FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS GONÇALO MONIZ

FIOCRUZ

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

TESE DE DOUTORADO

AVANÇOS NO CONHECIMENTO DA IMUNOPATOGÊNESE DA LEPTOSPIROSE E A APLICAÇÃO DO MÉTODO DO *IMPRINT* COMO FERRAMENTA QUALITATIVA E QUANTITATIVA DE LEPTOSPIRAS

ADENIZAR DELGADO DAS CHAGAS JÚNIOR

Salvador – Brasil
2014

CPqGM

Adenizar Delgado Das Chagas Júnior

Avanços no conhecimento da imunopatogênese da leptospirose e a aplicação do método do *imprint* como ferramenta qualitativa e quantitativa de leptospiras

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ADENIZAR DELGADO DAS CHAGAS JÚNIOR

Orientador: Prof° ALAN JOHN ALEXANDER MCBRIDE

Tese apresentada ao Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa para a obtenção do grau de Doutor**.**

Salvador – Brasil
2014

"AVANÇOS NO CONHECIMENTO DA IMUNOPATOGÊNESE DA LEPTOSPIROSE E A APLICAÇÃO DO MÉTODO DO IMPRINT COMO FERRAMENTA QUALITATIVA E QUANTITATIVA DE LEPTOSPIRAS"

ADENIZAR DELGADO CHAGAS JÚNIOR

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Dedico esta conquista ao meu pai Adenizar Delgado das Chagas

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RESUMO

A leptospirose é uma zoonose causada por espiroquetas patogênicas pertencentes ao gênero *Leptospira*. O modelo da doença em camundongos tem vantagens devido à ampla gama de ferramentas genéticas e imunológicas disponíveis para pesquisas básicas. A maior limitação na conduta clínica e na pesquisa experimental da leptospirose é o fraco desempenho dos métodos disponíveis para detecção direta e para quantificação de leptospiras. Foi incluído nesta tese um conjunto de três manuscritos que visam investigar o desfecho da infecção pela cepa virulenta de *Leptospira interrogans* nas linhagens de camundongos selvagens (A, CBA, BALB/c e C57BL/6), em camundongos óxido nítrico sintase induzível (iNOS) Knockout (KO), camundongos gene ativador de recombinação 1 (RAG1) KO , camundongos CB17 com imunodeficiência combinada grave (SCID), e os seus respectivos controles selvagens C57BL/6 e BALB/c. Investigar a confiabilidade do método de quantificação do *imprint* (IM)*, comparando os resultados obtidos com esta técnica aos obtidos com a utilização do PCR em tempo real (qPCR) para detectar e quantificar leptospiras em amostras de rim de ratos e hamsters experimentalmente infectados. Como esperado, nenhuma das linhagens de camundongos selvagens foram suscetíveis à leptospirose letal. A linhagem A e C57BL/6 exibiram altas cargas de *Leptospira* nas amostras de rim e as linhagens CBA e C57BL/6 desenvolveram lesões inflamatórias graves, enquanto a linhagem BALB/c provou ser a mais resistente apresentando leptospirose subclínica. Os camundongos iNOS KO e selvagem sobreviveram sem sintomas clínicos de leptospirose. A frequência e gravidade das nefrites foram significantemente menores nos camundongos iNOS KO. Todos os animais RAG1 KO e SCID morreram de leptospirose aguda, enquanto que todos os camundongos selvagens sobreviveram. A hemorragia pulmonar foi observada em 57 e 94% dos camundongos RAG 1 KO e em 83 e 100% dos camundongos SCID, usando doses de inóculos de 10^7 e 10^6 leptospiras, respectivamente. Não houve evidências de hemorragia pulmonar nos controles selvagens. Nos modelos de infecção agudo e crônico, houve correlação positiva estatisticamente significante ($P < 0,05$) na quantificação de leptospiras pelos métodos do qPCR e do IM. Como conclusão geral, a linhagem de camundongos A pode ser a linhagem de escolha em estudos na qual se pretende recuperar um grande número de leptospiras de rins colonizados. As linhagens CBA e C57BL/6 desenvolveram, com maior frequência, lesões inflamatórias e podem ser as mais adequadas para estudos de leptospirose associados com nefrite intersticial. A linhagem BALB/c é a mais indicada para estudar mecanismos que envolvam a imunidade inata e/ou a rápida resposta imune adaptativa. A ausência do gene do iNOS no modelo murino resultou em uma diminuição significativa da suscetibilidade para o desenvolvimento da nefrite intersticial. Além disso, a ausência de linfócitos B e T funcionais não impediu a ocorrência de hemorragia pulmonar. Estes dados fornecem fortes evidências de que a hemorragia pulmonar na leptospirose não está relacionada apenas a mecanismos autoimunes. Para a detecção e quantificação de leptospiras o método do *imprint* foi equivalente ao qPCR.

Palavras-chave: *Leptospirose, Camundongos, Hemorragia pulmonar, PCR em tempo real*

CHAGAS-JUNIOR, Adenizar Delgado das. Advances in the understanding of the immunopathogenesis of leptospirosis and the imprint method as a qualitative and quantitative tool for leptospires. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

ABSTRACT

Leptospirosis is a zoonosis caused by pathogenic spirochaetes belonging to the genus *Leptospira*. The mouse disease model is advantagous due to the broad array of immunological and genetic tools available for basic research. A major limitation in the clinical management and experimental research of leptospirosis is the poor performance of the available methods in the direct detection and quantification of leptospires. This thesis includes three manuscripts that investigate the outcome of infection by a virulent strain of *Leptospira interrogans* in wildtype mice strains: A, CBA, BALB/c and C57BL/6; in iNOS knockout (KO) mice, recombination activating gene 1 (RAG1) KO mice and CB17 severe combined immunodeficiency (SCID) mice. To investigate whether the imprint method (IM) of quantification was reliable we compared it with against real time PCR (qPCR) for the detection and quantification of leptospires in kidney samples from rats and hamsters. As expected, none of the wildtype mice were susceptible to lethal leptospirosis. The A and C57BL/6 strains exhibited high leptospiral loads in the kidney samples and the CBA and C57BL/6 strains developed severe inflammatory lesions, whilst the BALB/c strain proved to be the most resistant to subclinical leptospirosis. The iNOS KO mice survived with no clinical symptoms of leptospirosis. The frequency and severity of nephritis was significantly lower in the iNOS KO mice. All of the RAG 1 KO and SCID animals died of acute leptospirosis, whereas all of the wildtype mice survived. Pulmonary haemorrhage was observed in 57 and 94% of *Rag1* KO mice and in 83 and 100% of SCID mice, using inoculum doses of 10^7 and 10^6 leptospires, respectively. There was no evidence of pulmonary haemorrhage in the wildtype controls. In both the acute and chronic infection models, the correlation between quantification by qPCR and the IM was found to be positive and statistically significant ($P < 0.05$). In conclusion, the mouse A strain would be the strain of choice in studies requiring the recovery of a large number of leptospires from colonized kidneys. The CBA and C57BL/6 strains developed more inflammatory lesions and would be the most suitable for studies of leptospirosis associated with interstitial nephritis. The BALB/c strain appeared to be the most suitable for studying mechanisms involving innate immunity and/or rapid adaptive immune response. The absence of the iNOS gene in the murine model resulted in a significant decrease in susceptibility to the development of interstitial nephritis. Furthermore, the absence of functional B and T lymphocytes did not prevent the occurrence of pulmonary hemorrhage. These data provide strong evidence that pulmonary hemorrhage in leptospirosis is not only related to autoimmune mechanisms. For the detection and quantification of leptospires the imprint method was quivalent to that of qPCR.

Keywords: *Leptospirosis, mice, Pulmonary haemorrhage, Real-time PCR*

LISTA DE ABREVIATURAS E SIGLAS

iNOS	Óxido nítrico sintase induzível
IgG	Imunoglobulina tipo G
RAG-1	Gene ativador de recombinação 1
SCID	Imunodeficiência combinada grave
qPCR	Reação em cadeia da polimerase em tempo real
IM	Método do *imprint*
CI	Isolamento na cultura
KO	*Knockout*
WT	*Wildtype*
PH	Hemorragia pulmonar
μm	Micrometro
μg	Micrograma
ml	Mililitro
°C	Grau Celsius
Prkdc	Protein kinase, DNA-activated, catalytic polypeptide
%	Por cento
LPS	Lipopolissacarídeo
Th1	*T helper type 1*
IFN-y	Interferon gamma
NF-kB	Factor nuclear kappa B

MCP-1	Proteína de quimioatração de monócitos -1
TLR-4	Receptor toll-like 4
TLR-2	Receptor toll-like 2
lipL32	Lipoproteína 32
pi	Pós-infecção
Ca	Aproximadamente
PCR	Reação em cadeia da polimerase
DNA	Ácido desoxirribonucléico
VDJ	Regiões gênicas
NO	Óxido nítrico

SUMÁRIO

1 INTRODUÇÃO

A leptospirose é uma zoonose causada por bactérias patogênicas pertencentes ao gênero *Leptospira* (LEVETT, 2001). É uma doença emergente, com ampla distribuição mundial, negligenciada e uma grande ameaça à saúde pública, principalmente em países em desenvolvimento e subdesenvolvidos (BHARTI *et al.*, 2003; HARTSKEERL *et al.*, 2011; WHO, 2011).

As leptospiras são espiroquetas que possuem 0,1μm de diâmetro e 6-20μm de comprimento, e pertencem à ordem *Spirochaetales*, família *Leptospiraceae*, gênero *Leptospira* que é composto por espécies saprófitas e patogênicas (LEVETT, 2001). Atualmente são mais de 260 sorovares patogênicos e 60 saprófitas (FAINE, 1999; ADLER, 2009). Estas bactérias são finas, apresentam alta motilidade, crescimento lento, aeróbias obrigatórias e com crescimento ótimo a 30ºC (LI *et al.*, 2000).

A primeira descrição clínica da doença foi publicada por Weil em 1886 (FAINE, 1999). Em 1916, Inada e colaboradores isolaram leptospiras e as identificaram como o agente causador da leptospirose (INADA *et al.*, 1916). A leptospirose era conhecida tradicionalmente como uma doença ocupacional (LEVETT, 2001), mas também foi diagnosticada em pessoas que se envolvem em atividades recreativas, eventos esportivos, viagens e turismo de aventura (MCBRIDE *et al.*, 2005). Em países em desenvolvimento a doença é um grande problema de saúde para as populações da zona rural, e também se tornou um problema nos grandes centros urbanos (MCBRIDE *et al.*, 2005). Um bilhão de pessoas vive nas favelas, onde a falta de condições sanitárias básicas tem produzido condições ecológicas para que ocorra a transmissão de leptospiras a partir de roedores (KO *et al.*, 1999; MCBRIDE *et al.*, 2005). Mais de 500.000 novos casos são relatados todos os anos e com taxas de letalidade superiores a 10% (WHO, 1999). Entretanto estes dados provavelmente são subestimados devido à falta de programas coordenados de vigilância e diagnóstico da doença (MCBRIDE *et al.*, 2005). No Brasil mais de 10.000 casos são relatados anualmente (MCBRIDE *et al.*, 2005). Após o surto epidêmico de leptospirose associada à síndrome hemorrágica pulmonar severa que ocorreu na Nicaragua em 1995, o mundo começou a considerar a leptospirose como uma doença infecciosa emergente (MCBRIDE *et al.*, 2005).

A infecção de animais e humanos ocorre após o contato direto com a urina ou indiretamente através de fontes de águas contaminadas com leptospiras (LEVETT, 2001;

FAINE, 1999). A maioria das infecções é assintomática. Todavia, uma fração de casos (5 a 15%) pode progredir para formas graves como a síndrome de Weil (icterícia, insuficiência renal e fenômenos hemorrágicos) e para a síndrome hemorrágica pulmonar grave (LEVETT, 2001; FAINE, 1999; DAHER *et al.*, 1999). A infecção causa uma prolongada leptospiremia até que o hospedeiro possa desenvolver uma resposta imune efetiva, que ocorre uma ou duas semanas após a infecção (FAINE, 1957). Quando a infecção é instalada, pode ocorrer a evolução para uma doença aguda, o desenvolvimento de imunidade protetora e eliminação do agente ou o desenvolvimento do estado de portador crônico. Neste último caso, estudos indicam que o lúmen dos túbulos renais, local onde a concentração de anticorpos é baixa, é um local de colonização ideal para as leptospiras, sendo provavelmente uma forma de escape do sistema imune (ATHANAZIO *et al.*, 2008; FAINE, 1999). Os rins colonizados por leptospiras apresentam nefrite túbulo-intersticial, focos inflamatórios, necrose tubular e hemorragias. As leptospiras são encontradas em grande número nos túbulos contorcidos proximais, glomérulos e interstício (VAN DEN INGH *et al.*, 1986; NALLY *et al.*, 2004; COX, 1981).

O dano pulmonar principal na leptospirose ocorre devido às intensas hemorragias intra-alveolares, levando à insuficiência respiratória (NALLY, *et al.*, 2004; PEREIRA *et al.*, 2005; GOUVEIA *et al.*, 2008). Raras leptospiras são visualizadas nos pulmões, o que sugere um mecanismo diferente de patogenia, não correlacionada com a ação direta do microrganismo, assim como a presença do agente em tecidos renais e hepáticos não estão relacionados com lesão (CRODA *et al.*, 2010; NALLY *et al.*, 2004; VAN DEN INGH *et al.,* 1986). A severidade das infecções em humanos pode variar de acordo com o sorovar infectante, a idade, o estado de saúde e o estado imunológico do paciente (ADLER *et al.*, 2009; ADLER., 1977).

Diversos modelos experimentais em animais vêm sendo usados nas pesquisas de leptospirose. Porquinhos da índia e hamsters são os roedores de laboratório mais adequados para reproduzir a infecção letal aguda (NALLY *et al.*, 2004; SPICHLER *et al.*, 2007; SILVA *et al.*, 2008). Enquanto que os ratos representam um modelo de infecção persistente (NALLY *et al.*, 2005; ATHANAZIO *et al.*, 2008). Camundongos usualmente desenvolvem um estado de portador assintomático persistente, e são reconhecidos como portadores resistentes. Entretanto, esta possível resistência dos camundongos ainda não foi estudada detalhadamente em uma ampla variedade de linhagens. Os estudos em camundongos apresentam uma série de vantagens, devido à existência de uma ampla gama de ferramentas imunológicas e genéticas

para serem utilizadas em pesquisas básicas. Contudo, isso tem sido pouco explorado em leptospirose. Estudos em linhagens de camundongos transgênicos e/ou mutantes já foram desenvolvidos (NALLY *et al.*, 2005; VIRIYAKOSOL *et al.*, 2006; ATHANAZIO *et al.*, 2008), porém, uma maior utilização destas ferramentas é dificultada pela falta de entendimento dos resultados nas infecções experimentais de diferentes linhagens de camundongos. Além disso, não é conhecido até que ponto os camundongos desenvolvem nefrite intersticial após infecções experimentais com leptospiras. Em um estudo prévio, foram observadas discretas alterações inflamatórias em camundongos C57BL/6, ao passo que a linhagem BALB/c não desenvolveu nenhuma patologia sub-clínica (ATHANAZIO *et al.*, 2008). Algumas linhagens de camundongos como a CB17 SCID não produzem linfócitos B e T funcionais devido a uma mutação do gene *Prkdc* que codifica uma proteína quinase DNA dependente envolvida na reparação da dupla fita de DNA e recombinação gênica. Um anticorpo é composto por duas cadeias leves (L) e duas pesadas (H), e os genes que os especificam são localizam-se no locus da cadeia leve e pesada. No loci da cadeia pesada H existem três regiões, V, D e J, que se recombinam aleatoriamente, em um processo chamado recombinação VDJ, para produzir um único domínio variável na imunoglobulina de cada célula B, e os camundongos *knockout* (KO) para RAG1 não possuem o gene que desempenha este importante papel no rearranjo e recombinação dos genes de imunoglobulina e moléculas receptoras de linfócitos T durante o processo de recombinação VDJ. Assim, os camundongos RAG1 KO são incapazes de gerar linfócitos B e T específicos. Diante disto, estes animais podem ser utilizados como importantes ferramentas para o entendimento da imunopatogênese da leptospirose experimental.

A imunidade do tipo humoral é predominante em humanos com leptospirose e na maioria das espécies de animais incluindo cães, suínos, porquinhos da índia e hamsters. Esta evidência surgiu após uma série de estudos que utilizavam soros convalescentes de humanos ou de animais com leptospirose em experimentos de transferência passiva, com antissoro produzido experimentalmente, ou com anticorpos monoclonais produzidos diretamente contra o lipopolissacarídeo de leptospiras (JOST *et al.*, 1986; ADLER, 2009). Assim, demonstraram que existe proteção passiva contra a infecção letal em porquinhos da índia, hamsters, cães e macacos. Os anticorpos monoclonais em associação com outros anticorpos específicos garantem uma rápida fagocitose da leptospira tanto por macrófagos como por neutrófilos. Este fato foi demonstrado *in vivo* e *in vitro*. Entretanto, em bovinos os mecanismos de imunidade são diferentes, uma vez que animais com altos níveis de anticorpos aglutinantes anti-

Leptospira não estão protegidos contra a infecção. Essa diferença ocorre pois a imunidade nestes animais está correlacionada a uma resposta do tipo Th1 mediada por IFN-y (NAIMAN *et al.*, 2001). O mais intrigante é que os anticorpos de bovinos suscetíveis são capazes de conferir imunidade passiva para hamsters, demonstrando a grande divergência de mecanismos imunes nas diferentes espécies (ADLER, 2009).

Nally e colaboradores demonstraram em 2004 no modelo experimental, depósitos ao longo da membrana basal dos capilares septais de imunoglobulinas e complemento (C3) com padrão similar observado na síndrome de Goospasture, sugerindo fenômeno de auto-imunidade (CAMPANELLA, 1999; NALLY *et al.*, 2004) e, também em menor escala, em pacientes humanos com a síndrome hemorrágica pulmonar (CRODA *et al.*, 2009). Entretanto, o envolvimento de autoanticorpos na hemorragia pulmonar foi contraposto pela descrição de hemorragia pulmonar letal em camundongos com imunodeficiência combinada grave que foram infectados experimentalmente, pois os mesmos não possuem células T e B funcionais (VIRIYAKOSOL *et al.*, 2006). Os ratos são o modelo de resistência à infecção letal aguda (ATHANAZIO *et al.*, 2008), mas similarmente com o observado em camundongos SCID, ratos tratados com ciclofosfamida (que suprime a imunidade humoral) também desenvolvem hemorragia pulmonar (THIERMANN, 1980).

A principal lesão tecidual da leptospirose parece ser a lesão endotelial, levando às hemorragias nos tecidos (LEVETT, 2001). As hemorragias são uma importante complicação da leptospirose e contribuem para a letalidade da doença (TREVEJO *et al.*, 1998; GOUVEIA *et al.*, 2008). A hemólise apresenta vantagens para a bactéria, ao disponibilizar ferro e ácidos graxos que são essenciais para o seu crescimento (CRODA, 2008). A fonte de ferro mais abundante no hospedeiro é o heme e leptospiras são capazes de usar heme e hemoglobina *in vitro* (MAROTTO *et al.*, 1999). O completo mecanismo patogênico da síndrome da hemorragia pulmonar grave permanece, até o momento, não totalmente compreendido. Questões como quais seriam os fatores determinantes desta variedade de apresentação da doença continuam sem uma explicação clara (BHARTI *et al.*, 2003).

No Brasil, a hemorragia pulmonar constitui o principal fator de risco para o óbito com taxas de letalidade para essa forma da doença superior a 50% (KO *et al.*, 1999). Os fenômenos hemorrágicos são relativamente frequentes na síndrome de Weil podendo ocorrer na pele, mucosas ou órgãos internos (CRODA *et al.*, 2010). As hemorragias pulmonares podem variar desde leves, com presença de escarros hemoptoicos, até hemorragias maciças.

Também em graus variados de intensidade, podem ocorrer hemorragias gastrintestinais, tais como melena, hematemese ou enterorragia (CRODA, 2008). Apesar desse potencial hemorrágico da leptospirose ter sido descrito desde 1886 por Weil, a sua fisiopatologia ainda é obscura com várias teorias controversas. Apresentando maior evidência da participação das citocinas e mediadores inflamatórios na patogenia da doença, estudos tentaram avaliar esse padrão de resposta associado a injuria endotelial. Os níveis de TNF-α estão estritamente relacionados à severidade do quadro clínico e a letalidade da leptospirose (ANDRADE *et al*., 2008). O peptidoglicano extraído da parede celular de *Leptospira interrogans* induz a liberação de TNF-α de monócitos humanos e ativa a aderência de neutrófilos às células endoteliais (YANG *et al*., 2006). Em resumo, não há dados conclusivos que expliquem qual o grande desencadeador dos fenômenos hemorrágicos na leptospirose.

Em vários estudos as células endoteliais mostram-se como o alvo principal na leptospirose associada à hemorragia (CRODA *et al*., 2010). Dentre os achados patológicos a hemorragia e congestão são os achados mais comuns descritos na síndrome hemorrágica pulmonar. Infiltrado de monócitos e neutrófilos podem ocorrer, porém de forma discreta. Outros achados incluem edema pulmonar, deposição de fibrina, necrose e regeneração de pneumócito. Formação de membrana hialina pode ocorrer, em um cenário de dano alveolar difuso. O tecido pulmonar geralmente apresenta um menor número de leptospiras quando comparados com o fígado, rim ou vasos sanguíneos, porém quando presentes, elas estão associadas a células endoteliais, estando no septo alveolar, aderidas às células endoteliais capilares (CRODA, 2008). Material granular associado a macrófagos também foi evidenciado em tecidos pulmonares (YERSIN *et al*., 2000).

Recentemente, para elucidação da patogênese da síndrome hemorrágica pulmonar foram feitas investigações principalmente em modelos de estudo animal. Estes estudos focam principalmente a provável etiologia imune como o grande desencadeador desta grave forma de manifestação da leptospirose. Também foi evidenciada hemorragia alveolar associado à leve ou moderado infiltrado inflamatório com predomínio de neutrófilos e macrófagos e poucas leptospiras foram observadas no pulmão quando comparados ao rim e fígado (CAMPANELLA, 1999). Yang e colaboradores relataram em 2005 o caso de um paciente com leptospirose que apresentou apenas tosse e febre e rapidamente desenvolveu insuficiência respiratória aguda. Intensa hemorragia pulmonar foi observada após a intubação orotraqueal (YANG *et al*., 2005). Após esse evento o paciente veio a óbito em menos de 16 horas após a instalação da insuficiência respiratória. A autópsia revelou hemorragia pulmonar difusa com

formação de membrana hialina, aumento de macrófagos na luz alveolar e presença de deposição de imunoglobulina no septo alveolar e espaço alveolar (YANG *et al*., 2005). Os autores sugerem que a produção de óxido nítrico e deposição de imunoglobulina podem ser responsáveis pelos casos fatais de leptospirose (YANG E HSU, 2005). Avaliando os estudos relacionados à patologia e aos mecanismos patogênicos da síndrome hemorrágica pulmonar encontrada nos pacientes com leptospirose, percebemos que tais mecanismos ainda são pouco conhecidos.

Uma das principais características da leptospirose é o envolvimento renal com peculiaridades específicas. A lesão patológica básica é a nefrite intersticial (LEVETT, 2001). Nos rins as principais alterações são: nefrite intersticial e necrose tubular aguda, resultantes da migração das leptospiras pelos rins e deposição de antígenos em glomérulos e túbulos. Verifica-se a presença de uma proliferação das células mesangiais e nefrite intersticial representada por acúmulo de mononucleares, particularmente linfócitos e histiócitos, acompanhado de edema, vasodilatação, congestão e tumefação endotelial (SILVA *et al*., 2002). A necrose tubular aguda ocorre principalmente nos túbulos distais, caracterizada por túbulos dilatados, revestidos por células epiteliais baixas e de citoplasma basófilo, representando regeneração epitelial (TANGKANAKUL *et al*., 2005; REIS *et al*., 2008). A nefrite intersticial induz uma insuficiência renal aguda não oligúrica e frequentemente hipocalêmica (ALVES *et al*., 1986), com elevação da ureia e creatinina, aumento da fração de excreção de sódio e alterações variáveis no exame de urina, tais como leucocitúria, hematúria, proteinúria e cristalúria (FONSECA *et al*., 2006).

O óxido nítrico (NO) é um radical livre de vida curta sintetizado a partir da L-arginina pela reação catalítica da óxido nítrico sintase (NOS). As isoformas de NO de mamíferos incluem: duas enzimas expressas constitutivamente (cNOS), as neuronais (nNOS) e endotelial (eNOS) e uma isoforma NOS induzível (iNOS) (ALDERTON *et al*., 2001). As isoformas cNOS são reguladas a nível pós-traducional, enquanto a isoforma iNOS é regulada primariamente por taxa de transcrição (AKTAN, 2004; STUEHR, 1999). Em baixas concentrações o NO é vasodilatador e microbicida, e em altas concentrações pode ser citotóxico (KLEINERT, 2004). Já foi demonstrado que existe uma associação entre altos níveis séricos de NO e o grau de comprometimento renal em pacientes com leptospirose grave (MACIEL *et al*., 2006). A produção renal de NO poderia estar envolvida em falhas de transporte nas células tubulares renais (CERQUEIRA *et al*., 2008). Estudos anteriores relataram a ativação de uma ampla gama de genes inflamatórios, tais como os de fator de

transcrição NF-kB, iNOS, proteína de quimiotração de monócitos -1 (MCP-1) e fator de necrose tumoral alfa por células renais tubulares em resposta à exposição a produtos derivados de *Leptospira* spp. Estes achados têm sido interpretados como sendo um gatilho molecular para a nefrite intersticial (YANG *et al*., 2002; YANG *et al*., 2006). A deficiência genética de iNOS não foi investigada *in vivo*. O NO secretado durante a resposta imune atua como um radical livre e gera produtos tóxicos contra bactérias. Assim, em teoria, a deficiência genética de iNOS pode alternativamente promover cargas mais elevadas de leptospiras no sangue e nos tecidos ou causar menos lesões inflamatórias nos rins. A correlação entre a expressão de iNOS no tecido renal e as alterações histopatológicas não foi explorada na leptospirose.

O método diagnóstico considerado padrão-ouro na detecção de *Leptospira* spp. é o isolamento em cultura. Porém, este método tem pouca sensibilidade, apresenta alto risco de contaminação e é dificultado pelo crescimento lento das leptospiras que pode levar de quatro a seis meses de incubação (WHO, 2003; FAINE, 1999). A detecção direta de leptospiras pela microscopia de campo escuro é muito menos sensível e frequentemente resulta em falso positivos, devido a erros de interpretação (LEVETT, 2001). A utilização de PCR convencional ou em tempo-real (qPCR) para detecção de *Leptospira* spp. tem resultado em melhorias significativas na especificidade e sensibilidade do diagnóstico (AHMED *et al*., 2009). No entanto, a utilização generalizada de PCR na detecção de leptospiras tem sido comprometida pelo risco de contaminação com DNA exógeno, conferindo o risco de resultados falso-positivos (YANG, 2004), assim como os relatos de sensibilidades variáveis (BOURHY *et al*., 2011). Ensaios prévios de qPCR que utilizam genes alvo comuns a todas *Leptospira* spp. como os genes *rrs* (16S rDNA) (SMYTHE *et al*., 2002), *gyrB* (SLACK *et al*., 2006), e *secY* (AHMED *et al*., 2009), ou genes patogênicos específicos incluindo os genes *lipL32* (LEVETT, 2001), ou *ligA* e *ligB* (PALANIAPPAN *et al*., 2005) têm sido utilizados. A *lipL*32 é altamente conservado entre os sorovares patogênicos e é ausente nos saprófitos. Durante alguns anos a *lipL32* foi descrita como a maior proteína da membrana externa das leptospiras (HAAKE *et al*., 2000; HAAKE *et al*., 2004). Recentemente foi descrito que a *lipL*32 localíza-se a baixo da superfície celular das leptospiras, e este fato, vem para explicar a dificuldade encontrada para desenvolver uma vacina com a *lipL32*, devido ao difícil acesso dos componentes imunológicos à essa lipoproteína (HAAKE *et al*., 2013). Estes experimentos têm sido utilizados para monitorar a colonização renal em infecções experimentais (PALANIAPPAN *et al*., 2005; LOURDAULT *et al*., 2009), para avaliar a excreção de

leptospiras na urina de cães (ROJAS *et al*., 2010), e para confirmação de casos em seres humanos durante investigações de surtos (LAROCQUE *et al*., 2005; SEGURA *et al*., 2005; THAIPADUNGPANIT *et al*., 2011). Na avaliação de candidatos a vacinas e nas interações *Leptospira*-hospedeiro, a detecção e quantificação de leptospiras são essenciais. O qPCR tornou-se a ferramenta molecular padrão para fins de quantificação devido à sua alta precisão (LOURDAULT *et al*., 2009). No entanto, nem todos os laboratórios tem acesso à tecnologia do qPCR e os métodos microbiológicos padrão para quantificação não são aplicáveis para leptospiras (FAINE, 1999). Nós desenvolvemos previamente um método de imunofluorescência em *imprints* (IM) de tecidos para detecção direta de leptospiras patogênicas por microscopia (CHAGAS-JUNIOR *et al*., 2009). Esta técnica é utilizada rotineiramente para detecção da presença de leptospiras em modelos experimentais de leptospirose em nossos laboratórios (BANDEIRA *et al*., ; SANTOS *et al*., ; MURRAY *et al*., 2009). Levando em consideração que uma das grandes limitações na pesquisa experimental da leptospirose é o fraco desempenho dos métodos disponíveis para detecção direta e quantificação de leptospiras, e sabendo que o método do *imprint* demonstra ser eficaz na detecção de leptospiras. Avaliamos essa importante ferramenta como uma possível forma de quantificação de leptospiras em tecidos, devido a sua fácil aplicação e implantação em laboratórios que não possuem à sua disposição equipamentos sofisticados como as máquinas de PCR em tempo real.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Estudar diferentes desfechos da infecção experimental com *L. interrogans* em linhagens de camundongos selvagens, knockout e imunodeficientes, e avaliar a aplicabilidade do método do *imprint* como ferramenta quantitativa de leptospiras.

2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Investigar a frequência de doença aguda letal, a frequência e intensidade de lesões renais, a carga renal de leptospiras, os títulos de anticorpos aglutinantes séricos, os níveis séricos de IgG específicos anti-*Leptospira* nas linhagens de camundongos selvagens A, CBA, C57BL/6 e BALB/c infectados com inóculos alto (10^6) e baixo (10^3) de *L. interrogans* sorovar Copenhageni cepa Cop;

2.2.2. Investigar diferenças no desfecho (sobrevida, resposta imune e carga de leptospiras) da infecção em camundongos selvagens ou deficientes para o gene da enzima óxido nítrico sintase induzível (iNOS);

2.2.3. Investigar diferenças no desfecho (sobrevida, carga de leptospiras e frequência de hemorragia pulmonar) da infecção em camundongos imunodeficientes para células B e T (animais SCID), em camundongos gene ativador de recombinação 1 (RAG1) KO e nos controles selvagens;

2.2.4. Investigar se o método do *imprint* é eficiente na quantificação de leptospiras, comparando-o com a técnica padrão-ouro (qPCR) para quantificação de leptospiras;

3 LISTA DE ARTIGOS

- **<u>Artigo 1</u>**

Different outcomes of experimental leptospiral infection in mouse strains with distinct genotypes

Cleiton S. Santos, Júlio O. Macedo, Mauricio Bandeira, Adenizar D. Chagas-Junior, Alan J. A. McBride, Flávia W. C. McBride, Mitermayer G. Reis1, and Daniel A. Athanazio

Publicado no Journal of Medical Microbiology (2010), 59, 1101–1106.

- **<u>Artigo 2</u>**

Attenuated Nephritis in Inducible Nitric Oxide Synthase Knockout C57BL/6 Mice and Pulmonary Hemorrhage in CB17 SCID and Recombination Activating Gene 1 Knockout C57BL/6 Mice Infected with *Leptospira interrogans*

Maurício Bandeira, Cleiton S. Santos, Everton C. de Azevedo, Luciane Marieta Soares, Júlio O. Macedo, Samyra Marchi, Caroline Luane R. da Silva, Adenizar D. Chagas-Junior, Alan J. A. McBride, Flávia W. C. McBride, Mitermayer G. Reis, and Daniel A. Athanazio

Publicado na revista *Infection and Immunity*, July 2011, p. 2936–2940 Vol. 79, No. 7

- **<u>Artigo 3</u>**

Detection and Quantification of Leptospira interrogans in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR

Adenizar D. Chagas-Junior, Caroline L. R. da Silva, Luciane Marieta Soares, Cleiton S. Santos, Carlos D. C. M. Silva, Daniel A. Athanazio, Mitermayer G. dos Reis, Flávia W. Cruz McBride, Alan J. A. McBride

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ARTIGO 1

Título

Different outcomes of experimental leptospiral infection in mouse strains with distinct genotypes

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Publicado no Journal of Medical Microbiology (2010), 59, 1101−1106.

Neste estudo, relatamos o desfecho da infecção por *L. interrogans* sorovar Copenhageni em quatro linhagens murinas selvagens amplamente usadas em pesquisa básica: A, CBA, BALB/c e C57BL/6. Os desfechos avaliados foram sobrevida, presença de lesões renais, carga de leptospiras nos rins, títulos de anticorpos aglutinantes séricos e níveis séricos de IgG específica anti-*Leptospira*.

Different outcomes of experimental leptospiral infection in mouse strains with distinct genotypes

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The mouse disease model has the advantage of a broad array of immunological and genetic tools available for basic research. Some studies on transgenic and/or mutant mouse strains as models for experimental leptospirosis have been reported; however, the wider use of such models is hampered by a poor understanding of the outcome of experimental leptospiral infection among the different mouse strains available. Here, the outcome of infection by a virulent strain of *Leptospira interrogans* serogroup Icterohaemorrhagiae strain Cop was studied in four commonly used wild-type mouse strains: A, CBA, BALB/c and C57BL/6. The end points evaluated in this study were survival, presence of kidney lesions, leptospiral load in kidney samples, microscopic agglutination test titre and anti-leptospiral IgG antibody levels. As expected, none of the mouse strains were susceptible to lethal leptospirosis. However, these strains developed specific pathologies associated with sublethal leptospirosis. The A and C57BL/6 strains exhibited a high leptospiral load in kidney samples and the CBA and C57BL/6 strains developed severe inflammatory lesions, whilst the BALB/c strain proved to be the most resistant to subclinical leptospirosis.

INTRODUCTION

Leptospirosis is a widespread zoonosis with a broad clinical spectrum including fatal outcomes due to acute renal failure and pulmonary haemorrhage. Pathogenic leptospires are carried by diverse mammalian reservoirs, and peridomiciliary rodents are the most important source of infection in urban settings (McBride *et al.*, 2005). Despite major efforts to develop new vaccine strategies with a long-lasting effect and cross-protection against different serovars, our current knowledge of the immune determinants involved in host protection and pathogenesis remains limited (McBride *et al.*, 2005).

Diverse experimental animal models are used in leptospirosis research. Guinea pigs and hamsters are the most suitable laboratory rodents for reproducing acute lethal infection (Nally *et al.*, 2004; Silva *et al.*, 2008; Spichler *et al.*, 2007), whereas rats represent a prototype of resistance to acute disease and a potential model for persistent infection (Athanazio *et al.*, 2008b; Nally *et al.*, 2005b). Mice usually develop a persistent asymptomatic carrier state and are therefore recognized as resistant hosts, although this has not been studied in detail in a broad range of mouse strains. Swiss mice may succumb or develop subclinical

renal colonization depending on their age and on inoculum size (Faine, 1962). Mouse models have the advantage that a broad array of immunological and genetic tools is available for basic research. Several studies on transgenic and/or mutant mouse strains have been carried out (Athanazio *et al.*, 2008a; Nally *et al.*, 2005a; Viriyakosol *et al.*, 2006); however, the wider use of such tools is hampered by a lack of understanding of the outcome of experimental leptospiral infection among the different mouse strains. For instance, the lack of effect of a single gene knockout (such as interleukin-4 in BALB/c mice) could be due potentially to an intrinsic resistance to the disease in wild-type mice (Athanazio *et al.*, 2008a). In addition, the induction of higher expression levels of pro-inflammatory mediators in murine renal tubular cells *in vitro* implicates leptospire-derived products in interstitial nephritis (Yang *et al.*, 2000, 2002, 2006). This view ignores the fact that interstitial nephritis is a late feature of human leptospirosis, probably reflecting secondary lesions following acute tubular damage (Arean, 1962). Also, it is not known to what extent mice develop interstitial nephritis after experimental leptospiral infection. In a previous report, we observed mild inflammatory changes in convalescent C57BL/6 mice, whereas the BALB/c strain failed to exhibit any subclinical pathology (Athanazio *et al.*, 2008a).

Abbreviations: MAT, microscopic agglutination test; p.i., post-infection.

The aim of this study was to clarify the outcome of experimental leptospiral infection among four commonly used laboratory mouse strains. We tested low and high infective doses of a highly virulent *Leptospira interrogans* serogroup Icterohaemorrhagiae isolate and investigated five end points of infection: survival, renal pathology, renal colonization in the convalescent phase by immunofluorescence of imprint samples (Chagas-Junior *et al.*, 2009), immune responses by microscopic agglutination test (MAT) and an in-house IgG ELISA.

METHODS

***Leptospira* strain and culture conditions.** Leptospires were cultivated in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco Laboratories) at 29 °C and counted in a Petroff–Hausser counting chamber (Fisher Scientific). An isolate from Brazil, *L. interrogans* serogroup Icterohaemorrhagiae strain Cop, was used in all assays. This strain was passaged and reisolated from hamsters four times and stored at −70 °C. Frozen aliquots were thawed and passaged in liquid medium 14 times prior to use as a low-passage-number isolate in the infection experiments. In previous experiments, the virulence of this strain at this passage was evaluated in hamsters and the 50 % lethal dose was calculated to be ~164 leptospires.

Experimental infection of mouse models of leptospirosis. Four groups of wild-type mice strains (A, CBA, BALB/c and C57BL/6) were infected intraperitoneally with a low (10^3) or a high (10^6) infective dose of *L. interrogans* serogroup Icterohaemorrhagiae strain Cop. Control groups were inoculated with 1 ml sterile EMJH medium. All animals were females; the A, BALB/c and C57BL/6 strains were 4–5 weeks old, whilst the CBA strain mice were 6–7 weeks old. The A strain used in this study refers to the A/J Unib substrain derived from the A/J strain and maintained at Cemib (Multidisciplinary Centre for Biological Research), University of Campinas, Brazil. The same institution was the source of the CBA and BALB/c mice used in this study, whilst the C57BL/6 mice were supplied by CAECAL (Laboratory Animal Breeding Centre), Oswaldo Cruz Foundation (Fiocruz). All four strains were originally purchased from The Jackson Laboratory.

Light microscopy. Euthanasia was performed on animals at 28 days post-infection (p.i.) and necropsies were carried out immediately following euthanasia. One kidney was fixed in 4 % formalin, embedded in paraffin and sections of 4–5 μm were used for conventional histology. A semi-quantitative estimation of interstitial nephritis was used with the following criteria: grade +, infiltrates rich in macrophages and lymphocytes restricted to periarterial areas; grade + +, infiltrates extending to other renal parenchymal zones with one to two lesions per field of view at ×400 magnification; and grade + + +, the same lesion was detected in more than two areas per field of view at ×400 magnification (Athanazio *et al.*, 2008a). Grade + + and + + + lesions were considered to be severe for analysis purposes.

Immunofluorescence of imprint samples. Imprints were obtained by direct pressure of the cut surface of the kidney sample onto a poly-L-lysine-coated glass slide as described previously (Chagas-Junior *et al.*, 2009). Briefly, the imprint slides were dried at room temperature, fixed in acetone for 3 min and the smear regions on the slides were demarcated with a hydrophobic barrier pen. Imprint slides were incubated for 60 min with a primary rabbit polyclonal anti-leptospiral antibody at a dilution of 1 : 200. After three washes in PBS, the imprints were incubated with goat anti-rabbit IgG–FITC conjugate at a dilution of 1 : 500, washed three times in PBS and dried before visualization of stained organisms by fluorescence microscopy. Kidney samples from non-infected mice were used as negative controls. Counterstaining of nucleic acids or cell cytoplasm was achieved with 1 mg 4′,6-diamidino-2-phenylindole ml^{-1} (Sigma-Aldrich) at room temperature for 10 min or with 0.1 % Evans Blue (Sigma Aldrich), respectively. Leptospires were quantified in imprint samples as the mean number of leptospires in 10 fields of view at a magnification of ×400. Only intact spiral-shaped organisms were included in the calculation.

Serology assays. The MAT was performed as described previously (WHO, 2003) except that only the *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni reference strain M20 was used as the live antigen.

Cells from 7-day-old cultures of *L. interrogans* serogroup Icterohaemorrhagiae strain Cop were harvested by centrifugation (1500 ***g*** for 30 min at 4 °C) and washed three times by centrifugation in PBS. The cells were resuspended in 0.1 M Na_2CO_3 (pH 9.6), at a concentration of approximately 10^8 cells ml^{-1}. The cells were inactivated by heating at 56 °C for 20 min. The resulting material was stored at −20 °C and used as the antigen for ELISA. An in-house IgG ELISA was used to compare anti-*Leptospira* IgG levels among the various mouse strains used in this study. The optimal concentrations of the antigen, mouse sera and antibody conjugates were determined in a preliminary chequerboard analysis. The equivalent of ~10^7 inactivated whole leptospires (*L. interrogans* serogroup Icterohaemorrhagiae strain Cop) in 100 μl 0.1 M Na_2CO_3 (pH 9.6) was adsorbed onto the surface of each microtitre well at

Table 1. Presence of nephritis, leptospiral count and MAT titres in mice at 28 days p.i.

QD, Quartile deviation; ID, infective dose.

Mouse strain	% Nephritis (no./total)*			Leptospiral count (mean ± SD)		MAT titre (median ± QD)	
	Control	Low ID	High ID	Low ID	High ID	Low ID	High ID
A	0 (0/20)	66.7 (10/15)†	40.0 (8/20)†	20 ± 16	26 ± 21	800 ± 800	800 ± 1200
CBA	0 (0/20)	78.9 (15/19)†	60.0 (12/20)†	10 ± 11	10 ± 10	1200 ± 900	800 ± 300
BALB/c	0 (0/19)	0 (0/20)	0 (0/20)	5 ± 9	5 ± 7	400 ± 200	200 ± 300
C57BL/6	0 (0/20)	90.0 (27/30)†	92.0 (23/25)†	19 ± 27	15 ± 16	800 ± 600	800 ± 400

*Histopathological evidence for interstitial nephritis, grade + or + + (see Table 2 for further details).
†*P*<0.01 compared with the uninfected control group.

Table 2. Level and severity of interstitial nephritis in infected mice at 28 days p.i.

Grade: +, mild lesions; + +, moderate lesions; + + +, severe lesions.

Mouse strain	% Nephritis (no./total)								
	Uninfected controls			Low infective dose			High infective dose		
	+	+ +	+ + +	+	+ +	+ + +	+	+ +	+ + +
A	0 (0/20)	0 (0/20)	0 (0/20)	53.3 (8/15)	13.3 (2/15)	0 (0/15)	40.0 (8/20)	0 (0/20)	0 (0/20)
CBA	0 (0/20)	0 (0/20)	0 (0/20)	47.4 (9/19)	31.6 (6/19)	0 (0/19)	50.0 (10/20)	10.0 (2/20)	0 (0/20)
BALB/c	0 (0/19)	0 (0/19)	0 (0/19)	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)
C57BL/6	0 (0/20)	0 (0/20)	0 (0/20)	40.0 (12/30)	50.0 (15/30)	0 (0/30)	32.0 (8/25)	60.0 (15/25)	0 (0/25)

37 °C for 1 h. The wells were washed five times with 0.05 % Tween 20 in PBS (PBS-T) and incubated for 1 h at 37 °C with 200 µl blocking solution (PBS-T plus 1 % BSA). Mouse serum diluted 1:200 in blocking solution was added and incubated for 1 h at 37 °C. After five washes with PBS-T, anti-mouse horseradish peroxidase-conjugated antibody (1:2000; Dako Cytomation) was added and incubated for 1 h at 37 °C. After five washes with PBS, 100 µl substrate solution (1 mg 3,3′,5,5′-tetramethylbenzidine dissolved in 1 ml DMSO, 9 ml 0.05 M phosphate citrate buffer, 2 µl 30 % H_2O_2) was added to each well. The colour reaction was allowed to proceed for 15 min and stopped with 25 µl 1 M H_2SO_4. The plate was read in a microplate reader (GENios; Tecan) at A_{450}.

Statistics. Statistical analyses were performed using the GraphPad Prism 4.03 software package. Categorical data were compared by Fisher's exact test and numerical data were compared using a non-parametric Mann–Whitney test (two groups) or a Kruskal–Wallis test (more than two groups).
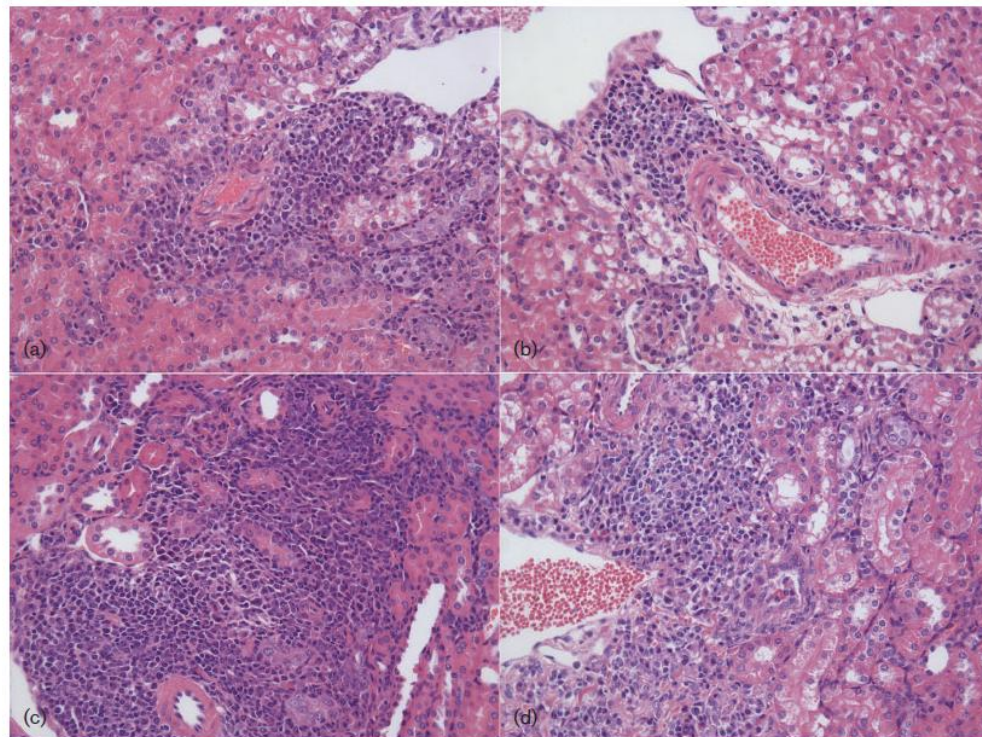

Fig. 1. Illustrative images of interstitial nephritis grading in infected mice. (a, b) Kidney sample from a C57BL/6 mouse infected with a low infective dose (10^3 leptospires) exhibiting nephritis grade +, characterized by infiltrates rich in macrophages and lymphocytes restricted to periarterial areas. (c, d) Kidney sample from a C57BL/6 mouse infected with a low infective dose exhibiting nephritis grade + +, characterized by inflammatory infiltrates extending to other renal parenchymal zones. Magnification, ×400.

RESULTS

None of the infected or control (uninfected) animals exhibited any clinical symptoms of leptospirosis; therefore, they were euthanized and necropsied at 28 days p.i. Kidney samples were examined microscopically for evidence of interstitial nephritis. None of the control animals, regardless of the mouse strain, exhibited lesions. Of note, none of the infected BALB/c mice, regardless of the size of the infective dose, exhibited inflammatory lesions. Table 1 summarizes the frequency of interstitial nephritis observed. The A, CBA and C57BL/6 strains presented with significantly more lesions than the controls ($P<0.01$) for both the lower and higher infective doses. However, the size of the infective dose in these strains had no significant effect on the frequency of interstitial nephritis observed. Detailed information on the severity of interstitial nephritis and the infective dose is presented in Table 2. Illustrative images of the interstitial nephritis grades $+$ and $++$ observed in this study are shown in Fig. 1.

The number of leptospires in kidney samples collected from the mouse strains at 28 days p.i. was determined by immunofluorescence-based detection in imprint samples (Table 1). No spirochaetes were detected in the uninfected controls. Strains A, CBA and C57BL/6 exhibited significantly higher ($P<0.001$) loads of leptospires compared with the BALB/c infected groups. This difference was observed for both infective doses used in this study. Significantly higher numbers of leptospires were detected in the A strain at the higher infective dose. Interestingly, the inverse was observed in the C57BL/6 strain, where a significantly higher leptospire count was seen in the group that received the lower infective dose. Representative images of the immunofluorescence-based detection of leptospires in imprint samples from mouse kidney samples are show in Fig. 2.

MAT quantification of agglutination antibodies against serovar Copenhageni is presented in Table 1. This revealed lower titres ($1:400$ and $1:200$) in the BALB/c groups, regardless of the size of the infective dose. In the other mouse strains, the median MAT titres were significantly higher and ranged from $1:800$ to $1:1200$. However, there was considerable variation of the MAT titres within the individual groups, as reflected in the quartile deviations presented in Table 1. The specific anti-leptospiral IgG levels in the infected mice strains were determined by ELISA (Fig. 3). The IgG levels were significantly higher in the infected animals than in the control groups. In the A and BALB/c strains, the higher infective dose stimulated significantly higher IgG levels ($P<0.05$) compared with the lower infective dose. When the IgG responses between strains were compared, the C57BL/6 mice, at the lower infective dose, exhibited significantly higher IgG levels ($P<0.01$), and the BALB/c mice, at the higher infective dose, had significantly higher IgG levels ($P<0.05$) (Fig. 3).
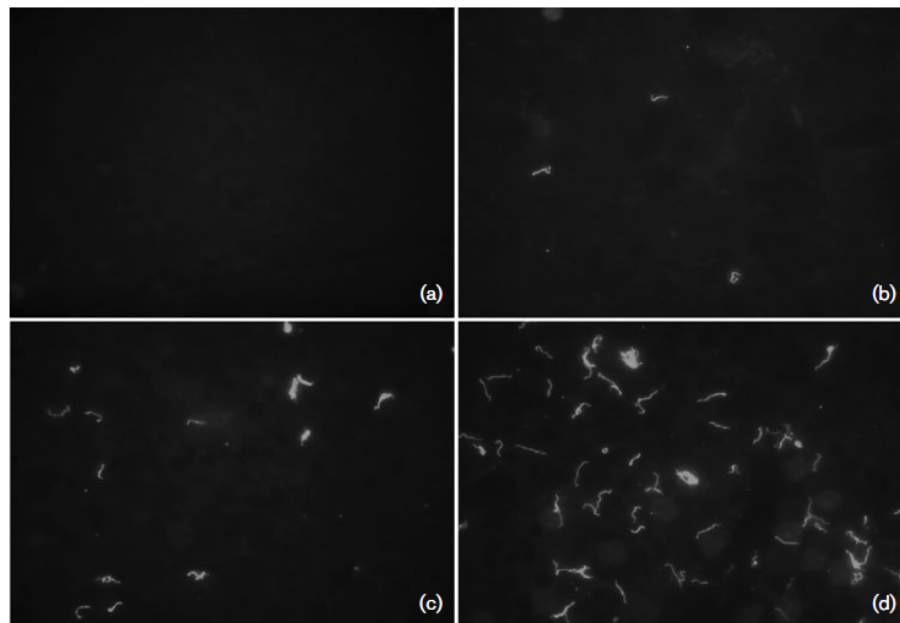

Fig. 2. Illustrative images of immunofluorescence-based detection of leptospires in imprint samples from representative mouse kidneys. (a) An A strain mouse inoculated with sterile EMJH medium (uninfected control). (b) BALB/c mouse infected with a low infective dose (10^3 leptospires): ~3 leptospires per ×400 field of view. (c) A CBA strain mouse infected with a low infective dose: ~12 leptospires per ×400 field of view. (d) An A strain mouse infected with a low infective dose: ~46 leptospires per ×400 field of view. Magnification, ×400.
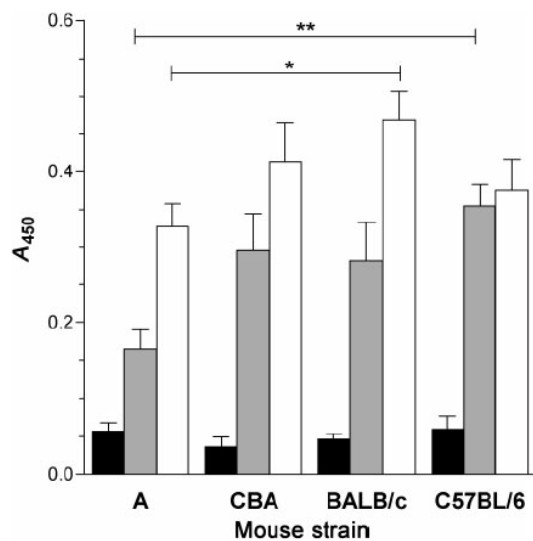
Fig. 3. Analysis of serum anti-leptospiral IgG levels in infected mice at 28 days p.i. An ELISA was used to determine IgG levels in sera collected from a control (uninfected) group (filled bars), a low infective dose (10^3 leptospires) group (shaded bars) and a high infective dose (10^6 leptospires) group of mice (open bars). Results are shown as means ± SEM for each group. The statistical significance between groups was calculated using a Mann–Whitney test: *, $P<0.05$; **, $P<0.01$.

DISCUSSION

Based on our observations in this study, the general concept that mice are relatively resistant to severe leptospirosis appears to hold true for many of the widely used laboratory strains. As expected, none of the mouse strains proved to be a model of lethal leptospirosis. However, our results indicated that certain strains could be useful for studies of experimental leptospirosis. The A and C57BL/6 mouse strains exhibited significantly higher leptospiral loads in kidney samples, and this may prove advantageous when studying *Leptospira* species recovered from renal tubules, as large numbers of leptospires are required in such studies. The A, CBA and C57BL/6 strains tended to develop more inflammatory lesions, suggesting that they may be the most suitable strains for studies on interstitial nephritis. Furthermore, the C57BL/6 strain displayed significantly more kidney lesions at the high infective dose than any of the other strains (Tables 1 and 2). This is in agreement with a previous study, where we reported higher susceptibility in the C57BL/6 strain compared with the BALB/c strain (Athanazio *et al.*, 2008a).

Our data on the immune responses support only preliminary interpretations; however, it is interesting to note that the A strain displayed one of the highest leptospiral loads observed in the kidneys and significantly lower levels of specific anti-*Leptospira* IgG. In contrast, BALB/c mice exhibited both significantly fewer kidney

lesions (zero) and a lower leptospiral load (independent of the infective dose), together with high levels of IgG, significantly so when compared with the A strain at the higher infective dose. Furthermore, the IgG response in both of these strains was dose dependent (Fig. 3). In addition, significantly higher levels of IgG were observed in the C57BL/6 strain compared with the A strain at the lower infective dose. Leptospiral load in the kidneys of both of these strains was comparable and, although the frequency of interstitial nephritis was higher in the C57BL/6 strain, it was not significant at the lower infective dose (Table 1). However, it was significant when only severe nephritis (grade + +) was evaluated at both the low and high infective dose when comparing the A and C57BL/6 strains (Table 2). It is possible that the lower MAT titres observed in the BALB/c strain may be due to the rapid clearance of leptospires during the initial stages of infection. However, the IgG levels observed in the BALB/c groups seem to contradict this conclusion.

Experimental data on leptospiral infection in mice are sporadic and fragmented. Passive immunization experiments and transfer of splenic B cell subsets in cyclophosphamide-treated BALB/c mice have suggested that humoral immunity is the key issue in natural resistance to disease. Athymic nude mice showed no difference in susceptibility when compared with wild-type animals (Adler & Faine, 1977). Transgenic and mutant murine models have been used to study leptospirosis, yet most studies have explored only the role of innate immunity. Toll-like receptor (TLR)-4 defective C3H/HeJ mice have been used to evaluate recombinant protein vaccine candidates (Koizumi & Watanabe, 2004). Double-knockout (TLR2 and TLR4) C57BL/6 mice were also found to be highly susceptible to lethal disease (Chassin *et al.*, 2009). TLR2-defective C57BL/6 mice were found to be resistant to the toxic effects of leptospiral LPS, but the role of TLR2 in host innate protection was not evaluated (Werts *et al.*, 2001). Furthermore, severe combined immunodeficiency C3H and C3H/HeJ mice are highly susceptible to lethal infection (Nally *et al.*, 2005a; Viriyakosol *et al.*, 2006). Recently, the BALB/c and C3H/HeJ strains were used as models of resistance and susceptibility, respectively (da Silva *et al.*, 2009). Similar results for wild-type mice have been reported for leishmaniasis, where a C57BL/6 strain was resistant whilst BALB/c mice were susceptible to infection (Barral-Netto *et al.*, 1987).

Our results indicate recommended strains for future research in experimental leptospirosis. Among those tested, the A strain may be the strain of choice for studies aiming to recover large numbers of leptospires from colonized kidneys. Mouse strains CBA and C57BL/6 most frequently developed inflammatory lesions and would be the most suitable for studies on leptospirosis-associated interstitial nephritis. BALB/c mice are the strain of choice for studying mechanisms that involve innate immunity and/or rapidly adaptive immune responses. Additionally, the availability of a wide range of genetically manipulated mouse strains

opens a wide range of possibilities for analysing leptospiral pathogenesis.

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ARTIGO 2

Título

Attenuated Nephritis in Inducible Nitric Oxide Synthase Knockout C57BL/6 Mice and Pulmonary Hemorrhage in CB17 SCID and Recombination Activating Gene 1 Knockout C57BL/6 Mice Infected with *Leptospira interrogans*

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Neste artigo estudamos a evolução da infecção pela *L. interrogans* sorovar Copenhageni em camundongos óxido nítrico sintase induzível (iNOS) knockout (KO), camundongos gene ativador de recombinação 1 (RAG1) KO, camundongos CB17 com imunodeficiência combinada grave (SCID), e os respectivos controles selvagens C57BL/6 e BALB/c com o objetivo de investigar a imunopatogênese da nefrite intersticial em camundongos iNOS KO e da hemorragia pulmonar em camungongos incapazes de produzir linfócitos B e T viáveis.

Attenuated Nephritis in Inducible Nitric Oxide Synthase Knockout C57BL/6 Mice and Pulmonary Hemorrhage in CB17 SCID and Recombination Activating Gene 1 Knockout C57BL/6 Mice Infected with *Leptospira interrogans*[▽]

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The aims of this study were to investigate the frequency of pulmonary hemorrhage (PH) in mice unable to produce functional B and T lymphocytes and to explore the effect of an inducible nitric oxide synthase gene (*Inos*) knockout (KO) on the frequency/severity of interstitial nephritis *in vivo*. We studied the outcome of infection by the virulent *Leptospira interrogans* serovar Copenhageni strain Cop. The animals used were *Inos* KO mice, recombination activating gene 1 (*Rag1*) KO mice, CB17 severe combined immunodeficiency (SCID) mice, and the respective wild-type (WT) C57BL/6 and BALB/c controls. The *Inos* KO and WT mice survived with no clinical symptoms of leptospirosis. The frequency and severity of nephritis was significantly lower in the *Inos* KO mice. All of the *Rag1* KO and SCID animals died of acute leptospirosis, whereas all of the WT mice survived. PH was observed in 57 and 94% of *Rag1* KO mice and in 83 and 100% of SCID mice, using inoculum doses of 10^7 and 10^6 leptospires, respectively. There was no evidence of PH in the WT controls. In conclusion, the loss of the *Inos* gene had a negligible effect on the outcome of leptospiral infection, although we observed a reduced susceptibility for interstitial nephritis in this group. Of note, the absence of functional B- and T-cell lymphocytes did not preclude the occurrence of PH. These data provide evidence that PH in leptospirosis may not be related only to autoimmune mechanisms.

Leptospirosis is a zoonosis with a wide clinical spectrum that includes fatal outcomes due to acute renal failure and pulmonary hemorrhage (PH). Pathogenic leptospires are carried by diverse mammalian reservoirs, and peridomiciliary rodents are the most important source of infection in urban settings (1). Major efforts in vaccine development and basic research on mechanisms of disease have been carried out in recent years; however, our knowledge of the genetic determinants involved in host protection and pathogenesis remains limited (10).

Among the diverse animal models used in leptospirosis research, guinea pigs and hamsters are the most suitable laboratory rodents for reproducing acute lethal infection (12, 16, 17). Rats are resistant to acute disease and are more suited to studies focusing on mechanisms of persistent infection (3, 13). The mouse model offers a broad array of immunological and genetic tools available for basic research; however, it has been poorly explored in leptospirosis. In previous reports, we described differences in the outcome of experimental leptospiral infection among distinct wild-type (WT) mouse strains (15) and the lack of significant effects on outcome of knockouts (KO) in the genes for tumor necrosis factor alpha receptor Rp55, gamma interferon, and interleukin 4 (2).

A potential role for autoimmunity in leptospirosis-associated PH was suggested based on observations in the guinea pig model of leptospirosis (12) and, to a lesser extent, in human patients with severe pulmonary hemorrhage syndrome (7). However, the involvement of auto-antibodies in PH was countered by a description of lethal PH in experimentally infected severe combined immunodeficiency (SCID) mice lacking functional B- and T-lymphocyte subsets (19). Rats are the prototype model of resistance to acute lethal infection (3), but consistent with the observation from SCID mice, rats treated with cyclophosphamide (which suppresses humoral immunity) develop PH (18). However, the observation of PH in SCID mice is not reliable because it was observed in the C3H/HeJ mouse strain background (19) but not the C3H background (14). In this study, we reproduced these experiments in the following murine models: CB17 SCID and C57BL/6 recombination activating gene 1 (*Rag1*) KO mice. CB17 SCID mice are unable to produce functional B and T lymphocytes due to a mutation in the *Prkdc* gene, which encodes a DNA-dependent protein kinase involved in DNA double-strand break repair and recombination. The strain is similar to the BALB/c strain except that it carries the *Igh-1b* allele from the C57BL/Ka strain. *Rag1* KO mice lack a gene that plays an important role in the rearrangement and recombination of the genes of immunoglobulin and T-lymphocyte receptor molecules during the process of VDJ recombination. Thus, *Rag1* KO mice are unable to generate specific B and T lymphocytes. Mutations in both *Prkdc* and *Rag-1* genes are listed as causes of human SCID. In

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TABLE 1. Evaluation of leptospirosis in iNOS-deficient mice infected with *L. interrogans* strain Cop

Mouse strain[a]	Expt	Size of inoculum	% of mice with nephritis[b] (no. with nephritis/total no.)		Leptospiral load [median (IQR)[c]]	MAT titer [median (IQR)]
			Positive	Severe		
Inos KO	1	10^3	57.1 (4/7)	28.6 (2/7)	10 (4.5)	400 (300)
WT			86.7 (13/15)	33.3 (5/15)	5 (7.5)	300 (200)
Inos KO	1	10^6	25.0 (2/8)	12.5 (1/8)	0 (2.5)	400 (350)
WT			73.3 (11/15)	40.0 (6/15)	3 (7.5)	400 (1,200)
Inos KO	2	10^6	33.3 (5/15)[d]	13.3 (2/15)	0 (4.0)	800 (800)
WT			80.0 (12/15)	40.0 (6/15)	3 (8.5)	800 (1,400)
Inos KO	3	10^6	40.0 (6/15)	20.0 (3/15)	4 (14)[d]	400 (600)
WT			66.7 (10/15)	26.7 (4/15)	1 (1.0)	400 (700)
Inos KO	Total	10^6	34.2 (13/38)[d]	15.8 (6/38)[d]	8.1 ± 17.9[e]	400 ± 600[e]
WT			73.3 (33/45)	35.6 (15/45)	7.6 ± 18.8[e]	400 ± 1,400[e]

[a] *Inos* KO, iNOS gene-deficient murine strain; WT, C57BL/6 wild-type control.
[b] Positive, grade + or higher nephritis; Severe, grade ++ or +++ nephritis.
[c] IQR, interquartile range.
[d] $P < 0.05$ compared to the control group.
[e] Median ± standard deviation.

our previous report, the C57BL/6 background mice exhibited high leptospiral loads in kidney samples and developed severe inflammatory lesions, while these features were not observed in the BALB/c mice (15).

We have also reported the association between high serum levels of nitric oxide (NO) and the severity of renal involvement in patients with severe leptospirosis (9) Renal production of NO could be involved in transport defects in renal tubular cells (4). *In vitro* studies have previously reported the activation of a broad range of inflammatory genes, such as those for transcription factor NF-κB, inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1, and tumor necrosis factor alpha, by renal tubular cells in response to exposure to leptospire-derived products. These findings have been interpreted as a molecular trigger for interstitial nephritis (20–22). The genetic deficiency of iNOS has not been investigated *in vivo*. Nitric oxide secreted during an immune response acts as a free radical and generates toxic products against bacteria. Thus, in theory, the genetic deficiency of iNOS could alternatively promote higher loads of leptospires in blood and tissues or result in less severe inflammatory lesions in kidneys.

The aims of this study are as follows: (i) to investigate the frequency of PH in mice unable to produce functional B and T lymphocytes in light of the hypothesis that PH in leptospirosis is related to immunopathogenesis/auto-antibodies and previous unreliable data on the frequency of this complication in SCID mice of the C3H and C3H/HeJ backgrounds and (ii) to explore the effect of iNOS gene (*Inos*) KO on the frequency and severity of interstitial nephritis *in vivo* in light of previous *in vitro* data suggesting that leptospiral products induce renal tubular cells to express proinflammatory genes, such as *Inos*.

MATERIALS AND METHODS

***Leptospira* strains and culture conditions.** *L. interrogans* serovar Copenhageni strain Cop was cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) modified Tween 80-bovine albumin medium (Difco Laboratories) at 29°C, and leptospires were counted in a Petroff-Hausser counting chamber (Fisher Scientific). This strain was passaged and reisolated from hamsters four

times and stored at −70°C. Frozen aliquots were thawed and passaged in liquid medium 14 times prior to use as a low-passage-number isolate in the infection experiments. The virulence of this strain was evaluated in hamsters as described previously, and the 50% lethal dose (LD_{50}) was calculated to be ~164 leptospires (15).

Experimental murine model of leptospirosis. The murine strains used in this study were C57BL/6 *Inos* KO (B6.129P2-Nos), C57BL/6 *Rag1* KO [129S(Cg)-Rag1], CB17 SCID, and the respective C57BL/6 and BALB/c WT controls. All mouse strains were purchased from The Jackson Laboratory and maintained in the animal unit at Fiocruz-BA. Animals were monitored daily for clinical signs of disease (loss of activity, jaundice, external hemorrhage, and moribund state). The *Inos* KO and the WT control mice (7 to 15 per group) were inoculated by intraperitoneal injection (10^3 and 10^6 leptospires in 1 ml phosphate-buffered saline [PBS]) in one experiment. The following endpoints of infection were evaluated: survival, renal pathology, leptospiral load, and immune response (Table 1). The immunodeficient mice and the WT controls (5 to 15 per group) were inoculated by intraperitoneal injection (10^6 and 10^7 leptospires in 1 ml PBS) in one experiment. The following endpoints of infection were evaluated: survival, time between infection and death, and the frequency of gross pulmonary hemorrhage (Table 2). The Ethics Committee of the Oswaldo Cruz Foundation approved all animal protocols used in this study.

Gross pathology and light microscopy. Animals presenting a moribund state were euthanized immediately, and convalescent survivors were euthanized 28 days postinfection. Necropsies were performed immediately after euthanasia. At necropsy, lungs were examined to detect macroscopic PH. Only macroscopic hemorrhages were reported as PH for purposes of this analysis. In all cases, microscopic examination was performed to confirm the presence of massive alveolar hemorrhaging. One kidney was fixed in 4% formalin and embedded in paraffin, and 4- to 5-μm-thick sections were used for conventional histology. A semiquantitative estimation of interstitial nephritis was used as previously described (2). Briefly, in grade + nephritis, infiltrate was rich in macrophages and lymphocytes and restricted to periarterial areas; in grade ++ nephritis, infiltrate extended to other renal parenchymal zones with 1 to 2 lesions per field of view at ×100 magnification; and in grade +++ nephritis, lesions were detected in more than 2 areas per field of view at ×100 magnification. For purposes of this analysis, grades ++ and +++ were considered to be severe nephritis.

Imprint detection of leptospires. Imprints were obtained by direct pressure of the cut surface of the tissue sample onto poly-L-lysine-coated glass slides, and leptospires were visualized by immunofluorescence as described previously (5). The immunofluorescence-based leptospiral detection in imprint samples is the detection method of choice in our laboratory, as it has proved to be reliable and has the advantages of simplicity and reduced time to result compared to immunofluorescence in frozen sections. Importantly, while renal colonization may lead to crowding of leptospires in tubular lumens, imprint-based visualization easily identifies isolated leptospires and, thus, has the additional advantage of allowing

TABLE 2. Lethal outcome, days between infection and death, and frequency of macroscopic pulmonary hemorrhages in immunodeficient and immunocompetent mice of the correspondent background

Expt[a]	Size of inoculum	No. of deaths/ total no. of mice (%)	No. of days between infection and death [median (IQR)][b]	No. of mice with macroscopic PH/ total no. (%)
1	10^7			
Rag1 KO		7/7 (100)	9 (0.5)	4/7 (57)
B6		0/15	NA	0/15
CB17 SCID		12/12 (100)	7 (0)	10/12 (83)
BALB/c		0/15	NA	0/15
2	10^6			
Rag1 KO		5/5 (100)	11 (0)	5/5 (100)
B6		0/15	NA	0/15
CB17 SCID		15/15 (100)	10 (0)	15/15 (100)
BALB/c		0/10	NA	0/10
3	10^6			
Rag1 KO		12/12 (100)	9 (0)	11/12 (91)
B6		0/14	NA	0/14
CB17 SCID		15/15 (100)	10 (0)	15/15 (100)
BALB/c		0/10	NA	0/10
Total	10^6			
Rag1 KO		17/17 (100)	9 (2)	16/17 (94)
B6		0/29	NA	0/29
CB17 SCID		30/30 (100)	10 (0)	30/30 (100)
BALB/c		0/10	NA	0/10

[a] Rag1 KO, recombination activating gene 1 knockout mice; B6, C57BL/6 strain; CB17 SCID, CB17 mice with severe combined immunodeficiency.
[b] IQR, interquartile range; NA, not applicable.

easier quantification. In a previous study, we used this assay to quantify leptospiral density in murine kidney samples (15). Leptospires were quantified in kidney imprints, and the results expressed as the mean value for 10 fields of view at ×400 magnification. Only easily identifiable, intact, spiral-shaped organisms were included. Imprint samples of lung and liver from animals that developed acute lethal disease were analyzed.

Serology assays. The microscopic agglutination test (MAT) was performed as described previously, except that only the *L. interrogans* serovar Copenhageni strain Cop was used as the live antigen (15). An in-house anti-*Leptospira* IgG enzyme-linked immunosorbent assay (ELISA) was performed as previously described (15).

Statistics. Statistical analyses and graphical presentation of the data were performed using the Prism version 4.03 software package (Graph Pad). Categorical data were compared by Fisher's exact test, and numerical data were compared by the nonparametric Mann-Whitney test; a *P* value of <0.05 was considered significant.

RESULTS

***Inos* gene-deficient murine model of leptospirosis.** Both *Inos* KO and WT mice, regardless of inoculum dose, survived with no clinical symptoms of leptospirosis. Furthermore, there were no significant differences between the reciprocal MAT titers for specific anti-*Leptospira* agglutinating antibodies or IgG antibodies in either group (Table 1 and Fig. 1). The data on renal pathology, the leptospiral load in kidney samples, and the MAT reciprocal titer are summarized in Table 1. Overall, the leptospiral load was slightly higher, but not significantly so, in kidney samples from the *Inos* KO mice than in kidney samples from the WT controls. Of note, in the third experiment (10^6 leptospires), a significantly higher leptospiral load was observed in the *Inos* KO group. The results from the three experiments at the 10^6 inoculum showed that the *Inos* KO mice were significantly less susceptible to interstitial nephritis (grade +) than the WT group (34 versus 73%, respectively;

$P < 0.01$) and, particularly, were less susceptible to severe nephritis (16 versus 36%, respectively; $P < 0.001$).

B- and T-lymphocyte-deficient murine model of leptospirosis. Both the CB17 SCID and *Rag1* KO murine strains were highly susceptible to acute lethal leptospirosis (Table 2). The median interval from infection to death was 9 days in *Rag1* KO mice, regardless of the inoculum dose. The median intervals to death for infected CB17 SCID mice were 7 and 10 days for inoculum doses of 10^7 and 10^6 leptospires, respectively. All animals developed severe jaundice and presented typical target organ pathology, including acute tubular damage and detrabeculation of hepatocytes (Fig. 2). The infected WT controls survived until 28 days postinfection, with no symptoms of leptospirosis.

Overall, macroscopic PH was observed in 57 and 94% of *Rag1* KO mice infected with 10^7 and 10^6 leptospires, respectively. PH lesions were observed in 82 and 100% of the CB17 SCID mice infected with 10^7 and 10^6 leptospires, respectively. When PH was noted macroscopically, microscopic evaluation was used to confirm the presence of massive recent intra-alveolar hemorrhaging (Fig. 2). The quantification of leptospires in the target organs of the immunodeficient mice found high loads of leptospires in all groups and experiments (Table 3). The leptospiral loads of the immunodeficient mice and WT controls were not compared because the immunodeficient mice died 7 to 10 days postinfection, while the WT mice survived and were only examined on day 28 postinfection. Thus, differences in leptospiral load could be attributed to the time point of infection (acute lethal disease versus convalescence) rather than the effect of immune status.

DISCUSSION

The loss of the *Inos* gene in mice had no apparent effect on their survival or development of agglutinating or specific IgG antibodies against *Leptospira*. In theory, impaired NO production during the immune response to leptospirosis could be
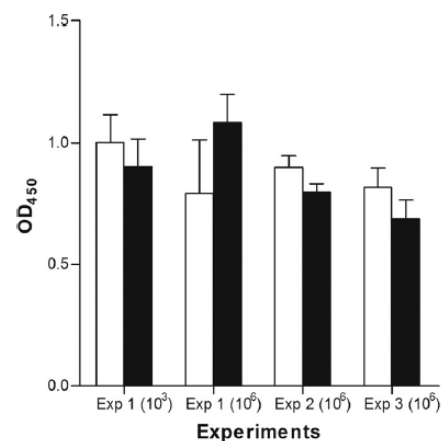

FIG. 1. ELISA analysis of serum anti-leptospiral IgG levels in infected mice 28 days postinfection. The graph compares antibody levels in C57BL/6 wild-type mice (solid bars) and inducible nitric oxide synthase knockout mice (open bars). The error bars represent the standard error of the mean for each group. OD$_{450}$, optical density at 450 nm.
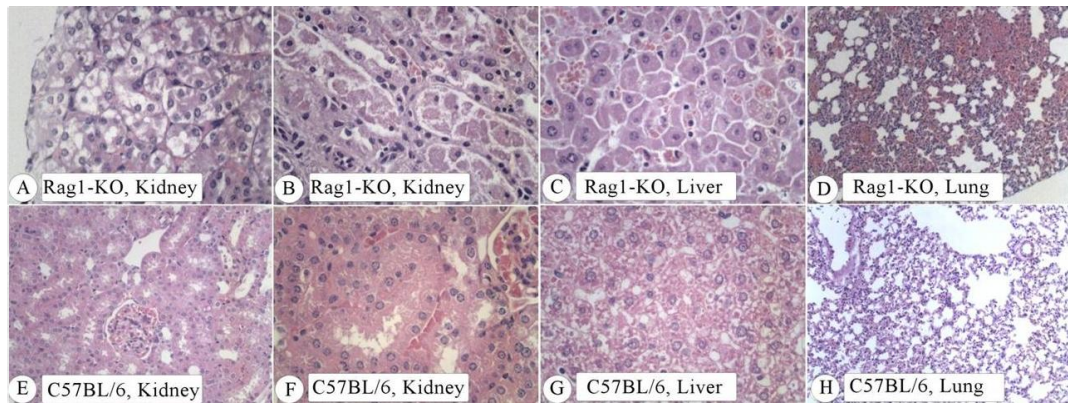
FIG. 2. Typical lesions of leptospirosis in a recombination activating gene 1 knockout C57BL/6 mouse that died 9 days after infection with strain Cop at a 10^6 inoculum. All tissue samples are stained with hematoxylin and eosin. (A) Marked cell swelling of epithelial cells of proximal tubules ($\times$400). (B) Advanced necrosis of proximal tubules ($\times$400). (C) Detrabeculation of hepatocytes ($\times$400). (D) Microscopic foci of a pulmonary hemorrhage ($\times$100). (E to H) For purposes of comparison, photomicrographs of tissue samples from a wild-type C57BL/6 mouse with no lesions are shown. (E and F) Kidney ($\times$200 and $\times$400, respectively). (G) Liver ($\times$400). (H) Lung ($\times$100).

related to the slightly higher loads of leptospires observed in the tissue samples. However, the significantly higher leptospiral load observed was not reproducible (Table 1). The only significant difference observed between the *Inos* KO and the WT C57BL/6 mice was that the transgenic animals did not develop interstitial nephritis to the same degree or severity as the WT control. This result is in accordance with the hypothesis that the expression of proinflammatory markers by renal tubular cells *in vitro* after exposure to leptospiral products may be related to the development of interstitial nephritis *in vivo* (20–22).

The present study confirms previous reports, using mice of other backgrounds (C3H and C3H/HeJ), that immunodeficiency results in high susceptibility to acute infection, rapidly progressing to death (14, 19). In addition, immunodeficiency resulted in high loads of leptospires in the target organs, as seen upon necropsy, in accordance with a previous report on C3H/SCID mice (14). We observed the typical target organ pathology associated with leptospirosis, including acute tubular damage and detrabeculation of hepatocytes, similar to the pathology described in humans and in other models of lethal leptospirosis, such as hamsters (16).

There are insufficient data to attribute the pathogenesis of leptospirosis-related PH to a single mechanism. Furthermore, it is reasonable to assume that the severe pulmonary forms result from a multifactorial response to the direct toxic effects of exposure to leptospires, the effects of systemic inflammation on the alveolar wall, hemostatic disorders, and uremia (11). Nally and colleagues described a linear deposition of antibodies and complement in the guinea pig model, suggesting a potential role for autoantibodies in the pathogenesis of leptospirosis-associated PH (12). This mechanism associates leptospirosis-associated pulmonary disease with Goodpasture's syndrome, where autoantibodies against the glomerular basement membrane (GBM) cross-react with the alveolar septal matrix, causing massive alveolar hemorrhaging. However, the original evaluation of serum anti-GBM antibodies in leptospirosis patients with and without PH found no association between anti-GBM antibodies and lung disease. There was no difference in serum anti-GBM antibody levels between patient and control groups for either acute-phase or convalescent-phase sera (8). In addition, Craig and colleagues found no evidence for anti-GBM antibodies in 40 leptospirosis patients (6). In the present study, mice that were unable to produce functional B and T lymphocytes developed severe PH. This finding suggests that autoimmunity is not a major mechanism for PH in experimental leptospirosis, at least in the murine model and/or in *L. interrogans* serovar Copenhageni infections.

Conclusion. The absence of a functional *Inos* gene in the murine model had a minimal effect on the outcome of leptospiral infection, except for a significantly reduced susceptibility to the development of interstitial nephritis. The absence of functional B and T lymphocytes does not preclude the occurrence of PH. These data provide strong evidence that PH in leptospirosis is not related only to autoimmune mechanisms.

TABLE 3. Quantification of leptospires in target organs of immunodeficient mice at necropsy

Mouse strain[a]	Expt	Size of inoculum	Median no. of leptospires (IQR[b]) in:		
			Kidney	Liver	Lung
Rag1 KO	1	10^7	154 (29)	60 (53)	49 (33)
CB17 SCID			26 (20)	20.5 (27.5)	2 (3.5)
Rag1 KO	2	10^6	220 (41)	258 (29)	159 (17)
CB17 SCID			141 (37)	181 (32)	155 (28.5)
Rag1 KO	3	10^6	29 (33)	24 (32)	29 (32.5)
CB17 SCID			160 (48)	143 (47.5)	123 (40.5)

[a] *Rag1* KO, recombination activating gene 1-deficient C57BL/6 strain; CB17 SCID, CB17 severe combined immunodeficiency strain.
[b] IQR, interquartile range.

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ARTIGO 3

Título

Detection and Quantification of Leptospira interrogans in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR

Adenizar D. Chagas-Junior, Caroline L. R. da Silva, Luciane Marieta Soares, Cleiton S. Santos, Carlos D. C. M. Silva, Daniel A. Athanazio, Mitermayer G. dos Reis, Flávia W. Cruz McBride, Alan J. A. McBride

Neste estudo comparamos o método do *imprint* com o PCR em tempo real (qPCR) que é a técnica padrão-ouro na quantificação de leptospiras, para investigar se a técnica do *imprint* além de eficiente na detecção, também seria eficiente na quantificação leptospiras em amostras renais de ratos e hamsters experimentalmente infectados com *L. interrogans*.

PLoS one

Detection and Quantification of *Leptospira interrogans* in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR

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Abstract

A major limitation in the clinical management and experimental research of leptospirosis is the poor performance of the available methods for the direct detection of leptospires. In this study, we compared real-time PCR (qPCR), targeting the *lipL32* gene, with the immunofluorescent imprint method (IM) for the detection and quantification of leptospires in kidney samples from the rat and hamster experimental models of leptospirosis. Using a virulent strain of *Leptospira interrogans* serovar Copenhageni, a chronic infection was established in the rat model, which were euthanized 28 days post-infection, while the hamster model simulated an acute infection and the hamsters were euthanized eight days after inoculation. Leptospires in the kidney samples were detected using culture isolation, qPCR and the IM, and quantified using qPCR and the IM. In both the acute and chronic infection models, the correlation between quantification by qPCR and the IM was found to be positive and statistically significant ($P<0.05$). Therefore, this study demonstrates that the IM is a viable alternative for not only the detection but also the quantification of leptospires, particularly when the use of qPCR is not feasible.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Leptospirosis is an emerging neglected disease and is a major threat to public health, especially in developing and under-developed countries [1,2,3]. The global burden of leptospirosis has been estimated to be 500,000 cases per year [2,4], although this is probably under-estimated due to the lack of coordinated surveillance programs and poor diagnosis [5]. The gold standard method for the detection of pathogenic *Leptospira* spp. is culture isolation (CI), however it has poor sensitivity, is hampered by the slow growth of leptospires (requiring four to six months incubation [6]) and there is a high risk of culture contamination [7]. Direct detection by darkfield microscopy is even less sensitive and often results in false-positives due to misinterpretation [8]. The use of PCR, conventional or real-time (qPCR), for the detection of *Leptospira* spp. has resulted in major improvements in specificity and sensitivity [9]. Nevertheless, the widespread application of PCR for the detection of leptospires has been hampered by the risk of contamination with exogenous DNA and the associated risk of false-positives [10], plus reports of variable sensitivity [11].

Previous qPCR assays targeted genes common to all *Leptospira* spp., including *rrs* (16S rDNA) [12], *gyrB* [13], and *secY* [9] genes, or pathogen-specific genes including *lipL32* [14], *ligA* and *ligB* [15]. The *lipL32* gene, which encodes the immunodominant lipoprotein located in the leptospiral outer membrane, is highly conserved

among the pathogenic serovars and is absent in the saprophytes [16,17]. These assays have been used to monitor renal colonization in experimental infection [15,18], to evaluate urinary shedding of leptospires in dogs [19] and for case confirmation in human subjects during outbreak investigations [20,21,22].

In the evaluation of vaccine candidates and leptospiral-host interactions, the detection and quantification of the leptospires is essential. qPCR has become the standard molecular tool for quantification purposes due to its high sensitivity [18]. However, not all laboratories have access to qPCR technology and the standard microbiological methods for quantification are not applicable to the pathogenic *Leptospira* spp. [7]. We previously developed an immunofluorescent imprint method (IM) for the direct detection of pathogenic *Leptospira* spp. by microscopy [23]. This technique is used routinely for detecting the presence of leptospires in the experimental models of leptospirosis used in our laboratories [24,25,26]. The aim of this study was to compare the IM with the standard method for quantification of leptospires, qPCR.

Methods

Ethics Statement

The Ethical Committee of the Oswaldo Cruz Foundation (Fiocruz) approved the animal protocols used in this study.

Leptospira strain and culture conditions

Leptospires were cultivated in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Becton Dickinson and Company, Franklin Lakes, NJ) at 29°C and counted in a Petroff–Hausser counting chamber. A highly virulent isolate from Brazil, *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Cop, was used in all assays. The strain was passaged in hamsters four times and virulent isolates from kidney samples were cultured *in vitro* and stored at −70°C, as previously described [7]. Frozen aliquots were thawed and passaged in EMJH medium up to 14 times prior to use as a virulent isolate in the infection experiments. In previous experiments, the virulence of this strain was evaluated in hamsters and the LD_{50} was calculated to be ~164 leptospires [24].

Experimental models of leptospirosis

Laboratory animals (n = 23), the rat and hamster models of leptospirosis, were used in these experiments. Twelve, four-five week-old female Wistar rats (*Rattus norvegicus*, Fiocruz) were infected intraperitoneally with 10^8 leptospires and were euthanized 28 days post-infection (pi) as described previously [27]. Ten, nine week-old female golden Syrian hamsters (Fiocruz) were infected intraperitoneally with 500 leptospires ($3 \times LD_{50}$) in 1 ml PBS, and euthanized 8 days pi. A hamster injected with PBS served as the negative control.

Collection of tissue samples and DNA extraction

Once euthanized, the abdominal cavity was opened and the kidneys were removed aseptically. Good laboratory practice was used in order to avoid DNA cross-contamination (including the use of a laminar airflow bench) and negative controls were included during all the DNA extraction procedures and qPCR steps. Total genomic DNA was extracted from approximately 25 mg tissue, using the QIAamp DNA Mini Kit (Qiagen, São Paulo, SP, Brazil). The tissue sample was a longitudinal section of the kidney that included the cortex and medulla regions, the same section was used in the IM method. The concentration of DNA obtained from tissues was determined with a spectrophotometer (NanoDrop ND 1000, NanoDrop products, Wilmington, DE).

Culture isolation of leptospires

CI was performed as previously described [27]. Briefly, whole kidney samples were homogenized in 5 ml EMJH, cell debris was allowed to settle for 10 min and 0.5 ml cleared homogenate was used to inoculate 5 ml EMJH. The cultures were incubated at 29°C and were examined regularly for growth, by darkfield microscopy, for up to 8 weeks.

Imprint detection

Imprints were produced by direct contact of the longitudinally cut surface of the kidney sample, the same region as used in the qPCR assay, onto a glass slide as described previously [23]. Briefly, the kidney imprints were dried, fixed in acetone for 3 min and incubated for 60 min with a primary rabbit polyclonal anti-leptospiral antibody at a dilution of 1:200. After washing in PBS, the imprints were incubated with a goat anti-rabbit IgG-FITC conjugate at a dilution of 1:500, washed in PBS and dried before visualization of stained organisms by fluorescence microscopy. Leptospires were quantified in imprint samples as the mean number of leptospires per 10 fields of view at a magnification of 1000×. Only intact spiral-shaped organisms were included in the calculation.

Real-time quantitative PCR

The *lipL32* gene was amplified using a previously described qPCR assay [19], with the following modifications. The qPCR reaction was performed using an Applied Bioscience 7500 thermocycler and the TaqMan Universal PCR Master Mix (Applied Biosystems, São Paulo, SP, Brazil). The standard curve was prepared from a *L. interrogans* serovar Copenhageni strain Cop culture (2×10^9 leptospires), centrifuged for 15 min at 10,000× g at 4°C. The recovered pellet was resuspended in PBS and washed by centrifugation (2×15 min, 10,000× g, 4°C). DNA was extracted from the pellet using a QIAamp DNA Mini Kit (Qiagen), as per the manufacturer's instructions. The concentration of the extracted DNA was calculated by spectrometry, optical density 260 and 280 nm (NanoDrop ND 1000), the standard curve was constructed by serial dilutions of the DNA stock. The samples were tested in duplicate, as was each dilution of the standard curve. Each run included a no-template negative control. Results were expressed as the number of genome equivalents per µg kidney DNA [18].

Statistical analysis

Statistical analyses were performed using the Prism v5 software package (GraphPad Software Inc., La Jolla, CA). The correlation between the methods was compared using the non-parametric Spearman's rank correlation (r_s), P values<0.05 were considered significant.

Results and Discussion

The end-point in the rat model of leptospirosis was a chronic non-lethal infection, as previously reported [27,28]. As expected, no deaths were observed, the animals were euthanized on day 28 pi and kidney samples were collected for evaluation by CI, IM and qPCR. In contrast, the hamsters developed an acute lethal leptospirosis and in previous reports we observed that symptoms/deaths due to leptospirosis typically occur from day 8 pi onwards [23,29]. Therefore, the hamsters were euthanized on day 8 pi and kidney samples were collected for evaluation of the presence and quantification of leptospires. All three methods were able to detect leptospires in the kidneys of all of the infected hamsters (10/10) and between 58 (7/12, qPCR) and 67% (8/12, CI and IM) of the infected rats (Table 1). Of note, two of the rats failed to establish a chronic infection. The uninfected controls were negative for the presence of leptospires.

Quantification of leptospiral load in the animal models was determined by qPCR, based on the assumption of one genome equivalent per spirochaete. The correlation coefficient of the standard curve was 0.999 and the efficiency was 92.4%, Fig. 1A. The limit of detection of the qPCR assay, based on serial dilutions

Table 1. Comparison of culture isolation (CI), the imprint method (IM) and real-time PCR (qPCR) for the detection of leptospires in animal models simulating chronic (rat) and acute (hamster) infection.

Animal model	Days post-infection	% Leptospire positive (No./total)		
		CI	IM	qPCR
Rat	28	66.6 (8/12)	66.6 (8/12)	58.3 (7/12)
Hamster	8	100 (10/10)	100 (10/10)	100 (10/10)

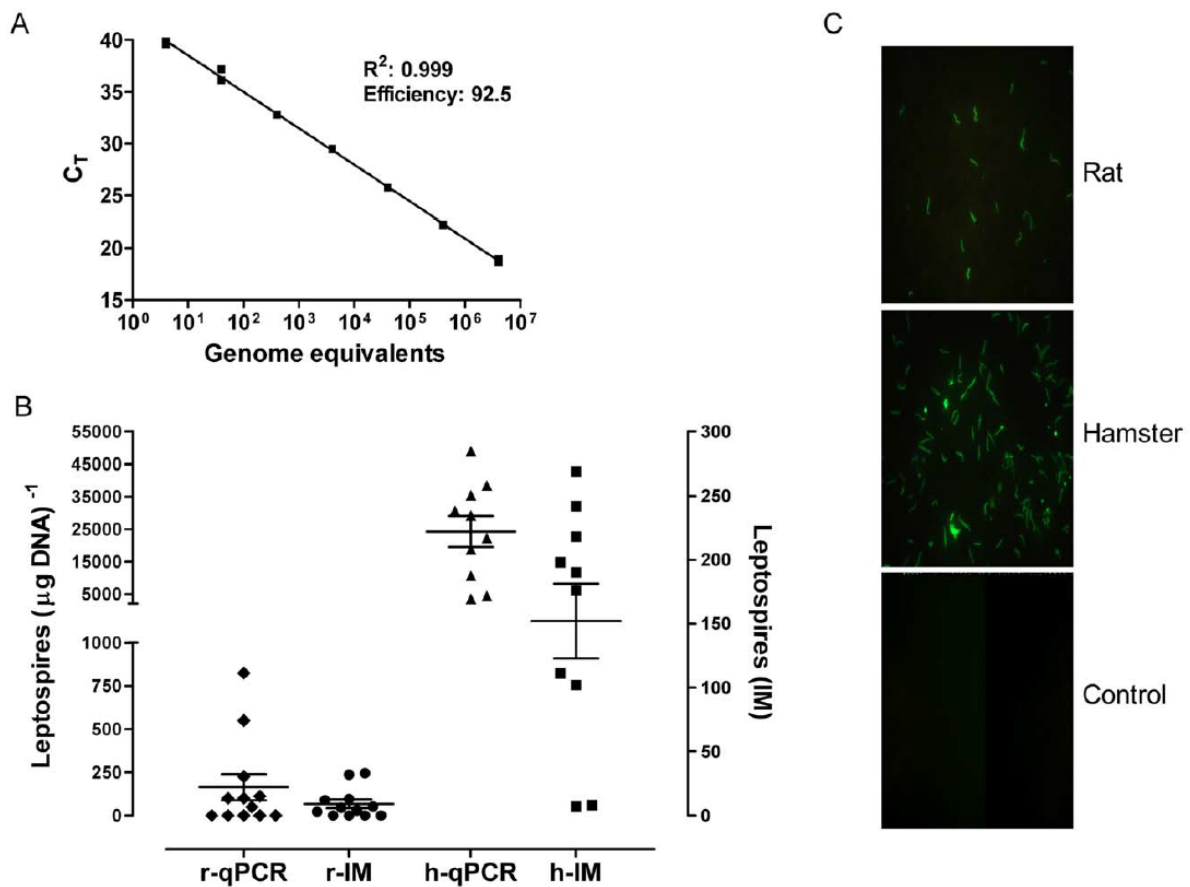doi:10.1371/journal.pone.0032712.t001

Figure 1. Quantification of leptospires by qPCR and the IM. A. Standard curve of the *lipL32* real-time PCR assay using DNA extracted from ten-fold serial dilutions of an *L. interrogans* strain Cop culture. Each DNA sample was quantified in duplicate and repeated twice. B. Quantification of the leptospiral load in the rat and hamster models. Rats were infected with 10^8 leptospires and were euthanized on day 28 pi. Hamsters were inoculated with 500 leptospires ($3 \times LD_{50}$) and euthanized eight days pi. The leptospiral load in the kidneys was determined by qPCR (open symbols) and the IM (solid symbols). The leptospiral loads for the qPCR (leptospires per μg kidney DNA) and the IM (leptospires per 10 fields-of-view, $\times 1000$ magnification) for the rat (r) and hamster (h) are presented as a scatter dot plot of the individual values for each animal, the horizontal line represents the mean value and the error bars the SEM. C. Representative examples of the imprint slides using kidney samples from an infected rat, a hamster and a non-infected control animal (magnification $1000 \times$).
doi:10.1371/journal.pone.0032712.g001

of leptospiral genomic DNA, was estimated to be 4 genome equivalents per reaction or ca. 50 leptospires per μg DNA. This is similar to previous reports for the use of *lipL32* in a qPCR assay [19]. In the hamster model, the leptospiral load ranged from 3.6×10^3 to 4.9×10^4 (mean 2.4×10^4) leptospires per μg DNA and 7 to 269 (mean 138) leptospires in the IM. The qPCR and the IM exhibited a significant positive correlation ($r_s = 0.65$, $P = 0.02$), see Fig. 2. The leptospiral loads observed among the rats were lower, ranging from 50 to 825 (mean = 163) leptospires per μg DNA and 3 to 33 (mean = 9) leptospires for the qPCR and the IM, respectively. The correlation between the two methods was the highest observed $r_s = 0.70$, $P = 0.01$, Fig. 2. The correlation coefficients observed in hamsters and rats in this study indicated there was a moderate level of correlation between the methods.

O note, the leptospiral load in the rat model was lower than expected, with a mean of 163 leptospires per μg kidney DNA or a mean 9 leptospires per field-of-view, depending on the method used. Previously, we estimated the leptospiral load in rat kidneys (7–9 days pi) to be ca. 9 leptospires per field-of-view using immunofluorescent microscopy [27], similar to that seen in the

current study using the IM. However, as the rat is the one of the main reservoir hosts for urban leptospirosis we expected a higher leptospiral load in the kidneys to allow for excretion to the environment and effective transmission of the disease [30]. A previous report found concentrations of up to 10^7 leptospires/ml urine 28 days p.i. [31]. A possible explanation is that the higher concentrations of leptospires are found in the renal tubules and not the surrounding kidney tissue in a chronic infection. The methodology used in the current study cannot determine the leptospiral load in renal tubules as the kidney sections used likely included only tubule cross-sections. Indeed, a limitation of the current study is that the concentration of leptospires in the urine of the infected rats was not determined.

The results reported in this study reinforce the usefulness of the IM for the detection of leptospires in commonly used experimental models of leptospirosis and confirm the results of the original imprint study [23]. Since its development, the IM has entered into routine use in our laboratories, in particular for evaluating the carriage status of animals used in the evaluation of potential vaccine candidates. A major drawback of the original study was
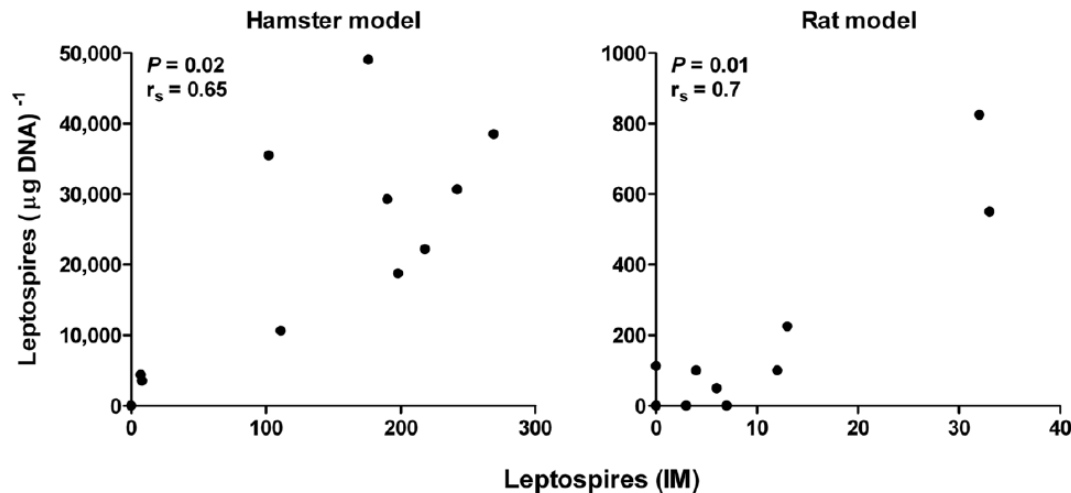
Figure 2. Correlation between the quantification of leptospires by qPCR and the IM. A significant ($P<0.05$), positive correlation was observed between the qPCR and the IM techniques in the experimental models of leptospirosis (rat and hamster) used in this study. The leptospiral loads for the qPCR are displayed as leptospires per µg DNA and in the IM as leptospires per 10 fields-of-view (×1000 magnification). doi:10.1371/journal.pone.0032712.g002

the lack of a comparison with a qPCR assay to compare sensitivity of detection and quantification of the leptospiral load. This has been addressed in the current study. In terms of detection of leptospires (positive or negative), both the qPCR assay and the IM were comparable to the gold standard method, CI, in the hamster and rat models (Table 1). Note, a potential limitation of the IM and qPCR is their inability to distinguish between viable and non-viable leptospires and this is particularly relevant in determining sterilizing immunity conferred by vaccine candidates.

Another advantage of the IM is the ability to count the leptospires in the imprint samples. However, it was not known how the leptospiral count determined by the IM correlated with the absolute leptospiral load based on qPCR. Therefore, this study evaluated how the two methods covaried by an analysis of correlation in two animal models of leptospirosis. The values determined by qPCR and the IM were analysed for correlation and a significant, positive correlation was observed between the two methods in the hamster and rat models of leptospirosis (Fig. 2). The highest correlation was found in the rat model.

In conclusion, the results of the current study show that for the detection and quantification of leptospires the IM is equivalent to

qPCR. In both acute and chronic infection models, the correlation between the IM and the qPCR methods was moderate. The imprint is a detection method that is cheap and is easily established in the laboratory. Furthermore, the fact that only intact leptospires are counted in the IM improves the probability that the observed leptospires are viable. Consequently, the IM is a valuable tool for use in evaluating secondary end-points, such as sterilizing immunity, during vaccine candidate trials and in determining the presence of leptospires in clinical samples.

Acknowledgments

Author Contributions

Conceived and designed the experiments: ADCJ AJAM. Performed the experiments: ADCJ CLRS LMS CSS CDCMS FWCM. Analyzed the data: ADCJ AJAM. Contributed reagents/materials/analysis tools: DAA MGR FWCM. Wrote the paper: ADCJ DAA MGR FWCM AJAM.

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4 DISCUSSÃO

Nesta seção são discutidos os resultados dos três artigos que fazem parte desta tese. Com base em nossas observações no primeiro artigo, o conceito geral de que os camundongos são relativamente resistentes à leptospirose grave foi comprovado em nossos experimentos. Como esperado, nenhuma das linhagens de camundongos desenvolveu a leptospirose letal. Entretanto, nossas avaliações indicaram que certas linhagens podem ser utilizadas em estudos de infecção experimental com leptospiras. As linhagens de camundongo A e C57BL/6 exibiram cargas de *Leptospira* significantemente maiores nas amostras de rim, quando comparadas ao grupo controle, o que pode ser vantajoso nos experimentos que visão fazer a recuperação de leptospiras dos túbulos renais, pois um grande número de leptospiras é necessário em tais estudos. As linhagens A, CBA e C57BL/6, tendem a desenvolver mais lesões inflamatórias, sugerindo que podem ser as linhagens mais adequadas para estudos da nefrite intersticial. Além disso, a linhagem C57BL/6 exibiu significantemente mais lesões renais quando a dose de infecção foi maior, quando comparada com as outras linhagens avaliadas. Isto está de acordo com um estudo anterior, onde foi demonstrada maior suscetibilidade da linhagem C57BL/6 comparada com a linhagem BALB/c (ATHANAZIO *et al*., 2008).

Nossos dados demonstraram que a linhagem A exibiu uma das maiores cargas de leptospiras nos rins e níveis significantemente menores de imunoglobulinas do tipo G anti-*Leptospira*. Em contraste, os camundongos BALB/c exibiram significantemente menos lesões renais e uma menor carga de *Leptospira* (independentemente da dose), juntamente com altos níveis de IgG, e de modo significativo, quando comparada com a linhagem A na dose infectante maior. Do mesmo modo, níveis altos e significantes de IgG foram observados na linhagem C57BL/6 quando comparada com a linhagem A com a dose infectante menor. A carga de leptospiras nos rins de ambas as linhagens foi comparada e embora a frequência de nefrite intersticial tenha sido maior na linhagem C57BL/6, isto não foi significante com a dose infectante menor. Entretanto, foi significativo quando somente as nefrites severas foram avaliadas em ambas as doses infectantes nas comparações das linhagens A e C57BL/6. É possível que os baixos títulos observados no MAT da linhagem BALB/c seja devido à rápida eliminação das leptospiras no estágio inicial da infecção. Contudo, os níveis de IgG observados no grupo de BALB/c parecem contradizer essa conclusão. Dados sobre leptospirose experimental em camundongos são esporádicos e fragmentados. Experimentos de

imunização passiva e transferência de subpopulações de células B esplênicas para camundongos BALB/c tratados com ciclofosfamida têm sugerido que a imunidade humoral é fundamental na resistência natural à doença. Camundongos nude atímicos não demonstraram diferença na suscetibilidade quando comparado com animais selvagens (ADLER *et al*. 1977).

Modelos murinos transgênicos e mutantes têm sido usados em estudos de leptospirose, entretanto até agora a maioria dos estudos tem explorado somente o papel da imunidade inata. Camundongos C3H/HeJ que não possuem o receptor Toll-like (TLR)-4 têm sido usados na avaliação de proteínas recombinantes candidatas a vacina (KOIZUMI *et al*. 2004). Camundongos C57BL/6 duplamente deficientes para TLR2 e TLR4 também demonstraram ser altamente suscetíveis à doença letal (CHASSIN *et al.*, 2009). Camundongos C57BL/6 deficientes em TLR-2 demonstraram ser resistentes ao efeito tóxico do LPS de *Leptospira*, mas o papel de TLR-2 na proteção inata do hospedeiro não foi avaliado (WERTS *et al.*, 2001). Além disso, camundongos C3H e C3H/HeJ com imunodeficiência combinada grave são altamente suscetíveis à infecção letal (NALLY *et al.*, 2005; VIRIYAKOSOL *et al.*, 2006). Recentemente, as linhagens BALB/c e C3H/HeJ foram usadas como modelos de resistência e suscetibilidade, respectivamente (DA SILVA *et al.*, 2009). Resultados semelhantes para camundongos selvagens têm sido demonstrados na leishmaniose, onde a linhagem C57BL/6 foi resistente, enquanto camundongos BALB/c são suscetíveis à infecção (BARRAL-NETTO *et al.*, 1987). Nossos resultados indicam as linhagens recomendadas para futuras pesquisas na leptospirose experimental. Além disso, a disponibilidade de uma ampla gama de linhagens de camundongos geneticamente manipulados abre uma ampla gama de possibilidades para análises da patogênese na leptospirose.

No segundo artigo desta tese observamos que a perda do gene iNOS em camundongos não teve efeito aparente na sua sobrevivência ou desenvolvimento de anticorpos aglutinantes ou IgG específicos contra *Leptospira* spp. Em teoria, a produção comprometida de óxido nítrico durante a resposta imune contra leptospirose poderia estar relacionada com a observação de cargas ligeiramente maiores de leptospiras nas amostras de tecidos. Entretanto, não foi observada uma carga de leptospiras significantemente maior. A única diferença significativa observada entre *Inos* KO e o camundongo C57BL/6 selvagem foi que os animais transgênicos não desenvolvem nefrites intersticiais com o mesmo grau ou severidade que o controle selvagem. Este resultado está de acordo com a hipótese de que a expressão dos marcadores pró-inflamatórios pelas células tubulares renais *in vitro* após exposição a produtos de *Leptospira* spp. pode estar relacionado com o desenvolvimento de nefrite intersticial

(YANG *et al*., 2002; YANG *et al*., 2006). O presente estudo confirma relatos anteriores, que utilizaram camundongos de outras linhagens (C3H e C3H/HeJ), onde a imunodeficiência resulta em alta suscetibilidade à infecção aguda, progredindo rapidamente para o óbito (NALLY *et al*., 2005; VIRIYAKOSOL *et al*., 2006). Do mesmo modo, a imunodeficiência resultou em altas cargas de leptospiras nos órgãos-alvo, como observado na necropsia, de acordo com relatos anteriores em camundongos C3H/SCID (NALLY *et al*., 2005). Nós observamos nos órgãos-alvo patologias típicas associadas com leptospirose, incluindo danos tubulares agudos e detrabeculação de hepatócitos, semelhante com a patologia descrita em humanos e em outros modelos de leptospirose letal, como em hamsters (SILVA *et al*., 2008).

Não existem dados suficientes para atribuir que a patogênese da hemorragia pulmonar na leptospirose está relacionada a um único mecanismo. Além disso, é razoável assumir que as formas pulmonares graves resultam de uma resposta multifatorial aos efeitos tóxicos diretos após a exposição às leptospiras, aos efeitos de inflamações sistêmicas na parede alveolar, aos distúrbios hemostáticos e à uremia (MEDEIROS *et al*., 2010). Nally e colaboradores descreveram uma deposição linear de anticorpos e complemento no modelo de porquinho da índia, sugerindo que os autoanticorpos poderiam ter um importante papel na patogênese da leptospirose associada à hemorragia pulmonar (NALLY *et al*., 2004). Entretanto, a avaliação original do soro anti-anticorpos da membrana glomerular basal em pacientes com leptospirose, com ou sem hemorragia pulmonar, não encontrou associação entre anticorpos anti-membrana basal glomerular e doença pulmonar. Não houve diferença nos níveis de anticorpos anti-membrana basal glomerular entre os soros do grupo de pacientes na fase aguda ou na fase convalescente, quando comparado ao grupo controle (CRODA, 2008). Além disso, Craig e colaboradores não encontraram evidências para anticorpos anti-membrana basal glomerular em 40 pacientes com leptospirose (CRAIG *et al*., 2009). No presente estudo, camundongos que não são capazes de produzir linfócitos B e T funcionais desenvolveram hemorragia pulmonar grave. Estes achados sugerem que a autoimunidade não é o mecanismo mais importante para hemorragia pulmonar na leptospirose experimental, ao menos no modelo murino e/ou na infecção por *L.interrogans* do sorovar Copenhageni.

No terceiro artigo observamos que os ratos infectados com leptospiras desenvolveram uma infecção crônica não letal, como descrito previamente (ATHANAZIO *et al*., 2008; BONILLA-SANTIAGO *et al*.). Como esperado, os ratos não foram a óbito, e os mesmos foram eutanaziados no dia 28 pós-infecção (pi). Amostras de rim foram coletadas para serem avaliadas pela cultura (CI), *imprint* (IM) e PCR em tempo real (qPCR). Ao contrário dos

ratos, os hamsters desenvolveram a leptospirose letal aguda, corroborando com dados anteriores nós observamos que os sintomas e a morte por leptospirose geralmente ocorrem a partir do dia 8 pós-infecção nestes animais (SILVA *et al.*, 2007; CHAGAS-JUNIOR *et al.*, 2009). Portanto, os hamsters foram eutanasiados no dia 8 pi e amostras de rim foram coletadas para serem avaliadas quanto a presença e quantidade de leptospiras. Todos os três métodos foram capazes de detectar leptospiras nos rins de todos os hamsters infectados (10/10) e entre 58% (7/12, qPCR) e 67% (8/12, CI e IM) dos ratos infectados (tabela 1). Observamos que dois dos ratos não estabeleceram a infecção crônica, e como esperado os controles foram negativos para a presença de leptospiras.

A quantificação da carga de leptospiras nos modelos animais foram determinadas por qPCR, com base no pressuposto de que um genoma corresponde a uma espiroqueta. O coeficiente de correlação da curva padrão foi 0,999 e a eficiência foi 92,4%, Fig. 1A. O limite de detecção do ensaio de qPCR, com base em diluições seriadas do DNA genômico de *Leptospira*, foi estimado em 4 genomas equivalentes por reação ou aproximadamente 50 leptospiras por μg DNA. Isto é semelhante aos estudos prévios com o uso de *lipL32* em ensaios de qPCR (ROJAS *et al.*, 2010). No modelo hamster, a carga de leptospiras variou de $3,6 \times 10^3$ a $4,9 \times 10^4$ (média de $2,4 \times 10^4$) leptospiras por μg de DNA e de 7 a 269 (média de 138) leptospiras no IM. O qPCR e o IM demonstraram correlação positiva e significante ($r_s = 0,65$ e $P = 0,02$), ver Fig. 2. As cargas de leptospiras observadas entre os ratos foram menores, variando de 50 a 825 (média = 163) leptospiras por μg de DNA e de 3 a 33 (média = 9) leptospiras para o qPCR e IM, respectivamente. A correlação entre os dois métodos foi a maior observada $r_s = 0,70$ e $P = 0,01$ (Figura 2). Os coeficientes de correlação observados em hamsters e ratos neste estudo indicaram que existe um nível moderado de correlação entre os dois métodos.

Nota-se que a carga de leptospiras no modelo rato foi inferior ao esperado, com uma média de 163 por μg de DNA renal ou uma média de 9 leptospiras por campo microscópico, dependendo do método utilizado. Anteriormente, estimamos uma carga de leptospiras no rim de ratos (7-9 dias pi) de 9 leptospiras por campo microscópico utilizando microscopia de imunofluorescência (ATHANAZIO *et al.*, 2008), semelhante ao observado no presente estudo, observando os *imprints*. No entanto, como o rato é o um dos principais hospedeiros das leptospiras nas regiões urbanas, espera-se encontrar altas cargas de leptospiras nos rins para ser excretada no meio ambiente e permitir uma transmissão eficaz da doença (ADLER, 2009). Um estudo prévio encontrou concentrações de até 10^7 leptospiras/ml de urina 28 dias

pi (NALLY *et al*., 2005). Uma possível explicação seria que as maiores concentrações de leptospiras são encontradas nos túbulos renais e não ao redor do tecido renal na infecção crônica. A metodologia aplicada neste estudo não determina a carga de leptospiras nos túbulos renais, pois as secções de rim usadas provavelmente só incluem secções transversais. Certamente, uma limitação deste estudo é que a concentração de leptospiras na urina de ratos infectados não foi determinada.

Os resultados demonstrados neste estudo reforçam a aplicabilidade do método do *imprint* para detectar leptospiras em modelos experimentais de leptospirose comumente usados e confirma os resultados do estudo original sobre o *imprint* (CHAGAS-JUNIOR, *et al*., 2009). Desde o seu desenvolvimento, a técnica do *imprint* é rotineiramente empregada em nossos laboratórios, em especial para avaliar o estado de colonização de animais usados em avaliações de potenciais candidatos a vacina. A principal desvantagem do estudo original foi a falta de uma comparação com o ensaio de qPCR para comparar a sensibilidade de detecção e quantificação da carga de leptospiras. Esta questão foi abordada neste estudo. Em termos de detecção de leptospiras (positivo ou negativo), ambos os ensaios qPCR e o IM foram comparados com o método padrão-ouro, a cultura, nos modelos hamster e rato (Tabela 1). Observe que uma potencial limitação do IM e do qPCR é a sua incapacidade de distinguir entre leptospiras viáveis e não viáveis e isso é particularmente relevante na determinação da imunidade esterilizante conferida por candidatos a vacina.

No entanto uma vantagem do IM é permitir a contagem leptospiras em *imprints* de amostras de tecidos. Entretanto, não era conhecido como a contagem de leptospiras encontrada no IM correlacionava-se com a quantificação absoluta de leptospiras encontrada no qPCR. Portanto, este estudo avaliou como os dois métodos covariariam em uma análise de correlação em dois modelos animais de leptospirose. Os valores determinados pelo qPCR e pelo IM foram analisados e uma correlação significativamente positiva foi observada entre os métodos no modelo hamster e no modelo rato de leptospirose (Figura 2). A melhor correlação foi encontrada no modelo rato. A aplicação do método do *imprint* poderá auxiliar em estudos que necessitem da avaliação da carga de leptospiras nos mais diversos tecidos. Auxiliando assim no conhecimento desta doença de grande importância para a saúde pública.

5 CONCLUSÕES

- A linhagem de camundongos A pode ser a linhagem de escolha em estudos na qual se pretende recuperar um grande número de leptospiras de rins colonizados;

- As linhagens CBA e C57BL/6 desenvolveram com maior frequência lesões inflamatórias e podem ser as mais adequadas para estudos de leptospirose associados com nefrite intersticial;

- A linhagem BALB/c é a mais indicada para estudar mecanismos que envolvam a imunidade inata e/ou a rápida resposta imune adaptativa;

- A ausência do gene do óxido nítrico sintase induzível no modelo murino teve um efeito mínimo sobre o desfecho da infecção por leptospiras, exceto pela diminuição significativa da suscetibilidade para o desenvolvimento da nefrite intersticial;

- A ausência de linfócitos funcionais B e T não impede a ocorrência de hemorragia pulmonar. Estes dados fornecem fortes evidências de que a hemorragia pulmonar na leptospirose não está relacionada apenas a mecanismos autoimunes;

- Para detecção e quantificação de leptospiras o *imprint* equivale ao PCR em tempo real;

- Houve moderada correlação entre o *imprint* e o PCR em tempo real nos modelos de infecção agudo e crônico;

- O *imprint* é um método de detecção barato que pode ser facilmente estabelecido no laboratório;

- O fato de apenas leptospiras intactas serem contadas no *imprint* é um bom indicativo que as mesmas sejam viáveis;

- O *imprint* é uma valiosa ferramenta para uso na avaliação de pontos secundários, tais como imunidade esterilizante, durante os ensaios de candidatos a vacina e na determinação de leptospiras em amostras clínicas;

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<u>APÊNDICE</u>

8.1. Outros trabalhos desenvolvidos no período do doutorado.

An imprint method for detecting leptospires in the hamster model of vaccine-mediated immunity for leptospirosis

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In determining the efficacy of new vaccine candidates for leptospirosis, the primary end point is death and an important secondary end point is sterilizing immunity. However, evaluation of this end point is often hampered by the time-consuming demands and complexity of methods such as culture isolation (CI). In this study, we evaluated the use of an imprint (or touch preparation) method (IM) in detecting the presence of leptospires in tissues of hamsters infected with *Leptospira interrogans* serovar Copenhageni. In a dissemination study, compared to CI, the IM led to equal or improved detection of leptospires in kidney, liver, lung and blood samples collected post-infection and overall concordance was good ($\kappa=0.61$). Furthermore, in an evaluation of hamsters immunized with a recombinant leptospiral protein-based vaccine candidate and subsequently challenged, the agreement between the CI and IM was very good ($\kappa=0.84$). These findings indicate that the IM is a rapid method for the direct observation of *Leptospira* spp. that can be readily applied to evaluating infection in experimental animals and determining sterilizing immunity when screening potential vaccine candidates.

INTRODUCTION

Leptospirosis is a widespread zoonosis with a global distribution, caused by pathogenic spirochaetes of the genus *Leptospira* (Adler & de la Pena Moctezuma, 2009; Levett, 2001). The major impact of leptospirosis is the high rate of case fatality due to its most severe complications: Weil's disease (>10 %) (Bharti *et al.*, 2003) and severe pulmonary haemorrhage syndrome (>50 %) (Gouveia *et al.*, 2008; Marotto *et al.*, 1999; Park *et al.*, 1989). A priority in current research on leptospirosis is the development of a vaccine that is able to elicit long-term immunity and to induce cross-protection against the serovars that are of greatest importance to public health (Adler & de la Pena Moctezuma, 2009; Koizumi & Watanabe, 2005; McBride *et al.*, 2005).

Several groups have reported on the use of various animal models, including mice, hamsters and gerbils, for vaccine candidate evaluation studies (Haake *et al.*, 1999; Koizumi & Watanabe, 2004; Palaniappan *et al.*, 2006; Silva *et al.*, 2007; Sonrier *et al.*, 2000). The primary goal of any vaccine is to induce a specific immune response such that the initial infection is prevented or subsequently eliminated (Seder & Mascola, 2003). Therefore, an important evaluation of vaccine efficacy is carriage status, as a successful vaccine should confer sterilizing immunity to the vaccinated animal. However, the gold standard protocol is culture isolation (CI), with the potential problems of contamination, incubator space requirements and delayed results (up to 8 weeks) (Palaniappan *et al.*, 2005).

In other disease conditions, tissue imprints (or touch preparations) are commonly used for the direct detection of micro-organisms. In leishmaniasis, the direct observation of trypanosomes in tissue imprints of canine liver and spleen samples is regarded as the definitive laboratory diagnostic test (Bahamdan *et al.*, 1996; Berger *et al.*, 1987;

Kar, 1995; Roscoe, 2005; Teixeira *et al.*, 2008; Volpini *et al.*, 2006). The technique has been applied to several infectious diseases (Jan *et al.*, 2008; Olsen & Stenderup, 1990; Silverman & Gay, 1995). The aim of this study was to adapt and evaluate the imprint method (IM) for the direct observation of leptospires in samples from experimentally infected hamsters, comparing its performance against CI and immunohistochemistry (IHC).

METHODS

***Leptospira* strain and culture conditions.** *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, isolated from a patient during an outbreak of leptospirosis in the city of Salvador, Brazil (Ko *et al.*, 1999; Nascimento *et al.*, 2004), was cultivated in liquid Ellinghausen–McCullough–Johnson–Harris medium (EMJH, Difco) at 29 °C (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) and counted in a Petroff–Hausser counting chamber (Fisher Scientific), as described by Faine *et al.*, (1999). The strain was passaged four times in Golden Syrian hamsters (*Mesocricetus auratus*) and stored at −70 °C. Before use, the aliquots were thawed and passaged in liquid EMJH seven times prior to use as a low-passage isolate in the infection experiments.

Experimental infection of hamsters. Female, 9-week-old hamsters (Fiocruz) were used in leptospiral dissemination studies and in evaluating the performance of the three methods for the direct detection of *Leptospira* spp. (Silva *et al.*, 2008). Groups of hamsters ($n=5$, days 3 and 6 post-infection (p.i.); $n=15$, day 9 p.i.) were inoculated intraperitoneally with 250 leptospires in 1 ml PBS, equivalent to $2.5 \times LD_{50}$ (Silva *et al.*, 2007), and were sacrificed at intervals ranging from 1 day to 21 days p.i. Control animals ($n=5$) were injected intraperitoneally with 1 ml sterile PBS. Kidney, liver, lung and blood samples were collected aseptically for evaluation. Collection of blood samples during the dissemination study was by phlebotomy of the retro-orbital venous plexus. The Ethical Committee of the Oswaldo Cruz Foundation approved all animal protocols used in this study.

Culture isolation of leptospires. CI was performed as previously described (Athanazio *et al.*, 2008). Briefly, tissue samples (kidney, liver and lung) were homogenized in 10 ml EMJH, cell debris was allowed to settle for 10 min and 0.5 ml cleared homogenate was inoculated into 5 ml EMJH. Blood samples (1–2 drops) were inoculated directly into 5 ml EMJH, and all cultures were incubated at 29 °C. The cultures were examined regularly for growth, by darkfield microscopy, for up to 8 weeks.

Immunohistochemistry. Necropsies were performed immediately upon sacrifice and kidney, lung and liver samples were stored at −70 °C in mounting medium (Tissue-Tek, Sakura Finetek). Frozen sections (4–5 μm) were thawed out onto poly-L-lysine-coated glass slides and incubated (1 h) with a primary polyclonal anti-leptospiral antibody (prepared by immunizing New Zealand White rabbits with a whole-cell preparation of *L. interrogans* serovar Icterohaemorrhagiae strain RGA (Athanazio *et al.*, 2008) at a dilution of 1:200. After three washes in PBS, the sections were incubated (1 h) with goat anti-rabbit IgG-FITC conjugate (Jackson ImmunoResearch) at 1:500, and washed three times in PBS before visualization of stained organisms by fluorescence microscopy. Frozen tissue sections from non-infected hamsters were used as negative controls.

Imprint detection. Imprints were obtained by direct pressure of the cut surface of the tissue sample onto poly-L-lysine-coated glass slides.

Blood smears for imprint were prepared by placing one drop of blood onto a poly-L-lysine-coated glass slide and a smear was formed using a spreader slide. Imprint slides were dried at room temperature, fixed in acetone for 3 min and the smear regions demarcated with a hydrophobic barrier pen. Imprint slides were incubated (1 h) with a primary rabbit polyclonal anti-leptospiral antibody at a dilution of 1:200. After three washes in PBS, the imprints were incubated (1 h) with goat anti-rabbit IgG-FITC conjugate at 1:500, washed three times in PBS and dried before visualization of stained organisms by fluorescence microscopy. Tissue imprints from non-infected hamsters and normal (uninfected) rabbit serum (NRS) were used as negative controls. Counterstaining of nucleic acids or cytoplasm was achieved with 1 mg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI, Sigma Aldrich) or 0.1 % Evans Blue (Sigma Aldrich) at room temperature for 10 min.

Hamster immunization and challenge studies. Recombinant LigA C-terminal protein fragments were cloned, expressed and purified as described previously (Silva *et al.*, 2007). The subunit vaccine preparations were prepared by adsorption of the recombinant leptospiral immunoglobulin-like (Lig) proteins to aluminium hydroxide (alhydrogel) adjuvant. Immunization protocols were performed as described previously (Silva *et al.*, 2007) with the following modifications. Briefly, female, 4-week-old hamsters were immunized by intramuscular injection on day 0 with a second immunization on day 14; control animals were injected with alhydrogel at the same intervals. The hamsters were challenged with an intraperitoneal inoculum of 250 leptospires (strain Fiocruz L1-130) in 1 ml PBS. Hamsters were monitored three times a day during the post-challenge period and euthanized when clinical signs (prostration and jaundice or pulmonary haemorrhage) of terminal disease appeared. Survivors were euthanized 21 days p.i. Kidney samples were collected from all surviving hamsters in eight independent experiments. Blood, liver and lung samples were collected from a random selection of immunized animals. The kidney samples were analysed using both CI and the IM detection methods. The IM was used to screen the remaining blood, liver and lung samples.

Statistical analysis. Statistical analysis was carried out using Epi Info, ver 6.04 (Centers for Disease Control and Prevention, USA). The chi-squared test was used to compare proportions; $P<0.05$ was considered significant. Concordance between detection methods was calculated using Cohen's Kappa (Landis & Koch, 1977).

RESULTS AND DISCUSSION

Direct observation of leptospires in a dissemination study

The IM permitted the direct observation of leptospires by using specific anti-*Leptospira* whole-cell-antigen antibodies and an FITC conjugate or DAPI in kidney, liver, lung and blood samples (Fig. 1a, b). Table 1 summarizes how the three different methods performed in the detection of leptospires in kidney, liver, lung and blood samples collected from infected hamsters. Leptospires, although present, could not be detected until day 6 p.i. The IM had a higher, but not significant, rate of detection than CI on day six p.i. in the kidney (100 vs 60 %, respectively), liver (100 vs 80 %) and lung (40 vs 20 %) from infected hamsters, while the two methods exhibited a similar capacity to detect leptospires in blood samples (80 vs 80 %). By day 9
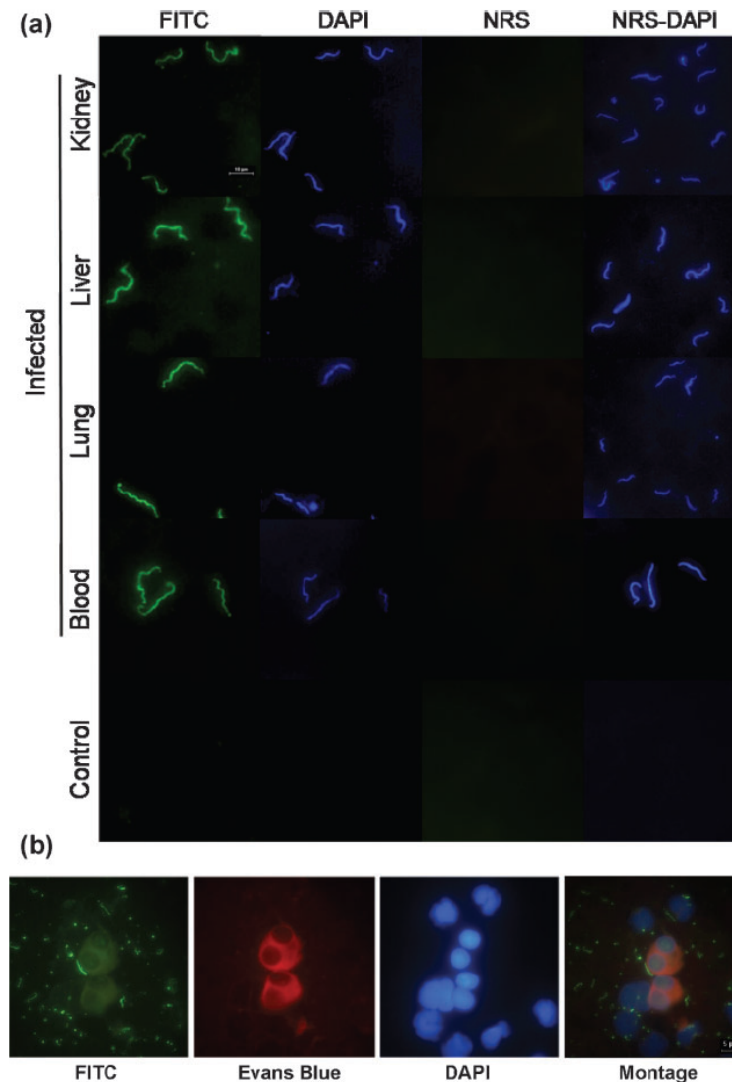
Fig. 1. Direct observation of leptospires in imprint samples. (a) Isolated leptospires were detected in kidney, liver, lung and blood imprint samples from hamsters infected with *L. interrogans* serovar Copenhageni strain Fiocruz L1-130; the negative control kidney sample was from an uninfected hamster. Normal rabbit serum (NRS) was included as a negative control. Bar, 10 μm. (b) Leptospires closely associated with mammalian cells were observed using FITC, cytoplasm was stained with Evans Blue, and DNA with DAPI (note the quenching of fluorescent signal from the leptospires). A montage of the superimposed images is shown. Bar, 5 μm.

p.i., the methods detected leptospires in all 15 of the kidney samples analysed. Both the IM and IHC offered a significant improvement in detection over CI in liver (100 and 100 % vs 53.3 %, respectively, $P<0.01$) and lung samples (80.0 and 73.3 % vs 18.2 %, $P<0.01$). The IM and CI performed similarly for blood samples (53.3 vs 46.6 %, respectively). When blood samples were collected daily from day 2 to day 10 p.i., leptospires were detected by day 6 p.i. and overall IM performance was equal or superior to CI. On day 9 p.i., the IM detected leptospires in 80 % (4/5) compared to 20 % (1/5) for CI; however, this difference was not significant (data not shown). Concordance was good for all three tests ($\kappa=0.61$), and for CI and the IM ($\kappa=0.64$), agreement between CI and IHC was moderate ($\kappa=0.49$), while between the IM and IHC concordance was very good ($\kappa=0.96$). When stratified according to sample type, the agreement between CI and the IM was very good in kidney ($\kappa=0.86$) and blood samples ($\kappa=0.93$), moderate in liver samples ($\kappa=0.50$) and fair in lung samples ($\kappa=0.23$).

Detection of leptospires in immunized hamsters post-challenge

Summarizing the eight experiments performed (Table 2), survival was 85.0 % (68/80) among immunized hamsters compared to 2.5 % (2/80) among control hamsters immunized with adjuvant only. Among the immunized hamsters which survived, cultures from eight animals could not be evaluated due to contamination. In an evaluation of sterilizing immunity, leptospires were isolated (CI) from

Table 1. Direct detection of leptospires in a dissemination study of hamsters infected with *L. interrogans* strain Fiocruz L1-130

Days p.i.	Method*	Number of positives/total (% positive)			
		Kidney	Liver	Lung	Blood
3	CI	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
	IM	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
6	CI	3/5 (60)	4/5 (80)	1/5 (20)	4/5 (80)
	IM	5/5 (100)	5/5 (100)	2/5 (40)	4/5 (80)
9	CI	15/15 (100)	8/15 (53)	2/11 (18)†	7/15 (47)
	IHC	15/15 (100)	15/15 (100)	11/15 (73)	ND
	IM	15/15 (100)	15/15 (100)	12/15 (80)	8/15 (53)
Uninfected controls	CI	0/5 (0)	0/5 (0)	0/4 (0)‡	0/5 (0)
	IHC	0/5 (0)	0/5 (0)	0/5 (0)	ND
	IM	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)

ND, Not determined.
*CI, culture isolation; IHC, immunohistochemistry; IM, imprint method.
†Four cultures were excluded due to contamination.
‡One culture was excluded due to contamination.

the kidneys of 23.3 % (14/60) of surviving hamsters, whereas leptospires were detected by the IM in 26.5 % (18/68) of surviving hamsters. In a random evaluation of the IM, leptospires were detected in 42.8 % (9/21) liver samples and 20.0 % (1/5) lung samples, while none of the blood samples (0/43) were positive. Based on the kidney samples, there was very good agreement between CI and the IM ($\kappa=0.84$). It is of note that all samples that were CI positive were also positive by the IM.

Sterilizing immunity is an important secondary end point in vaccine candidate evaluations, particularly since it is known that commercial vaccines for animal use do not prevent leptospiruria (Adler & de la Pena Moctezuma, 2009). The gold standard for the direct detection of leptospires in biological samples is culture isolation (Faine *et al.*, 1999; WHO & ILS, 2003). However, this method is not ideal due to the fastidious nature of pathogenic *Leptospira* spp., the need to incubate the cultures for up to 8 weeks, the considerable demand on laboratory resources

when large numbers of cultures are involved, and the risk of contamination (Palaniappan *et al.*, 2005). The main aim of this study was to adapt and evaluate the imprint method for the direct detection of leptospires in biological samples. To our knowledge, this is the first report on the application of this technique to the field of leptospirosis.

A potential limitation in the use of the IM is that although the technique identifies intact *Leptospira* spp. it may not discriminate between intact-viable and intact-non-viable leptospires. However, the IM only demonstrated significantly increased detection of leptospires, when compared to CI, in the dissemination study. It is of note that in the dissemination study the presence of leptospires can be presupposed, as 100 % of the infected animals died. It is known that leptospires are cleared from the blood during the first 2 weeks of infection (Faine *et al.*, 1999; Levett, 2001); therefore if the IM was detecting intact non-viable leptospires it should have identified more positive samples by day 9 p.i. and this was not the case (Table 1). It is

Table 2. Evaluation of the IM for the detection of sterilizing immunity in the hamster model of leptospirosis

Outcome	Sample	Immunized hamsters* % Positive (no./total)			Non-immunized hamsters* % Positive (no./total)		
		CI	IM	Total	CI	IM	Total
Survival	Kidney	23 (14/60)†	26 (18/68)	68	100 (2/2)	100 (2/2)	2
	Liver	ND	43 (9/21)		ND	ND	
	Lung	ND	20 (1/5)		ND	ND	
	Blood	ND	0 (0/43)		ND	ND	
Death	NA	NA	NA	12	NA	NA	78

ND, Not determined; NA, not applicable.
*Pooled results from eight independent experiments.
†Eight cultures lost to contamination.

possible that the antigenic determinants targeted in the IM are not readily detected in intact non-viable leptospires, possibly due to loss of the outer membrane or changes in conformation or degradation of the antigens.

The leptospiral immunoglobulin-like (Lig) proteins are highly conserved, unique to pathogenic *Leptospira* spp. (Matsunaga *et al.*, 2003; Cerqueira *et al.*, 2009; McBride *et al.*, 2009), and their use in vaccine preparations has been reported by several groups (Koizumi & Watanabe, 2004; Palaniappan *et al.*, 2006). We reported that hamsters immunized with a LigA C-terminal fragment in Freund's adjuvant were significantly protected (67–100 %), but that all survivors exhibited evidence of renal colonization (Silva *et al.*, 2007). In addition, LigA vaccine formulations were reported to confer 75–100 % protection, with sterilizing immunity ranging from 25 to 90 % (Faisal *et al.*, 2008, 2009; Palaniappan *et al.*, 2006). A vaccine preparation of LigB and alhydrogel induced significant protection in hamsters (54–83 %) but did not confer sterilizing immunity (Yan *et al.*, 2009). In C3H/HeJ mice, immunization with Lig proteins elicited significant protection (100 %), but not sterilizing immunity (Koizumi & Watanabe, 2004). Haake *et al.*, (1999) found that hamsters immunized with recombinant OmpL1 and LipL41 were significantly protected (71–100 %) and sterilizing immunity was 100 %. Such variability in sterilizing immunity may be associated with the virulence of the challenge strain, as the more virulent challenge strains resulted in kidney colonization (Silva *et al.*, 2007; Faisal *et al.*, 2008, 2009; Yan *et al.*, 2009); however, this was not always the case (Haake *et al.*, 1999). Yet another factor could be the poor reproducibility of CI, highlighting the urgent need for an improved standardized method. Among the eight cultures that were contaminated during CI, three were imprint positive. This suggests that the imprint technique could provide more reproducible results than CI in evaluating sterilizing immunity.

The IM proved to be a robust and sensitive method for the direct observation of intact leptospires in blood and tissue samples. Although the technique was equal to IHC in terms of detection, the IM had advantages in terms of its relative simplicity and reduced time to result. More importantly, the IM performed equally to CI in evaluating sterilizing immunity (Table 2) in the hamster model of leptospirosis. The use of the IM could also be applied to the direct examination of urine, as this is traditionally evaluated by darkfield microscopy and is unreliable (WHO & ILS, 2003). This could lead to the application of the IM in field evaluations of leptospiral carriage among natural reservoirs; this is currently being evaluated.

In conclusion, we report the successful adaptation and evaluation of the imprint method for the direct detection of pathogenic *Leptospira* spp. in blood and tissue samples collected from an experimental animal model. Furthermore, the technique was readily applied to the evaluation of sterilizing immunity in vaccine-mediated immunity studies. We therefore propose that the imprint technique should be

considered as a complementary method for the detection of leptospires in animal models of leptospirosis.

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Ionic imbalance and lack of effect of adjuvant treatment with methylene blue in the hamster model of leptospirosis

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Leptospirosis in humans usually involves hypokalaemia and hypomagnesaemia and the putative mechanism underlying such ionic imbalances may be related to nitric oxide (NO) production. We previously demonstrated the correlation between serum levels of NO and the severity of renal disease in patients with severe leptospirosis. Methylene blue inhibits soluble guanylyl cyclase (downstream of the action of any NO synthase isoforms) and was recently reported to have beneficial effects on clinical and experimental sepsis. We investigated the occurrence of serum ionic changes in experimental leptospirosis at various time points (4, 8, 16 and 28 days) in a hamster model. We also determined the effect of methylene blue treatment when administered as an adjuvant therapy, combined with late initiation of standard antibiotic (ampicillin) treatment. Hypokalaemia was not reproduced in this model: all of the groups developed increased levels of serum potassium (K). Furthermore, hypermagnesaemia, rather than magnesium (Mg) depletion, was observed in this hamster model of acute infection. These findings may be associated with an accelerated progression to acute renal failure. Adjuvant treatment with methylene blue had no effect on survival or serum Mg and K levels during acute-phase leptospirosis in hamsters.

Key words: leptospirosis - methylene blue - models - animal

Leptospirosis is a widespread zoonosis in which the most important and life-threatening complications are acute renal failure and pulmonary haemorrhage (Cerqueira et al. 2008, Medeiros et al. 2010). The beneficial effect of antibiotics against severe leptospirosis when treatment is initiated within four days after its clinical onset is undisputed. Although antimicrobial treatment is recommended even if it is delayed, the clinical benefits in this case are controversial (WHO 2003). When severe manifestations develop, the most important issue in clinical management is supportive therapy, including dialysis and mechanical ventilation (McBride et al. 2005). In clinical practice, leptospirosis is usually not initially suspected or is diagnosed late because its features overlap with other diseases (e.g., hepatitis and dengue) and because of the limited performance of confirmatory serological tests. Thus, despite aggressive supportive treatment, fatalities from severe forms of leptospirosis remain high and adjuvant therapies that, in association with antibiotics, could benefit patient outcomes are urgently needed. We previously developed a model of antibiotic therapy initiated late after the infec-

tion of hamsters. In the first evaluated adjuvant therapy, the antioxidant effects of N-acetylcysteine did not yield any additional benefit compared with ampicillin treatment alone (Spichler et al. 2007).

Leptospirosis causes a peculiar form of non-oliguric renal failure [characterised by potassium (K) depletion] that rapidly evolves to an oliguric hyperkalaemic form, indicative of a poor outcome (Cerqueira et al. 2008). Several reports have described patients with severe leptospirosis who developed hypomagnesaemia during the acute phase of the disease (Khositseth et al. 2008, Spichler et al. 2008, Craig et al. 2009). Within the kidneys, the major site of magnesium (Mg) transport is the thick ascending limb (TAL), where 65% of filtered Mg is reabsorbed, while 20-25% returns to the blood through the proximal tubule and 5-10% returns to the distal tubule (Berkelhammer & Bear 1985). Regardless of which major molecular targets of leptospirosis lead to tubular dysfunction, impaired ion transport results in sodium (Na) and K wasting (Covic et al. 2003, Cerqueira et al. 2008). The gradient of K and Na is the major driving force for the paracellular reabsorption of Mg in the TAL. Thus, some degree of Mg loss may be expected in K/Na-wasting states. Because hypomagnesaemia is a common feature of critically ill patients, is correlated with a poor prognosis and has been increasingly recognised in association with severe leptospirosis, we inferred that this ionic imbalance might represent a promising target for adjuvant therapy to treat leptospirosis. Therefore, we were interested in determining whether our hamster model of leptospirosis reproduces ionic changes, such as hypomagnesaemia or hypokalaemia and how these

changes correlate with disease outcomes. Studies on experimental leptospirosis focusing on ionic changes are scarce and have been restricted to in vitro models (Wu et al. 2004), microperfusion analyses (Magaldi et al. 1992) and evaluation of tubular transporter expression via immunohistochemistry (Spichler et al. 2007). To the best of our knowledge, no study has previously evaluated the possible reproduction of serum Mg changes in experimental leptospirosis.

Clinical studies based on clearance tests suggest that the main tubular defect involved in leptospirosis is impaired function of the Na,2Cl,K cotransporter (NKCC2) in the TAL (Lin et al. 1999, Wu et al. 2004). In vitro, the NKCC2 of murine TAL cells can be inhibited using leptospiral outer membrane extracts (Wu et al. 2004). In our hamster model, we demonstrated that NKCC2 expression is reduced in TAL cells during acute infection and the downregulation of NKCC2 can be reversed by antimicrobial therapy (Spichler et al. 2007). Taken together, these data suggest a potential direct toxic effect of leptospires on tubular transporters. Furthermore, the renal loss of Mg, Na and K may be related to the production of nitric oxide (NO), which is a known inhibitor of NKCC2 (Ortiz & Garvin 2002, Beltowski et al. 2003). Inducible NO synthase (iNOS) is stimulated in vitro when tubular cells are exposed to leptospirally derived products (Yang et al. 2000, 2002, 2006) and we demonstrated that serum levels of NO correlate with a laboratory marker of renal dysfunction (serum creatinine) in patients (Maciel et al. 2006). Recently, Prêtre et al. (2011) reported increased expression of iNOS in vivo in the kidneys of hamsters and C3H/HeJ mice during acute infection (as determined via immunoblot and immunohistochemistry analyses) as well as elevated nitrite/nitrate concentrations in serum samples from these animals. Thus, inhibition of NO production represents a potential therapeutic target for adjuvant therapy in severe leptospirosis. Methylene blue is a known inhibitor of soluble guanylyl cyclase (which is downstream of the action of any NOS isoform) that shows encouraging results in patients with sepsis (Kirov et al. 2001, Kwok & Howes 2006, Heemskerk et al. 2008, Paciullo et al. 2010).

The aims of this study were to test the following parameters in our hamster model of leptospirosis (i) whether the acute form of the disease is associated with hypokalaemia and hypomagnesaemia and (ii) whether adjuvant therapy using methylene blue has beneficial effects on survival or ionic imbalance during acute experimental leptospirosis in hamsters.

MATERIALS AND METHODS

Bacteria - Leptospires were cultured in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco Laboratories, Detroit, MI) at 29ºC and were counted in a Petroff-Hausser counting chamber (Fisher Scientific, Pittsburgh, PA). An isolate from Brazil, *Leptospira interrogans* serovar Copenhageni strain L1-130, was used in all of the assays (Nascimento et al. 2004). This strain was passaged and re-isolated four times from the hamsters and was stored at -70ºC. Frozen aliquots were thawed and passaged in liquid medium eight times prior to use as a low-passage-number isolate in the infection experiments.

Study design - Nine-week-old female Golden Syrian hamsters [Oswaldo Cruz Foundation (Fiocruz), state of Bahia] were used in all of the experiments. The Ethical Committee of the Fiocruz approved the animal protocols employed in this study. Based on previous experiments in which an inoculum of 10^3 leptospires was found to cause 100% lethality, the interval between infection and death was estimated to be 10-14 days. In three preliminary experiments, late ampicillin treatment (100 mg/kg/bid) was tested to determine the day on which the initiation of antimicrobial therapy would yield an approximately 50% survival rate (Table I) and that day was chosen to test the adjuvant methylene blue therapy.

TABLE I

Effect of the interval between infection (*Leptostpira interrogans* strain L1130) and the initiation of ampicillin (100 mg/Kg/bid) treatment on the survival of nine-week-old hamsters

Experiment	1[a]		2[b]		3[b]	
Starting day	Deaths n/N (%)	Days to death	Deaths n/N (%)	Days to death	Deaths n/N (%)	Days to death
Untreated	ND	-	7/11 (64)	10, 10, 10, 10, 11, 11, 13	7/7 (100)	9, 9, 10, 13, 13, 13, 13
6	0/6	-	ND	-	0/7	-
7	0/6	-	ND	-	ND	-
8	0/6	-	0/7	-	0/7	-
9	2/6 (33)	10, 10	ND	-	ND	-
10	ND	-	2/6 (33)	12, 13	2/5 (40)	10, 15
11	ND	-	ND	-	ND	-
12	ND	-	3/4 (75)	12, 12, 12	ND	-

a: inoculum size, 500 leptospires; *b*: inoculum size, 1,000 leptospires; ND: not done; n/N: number of deaths/total number of evaluated hamsters.

The hamsters were inoculated intraperitoneally with 10^3 bacteria from virulent strain L1130. The experiment began with 80 infected animals that were observed and euthanised in groups of 20 on days 4, 8, 16 and 28. After treatment was initiated, the 20 animals from each time point were further divided into four groups according to the type of treatment initiated on the 10th day, which was ampicillin alone, methylene blue alone, ampicillin and methylene blue together or no treatment.

In a second experiment, hamsters were infected with a high inoculum dose of 10^6 leptospires and assigned to groups (of 9-11 animals) that were treated with ampicillin alone, methylene blue alone, ampicillin and methylene blue together or received no treatment. The treatments were planned to begin when the first death was observed. This study design was used as an alternative strategy to reproduce the late initiation of therapy during acute lethal leptospirosis.

Blood tests - Serum Na, K and Mg levels were measured using a Labmax 240 device (Labtest Diagnostica SA, Minas Gerais, Brazil). Na and K were quantified using ion-selective electrodes, while Mg was measured using a colorimetric method. Creatinine analyses were performed in serum with an immunochemistry assay (A25 system, Biosystems SA, Barcelona, Spain). The reference values presented in Figs 1-4 were obtained from previous clinical chemistry reports on laboratory hamsters (Tomson & Wardrop 1987). Four or five uninfected animals were also tested to serve as controls.

Histopathological analysis - Necropsies were performed immediately after euthanasia. Kidneys were fixed in 4% formalin and embedded in paraffin and 4-5 μm-thick sections were subjected to conventional histological analyses. Semi-quantitative estimation of interstitial nephritis was performed as previously described (Bandeira et al. 2011). Briefly, grade + nephritis was defined as an infiltrate that was rich in macrophages and lymphocytes restricted to periarterial areas, grade ++

Fig. 2: serum levels of magnesium (Mg) in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mg/dL. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).

Fig. 3: serum levels of sodium (Na) in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mEq/L. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).
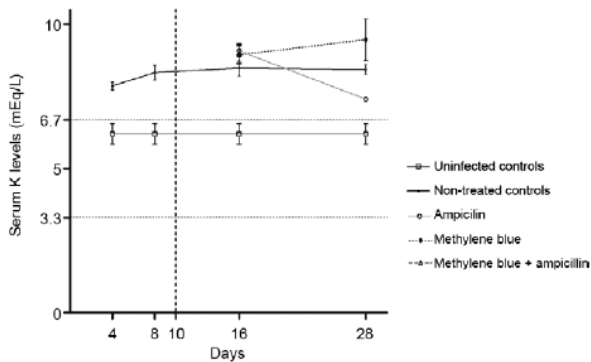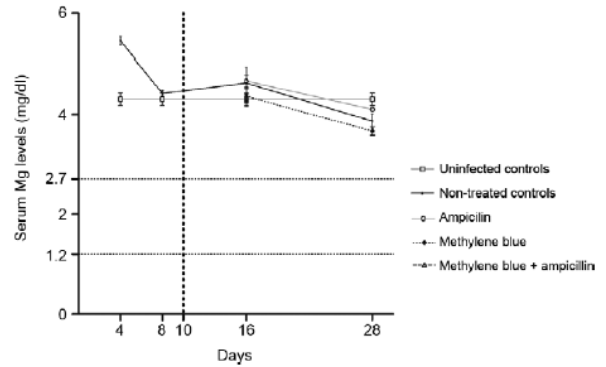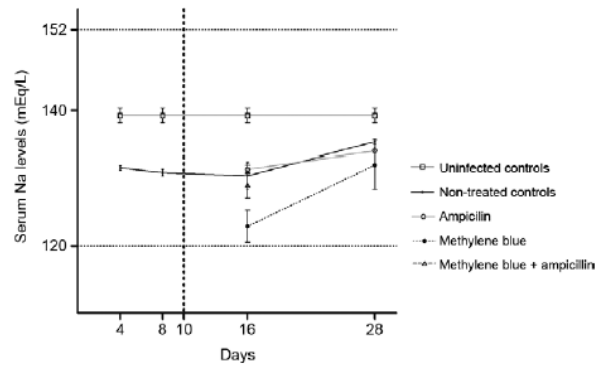

Fig. 1: serum levels of potassium (K) in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mEq/L. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).
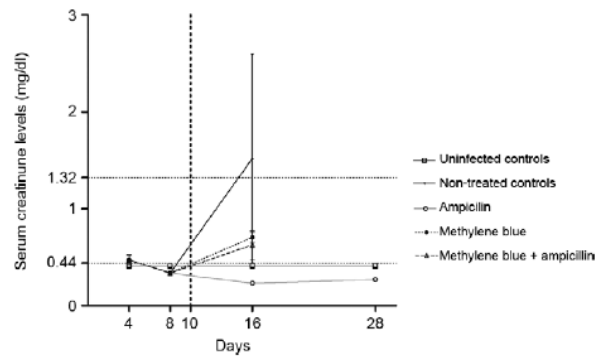

Fig. 4: serum levels of creatinine in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mg/dl. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).

nephritis was characterised as an infiltrate that extended to other renal parenchymal zones with one-two lesions per field of view at 100X magnification and grade +++ nephritis was characterised by lesions detected in more than two areas per field of view at 100X magnification. In the present study, the regeneration of the tubular epithelium was quantified using the criteria for foci of interstitial nephritis. Acute tubular damage (tubular cell swelling) was also estimated semi-quantitatively, as mild, moderate or severe.

Statistical analyses - Statistical analyses and graphical presentation of the data were performed using the Prism v4.03 software package (GraphPad Software Inc, La Jolla, CA USA). Numerical data were compared using the non-parametric Mann-Whitney U test when comparing two groups and using the non-parametric Kruskal-Wallis U test when comparing more than two groups. Survival curves were compared using the log-rank Mantel-Cox test. A p value < 0.05 was considered significant.

RESULTS

We selected the 10th day for the initiation of treatment, as this resulted in the survival rate closest to 50% (Table I). During the second and third preliminary experiments, in which the selected inoculum of 1,000 leptospires was used, ampicillin treatment initiated on day 10 resulted in survival rates of 33% and 40%, respectively.

The first experiment involved 80 hamsters. At both the day 4 and day 8 scheduled time points, 20 animals were euthanised and necropsied. Treatments were initiated on day 10. Another 20 hamsters were evaluated at the day 16 and day 28 time points, now assigned to four different treatment groups. Prior to day 16, deaths were observed in two untreated hamsters (both on day 11), four ampicillin-treated hamsters (on days 10, 10, 10 and 11) and five ampicillin + methylene blue-treated hamsters (on days 10, 10, 10, 11 and 13). At day 16, five animals were evaluated in each group. Then, prior to day 28, two additional animals from the methylene blue-treated group died (both on day 27). Thus, on day 28, all of the survivors were euthanised, which included three in the untreated group, three in the methylene blue only group and one in the ampicillin only group. No hamsters treated with both ampicillin and methylene blue survived until day 28. Blood and tissue samples were only collected from animals that survived until the scheduled time points.

At days 4 and 8, serum K levels were found to be significantly higher (Mann-Whitney U, p < 0.05) among the infected hamsters compared with uninfected hamsters. At day 16, all of the infected groups displayed higher serum K levels compared with the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the methylene blue and no-treatment groups still exhibited significantly higher levels of serum K compared with the controls. The dynamics of the serum K levels in the different treatment groups are shown in Fig. 1.

Both the uninfected and infected animals (regardless of the treatment group) showed higher serum Mg levels compared to historical (literature-based) reference values. For this reason, serum Mg measurements were repeated twice for each sample and samples from other uninfected hamsters collected after the end of experiment were found to show consistent serum Mg levels of 4.0-4+5 mg/dl. In all cases, these measurements confirmed similar results. Infected animals showed higher Mg levels compared to uninfected controls on day 4 and there was a trend toward decreasing levels of Mg detected on days 8, 16 and 28 among the infected hamsters. Serum Mg levels were significantly higher among the infected hamsters compared with the uninfected hamsters on day 4 (Mann-Whitney U, p < 0.05). None of the other differences were significant. The dynamics of the serum Mg levels in the different treatment groups are shown in Fig. 2.

Serum Na levels were within the normal range in all hamsters, though there was a trend toward lower Na levels among the infected animals compared with the uninfected controls. The serum Na levels were significantly lower among the infected hamsters compared with the uninfected hamsters on days 4 and 8. At day 16, all of the infected groups exhibited lower serum Na levels compared with the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the no-treatment group still presented significantly lower Na levels compared with the controls and the group receiving methylene blue exhibited a trend toward lower Na levels (Mann-Whitney U, p = 0.06). The dynamics of the serum Na levels in the different treatment groups are shown in Fig. 3.

The only difference observed between the groups of infected animals was that the methylene blue treatment group presented lower serum Na levels at day 16 compared with the other infected groups (Kruskal-Wallis, p = 0.04).

Serum creatinine levels peaked at day 16 and were higher in the untreated group. The serum creatinine levels in the untreated group were higher than normal reference limits and significantly higher than the levels in the hamsters in all of the treatment groups (Kruskal-Wallis, p = 0.02). Ampicillin administration prevented the elevation of serum creatinine at days 16 and 28. The dynamics of the serum creatinine levels in the different treatment groups are shown in Fig. 4.

The kidney samples from the hamsters euthanised at day 4 were uniformly normal when examined under light microscopy (data not shown). In contrast, the hamsters euthanised on day 8 presented the typical features of acute disease, such as diffuse massive tubular cell swelling with mild or no interstitial nephritis. Foci of tubular cell swelling were still detectable at days 16 and 28, but were not as large or as diffusely distributed as on day 8. Regenerative tubules and interstitial inflammation were detected to various degrees on days 16 and 28, but not on day 8. The frequency and severity of renal lesions did not differ in the infected hamsters on day 16 and 28. Illustrative images of the renal histopathology of these hamsters are displayed in Fig. 5.

A second experiment evaluated the outcome of infection when treatment was initiated immediately after the first death was observed among the infected animals (regardless of the assigned group). In this case, only 10% of the tested animals survived in the ampicillin group, whereas infection was uniformly lethal in the untreated, methyl-

ene blue-treated and methylene blue + ampicillin-treated groups (Table II). Additional methylene blue administration resulted in no improvement of outcomes, evaluated either in terms of survival or the period between infection and death (Log-rank Mantel-Cox test, p = 0.35).

DISCUSSION

The hamster model employed in this study did not reproduce the hypokalaemia that is commonly observed in human leptospirosis. The rapid and progressive development of hyperkalaemia most likely reflects experimental conditions in which supportive therapy is not feasible, such as venous rehydration. Even the surviving hamsters that were euthanised at day 28 presented elevated levels of serum K (Fig. 1). Antibiotic treatment and/or methylene blue treatment had no effect on serum K levels in the infected hamsters. Serum K levels were significantly higher among the infected hamsters compared with the uninfected hamsters on days 4 and 8. At day 16, all of the infected groups exhibited higher serum K levels than the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the methylene blue and no-treatment groups still exhibited significantly higher levels of serum K than the controls.

A similar pattern of Mg elevation was observed among the infected hamsters (Fig. 2). Although some Mg depletion might be expected based on clinical studies, rapid progression to severe renal failure could explain the retention of Mg. In contrast to the K dynamics, there was a trend toward decreasing levels of Mg observed on days 8, 16 and 28. The baseline serum Mg levels in the uninfected hamsters were the only measurements that were considerably different (higher) compared with the reference values for laboratory hamsters. The serum Mg levels were significantly higher among the infected hamsters compared with the uninfected hamsters at day 4. None of the other differences were significant.

High baseline serum Mg levels were not expected. Reference values for serum Mg (Fig. 2) in hamsters were obtained from a textbook (Tomson & Wardrop 1987), which were based in two previous studies using 164 and 19 hamsters (mean ± standard deviation of up to 2.5 ± 0.2 mg/dl in males from one study and 1.6 ± 0.4 mg/dl in females from the other). The range suggested from these data is indeed close to the reference serum Mg levels in humans. Another reference textbook indicated values with a considerably wider range, of 1.9-3.5 mg/dl (Gad 2007). However, a more recent textbook apparently ig-
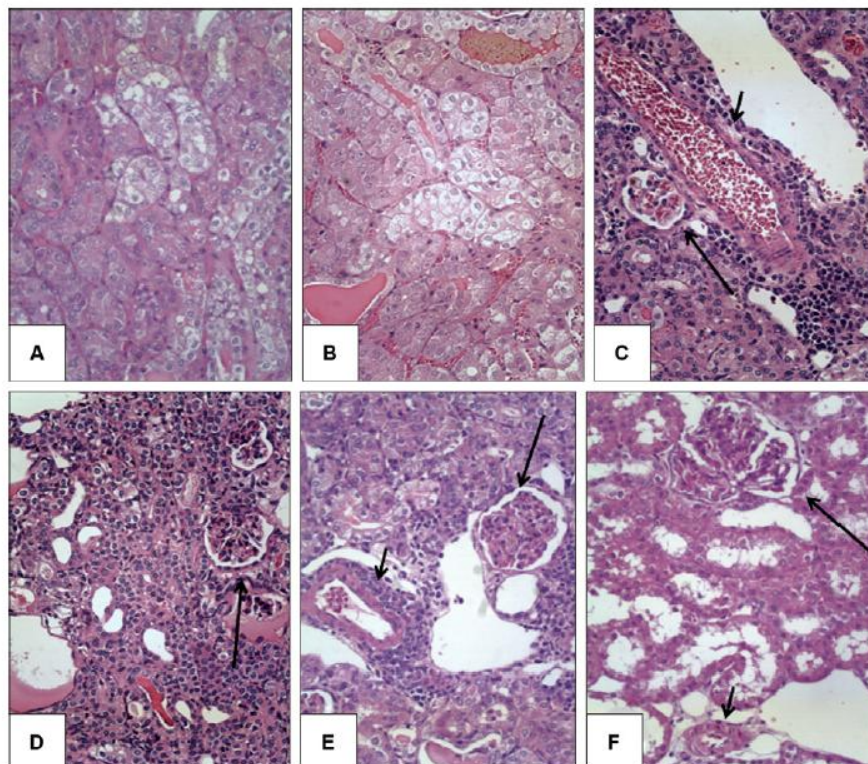

Fig. 5: typical lesions of leptospirosis in the infected hamsters. A: marked swelling of the epithelial cells of the proximal tubules in an untreated hamster at day 8; B: other focus of tubular cell swelling in an untreated hamster at day 16; C: mild interstitial nephritis with infiltrates rich in lymphocytes, plasma cells and macrophages surrounding an artery of an untreated hamster at day 16; D: regenerative changes of cortical tubular epithelium of an untreated hamster at day 16; E: mild interstitial nephritis with infiltrates rich in lymphocytes, plasma cells and macrophages surrounding the glomeruli and small arteries in the renal cortex of an ampicillin-treated hamster at day 28; F: normal kidney of an uninfected hamster at day 8. The short arrows indicate small arteries and the long arrows indicate the glomerulus. Haematoxylin-eosin (A-F) 200X.

nores these previous data and concludes that reference serum Mg values are not available for hamsters. Strikingly, the indicated reference values for other laboratory animals are as high as 2.0-5.4 mg/dl in rabbits, 3.5-4.1 mg/dl in guinea pigs and 3.6-4.0 mg/dl in chinchillas (Washington & van Hoosier 2012). In comparison to the normal range for guinea pigs, the baseline serum Mg levels found in the uninfected controls in the present study would considered be normal, while in comparison to the normal range in rabbits, the serum Mg levels found in all of our experimental groups would be within normal limits. For the purpose of the present study, the analysis was based on the comparison between all groups with the serum Mg levels detected in our uninfected controls. These values consistently ranged from 4.0-4.5 mg/dl in samples from the uninfected animals used in this study and from other uninfected hamsters in our laboratory. Although this is beyond the scope of our study, we speculate that reference values for serum Mg in hamsters should be reviewed or further evaluated based on the following observations: (i) the available data in the literature are scarce and show a wide range, (ii) the present results, both from animals evaluated during the study period and in different independent experiments afterwards, indicate considerably higher levels than have been previously reported and (iii) some related laboratory rodents are known to display considerably higher serum Mg levels.

There was a trend toward lower Na levels among the infected animals compared with the uninfected controls. However, all of the measurements were within the normal range of serum Na levels for hamsters (Fig. 3). The serum Na levels were significantly lower among the infected hamsters compared with the uninfected hamsters at days 4 and 8. On day 16, all of the infected groups exhibited lower serum Na levels compared with the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the no-treatment group still presented significantly lower Na levels compared with the controls and the group receiving methylene blue exhibited a trend toward lower Na levels.

The methylene blue treatment group presented lower serum Na levels on day 16 compared with the other infected groups (Fig. 3). Any discussion regarding this finding is merely speculative, but it might be explained based on the combination of the following factors: (i) a syndrome of inappropriate antidiuretic hormone hypersecretion causes hyponatremia and is known to occur in some severe infectious diseases such as pneumonia and meningitis (Mendoza 1976) and (ii) local inhibition of NO production in the kidney interferes with Na reabsorption and may result in either depletion or retention (Manning & Hu 1994).

In this study, the dynamics of ionic changes were not found to be associated with any peculiar histopathologic features in the kidneys. The kidney samples from the hamsters that were euthanised at day 4 were uniformly normal when examined via microscopy (data not shown), despite the high serum levels of Mg detected in these animals (Fig. 2). The hamsters that were euthanised at day 8 presented the typical features of acute disease, such as diffuse and massive tubular cell swelling with mild or no interstitial nephritis. At this time point, marked hyperkalaemia was observed (Fig. 1); however, it was even higher on day 28, when the tubular changes were resolved and more severe manifestations of interstitial nephritis dominated the microscopic observations (Fig. 5E). In the present study, severe ionic changes persisted after recovery from the acute tubular changes. This last finding confirmed a previous clinical report, suggesting that tubular defects, such as impaired urinary concentration capacity, may last for months in patients (Daher Ede et al. 2004).

The hamsters that were euthanised on days 16 and 28 exhibited variable degrees of acute tubular changes, regeneration of tubular epithelia and interstitial nephritis. In a previous report on experimental leptospirosis in guinea pigs, de Arriaga et al. (1982) did not observe any association between the frequency or severity of interstitial nephritis and renal failure. In the same study, regenerative tubular changes were found to be associated with renal dysfunction, which may imply previous tubular necrosis. As shown in Fig. 4, serum creatinine levels peaked at day 16, when regenerative changes in the epithelial tubular cells were first detected. The serum creatinine levels in the untreated group were higher than normal reference limits and significantly higher than the levels in the hamsters from all of the treatment groups. In contrast, serum creatinine levels were not significantly different from other treatment groups. In this study, the measured serum creatinine levels were likely biased

TABLE II

Outcome of hamsters infected by *Leptospira interrogans* strain L1130 and treated with ampicillin, methylene blue, both or no treatment

Treatment	Survivors n/N (%)	Days to death
Untreated controls	0/11 (0)	7, 7, 8, 8, 9, 9, 10, 13, 14, 16, 16
Ampicillin	1/10 (10)	8, 10, 10, 10, 11, 11, 12, 14, 15
Methylene blue	0/10 (0)	7, 7, 9, 9, 9, 10, 10, 11, 11, 13
Ampicillin + methylene blue	0/9 (0)	8, 9, 9, 9, 10, 10, 10, 11, 15

n/N: number of survivors/total number of evaluated hamsters.

because serum samples were only collected from the hamsters that were euthanised at scheduled time points. Hamsters that died before the scheduled euthanasia date likely exhibited more severe ionic imbalances and renal dysfunction. Importantly, the histopathology was similar in all groups at day 16, even though the serum creatinine levels were higher in the untreated animals. We have previously demonstrated that late antibiotic treatment in this hamster model can prevent or reverse the loss of Na+/H+ exchanger 3 and NKCC2 expression in renal tubular cells (Spichler et al. 2007), which is a finding that may be linked to the effect of antimicrobial treatment on the renal dysfunction markers observed in this study.

As shown in Table I, deaths were not expected to occur in the treatment assay after 15 days of infection. Thus, we defined survivors as those animals that survived to the 16 and 28 day time points. There was no observable benefit of adding methylene blue to standard ampicillin treatment. We previously reported a similar lack of an effect for the antioxidant N-acetylcysteine as an adjuvant therapy (Spichler et al. 2007). However, the strict methodology applied in these studies is the best way to reproduce the clinical conditions related to late treatment of patients. Models of delayed experimental treatment avoid obtaining promising results associated with experimental treatments that may be initiated too early during experimental infection to yield reproducible benefits in clinical practice.

Additionally, we have previously reported that *inos*-knock-out C57Bl/6 mice display lower rates and severities of interstitial nephritis and that the absence of iNOS is not associated with increased bacterial dissemination in tissues (Bandeira et al. 2011). Prêtre et al. (2011) have reported that treatment with another NOS inhibitor, 4-aminopyridine (0.3 mg/kg daily, starting on the day of infection), without co-administration of antibiotics, results in an accelerated lethal outcome in hamsters and a higher mortality rate in C3H/HeJ mice as well as a higher leptospiral burden in their tissues. The design of this previous study was different from the one presented here, as the former did not test NOS inhibition as an adjuvant treatment in the late stage of acute infection. In contrast with the effects observed in other models of sepsis (Kirov et al. 2001, Kwok & Howes 2006, Heemskerk et al. 2008, Paciullo et al. 2010), methylene blue does not appear to be beneficial in this model of leptospirosis. Blocking NO production also had no effect on the ionic imbalance observed during acute leptospirosis in the hamster model.

One limitation of this study is the lack of information on the bacterial burden. While it is important to understand the dynamics of infection, the examination of leptospiral loads does not directly interfere with analyses of survival, ionic changes and the frequency of renal lesions. We have previously shown that ampicillin treatment is associated with the clearance of leptospiral antigens, which parallels the preservation of renal tubule transporter expression (Spichler et al. 2007). In addition, we have demonstrated that genetic deficiency of iNOS is not associated with differences in the leptospiral load in tissues from the C57BL/6 mouse model (Bandeira et al. 2011).

The present study also revealed that the hamster model is not practical for reproducing and therefore studying the common ionic disturbances (such as hypokalaemia and hypomagnesaemia) that are observed in patients with severe leptospirosis. These ionic changes may occur during experimental infection, but progress so quickly to typical acute renal failure that they could not be detected at the time points selected in the present study. Patients with leptospirosis usually receive aggressive supportive therapy (including fluid expansion) that blocks or retards progression to an oliguric/hyperkalaemic state. Such supportive therapy was not provided in this study, which may explain the rapid progression to a severe form of renal failure.

In our second experiment, a different strategy was employed to reproduce the late initiation of treatment of experimental leptospirosis. Infected hamsters were followed to detect clinical signs and treatment was initiated one day after the first death was observed. In this model, even antibiotic therapy had almost no effect on survival (only 1 out of 10 animals survived) and the addition of methylene blue to the treatment regime provided no improvement of outcomes (Table II). Notably, although antibiotics are highly recommended, even in late-stage leptospirosis in clinical settings, there is no evidence demonstrating positive effects on survival when antibiotic treatment is initiated in late-stage leptospirosis. Such studies will likely not be performed because of ethical concerns about denying patients antibiotic treatment, even if no clinical benefit has been demonstrated.

Hypokalaemia was not reproduced in our hamster model: all of the groups developed increased levels of serum K. Furthermore, in this model of acute infection, hypermagnesaemia (rather than Mg depletion) was observed. These findings may be associated with an accelerated progression to acute renal failure. Furthermore, adjuvant methylene blue treatment had no effect on serum Mg and K levels during acute-phase leptospirosis in hamsters. Late antibiotic treatment prevented the accelerated elevation of serum creatinine levels in this model.

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8.2. Aprovação pela Comissão de Ética no Uso de Animais
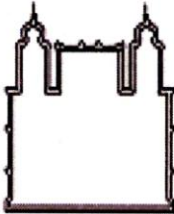
MINISTÉRIO DA SAÚDE / FUNDAÇÃO OSWALDO CRUZ
VICE-PRESIDÊNCIA DE PESQUISA E DESENVOLVIMENTO TECNOLÓGICO
Comissão de Ética no Uso de Animais
CEUA-FIOCRUZ

C E R T I F I C A D O

Certificamos que o protocolo intitulado :

" *Avaliação de resposta imune contra leptospirose em modelos animais.* "

número P-445/07, proposto por Alan John Alexander McBride, foi licenciado pelo Nº L-055/08.

Sua licença de Nº L-055/08 autoriza o uso anual de :

- 396 *Mesocricetus auratus*   - 100 *Rattus norvegicus*
- 10 *Oryctolagus cuniculus*   - 80 *Mus musculus*

Esse protocolo está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi APROVADO pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA - FIOCRUZ). Na presente formatação, este projeto está licenciado e tem validade até 21 de julho de 2012 .

Rio de Janeiro, 24/09/2008

Dra. Norma Vollmer Labarthe
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