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Programa de Pós-Graduação em Biotecnologia

Tese

Leptospira interrogans: cinética da infecção e avaliação da proteção conferida por LigA

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Resumo

COUTINHO, Mariana Loner. ***Leptospira interrogans*: cinética da infecção e avaliação da proteção conferida por LigA.** 2011. <89>f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A *Leptospira interrogans* é o agente causador da leptospirose mais comum, uma zoonose que é endêmica em países tropicais como Brasil e Índia. As vacinas hoje existentes se baseiam em bacterinas de sorovares mais comumente encontrados na espécie a ser vacinada, mas essas vacinas não desenvolvem uma imunidade duradoura ou de proteção cruzada. As vacinas de subunidade com proteínas recombinantes possuem potencial para produção de uma vacina eficiente contra a leptospirose uma vez que podem oferecer proteção cruzada. LigA é uma proteína que possui função de adesão ao tecido do hospedeiro e já foi relatada como protetora contra o desafio letal por *Leptospira interrogans*. A primeira parte deste documento versa sobre a definição de uma região protetora de três segmentos constituída pelos domínios 11 e 12, acrescidos do domínio 10 ou 13. O efeito da vacina foi avaliado com base na patologia, resposta imune humoral e quantificação da carga microbiana nos rins. A segunda parte deste trabalho descreve a cinética da infecção por *Leptospira interrogans* em hamster, de forma a apontar como o organismo reage durante a primeira fase da infecção leptospírica. Essa avaliação foi realizada por meio de quantificação da carga microbiana em órgãos como rins, fígado, pulmões e baço, além de acompanhamento da evolução da doença por intermédio de hemograma e análises bioquímicas. Os dados analisados mostram que a maior carga bacteriana é observada após 6 dias de infecção em ambas as rotas.

Palavras-chave: Leptospira. Leptospirose. Tempo real. Quantificação.

Abstract

COUTINHO, Mariana Loner. ***Leptospira interrogans*: cinética da infecção e avaliação da proteção conferida por LigA.** 2011. <89>f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Leptospira interrogans is the usual causative agent of leptospirosis, a zoonosis that is endemic in tropical countries like Brazil and India. The available vaccines are bacterin-based from the more prevalent serovars to the vaccinated specie, but these vaccines do not develop long-lasting immunity or cross-protection. Subunit vaccines with recombinant proteins have the potential to generate an efficient leptospirosis vaccine. LigA is a protein that has adhesin function to the host and has been reported as protective against lethal challenge of *Leptospira interrogans*. The first part of this document defines a three-segment protective region of LigA constituted as domains 11 and 12, added of domains 10 or 13. The vaccine effect was evaluated based on pathology, humoral immune response and kidney leptospiral burden. The second part of this work aims to point out the importance of the correct understanding of the *Leptospira interrogans* infection kinectics to elucidate how the organism reacts in the first phase of the leptospiral infection. This evaluation is accessed by leptospiral load quantification in key-organs such as kidneys, liver, lungs and spleen, and by the evolution of the disease by hemmogram and biochemical analysis.

Keywords: Leptospira. Leptospirosis. Real-time. Quantification.

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Sumário

1 Introdução

A leptospirose é causada por bactérias do gênero *Leptospira, a qual pretence à ordem das Spirochaetales*, e difere das outras espiroquetas pela presença de ñganchosò nas extremidades (WHO, 2003). A leptospirose é considerada uma doença zoonótica que atinge principalmente países tropicais. No Brasil o número de casos é variável, com 4.000 casos confirmados laboratorialmente a cada ano, mas esse número pode variar muito uma vez que a notificação não é obrigatória e que muitos casos não são confirmados e/ou apresentam sintomatologia clínica muito branda. As leptospiras patogênicas são mantidas na natureza nos hospedeiros reservatórios que são espécies nas quais a infecção é endemica e é perpetuada pelo contato direto dos animais (LEVETT, 2001).

A classificação antiga por patogenicidade agrupava as leptospiras em duas espécies diferentes denominadas *Leptospira interrogans* (patogênica) e *Leptospira biflexa* (saprofítica). Este sistem foi substituído por uma classificação genetic que contém 7 espécies patogênicas, 6 espécies de patogenicidade intermediária, e 6 espécies saprófitas.

A classificação genetic dos sorovares é determinada pelo perfil de hibridização DNA-DNA obtido e confirmado pela técnica de multiloccus enzyme electrophoresis (LEVETT, 2001). Este método de classificação permite que um serovar seja classificado em duas species diferentes ao mesmo tempo dependendo da cepa utilizada (BRENNER *et al*., 1999).

Tabela 1: Espécies descritas

	Patogênicas	Patogenicidade Intermediária	Saprofíticas
1	*L. alexanderi*	*L. alstonii*	*L.biflexa*
2	*L. santarosai*	*L. kmetyl*	*L. meyeri*
3	*L. borgpetersenii*	*L. wolffii*	*L. yanagawae*
4	*L. noguchi*	*L. licerasiae*	*L. tersptrae*
5	*L. interrogans*	*L. broomii*	*L. vanthielii*
6	*L. weilii*	*L. inadai*	*L. wolbachii*
7	*L. kirschneri*		

1.1 Epidemiologia da leptospirose

Os reservatórios naturais das espécies patogênicas são roedores como *Rattus norvegicus* e animais selvagens, os quais carreiam a bactéria em seus túbulos renais e disseminam os organismos na sua urina. Humanos e animais domésticos são infectados com leptospiras pelo contato com urina ou tecidos de animais infectados ou pela exposição indireta à agua contaminada. Em países desenvolvidos, é normalmente associada com atividades recreacionais, como canoagem (SEIVAR, *et al*, 2003) ou atividades profissionais que envolvem exposição ao sistema de esgoto ou trablhadores de abatedouros (CENTERS FOR DISEASE CONTROL AND PREVENTION, 1998). Em países em desenvolvimento, a doença primariamente ocorre em áreas com pouco saneamento, nas quais há a ocorrência de esgotos abertos ou áreas que são propensas à inundações após chuva severa (KO *et al*., 1999).

As leptospiras provavelmente não podem penetrar a pele intacta; elas normalmente penetram no hospedeiro através de pequenas lesões na pele, conjuntiva ou sistema reprodutivo, apesar de existirem alguns relatos de infecções iniciadas no sistema digestório ou através de mordidas de ratos (LUZZI, G. A, *et al*, 1993 and GOLLOP *et al*, 1987), mas estas não são freqüentes e provavelmente são acidentais. Uma vez dentro do hospedeiro, as leptospiras se espalham por órgãos como fígado, coração, rins, pulmões, cérebro, músculo esquelético, glândulas adrenais e sangue. A imunidade desenvolvida pela infecção é principalmente humoral a qual opsoniza as bactérias e induz fagocitose por macrófagos. A maioria dos anticorpos produzidos reage contra o LPS da leptospira e a imunidade conferida é sorovar-específica (FAINE *et al*., 1999).

A leptospirose é normalmente classificada como uma doença emergente, e isso se deve ao fato de o número total de casos de leptospirose humana não ser conhecido. Na maior parte dos países a leptospirose não é uma doença de notificação obrigatória às autoridades, resultando em dados epidemiologicos não confiáveis. É estimado que entre 350.000 à 500.000 pessoas são acometidas pela forma severa da doença, mas o número pode ser muito maior uma vez que a maioria dos casos de leptospirose não são severos (HARTSKEERL *et al.* 2011). No Brasil o número de pacientes confirmados de leptospirose é em torno de 4.000 por ano, com picos óbvios em áreas que sofreram inundações recentes. A taxa de mortalidade é entre 10 à 15% (DADOS EPIDEMIOLOGICOS, MINISTÉRIO DA SAÚDE, 2010).

1.1.1 Hospedeiro Reservatório

Ratos e camundongos desempenham um importante papel na disseminação das leptospiras no ambiente uma vez que albergam as espiroquetas nos seus rins sem demonstrar a presença de nenhuma patologia séria além de nefrite intersticial. A disseminação das leptospiras pode ocorrer a partir dos sexto dia após a infecção com uma carga microbiana de $1x10^5$ à $1x10^7$ leptospiras/ mL (MONAHAN *et al*, 2008) e durar por vários meses, sem apresentar uma perda significativa de peso ou sinal clínico (NALLY *et al*, 2005). Foi demonstrado que nefrite intersticial é o achado patologico mais proeminente em ratos infectados experimentalmente ou capturados no sistema de esgoto da cidade de Salvador (Brasil) e que tinham culturas renais positivas (FARIA *et al*. 2007).

Algumas species são consideradas hospedeiros de manutenção para um serovar específico tais como Icterohaemorrhagiae e Ballum em ratos, sorogrupo Ballum em camundongos, Pomona, Tarassovi e Bratislava em suínos, Hardjo e Pomona em ovinos e canicola em caninos (LEVETT, 2001). Em bovinos o sorovar mais importante é o Hardjo, que geralmente causa uma infecção subclínica. A adaptação da bacteria ao seu hospedeiro é exemplificada pela adaptação de *L. borgpetersenii* sorovar Hardjo ao gado europeu. Neste caso, a adaptação à trasmissão direta resultou emu ma redução genômica (BULACH *et al*., 2006), levando à perda da capacidade de sobrevivência no meio-ambiente e limitando a sua transmissão ao contato direto entre hospedeiros.

1.1.2 Hospedeiro acidental

Ao contrário do hospedeiro reservatório, a leptospirose em um hospedeiro accidental normalmente causa manifestações clínicas mais severas. A infecção leptospírica em bovinos recebe muita atenção devido à sua importância econômica devido à problemas associados com reprodução, lactação e riscos aos humanos.

A infecção bovina pelos sorovares Hardjo, Pomona e Grippotyphosa geralmente resulta em infecção subclínica a qual, quando não tratada, pode levar à infecção crônica e perdas econômicas como baixa taxa de crescimento, agalactia e aborto. O estado crônico resulta emu ma eliminação continua de bactéria na urina e, consequentemente, disseminação aos animais não infectados e descendentes. Entretanto, quando o gado é infectado com outros sorovares, uma infecção mais aguda e severa é gerada com uma pletora de sintomas, que tipicamente incluem febre, anemia, hemoglobinúria e congestão pulmonar (FAINE, 1999).

A leptospirose aguda bovina geralmente ocorre em animais jovens e apresenta sintomas severos como septicemia com febre, anorexia e anemia hemolítica aguda, hemoglobinúria e icterícia, e em alguns casos pode progredir para meningite e morte. Diminuição severa na produção de leite e hemolactia podem ocorrer em vacas em lactação infectadas com os sorovares hardjo-pratijno ou hardjo-bovis; a diminuição na produção de leite pode durar de 2 à 10 dias com o leite apresentando uma coloração amarela, um alto númeor de células somáticas e pode até mesmo conter coágulos sanguíneos. Apesar da recuperação dos animais ocorrer após 10 dias, a produção de leite pode não retornar ao nível original até o final daquele período de lactação (ELLIS, *et al*, 1984).

A leptospirose possui uma variação grande de sintomas em humanos, que podem variar entre sintomas parecidos com uma gripe até falência renal aguda, hemorragia pulmonary e morte, os sinais mais comuns incluem febre, calafrios, cefaléias, mialgias severas, náusea, emese e prostração (DOLHNIKOFF *et al*. 2007), com 20 à 70% dos casos apresentando envolvimento pulmonar com hemorragia alveolar. Foi relatado que o envolvimento pulmonar é indicative de mau prognóstico (SPICHLER, *et al*. 2008) e é observado 4 à 6 dias após a infecção e pode levar rapidamente à morte. Outros indicadores de mau prognóstico em humanos são oligúria, baixa contagem de plaquetas (<70,000), creatinine elevada (>3mg/dL) e idade (>40 anos).

1.1.3 Apresentação

Este documento é composto por três artigos que foram ou serão submetidos à publicação em periódicos científicos e indexados. O primeiro artigo é uma introdução ao tema de modelos animais atualmente usados em pesquisa de leptospirose. Este artigo discute a epidemiologia da leptospirose nos hospedeiros reservatório e acidental e, posteriormente, foca nos modelos animais utilizados em pesquisa tal como hamsters, porquinho-da-índia, camundongos e ratos; considerando a condição de hospedeiro acidental ou reservatório. Considerações são feitas sobre o uso de ratos e camundongos, uma vez que eles não são acometidos por leptospirose e, portanto, podem ser usados como modelos de infecção crônica ou em testes de virulência pela utilização de camundongos *knock-out.* Como modelos de infecção aguda utilizam-se mais frequentemente os hamsters e porquinhos-da-índia, que apresentam sintomas característicos da doença. É ainda objeto desta revisão uma abordagem sobre a patogênese da infecção e os mecanismos de resposta imune da leptospira em diferentes ambientes. Ao final é feita uma abordagem das vacinas existentes comercialmente formuladas de bacterinas e as vacinas de subunidade recombinantes.

Existem várias vacinas de bacterinas comercialmente disponíveis para uso em animais, mas infelizmente elas não induzem proteção longa ou cruzada, o que demanda a revacinação anual mais prevalentes na região (SRIKRAM *et al*, 2011). Atualmente o foco das pesquisas em vacinas se dá em entender as razões que levam à proteção conferida pelas bacterinas (BROWN *et al*, 2003 e SRIKRAM *et al*, 2008), ou ao desenvolvimento de vacinas de subunidade recombinante composta por proteínas de membrana como a LigA presentes em vários sorovares como a LigA (SILVA *et al*, 2007), que protege embora ainda não seja conhecida quão severa seja a infecção em animais immunizados. O primeiro artigo referente à pesquisa desenvolvida foi submetido e aceito pelo periódico PloS Neglected Tropical Diseases e versa sobre a produção de vacina recombinante contra LigA de *Leptospira interrogans*, mais especificamente sobre a identifição da menor região protetora. É descrito a avaliação da patologia e funções renais e hepáticas dos animais sobreviventes, bem como determinado a carga bacteriana nos rins e a resposta immune obtida.

A Leptospira penetra na pele por abrasões ou em membranas intactas (LEVETT, 2001), porém a rota mais utilizada para infecção experimental é a intraperitoneal (IP) pois reproduz a doença mais facilmente e é de fácil execução técnica. A desvantagem da utilização dessa rota no estudo da leptospirose é a eliminação da proteção immune conferida pela pele, podendo levar à presunções errôneas de como a infecção natural se desenvolve. O segundo artigo desta tese, a ser submetido ao periódico PloS Neglected Tropical Diseases, dedica-se ao estudo de differentes formas de inoculação de leptospira utilizando duas rotas: intradérmica e sub-cutânea. A análise foi feita através do acompanhamento da cinética da disseminação da infecção em órgãos e das funções renal e hepática no momento da eutanásia. A produção de anticorpos foi avaliada de forma a identificar o momento no qual a resposta imune humoral começa a ser eficaz.

2 Artigo 1: Animal Models of Leptospirosis

2.1 Animal Models ï How well do they reproduce infection

2.1.1 Immunology

In experimental infections, rats usually require several folds more of leptospira inoculum than hamsters or guinea pigs. One important immunological feature is that mouse Toll-like receptor 4 (TLR4) and TLR2 recognizes LPS, while in humans the leptospiral LPS is only recognized by the TLR2 (CHASSIN *et al*., 2009). TRL2 knock-out mice are resistant to leptospiral infection, while TLR4 has an intermediate survival curve when compared to double knock-out mice. Another finding is that the TLR4-/- mice had higher liver bacterial load than the wild type, but both TLR2 -/- and TLR4 -/- had comparable kidney bacterial loads to WT.

2.1.1.1 Histopathology

Both hamsters and guinea pigs are considered good animal models for leptospirosis. Hamsters present renal pathology before 10 days after the inoculation, with damage of tubular epithelia with cortical cellular necrosis (SILVA *et al*, 2008), shrunken glomeruli with the presence of protenaiceous material (BARNETT *et al*, 1999), and petechial hemorrhages in the glomeruli and proximal tubules. At necropsy, organs are usually discolored due to icterus and kidneys can present areas of capsular depression. The major finding in kidneys is interstitial nephritis (LEVETT 2001).

Liver can present dissociation of hepatic trabecula with cytoplasmic size variation (SILVA *et al*, 2008); and edema of liver cells with deformity of hepatic cords (HE *et al*, 2008), lungs can show widespread bleeding with pulmonary congestion and alveolar hemorrhage (DOLHNIKOFF *et al*, 2007).

2.1.1.2 Challenge routes

Leptospira usually infects an organism by a breakage in the skin or intact membranes, only a few minor reports have included the oral route of infection or infection through bites (LEVETT, 2001). It is commonly used and widely accepted that the intraperitoneal (IP) route is the route of choice, since it is easy to perform and does reproduce infection more easily and faster than other routes.

In an early study (STAVITSKY, 1945), guinea pigs and hamsters were used to test different routes of infection and it was shown that blood cultures were positive with IP and intracardiac (IC) after only one hour, subcutaneous (SQ) after 24 h and oral (O), intradermal (ID) and intraocular (IO) after 48 h with the onset of jaundice between 5 to 10 days post infection, for SQ and O routes respectively. In this report, with the IP route, it was possible to isolate leptospires from blood, kidneys, bone marrow, liver and adrenal glands from guinea pigs, but hamsters only presented positive cultures in blood at euthanasia.

A recent kinetics study that used the IP route in hamsters showed a high liver and kidney colonization, with leptospires reaching 1×10^5 and 5×10^4 copies /µg of tissue DNA, respectively.

Although the IP route has been used to study leptospirosis, it has a caveat of eliminating the protection conferred by the immune system of skin and membranes which can lead to erroneous assumptions on how the natural infection really works. Also, leptospires have OMPs that can undergo transcriptional changes depending on the environment (as discussed later), therefore eliminating the invasion step that leptospires go through when entering a body can result in underexpression or overexpression of different proteins that do not mimic a natural infection.

2.2 Pathogenesis of infection

2.2.1 Leptospiral adhesion

The first step in the leptospiral infection of the organism is the adhesion to the tissue, either intact membranes or broken skin. *Leptospira* has the ability to adhere both to host cells as well as extra-cellular matrix (EMC); it was demonstrated that the adherent leptospires rapidly invade fibroblasts, renal cells and macrophages *in vitro* (MÉRIEN et al, 1997 and 2007).

Several leptospiral outer membrane proteins have been proven to adhere to ECM structures such as collagen type I and IV, laminin and fibronectin (BARBOSA *et al*, 2006, CHOY *et al* 2007). LigB has been shown to bind fibronectin and fibrinogen and inhibits fibrin formation, which might help the leptospiral adhesion at the site of infection (CHOY *et al*, 2011). OmpL37 is a new protein that was recently found to bind to human skin and aorta elastin (table 2), both of which could help in the host adhesion process (PINNE *et al*, 2010).

Table 2: Leptospiral proteins that bind to ECM

Protein	Ligand	Reference
LigB	Collagen, laminin, tropoelastin and elastin Fibronectin modulated by calcium and fibrinogen	CHOY *et al*, 2007 ; LIN YP, CHANG YF 2007; LIN *et al*, 2009; LIN YP, CHANG YF 2008; LIN *et al*, 2009;
OmpL37	Skin and aorta Elastin	PINNE *et al*, 2010

LenA	collagen IV, laminin and fibronectin, human plasminogen and complement regulator factor H	ATZINGEN M.V.,*et al*, 2008 and VERMA, *et al* 2010

2.2.2 Complement evasion

After the entry and adhesion to the host tissue, leptospires have to overcome the hostôs innate immune system (CARROLL, M. C. 2004). Non-pathogenic leptospires are susceptible to the human complement (CINCO *et al*., 1983). Pathogenic leptospires express Len proteins (STEVENSON *et al*, 2007, VERMA *et al*, 2006 and BARBOSA *et al*, 2006), which are proteins that binds to factor H, a complement component that signals self-proteins and inactivates C3b bound to the membrane. The Len family is composed of 6 proteins named Len A, B, C, D, E and Len F and bind to fibronectin. Pathogenic leptospires also bind C4BP, from the classical complement pathway, which is a cofactor of factor I that inactivates C4b (BARBOSA *et al*, 2009).

2.3 Leptospiral response to the host environment

2.3.1 Outer membrane proteins ï reviews definition, types

As mentioned before the leptospiral LPS has unique features that are highly variable amongst serovars due to O-side chains sugars variability (HAAKE and MATSUNAGA, 2010), and it is the major component of the leptospiral membrane. There are several outer membrane proteins (OMPs) and transmembrane OMPs that have been the focus of intense research in the last decade, some of them are only expressed in pathogenic serovars (CULLEN *et al*., 2004; MATSUNAGA *et al*., 2005;

CULLEN *et al*., 2005). A large group of OMPs are the lipoproteins such as LipL32, LipL21, LipL36, LipL48 e LipL41. LipL32 is definitely the most abundant protein in the leptospiral membrane although it is not entirely expressed on the surface; most of it can be found after membrane permeabilization (PINNE *et al*, 2009). While LipL32, LipL41, LipL21 are expressed both *in vivo* and *in vitro*, LipL36 is anchored to the inner face of the outer membrane and is only expressed *in vitro* (HAAKE *et al*., 2000).

Other proteins are the OmpL1, a transmembrane protein with porin function, and P31LipL45, is a peripherical protein that uses the lipoprotein secretory channel to reach the inner and outer membranes (CULLEN *et al*., 2003).

The Leptospiral Immunoglobulin-Like (Lig) protein family is composed by 2 proteins named LigA (PALANIAPPAN *et al*., 2002) and LigB (MATSUNAGA *et al*., 2003) and a pseudo-gene, *ligC*. LigA has a secreted form and both proteins have adhesion and invasion function. These proteins are recognized by the sera of naturally infected patients and their expression occurs mostly *in vivo* with a marked decrease in expression after a few rounds of culture (MATSUNAGA *et al*, 2003).

2.3.2 Regulation of expression in response to host-like conditions

Leptospires have an unusually large number of transcriptional regulators indicating their ability to respond to environmental conditions. In terms of environments, leptospires encounter outside a wide range, like mud, to eye conjunctiva. Lig proteins have been shown to superexpress *in vitro* when osmolarity is equal to physiological osmolarity (MATSUNAGA *et al*, I&I 2007).

In a recent study, iron limitation was found to be a major transcriptional regulator for almost 100 genes (LO *et al*, 2010). Another relevant factor is the temperature shift from environment to host, with hundreds of proteins being up-or down-regulated, most of which are not characterized yet (LO *et al*, 2006)

2.4 Progress Towards a Subunit Vaccine

2.4.1 Where are we now: whole cell bacterins

There are several commercially available vaccines against leptospirosis both for animals and humans. They are all made with bacterins from the most important serovars that affect each species (FAINE, 1999). Although they protect well against infection they lack several characteristics of an ideal vaccine, e.g., they do not provide long-lasting immunity which requires annual boosters, they do not protect against several serovars or serogroups, and they lack coverage if there is a geographical niche for a specific serovar not predicted by the manufacturer. Human vaccines are only available in a few countries such as China, Japan, Vietnam and Cuba (ADLER and MONTECZUMA, 2010) and have the same shortcomings as veterinary vaccines.

2.4.2 Research on new vaccines (including recombinant)

The use of live attenuated or bacterin vaccines in animals has focused on how the vaccine works and what are the requirements for cross-protection (SRIKRAM *et al*, 2011) and efficient immune response (BROWN *et al*, 2003), like the induction of Th1 immune response is more efficient in preventing leptospiral infections. The development of inflammatory cytokines such as TNF-Ŭ and IFN-ɔ during infection seem to correlate to a poor outcome, while IL-4 and IL-10 are favorable to disease resolution (SRIKRAM *et al*, 2008).

A lot of effort has been put on developing recombinant vaccines against leptospirosis; so far the main focus of research has been the discovery of a protective protein that is expressed in multiple pathogenic serovars such as LipL32. Despite LipL32 being the most abundant protein on the leptospiral membrane and generating a high antibody titer, it does not completely protect against lethal infection

(BRANGER *et al*., 2001, BRANGER *et al*., 2005 SEIXAS *et al*., 2007) which suggest that a LipL32 vaccine could be used in combination with other protective proteins.

Since the first report of partial protection with a recombinant leptospiral protein (HAAKE *Et al*, 1999), a lot of effort has been put into finding the protein that would not only prevent from lethal infection, but other less severe forms of the disease and shedding.

In this matter, the final portion of LigA has been the most successful, several unrelated groups reporting protection in hamsters, LigA was tested under multiple forms such as recombinant protein (SILVA *et al*, 2007), DNA vaccine (FAYSAl *et al*, 2007) or liposomes (FAISAL SM *et al*,2009). Although it has been reported as a protective protein, there is an urgent need to clarify other features of the protection conferred by this protein. It is known that it does not prevent leptospiral shedding in urine, but it remains unclear how well does LigA provides cross-protection against other serovars, how long does immunity last and what would be the best adjuvant to be used for this protein.

3 Objetivos

3.1 Geral

Conhecer a região protetora de LigA de *Lepstospira interrogans* e avaliar a cinética da infecção experimental.

3.2 Objetivos Específicos

- Determinar a região minima requerida de LigA recombinante para eficácia da vacina;
- Estabelecer um critério quantitativo de ponto final em experimentos de pesquisa em leptospirose que utilizam hamsters;
- Avaliar a prevenção de leptospirose em tecidos e sangue em hamsters vacinados;
- Avaliar a cinética da infecção leptospírica em rotas de infecção mais naturais que a intraperitoneal em hamsters;
- Analisar a progressão da leptospirose em hamsters;

4 Paper 1 - A LigA Three-Domain Region Protects Hamsters from Lethal Infection by *Leptospira interrogans*

(Text formatted as used by the scientific journal PLoS Neglected Tropical Diseases)

A LigA Three-Domain Region Protects Hamsters from Lethal Infection by *Leptospira interrogans*†

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Abstract

The leptospiral LigA protein consists of 13 bacterial immunoglobulin-like (Big) domains and is the only purified recombinant subunit vaccine that has been demonstrated to protect against lethal challenge by a clinical isolate of *Leptospira interrogans* in the hamster model of leptospirosis. We determined the minimum number and location of LigA domains required for immunoprotection. Immunization with domains 11 and 12 was found to be required but insufficient for protection. Inclusion of a third domain, either 10 or 13, was required for 100% survival after intraperitoneal challenge with *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130. As in previous studies, survivors had renal colonization; here we quantitated the leptospiral burden by qPCR to be 1.2 x 10^3 to 8 x 10^5 copies of leptospiral DNA per microgram of kidney DNA. Although renal histopathology in survivors revealed tubulointerstitial changes indicating an inflammatory response to the infection, blood chemistry analysis indicated that renal function was normal. These studies define the Big domains of LigA that account for its vaccine efficacy and highlight the need for additional strategies to achieve sterilizing immunity to protect the mammalian host from leptospiral infection and its consequences.

4.1 Non-technical Author Summary

Leptospirosis is the most widespread bacterial infection transmitted to man from host animals that harbor the bacteria in their kidneys. Human infections caused by the bacterium, *Leptospira interrogans*, frequently result in a life-threatening illness characterized by jaundice and kidney failure. Vaccines are urgently needed to prevent leptospirosis in populations at risk. The leptospiral protein, LigA, is a promising vaccine candidate because it is the first purified protein to be shown to protect animals from fatal leptospirosis. The goal of this study was to determine which of LigA's 13 domains are required for the protective effect. Immunization with domains 11 and 12 was found to be required, but insufficient, for protection. A third domain, either 10 or 13, was required for 100% survival. As in previous studies, residual bacteria were cultured from the kidneys of survivors. However, in contrast to previous studies, we determined the amount of bacterial DNA in the kidneys as a measure of vaccine efficacy. We also examined the kidneys microscopically for signs of damage and measured blood chemistries to assess kidney function. These are important steps towards developing vaccines that provide protection from kidney damage and infection.

4.2 Introduction

Pathogenic *Leptospira* species are globally distributed spirochetes that cause 350,000-500,000 severe human infections annually with an incidence of >10 cases per 100,000 population in humid, subtropical regions of the world and a mortality rate of 10% [1,2,3]. These figures are likely to be underestimates because leptospirosis is a neglected tropical disease that occurs more commonly among medically underserved populations [4,5]. The infection is endemic wherever there is exposure to urine of reservoir host animals that harbour the organism in their renal tubules [6]. At least 18 species and more than 200 leptospiral serovars have been described, many of which were isolated by cultivation of kidneys from a wide diversity of infested wild and domestic animals [1,7]. Environmental contamination of water and soil results in frequent outbreaks of leptospirosis among the poor in developing countries. Leptospirosis is also emerging among participants of aquatic sports and adventure tourism [8,9]. In the urban setting, *Rattus norvegicus* is the most important vector of human leptospirosis [5]. Serovars of *Leptospira interrogans* carried by rats cause life-threatening hepatorenal failure and pulmonary hemorrhage syndromes in tropical regions, especially where heavy rainfall occurs in urban areas with poor sanitation and flood control infrastructure [10]. Commercially available whole-cell bacterin vaccines for prevention of leptospirosis in animals provide relatively short-term serovar-specific protection and require frequent boosters [11]. Although inactivated whole-cell vaccines have been administered to humans, they are rarely used today because of their reactogenicity. Thus, there is an urgent need for development of novel vaccine strategies that provide safe, long-term, cross-protective immunity.

Recombinant surface-exposed outer membrane proteins (OMPs) are attractive subunit vaccine candidates because in contrast to the lipopolysacchride, leptospiral OMPs are relatively well conserved and those that are surface-exposed represent potential targets for immune-mediated defense mechanisms. We have developed a suite of complementary approaches for determining which leptospiral OMPs are surface-exposed, including surface immunofluorescence, surface biotinylation, surface proteolysis, surface immunoprecipitation, and surface ELISA [12,13,14,15]. Using these approaches, a number of transmembrane OMPs and surface lipoproteins have been identified [16,17]. Despite the rapid increase in knowledge about leptospiral OMPs, progress in understanding their vaccine potential has been slow. Although LipL32 is the most abundant pathogenic leptospiral OMP [18], purified, recombinant LipL32 has no detectable vaccine efficacy [19]. Nevertheless, hamsters immunized with recombinant bacillus Calmette-Guerin expressing LipL32 were partially protected from lethal challenge [20] and there is evidence for immunoprotection employing *lipL32*-containing viral or DNA-based vectors [21,22]. Synergistic immunoprotection has been observed using a combination of leptospiral OMPs, OmpL1 and lipidated LipL41, expressed as membrane proteins in *E. coli* [23].

Leptospiral immunoglobulin-like (Lig) proteins are of great interest as mediators of leptospiral pathogenetic mechanisms, as serodiagnostic antigens, and as effective recombinant vaccinogens [24,25,26,27,28]. At least two of the three members of the Lig protein family are outer membrane lipoproteins containing a tandem series of bacterial immunoglobulin-like (Big) domains [29]. Lig protein expression is associated with virulence and is strongly and rapidly induced by increasing the osmolarity of the culture medium to physiologic levels found in the mammalian host, suggesting that they may be involved in the initial stages of host tissue colonization [30,31]. LigA consists of 13 Big domains, the first six of which are nearly identical in sequence to those in LigB, while the last seven are unique to LigA [32] and mediate interactions with host extracellular matrix proteins and fibrinogen [24,33]. One study has found that the region shared by LigA and LigB was not immunoprotective [27], while another study reported that this region conferred some immunoprotective activity [34]. In contrast, several groups have reported that immunization with the LigA-unique region induced protection from lethal infection either in a mouse model [28] or in the hamster model [27,35] of leptospirosis.

Although hamsters surviving leptospiral challenge were found to have sublethal kidney infection, both the extent of infection and its effects on the kidney, the key target organ in leptospirosis, were not well understood. In this study, we determined which LigA domains are most strongly associated with immunoprotection and the effect of LigA immunization on the burden of infection and the histopathology in the kidney. Our results show that protection from lethal infection required immunization with domains 11 and 12 along with a third domain, either 10 or 13.

4.3 Materials & Methods

Leptospiral strain and cultivation. *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium [36] supplemented with 1% rabbit serum (Rockland Immunochemicals, Gilbertsville, PA) and 100 µg/ml 5-fluorouracil at 30°C in a shaker incubator. Organisms were passaged no more than five times prior to hamster challenge. Hamster tissues were cultured in semi-solid EMJH or semi-solid Probumin Vaccine Grade Solution (Millipore, Billirica, MA) containing 0.2% Bacto agar (BD, Franklin Lakes, NJ) and 100 µg/ml 5-fluorouracil in a stationary incubator at 30°C and were examined for leptospiral growth for up to two months.

Preparation of recombinant proteins. PCR primers were designed to amplify gene fragments encoding various immunoglobulin-like domains from *ligA* of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (Table 1). DNA amplicons, which included *Nde* I and *Xho* I restriction endonuclease sites, were ligated into pET-20b(+) (Novagen), providing a carboxy-terminal His6 tag, and used to transform *Escherichia coli* BLR(DE3)pLysS (Novagen). Protein expression was induced with isopropyl-ß-D-thiogalactopyranoside at 30°C and soluble proteins were released with BugBuster (Novagen) and purified with nickel-affinity chromatography as previously described [25]. All proteins were stored at 4°C after dialysis in PBS.

Hamster immunization. Groups of four female Syrian hamsters, 5 to 6 weeks of age (Harlan Bioscience, Indianapolis, IN), were immunized subcutaneously with 100 µg of recombinant protein, PBS, or 1 x 10^8 heat-killed (56°C for 1 h) leptospires (HKL) in a total volume of 0.5 mL on days 0, 14 and 28 with Freündôs adjuvant

(complete adjuvant for the first immunization, incomplete adjuvant for subsequent immunizations). Blood samples were obtained two days before the first immunization and 10 to 12 days after each immunization *via* the retro-orbital route. All animal procedures were approved by the Veterans Affairs Greater Los Angeles Healthcare System Institutional Animal Care and Use Committee and adhere to the United States Health Research Extension Act of 1985 (Public Law 99-158, November 20, 1985, ñ*Animals in Research*ò), the National Institutes of Healthôs *Plan for Use of Animals in Research* (Public Law 103-43, June 10, 1993), U.S. Government Principles for the Utilization and Care of Veterbrate Animals Used in Testing, Research, and Training, Public Health Service Policy on Humane Care and Use of Laboratory Animals, the United States Department of Agricultureôs Animal Welfare Act & Regulations, and Veterans Health Administration Handbook 1200.7.

ELISA. Ninety-six-well ELISA microtiter plates (Immulon 4HBX,Thermo Fisher, Waltham, MA) were coated either with 100 µL of 10 µg/mL of recombinant LigA protein or 1 x 10^9 heat-inactivated leptospires/mL diluted in PBS, pH7.2 (Invitrogen, Carlsbad, CA), by overnight incubation at 4°C. The plates were allowed to warm to room temperature (RT), washed once with 200 µL of PBS, and blocked with Protein-Free Blocking Buffer (PFBB, Thermo Fisher, Rockford, IL) for 1 to 2 h at RT. Wells were washed with PBS, sera diluted with PFBB were added in a volume of 100 µL, and plates were incubated for 1 h at 37°C. Non-binding antibodies were removed with three PBS washes, and Horseradish peroxidase (HRP)-conjugated anti-Syrian hamster immunoglobulin secondary antibody (Jackson ImmunoResearch, West Grove, PA) 1:5000 was incubated for 30 min at RT. Following three washes with PBS, 100 µL of 1-Step Turbo TMB HRP substrate (Thermo Fisher) was added and incubated for 30 min at RT with shaking. The reaction was stopped by the addition of 50 µL of 2 M H_2SO_4, and plates were immediately read in a Bio-Rad 550 Microplate Reader at 450 nm. End-point titers were defined as the highest titer that yielded a reading two standard deviations above the result with sera from PBS-immunized hamsters. Geometric mean end-point titers were calculated as previously described [37].

Challenge and sample collection. Fourteen days after the third immunization (day 42), hamsters were challenged intraperitoneally with 1 x 10^3 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 in 0.5 mL of EMJH. The

animals were weighed daily and observed for end-point criteria, including loss of appetite, gait or breathing difficulty, prostration, ruffled fur, or weight loss of Ó10% of the animalôs maximum weight. Animals that reached end-point criteria were euthanized with isoflurane and tissue samples were collected in formalin for histopathology or incubated overnight at 4°C in RNAlater (Ambion, Austin, TX) and stored at -80°C. Processing tissues for histopathology involved formalin fixation, paraffin embedding, sectioning, and periodic acid Schiff (PAS) stains in a Dako automated slide processor. Blinded scoring of kidney sections used a scale of 0 to 5 for the extent of histopathology, ranging from normal to severe renal tubular damage, based on the degree of hyaline cast deposition, interstitial inflammation, mitosis, Bowman's space dilation, tubular atrophy and associated capsular depression. Blood was collected for serology and chemistry analysis (Antech Diagnostics, Irvine, CA). 100 µL of blood or pulverized kidney or liver were inoculated into semi-solid medium at dilutions of 1:100 and 1:10,000 and incubated at 30°C.

Microscopic agglutination test (MAT). Sera collected at euthanasia were examined at a 1:50 dilution by MAT as previously described [38] with live *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. Briefly, heat-inactivated serum, diluted in physiologically buffered water, pH7.6, was incubated overnight at 4°C with 2 to 4 x 10^8 leptospires/ mL and examined under dark-field microscopy for > 50% reduction in the number of free leptospires when compared with serum from uninfected animals.

Quantitative PCR (qPCR). Kidneys were stored in RNAlater and DNA was extracted with DNeasy Blood and Tissue kit according to the manufacturer instructions (Qiagen, Valencia, CA) with modifications. 15 to 25 mg of kidney were immersed in 360 µL of ATL buffer and the tissue was homogenized in a 24-Fast Prep tissue homogenizer (MP Biomedicals, Solon, OH) using lysing matrix A with a setting of 6 m/s for 40s. 40 µL of proteinase K at a concentration of 15 mg/mL of protein were added and the samples were incubated for 3 h at 37°C. Two volumes of AL buffer-ethanol (1:1) were added and the mixture was applied to a spin column, on which the bound DNA was washed with washing solutions 1 and 2 and eluted with 200 µL of AE buffer-water (1:4). The purified DNA was stored at -80°C until use.

The extracted DNA was used in a qPCR using the Bio-Rad iQ5 Real-time System (Bio-Rad, Hercules, CA). 100 ng of total DNA was combined with 1 µM of each primer and 12.5 µL iQ SYBR Green Supermix (Bio-Rad) and brought to a final volume of 25 µL with nuclease-free water (Ambion). 4 samples were run per group and each sample was run in duplicate. qPCR primer pairs were LipL32-f, 5-CGCGTTACCAGGGCTGCCTT-3ǒ and LipL32-r, 5ǒCGCTTGTGGTGCTTTCGGTG-3ǒ and hamster GAPDH-f, 5ǒCTGGTTACCAGGGCTGCCTT-3ǒ and GAPDH-r, 5ǒ CCGTTCTCAGCCTTGACTGTGC-3ǒ resulting in amplicons of 152 bp and 146 bp, respectively. The PCR protocol consisted of an initial incubation step at 95°C for 12.5 min followed by 40 cycles of amplification (95°C for 15 s, 57°C for 30 s and 72°C for 30 s). The level of the *lipL32* gene of L*. interrogans* was normalized to that of hamster *gapdh*, using Bio-Rad iQ5 software and Microsoft Excel. Standard curves were generated for each gene ranging from 10 to 1.6 x 10^6 copies of *Leptospira* (20-fold dilutions) and 0.02 to 200 ng (10-fold dilutions) of hamster DNA.

Statistics. Survival differences between groups were analyzed by Fisherôs Exact Test using GraphPad InStat version 3.10 (GraphPad Software Inc., La Jolla, CA). One-way analysis of variance (ANOVA) was used to test for differences between multiple (Ó3) groups using a P value < 0.05. For ordinal data, such as the histopathology scores, the Kruskal-Wallis one-way ANOVA with Dunn's post-test was included. The unpaired, two-tailed Studentôs t-test assuming unequal variance was used to test for differences between two groups using a P value < 0.05.

4.4 Results

Recombinant LigA proteins and hamster response to immunization. Eight clones were designed to express recombinant proteins corresponding to various LigA domains from the second half of domain 7 to domain 13 (Table 1) of *L. interrogans* serovar Copenhageni. All proteins were expressed and purified as soluble proteins and found to be stable at 4°C after dialysis in sterile PBS. These proteins were employed as hamster immunogens in two independent experiments (#1 and #2) and as antigens in an indirect ELISA to measure the corresponding antibody response. As shown in Figure 1, hamsters had higher antibody titers after the third immunization than after one or two immunizations (one-way ANOVA with test for linear trend, $P < 0.05$), except in the HKL (experiment #2) and LigA7ϴ11 groups. There was no correlation between the antibody titer and the number of domains in the LigA protein (Pearson correlation coefficient 0.29, $P > 0.05$).

Immunoprotective LigA domains. Hamsters were challenged with virulent *L. interrogans* via the intraperitoneal route and observed daily, with a 10% decrease in body weight included as an end-point criterion. Body weight was found to be a useful measure of the response of animals to challenge; a decrease in body weight was the earliest observable sign of clinical leptospirosis. In contrast to animals that were immunized with LigA7ϴ13 and exhibited 100% challenge survival (Figure 2A, Table 2), non-surviving animals that were sham-immunized with PBS began to lose weight on day 8 after the challenge and reached -10% of peak weight within 48 h (Figure 2B).

Immunization with different recombinant LigA protein constructs (Table 1) resulted in dramatically different challenge outcomes (Table 2 and Figure 3). In both experiments, there was 100% survival in hamsters immunized with either the LigA7ϴ 13 or LigA10-13 proteins. In experiment #1, immunization with either the LigA7ϴ11 protein or the LigA12-13 protein resulted in < 50% survival. This result indicated that no single LigA domain was sufficient to afford 100% immunoprotection. For this reason, a second experiment was performed to identify the LigA domain(s) and the minimum number of domains required to protect hamsters from lethal challenge. Interestingly, both the LigA10-12 and the LigA11-13 proteins were both effective immunogens, while the LigA11-12 protein consisting of their shared domains

afforded only 25% survival. Taken as a whole, these data indicate that LigA domains 11 and 12 are required but not sufficient to induce 100% survival. A recombinant LigA protein construct consisting of at least three specific Big domains is needed to induce a maximally protective immune response. The protective effect was not merely a reflection of antibody titer; as there was no correlation between survival and geometric mean end-point titer (Figure 1, one-way ANOVA, $P > 0.05$).

Effect of LigA immunization on organ colonization. As previously reported [27], immunization with LigA proteins provided non-sterilizing immunity, as organisms were isolated from the kidneys of animals surviving challenge. Cultures of kidney tissue from all hamsters surviving to 28 days were positive (Table 2). In contrast, only 3 and 10 of 56 animals had positive liver and blood cultures, respectively (data not shown). One non-surviving animal immunized with LigA11-12 had a positive blood culture but negative cultures of the kidney and liver. The residual kidney infection was reflected in lower weight gain of hamsters after challenge (Figure 4). Among the surviving hamsters, those immunized with LigA10-13 had a non-statistical trend of gaining less weight after challenge than those immunized with LigA7ô13 or heat-killed leptospires. Infection resulted in the formation of agglutinating antibodies; the MAT was positive in nearly all LigA-immunized animals surviving for 28 days (Table 2). The only exceptions were one animal from the HKL control group and two from the LigA12-13 group that met end-point criteria early, the latter presumably because these animals had insufficient time to develop agglutinating antibodies.

To more accurately assess the leptospiral burden, DNA from kidneys was analyzed by qPCR. As shown in Table 2 and Figure 5, groups immunized with LigA fragments had a mean of 1.2×10^3 to 8×10^5 copies of leptospiral DNA per microgram of kidney DNA. As expected, kidneys from animals immunized with heat-killed leptospires had a lower leptospiral burden than groups immunized with LigA proteins such as LigA10-12, LigA11-13, LigA10-13 (experiment #1) and LigA7´-13 (experiment #1) (non-parametric ANOVA, Dunn´s post-test, $P < 0.05$). Leptospiral burden appeared to have a significant effect on animal health as reflected in the weight of surviving hamsters; there was an inverse correlation (Pearson correlation coefficient -0.51, $P < 0.05$) in experiment #2 between the percent weight gain during the last week of the experiment and the copies of leptospiral DNA per μg of kidney tissue DNA. However, there were no significant differences in the leptospiral burden

among groups with 100% survival immunized with different LigA proteins (Non-parametric ANOVA, $P > 0.05$).

Pathology. Hemorrhagic areas were frequently noted on gross examination of the kidney and lungs of animals that did not survive challenge. Organs of survivors were usually normal in appearance but the kidneys occasionally appeared shrunken, pale, or had surface depressions indicating underlying infarction. Histopathological changes in the kidneys were largely limited to tubulointerstitial damage. Glomeruli were uniformly unaffected, except for one case of hyaline deposition seen in an HKL-immunized hamster. Although Bowman's space was dilated in some cases, the cells of the glomerulus were unaffected. Tubulointerstitial changes included renal tubular damage, encompassing changes of thinning of renal tubular epithelial cells (compare Figures 6A and 6B), increasing hyaline cast deposition, mitosis, tubular atrophy (Figure 6C), interstitial inflammation (Figure 6D), and associated capsular retraction (Figure 6E). Renal tubular obstruction was the most likely cause of hyaline cast deposition of the material staining intensely with PAS (Figure 6F). Other changes due to tubular obstruction were dilated Bowman's space with or without hyaline casts. Mitoses were seen in only 2 cases, which further supported tubular injury because the rate of tubular cell turnover is normally close to zero. As shown in Table 2, scores based on the extent of renal tubular damage were higher in groups immunized with the LigA10-12 and LigA11-12 proteins, suggesting that immunization with these constructs was associated with relatively more histopathology than other LigA constructs. Groups immunized with HKL and the LigA7ô13 protein had lower renal histopathology scores (Table 2) and there was an inverse correlation between renal histopathology score and weight gain (Pearson correlation coefficient -0.75, $P < 0.01$). There was also an inverse correlation between renal histopathology score and leptospiral burden (Pearson correlation coefficient -0.84, $P < 0.01$) for animals with $> 1.5 \times 10^4$ copies of leptospiral DNA/µg of tissue DNA, suggesting that a more intense immune response (reflected by interstitial nephritis) may be partially effective at clearing residual infection.

Serum chemistries were measured to evaluate liver and kidney function of the hamsters (Table 3). Alanine aminotransferase and alkaline phosphatase levels were moderately elevated in all groups, consistent with hepatitis and cholestasis, respectively. However, bilirubin levels were universally normal, indicating that hepatic

cholestasis had not progressed to biliary obstruction. Blood urea nitrogen (BUN) levels were increased in all groups and extremely elevated in the PBS control, while creatinine was low in all groups and elevated in the PBS control group (one-way ANOVA with Dunnôs post test, $P < 0.05$), indicating that renal dysfunction and/or dehydration contributed to mortality in these animals. In contrast, serum creatinine and BUN levels were universally normal in survivors, indicating that the renal tubular damage observed by histopathology had not progressed to frank kidney failure.

4.5 Discussion

In this study, we identified the LigA domains involved in protecting hamsters from lethal leptospiral infection. Intraperitoneal inoculation was performed with 1000 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, resulting in a lethal infection in all control animals (Table 2, Figure 3). This is the same challenge dose used in a previously successful LigA protection study and is estimated to be ~ 20-fold over the LD_{50} for this strain [27]. We found that a LigA protein construct consisting of at least three Big domains are required for immunoprotection and that the 11^{th} and 12^{th} specific Big domains must be included in this construct. Given that the average pairwise sequence identity among LigA Big domains is only 37% [32], the domains identified here are likely to be antigenically unique and contain unique immunoprotective epitopes. Compared to maximally protective proteins, less protective LigA proteins elicited similar antibody titers in hamsters (Figure 1), suggesting that protection was not solely due to the antigenicity of the respective LigA vaccine. The mechanism of LigA mediated immunoprotection has not been elucidated, but may involve the disruption of a key function of LigA in leptospiral pathogenesis and/or the enhancement of host defense mechanisms. One key function of LigA is to mediate binding of *Leptospira* to host molecules such as fibronectin and fibrinogen [24]. Fibronectin- and fibrinogen-binding activity is found within domains 7 through 13 of LigA, with the carboxy-proximal domains 10 to 13 being required for fibronectin binding (unpublished study, H. A. Choy). Finer mapping of the LigA binding activities may give clues as to the possible immunoprotective mechanism.

As noted previously, LigA immunization converts an otherwise lethal infection into a sublethal kidney infection [27]. The burden of infection and its effects on vaccinated hamsters, qPCR and a histopathology scoring system were included as quantitative outcome measures. To our knowledge, this is the first vaccine study to use qPCR to quantitate leptospiral burden in animals after challenge. The application of qPCR to leptospiral vaccine studies allows for the accurate determination of the leptospiral burden, especially in the kidney, where colonization can lead to kidney damage and/or urinary shedding of the pathogen. We found that the heat-killed leptospires may not confer sterilizing immunity. Although the kidneys from the immunized animals were culture negative, leptospiral DNA was detected by qPCR. Reverse transcription-qPCR studies are needed to determine whether the low levels of DNA in these kidneys represent viable spirochetes or are remnants of leptospires killed by the host immune system. Comparison of quantitation results among surviving hamsters shows that immunization with as few as three LigA domains did not result in significantly higher levels of renal colonization than immunization with longer constructs such as the seven-domain LigA7ô13 protein (Figure 5). However, immunization with LigA10-12 did lead to greater histopathology, indicating different protective effects of the LigA10-12 and LigA11-13 constructs (Table 2).

Histopathology analysis of kidney sections was performed using PAS staining, which is useful for evaluating many different types of nephropathology, including the severity of tubulointerstitial damage in our study. PAS staining facilitated identification of proximal tubules by their carbohydrate-containing brush border, evaluation of tubular basement membrane changes, as well as tubular atrophy (Figures 6A, B, and C). A striking finding of our study was the identification of intensely staining protein casts in the tubules of 32% of animals, both in solid and ñbubblyò deposition patterns (Figure 6E). These protein casts probably represent Tamm-Horsfall glycoprotein (THP), also known as uromodulin or TAMM protein, a glycoprotein that is produced by renal tubular epithelial cells [39]. THP is the most abundant protein in mammalian urine and though its deposition, in and of itself, is not pathologic, the high frequency of THP deposition in our study, including one case with extensive tubular deposition that occurred in an animal that succumbed to acute leptospirosis, suggests that increased THP deposition is related to the pathogenesis of leptospiral renal pathology. These physiologic hyaline deposits are usually solid, but in our study all cases demonstrated both a solid and "bubbly" deposition pattern.

This "bubbly" pattern appeared to be due to a pathological process rather than an artifact of fixation and/or embedding, but further studies are needed to confirm this conclusion.

Insufficient information is currently available to understand how broadly LigA immunoprotection can be applied. Whereas *ligB* has been found in all pathogenic *Leptospira* species, *ligA* has been found in only *L. interrogans* and *L. kirschneri* [32]. *L. interrogans* serovar Lai is the only *L. interrogans* isolate found not to contain *ligA* [40]. If *ligA* deficiency is confirmed in other Lai isolates, this would be a notable exception because the organism is both highly virulent and epidemiologically important. Recently, it was reported that homologous immunization with LigA7-13 that was expressed and purified under denaturing conditions did not protect hamsters against lethal infection by *L. interrogans* serovar Manilae strain L495, an organism that expresses LigA [19]. This result stands in stark contrast to previously successful immunization studies involving *L. interrogans* serovars Manilae (strain UP-MMC-NIID), Copenhageni and Pomona [27,28,41]. Although there were differences in the strains and adjuvants used, the finding that denatured LigA did not protect against lethal challenge could indicate that the protective epitope is conformational rather than linear. Accordingly, our finding that protective segments include domains 11 and 12 plus a third domain (10 or 13) on either end, suggests that three domains may be required for proper conformational folding. Additional research is needed to further define the structural requirements for LigA vaccine efficacy.

We strongly recommend daily weighing of animals in leptospiral challenge experiments, including studies evaluating vaccine efficacy. We found that 10% weight loss effectively identified animals with leptospiral infection that had advanced to a premorbid condition. A similar result was observed in a recent study of leptospirosis in guinea pigs [42]. Weight loss is an objective end-point criterion that avoids uncertainty about whether an animal is able to eat and drink sufficient amounts of food and water. Thus, weight should be monitored along with other clinical parameters as different challenge doses or different strains may not present the same pattern of disease.

In summary, we have mapped the immunoprotective segment of LigA and determined the minimal number of domains necessary to protect hamsters from lethal infection. This work also extends previous studies by quantifying the sublethal burden of infection and by defining the renal histopathological consequences of infection. It is worth noting that the immunoprotective domains we identified are contained within a segment that is known to mediate interactions with host extracellular matrix proteins [24]. This suggests that LigA-mediated immunoprotection may involve interference with key leptospiral-host interactions rather than a bactericidal mechanism. Further studies to define the kinetics of leptospiral infection in immunized animals may provide insight into both the mechanism of LigA-mediated immunoprotection and the development of vaccines for sterilizing immunity against leptospirosis.

4.6 Acknowledgements

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4.8 FIGURES

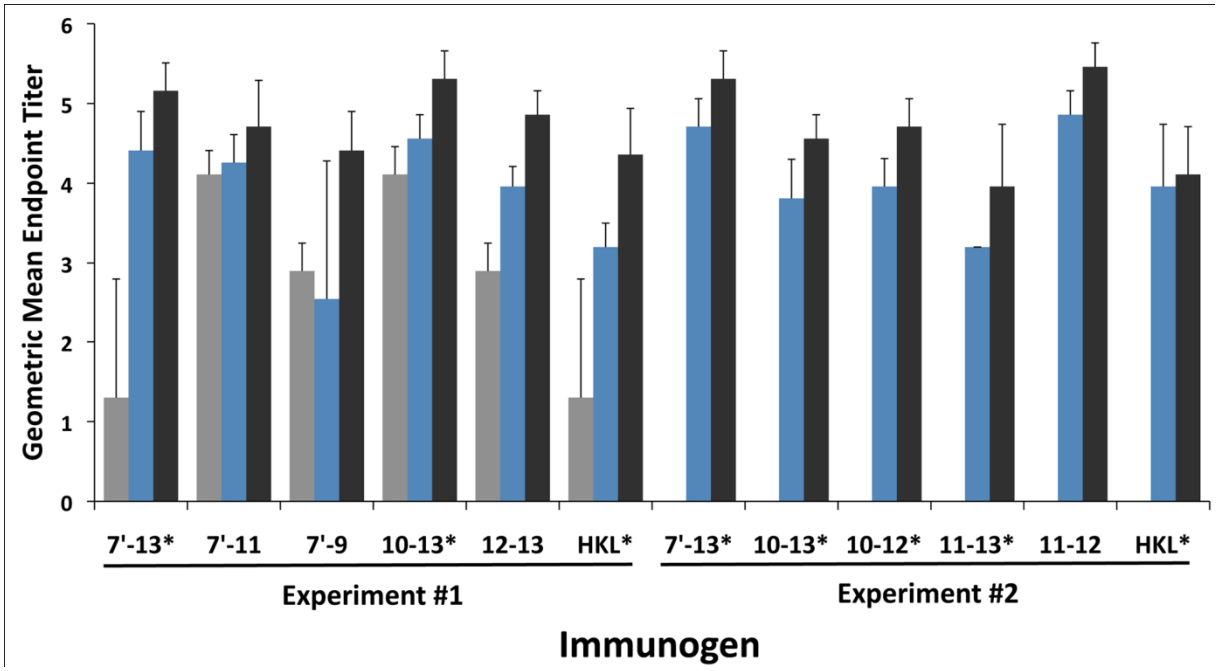


Figure 1: Antibody response in hamsters immunized with recombinant LigA proteins or heat-killed leptospires (HKL).

Total hamster immunoglobulin responses to immunogens were measured by ELISA. Geometric mean end-point titers and standard deviations (n= 4) are shown after the first (gray), second (blue), and third (black) immunizations. In all cases, pre-immune sera were negative. Recombinant LigA proteins are represented by their domain numbers. Asterisks indicate proteins that provided 100% protection against lethal challenge.

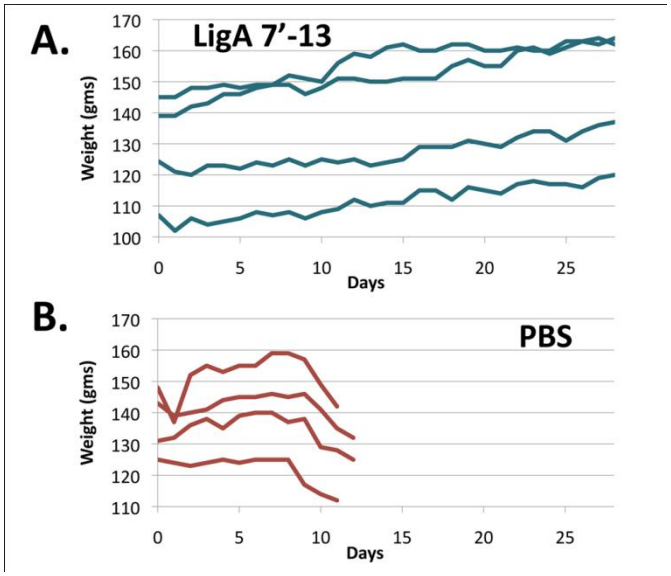
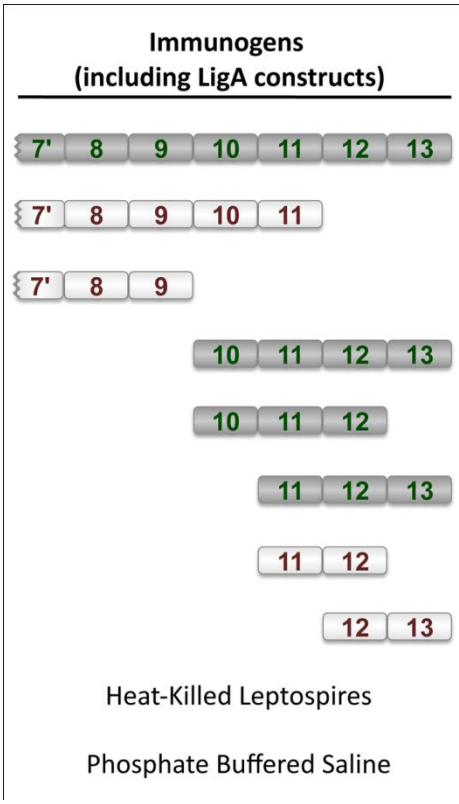


Figura 2: . Hamster weight as an end-point for leptospiral infection. Animals were weighed at the time of challenge and daily thereafter for 28 days.

Data are shown for experiment #2. A. Animals immunized with recombinant LigA7ô13 progressively gained weight (lines represent individual animals). B. Control animals sham-immunized with phosphate-buffered saline (PBS) had stable or increasing weights until day 8 or 9 after challenge, after which they lost weight and met the end-point criterion of a 10% weight decrease (lines represent individual animals).



Immunogens (including LigA constructs)	Experiment 1		Experiment 2	
	Survival	Endpoint Days	Survival	Endpoint Days
7' 8 9 10 11 12 13	4/4	28,28,28,28	4/4	28,28,28,28
7' 8 9 10 11	2/4	12,13,28,28	-	-
7' 8 9	0/4	10,10,11,11	-	-
10 11 12 13	4/4	28,28,28,28	4/4	28,28,28,28
10 11 12	-	-	4/4	28,28,28,28
11 12 13	-	-	4/4	28,28,28,28
11 12	-	-	1/4	11,12,27,28
12 13	2/4	12,12,28,28	-	-
Heat-Killed Leptospires	4/4	28,28,28,28	4/4	28,28,28,28
Phosphate Buffered Saline	0/4	9,10,10,10	0/4	11,11,12,12

Figura 3 Mapping of the immunoprotective segment of LigA.

Recombinant LigA proteins were tested for protective efficacy. The number of animals surviving (survivors/total) and days to endpoint after challenge are shown. Surviving animals were observed for up to 28 days. Ig-like domains of fully protective proteins are represented by dark symbols with green numbers while Ig-like domains of partially protective and non-protective proteins are represented by white symbols with red numbers.

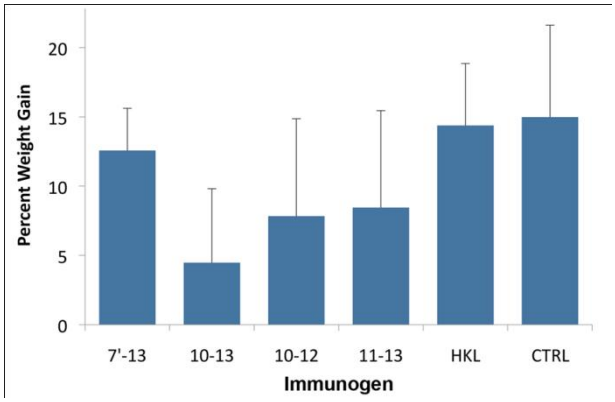


Figura 4: Percentage weight gain in hamsters immunized with protective immunogens.

Mean and standard deviation (n=4) of percent weight gain from challenge to 28 days in groups that had 100% survival, including hamsters immunized with recombinant LigA proteins (represented by their domain numbers), heat-killed leptospires (HKL) and a non-immunized and unchallenged control (CTRL) group.

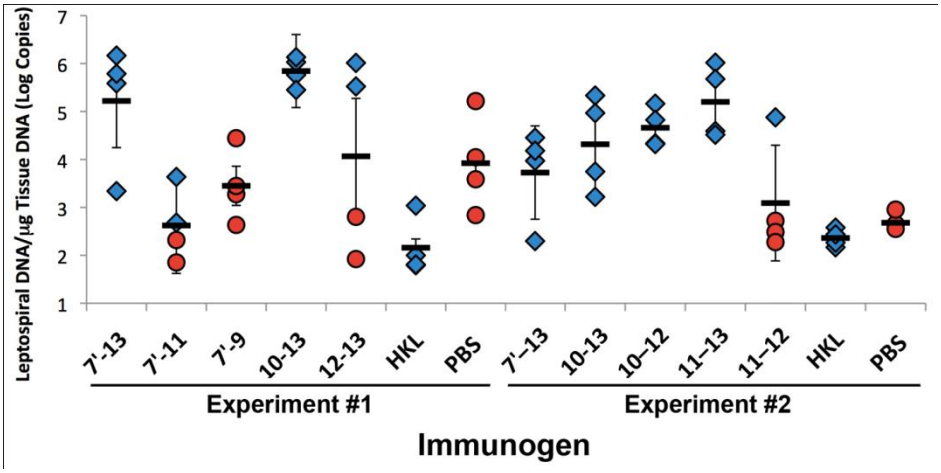


Figura 5: Leptospiral burden in kidney tissue.

Kidney tissue was subjected to DNA extraction and real-time PCR to measure the leptospiral burden per microgram of tissue DNA. Means are depicted as bold horizontal bars along with standard deviations. Animals that survived to 28 days (blue diamonds) had higher leptospiral burdens than those that met end-point criterion early (red circles). Of the groups with 100% survival to 28 days, animals immunized with heat-killed leptospires (HKL) had lower bacterial burdens than those immunized with LigA fragments. LigA-immunized groups that survived up to 28 days were used for statistical comparisons (one-way ANOVA, $P < 0.05$).

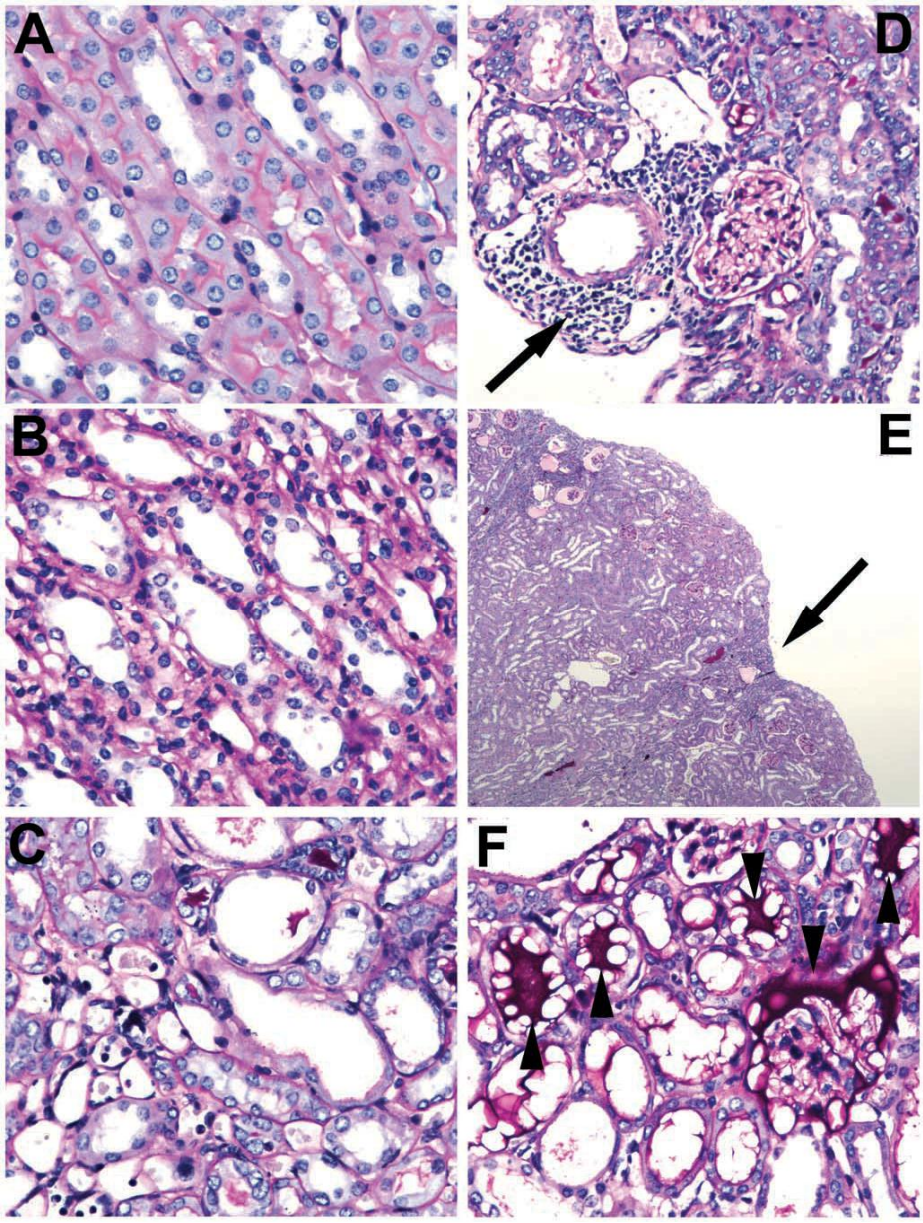


Figura 6: Renal histopathology showing tubulointerstitial changes.

Representative PAS-stained kidney sections obtained from hamsters 28 days after leptospiral challenge showing, A. Normal tubular epithelium (40x); B. Moderate tubular damage (40x); C. Severe tubular atrophy (40x); D. Interstitial inflammation (arrow, 40x); E. Tubular scarring with depressed renal capsule (arrow, 4x); and F. Tubular deposition of intensely PAS-positive material consistent with Tamm-Horsfall glycoprotein (arrowheads, 40x).

4.9 TABLES

Table 1. Recombinant LigA Proteins

Protein[a]	Amino acid coordinates[b]	MW (Da)	Primers[c]
LigA7ô-13	L631-P1224	63,422	f-AACATATCTCATATGCTTACCGTTTCCAACACAAACGCCAA
			r-TTCCTCGAGTGGCTCCGTTTTAATAGAGGCTAAT
LigA7ô-11	L631-A1033	42,991	f-AACATATCTCATATGCTTACCGTTTCCAACACAAACGCCAA
			r-GACGTCCTCGAGAGCAGAAGTGACATACAAGGTAGTAGA
LigA7ô-9	L631-A851	24,034	f-AACATATCTCATATGCTTACCGTTTCCAACACAAACGCCAA
			r-AGTCTCGAGCGCTGCGGTAACGGATAATTTGGA
LigA10-13	E852-P1224	40,602	f-ACGCTTACGCATATGGAACTTACTGAGATTGTGCTAAATCC
			r-TTCCTCGAGTGGCTCCGTTTTAATAGAGGCTAAT
LigA10-12	E852-E1124	30,085	f-ACGCTTACGCATATGGAACTTACTGAGATTGTGCTAAATCC
			r-GCGTAGCTCGAGCTCGTCATTGACGAATATCCA
LigA11-13	R943-P1224	31,233	f-CATCAATGACATATGAGAATAGCTTCAATCGAAGTAACACC
			r-TTCCTCGAGTGGCTCCGTTTTAATAGAGGCTAAT
LigA11-12	R943-E1124	20,716	f-CATCAATGACATATGAGAATAGCTTCAATCGAAGTAACACC
			r-GCGTAGCTCGAGCTCGTCATTGACGAATATCCA
LigA12-13	V1034-P1224	21,645	f-ATACAGTCTCATATGGTCCTTATTGACATAGAAGTCAAGCC
			r-TTCCTCGAGTGGCTCCGTTTTAATAGAGGCTAAT

[a]LigA protein designations list the first and last domains included in the construct; 7ô indicates a half domain.

[b]Coordinates refer to the first and last amino acids in the LigA protein of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130.

[c]Forward (f) and reverse (r) primer sequences, including an *Nde* I or *Xho* I site, respectively, are listed in the 5ôto 3ôdirection.

Table 2 – Summary of Immunoprotection Outcomes[a]

Immunogen (LigA Domains)	**Survival[b]** (%)	**MAT** (Positives/Total)	**Culture[c]** (Positives/Total)	**Histology[c]** (Mean Score)	**qPCR[c,d]** (Log10)
Experiment 1					
7–13	100	3/3	4/4	ND	5.79 ± 2.0
7–9	0*	1/3	4/4	ND	3.92 ± 0.3
7–11	50	2/4	2/4	ND	3.10** ± 0.8
10-13	100	4/4	4/4	ND	5.92 ± 0.8
12-13	50	0/4	4/4	ND	5.53 ± 1.3
HKL	100	3/3	0/4	ND	2.48** ± 0.6
PBS	0*	0/1	4/4	ND	4.66 ± 1.0
Experiment 2					
7–13	100	4/4	4/4	1.0	4.13 ± 0.2
10-13	100	4/4	4/4	2.5	4.90 ± 1.0
10-12	100	4/4	4/4	3.3**	4.81 ± 0.4
11-13	100	4/4	4/4	1.75	5.61 ± 1.2
11-12	25	4/4	1/4	3.3**	4.29 ± 0.8
HKL	100	3/4	0/4	1.25	2.39** ± 0.2
PBS	0*	3/3	2/4	ND	2.72** ± 0.2

[a]Abbreviations: HKL = heat-killed leptospires, PBS = phosphate-buffered saline, ND = not done

[b]Four animals per group. *Statistically different from the LigA7–13 group (Fisher's exact test, $P < 0.05$).

[c]Data refer to kidney analysis. Means are shown for Histology and qPCR (n=4).

**Statistically different from the LigA7–13 group (ANOVA, Dunn post-test, $P < 0.05$).

[d]Expressed as copies per microgram of tissue DNA.

Table 3 ï Chemistry Results[a]

Group (LigA Protein)	BUN[b] (mg/dL)	AlkPhos[b] (IU/L)	Calcium (mg/dL)	Creatinine (mg/dL)	Phos[b] (mg/dL)	SGPT[b] (IU/L)	Total bilirubin (mg/dL)	Total protein (g/dL)
7ô13	21 ± 2	**127 ± 8***	13 ± 0.7	0.3 ± 0.1	7.6 ± 0.5	64 ± 11	0.1 ± 0	6.6 ± 0.4
10-13	25 ±5	78 ± 14	12.8± 2.2	0.2 ± 0	6.8 ± 1.5	57 ± 26	0.1 ± 0	5.6 ± 1
10-12	26 ± 6	72 ± 4	11.6± 1.8	0.2 ± 0	6.6 ± 1	39 ± 20	0.1 ± 0	5.3 ± 0.6
11-12	67 ±77	71 ± 18	9.7 ± 1.3	0.8 ± 1.2	7.5 ± 3.3	49 ± 10	0.1 ± 0	**4.6 ± 0.6**
11-13	22 ± 2	**98 ± 4**	13± 0.9	0.2 ± 0.1	7.6 ± 0.6	51 ± 16	0.1 ± 0	6.2 ± 0.5
HKL[c]	23 ±2	73 ± 10	12.5 ± 0.9	0.2 ± 0	6.8 ± 1	42 ± 2	0.1 ± 0	6.2 ±0.5
PBS[d]	**235 ± 49***	**130 ± 15***	13.7± 1.4	**6.8 ± 1.6***	18.1 ± 1	48 ± 5	0.3 ± 0.1	6.7 ± 0.4
Ref. range	9-30	15-45	8-12	0.5-2.2	4.2-8.5	10-35	0-1	4.5-6.5

[a]Means with standard deviations from Experiment 2 are displayed (n=4). Bold numbers: Significantly different from group immunized with heat-killed leptospires (one-way ANOVA with Dunnôs post test, P < 0.05). *Significantly different from group immunized with heat-killed leptospires (one-way ANOVA with Dunnôs post test, P < 0.01)

[b]Abbreviations: BUN, blood urea nitrogen; AlkPhos, alkaline phosphatase; Phos, phosphorus; SGPT, serum glutamic pyruvic transaminase

[c]Heat-killed leptospires

[d]Phosphate buffered saline

5 Paper 2 ï Kinectics of the Hamster Model of *Leptospira interrogans* infection

(Text format as used by the scientific journal PLoS Neglected Tropical Diseases)

Kinetics of the Hamster Model of *Leptospira interrogans* Infection†

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5.1 Abstract

Leptospirosis is a zoonosis caused by *Leptospira*. The organism penetrates the skin through lesions or abrasions and rapidly disseminate to the kidney, liver and other organs. In humans, leptospirosis ranges from asymptomatic to a life-threatening infection characterized by hepatorenal failure and pulmonary hemorrhage. The intraperitoneal route of infection is the most widely used to experimentally inoculate hamsters, but it does not represent the natural route of infection. Here we report the use of more natural routes of infection such as intradermal or subcutaneous inoculation of leptospires and how the disease progresses during time. Animals showed hepatic and renal failure that started at day 7 post infection and a peak of leptospiral burden in the key-organs of leptospirosis at day 6, after which leptospires tended to remain in the kidneys.

5.2 Introduction

Leptospirosis is a zoonosis with worldwide distribution caused by spirochetes belonging to the genus *Leptospira* (Haake, 2009). Up to 500,000 severe human infections are documented annually in tropical areas with an incidence of >10 cases per 100,000 population and a mortality rate of 10% (Hartskeerl et al., 2011). Human infection typically occurs in persons involved in water-related occupations such a riceï field workers, or that deal with animals or their carcasses, or recreational activities in humid, subtropical regions where conditions are favorable for survival of the organism. In developing countries, often patients get infected by direct exposure to rats or open sewer. In these areas, transmission is thought to involve cutaneous or mucous membrane exposure to water contaminated with urine from infected animals (KO, 1999). Rodents are frequently the source of organisms causing human infections, but many mammalian species have been found to harbor infection in their kidneys (Levett 2001). Once the organisms reach the bloodstream they rapidly disseminate to the kidney, liver and other organs. In humans, leptospirosis ranges from asymptomatic to a life-threatening infection characterized by hepatorenal failure and pulmonary hemorrhage.

Severe leptospirosis occurs as a dual-phase disease with a leptospiremic phase in the first week after infection followed by an immune phase during which antibody is produced and organisms appear in the urine (Levett 2001). Symptoms of organ dysfunction, such as jaundice and azotemia, are absent during the initial presentation of infection and typically begin as organisms are cleared from the bloodstream and the immune phase begins (Levett, 2001).

The kinetics of leptospirosis has been described, examining the time to appearance of organisms in various tissues depending on the challenge route (Stavitsky 1945). These initial studies primarily involved dark-field observations and leptospires isolation from experimentally infected guinea pigs and hamsters which are highly susceptible to infection with virulent leptospires. More recently, quantitative PCR has been used to measure the leptospiral burden in various organs after intraperitoneal challenge (Lourdault *et al*, 2009).

The IP infection has been successfully used to reproduce leptospirosis in animal models such as hamsters and guinea pigs, and although it has advantages this is not likely a natural route of infection. Other routes of infection such as conjuntival, subcutaneously, intradermal, oral, intracardiac and intracranial have been reported, but somehow the quantification of the leptospiral burden through these alternate routes of infection remains not well studied. The understanding of how a natural infection progresses can be used to help the development of new vaccines and the clinical treatment of leptospirosis.

In this study we report the inoculation of hamsters with intradermal and subcutaneous routes of infection and the harvesting of the organs at different time-points to provide a better understanding of the kinetics of leptospiral infection.

5.3 Materials & Methods

Leptospiral strain and cultivation. *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Haake t al., 2002) supplemented with 1% rabbit serum (Rockland Immunochemicals, Gilbertsville, PA) and 100 µg/ml 5-fluorouracil at 30°C in a shaker

incubator. Leptospiral cultures (passage 2) in log phase of growth were centrifuged at 5,000g for 5 minutes and diluted in fresh EMJH prior to hamster inoculation.

Hamster infection and sample collection. Groups of three to four female Syrian hamsters, 5 to 6 weeks of age (Harlan Bioscience, Indianapolis, IN), were inoculated either subcutaneously (0.5 mL) or intradermally (0.05 mL) with 10^7 leptospires or EMJH alone on day 0. Animals were euthanized at different time-points ranging from 3 hours to 12 days post-infection. Hamsters were randomly selected for euthanasia unless they presented clinical signs of leptospirosis, such as loss of appetite, gait or breathing difficulty, prostration, ruffled fur, or weight loss of Ó10% of the animalôs maximum weight. Kidney, liver, lungs and spleen were collected in formalin for histopathology or incubated overnight at 4°C in RNAlater (Ambion, Austin, TX) and stored at -80°C. Paraffin embedded tissues were sectioned and stained with periodic acid Schiff (PAS) in a Dako automated slide processor. Kidney sections were scored on a scale of 0 (normal tissue) to 5 (severe renal tubular damage) based the severity of histopathology. Blood was collected for serology and chemical analysis (Antech Diagnostics, Irvine, CA). All animal procedures were approved by the Veterans Affairs Greater Los Angeles Healthcare System Institutional Animal Care and Use Committee and adhere to the United States Health Research Extension Act of 1985 (Public Law 99-158, November 20, 1985, ñ*Animals in Research*ò), the National Institutes of Healthôs *Plan for Use of Animals in Research* (Public Law 103-43, June 10, 1993), U.S. Government Principles for the Utilization and Care of Veterbrate Animals Used in Testing, Research, and Training, Public Health Service Policy on Humane Care and Use of Laboratory Animals, the United States Department of Agricultureôs Animal Welfare Act & Regulations, and Veterans Health Administration Handbook 1200.7.

DNA extraction and Quantitative PCR (qPCR). Tissue DNA was extracted using either the FastDNA SPIN Kit (MP Biomedicals, Santa Ana, CA) according to the manufacturer instructions or with DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) with modifications as previously described (Coutinho, *et al*. 2011). The purified DNA was stored at -80°C until use.

DNA was amplified in a PCR using the 16S primers 16sfor, 5ó GGCGGCGCGTCTTAAACATG-3ô and 16srev, 5óTTCCCCCCATTGAGCAAGATT-3ô as described before (Lourdault *et al*., 2009). Two µL of sample DNA was added to a mix containing forward and reverse primers (0.4 µM), dNTPs (200 µM), PCR buffer (1x) and Hot Start Taq DNA polymerase (Qiagen, Germantown, MD). The reaction was performed in a Vapoprotect Thermocycler (Eppendorf) under the following conditions: initial denaturation at 95°C for 15 min followed by 30 cycles of amplification (94°C for 30 s, 57°C for 30 s, 72°C for 30 s) and a final cycle of 72°C for 1 min. The amplicons were examined by agarose gel electrophoresis to screen for *Leptospira*-positive samples in experiment #1.

PCR-positive samples from experiment #1 and all samples from experiment #2 were tested by qPCR using the Bio-Rad iQ5 Real-time System (Bio-Rad, Hercules, CA). One hundred nanograms of total DNA was combined with 1 µM of each primer and 12.5 µL iQ SYBR Green Supermix (Bio-Rad) and brought to a final volume of 25 µL with nuclease-free water (Ambion, Austin, TX). Each sample was run in duplicate. qPCR primer pairs were LipL32-f, 5ó CGCGTTACCAGGGCTGCCTT-3ô and LipL32-r, 5ôCGCTTGTGGTGCTTTCGGTG-3ô and hamster GAPDH-f, 5ôCTGGTTACCAGGGCTGCCTT-3ô and GAPDH-r, 5ô CCGTTCTCAGCCTTGACTGTGC-3ô the latter being used as a reference gene to which *lipL32* was normalized using Bio-Rad iQ5 software and Microsoft Excel. The

resulting amplicons were 152 bp and 146 bp, respectively. The PCR protocol consisted of an initial incubation step at 95°C for 12.5 min followed by 40 cycles of amplification (95°C for 15 s, 57°C for 30 s and 72°C for 30 s). Standard curves were generated for each gene ranging from 10 to 1.6 x 10^6 copies of *Leptospira* (20-fold dilutions) and 0.02 to 200 ng (10-fold dilutions) of hamster DNA.

ELISA. Ninety-six-well ELISA microtiter plates (Immulon 4HBX,Thermo Fisher, Waltham, MA) were coated with 1 x 10^9 heat-inactivated leptospires/mL diluted in PBS, pH7.2 (Invitrogen, Carlsbad, CA), by overnight incubation at 4°C as described previously (Choy et al. 2007). Briefly, the plates were blocked with Protein-Free Blocking Buffer (PFBB, Thermo Fisher, Rockford, IL) for 1 to 2 h at room temperature (RT). Sera were tested in triplicate after dilution with PFBB, to wells in a volume of 100 µL, and plates were incubated for 1 h at 37°C. Non-binding antibodies were removed, and Horseradish peroxidase (HRP)-conjugated anti-Syrian hamster secondary antibody (Jackson ImmunoResearch, West Grove, PA) 1:5000 was incubated for 30 min at RT. One hundred µL of 1-Step Turbo Ultra TMB HRP substrate (Thermo Fisher) was added to the wells and incubated for 30 min at RT with shaking. The reaction was stopped by the addition of 50 µL of 2 M H_2SO_4, and plates were immediately read in a Bio-Rad 550 Microplate Reader at 450 nm. End-point titers were defined as the highest titer that yielded a reading two standard deviations above the result with sera from mock-infected hamsters. Geometric mean end-point titters were calculated as previously described (Perkins, 1958).

Statistics. Differences between variances were analyzed by the f-test. All other analises were done by GraphPad InStat version 3.10 (GraphPad Software Inc., La Jolla, CA). One-way analysis of variance (ANOVA) was used to test for differences between multiple (Ó3) groups using a P value < 0.05. The Kruskal-Wallis one-way ANOVA with Dunn's post-test was included. The unpaired, two-tailed Student's t-test assuming unequal variance was used to test for differences between two groups using a P value < 0.05.

5.4 Results

End-point criteria and the infection effect on body weight. All animals were observed for the presence of clinical signs of disease and, in experiment #1, two animals (one from each route), succumbed to leptospirosis and were found dead in their cage on day 12. All other animals were euthanized (three animals/time-point/route).

In experiment #2 hamster body weight was measured at time of infection and each day thereafter. Uninfected hamsters gained an average of 3.6% of body weight per day during the course of the study. As shown in Figure 7, weight gain in infected hamsters was comparable with uninfected hamsters until the sixth day after challenge. A decrease in body weight was the earliest observable sign of clinical leptospirosis and a cumulative 10% weight loss from peak weight was used as an endpoint criterion. As in our previous study [Coutinho 2011], spontaneous death was not observed in any animals in which the 10% weight loss criterion was employed. By day 7 after SQ challenge, 10/13 (77%) hamsters had lost Ó10% of peak weight. Weight loss among ID challenged hamsters was not as rapid; by day 7 after challenge, 6/13 (46%) animals had lost Ó10% of peak weight. The difference in weight loss on day 7 between the hamsters challenged ID vs. SQ was significant (T-test, $P<0.02$). Comparing each day with day 0, there is a significant difference (P ’ 0.001, Anova with Bonferroniôs multiple comparison test) in both routes that was noticed from day 6 until the end of the experiment.

Leptospiral burden on organs. The leptospiral burden in kidneys, lungs, spleen and liver was accessed in both intradermal and subcutaneous route (table 1 and 2).

Subcutaneous route. Leptospires were detected in the spleen within 24 to 48 hours after infection and the bacterial load seemed to have peaked on days 5 and 6 and declined afterwards. In both experiments leptospiral burden was only seen from day 5 on and declined after day 6 or 7. The leptospiral load on lungs was detected on day 5 and lasted for 2 to 4 days presenting infection levels much lower than spleen.

Although the presence of leptospires was detected very early in liver, it only rose after 4 days and reached their maximums load on day 6 with a fast decline afterwards.The most affected organ was kidney, in which the bacteria seems to colonize fast and showed a much higher load than the other organs, but it also showed a peak around days 5 and 6 followed by a decline which could explained by the elimination of leptospires in the urine.

Intradermal route. The spleen of animals infected through the ID route showed that leptospires took 48 to 72 h to infect the organ with a peak on day 5, with similar pattern of decline in bacterial load after as observed in the SQ route. It was observed a difference when routes were compared in both experiments denoting that the SQ route allows more leptospires to reach the spleen on days 5 (exp #2, $P<0.001$) and 6 (exp #1, $P<0.05$) than the ID infection (Parametric ANOVA with Bonferroni post-test).

In exp #1 the lung bacterial load was not possible to be quantified due to low amounts of bacterial DNA, however exp #2 showed a peak of leptospiral DNA in lungs on days 5 and 6 with a decline after this low peak. Both routes seemed not to differ from one another except on day 6 from exp #1(Parametric ANOVA with Bonferroni post-test, $P<0.01$).

In a screening PCR on experiment 1 the leptospiral DNA in liver was not detected and therefore not used in qPCR, experiment #2 however showed very low

leptospiral burden with ID route and which was different than the SQ route on day 6 (Parametric ANOVA with Bonferroni post-test, P<0.001). The kidneys were highly infected, although not as high as the SQ route (Parametric ANOVA with Bonferroni post-test, P<0.001) with a peak on day 5 followed by a decline that still kept the leptospiral load much higher than any of the other organs tested.

Blood chemistries. The serum collected at time of euthanasia was submitted to chemical analysis to evaluate liver and kidney function. Alanine aminotransferase was a little bit elevated in all groups, and alkaline phosphatase was extremely elevated on day 6 in both routes ($p<0.05$) when compared to controls, pointing to cholestasis without biliary obstruction since total bilirubin level was normal for all samples except three animals on day 7 of the SQ route which were 2 to 8 times higher than other animals.

Blood urea nitrogen was very elevated on day 7 of both routes ($p<0.05$) and day 8 of ID group ($p<0.01$). Elevated creatinine (>3mg/dL) level has been described as a prognosis indicator in humans. In hamsters it was observed increased creatinine in both routes on days 7 and 8 post infection ($p<0.05$), indicating renal malfunction at day 7 and 8. Although calcium level was mildly elevated, phosphorous level seem to have a tendency to increase from day 1 on in both routes, it was statistically different from controls on days 6 and 7 ($p<0.01$), suggesting that rhabdomyolysis might have started early in the disease.

Antibody Response. Serum collected from hamsters at time of euthanasia were used in an ELISA at a dilution of 1:6400, chosen as the best signal-to-noise

ratio (data not shown). The SQ route didn't show any improvement on antibody level during the 8 days of experiment, however, in the ID route it was observed an increase in antibody levels toward days 8 and 9 (Parametric ANOVA with Dunnett post-test, $P<0.01$ and $P<0.05$ respectively). Antibody levels against leptospires in a natural infection are usually only detected at the end of the first week of symptoms when the immune phase of the disease begins.

5.5 Discussion

Hamsters is one of most well characterized animal models used in leptospirosis, unlike rats and mice it shows signs of severe disease. It is not clear how leptospires distributes itself in the body after infection and how much time it takes to reach the key organs.

Our results show that the skin plays a major role in preventing the leptospiral burden that reaches the organs, with a much lower number of copies in the intradermal route when compared to subcutaneous inoculation. It is worth noting that the outcome on the body weight and blood analysis was the same in both routes. While it can be accepted that the leptospiral burden in the kidneys decreases when the leptospires start to be shed, however it is intriguing that they also decrease in target organs such as lung and liver at the same time.

Our approach and results are consistent with the report by Lourdault et al.(2009), which described the kinetics of infection by *L. interrogans* sv Copenhageni, strain L1-130, the same strain used here, inoculated into guinea pigs by the intraperitoneal (i.p.), subcutaneous (s.c.), and conjunctival routes. In that study, leptospiral burden in the liver, kidneys, and lungs peaked on day six, shortly before

guinea pigs succumbed to infection. In contrast, we were able to follow hamsters for a total of 8-9 days and observed a drop in leptospiral burden during the last 2-3 days. Lourdault et al.(2009), reported that the kinetics of infection in guinea pigs challenged via the i.p., s.c., and c.j. routes were similar. A novel aspect of our study was the comparison of the s.c. and i.d. routes with the finding of significant differences in the kinetics of infection between these routes. We believe that the i.d. challenge route is a biologically relevant route of infection and worthy of further study because it has the potential of incorporating interactions between leptospires and defense mechanisms of the skin, such as the dendritic cells. Antigen-presentation by dendritic cells has the potential of ñjump-startingò the host immune response at an early stage of infection.In this study we report the kinectics of the leptospiral infection during the first phase of the disease by two different routes of infection that are more natural than intraperitoneal inoculation. The finding that both routes produce the same result at practically the same time with intradermal injection resulting in a lower leptospiral burden is very useful in the development of better vaccines and treatment evaluation strategies.

5.6 Acknowledgements

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5.8 Figures

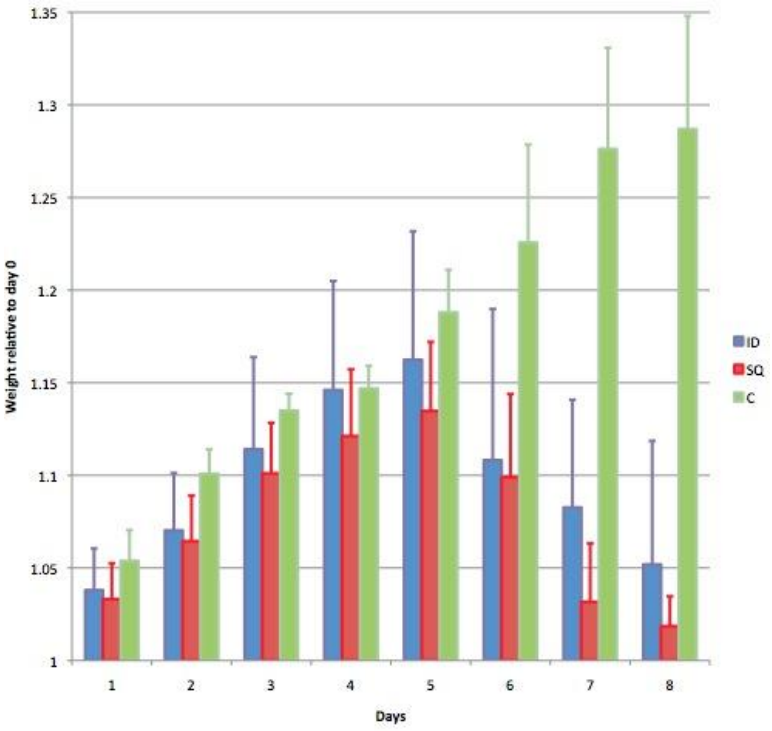

Figura 7: Hamster weight as a marker for disease progression.

Animals were weighed at the time of challenge (day zero) and daily thereafter. Data are shown for experiment #2. Bars represent weight relative to day zero for animals challenged subcutaneously (red), intradermally (blue), or the control group (green). Infected animals gained weight until day six, after which they progressively lost weight.

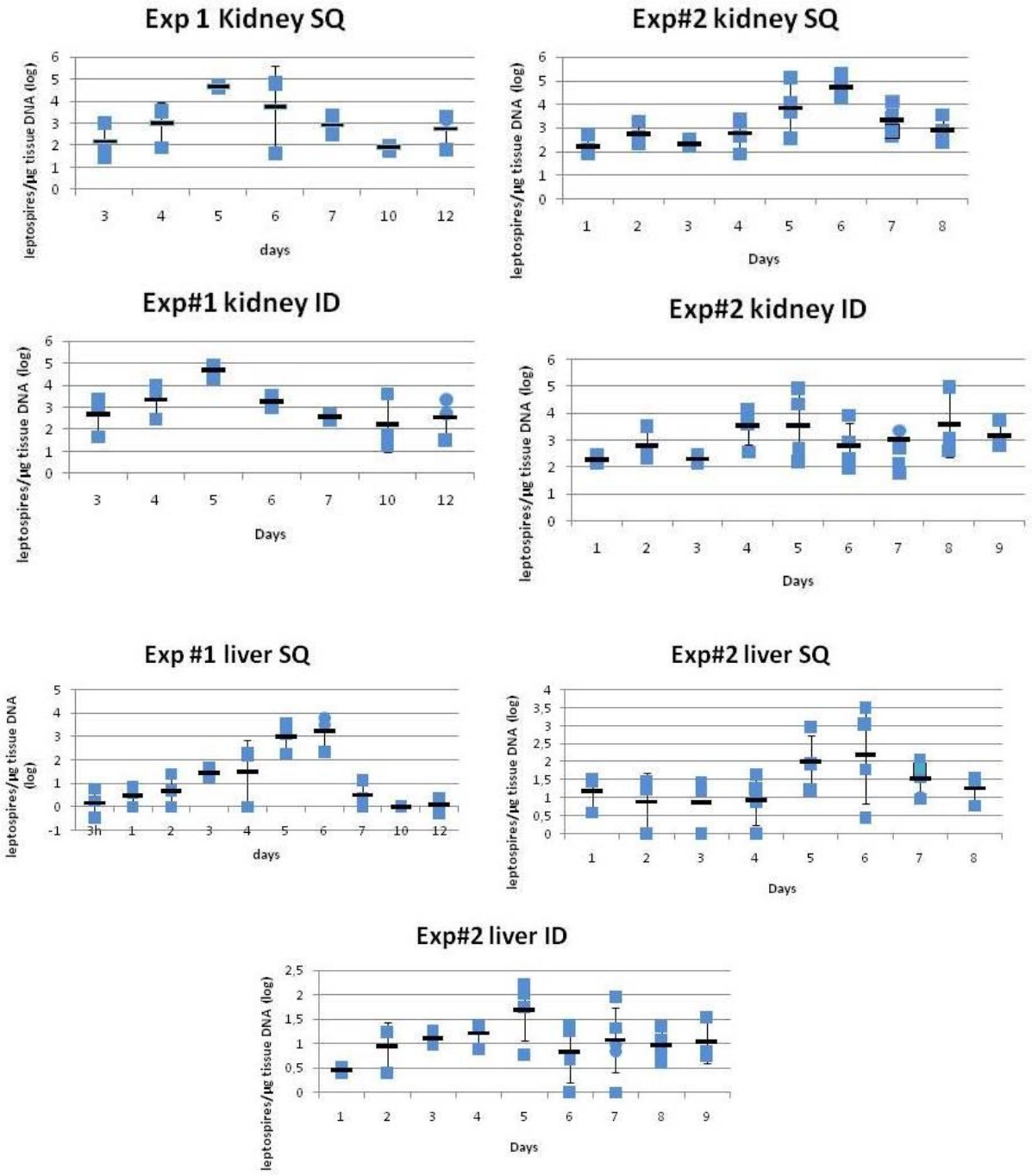

Figura 8 : Leptospiral tissue burden.

Liver and kidney tissue were subjected to DNA extraction and real-time PCR to measure the leptospiral copies per microgram of tissue DNA. Means are depicted as bold horizontal bars along with standard deviations for animals challenged subcutaneously or intradermally. Leptospiral tissue burdens after subcutaneous challenge were significantly higher than after intradermal challenge on days 5-6 in the liver and on day 6 in the kidney (One way ANOVA, $P < 0.05$).

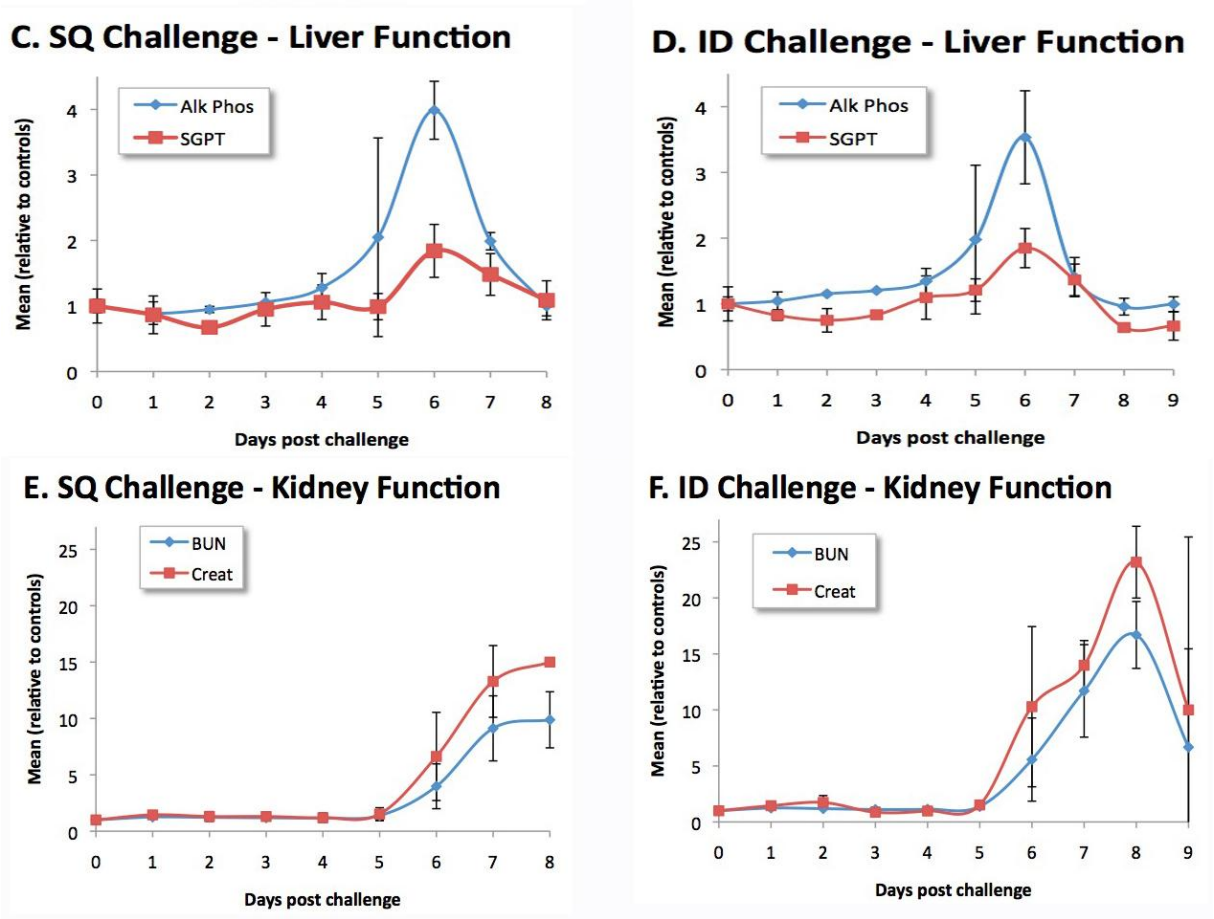

Figura 9: Kinetics of liver and kidney function

Mean and standard deviations are shown for blood samples obtained from hamsters challenged intradermally. Markers of liver function (alkaline phosphatase and SGPT) peaked on day 6 after challenge. Markers of kidney function (serum creatinine and blood urea nitrogen) became abnormal on day 6 and peaked on day 8. Results for hamsters challenged subcutaneously were similar.

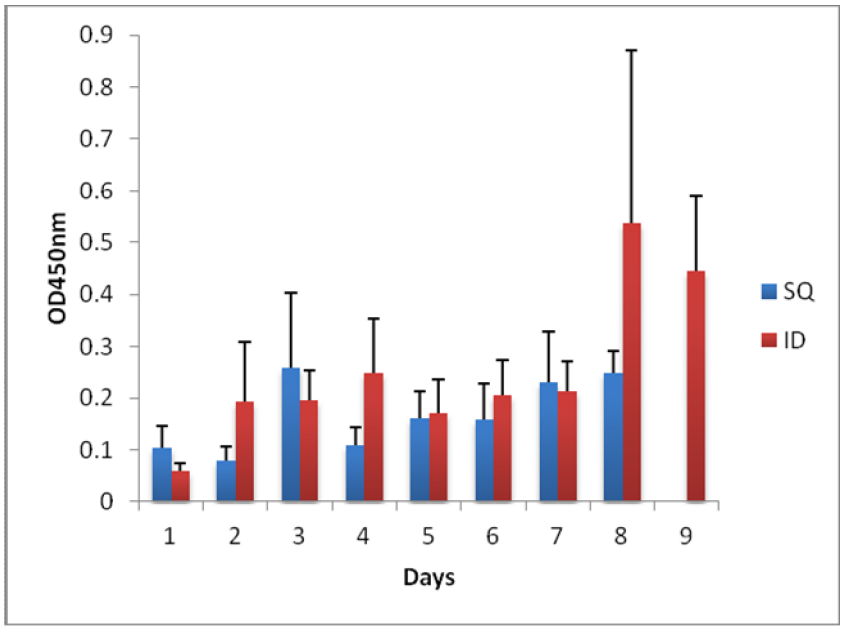

Figura 10: Antibody response in infected hamsters

Hamster immunoglobulin responses to heat-killed leptospires were measured by ELISA. Mean and standard deviations are shown for serum samples (tested in triplicate at a dilution of 1:6400) obtained from hamsters challenged intradermally (red) or subcutaneously (blue). Dotted gray line represents two standard deviations above background ELISA for sera from six uninfected control hamsters. Anti-leptospiral antibody levels in sera from hamsters challenged intradermally were higher than those from control hamster and hamsters challenged subcutaneously beginning on day 8 after challenge ($P < 0.05$).

Table 1 ï Leptospiral burden in various organs Exp# 1

Time-points	Organ/ Experiment (mean ± SD)*							
Days	Spleen		Lungs		Liver		Kidneys	
	SQ	ID	SQ	ID	SQ	ID	SQ	ID
3h					< 20			
1		<20		< 20	< 20	22.1 (± 16.0)		233.7 (± 234.4)
2	< 20	252.9 (± 118.3)		< 20	< 20	23.4 (± 7.8)		849.4 (± 895.9)
3	< 20	242.8 (± 197,2)	< 20	< 20	30.5 (± 13.5)	21.9 (± 7,9)	383.8 (± 552.6)	231.2 (± 72.0)
4	86.2 (± 100.8)	958.7 (± 1,772.6)	< 20	< 20	126.4 (± 112.2)	23.2 (± 19.2)	2,418.1 (± 2,036.2)	1,174.3 (± 1,107.1)
5	8,28.8 (± 556.4)	1,535.9 (± 2,867.3)	123.1 (± 137.1)	110.6 (± 8.8)	1,678.3 (± 197.2)	279.2 (± 427.3)	49,538.0 (± 8,176)	36,306.0 (± 61,300.8)
6	**2,939.0 (± 2,801.5)**	**164.2 (± 238,0)**	**396.4 (± 292.3)**	**398.5 (± 514.7)**	3,399.1 (± 233.6)	1,126.3 (± 1,550.5)	43,885.4 (± 37,983)	82,082.6 (± 78,750.9)
7	90.6 (± 34.2)	109.6 (± 131.8)	< 20	236.8 (± 322.2)	< 20	47.8 (± 33.9)	1,095.1 (± 916)	3,627.9 (± 3,930.5)
8		64.5 (± 58.3)		127.5 (± 10.9)		24.6 (± 16.1)		1,523.2 (± 1,812.3)
10	187 (± 316.6)				< 20		81.6 (± 21.8)	
12					< 20		1,149.4 (± 952.5)	

Table 1: Kinectics of leptospiral infection in experiment #1. Numbers represent the average of three animals at each time-point. Bold numbers depict statistical difference between routes within the same day.

Table 2 ï Leptospiral burden in various organs Exp# 2

Time-points	Organ/ Experiment (mean ± SD)*							
Days	Spleen		Lungs		Liver		Kidneys	
	SQ	ID	SQ	ID	SQ	ID	SQ	ID
1	<20	< 20	< 20	66.2 (± 2.0)	22.1 (± 16.0)	< 20	233.7 (± 234.4)	183.9 (± 59.8)
2	252.9 (± 118.3)	350.1 (± 591.3)	< 20	58.6 (± 43,0)	23.4 (± 7.8)	< 20	849.4 (± 895.9)	1,228.7 (± 1,686.5)
3	242.8 (± 197,2)	< 20	< 20	< 20	21.9 (± 7,9)	< 20	231.2 (± 72.0)	197.8 (± 56.4)
4	958.7 (± 1,772.6)	36.3 (± 22.6)	< 20	< 20	23.2 (± 19.2)	< 20	1,174.3 (± 1,107.1)	6,105.3 (± 5,595.1)
5	1,535.9 (± 2,867.3)	< 20	110.6 (± 8.8)	296.32 (± 269.0)	279.2 (± 427.3)	84,57 (± 68,78)	36,306.0 (± 61,300.8)	26,027.5 (± 39,146.3)
6	164.2 (± 238,0)	261.2 (±105.8)	398.5 (± 514.7)	119.2 (± 55.9)	**1,126.3 (± 1,550.5)**	**< 20**	**82,082.6 (± 78,750.9)**	**2,205.1 (± 3,671.2)**
7	109.6 (± 131.8)	152.7 (± 204.5)	236.8 (± 322.2)	84.7 (± 107.1)	47.8 (± 33.9)	25,25 (± 34,1)	3,627.9 (± 3,930.5)	709.6 (± 814.4)
8	64.5 (± 58.3)	61.3 (± 20.3)	127.5 (± 10.9)	66.3 (± 35.1)	24.6 (± 16.1)	< 20	1,523.2 (± 1,812.3)	23,165.9 (± 44,985.1)
9		54.6 (±56.7)		84.8 (± 93.1)		< 20		

Table 2: Leptospiral load in organs in experiment #2. Bold numbers represent statistical difference between routes within the same day.

5.9 Conclusões

5.9.1 Paper 1

- A região minima requerida para proteção contra desafio letal de L*eptospira interrogans* inclui os domínios 11 e 12 sendo necessária a inclusão de um terceiro domínio que pode ser tanto a região 10 quanto a 13.
- Os hamsters sobrevivem apesar de apresentarem uma alta carga microbiana renal;
- O acompanhamento do peso e observância da sua perda pode ser utilizada como auxiliar na determinação de ponto-final em experimentos que usam hamsters como modelo experimental

5.9.2 Paper 2

- As leptospiras são rapidamente disseminadas no organismo e alcançam densidades máximas 6 dias após a infecção de hamsters SC ou ID;
- A rota intradérmica de inoculação prove o mesmo resultado que a rota subcutânea em hamsters em termos de sinais clinicos e cinética da infecção, mas com uma carga microbiana que atinge os órgãos muito menor.

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