). Genomes used for analysis: *L. interrogans* sv. Lai strain 56601, *L. borgpetersenii* sv. Hardjo strain L550, *L. santarosai* sv. Shermani strain LT821; *L. licerasiae* sv. Varillal strain VAR010; and *L. biflexa* sv. Patoc strain Patoc1 Ames, respectively. The color coding used in the heat map is as follows: blue, 95–100% identity; green, 90–94% identity; orange, 85–89%; and yellow, 80–84%.
doi:10.1371/journal.ppat.1004004.g003

Virulence-associated genes. Eight DMC-upregulated genes encode proteins implicated in pathogenicity and/or virulence. *LIC12760/colA*, the most significantly upregulated gene (49-fold) in our studies, encodes a collagenase precursor. Degradation of host tissues by this enzyme is thought to promote bacterial colonization and/or dissemination as well as provide an additional source of nutrients (*e.g.*, amino acids) [49]. *LIC12631*, *LIC12632* and *LIC13198*, respectively, encode Sph2, Sph1 and Sph3. Lysis of host erythrocytes by Sph2 may enhance acquisition of fatty acids and heme/iron from the host. Narayanavari *et al.* [33] also raised the possibility that the non-catalytic Sphs (Sph1 and Sph3) function as adhesins *via* their interaction with host sphingomyelin. *LIC10465* encodes leptospiral immunoglobulin-like (Lig) protein A; this multifunctional, outer membrane-associated lipoprotein has been shown to promote binding to host molecules, including fibronectin, fibrinogen and extracellular matrix [50,51,52]. Moreover, antibodies against LigA are protective in a hamster model of acute infection [53]. *LIC12659/vapB* encodes a putative virulence-associated protein with similarity to the AbrB-like family of transcriptional regulators [54]; ArbB-like transcription factors, also referred to as transition state regulator proteins, have been identified in diverse bacteria but only orthologs from *Bacillus* have been characterized with respect to function and DNA-binding capabilities [55,56]. *LIC11219* and *LIC12927*, encoding a peroxiredoxin (AhpC) and cytochrome c peroxidase, respectively, are discussed below.

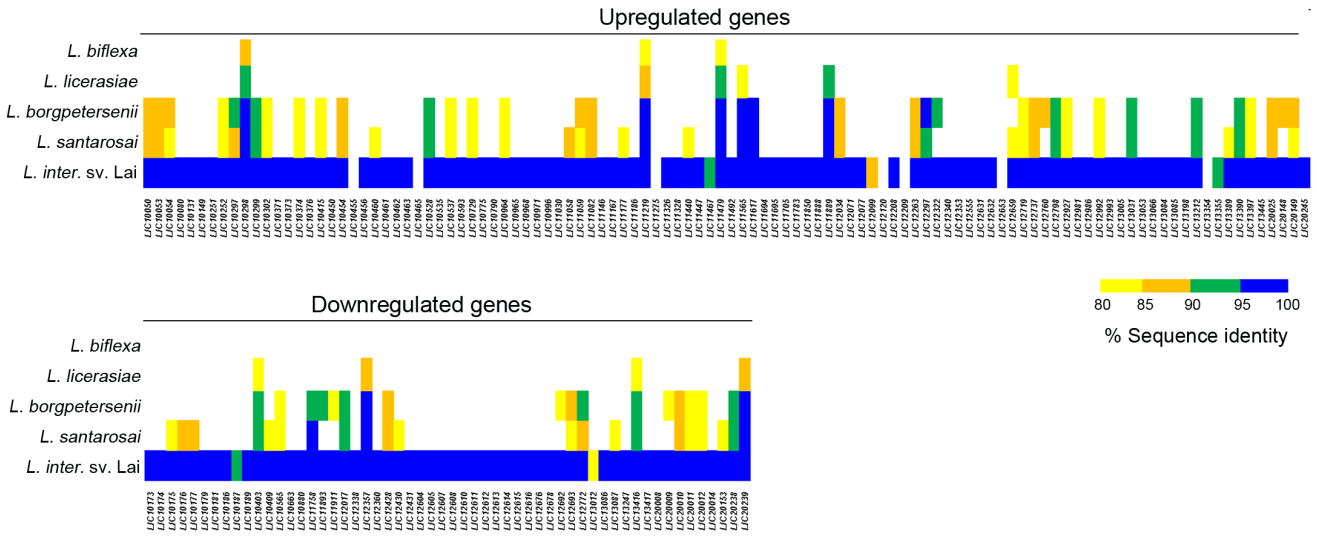

Figure 4. Functional categories of genes differentially-expressed by *L. interrogans* sv Copenhageni strain Fiocruz L1-130 within DMCs. Functional categories are based on those of [11,12] and the *Leptospira interrogans* sv. Copenhageni Genome Project database (http://aeg.lbi.ic.unicamp.br/world/lic/). The number of upregulated (Ups) and downregulated (Down) genes within each category are indicated in red and blue, respectively.
doi:10.1371/journal.ppat.1004004.g004

Motility-related genes. Consistent with the highly invasive nature of leptospiral infection, seven motility-related genes were upregulated within DMCs (Table 2), including three (*LIC10299/flgB*, *LIC10298/flgC* and *LIC10297/fliE*) involved in flagellar basal body formation (Figure 5). The *L. interrogans* genome contains five copies of *flaB* (*LIC11889*, *LIC11890*, *LIC11531*, *LIC11532* and *LIC12947*), which encode the flagellar core subunit flagellin. Of these, only *LIC11889* was differentially-expressed within DMCs. Interestingly, based on the number of uniquely mapped reads determined by DESeq, *LIC12947* was expressed at substantially lower levels than the other four *flaB* paralogs under both growth conditions (Table S2), suggesting that this gene product may not contribute significantly to the formation of flagella *in vitro* or *in vivo*.

Uptake and utilization of iron/heme. Unlike *B. burgdorferi* [57], *L. interrogans* requires iron for growth *in vitro* and, presumably, within the mammalian host. In EMJH medium, leptospires obtain iron from Fe(II) sulphate, while organisms in the mammal acquire iron from heme and/or heme-containing proteins [58]. Heme (free or complexed with hemoglobin) is appropriated from the host by high-affinity TonB-dependent outer membrane receptor (TB-DR) proteins. Based on bioinformatic analysis, *L. interrogans* encodes at least 13 putative TB-DRs [58], however, only two (*LIC10964* and *LIC11694*) were upregulated within DMCs (Table 3 and Table S4). While most often associated with iron uptake, TB-DRs also may bind vitamin B_{12}, a nutrient essential for leptospires *in vitro* and, presumably, *in vivo* [59]. Only one TB-DR in *L. interrogans* sv. Copenhageni (*LIC12374/btuB*) is annotated as being specific for vitamin B_{12}, and the gene encoding this transporter component was not differentially-expressed in DMCs compared to *in vitro* (Table S4). Transport of heme and/or iron across the outer membrane requires energy produced by an inner membrane complex of the energy transduction protein TonB and two accessory proteins, ExbB and ExbD [59]. *L. interrogans* encodes at least two TonB-ExbB-ExbD complexes, arranged in separate operons, one on each chromosome. Interestingly, the transporter on Chromosome 2 (*LIC20216-20218*) was expressed at much higher levels (>14-fold) than its counterpart on Chromosome 1 (*LIC10889-10892*) under both growth conditions. Neither operon, however, was differentially-expressed in DMCs.

Consistent with an increased requirement for iron and/or heme *in vivo*, we detected increased expression (3.27-fold) of heme oxygenase (*LIC20148/hol*) [60] within DMCs. Once released, iron would be stored in the cytoplasm by bacterioferritin (*LIC11310*) and/or ferredoxin (*LIC13258* and *LIC13209*) gene products, all of which were well expressed by leptospires *in vitro* and in DMCS (Table S5).

When in excess, iron can lead to toxicity *via* the production of reactive oxygen species (ROS). As such, bacterial genes associated with iron homeostasis often are regulated by the ferric uptake regulator protein Fur, a global iron-responsive transcriptional repressor [61]. *L. interrogans* encodes at least four putative Fur paralogs (*LIC11006*, *LIC11158*, *LIC12034* and *LIC20147*). We used SLiMSearch [62] to survey the *L. interrogans* genome for "fur boxes" ([GC]AT[AT]AT[GC]AT[AT]AT[GC]AT[AT]AT[GC]) [61], and were unable to identify any obvious Fur-regulated genes (data not shown). Fur proteins, including those encoded by *Leptospira* spp. [58], share significant sequence similarity with orthologs for Zur, a zinc uptake regulator, and Per, an oxidative stress response regulator [63]. Based on bioinformatics and/or experimental evidence, two of Furs identified in *L. interrogans* sv. Copenhageni (*LIC12034* and *LIC20147*) appear to encode Per orthologs [22,58]. One of these (*LIC12034*) was upregulated in DMCs (Table 2), suggesting that leptospires within DMCs are under some degree of oxidative stress.

Oxidative and thermal stress-related genes. Leptospires must cope with numerous stressors within the host, most notably, oxidative stress. Incomplete reduction of oxygen by iron-containing cytochromes is one potential source of endogenous ROS [64]. Leptospires likely encounter exogenously-derived ROS within the proximal renal tubules, a highly oxygenated tissue niche. Not surprisingly, *L. interrogans* encodes a more diverse repertoire of antioxidant proteins than either *Treponema pallidum* or *B. burgdorferi* (Table S5). Although *L. interrogans* encodes a functional catalase [65], it lacks superoxide dismutase (the enzyme typically associated with detoxification of $O_2^{\bullet-}$) and the regulatory proteins OxyR and SoxR. Interestingly, only two oxidative stress-associated genes (*LIC12927* and *LIC11219*) were upregulated within DMCs (Table 2). The former encodes a cytochrome c peroxidase while the latter encodes an AhpC-type peroxiredoxin. In *E. coli*, AhpC scavenges basal levels of endogenous peroxide generated as a metabolic by-product [66]. Increased expression of AhpC within DMCs is consistent with increased uptake of exogenously-derived heme (see above) and increased potential for Fenton chemistry within the cytoplasm. Like *T. pallidum* [67], *L. interrogans* does not

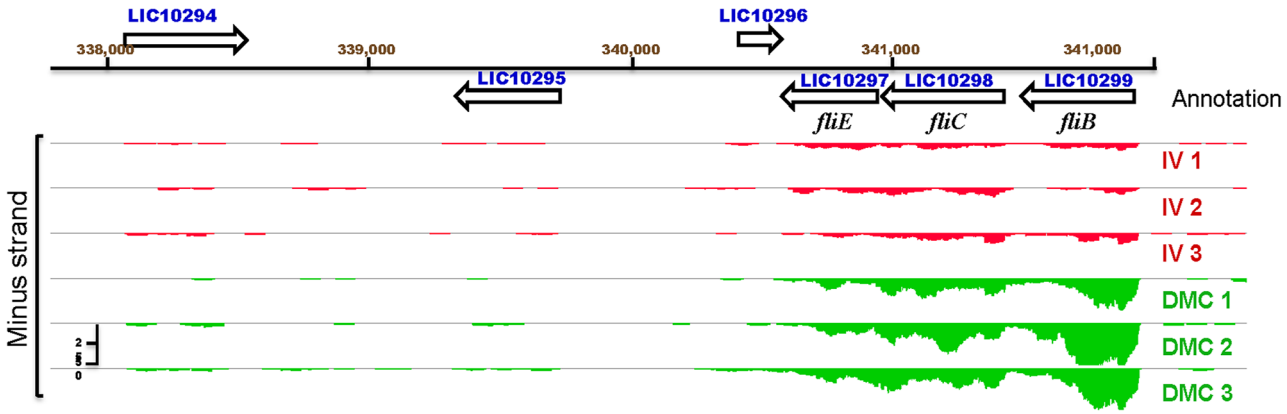

Figure 5. IGB viewer of normalized gene expression data for the flagellar genes *fliE, flgB* and *flgC*. Visualization of normalized mapped reads for minus (-) strand of an operon encoding genes *fliE*, *flgB* and *flgC* of the flagellar proximal rod shows increased expression by leptospires cultivated in dialysis membrane chambers (DMC, green) compared to those cultivated *in vitro* (IV, red). Annotated genes on Chromosome 1 are in blue. The vertical "read count" scale is 0–50.
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encode an AhpF, the usual reducing partner for AhpC, and most likely uses thioredoxin/thioredoxin reductase and/or glutaredoxin for this purpose, all of which were well expressed by DMC-cultivated leptospires (Table S5).

In addition to oxidative stress, increased temperature within the host might induce a stress response by leptospires *in vivo* [18,19]. However, consistent with previous reports [19,68,69], none of the classical heat shock response genes encoded by *L. interrogans* were upregulated within DMCs, compared to *in vitro* growth at 30°C (Table S4).

Regulators of transcription. The leptospiral genome encodes >200 gene products with the potential to directly regulate transcription (Figure 3), including numerous two-component sensor histidine kinases (HKs) and/or response regulators (RRs), alternate sigma factors, sigma factor regulators, anti-sigma factor antagonists, and trans-acting factors [11,12]. Only a few of these were upregulated within DMCs. One (*LIC11440*) encodes a hybrid sensor kinase/response regulator (HK/RR) protein; the sensor for this HK/RR contains a PAS-type sensor domain, which typically recognize small molecules, including heme [70]. Three additional genes (*LIC12798*, *LIC11146* and *LIC11617*) were DMC-upregulated and encode putative transcriptional regulators belonging to the TetR [71], DeoR [72], and ArsC [73] families of repressor proteins.

Genes whose expression was significantly downregulated in DMCs compared to *in vitro*

By RNA-Seq, we identified 56 genes (47 on Chromosome 1 and 9 on Chromosome 2) that were downregulated in DMCs (Tables 3 and 4). All of the downregulated genes are pathogen-specific (*i.e.*, not found in *L. biflexa*); almost half (26/56) are unique to *L. interrogans* (*i.e.*, not in *L. borgpetersenii*, *L. santarosai* or *L. licerasiae*) (Figure 3). As with the upregulated gene subset, more than half (35/56) of the DMC-downregulated genes encode hypothetical proteins (Figure 4 and Table 3); of note, almost half (43%) of these appear to be transcribed in two polycistronic operons (*LIC10173-10177* and *LIC12604-12616*). Interestingly, all of the genes within these two putative operons are pathogen-specific. Only one lipoprotein (*LIC20153*) was expressed at lower levels in DMCs (compared to 16 upregulated).

Five genes related to *de novo* heme biosynthesis (*LIC20008/hemA*, *LIC20009/hemCD*, *LIC20010/hemB*, *LIC20011/hemL* and *LIC20014/hemE*) [74] were DMC-downregulated these findings imply that leptospires can scavenge heme from the mammalian host. The heme biosynthetic operon also contains genes encoding a two component system (TCS). Signal transduction by the orthologous TCS in *L. biflexa* is required for regulation of heme biosynthesis [75]. Although both the histidine kinase (HK; *LIC20012*) and the response regulator (RR; *LIC20013*) were downregulated (2.50- and 2.22-fold; respectively) in DMCs, the fold-change for the RR was not significant ($p = 0.097$). Based on their tandem arrangement and similar expression profiles, these heme biosynthetic genes appear to be transcribed as a single operon. *LIC20017/hemG* and *LIC20018/hemH*, encoding enzymes responsible for the last two steps in heme biosynthesis, respectively, are located downstream of the larger biosynthetic operon; both of these genes appear to be transcribed as monocistronic messages at similar levels *in vitro* and in DMCs (Table 4 and data not shown).

Identification of novel candidate small RNAs

One of the advantages of RNA-Seq is that it allows visualization of uniquely mapped reads within non-annotated regions of the genome. Using the IGB browser, we detected at least 11 regions that were transcriptionally-active but not protein coding; these non-coding RNA (ncRNA) transcripts are novel candidate small regulatory RNAs (sRNAs) within *L. interrogans* (Table 5 and Figure S3). Five of these are homologous to known sRNA families (tmRNA, RNaseP, PyrR binding site and two cobalamin sRNAs) (http://rfam.sanger.ac.uk/) [76,77,78,79]. The expression of 8 of the 11 putative sRNAs was validated by reverse-transcriptase PCR in *L interrogans* sv. Copenhageni strain RJ16441 (Table 5) and all predicted sRNAs were highly conserved in the closely-related virulent serovar type strain Lai [80]. One of the predicted sRNAs, *LIC1nc80* (Figure 6), was significantly DMC-upregulated (4.39-fold) compared to *in vitro*-cultivated leptospires (Table S2). Further characterization of these candidate sRNAs (*i.e.*, by Northern blot) is required to understand their function(s) and relationships to the surrounding genes (*i.e.*, 5′ UTR verses bone fide sRNA).

Validation of RNA-Seq data by quantitative RT-PCR

To validate our RNA-Seq data, we performed quantitative reverse transcription-PCR (qRT-PCR) on a panel of 14 genes that were, according to DESeq analysis, upregulated (*LIC12631/sph2*, *LIC11888* and *LIC11889/flaB*), downregulated (*LIC10175*, *LIC10179* and *LIC12615*), or unchanged (*LIC10191/loa22*, *LIC12966/lipL41*, *LIC13166/ompL36*, *LIC10787/flaA-2*, *LIC10068*, *LIC10421*, *LIC12339*, and *LIC20001*) in DMCs compared to *in vitro*.While there is some debate regarding the most appropriate leptospiral gene to use for normalization [21,81], we selected *LIC11352/lipL32* based on studies demonstrating that its expression was relatively unchanged under a wide-range of growth conditions, including increased temperature, increased osmolarity, and/or exposure to serum [21,38,68]. Representative results are shown in Figure 7A; data for the entire panel are presented in Figure S4. Overall, we saw strong agreement between our RNA-Seq and qRT-PCR datasets; the correlation coefficient (R^2) between RNA-Seq and qRT-PCR data across the entire panel was 0.8881 (Figure 7B). We also used qRT-PCR to confirm the relative expression for two (*LIC1nc60*/RNase P and *LIC2nc10*/cobalamin) of the putative sRNAs (Figure S4); of these, only *LIC1nc60*/RNaseP was upregulated (2.65-fold; $p = 0.0054$) within DMCs.

Discussion

The identification of genes/proteins that are differentially-expressed by microorganisms only during infection and/or within specific host niches often provides insight into the parasitic strategies of pathogens. During natural and experimental infection in rats, *L. interrogans* rapidly disseminate hematogenously to all tissues but are cleared by 7 days post-inoculation from all sites except the kidneys [7,82]. The ability of leptospires to colonize and persist within renal tubules almost certainly involves unique virulent determinants [1]; however, the paucilbacillary nature of leptospiral infection, even within this preferred niche, hinders our ability to perform global gene expression studies on *L. interrogans* within host tissues. Prior studies, including several using micro-array-based approaches [18,19,21,22,83], have manipulated *in vitro* growth conditions to simulate the environmental signals encountered by leptospires within the mammal. Based on extensive studies with *B. burgdorferi*, another pathogenic spirochete, we and others have demonstrated that *bone fide* mammalian host adaptation is a complex and dynamic process that cannot be fully reproduced *ex vivo* [23,27,32]. We therefore used a rat peritoneal dialysis membrane chamber (DMC) model to generate sufficient *L. interrogans* in a mammalian host-adapted state to perform global transcriptional studies. Cultivation of leptospires within DMCs, in conjunction with next generation sequencing, enabled us to define

Table 4. *L. interrogans* sv. Copenhageni genes downregulated in DMCs compared to *in vitro*.

Gene ID[1]		Product[1]	Fold (DMC vs IV)	P value (adjusted)
Hypothetical Proteins				
LIC10173		Hypothetical protein	−31.35	2.28E-09
LIC10174		Hypothetical protein	−29.41	4.12E-02
LIC10176		Hypothetical protein	−16.09	1.52E-05
LIC10175		Hypothetical protein	−12.71	4.24E-15
LIC12611		Hypothetical protein	−11.09	2.02E-11
LIC12616		Hypothetical protein	−9.22	3.54E-03
LIC12615		Hypothetical protein	−8.46	2.01E-12
LIC12614		Hypothetical protein	−8.13	1.59E-05
LIC12610		Hypothetical protein	−6.99	2.07E-06
LIC10179		Hypothetical protein	−6.81	5.27E-03
LIC13417		Hypothetical protein	−6.59	2.38E-08
LIC11893		Hypothetical protein	−6.21	4.89E-04
LIC12612		Hypothetical protein	−6.19	2.50E-03
LIC13247		Hypothetical protein	−6.01	1.79E-02
LIC10177		Hypothetical protein	−6.01	4.11E-06
LIC12608		Hypothetical protein	−5.54	8.50E-04
LIC12613		Hypothetical protein	−5.46	1.09E-05
LIC13416		Hypothetical protein	−5.04	5.73E-06
LIC12604		Hypothetical protein	−4.94	2.02E-03
LIC10189		Hypothetical protein	−4.83	9.23E-03
LIC12692		Hypothetical protein	−4.76	2.40E-05
LIC12338		Hypothetical protein	−4.75	3.31E-02
LIC10880		Hypothetical protein	−4.49	3.89E-02
LIC10186		Hypothetical protein	−4.27	1.17E-02
LIC12430		Hypothetical protein	−3.51	1.99E-02
LIC10187		Hypothetical protein	−3.38	2.03E-02
LIC12693		Hypothetical protein	−3.36	2.65E-03
LIC13012		Hypothetical protein	−3.34	8.21E-03
LIC12678		Hypothetical protein	−3.30	4.75E-02
LIC12607		Hypothetical protein	−3.21	3.49E-02
LIC12605		Hypothetical protein	−3.14	8.38E-03
LIC10181		Hypothetical protein	−3.13	4.52E-02
LIC12676		Hypothetical protein	−2.87	1.49E-02
LIC13086		Hypothetical protein[2]	−2.77	2.11E-03
LIC10663		Hypothetical protein	−2.65	1.69E-02
Putative or Probable Lipoproteins (unknown function)				
LIC20153		Lipoprotein[2]	−2.90	2.27E-02
Regulatory Functions				
LIC13087		Histidine kinase sensor protein	−4.55	2.15E-02
LIC12431		TetR family transcriptional regulator	−2.87	1.49E-02
LIC20012		Histidine kinase sensor protein	−2.52	4.03E-02
Cellular Processes/Transport				
LIC12428	*phnL*	ABC transporter ATP-binding protein	−7.96	2.03E-02
Intermediary Metabolism and Biosynthesis of Small Molecules				
LIC20008	*hemA*	Glutamyl-tRNA reductase	−6.31	2.78E-09
LIC11758		Acyl-CoA hydrolase	−5.56	2.23E-02
LIC12772	*proB*	Gamma-glutamyl kinase	−3.97	2.53E-02
LIC20009	*hemC*	Porphobilinogen deaminase	−3.74	1.82E-04

Table 4. Cont.

Gene ID[1]		Product[1]	Fold (DMC vs IV)	P value (adjusted)
LIC10409	*leuA*	2-isopropylmalate synthase	−3.34	1.93E-03
LIC20014	*hemE*	Uroporphyrinogen decarboxylase	−3.33	3.41E-03
LIC10565	*hbd1*	Enoyl-CoA hydratase	−3.28	2.26E-02
LIC20238	*speH*	S-adenosylmethionine decarboxylase like protein	−3.28	1.19E-03
LIC10403	*ribH*	6,7-dimethyl-8-ribityllumazine synthase	−2.86	3.06E-02
LIC20011	*hemL*	Glutamate-1-semialdehyde aminotransferase	−2.44	1.49E-02
LIC20239	*speD*	S-adenosylmethionine decarboxylase proenzyme	−2.31	1.99E-02
LIC20010	*hemB*	Delta-aminolevulinic acid dehydratase	−2.29	3.57E-02
Macromolecular Metabolism and Cell Structure				
LIC12017	*clpB*	ATP-dependent protease	−4.71	1.11E-02
LIC12360	*pirin*	Pirin	−2.83	6.60E-03
LIC12357	*fusA*	Elongation factor EF-G	−2.71	3.53E-03
LIC11911		Glycosyltransferase	−2.65	4.04E-02

[1]Gene designations and protein product descriptions are based on those of [11,12] and the *L. interrogans* sv. Copenhageni Genome Project database (http://aeg.lbi.ic.unicamp.br/world/lic/) except where indicated.
[2]Annotation based on Setubal *et al.* [47].
doi:10.1371/journal.ppat.1004004.t004

for the first time the transcriptome of *L. interrogans* within the mammalian host.

In order to transition from a free living to infectious state, leptospires must adjust their metabolism to utilize nutrients available within the mammalian host. Quite surprisingly, we found that the majority of genes implicated in central and intermediary metabolism were expressed by leptospires at similar levels in DMCs and *in vitro*. We interpret these data to suggest that EMJH, the medium commonly used to cultivate pathogenic and saprophytic leptospires *in vitro*, reflects the overall composition of nutrients available within mammalian host fairly well. Nonetheless, leptospires cultivated within DMCs differentially-regulated a handful of genes whose products are involved in metabolic and biosynthetic pathways, most notably, heme uptake and utilization (see below). Although increased temperature often is implicated as an important stimulus for host adaptation, we observed very little overlap (<10%) between the cohort of genes that were upregulated in DMCs and those previously identified as being temperature-regulated *in vitro* [18,19,21,68]. Thus, differential gene regulation by leptospires within DMCs appears to be driven primarily by non-thermal mammalian host-specific stimuli. The relatively small pore size of the dialysis tubing used to cultivate leptospires within rat peritoneal cavities would exclude macromolecules and most serum proteins but allow for efficient exchange of nutrients (*i.e.*, glucose, ions, and free amino acids) present within serum. These are the same types of small molecules that leptospires likely encounter within proximal convoluted tubules, where the composition of the glomerular ultrafiltrate most closely resembles that of interstitial fluid [84]. Further experimentation is required to assess how closely DMC-cultivated

Table 5. Candidate small non-coding RNAs identified by RNA-Seq.

Transcript[1]	Homology[2]	E-value	Chr	Genome Coordinates		sRNA size	Validated[3]
LIC1nc10	tmRNA	1.50E-62	1	175,606	175,960	355	+
LIC1nc20	PyrR binding site	1.50E-04	1	263,598	264,013	416	+
LIC1nc30	–		1	849,634	849,900	267	+
LIC1nc50	–		1	2,109,156	2,109,444	289	+
LIC1nc55	Cobalamin	3E-25	1	2,878,556	2,878,746	191	–
LIC1nc60	RNaseP	5.50E-31	1	3,031,445	3,031,846	402	+
LIC1nc80	–		1	4,015,037	4,015,237	201	–
LIC2nc10	Cobalamin	6.80E-19	2	159,019	159,243	225	+
LIC2nc20	–		2	242,735	243,092	358	+
LIC2nc30	–		2	246,062	246,477	416	+
LIC2nc40	–		2	348,946	349,168	223	–

[1]Predicted sRNAs are annotated according to the genome of *L. interrogans* sv. Copenhageni (LIC) chromosome number followed by non-coding RNA designation as included in Supplementary Table S2.
[2]Homology to known sRNA families is indicated as is the E-value when transcripts were searched against the Rfam database.
[3]Expression was validated by reverse-transcriptase PCR in *L. interrogans* sv. Copenhageni strain RJ16441.
doi:10.1371/journal.ppat.1004004.t005

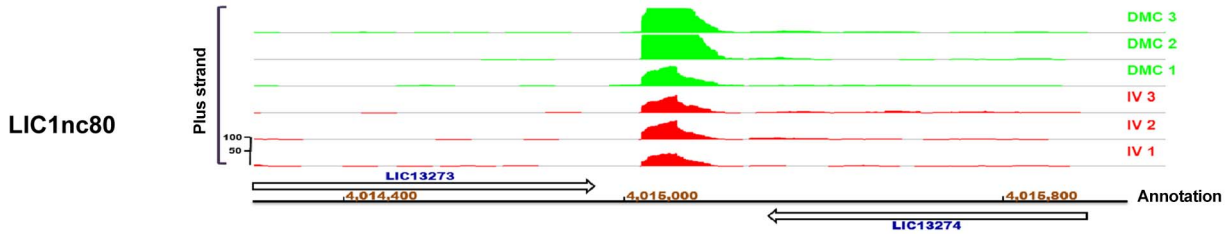
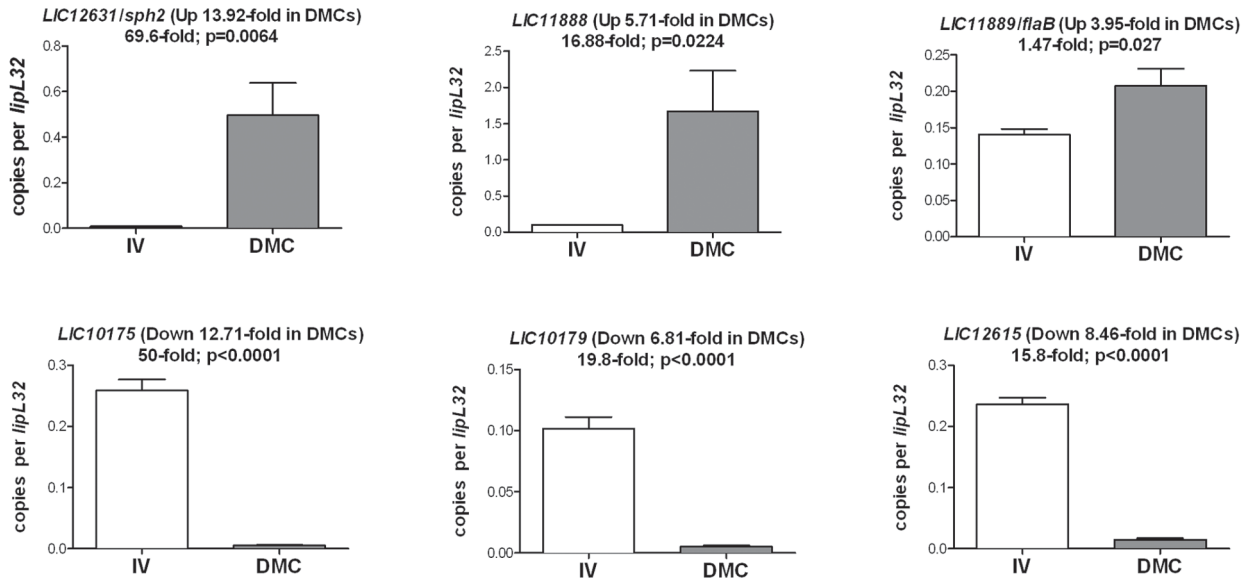


Figure 6. IGB viewer of candidate sRNA *LIC1nc80*. *LICnc80* was identified as an area of high transcriptional activity within an intergenic region of the genome of *L. interrogans* sv. Copenhageni Fiocruz L1-130. Expression data for leptospires cultivated in DMCs (green) compared to those cultivated *in vitro* (IV, red) are indicated on the plus strand of the genome. Annotated genes on the relevant chromosome and nucleotide co-ordinates are indicated. The vertical "read count" scale is 0–100.
doi:10.1371/journal.ppat.1004004.g006

A



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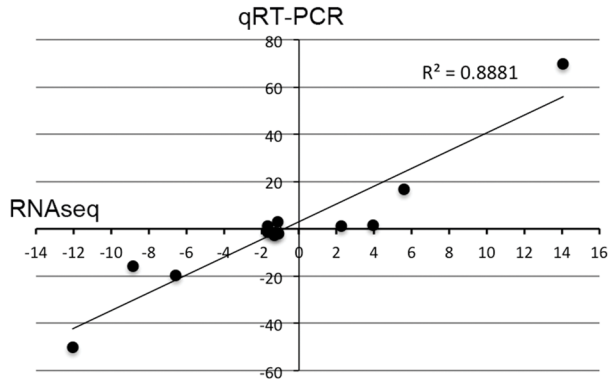

Figure 7. Validation of comparative RNA-Seq analysis. (A) qRT-pCR analysis of representative genes identified by RNA-Seq. Values represent the average transcript copy numbers for each gene normalized per *lipL32* transcript. Bars indicate the standard error of the mean (SEM). Results presented are mean values from at least 3 biologically-independent samples of leptospires for each growth condition. The fold-regulation for each gene determined by RNA-Seq is indicated in parentheses. The fold-regulation between *in vitro*- (IV) and DMC-cultivated leptospires determined by qRT-PCR are indicated. P values were calculated using an unpaired *t*-test. (B) Correlation coefficient (R^2) between RNA-Seq and qRT-PCR data.
doi:10.1371/journal.ppat.1004004.g007

leptospires resemble their counterparts within host tissues during acute and/or chronic infection. The DMC model does have some limitations. For instance, virulence genes associated with pulmonary haemorrhage may be expressed only within the context of lung tissue. Because bacteria within DMCs are prevented from interacting with host immune cells and immunoglobulin [85], this model does not enable us to identify genes that are differentially-regulated in response to specific pathogen-host interactions and/or immune evasion.

Although increased temperature often is implicated as an important stimulus for host adaptation, we observed very little overlap (<10%) between the cohort of genes that were upregulated in DMCs and those previously identified as being temperature-regulated *in vitro* [18,19,21,68]. We observed a similarly limited overlap between our RNA-Seq data and genes found to be differentially regulated *in vitro* in response to exposure to serum [21] and low iron [22]. We observed a somewhat higher, but nonetheless small, degree of overlap (16%) between our RNA-seq dataset and genes identified by Matsunaga *et al.* [20] as being upregulated by physiologic osmolarity (EMJH supplemented with 120 mM NaCl); included in this overlap are *lipL53* (*LIC12099*), *sph2* (*LIC12631*), a putative CoA-transferase (*LIC12322*), *phoD* (*LIC13397*) and *hol* (LIC20148; see below). Thus, differential gene regulation by leptospires within DMCs appears to be driven by mammalian host-specific stimuli that are not readily reproduced *in vitro*.

The relatively small pore size of the dialysis tubing used to cultivate leptospires within rat peritoneal cavities would exclude macromolecules and most serum proteins but allows for efficient exchange of nutrients (*i.e.*, glucose, ions, and free amino acids) present within serum. These are the same types of small molecules that leptospires likely encounter within proximal convoluted tubules, where the composition of the glomerular ultrafiltrate most closely resembles that of interstitial fluid [84]. Further experimentation is required to assess how closely DMC-cultivated leptospires resemble their counterparts within host tissues during acute and/or chronic infection. The DMC model does have some limitations. For instance, virulence genes associated with pulmonary haemorrhage may be expressed only within the context of lung tissue. Because bacteria within DMCs are prevented from interacting with host immune cells and immunoglobulin [85], this model does not enable us to identify genes that are differentially-regulated in response to specific pathogen-host interactions and/or immune evasion.

To date, >20 named species of *Leptospira* have been identified based on molecular taxonomic analyses [86]. *Leptospira* spp. can be further divided into three major groups based on pathogenicity: pathogenic (9 species), intermediate virulence (5 species) and free-living saprophytes (6 species). The vast majority (69%) of genes upregulated by leptospires in response to mammalian host signals are found only in pathogenic and intermediate virulence species (*i.e.*, absent in *L. biflexa*), suggesting that their gene products may help promote infection and/or colonization within mammal. However, more than half (64/110) of these upregulated genes encode either hypothetical proteins or lipoproteins of unknown function without any obvious conserved/functional domains. While their functions remain to be determined, our finding that these protein-coding genes are differentially-regulated in response to mammalian host-specific signals make them attractive candidates for further experimentation in animals model and, in particular, their potential use as part of a mono- or multi-valent protein-based vaccine. Thirty-five of the 56 genes downregulated in DMCs encode hypothetical proteins. Interestingly, all but 7 of these are unique to pathogenic and intermediate virulence species, raising the possibility that these genes products, while not required for survival within the host, facilitate the transition from a free-living to infective state.

Heme is the major source of iron in *L. interrogans* and also serves as a cofactor for proteins essential for respiration (*i.e.*, cytochromes), biosynthesis of vitamin B_{12}, and detoxification of reactive oxygen intermediates (*i.e.*, catalase). Unlike *B. burgdorferi* [87] and *T. pallidum* [88], *L. interrogans* possess a complete set of genes required for *de novo* heme biosynthesis as well as the uptake and utilization of exogenous heme [58,74,89]. By RNA-Seq, expression of 6 heme biosynthesis genes was significantly downregulated in DMCs compared to *in vitro*, while heme oxygenase (*LIC20148/hol*) and *phuR*, encoding a TonB-dependent heme receptor, were upregulated; these data support the notion that pathogenic leptospires preferentially use exogenously derived heme within the mammal. Of the four putative *fur* orthologs encodes by *L. interrogans*, only one (*LIC12034*) was upregulated in DMCs. Recently, Marcsisin *et al.* [46] demonstrated that inactivation of this gene had no effect on virulence in a hamster acute infection, implying that this Fur paralog is not responsible for downregulation of the heme operon within DMCs. Alternatively, downregulation of heme biosynthesis is not a prerequisite for survival *in vivo*. Because heme is highly toxic [90], there is relatively little, if any, free heme within plasma [91]. In the glomerulus, the molecular weight cut-off for ultrafiltration is ~70 kDa [92]. Thus, while *L. interrogans* is able to use haemoglobin (64 kDa) as a source of heme *in vitro* [60], this micronutrient is likely present in only minute amounts within the proximal tubules. Smaller molecules (≤20 kDa), only the other hand, easily pass through the glomerulus into Bowmen's capsule; it is worth noting that this molecular weight cut-off is essentially equivalent to that of the dialysis tubing used for our DMCs (8 kDa MWCO). Instead, leptospires may be using myoglobin (16.7 kDa), which is present in human plasma at concentrations similar to that of haemoglobin [93]. Both hemoglobin and myoglobin, released by red blood cell turnover and muscle tissue damage, respectively, are filtered by the kidneys and would be available to leptospires within the renal tubules.

Small non-coding RNAs (sRNAs) are increasingly recognized as essential post-transcriptional gene expression regulators that enable bacteria to adjust their physiology in response to environmental cues [94]. Bacterial sRNAs range from 50 to 500 nucleotides and frequently are located within intergenic regions [95]. By diverse mechanisms, including changes in RNA conformation, interactions with DNA, other RNAs and proteins, sRNAs can modulate transcription, translation, mRNA stability and DNA maintenance or silencing [96,97]. Five of the 11 candidate sRNAs identified as part of this study are conserved in bacteria and known to carry out specific housekeeping functions, including RNase P (*LIC1nc60*), responsible for processing of tRNAs and other RNAs, and tmRNA (*LIC1nc10*), which acts as both a transfer RNA (tRNA) and mRNA to tag incompletely-translated proteins for degradation and to release stalled proteins [76,77]. We also identified two cobalamin riboswitches (*LIC1nc55* and *LIC2nc10*), which act as *cis*-regulatory elements in 5′ untranslated regions of vitamin B_{12}-related genes; allosteric rearrangement of mRNA structure is mediated by ligand binding resulting in modulation of gene expression or translation of mRNA [78]. *LIC1nc55* lies upstream of *LIC121374/btuB*, which encodes a constitutively-expressed TonB-dependent outer membrane cobalamin receptor protein [98]. We also identified a candidate sRNA (*LIC2nc10*) upstream of *LIC20135*; although annotated as a ferredoxin, LIC20135 contains a domain conserved within sirohydrochlorinin cobalt chelatases, an important enzyme

involved in biosynthesis of vitamin B_{12}. Finally, *LIC1nc20* contains a conserved PyrR binding site; this RNA element is found upstream of genes involved in pyrimidine biosynthesis and transport in *Bacillus subtilis* [79]. In *L. interrogans*, this sRNA was found downstream of genes encoding hypothetical proteins. In addition to these known sRNAs, we identified six transcriptionally-active, non-coding regions that encode novel candidate regulatory sRNAs. *LIC1nc30*, *LIC1nc50*, *LIC2nc30* and *LIC2nc40* were all identified in the 5′ untranslated regions for *LIC14007*, *LIC10702*, *LIC20192* and *LIC20276*, respectively, all of which encode proteins of unknown function. The remaining two putative sRNAs (*LIC1nc80* and *LIC2nc20*) are located in the 3′ untranslated region of genes, which are known to be a repository of sRNAs in other bacterial species [99].

The *L. interrogans* genome encodes >200 proteins whose annotations suggest a role in transcriptional regulation (*i.e.*, sigma factors, anti-sigma factors and trans-acting factors), two-component signal transduction and the synthesis/degradation of cyclic nucleotides [11,12]. By RNA-Seq, the vast majority of these putative regulatory proteins were expressed at similar levels *in vitro* and in DMCs; this finding is not unexpected given that these types of regulatory factors typically are activated at the protein level by endogenously- or exogenously-derived small molecules and environmental stimuli [100,101,102].

Recent advances in *Leptospira* molecular genetics, including the development of site-directed [103] and transposon-mediated [104,105,106] mutagenesis techniques, now make it possible to determine the contribution(s) of genes that are regulated within DMCs. We anticipate that this approach will identify proteins involved in environmental sensing, mammalian host adaptation and/or the expression of specific virulence determinants *in vivo*.

Materials and Methods

Ethics statement

All animal experimentation was conducted following the Guide for the Care and Use of Laboratory Animals (Eighth Edition) and in accordance with protocol (ACC# 100570-0116) reviewed and approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee. The UCHC laboratory animal care program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The USDA Site ID: Customer Number 44, Certificate Number 16-R-0025, PHS Assurance Number A3471-01.

Bacteria

Virulent low-passage *Leptospira interrogans* sv. Copenhageni strains Fiocruz L1-130, kindly provided by Dr. David Haake (UCLA), and RJ16441 were cultivated *in vitro* under standard conditions at 30°C in EMJH medium [107] supplemented with 1% rabbit serum (Pel-Freez Biologicals, Rogers, AR) with 100 μg/ml 5-fluorouracil. Cultures were passaged *in vitro* no more than 3 times before being used for experimentation.

Cultivation of virulent *L. interrogans* within dialysis membrane chambers

To obtain *L. interrogans* in a mammalian host-adapted state, organisms were cultivated in dialysis membrane chambers (DMCs) as previously described [23,24]. Briefly, DMCs were constructed using standard dialysis membrane tubing (Spectra-Por; 8000 MWCO). Prior to use, 8-inch strips of dialysis tubing were tied off at one end and then sterilized by boiling for 20 min in sterile water containing 5 mM EDTA, followed by two successive boiling washes in water alone. Dialysis bags were cooled to room temperature and then filled with ~8–9 mls of EMJH medium (supplemented with 10% vaccine-grade bovine serum albumin to maintain osmotic pressure) containing 10^4 organisms per ml. Once filled, the tubing was tied and excess membrane removed from both ends. For implantation, female Sprague-Dawley rats (150–174 g) were anesthetized by intramuscular injection of a mixture of ketamine (50 mg/kg), xylazine (5 mg/kg), and acepromazine (1 mg/kg). Using strict aseptic technique, a DMC was implanted into the peritoneal cavity of each rat. Analgesia (carprofen; 5 mg/kg) was administered on the day of surgery and once the following day. At designated time points (typically 9–10 days post-implantation), rats were euthanized by CO_2 narcosis and DMCs recovered. The contents of each chamber were removed by gentle syringe aspiration with an 18G needle; the needle was removed prior to expelling the DMC dialysate into a sterile 15 ml conical bottom tube. Bacteria were enumerated by dark field microscopy immediately following explant using a Petroff-Hausser counting chamber (Hausser Scientific Co., Horsham, PA).

Gel electrophoresis and immunoblotting

In vitro-cultivated *L. interrogans*, harvested at late-log phase (5×10^8–1×10^9 per ml) and leptospires explanted from DMCs were processed for one- and two-dimensional SDS-polyacrylamide gel electrophoresis (1D and 2D SDS-PAGE, respectively) as previously described [8]. Protein concentrations were determined using the DC protein assay kit (Bio-Rad). Total protein separated by 1D SDS-PAGE was detected by SYPRO Ruby protein gel stain (Sigma-Aldrich Inc, Ireland) as per manufacturer's instructions. Images were visualized with the BioSpectrum AC Imaging System (Ultra-Violet Products Ltd, UK). For immunoblotting, proteins were transferred to nylon-supported nitrocellulose, incubated with rabbit polyclonal antiserum directed against Sph2 [34], LipL32 [38] and LipL41 [39] followed by goat anti-rabbit secondary antibody (Southern Biotechnology Associates, Birmingham, Ala.). Blots were developed using the SuperSignal West Pico chemiluminescence substrate according to the manufacturer's instructions (Pierce, Rockford, Ill.). 2D gels were loaded with 500 μg total protein and stained with silver as previously described [8].

RNA isolation, library preparation and RNA-Seq

Total RNA was extracted using TRIzol reagent (Invitrogen) from three biologically-independent samples of (i) *in vitro*-cultivated leptospires or (ii) leptospires cultivated in DMCs (2 rats per sample) for 10 days as described above. Purified RNA was treated with Turbo DNA*free* (Ambion, Inc. Austin, TX) as previously described [108] to remove contaminating genomic DNA. The integrity of DNase-treated RNAs use for RNA-Seq were assessed using the Agilent Bioanalyzer RNA NanoChip (Agilent Technologies, Wilmington, DE) to ensure that each had an RNA integrity (RIN) value ≥8. One-hundred ng of total RNA was used for library generation according to Illumina standard protocols (TruSeq RNA Sample Preparation Guide, Low-Throughput Protocol, Part # 15008136 Rev. A). cDNAs were normalized using a duplex-specific nuclease (DSN) approach according to the DSN Normalization Sample Preparation Guide, Early Access Protocol, Part # 15014673 Rev. C, which decreases the prevalence of highly abundant transcripts, such as rRNAs. 76-bp paired-end sequencing was carried out by Sequensys (Prognosys Biosciences, La Jolla, USA) on an Illumina Genome Analyzer IIx according to the manufacturer's instructions.

RNA-Seq data analysis

Mapping of sequenced reads to Chromosome 1 and 2 of the reference genome of *Leptospira interrogans* sv. Copenhageni strain

Fiocuz L1-130 (NCBI Reference Sequence: NC_005823.1 and NC_005824.1 respectively) [11] was carried out using the software tool segemehl [109] with accuracy set to 100%. To increase coverage, mismatched nucleotides at the lower-quality 3′ end were removed from the reads and the mapping was repeated until a match was found or the read length decreased below 20 nucleotides (see [110]). Reads that mapped to (i) ribosomal or transfer RNAs or (ii) more than one reference genome location (*e.g.*, paralogous genes) were discarded. Uniquely mapped reads (*i.e.*, mapped to a single genomic location) were selected for further analysis, such as data visualisation and determination of differential gene expression. Normalization, differentially-expressed genes, regulatory fold-changes and statistical significance were determined using DESeq [43]. Read coverage used for graphical display was normalized as follows to compensate for different library sizes: the number of reads covering each nucleotide position was divided by the total number of mapped reads in the library and then multiplied with the number of mapped reads from the smallest library. Mapped unique reads were visualised with the Integrated Genome Browser (IGB, version 5.5) (http://bioviz.org/igb/releases.html) [111].

Bioinformatics

Putative orthologous relations between proteins in other *Leptospira* serovars and/or species were determined using BlastP alignment (≥40% amino acid identify over ≥80% of the length of the smallest protein) as previously described [14]. Protein sequence similarity between differentially-expressed genes identified in *L. interrogans* sv. Copenhageni and other *Leptospira* spp. (*L. interrogans* sv. Lai strain 56601 [80]; *L. borgpetersenii* sv. Hardjo strain L550 [13]; *L. santarosai* sv. Shermani strain LT821 [15]; *L. licerasiae* sv. Varillal strain VAR010 [16]; and *L. biflexa* sv. Patoc strain Patoc1 Ames [14]) was determined using GLSEARCH (version 35.04) from the FASTA package [112]. GLSEARCH identifies the optimal alignment across the entire genome of each strain, translated into all six reading frames, and calculates the percent identity across the whole length of the corresponding sequence. Conserved domain searches were performed on full length protein coding sequences using the NCBI Conserved Domain Database interface [44,45]. The presence of fur boxes was investigated using the predictive computational tool SLiMSearch [62]. SLiMSearch, which can be used to determine the occurrences of a predefined motif in DNA and protein sequences, makes use of disorder and conservation masking to reduce the number of false positives. The fur box consensus sequence ([GC]AT[AT]AT[GC]AT[AT]AT[GC]AT[AT]AT[GC]) used to search the genome of *Leptospira interrogans* sv. Copenhageni was based on that of [61]. Putative functions of candidate sRNAs were identified by BLAST using the Rfam database, Wellcome Trust Sanger Institute (http://rfam.sanger.ac.uk/).

Quantitative RT-PCR

DNase-treated RNAs (~1 µg per sample), isolated from leptospires grown to late-logarithmic phase at 30°C *in vitro* and within DMC, were prepared as described above and converted to cDNA using SuperScript III (Invitrogen) in the presence and absence of reverse transcriptase (RT) according to the manufacturer's instructions. cDNAs were assayed in quadruplicate using iQ Supermix (Bio-Rad) using the primer pairs described in Table S1. For relative quantitation of transcript levels, amplicons corresponding to each gene of interest were cloned into the pCR2.1-TOPO cloning vector (Invitrogen), then purified recombinant plasmid DNAs for each amplicon were diluted (10^7–10^2 copies/µl) to generate a standard curve. Reaction conditions for each primer pair were optimized to ensure that each had an amplification efficiency of >90%. Transcript copy numbers for each gene of interest were calculated using the iCycler post-run analysis software based on internal standard curves then normalized against copies of *lipL32* (*LIC11352*) present in the same cDNA. Normalized copy number values were compared within Prism v5.00 (GraphPad Software, San Diego, CA) using an unpaired *t*-test with two-tailed *p* values and a 95% confidence interval.

Supporting Information

Figure S1 Comparison of leptospires cultivated *in vitro* and within DMCs by two dimensional SDS-PAGE revealed numerous polypeptide differences. Protein lysates prepared from *L. interrogans* sv. Copenhageni strain Fiocruz F1-130 grown at 30°C in EMJH medium (top) or within dialysis membrane chambers (DMCs; bottom). Total protein (500 µg per gel) was solubilized in 7 M urea, 2 M Thiourea and 1% ASB-14 and separated by two-dimensional gel electrophoresis as previously described [8]. Proteins were visualized with Lavapurple.
(TIFF)

Figure S2 Clustering of biological replicates. Heatmap representing the expression data for genes whose expression was either positively- or negatively-regulated by ≥Log_2-fold (adjusted p-value≤0.05) in DMC- versus *in vitro*-cultivated *L. interrogans* sv. Copenhageni strain Fiocruz L1-130.
(TIF)

Figure S3 IGB viewer of putative sRNAs (*LIC1nc10* - *LIC2nc40*) mapping to non-annotated regions of the genome. Candidate sRNAs were identified as areas of high transcriptional activity in intergenic regions of the genome of *L. interrogans* sv. Copenhageni Fiocruz L1-130. Expression data for leptospires cultivated in DMCs (green) compared to those cultivated *in vitro* (IV, red) are indicated on plus strand of the genome. Annotated genes on the relevant chromosome and nucleotide coordinates are indicated. The vertical "read count" scale is 0–100.
(TIFF)

Figure S4 Validation of RNA-Seq analysis. qRT-PCR analysis of the entire panel of genes used to validate RNA-Seq data derived from *L. interrogans* sv. Copenhageni cultivated in EMJH at 30°C *in vitro* (IV) and within DMCs. Values represent the average transcript copy number for each gene normalized per copy of *lipL32*. Bars indicate the standard error of the mean (SEM). Results presented are mean values from at least 3 biologically-independent samples of leptospires for each growth condition. The fold-regulation for each gene determined by RNA-Seq is indicated in parentheses. The folds of regulation between *in vitro*- and DMC-cultivated leptospires determined by qRT-PCR are indicated. P values were calculated using an unpaired *t*-test.
(TIF)

Table S1 Oligonucleotide primers used in these studies.
(DOCX)

Table S2 Gene expression data for Chromosome 1 and 2. Column A: Gene identification, Column B: Mean number of DESeq values for each gene in all 6 biological replicates, Column C: Mean DESeq values for each gene in 3 biological replicates of leptospires cultured *in vitro* (IV), Column D: Mean DESeq values for each gene in 3 biological replicates of leptospires cultured in dialysis membrane chambers (DMC), Column E: Fold change gene expression by DMC leptospires compared to IV leptospires,

Column F: Log_2 fold change gene expression by DMC leptospires compared to IV leptospires, Column G: p-value of differential gene expression, Column H: adjusted p-value of differential gene expression, Column I: Residual variance of DESeq values for each gene in three biological replicates of IV, Column J: Residual variance of DESeq values for each gene in three biological replicates of DMC, Column L-Q: DESeq values for each gene in each biological replicate, Column S: DNA strand location for each gene, Column T: location of gene on positive or negative strand, Column U: Start position for each gene, Column V: End position for each gene, Column W: gene length, Column X: gene name, Column Y: gene product. Datasheets are arranged to present (1) data for all genes sorted according to the expression values in DMCs; (2) data for all genes sorted according to the expression values *in vitro*; (3) data for genes whose expression was upregulated within DMCs compared to *in vitro*; and (4) data for genes whose expression was downregulated within DMCs compared to *in vitro*.
(XLSX)

Table S3 Top 100 protein–coding genes expressed by *L. interrogans* within DMCs.
(DOCX)

Table S4 Expression data for individual genes and pathways highlighted in the manuscript.
(DOCX)

Table S5 Redox-relevant proteins encoded within the genomes of the pathogenic spirochetes *Treponema pallidum*, *Borrelia burgdorferi* and *L. interrogans* sv. Copenhageni.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MJC JEN. Performed the experiments: MJC SKS AA DH KH AAG JEN. Analyzed the data: MJC SKS DH KH AAG JCDH JEN. Contributed reagents/materials/analysis tools: MJC SKS KH JCDH JEN. Wrote the paper: MJC SKS KH JCDH JEN.

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4.4 Artigo 4

Hamster Infection with *Leptospira interrogans* by the Transcutaneous Route: An Attempt to Mimic Natural Route of Infection

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Hamster Infection with *Leptospira interrogans* by the Transcutaneous Route: An Attempt to Mimic Natural Route of Infection

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Abstract

Pathogenic *Leptospira* spp., the causative agents of the neglected tropical disease leptospirosis, are spirochaetes maintained by reservoir hosts, with few or no clinical manifestations, and that shed large numbers of leptospires in their urine. They can survive in environments outside the host, until they infect another host via abraded/damaged skin or mucosa. In animal models, the most commonly used route of infection is intraperitoneal (IP) injection. However, this do not mimic natural infection and the median lethal dose (LD_{50}) used depends on the virulence of the serovar, often resulting in infective doses of $>10^8$ leptospires. In this work, we describe a new protocol for the infection in the hamster model of lethal leptospirosis. Infection by *L. interrogans* usually results from contact with a contaminated source (water, mud, blood etc.) to the host, termed the transcutaneous (TC) route. To mimic this in the experimental model, the skin of the rear leg was slightly abraded prior to exposure to *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni. After infection, hamsters developed severe leptospirosis with the endpoint criteria reached 9-10 days post infection (d.p.i.). Leptospires were detected by culture of kidney, liver, lung and blood samples 3 d.p.i. the LD_{50} for the TC route of infection was calculated to be 1.7×10^7 leptospires/mL for females and $3.9 \times 10^7 \pm 0.74$ leptospires/mL for males. The TC route of infection proved to be a straightforward, reliable and reproducible approach for the induction of lethal leptospirosis and one that potentially simulated a natural route of infection. This protocol could potentially replace IP injection in experimental leptospirosis studies.

Keywords: *Leptospira interrogans*; golden Syrian hamster; route of infection; intraperitoneal; transcutaneous route; vaccines.

Introduction

Leptospirosis is a widespread, neglected tropical zoonotic disease, estimated to be responsible for 500,000 severe cases in 2000 rising to over 873,000 severe cases and 49,000 deaths in 2013, according to the World Health Organization [1,2]. *Leptospira* spp. are a unique and genetically and antigenically diverse group of spirochetes classified into 9 pathogenic *Leptospira* species, that include 24 serogroups and more than 250 serovars [3,4]. Leptospires can infect virtually any mammal species [5]. The so-called maintenance hosts, especially rodents, are not susceptible, can carry spirochetes in the renal tubules and shed number of this organism in the urine, the main source of leptospires for new infections. This spirochete can survive in wet soil and water pounds from where new hosts become infected. In susceptible hosts, or accidental hosts including humans, pathogenic leptospires spread throughout the body, causing a febrile icteric illness that, if untreated, can result in renal, hepatic and cardiac failures and eventually death [6].

The first isolation of pathogenic *Leptospira* spp. was over 100 years ago [7], however, the basic pathogenic mechanisms for host entry remain poorly understood. It seems likely that leptospires infect through the mucosa or abraded/damaged skin [8]. However, there are reports of experimental infection of hamsters with undamaged skin [9-11]. Historically, intraperitoneal (IP) injection of virulent leptospires is the gold standard route for challenge and infection studies [12-14]. This infection route is reproducible and easily performed. However, it is not a natural route of infection and circumvents the mucosal and cutaneous defence mechanisms of the host [15]. A small number of studies infected susceptible animal models using approaches closer to what happens during natural infection [9,16-19]. Conjunctival infection route (CJ) appeared promising, it is based solely in the leptospires ability to survive in the eye until they can naturally penetrate the conjunctival mucosa and get into the vascular system [16,17,20]. However, it required a large number of leptospires in a small volume (microliters), necessitating increased manipulation resulting in significant loss of leptospires and potentially virulence [21]. Other approaches, such as intradermal or subcutaneous infection [16,17,19] are similar to IP injection, in that the skin barrier was bypassed.

Vaccination of humans and the reservoirs that transmit leptospirosis represents the most effective prophylactic measure available for the prevention of leptospirosis. Approved human vaccines for leptospirosis are available in only a handful of countries and have several major limitations, reviewed in [6,22]. Over the last 15 years, several research groups have evaluated potentially new vaccine candidates, the majority were subunit based and used the IP challenge route, with mixed success [22-25]. Recent advances have provided new tools for the discovery of novel virulence factors and potential vaccine candidates [26-28]. Some of these candidates could be involved in the primary invasion of the host through the skin and the use of an IP infection route in the experimental model used to evaluate their efficacy could underestimate their usefulness. Therefore, the aim of this study was to develop a viable alternative to IP infection and one that simulated a natural route of infection.

Materials and Methods

Leptospira culture and bacterin preparation

L. interrogans serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) (DIFCO BD) liquid medium (Difco, BD Diagnostics) supplemented with 10% *Leptospira* Enrichment EMJH (Difco, BD Diagnostics) and the cultures were incubated at 28-30 °C. Weekly passages were made, with counting of spirochete density every other day using a Petroff-Hausser counting chamber (Hausser Scientific). All animal experiments were performed with a culture with less than six passages *in vitro* and before the culture entered the stationary phase of growth. For bacterin preparation, *L. interrogans* cells were harvested by centrifugation (8000 × g; 15 min; 4 °C), washed three times in PBS and counted. Each dose of 10^8 leptospires in PBS was heat inactivated at 56 °C for 30 min as described previously [25].

Animals and ethics statement

Adult golden Syrian hamsters (*Mesocricetus auratus*) aging from 4 to 10 weeks old, depending on the experiment were maintained in cages with a maximum 5 animals each, with food and water *ad libitum* and acclimatized environmental at the UFPel Animal Facility. Animals were weighed daily following infection. Infected hamsters that showed signs of leptospirosis (loss of appetite, gait difficulty, dyspnoea, prostration, ruffled fur and jaundice) combined with loss of 10% or more of corporal weight were euthanized. The combination of these criteria provided a reliable endpoint from which animals were unlikely to recover from the clinical disease [29]. Surviving animals were euthanized 28 days after infection. After euthanasia, kidney, liver, lung and blood samples were collected for maceration into EMJH for detection of leptospires in the different organs.

All animal procedures were carried out in accordance with the National Council of Animal Experimentation Control (CONCEA). This work was reviewed and approved

by the Federal University of Pelotas Committee for Ethics in Animal Experimentation (Process number 3782).

Transcutaneous (TC) infection route

Phosphate buffered saline (PBS) containing either 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 or 10^2 leptospires per mL (final volume 10 ml) was prepared and transferred to a 1 L glass beaker immediately before animal exposure. One hamster was placed inside the beaker to simulate the conditions of natural infection, which was covered with a plastic lid containing 2 mm diameter breathing holes (Fig. 1A and 1B). The exposure time was 5 min after which the animal returned to its cage. The beaker is cleaned 3× with 70% ethanol and allowed to dried before the next hamster was infected. Once the routine was established, we could infect up to four animals at a time.

Before infection, specific groups of animals were pre-treated to weaken the skin barrier. The lesion (LE) group: the right footpad of each hamster was perforated with a 0.45×13 mm needle to mimic a lesion. A group of hamsters (WW) were exposed warm water (~30 °C) in an attempt to soften the skin and improve penetration by leptospires during exposure. In the LS group, the inner surface of the lower right leg of each hamster was slightly abraded using a scalpel blade scraped lightly (12-15 ×) across the skin, with no bleeding. A no lesion (NL) group was also included. The control groups were infected with an IP injection of 10^3 leptospires.

Pilot experiments

A pilot experiment was performed to determine whether the various TC protocols could establish lethal leptospirosis. Thirty-six, nine-week-old, male hamsters were separated into nine groups of four animals each, and each animal was pre-treated: LE (n = 16) or NL (n = 16) prior to infection with 10^8, 10^6, 10^4 or 10^2 leptospires/ml. A control group (n = 4) were infected by the IP route to verify virulence.

In a second pilot experiment, the WW and LS pre-treatments were included as well. Eighteen hamsters were distributed in four groups (LE, NL, WW and LS) of four

animals each. Only the 10^8 leptospires/ml infective dose was evaluated. A control group (n = 2) was infected IP with 10^3 leptospires.

A third pilot experiment was conducted to test the NL and LS pre-treatment protocols using a higher concentration of leptospires. Female hamsters were exposed to 10^9 leptospires/ml as described above (n = 5 for each, NL and LS).

LD_{50} for IP and TC infection routes

Fifteen, nine-week-old, female hamsters were divided into five groups of 3 animals each that were infected IP with 10^5, 10^4, 10^3, 10^2 or 10^1 leptospires . The LD_{50} for the TC infection route was determined for both male (in two experiments) and female hamsters. For each LD_{50} experiment, hamsters were separated into five groups of four animals/group. Following LS pre-treatment, each group was exposed to 10^9, 10^8, 10^7, 10^6 or 10^5 leptospires/ml. The LD_{50} was calculated as described previously [30].

Qualitative evaluation of the dynamics of infection

Forty, nine-week-old, female hamsters were distributed into 10 groups of four animals/group. Twenty animals were infected by IP injection of 10^3 leptospires in 1 mL of PBS and the remainder were LS pre-treated and infected with 10^9 leptospires/ml. One group from each infection route (IP and LS) was euthanized at 1, 3, 5, 7 and 9 days post infection (d.p.i.). Blood was collected by cardiac puncture and ca. 100 µl was added to EMJH medium, while kidney, liver, lung samples were collected and macerated into the same medium. After 1 h of incubation at 30 °C, 500 µl was inoculated into 4.5 mL of fresh EMJH medium and incubated at 30 °C. Cultures were checked weekly for growth using dark field microscopy.

Test of TC in challenge of vaccine experiments

Ten, five-week-old, male hamsters were divided in two groups of five, followed of either an intramuscular injection of PBS or immunized with bacterin. After 14 days,

a second dose was administered and the animals were challenged 14 days after the booster by the LS TC route. This experiment was repeated using female hamsters.

Results

Infection through transcutaneous route is possible

Only groups of animals exposed to higher densities of spirochetes, *i.e.* 10^8 and 10^9 leptospires/mL of PBS (10^9 and 10^{10} spirochetes total) were infected and some of them were euthanized after reach endpoint criteria (Fig. 2). Fifty percent (2/4) of the LE group developed severe leptospirosis compared to 25% (1/4) of the NL group when exposed to 10^8 leptospires/ml (Fig 2A and 2B). The endpoint criteria were fulfilled and the animals were euthanized on 12 and 19 dpi for the LE group and 12 dpi in the NL group (Fig. 2C). Increasing the concentration to 10^9 leptospires/ml during exposure resulted in 100% infection in the LS group comparted to 80% in the NL group (Fig. 2D). As the SL pre-treatment was the only TC protocol that established lethal leptospirosis in 100% of exposed animals this was adopted as the TC protocol of choice for all future experiments. In addition, 100% of euthanized animals were positive for leptospires as determined by kidney culture. Those animals that survived to 28 dpi, gained weight and were negative for leptospires following kidney culture.

Fiocruz L1-130 strain is virulent for both IP and TC routes of infection

All (15/15) animals developed lethal leptospirosis following IP injection of leptospires, regardless the inoculum size and were euthanized 8 – 13 d.p.i. (Fig. 3A). The LD_{50} calculated for *L. interrogans* Fiocruz L1-130 strain for IP injection was 3.2 leptospires. In agreement with the pilot experiments, 100% (12/12) of hamsters infected via the SL route after exposure to 10^9 leptospires/ml developed lethal leptospirosis (Fig. 3B-3D). The LD_{50} for exposure via the SL route was calculated as 1.7×10^7 leptospires/ml and $3.9 \times 10^7 \pm 0.74$ leptospires/ml for female and male hamsters, respectively.

Host dissemination dynamics is similar in TC and IP routes

Culture of all kidney, liver, lung and blood samples were positive at 5, 7 and 9 d.p.i. for both IP and the SL TC routes of infection (Table 1). Of note, two animals exposed via the TC SL route were culture positive in at least two samples tested at 3 dpi. No leptospires were detected prior to 3 dpi.

TC infection and vaccine challenge experiments

One of the main objectives of this study was to develop a protocol for establishing lethal leptospirosis in hamster that mimicked a natural route of infection. The hypothesis being that this would allow for improved evaluation of novel vaccine candidates. This was evaluated in an experiment using a bacterin vaccine and compared the IP and TC-SL routes of infection. Hamsters immunized with two doses of a bacterin vaccine survived (100%) challenge. Furthermore, all of the surviving animals were culture negative, suggesting that the vaccine induced sterilizing immunity. The negative control group developed severe leptospirosis and the endpoint criteria were reached at 9-10 d.p.i. (Fig. 4A and 4B). All these animals had leptospires in the kidneys as shown by culture.

Discussion

Since the original experiments in the field of leptospirosis [12,13], the standard methodology for establishing experimental leptospirosis in animal models for pathogenesis investigations and evaluations of vaccine efficacy is IP injection of virulent leptospires [14,31]. The first studies used guinea pigs as animal model for leptospirosis [12,13], however, the hamster soon became the most common animal model [32], and the IP route has been used almost exclusively to establish infection, see e.g. [19,22-25]. Following IP injection of virulent *Leptospira* spp., they disseminate quickly throughout the host via the bloodstream, and when a lethal dose is used, leptospirosis ideally causes death in 7 - 12 d.p.i. [14,17]. The indubitable technical advantages inherent to the IP route of infection and its reproducibility have made it the standard approach for induction of lethal leptospirosis and for evaluating vaccine efficacy, for either bacterin [33] or subunit vaccines [22]. However, this port of entry for leptospires into the host does not reproduce a natural route infection, generally regarded as via abraded skin (microlesions) and/or mucosal membranes [8]. The IP route circumvents the natural non-specific defence mechanisms presented by the skin and mucosal barriers. Leptospiral pathogenicity mechanisms and virulence factors associated with adhesion to skin or mucosa might be underrepresented in leptospires directly injected into the host. This raises the possibility that some antigens that are involved in early host invasion could demonstrate low efficacy when evaluated against an IP route of infection. Therefore, to evaluate virulence factors used in vaccine preparations a more natural route of infection is justified.

In this study, we developed a model for lethal leptospirosis in hamsters based on TC exposure to *L. interrogans* strain Fiocruz L1-130 and showed that we could establish lethal leptospirosis in 100% of animals with lightly abraded skin. Although we found that it was possible to infect hamsters without micro-lesions, infection was not as reliable when compared to the abraded skin TC protocol. A higher density of spirochetes (10^{10}-10^{11}/ml) could improve the reliability of infection. However, leptospiral cultures reach stationary phase of *in vitro* growth at ≥10^9 leptospires/ml. Thus, the need for highly concentrated leptospires would likely result in the use of older cultures, with altered protein expression profiles [4], and a potentially disrupted outer

membrane because of the *in vitro* processing required [21]. Potentially resulting in a less virulent inoculum and poor reproducibility.

Several studies evaluated alternative protocols for infection using routes closer to that of the natural infection. Lourdault and colleagues [17] studied the dissemination of *L. interrogans* in the guinea pig model of experimental leptospirosis using three routes of infection, namely: IP, conjunctival (CJ) and subcutaneous (SC) inoculation. They reported LD_{50} values of 133, 251 and 2×10^5 leptospires for IP, SC and CJ inoculation, respectively. The need for a high number of leptospires by the CJ route was not unexpected, this is a more challenging route with the need to overcome natural defence mechanisms of the conjunctiva. Due to the small size of the eye, this procedure requires a high concentration of leptospires in a small volume (microliters), requiring extensive processing of the culture and potential reduction in virulence. In addition, hamsters need to be anesthetized, as it is difficult to deliver the full dose to the eye. The SC route does not differ significantly from IP, as the leptospires are administered underneath the skin barrier.

More recently, a report demonstrated that epicutaneous administration of leptospires in a guinea pig model induced leptospirosis, however, less than 30% of infected animals developed lethal leptospirosis [18]. Two other works, available only in Portuguese [9,16], described infection routes similar to the SL route reported here. Batista *et al.* found that TC infection after skin scarification induced lethal leptospirosis in 98.3% of animals compared to 15.8% following exposure to intact skin. Macedo and colleagues evaluated CJ, SC and skin scarification routes, however, they could not induce 100% lethal leptospirosis. Their protocols involve excessive manipulation of the animals and a concentration of leptospires to a small volume. As in the natural transmission of leptospires, the TC/LS route of infection elegantly reproduced water contaminated with leptospires that could penetrate abraded skin with minimum manipulation of animals in a reliable and reproducible way.

TC challenge after skin abrasion was efficient in inducing lethal leptospirosis in hamsters while immunization with a bacterin stimulated a sterilizing immune response. The challenge dose used was equivalent to 21-58× the LD_{50}, above the recommended minimum value for approval of animal vaccines [23,25,34]. Many leptospiral proteins were reported to bind extracellular matrix and cell surface proteins *in vitro*: LigA/B, Lsa21, Lsa27, LenA to F, LipL32, OmpL37, TlyC and LipL53 [35-44]. Some of these

proteins, e.g. OmpL37, have a strong affinity for skin elastin and may mediate leptospiral attachment to the elastin-rich inner layer of the skin [40]. When evaluated as vaccine antigens, followed by IP challenge, proteins like LipL32 [45] and OmpL37 (unpublished work) failed to induce a significant protective immune response. These proteins are examples of potential vaccine candidates that might show increased efficacy if the TC/LS challenge route was used. Recent advances have improved our understanding of pathogenic mechanisms of pathogenic *Leptospira* spp. [27,28,46]. New tools for genetic manipulation are still required in order to help identify new targets for new vaccine development.

Conclusions

We described a route of infection that mimics not only the clinical presentation of severe leptospirosis in patients but also a route of natural entry of leptospires into the host. TC infection following skin abrasion and exposure to 10^9 leptospires/ml induced lethal leptospirosis in 100% of nine-week-old hamsters, regardless of gender. We recommend the TC infection of hamsters as an alternative to the IP route for administration of the challenge dose in evaluations of vaccine efficacy.

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Figures

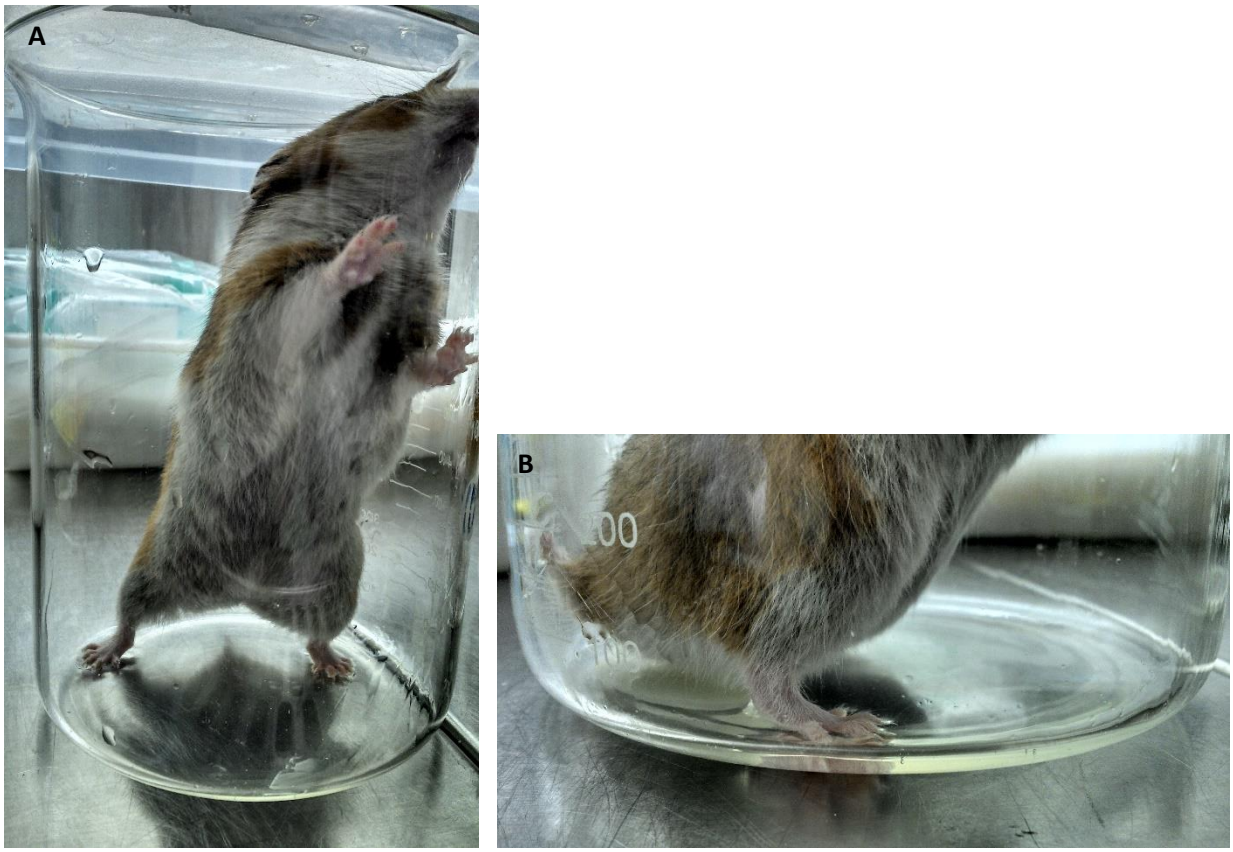

Fig.1. The TC route of infection through the exposure to PBS containing leptospires. **A.** General view of the beaker during infection, hamsters can be raised up on two legs or in normal standing position with all four legs in contact with the liquid. They usually cannot reach the top border of the beaker. **B.** Close-up of hamster legs in contact with the liquid containing leptospires.

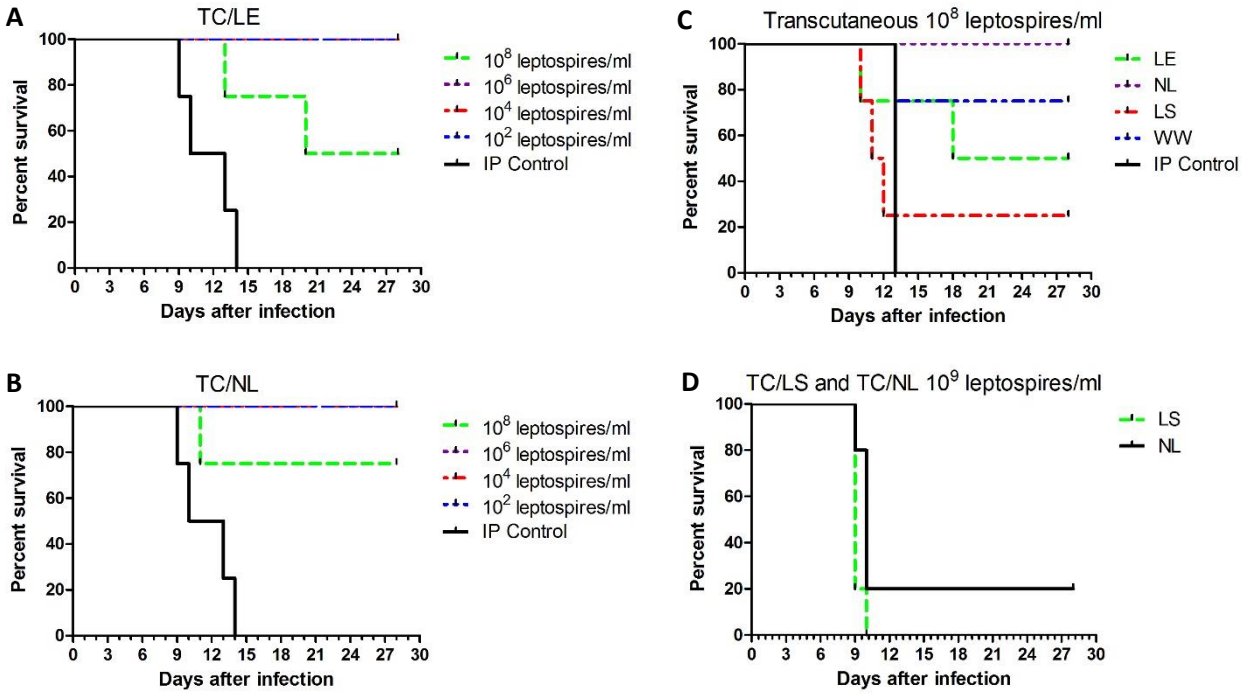

Fig. 2. Mortality of hamsters following exposure to varying concentration of *L. interrogans* by the TC route. **A.** TC infection of hamsters with a lesion (LE). **B.** TC infection of hamsters without any lesions (NL). **C.** TC infection of hamsters exposed to 10^8 leptospires/ml in the LE, SL, WW and NL groups. **D.** TC infection in hamsters after exposure to 10^9 leptospires/ml in the LS and NL groups. The control group were IP injected with 10^3 leptospires (4 animals in A and B, and 2 animals in C).

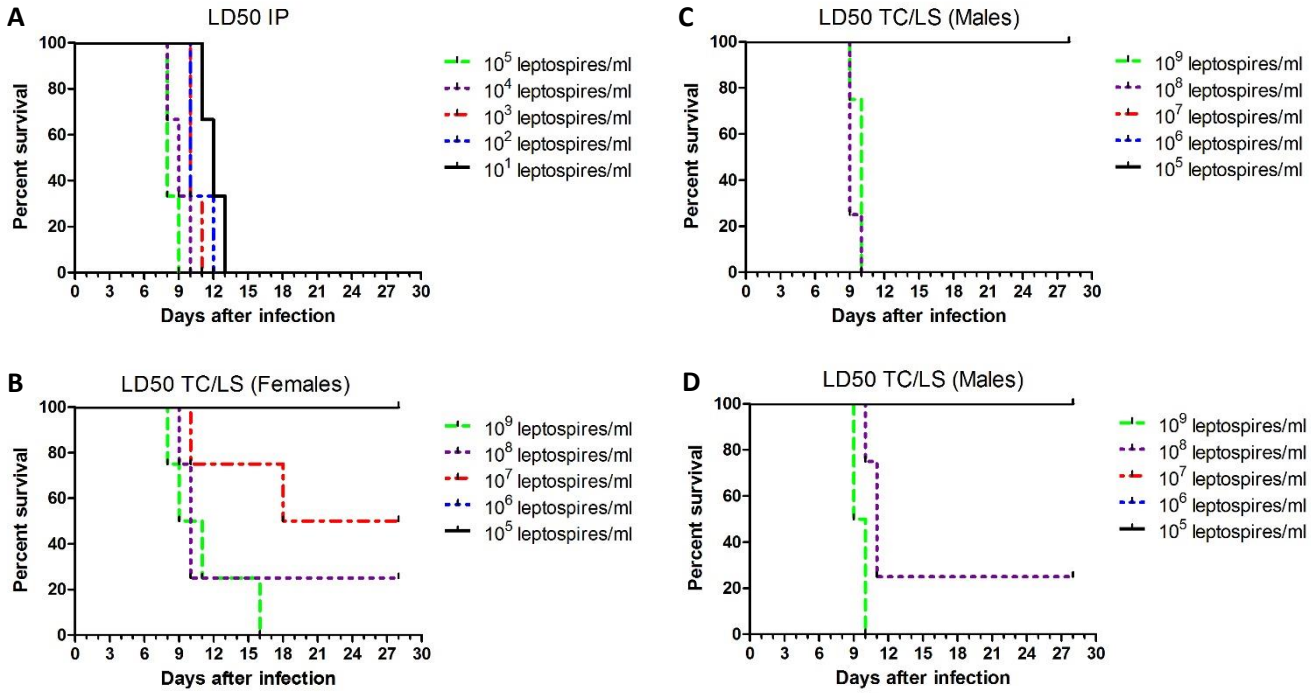

Fig. 3. Comparison of the TC and IP routes of infection on the LD_{50} for *L. interrogans* strain Fiocruz L1-130 in nine-week-old hamsters. **A**. IP administration of leptospires (n=3 per group). **B-D**. TC infection of hamsters following exposure to abraded skin (SL group) for female (**B**) and male (**C and D**) hamsters (n=4).

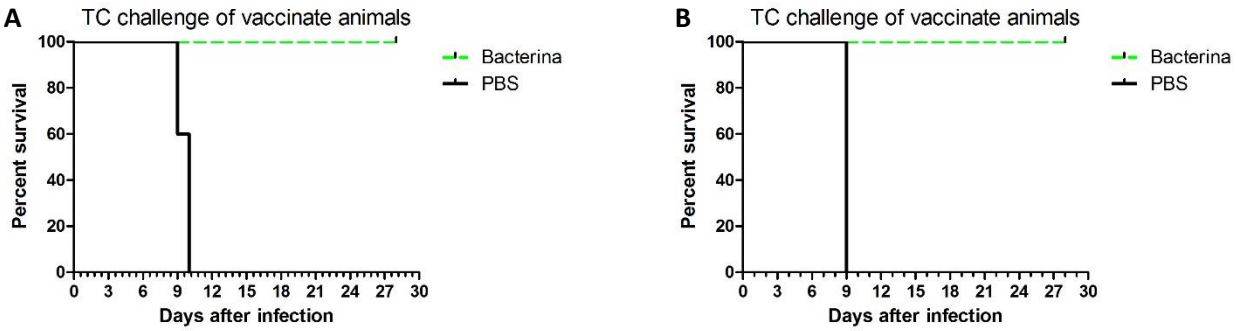

Fig. 4. TC challenge of bacterin immunized hamsters. Animals were injected intramuscularly with bacterin or PBS (n=5 per group) and challenged following exposure to abraded skin (10^9 leptospires/ml). **A.** First experiment (males). **B.** Second experiment (females).

Tables

Table 1. Culture of hamster tissues collected at different time point's post-infection with *L. interrogans* by TC or IP routes.

Tissue cultures in EMJH medium					
Infection route	d.p.i.	Tissues culture (+/-)			
		Kidney	Liver	Lung	Blood
Transcutaneous	1	0/4	0/4	0/4	0/4
	3	1/3	2/2	1/3	1/3
	5	4/0	4/0	4/0	4/0
	7	4/0	4/0	4/0	4/0
	9	4/0	4/0	4/0	4/0
Intraperitoneal	1	0/4	0/4	0/4	0/4
	3	0/4	0/4	0/4	0/4
	5	4/0	4/0	4/0	4/0
	7	4/0	4/0	4/0	4/0
	9	4/0	4/0	4/0	4/0

d.p.i = days post infection

(+/-) = culture negative or positive for leptospires by dark-field microscopy

n = 4 per group

4.5 Artigo 5

Isolation and characterization of *Leptospira interrogans* serogroup Icterohaemorrhagiae strain UFPEL-RCA from a dog with clinical leptospirosis

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Isolation and characterization of *Leptospira interrogans* serogroup Icterohaemorrhagiae strain UFPEL-RCA from a dog with clinical leptospirosis

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Abstract

Leptospirosis is an important zoonosis caused by pathogenic *Leptospira* species. We report the isolation, molecular typing and virulence analysis of a *Leptospira interrogans* serogroup Icterohaemorrhagiae strain from a dog presenting clinical leptospirosis. This strain, named UFPEL-RCA is virulent in the hamster model of lethal leptospirosis and is available for the diagnosis of leptospirosis using the microscopic agglutination test (MAT), epidemiological studies as well as basic microbiology and pathology investigations and vaccine development.

Graphical Abstract:

Isolation and characterization of *Leptospira interrogans* serogroup Icterohaemorrhagiae strain UFPEL-RCA from a dog with clinical leptospirosis

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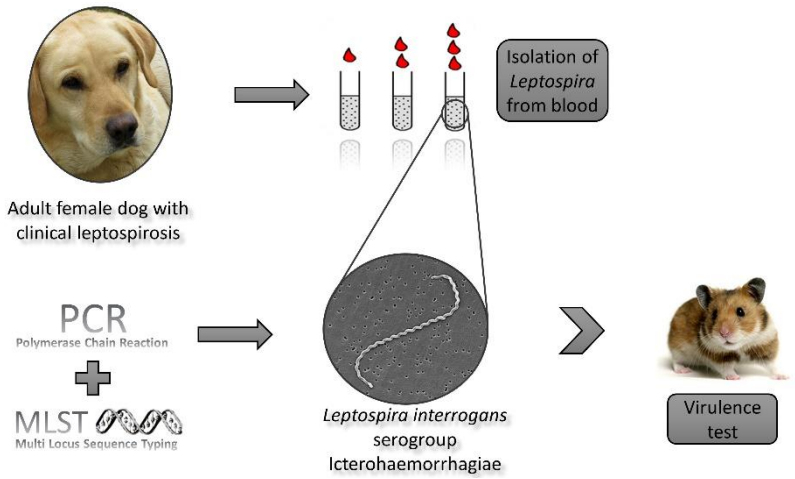
Summary:

A *Leptospira interrogans* serogroup Icterohaemorrhagiae isolated from a dog with acute leptospirosis was characterized by MLST and is virulent in the hamster model of leptospirosis.



1 Introduction

Pathogenic spirochetes from the *Leptospira* genus are the causative agent of leptospirosis, a worldwide spread zoonosis that is considered a re-emerging neglected tropical disease (McBride et al., 2005). The World Health Organization estimates the annual incidence of leptospirosis to be ca. 873,000 severe human cases, with ca. 49,000 deaths (Picardeau et al., 2014). In addition, this disease is responsible for major losses in livestock production (Ellis, 2015). Domesticated dogs occupy a central position in transmission of leptospires due to their close proximity with humans (Gay et al., 2014). There are nine pathogenic species of *Leptospira* (Bourhy et al., 2014), at least 250 serovars and 24 serogroups (Cameron, 2015). Dogs tend to be symptomless reservoir hosts for serovars of *L. interrogans* serogroup Canicola, however, they can develop serious clinical complications when infected by other serogroups, e.g. Icterohaemorrhagiae, Pomona and Grippotyphosa (Andre-Fontaine, 2006).

The clinical manifestations of leptospirosis in dogs are very similar to those of humans, presenting with either the sudden onset of an acute anicteric febrile illness or an icteric disease. Icteric leptospirosis usually presents fever, vomiting, jaundice and muscular pain. If untreated, canine leptospirosis can result in renal and liver failure, haemorrhaging, cardiovascular collapse and ultimately, death (Ellis, 2015). Prophylaxis of canine leptospirosis is based on vaccination (Andre-Fontaine, 2006; Ellis, 2010). The currently recommended vaccine is based on an inactivated whole-cell preparation (bacterin), that should include the most prevalent local serogroups (Dellagostin et al., 2011). The inclusion of the most prevalent serogroups/serovars in vaccine preparation can avoid not only canine leptospirosis in synanthropic dogs but also contribute towards the prophylaxis of human leptospirosis (Gay et al., 2014). In this work, we report the isolation and molecular characterization of a virulent strain or *L. interrogans* serogroup Icterohaemorrhagiae.

2 Materials and Methods

2.1 Clinical case and sample collection

A ten-month-old female dog was received in a veterinary clinic with suspected leptospirosis in Pelotas, RS, Brazil (31.7719° S, 52.3425° W). The dog lived in the backyard of a house and four days before veterinary examination presented with jaundice, fever (41 °C), vomiting, loss of weight, dehydration and haematuria. The blood sample was collected from the cephalic vein. Standard clinical antimicrobial treatment, intramuscular streptomycin and penicillin, was started following blood collection and was continued for 14 days.

2.2 *Leptospira* isolation and culture in EMJH

Approximately 0.1 ml of whole blood was inoculated under aseptic conditions into 5 ml of Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium (Difco, BD Diagnostics) supplemented with *Leptospira* Enrichment EMJH (Difco, BD Diagnostics). After incubation at 30 °C for 1 h, 0.5 ml were transferred to a new tube containing 4.5 mL of supplemented EMJH medium. Cultures were incubated at 30 °C and examined weekly by dark-field microscopy. After three passages, dimethyl sulphoxide (DMSO, 0.25% final concentration) was added to culture and the isolates were stored in liquid nitrogen.

2.3 Molecular characterization

Leptospires were collected by centrifugation, 8,000 × g for 15 min at 4 °C, the pellet was washed three times in PBS and genomic DNA was extracted using the GenomicPrep Mini Spin Kit (GE Healthcare). The presence of the *lipL32*, *rpoB* and 16S genes was determined by PCR as previously described (Miraglia et al., 2013; Vedhagiri et al., 2010). The bacterial genome was sequenced using the Illumina HiSeq 2000 paired-end sequencing platform. After *de novo* genome assembly, housekeeping gene loci were identified and used in a Multi-locus Sequencing Typing (MLST) scheme as previously described (Boonsilp et al., 2013), whereby seven loci sequences are used to discriminate up to seven *Leptospira* spp. by sequence type (ST), permitting identification of the serogroup and occasionally the serovar.

2.4 Virulence test

Three adult (28 days old) golden Syrian hamsters were inoculated intraperitoneally with 10^8 leptospires in 1ml of EMJH media. In the control group, two hamsters were inoculated with 1ml of sterile EMJH media only. Animals were monitored twice daily for signs of leptospirosis and weighed daily. Animals were humanly euthanized when presenting clinical signs of disease (loss of appetite, gait difficulty, dyspnea, prostration, ruffled fur and jaundice) and ≥10% loss of body weight (Coutinho et al., 2011). Control animals were euthanized 28 days post injection. After euthanasia, animals were necropsied and examined macroscopically for signs of organ damage. One kidney from each animal was aseptically removed, macerated into 5ml of EMJH and cultured as described in section 2.2. The presence of leptospires in tissue samples from the lungs, liver and the remaining kidney was detected using the immunofluorescent imprint method (IM) as previously described (Chagas-Junior et al., 2009).

2.5 Histopathology

Kidney, lung and liver tissue samples were collected, fixed in 10% formalin and paraffin embedded. Sections, 5 µm thick, were stained with haematoxylin and eosin (HE) and analysed by a pathologist. Samples from the two hamsters injected with 1 mL sterile EMJH media only were also included.

2.6 Ethical statement

All animal procedures were in accordance with the National Council of Animal Experimentation Control (CONCEA). This work was reviewed and approved by University's Committee for Ethical in Animal Experimentation (process number 6843). The dog owner understood and consented to donate the blood sample used in this work.

3 Results

After one month of culture in EMJH, the presence of leptospires was confirmed by dark-field microscopy, confirming that the dog had leptospirosis. Antibiotic treatment resulted in full recovery from the disease. After three *in vitro* passages, the isolate, identified as the UFPEL-RCA strain, was stored in liquid nitrogen and maintained in the Biotechnology Strain Collection at the Federal University of Pelotas. The isolate was identified as *Leptospira* spp. after positive PCRs for the *lipL32*, *rpoB* and 16S genes. MLST of the UFPEL-RCA strain resulted in a match with the ST 17 molecular profile. ST 17 includes *L. interrogans* serogroup Icterohaemorrhagiae serovar Icterohaemorrhagiae and serovar Copenhageni (strains RGA and Ictero No. 1, and FIOCRUZ L1-130 and M20, respectively). Although MLST identified UFPEL-RCA as belonging to the Icterohaemorrhagiae serogroup, we were unable to determine the serovar.

All animals inoculated with the UFPEL-RCA strain presented clinical features consistent with leptospirosis and lost at least 10% of their corporal weight at 4 (2 animals) and 6 days post infection, when they were humanly euthanized. The control animals remained healthy until 28 days post injection when they were euthanized. Necropsy of the infected animals revealed macroscopic signs consistent with leptospirosis, *i.e.* pulmonary haemorrhaging, splenomegaly, hepatomegaly and jaundice. Histopathology evaluation showed altered organ anatomy, with haemorrhage and congestion of the kidneys (Figure 1) and lungs. Hyaline cast formation was also found in kidneys. The renal tubules were degenerated and necrotic. Lungs showed signs of emphysema, leucocytes in the alveolar septa, fibrin in the alveolus, and presence of high amounts of haemosiderin, confirming haemorrhage and haemolysis. IM and culture isolation from the infected hamster kidney samples were positive for the presence of leptospires.

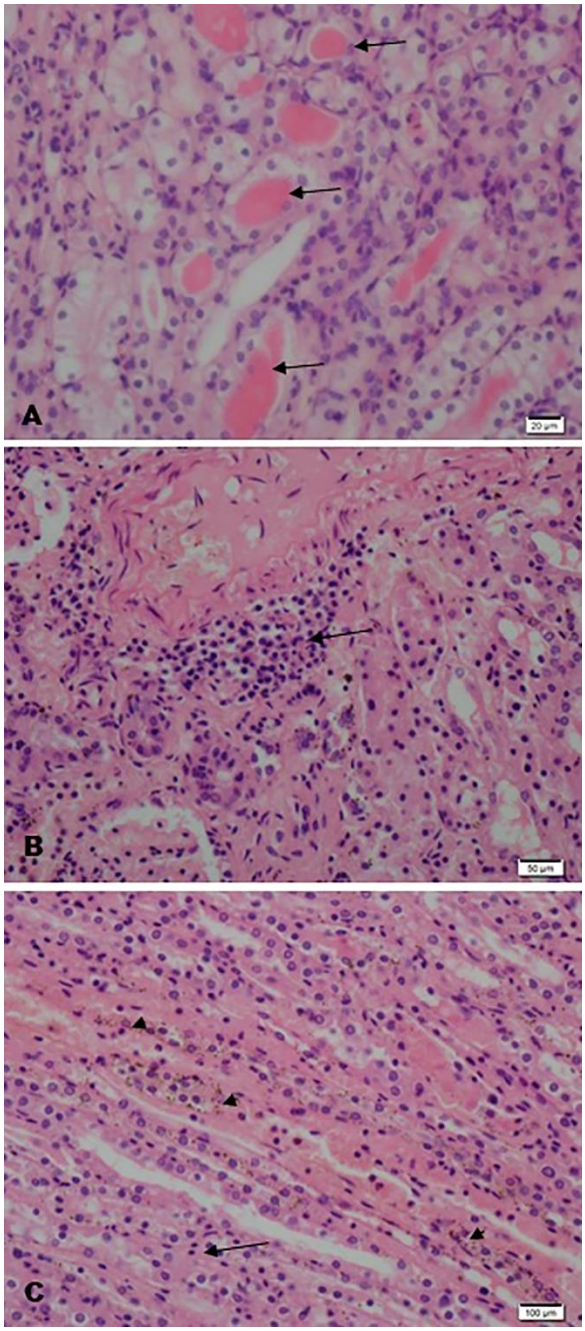


Figure 1. Histopathological evaluation of kidney sections taken from hamsters infected with *L. interrogans* serogroup Icterohaemorrhagiae UFPEL-RCA strain. A) Arrows indicate hyaline cast formation. B) Perivascular lymphocytes infiltration. C) Tubular degeneration and necrosis (long arrow), with deposits of hemosiderin (arrowheads).

4 Discussion

Infection with pathogenic *Leptospira* spp. is responsible for hundreds of thousands deaths and severe disease cases in both humans and animals and untold economic losses every year. Human leptospirosis is endemic in tropical areas and is mainly associated with urban poverty in developing countries, and occupational or recreational activities in developed nations (McBride et al., 2005). Domestic animal leptospirosis is a significant problem regardless of a countries developmental status (Ellis, 2015). In this work, we report the isolation of a virulent *L. interrogans* serogroup Icterohaemorrhagiae strain from a dog that presented with acute clinical leptospirosis.

The MLST scheme used to characterise the isolate is based on the sequence of seven loci in order to assign a specimen into one of the seven major pathogenic *Leptospira* spp. Although congruence is low at the serovar level, this molecular typing approach provides reliable species classification and the correct identification of the majority of serogroups. MLST is rapidly becoming the standard for molecular typing of new isolates of pathogenic *Leptospira* spp. The loci sequence data from the UFPEL-RCA strain matched ST17, that includes the *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strains FIOCRUZ L1-130 and M 20 and serovar Icterohaemorrhagiae strains RGA and Ictero No.1.

Leptospirosis in dogs represents a risk to humans and other animals due to proximity of this species and the asymptomatic carrier role that dogs can play (Andre-Fontaine, 2006; Ellis, 2015). The precise events that determine whether infection by a given pathogenic *Leptospira* serovar is asymptomatic or result in clinical manifestations remain unclear. The infecting serovar, host species and immunity play an important role in this scenario (Murray, 2015). It is well established that serovar Canicola is maintained by dogs throughout the world, while Icterohaemorrhagiae seems to be maintained by rodents (Andre-Fontaine, 2006; Ellis, 2010, 2015; McBride et al., 2005), however, Icterohaemorrhagiae serogroup infections in dogs are increasing in number around the world (Calderon et al., 2014; Ellis, 2010). Some studies show leptospirosis rates as high as ~70% in urban canine populations in Brazil (Brod et al., 2005; Freire et al., 2007; Tesserolli et al., 2008), while seroprevalence of Icterohaemorrhagiae serogroup in the city of Pelotas was reported to be ~10% (Brod et al., 2005; Felix, 2013). To the best of our knowledge, this is the first report of the

isolation of a strain belonging to the Icterohaemorrhagiae serogroup from a dog in the south of Brazil.

Commercial vaccines in Brazil tend to be imported from USA and Europe and, while they usually include serogroup Icterohaemorrhagiae, the inclusion of foreign isolates is prejudicial to the effectiveness of the vaccine due to genetic variation, even within the same serovar (Arent et al., 2013; Ellis, 2010). Furthermore, imported vaccines rarely include the most prevalent local serovars. The inclusion of local isolates in bacterin-based vaccine preparations for animals, like UFPEL-RCA strain, in vaccines administered in the south of Brazil, represents an optimal strategy for the prophylaxis of leptospirosis. Further applications of the new isolate also include its use in vaccine trial experiments, especially those designed to prevent canine leptospirosis. In addition, the pathological findings after infection with this strain make it possible to better understand virulence factors and pathogenic mechanisms.

Our group recently reported the isolation of *L. interrogans* serogroup Icterohaemorrhagiae from capybara (*Hidrochaerys hidrochaerys*) (Jorge et al., 2012) and *Cavia aperea* (Brazilian guinea pig) (Monte et al., 2013) both rodents captured in the wild. The same serogroup was also isolated from a rat found in an abandoned swimming pool (Forster et al., 2013). These data suggests that this serogroup is a common pathogen in Pelotas. This city is near sea level in the southernmost part of Brazil and is surrounded by lakes with thousands of people living in slum communities with a large numbers of stray dogs. The urban setting, humid climate, frequent rainfall and hot summer temperatures provide ideal environmental conditions for pathogenic *Leptospira* spp. transmission among rodents and urban dogs and from them to humans. We are currently undertaking a wide serological study in poor slum communities in Pelotas, aiming to better understand the true burden of human leptospirosis in this city and the influence of the canine population.

In conclusion, we report the isolation and characterization of a new strain of *L. interrogans* serogroup Icterohaemorrhagiae named UFPEL-RCA from a dog that presented with acute clinical leptospirosis. This strain is virulent in the hamster model of leptospirosis.

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6 CONSIDERAÇÕES FINAIS

- A bacterina utilizada em vários países apresenta problemas. Diversas preparações vacinais experimentais, baseadas majoritariamente em vacinas recombinantes apresentou sucesso apenas parcial, com destaque para LigANI. Ainda não há uma preparação vacinal totalmente protetora contra os vários sorovares de *Leptospira* spp. que induza imunidade esterilizante sem efeitos adversos.
- A obtenção de leptospiras adaptadas ao hospedeiro a partir do cultivo em DMCs implantadas no peritônio de ratos é uma metodologia promissora para estudos buscando entender as respostas desta espiroqueta aos estímulos do hospedeiro. O papel de RNAs não codificadores em *Leptospira* precisa ser demonstrado.
- A infecção de hamster por *L. interrogans* virulenta pela exposição natural da pele escarificada e musocas a líquido contendo leptospiras pode substituir a inoculação intraperitoneal como metodologia para estabelecimento da doença.
- A cepa de *Leptospira* patogênica isolada de um caso clínico de leptospirose canina pertence a *L. interrogans* sorogrupo Icterohaemorrhagiae, é virulenta em modelo hamsters e foi nomeada cepa UFPel-RCA.
- Ainda são necessários mais estudos para o descobrimento de antígenos protetores para utilização no desenvolvimento de novas vacinas contra leptospirose.

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8 ANEXOS

Anexo A – Paper publicado durante o doutorado, referente ao trabalho realizado durante o mestrado em Biotecnologia na UFPel.

Clinical and Vaccine Immunology

Protection against Lethal Leptospirosis after Vaccination with LipL32 Coupled or Coadministered with the B Subunit of Escherichia coli Heat-Labile Enterotoxin

André A. Grassmann, Samuel R. Félix, Carolina Ximendes dos Santos, Marta G. Amaral, Amilton C. P. Seixas Neto, Michel Q. Fagundes, Fabiana K. Seixas, Éverton F. da Silva, Fabricio R. Conceição and Odir A. Dellagostin

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Protection against Lethal Leptospirosis after Vaccination with LipL32 Coupled or Coadministered with the B Subunit of *Escherichia coli* Heat-Labile Enterotoxin

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Leptospirosis, a worldwide zoonosis, lacks an effective, safe, and cross-protective vaccine. LipL32, the most abundant, immunogenic, and conserved surface lipoprotein present in all pathogenic species of *Leptospira*, is a promising antigen candidate for a recombinant vaccine. However, several studies have reported a lack of protection when this protein is used as a subunit vaccine. In an attempt to enhance the immune response, we used LipL32 coupled to or coadministered with the B subunit of the *Escherichia coli* heat-labile enterotoxin (LTB) in a hamster model of leptospirosis. After homologous challenge with 5× the 50% lethal dose (LD_{50}) of *Leptospira interrogans*, animals vaccinated with LipL32 coadministered with LTB and LTB::LipL32 had significantly higher survival rates ($P < 0.05$) than animals from the control group. This is the first report of a protective immune response afforded by a subunit vaccine using LipL32 and represents an important contribution toward the development of improved leptospirosis vaccines.

Spirochetes from the genus *Leptospira* are the causative agents of leptospirosis, a zoonosis with a worldwide distribution. Leptospirosis is recognized as an emerging infectious disease and affects humans and wild and domestic animals (1). Leptospires colonize the proximal renal tubules of carrier animals (34) and are shed in the urine. The disease is associated with direct or indirect contact with contaminated urine (1, 25). Due to the impacts on animal production and public health and the severity of the disease, an efficient prophylactic measure is urgently needed. Current vaccines against leptospirosis are whole-cell preparations that produce only short-term immunity, with adverse reactions due to both leptospiral lipopolysaccharide (LPS) and residual medium components (1). Furthermore, the protection conveyed by these whole-cell preparations is serovar specific, with limited or no cross-protection (10) among the more than 260 serovars of *Leptospira* reported (1). Therefore, an effective multiserovar vaccine against leptospirosis with no collateral effects remains a challenge.

Efforts to develop recombinant vaccines against leptospirosis have focused on outer membrane proteins (OMPs) (10). The most abundant protein in the entire leptospiral proteome is an outer membrane lipoprotein of 32 kDa, LipL32 (27), accounting for 75% of the outer membrane proteome (7). This protein can be considered a promising antigen for the development of a multiserovar vaccine. LipL32 is expressed in all pathogenic *Leptospira* spp., and it is highly conserved (19) and not expressed in the saprophytic *L. biflexa* (29). This protein binds to extracellular matrix components, as indicated by *in vitro* assays (22, 23) and crystal structure analyses (36). Moreover, LipL32 is expressed during mammalian leptospiral infection (18). Different immunization strategies that have been tested with LipL32 have shown some immune protection when administered with naked-DNA (4), recombinant adenoviral (3), and *Mycobacterium bovis* BCG (30) delivery systems. However, LipL32 produced no protection by recombinant subunit protein vaccination with either a Freund or aluminum hydroxide adjuvant (4, 26). These findings suggest that the immune protection induced by LipL32 is correlated with a modulation of the immune system.

The *Escherichia coli* heat-labile enterotoxin (LT), and its closely related homologue *Vibrio cholerae* cholera toxin (CT), consists of one A subunit with ADP-ribosyltransferase activity linked to five B subunits (8). The B subunit of *Escherichia coli* heat-labile enterotoxin (LTB) is highly immunogenic upon systemic (6, 9, 15) and mucosal (14, 37) immunizations. Its adjuvant activity has been demonstrated with unrelated antigens, both coadministered (14, 15) and linked by chemical conjugation or genetic fusion (6, 14, 37), exhibiting no toxic effect (8). LTB has a pentameric structure that binds to the ubiquitously expressed monosialotetrahexosylganglioside (GM1-ganglioside) receptor on the surface of mammalian cells, and this binding is essential for adjuvant properties (8). Therefore, the aim of the present study was to assess the immune protection induced by recombinant LipL32 coadministered or coupled with recombinant LTB. Our findings reveal the protective potential of LipL32 and suggest a new vaccine against leptospirosis using LTB and LipL32.

MATERIALS AND METHODS

***Leptospira* culture.** *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco Laboratories) at 29°C. The procedures for the maintenance of the culture and challenge experiments were conducted as previously described (33).

Cloning, expression, and purification of recombinant proteins. Three recombinant vectors were used in this study. Two of them had been

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previously constructed, pAE/*ltb* (16) and pAE/*lipL32* (31), and one was generated as follows: the *lipL32* coding sequence from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was amplified by PCR from pAE/*lipL32*. The following primers were used: LipL32-For (5′-GGGGTACCG GCGGCGGTGGTCTGCCAAGCCT) and LipL32-Rev (5′-GGAATTCT TACTTAGTCGCGTCAGAAGC). After amplification, the 771-bp fragment was cut with the KpnI and EcoRI (New England BioLabs) restriction enzymes and cloned into pAE/*ltb* cut with the same enzymes. The KpnI restriction site was modified to allow the insertion of *lipL32* in the correct reading frame of the *ltb* coding sequence. The forward primer was constructed to allow a 4-amino-acid (aa) linker/spacer between *ltb* and *lipL32* (Gly-Thr-Gly-Gly). The resulting plasmid, pAE/*ltb*::*lipL32*, was confirmed by PCR and restriction digestion. The recombinant vectors pAE/*ltb*, pAE/*lipL32*, and pAE/*ltb*::*lipL32* were used to transform *E. coli* BL21 Star(DE3) cells (Invitrogen). The 6×His-tagged recombinant LTB (rLTB), recombinant LipL32 (rLipL32), and rLTB::LipL32 proteins were expressed and purified by affinity chromatography as previously described (32).

Characterization of recombinant proteins by Western blotting and GM1-ELISA. Western blot characterization was conducted as described elsewhere previously (32). The antibodies used were anti-LipL32 monoclonal antibody (MAb) 1D9 (13), diluted 1:5,000; rabbit anti-cholera toxin IgG (Sigma-Aldrich), diluted 1:6,000; sera from a human leptospirosis patient (21), diluted 1:500; a goat IgG–anti-mouse Ig–peroxidase conjugate (Sigma-Aldrich), diluted 1:6,000; a goat IgG–anti-rabbit IgG–peroxidase conjugate (Sigma-Aldrich), diluted 1:6,000; and a rabbit IgG–anti-human Ig–peroxidase conjugate (Abcam), diluted 1:2,000.

The abilities of rLTB and rLTB::LipL32 to bind to GM1-ganglioside were determined by an enzyme-linked immunosorbent assay (ELISA) as previously described (16), with minimal modifications. Briefly, plates were coated with 100 ng/well of bovine GM1-ganglioside (Sigma-Aldrich), and after blocking, the plates were incubated with 100 ng/well of rLTB, rLipL32, rLTB::LipL32, or choleric toxin (Sigma-Aldrich). The plates were then incubated with anti-LipL32 MAb 1D9 diluted 1:5,000 or rabbit IgG anti-cholera toxin antibody diluted 1:6,000, followed by a goat IgG anti-mouse- or anti-rabbit IgG-peroxidase conjugate diluted 1:6,000, respectively. The reactions were revealed with *O*-phenylenediamine dihydrochloride (Sigma-Aldrich) and hydrogen peroxide (Sigma-Aldrich) and read at 492 nm. Wells with GM1 but without proteins and wells without GM1 but with proteins were used as controls.

Animal immunization. Four- to five-week-old female Golden Syrian hamsters were individually identified and distributed into three treatment groups. Each treatment group was composed of five animals, and three independent experiments were conducted, for a total of 45 animals. Hamsters were inoculated in the quadriceps muscle with 60 μg of rLTB::LipL32 (coupled) or 16.5 μg of rLTB and 43.5 μg of rLipL32 (coadministered), and the control group received 16.5 μg of rLTB (control). This dose design was used to administer equal amounts of adjuvant and antigen as coupled and coadministered proteins. Each animal received two doses, administered at days 0 and 14. The animals were inoculated with a maximum of 300 μl per injection site. Serum samples were collected from each animal by phlebotomy of the retro-orbital venous plexus on the day before the first immunization (preimmune; day zero) and on the day before challenge (postimmune; day 34). The animals were manipulated in accordance with the guidelines and approval of the Federal University of Pelotas Ethics Committee in Animal Experimentation.

Antibody response determination by rLipL32 ELISA. For the determination of the humoral immune response induced by rLipL32 coupled or coadministered with rLTB, the serum from each animal was serially diluted and tested by a recombinant LipL32 ELISA (30). A preliminary checkerboard analysis was performed to determine ideal antigen concentrations and primary and secondary antibody dilutions. Polystyrene microtiter plates were coated with 100 ng/well of rLipL32 diluted in carbonate-bicarbonate buffer (pH 9.6) at 4°C overnight. After three washes with phosphate-buffered saline (PBS) with 0.05% Tween 20 (PBS-T), the serum from each animal, diluted 1:800 to 1:25,600, was added in triplicate and incubated for 1 h at 37°C. Following three washes with PBS-T, the reaction mixtures were incubated for 1 h at 37°C with a 1:3,000 dilution of a goat polyclonal anti-hamster IgG–peroxidase conjugate (Abcam). After five washes with PBS-T, the reactions were revealed with *O*-phenylenediamine dihydrochloride (Sigma-Aldrich) and hydrogen peroxide (Sigma-Aldrich). The color reaction was allowed to develop for 15 min and stopped by the addition of 25 μl of 4 N H_2SO_4 to the mixture, and the optical densities were read at 492 nm.

Challenge of vaccinated animals. To determine vaccine-induced protection, the same animals used for the serological analysis were challenged 21 days after the second dose. The animals received an intraperitoneal injection of 10^2 cells of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (5× the 50% lethal dose [LD_{50}]) (30). Two additional control groups of five animals were included in each of the three independent experiments. Similar to the treatment groups, on days 0 and 14, one group received 300 μl of PBS (negative control), and another received homologous bacterin (10^8 cells in 300 μl of PBS). The hamsters were observed daily for mortality. Survivors were euthanized at 21 days postchallenge.

Statistical analysis. Statistical analyses for ELISAs were carried out with Student's *t* test. The Fisher exact test and log-rank test were used to determine significant differences in mortality and survival rates, respectively, among the experimental groups. *P* values of <0.05 were considered to be indicative of statistical significance. All analyses were carried out with GraphPad Prism 4 software (GraphPad Software).

RESULTS

Heterologous expression of recombinant proteins. The construction of the recombinant vector carrying the fusion gene was successful. The *lipL32* coding sequence without its signal sequence was ligated onto the 3′ end of *ltb*. rLTB, rLipL32, and rLTB::LipL32 were expressed in *E. coli* BL21 Star(DE3) cells. Purified recombinant proteins were analyzed by SDS-PAGE (Fig. 1A). The apparent molecular masses were as expected for each protein: 13 kDa, 30 kDa, and 41 kDa for rLTB, rLipL32, and rLTB::LipL32, respectively. rLTB and rLTB::LipL32 were expressed as inclusion bodies and required the addition of the denaturing agent *N*-lauroyl-sarcosine for purification, while rLipl32 was expressed and purified as a soluble protein. The yield of purified proteins varied from 3 to 10 mg per liter of culture. The pentameric form of rLTB was easily identified when the sample was not heated before SDS-PAGE was performed (data not shown). The pentamerization of rLTB::LipL32 (205 kDa) was not visualized.

Antigenic and functional characterization of purified proteins. The antigenic characterization of recombinant proteins was performed by Western blot analysis with antibodies specific for rLTB (Fig. 1B) and rLipL32 (Fig. 1C). Recombinant LTB was recognized by anti-CT antibodies. This serum did not react with rLipL32. MAb 1D9, as well as human convalescent-phase sera, reacted with rLipL32. As expected, the fusion protein was recognized by all tested antibodies. The negative-control *E. coli* extract did not react with any antibody, and the *L. interrogans* whole-cell extract reacted with human convalescent-phase sera (Fig. 1D). The GM1-ELISA with the recombinant proteins (Fig. 2) revealed the GM1-binding affinity of rLTB and rLTB::LipL32. These proteins showed a binding affinity as high as that of commercial choleric toxin, while rLipL32 did not bind to GM1-ganglioside. The binding activity obtained for rLTB::LipL32 was the same when anti-LipL32 or anti-CT antibody was used.

Humoral immune response in vaccinated hamsters. In order to assess whether rLipL32 coupled or coadministered with rLTB was able to promote IgG anti-LipL32 antibody responses in ham-

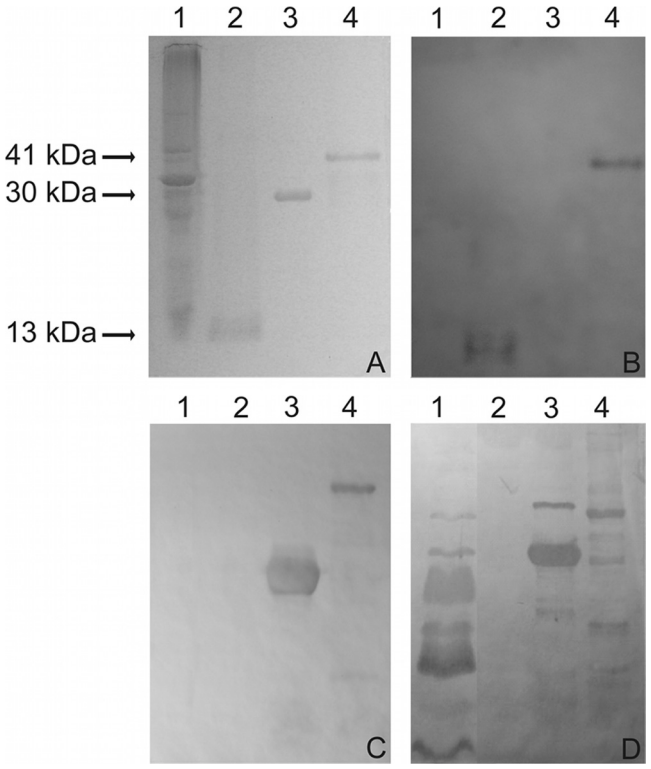

FIG 1 Characterization of purified recombinant proteins. (A) SDS-PAGE analysis; (B) anti-LTB Western blot analysis; (C) anti-LipL32 Western blot analysis; (D) Western blot analysis of human convalescent-phase sera. Lanes: 1, *E. coli* whole-cell extract; 2, rLTB; 3, rLipL32; 4, rLTB::LipL32 (A to C). Lanes: 1, *L. interrogans* strain L1-130 whole-cell extract; 2, rLTB; 3, rLipL32; 4, rLTB::LipL32 (D).

sters, preimmune and postimmune sera from each animal were evaluated by an indirect ELISA with rLipL32 as the immobilized antigen. The mean absorbances of the sera from each experimental group are shown in Fig. 3. The highest titer (over 1:25,600) was observed for animals receiving two doses of rLTB::LipL32, significantly higher than those of the other groups ($P < 0.01$ at a titer of 1:25,600). The coadministration of rLTB and rLipL32 also induced high anti-LipL32 titers.

Protection from leptospirosis. Animals were monitored for 21 days postchallenge for the occurrence of death. Three independent experiments were accomplished, and statistical analyses of lethality rates are shown in Table 1, while survival rates (which also consider days to death) are shown in Fig. 4. In the first experiment, three and two animals died in the groups treated with rLTB::LipL32 and rLTB plus rLipL32, respectively. In subsequent experiments, no death occurred in these groups. All animals receiving rLTB and PBS in experiment 1 died, while just two deaths in each subsequent experiment with rLTB and two and four deaths with the PBS treatment in experiments 2 and 3, respectively, were registered. The survival analyses showed statistically significant results (log-rank test) when either of the experimental groups (rLTB::LipL32 or rLTB plus rLipL32) was compared to control groups (PBS or rLTB) in the first experiment and in the grouped results. Furthermore, in the third experiment, both experimental groups were statistically different from the PBS-treated negative-control group; however, no statistically significant survival was observed for the second experiment. Regarding lethality, no significance (Fisher exact test) was found by the statistical analysis of each experiment alone. rLipL32 coadministered with rLTB protected 87% of the animals, which is statistically different from the 40% of animals protected in the rLTB group ($P = 0.02$). The group receiving the fusion protein rLTB::LipL32 had a combined level of protection of 80%, which is statistically different from the level of protection in the PBS group ($P < 0.01$). When survival or lethality was considered, no difference was observed between the rLTB- and PBS-treated groups. Similarly, no difference could be attributed to the groups treated with rLTB plus rLipL32, rLTB::LipL32, and bacterin among themselves.

DISCUSSION

A truly effective and cross-protective leptospirosis vaccine is yet to be developed. At present, the highest level of protection afforded by a recombinant vaccine was observed with the recombinant leptospiral immunoglobulin-like protein LigA from *L. interrogans* (10, 32). However, even after six full genome sequences, molecular studies of multiple strains (1, 25), and several vaccine trials (5,

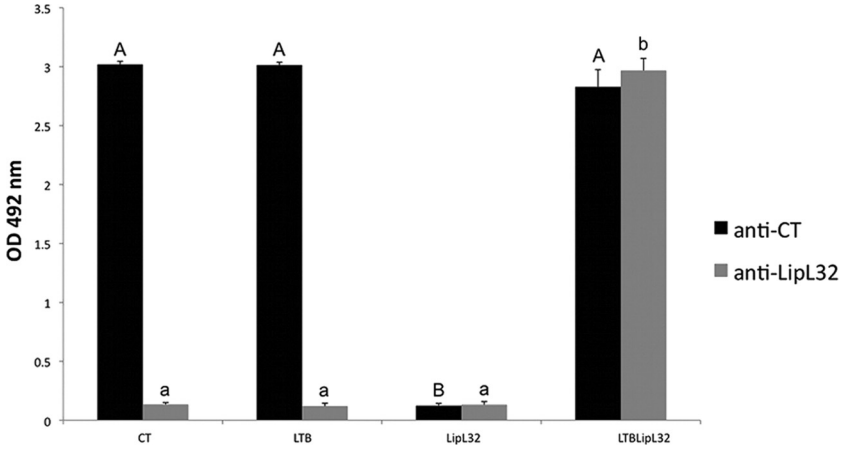

FIG 2 Recombinant protein GM1-binding ELISA. Different letters represent statistical differences ($P < 0.05$). Uppercase letters relate to significance for the anti-CT antibody, and lowercase letters relate to significance for the anti-LipL32 antibody. OD, optical density.

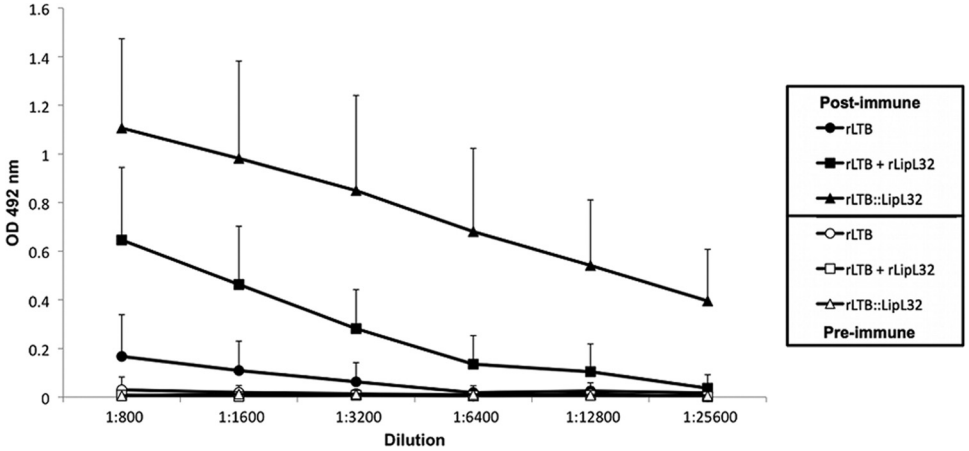

FIG 3 Humoral immune response against rLipL32 examined by ELISA. Error bars represent standard deviations. Both postimmune treatment groups were different from the preimmune group and control serum at all dilutions.

11, 12) have been reported, LipL32 remains the most promising recombinant vaccine candidate. In this work, we assessed the immunogenic properties of recombinant LipL32 in different subunit preparations using LTB as an adjuvant. To our knowledge, this is the first time that LTB has been used with a leptospiral antigen and the first study to report significant protection afforded by LipL32 administered as a subunit vaccine.

Challenge experiments are the most reliable assays to measure vaccine effectiveness (32). LipL32 has been extensively studied, with promising results when vaccine vectors or naked DNA was used (3, 4, 30). However, studies that used purified protein have thus far failed to produce significant protection with either Freund's adjuvant (4), aluminum hydroxide (26), or aluminum hydroxide and QS21 saponin (4). Our results show high survival rates for animals receiving recombinant LipL32 coadministered with or coupled to an LTB adjuvant, representing significant protection compared to that for any of the control groups (log-rank test). The level of significance of the results would be even higher if there were no surviving animals in the control groups; however, survival of a few animals from negative-control groups challenged with *L. interrogans* strain L1-130 is not uncommon (2, 10–12, 30).

Several studies have described LTB adjuvant efficiency when coupled (6, 37) or coadministered (15, 37) with different antigens; however, few studies have compared these two delivery systems (37). Our study shows that the rLTB::LipL32 protein was capable of stimulating significantly higher antibody titers than those elicited by the coadministration of the rLTB and rLipL32 proteins. Similar results were found for LipL32 coupled to the B subunit of CT (CTB), which induced higher antibody titers than those elicited by treatment with recombinant CTB (rCTB) plus rLipL32 (20). The animals vaccinated with rLTB::LipL32 had the highest antibody titers among all groups, but no statistical difference in survival rates was observed between the rLTB::LipL32 and rLTB-plus-rLipL32 treatment groups. Furthermore, within the same group, surviving animals did not necessarily have the highest antibody titers (data not shown). The demonstration that protection against leptospirosis is not necessarily associated with antibody titers is important, since most studies aimed at finding effective immunogens have been based solely on antibody responses (17, 31). Protection with LipL32 has been shown by use of recombinant BCG, adenovirus, and naked-DNA delivery systems (3, 4, 30), which are effective stimulants of cellular immunity. Therefore, not only humoral immunity but also cell-mediated immunity seems to play an important role in protection against leptospirosis (28, 35). Some studies have shown that cattle vaccines stimulating cellular immune responses are able to provide protection, while those stimulating high antibody titers are not (38). This phenomenon is not well understood for other species. In our study, protection may have occurred because LTB presents pow-

TABLE 1 Protection against lethal leptospirosis in hamsters conferred by experimental treatments

Treatment	No. of surviving hamsters/total no. of hamsters (% survival)[a]			
	Expt 1	Expt 2	Expt 3	Total
rLTB::LipL32	2/5 (40)^{ab}	5/5 (100)^{a}	5/5 (100)^{a}	12/15 (80)^{AB}
rLTB + rLipL32	3/5 (60)^{ab}	5/5 (100)^{a}	5/5 (100)^{a}	13/15 (87)^{A}
rLTB	0/5 (0)^{b}	3/5 (60)^{ab}	3/5 (60)^{ab}	6/15 (40)^{BC}
Bacterin	5/5 (100)^{a}	5/5 (100)^{a}	5/5 (100)^{a}	15/15 (100)^{A}
Negative control	0/5 (0)^{b}	3/5 (60)^{ab}	1/5 (20)^{b}	4/15 (27)^{C}

[a] Different superscript letters represent a statistical difference ($P < 0.05$). Uppercase letters relate to grouped results only, and lowercase letters relate to individual experiments.

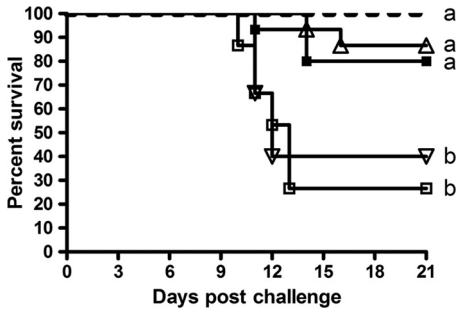

FIG 4 Hamster survival timeline (grouped results of three independent experiments). Different letters represent statistically different results ($P < 0.05$). Timelines represent the rLTB::LipL32 (■), rLTB-plus-rLipL32 (△), rLTB (▽), bacterin (----), and negative-control (□) groups. Statistical analyses and graph generation were carried out with GraphPad Prism 4 software systems (GraphPad Software).

erful immunostimulatory and immunomodulatory effects, such as enhancing antigen presentation via both major histocompatibility complexes, activating the selective differentiation of lymphocytes, increasing the expression levels of activation markers on B lymphocytes, and influencing the maturation and activation of dendritic cells (8). However, these effects must still be assessed.

The Western blot data (Fig. 1) suggest that the recombinant proteins retained antigenic epitopes present on native proteins. In addition, rLTB::LipL32 and rLipL32 were identified in serum from human leptospirosis patients, indicating that the immune response induced by these proteins is able to recognize native LipL32. The GM1-ELISA (Fig. 2) data show that the fusion did not impair the binding affinity of LTB. Variable numbers of amino acids in spacers/linkers between subunits in fusion proteins have been tested, and most fusions reported were successful (24, 37). In a recent study, Chen and coworkers (6) reported that 10 but not 6 amino acids in the flexible linker between LTB and the antigen were necessary to induce prolonged protection against BCL1 lymphomas. Therefore, we believe that a linker longer than 4 amino acids in LTB::LipL32 could allow the further folding and/or pentamerization of the coupled protein, thus affording a higher level of protection against leptospirosis.

In this study, we described a leptospirosis vaccine using a recombinant LipL32 antigen and rLTB as an adjuvant. We showed that rLipL32 coadministered or coupled with rLTB is highly immunogenic and protects hamsters from lethal leptospirosis. Studies are being carried out to assess the optimum dose, protection against other serovars, and vaccine dynamics. This approach may result in a formulation that could replace traditional vaccines against leptospirosis.

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