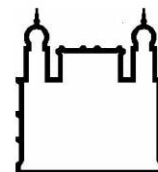**UNIVERSIDADE FEDERAL DA BAHIA**
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
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Curso de Pós-Graduação em Patologia

DISSERTAÇÃO DE MESTRADO

LEPTOSPIRA EM MAMA E LEITE DE *RATTUS NORVEGICUS* DE ÁREAS URBANAS: POSSÍVEL VIA DE TRANSMISSÃO VERTICAL?

DAIANA SANTOS DE OLIVEIRA

Salvador - Bahia
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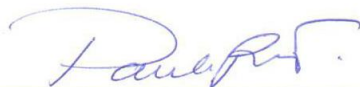
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**"LEPTOSPIRA *EM MAMA E LEITE DE* RATTUS NORVEGICUS *DE ÁREAS URBANAS:*
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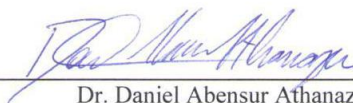
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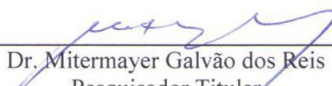
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AGRADECIMENTOS

Habilidade é o que você é capaz de
fazer. Motivação determina o que
você faz. Atitude determina o
quanto você faz isso bem feito!

Lou Holtz

OLIVEIRA, Daiana Santos de. *Leptospira* em mama e leite de *Rattus norvegicus* de áreas urbanas: possível via de transmissão vertical? 79 f. il. Dissertação (Mestrado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

RESUMO

A leptospirose é uma zoonose distribuída globalmente causada por bactérias do gênero *Leptospira*. *Rattus norvegicus* é o principal reservatório de *Leptospira* em comunidades urbanas do Brasil e em outros países. As vias de infecção por *Leptospira* nas populações de roedores são desconhecidas. O objetivo deste trabalho foi identificar a presença de *Leptospira* em mama e leite como indicadores da transmissão vertical em *R. norvegicus*. As capturas de roedores foram realizadas em 2013 e 2014 em uma comunidade urbana de Salvador, Brasil. Nós pesquisamos a presença de *Leptospira* na mama, leite e rins de fêmeas em lactação utilizando testes de Imunofluorescência (IFA), Imunohistoquímica (IHQ), qPCR e exame no microscópio eletrônico de varredura (MEV). Nós examinamos 24 ratas em lactação. Todas as fêmeas foram positivas no rim em pelo menos uma das técnicas utilizadas. 18/24 (75%) foram positivas no leite por IFA e 04/28 (17%) foram confirmadas por qPCR. Na mama 16/24 (67%) ratas foram positivas por IFA ou IHQ. Observamos a presença de *Leptospira* em 1/4 ratas no exame com MEV. Na mama foram encontradas *Leptospira* em áreas sem ou com alterações patológicas. A presença de *Leptospira* na mama (67%) e no leite (75%) sugere que ocorra transmissão vertical pela amamentação nas populações urbanas de *R. norvegicus*. Estes achados precisam ser confirmados por estudos experimentais. A caracterização das vias de transmissão e manutenção de *Leptospira* é fundamental para entender a dinâmica do patógeno nos reservatórios, permitindo o desenvolvimento de novos modelos preditivos de risco para leptospirose em roedores e em seres humanos.

Palavras-chave: *Leptospira interrogans*, imunofluorescência. imunohistoquímica, microscopia eletrônica de varredura, e amamentação.

OLIVEIRA, Daiana Santos de. *Leptospira* in breast and milk of *Rattus norvegicus* from urban areas: possible route of vertical transmission? 79 f. il. Dissertação (Mestrado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2015.

ABSTRACT

Leptospirosis is a zoonosis distributed globally caused by bacteria of the genus *Leptospira*. *Rattus norvegicus* is the main reservoir of *Leptospira* in urban communities in Brazil and other countries. The routes of *Leptospira* infection in the rodent populations are unknown. The objective of this study was to identify the presence of *Leptospira* in breast milk and as an indicator of vertical transmission in *R. norvegicus*. The rodents were trapped in 2013 and 2014 in an urban community of Salvador, Brazil. We research the presence of *Leptospira* in the breast, milk and kidney of lactating females using immunofluorescence test (IFA), Immunohistochemistry (IHC), qPCR and examination in Scanning Electron Microscope (SEM). 24 rats were examined lactating. All were positive in at least one kidney of techniques. 18/24 (75%) tested positive in milk by IFA and 4/28 (17%) were confirmed by qPCR. In breast tissue 16/24 (67%) rats were positive by IFA or IHC. In a breast tissue sample from a rat was examined under SEM four *Leptospira* presence observed. In the presence of breast *Leptospira* was observed in areas with or without pathological changes. The presence of *Leptospira* breast (67) and milk (75%) suggests that vertical transmission occurs by feeding in urban populations of *R. norvegicus*. These findings need to be confirmed by experimental studies. The characterization of *Leptospira* transmission routes and maintenance is fundamental to understanding the dynamics of the pathogen in the reservoir allowing the development of new predictive models risk for leptospirosis in rodents and in humans.

Keywords: *Leptospira interrogans*, immunofluorescence. immunohistochemistry, scanning electron microscopy, breastfeeding.

LISTA DE ABREVIATURAS

EMJH   Meio de Ellinghausen e McCullough modificado por Johnson e Harris

IFA   Imunofluorescência

IHQ   Imunohistoquímica

MET   Microscopia eletrônica de transmissão

MEV   Microscópio eletrônico de varredura

qPCR   Reação em cadeia da polimerase quantitativa

SPHS   Síndrome de hemorragia pulmonar grave

SUMÁRIO

1 INTRODUÇÃO

A leptospirose é uma zoonose distribuída globalmente causada bactérias do gênero *Leptospira* (LEVETT, 2001). Esta doença tem um alto impacto na saúde pública, sendo responsável por mais de um milhão de casos anuais em humanos no mundo (COSTA et al., 2015). Sua transmissão ocorre através de contato direto com a urina de animais infectados ou indireto, pela exposição à água ou ao solo contaminado com a urina dos reservatórios (FAINE; ADLER; PEROLAT, 2000). Nos países em desenvolvimento a maior carga da doença está associada às comunidades carentes urbanas onde os surtos durante a estação de chuvas afetam a parcela mais pobre da população (KO et al., 1999).

O *Rattus norvegicus* é o principal reservatório de *Leptospira* nas comunidades urbanas no Brasil (BAROCCHI et al., 2001; DE FARIA et al., 2008) ou em outros países em desenvolvimento (HIMSWORTH et al., 2014). Apesar dos numerosos estudos que descrevem a prevalência de *Leptospira* em roedores (COSTA et al., 2014a; DE FARIA et al., 2008), pouco se sabe sobre a manutenção e propagação da infecção nestas populações (HIMSWORTH et al., 2014; HOLT; DAVIS; LEIRS, 2006). Acredita-se que nos roedores, como nas populações humanas, a transmissão ambiental é a principal via de infecção por *Leptospira* (FAINE; ADLER; PEROLAT, 2000). No entanto, estudos de prevalência de *Leptospira* em roedores sugerem que é provável que o meio ambiente não seja a única via pela qual a infecção é mantida nessas populações (HOLT; DAVIS; LEIRS, 2006). O aumento da prevalência de colonização associado à idade que o rato inicia sua atividade sexual sugere que esta última representa uma potencial via de transmissão horizontal (COSTA et al., 2014a). Adicionalmente, é importante ressaltar que a infecção nos primeiros estágios de vida (<100g), sugere também que a transmissão poderia acontecer por transmissão vertical ou no ninho (COSTA et al., 2014a).

Até o presente não há nenhuma evidência conclusiva do papel da via transplacentária (LAHIRI, 1941; WARD; TURNER, 1940) ou da amamentação na transmissão de *Leptospira* em população de roedores. A transmissão por aleitamento materno tem sido informalmente descrito em apenas um caso em humano (BOLIN; KOELLNER, 1988). Também já foi demonstrada presença de anticorpos anti-*Leptospira* no leite de rebanhos leiteiros (DOM et al., 1991; TABATABAEIZADEH et al., 2011); entretanto, ainda não foi elucidada a contribuição da transmissão de leptospiras pela amamentação em reservatórios naturalmente infectados.

Este estudo tem como objetivo identificar a presença de *Leptospira* na mama e leite de *R. norvegicus* para contribuir na caracterização do papel da amamentação na transmissão vertical da leptospirose em roedores, e gerar novas hipóteses sobre a manutenção de *Leptospira* em populações de reservatórios. A identificação de vias de transmissão na leptospirose pode contribuir ainda para criação de modelos matemáticos preditivos do risco da transmissão (HOLT; DAVIS; LEIRS, 2006) e no desenvolvimento de novas estratégias mais eficazes para o controle da doença (COSTA et al., 2014a).

2 REVISÃO DE LITERATURA

2.1 ASPECTOS HISTÓRICOS DA LEPTOSPIROSE

Historicamente, a leptospirose é considerada uma doença ocupacional sendo conhecida por algumas sinonímias como febre dos pântanos, febre dos arrozais, febre outonal, tifo canino e febre dos nadadores (BRASIL, 2010). A doença foi formalmente descrita pela primeira vez em 1886, por Adolf Weil, na Alemanha, ao observar em seres humanos sinais clínicos como ictérica, comprometimento hepático e a falência renal. Após esta caracterização, a leptospirose passou a ser conhecida como "Doença de Weil" ou "Tríade de Weil" (FAINE; ADLER; PEROLAT, 2000).

Somente em 1915, no Japão, a bactéria foi isolada pela primeira vez pelos médicos Inada e Ido (LEVETT, 2001). Os pesquisadores japoneses isolaram a bactéria a partir de sangue de trabalhadores em minas que sofriam da doença de Weil. Adicionalmente, Ido et al., em 1917, demonstraram o papel dos ratos como hospedeiros das leptospiras (LEVETT, 2001). Em seguida, no ano de 1918, Hideyo Noguchi propôs a criação do gênero *Leptospira*, denominando a espécie de *Leptospira icterohaemorrhagiae* (FAINE; ADLER; PEROLAT, 2000).

No Brasil, os primeiros casos da leptospirose foram relatados no período de 1910 e 1940, sendo inicialmente confundidos com a febre amarela e outras doenças que apresentavam sinais e sintomas semelhantes. Em 1917, Aragão identificou a presença do sorovar icterohaemorrhagiae em *Rattus norvegicus* no Rio de Janeiro (ARAGÃO, 1917). As diversas formas clínicas da leptospirose foram caracterizadas somente na década de 1930-40 (FAINE; ADLER; PEROLAT, 2000; LEVETT, 2001). Posteriormente, na década de 1960, vários surtos urbanos foram relatados nas grandes cidades brasileiras (GONÇALVES et al., 2006).

2.2 TAXONOMIA E MICROBIOLOGIA DO AGENTE ETIOLÓGICO

O agente etiológico causador da leptospirose é uma bactéria pertencente ao gênero *Leptospira*, ordem Spirochaetales, família Leptospiraceae (FAINE; ADLER; PEROLAT, 2000). A classificação taxonômica clássica, baseada em reações sorológicas, dividia o gênero *Leptospira* em duas espécies, *Leptospira interrogans*, que compreendia todas as leptospiras

patogênicas, com ciclo de vida associado à infecção animal e a *L. biflexa*, de vida livre, que englobava às leptospiras saprófitas isoladas no ambiente. Ambas as espécies patogênicas e as não patogênicas são divididas em diversos sorovares antigenicamente diferentes (BHARTI et al., 2003; FAINE; ADLER; PEROLAT, 2000). Os sorovares variam de região para região e são diferentes em graus de virulência e hospedeiros preferenciais. No Brasil, os sorovares Icterohaemorrhagiae e Copenhageni estão relacionados frequentemente aos casos mais graves da doença e são responsáveis pelas epidemias urbanas (BAROCCHI et al., 2001; KO et al., 1999).

Adicionalmente, além da classificação sorológica das leptospiras foi desenvolvida a classificação com base molecular, que propõe a divisão do gênero *Leptospira* em genomoespécies estabelecidas com bases genéticas (YASUDA et al., 1987). Essa classificação genotípica tem gradativamente ganhado espaço na classificação taxonômica. Análises filogenéticas demostraram que as 20 genomoespécies descritas são agrupadas em três grandes grupos que compreendem as cepas patogênicas, intermediárias e saprofíticas (KO; GOARANT; PICARDEAU, 2009; LEVETT, 2001).

As leptospiras apresentam morfologia helicoidal e medem em média de 0,1 μm de diâmetro por 6,0 a 20 μm de comprimento e em suas extremidades possuem ganchos e além de endoflagelos (FAINE; ADLER; PEROLAT, 2000). Essas bactérias apresentam características da parede celular semelhantes às bactérias Gram-negativas, no entanto não são coradas com corantes convencionais, mas podem ser visualizadas diretamente em microscopia de campo escuro. São microrganismos aeróbios obrigatórios e crescem satisfatoriamente em temperaturas ambientais de 28 a 30° C. Possuem crescimento lento, além de serem exigentes no que diz respeito às fontes de nutrientes, pois necessitam de meios enriquecidos com vitaminas B2 e B12, ácidos graxos de cadeia longa e sais de amônia (FAINE; ADLER; PEROLAT, 2000; LEVETT, 2001).

2.3 PATOGENIA

Devido às suas características morfológicas e fisiológicas, por serem finas e apresentarem alta motilidade, as leptospiras têm a capacidade de penetrar no homem através da pele lesionada (LEVETT, 2001; BRASIL, 2009) ou inclusive diretamente na pele integra quando esta se encontra em contato com o ambiente contaminado por tempo prolongado, como descrito em um estudo experimental com hamster (BATISTA, 2007). Há casos em que

as espiroquetas podem penetrar também pelas mucosas bucal, ocular, nasal, e genital (BRASIL, 2009).

Após a entrada de *Leptospira* na corrente circulatória, a mesma multiplica-se e dissemina-se por órgãos e tecidos, principalmente nos rins, fígado e pulmão, caracterizando a fase de leptospiremia (FAINE; ADLER; PEROLAT, 2000). O aspecto patológico na leptospirose tanto é caracterizado por danos na parede dos vasos sanguíneos, como também em alguns órgãos internos, isso explica a grande variedade de manifestações clínicas (WORLD HEALTH ORGANIZATION, 2003). A patogenicidade em humanos ainda não foi totalmente elucidada, porém é possível que seja diferenciada de acordo com a carga de microrganismo infectante, a espécie de *Leptospira*, a saúde e o estado imunológico do hospedeiro (BHARTI et al., 2003; CINCO, 2010). Os principais achados patológicos incluem, comprometimento nos rins com a presença de focos inflamatórios, nefrite túbulo-intersticial, necrose tubular, edema intersticial e hemorragias, ocasionados pela presença das leptospiras nos túbulos contorcidos proximais, glomérulos e interstício (AREAN, 1962; BARNETT et al., 1999; SITPRIJA et al., 1980). Os pulmões apresentam intensas hemorragias intra-alveolares, que pode levar à insuficiência respiratória (GOUVEIA et al., 2008).

Assim como nos humanos, existem outros hospedeiros acidentais que também apresentam manifestações clínicas quando infectados. Entre eles podemos citar bovinos (MINEIRO et al., 2011), cães (NETTO, 2008) e cobaias (HAAKE, 2006). Os hamsters são considerados como modelos animais suscetíveis à doença e apresentam um quadro agudo letal (SILVA et al., 2007), sendo utilizados em estudos experimentais para avaliar patogenia e testes de vacinas (HAAKE, 2006).

Espécies de roedores como do gênero *Rattus* são utilizados como modelo de reservatórios para *Leptospira*. Os ratos não desenvolvem a doença e albergam as leptospiras em seus rins por um longo período, principalmente nos lúmen dos túbulos renais, estabelecendo uma infecção crônica (ATHANAZIO et al., 2008; THIERMANN, 1981). O rim dos ratos é considerado um órgão de escape do sistema imune favorecendo a colonização e a permanência das leptospiras (ATHANAZIO et al., 2008). Entretanto não se conhecem ao certo quais são os mecanismos que estão envolvidos na resistência dos ratos à leptospirose.

2.4 ASPECTOS CLÍNICOS

O espectro da doença causada pela bactéria *Leptospira* é muito amplo em humanos e as manifestações clínicas variam desde formas discretas até formas graves (FAINE; ADLER; PEROLAT, 2000; LEVETT, 2001). Nas formas discretas os principais sintomas são calafrios, febre alta, cefaleia intensa, mialgia, náuseas, dores abdominais e vômitos (LEVETT, 2001). Como a sintomatologia é inespecífica, a leptospirose é freqüentemente confundida com outras doenças que ocorrem no mesmo período, como dengue ou influenza. Uma parte destes pacientes pode progredir para formas graves e potencialmente fatais da doença (FAINE; ADLER; PEROLAT, 2000; BRASIL, 2009). Em geral, na forma grave observa-se icterícia, insuficiência renal aguda e hemorragia caracterizada como síndrome de Weil (BHARTI et al., 2003; FAINE; ADLER; PEROLAT, 2000). Nas últimas décadas têm sido observados casos graves de leptospirose com grau variável de hemorragia pulmonar. Os casos com hemorragia pulmonar maciça são classificados como síndrome de hemorragia pulmonar grave. Nesses casos, a taxa de letalidade pode ultrapassar o 50% (GOUVEIA et al., 2008).

2.5 DIAGNÓSTICO

A identificação de leptospiras em tecidos pode ser realizada por diferentes técnicas. A visualização direta da bactéria por meio da microscopia de campo escuro, apesar de ser possível, é pouco usada por ter baixa sensibilidade e especificidade (FAINE; ADLER; PEROLAT, 2000). Alternativamente, é possível verificar a presença da bactéria através de isolamento em meio de cultura. O meio de cultura apropriado para o cultivo de *Leptospira* é o Ellinghausen, McCullough, Johnson e Harris (EMJH) (ELLINGHAUSEN; MC CULLOUGH, 1965; JOHNSON; HARRIS, 1967). Este método possui alta especificidade, porém o crescimento lento destas bactérias, além de serem facilmente contaminadas, torna inviável o uso desta metodologia como método diagnóstico de triagem.

Em geral, os testes de imunoensaio como imunoflorêscencia (IFA) ou imunohistoquímica (IHQ) são utilizados em estudos experimentais (CHAGAS-JUNIOR et al., 2009). A IFA pode ser utilizada para identificar leptospiras em tecidos frescos (fígado, pulmões, rins entre outros) (CHAGAS-JUNIOR et al., 2012) ou sedimentos urinários e sanguíneos. A técnica de IHQ também apresenta uma alta especificidade e permite detectar

tanto a presença quanto a localização do patógeno no tecido (BARNETT et al., 1999; ROSS et al., 2009).

Na leptospirose, as técnicas de biologia molecular a exemplo da PCR (Reação em Cadeia da Polimerase) e do PCR quantitativo (PCR em Tempo Real) vêm sendo utilizadas como ferramentas de diagnóstico e pesquisa. Estas técnicas apresentam uma alta sensibilidade e especificidade (CRODA et al., 2007), demonstrando ter mais eficiência na detecção de positivos que quando comparadas à cultura e ensaios sorológicos (VAN EYS et al., 1989). O qPCR além de permitir a detecção e amplificação do gene alvo permite quantificar da carga bacteriana presente na amostra (AHMED; ANTHONY; HARTSKEERL, 2010; SMYTHE et al., 2002). Esta técnica tem como limitação o custo mais elevado dos equipamentos e insumos essenciais na estrutura física necessária para a realização da técnica assim como o treinamento das pessoas que a realizam.

2.6 EPIDEMIOLOGIA

A leptospirose apresenta uma ampla distribuição mundial, no entanto é mais frequente em regiões tropicais do que em regiões de clima temperado (LEVETT, 2001). Nos países de clima tropical, a doença apresenta um caráter sazonal, com epidemias observadas durante as estações chuvosas. Isso ocorre, sobretudo, em razão das condições ambientais de calor e umidade que favorecem a permanência da bactéria no meio ambiente (COSTA, 2010; KO et al., 1999; SARKAR et al., 2002)

Algumas profissões contribuem para o contato dos humanos com as leptospiras, como garis, desentupimento de esgotos, catadores de lixo, agricultores, veterinários, tratadores de animais, pescadores, e militares, dentre outras (BRASIL, 2010). Em países em desenvolvimento, a epidemiologia da leptospirose está intimamente relacionada às condições socioeconômicas da população. No Brasil, a leptospirose representa um sério risco à saúde pública, principalmente em áreas com precárias condições ambientais de saneamento básico e grande aglomeração populacional (KO et al., 1999). Nestas comunidades a transmissão está associada a condições precárias do peridomicílio (COSTA et al., 2014b; FELZEMBURGH et al., 2014; REIS et al., 2008).

O perfil epidemiológico da população afetada no Brasil consiste de adultos jovens, do sexo masculino, residentes em áreas urbanas e com relatos de exposição a chuvas (PAPLOSKI, 2013). Os casos urbanos, geralmente, estão relacionados à infecção por cepas

do sorogrupo Icterohaemorrhagiae, tendo o rato de esgoto como principal transmissor, visto que *R. norvergicus* é o reservatório mais comum desse sorogrupo (KO et al., 1999). Este perfil é mais evidente em algumas cidades tais como Salvador, São Paulo e Recife (BAROCCHI et al., 2001; CÔRREA; HYAJUTAKE; AZEVEDO, 1972; KO et al., 1999; SAKATA et al., 1992).

2.7 CICLO DE TRANSMISSÃO

A infecção em humanos está relacionada ao contato direto ou indireto com a água e solo contaminados pela urina de animais portadores da *Leptospira*. No ciclo de transmissão da bactéria, o homem é considerado hospedeiro acidental e terminal uma vez que o mesmo não é eficiente na excreção do patógeno viável (FAINE; ADLER; PEROLAT, 2000; BRASIL, 2002; SAMPAIO et al., 2011).

A manutenção do ciclo de transmissão depende da interação de elementos importantes inerentes à fonte de infecção, ao ambiente e ao hospedeiro. Este último pode ser influenciado diretamente pela criação e disposição de um ambiente antrópico favorável (OLIVEIRA, 2009). As leptospiras podem ser liberadas na natureza através de vários hospedeiros. Estes incluem animais silvestres, domésticos e sinantrópicos (OLIVEIRA, 2009; VIJAYACHARI P, SUGUNAN AP, 2008). Podemos destacar os caninos, suínos, bovinos, eqüinos, ovinos e caprinos. Entretanto, dentre estes hospedeiros, em áreas urbanas, os roedores sinantrópicos são considerados os principais responsáveis por sua transmissão. O *R. norvergicus* (ratazana de esgotos) e o *Rattus rattus* (rato de telhado ou rato preto) são as espécies mais comuns. Porém, estudos epidemiológicos e de biologia molecular indicam que *R. norvergicus* é a principal espécie transmissora da doença em ambientes urbanos no Brasil (COSTA et al., 2015; KO et al., 1999; OLIVEIRA, 2009; TUCUNDUVA DE FARIA et al., 2007).

Os ratos apresentam características fundamentais à manutenção da *Leptospira* tais como: elevada receptividade à infecção; infecção renal com leptospiúria; urina alcalina; além de não manifestarem sintomas clínicos. Estes fatores permitem o alojamento das leptospiras nos rins, eliminando-as vivas no ambiente (KO et al., 1999; LEVETT, 2001; OLIVEIRA, 2009; SAMPAIO et al., 2011), em elevadas concentrações (COSTA et al., 2015). No entanto, é limitado o conhecimento de como ocorre à transmissão entre os ratos apesar de estudos prévios sugerirem que está associada à excreção de urina contaminada devido a elevada colonização renal (ATHANAZIO et al., 2008; FAINE; ADLER; PEROLAT, 2000), porém

essas informações não elucidam completamente como os ratos juvenis apresentam altas taxas de infecção (COSTA et al., 2014a).

2.8 VIAS DE TRANSMISSÃO DE "LEPTOSPIRA"

O animal infectado por *Leptospira* ou outro agente infeccioso pode transmitir o patógeno direto ou indiretamente para uma ou mais espécies de hospedeiros. Na via "indireta" os animais podem ser infetados por transmissão ambiental através de solo ou água contaminada (BHARTI et al., 2003), enquanto na forma de transmissão "direta" ela pode se dar através da transmissão horizontal ou da transmissão vertical. Na transmissão horizontal ocorre a passagem de infecção de um indivíduo hospedeiro para outro, por exemplo, por ingestão de material infeccioso, mordida (ROCZEK et al., 2008) ou através da saliva no ato de lamber ou na atividade sexual (PIRES, 2005). A transmissão vertical é a transferência direta de uma doença, a partir mãe, para sua prole (filhos) durante a gestação (ex. transplacentária), ou logo após o nascimento pela transmissão pós-natal (ex. amamentação) (PIRES, 2005).

- **Transmissão ambiental**

Na transmissão ambiental, outras espécies de animais se infectam de maneira similar aos humanos quando entram em contato com solo ou água contaminados com a bactéria. Estudos de prevalência sugerem que esta via é a principal forma de transmissão de *Leptospira* (FAINE; ADLER; PEROLAT, 2000). No entanto, para a sobrevivência das leptospiras no meio ambiente são necessárias condições especiais no solo ou na água, como elevada umidade e temperatura, pH neutro à levemente alcalino, onde a bactéria possa sobreviver por meses (ESCÓCIO et al., 2010). Não se conhece a dose infectante necessária para produzir infecção ambiental em roedores, ou se a presença de feridas facilita esta via de transmissão (HIMSWORTH et al., 2014).

- **Transmissão sexual**

Estudos sobre transmissão sexual em leptospirose sugerem que a infecção pode estar ligada diretamente a fluidos sexuais contaminados com o patógeno (LILENBAUM et al.,

2008). Um dos primeiros indícios sobre a importância do sêmen como via de transmissão da leptospirose foi a partir de estudos realizados por KIKTENKO et al. (1976), que isolaram *Leptospira* de 26% das amostras de sêmen de coelhos e 7% das amostras de touros examinados (KIKTENKO; BALASHOV; RODINA, 1976). Adicionalmente foi detectado DNA de *Leptospira* por PCR em amostras de sêmen de cavalos (HAMOND et al., 2013) e de carneiros assintomáticos (42,8%), apesar dos autores não descartarem a possibilidade de contaminação das amostras com a urina presente na uretra (LILENBAUM et al., 2008). O ducto excretor nos machos é comum ao sistema urinário e genital, isso favorece a contaminação do sêmen por leptospiras quando excretadas pela urina (SLEIGHT; WILLAMS, 1961). Um estudo de cães infectados com *Leptospira interrogans* sorovar Canicola sugere que estes podem transmitir a bactéria (identificada por PCR) por meio de sêmen nos primeiros dias pós-infecção, antes da transmissão urinária (NETTO, 2008).

A *Leptospira* também tem sido identificada no trato reprodutor feminino de várias espécies. *Leptospira interrogans* Hardjoprajitno foi isolada de fluido vaginal de uma ovelha clinicamente saudável (DIRECTOR et al., 2014). ELLIS et al. (1986) avaliaram no útero, nas tubas uterinas, nos ovários e na vagina de vacas não prenhes a presença de *Leptospira interrogans* sorovar Hardjo. Das 60 vacas experimentalmente infectadas, 39 (65%) apresentaram leptospiras no trato genital (MAGAJEVSKI, 2007). Em roedores, BRANDESPIM et al. (2004), em um estudo experimental, identificou *Leptospira interrogans* sorovar Pomona em órgãos do aparelho reprodutor de hamsters pela técnica de Levaditi (MAGAJEVSKI, 2007). Ainda em roedores, a técnica de sais de prata identificou nos ovários de hamsters infectados experimentalmente a presença de *Leptospira interrogans* sorovar Pamona (CAMARGO et al., 1993).

- **Transmissão transplacentária**

Em bovinos, equinos e caprinos existem indícios que a via de transmissão transplacentária ocorra, uma vez que estes animais podem sofrer abortos devido à infecção por leptospiras (HIGGINS et al., 1980; LILENBAUM et al., 2007; MARTINS, 2012; POONACHA et al., 1993). Em humanos também têm sido relatados casos de abortos potencialmente relacionados à leptospirose (COGHLAN; BAIN, 1969). Shaked *et.al.* (1993), relatou um caso humano por transmissão via transplacentária e também revisaram outros 15 casos prévios, diagnosticados por meio do exame da placenta ou do recém-nascido (SHAKED

et al., 1993). Foram detectadas *Leptospira* sorovar Hardjo cultivadas a partir da placenta e líquido amniótico da mãe (CHUNG et al., 1963). Nos roedores, ainda não está claro o papel da transmissão transplancentária. Por exemplo, dois estudos realizados com *R. norvegicus* mostram evidencias contrastantes, sendo em um dos trabalhos foi possível isolar leptospira a partir dos fetos (LAHIRI, 1941) enquanto no outro não foi encontrado (WARD; TURNER, 1940).

- **Transmissão por aleitamento materno**

Existem poucos trabalhos dedicados ao estudo da transmissão por aleitamento materno em leptospirose. Este mecanismo foi informalmente descrito em apenas um caso, relatado em humanos (BOLIN; KOELLNER, 1988). O relato é sobre uma veterinária que ao realizar atividades com animais infectados com *Leptospira interrogans* sorovar Hadjo ficou doente e que potencialmente infectou o filho de quatro meses de vida através do leite. Visto que foram identificadas leptospiras por fluorescência e isolada a partir da cultura de urina do bebê. A confirmação da transmissão pelo leite não foi possível, porém a transmissão pela urina ou ambiental foi descartada (BOLIN; KOELLNER, 1988). Contudo, não há nenhuma evidência de que este é um mecanismo comum. Há outros estudos que isolaram *Leptospira* (*L. australis*) a partir de sangue e leite de uma mulher em amamentação (CHUNG et al., 1963). A amamentação foi interrompida durante a doença da mãe e a transmissão para a criança não foi evidenciada. Da mesma forma, há indicios de que leptospiras são eliminadas pelo leite de vacas com quadro de mastite infectadas com sorovar Hardjo (ELLIS et al., 1976). Adicionalmente a presença de anticorpos anti-*Leptospira* no leite têm sido descritas em rebanhos leiteiros (DOM et al., 1991; TABATABAEIZADEH et al., 2011). Porém, a contribuição da transmissão pela amamentação em roedores reservatórios naturalmente infectados ainda não foi esclarecida.

2.9 MEDIDAS DE CONTROLE DA LEPTOSPIROSE

O controle da leptospirose em humanos depende, sobretudo, de melhoras no ambiente. Em áreas urbanas, a fração da população com baixas condições socioeconômicas está mais vulnerável à doença. Mudanças ambientais, como investimento em infraestrutura básica (saneamento), são imprescindíveis, na prevenção da leptospirose assim como de outras

doenças (DE MASI; VILAÇA; RAZZOLINI, 2009). Porém, a implementação de saneamento básico em curto prazo apresenta desafios já que 33% da população brasileira vive em comunidades carentes com condições sociais e ambientais precárias (IBGE, 2010). Outras estratégias têm sido implementadas incluindo: alertar a população para que evite entrar em contato com áreas alagadas ou enlameadas, realizar medidas de limpezas de domicílios após as enchentes, evitar o acúmulo de lixo ou outros fatores que favorecem a proliferação de roedores, e a utilização de equipamentos de proteção individual EPIs (BRASIL, 2002). Ações continuadas de informação, comunicação e educação em saúde devem ser realizadas com o intuito de repassar à população informações sobre as rotas de transmissão, reservatórios animais envolvidos, situações de risco e sinais/sintomas da leptospirose (BRASIL, 2002).

Dentre as ações preventivas, talvez a mais frequentemente utilizada é o controle de roedores, principalmente em áreas urbanas endêmicas sujeitas a inundações (COSTA, 2010). Porém o controle de roedores não tem provado ser efetivo, como pode ser demonstrado pelos elevados índices de morbimortalidade da leptospirose (COSTA, 2010). Todavia, o uso do controle químico e/ou mecânico reduz o número de indivíduos em uma população, mas não necessariamente a população reprodutora como um todo e não restringem à disposição de um ambiente antrópico favorável as necessidades dos roedores remanescentes ou provenientes de outras áreas (OLIVEIRA, 2009). Quando esses métodos de controle são aplicados de maneira descontínua e de forma inadequada, há uma rápida re-infestação da população de roedores, assim o controle torna-se apenas temporário (DE MASI; VILAÇA; RAZZOLINI, 2009; SÃO PAULO, 2013). A baixa efetividade no controle pode ser atribuída principalmente à ausência de melhorias ambientais e à falta de conhecimentos sobre a ecologia e as vias de transmissão do patógeno no roedor reservatório (*R. norvegicus*). De modo que a identificação das vias de transmissão pode estar diretamente ligada à manutenção do ciclo da *Leptospira* nos roedores sobreviventes ou imigrantes após o tratamento químico com rodenticida.

Novas abordagens no controle de roedores incluem a elaboração de modelos matemáticos preditivos da contaminação ambiental produzida pelas populações de roedores (HOLT; DAVIS; LEIRS, 2006). Estes modelos integram fatores demográficos (abundância, mortalidade, sazonalidade e migração), proporção de animais infectados e susceptíveis, taxas de infecção para cada via de transmissão (vias indiretas ou diretas [sexual, transplacentária, aleitamento, etc.]) (HOLT; DAVIS; LEIRS, 2006), e taxas de leptospiras excretados no ambiente (COSTA et al., 2015). Assim a caracterização destes parâmetros é necessária para

informar estes modelos e produzir estimativas espaciais e temporais do risco de leptospirose em humanos para o desenvolvimento de estratégias de prevenção mais efetivas.

Para identificar medidas mais eficientes de controle da leptospirose, é necessário a compreensão do ciclo de transmissão intraespecífica na população do roedor reservatório. O presente estudo tem o intuito de produzir informações sobre a dinâmica de *Leptospira* entre os roedores e sua possível transmissão através da rota de aleitamento materno. A identificação e a caracterização das vias de transmissão de *Leptospira* pelos roedores são parâmetros imprescindíveis para gerar novas hipóteses sobre a manutenção e resistência de *Leptospira* em modelos animais portadores. Podem contribuir também no desenvolvimento de modelos preditivos de risco da leptospirose (HOLT; DAVIS; LEIRS, 2006).

3 OBJETIVOS

3.1 OBJETIVO GERAL

- Identificar a presença de leptospiras na mama e leite, em populações urbanas de *Rattus norvegicus*.

3.1 OBJETIVOS ESPECÍFICOS

- Identificar a presença da *Leptospira* na mama através das técnicas de imunofluorescência, imunohistoquímica e microscopia eletrônica;
- Determinar a localização de *Leptospira* na mama de fêmeas lactantes de *R. norvegicus* através da imunohistoquímica;
- Quantificar DNA de *Leptospira* na mama e no leite de ratas através do qPCR;
- Descrever as alterações histopatológicas na mama de fêmeas lactantes de *R norvegicus* e verificar se estão associadas à presença de *Leptospira*.

4. ARTIGO

Leptospira **in breast tissue and milk of urban Norway rats (*Rattus norvegicus*)**

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Artigo a ser submetido ao Periódico "Epidemiology and Infection"

APRESENTAÇÃO: Este artigo contempla a investigação proposta na introdução, justificativa e nos objetivos da presente dissertação, que consiste em identificar a presença de *Leptospira* na mama e no leite como indicadores de transmissão vertical em *R. norvegicus*.

1   **Title:** *Leptospira* in breast tissue and milk of urban Norway rats (*Rattus norvegicus*)

2

3   **Journal**: Epidemiology and Infection

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28 **Short title:** *Leptospira* spp. in breast tissue and milk of urban rats

29 **Keywords**: vertical transmission, Scanning Electronic Microscopy, immunofluorescence,

30 immunohistochemistry breastfeeding, *Rattus norvegicus*

31   **SUMMARY**

32

33   Leptospirosis is a zoonosis caused by bacteria of the genus *Leptospira*. The disease is

34   globally distributed and a major public health concern. The Norway rat (*Rattus norvegicus*) is

35   a principal reservoir of the leptospire pathogen in the urban slums of developing and

36   developed countries. The potential routes of intra-specific leptospires transmission among

37   rats are largely unknown. Herein, we identified pathogenic *Leptospira* spp. in breast and milk

38   of naturally infected rats. We examined kidney, breast tissue and milk from 24 rats for the

39   presence of leptospires using immunofluorescence, immunohistochemistry, PCR and

40   scanning electronic microscopy. All 24 rats had evidence for *Leptospira* in the kidneys,

41   indicating chronic carriage. The majority of kidney-positive rats had detectable leptospires in

42   milk (18, 75%) and in breast tissue (16, 67%), as evidenced by IFA and IHC. Four milk

43   samples (17%) and two breast tissues (8%) were positive by qPCR. Scanning electron

44   microscopy confirmed the presence of leptospires in one of four breast tissue samples that

45   were examined. No major pathological changes in breast tissue were found. Our study, for

46   the first time, identified leptospires in the milk and breast tissue of Norway rats, suggesting

47   the possibility of milk-borne transmission of leptospirosis to neonates.

48

49   **INTRODUCTION**

50   Leptospirosis is a globally distributed zoonosis caused by a bacteria of the genus

51   *Leptospira* (KO; GOARANT; PICARDEAU, 2009). This disease has a large impact on public

52   health, accounting for at least 1.2 million annual humans cases worldwide (COSTA et al.,

53   2015a). Leptospirosis is transmitted through direct contact with the urine of infected animals

54   and occasionally through rodent bites (ROCZEK et al., 2008), and indirectly by exposure to

55   water or soil contaminated with leptospires shed in the urine of infected hosts (KO;

56   GOARANT; PICARDEAU, 2009; LAU et al., 2012; MCBRIDE et al., 2005). In developing

57   countries the burden of disease is associated with residents of urban slums, as exemplified by

58   studies conducted in Salvador, Brazil, where outbreaks during the rainy season affect the

59   poorest fraction of the population (FELZEMBURGH et al., 2014; KO et al., 1999; SARKAR

60   et al., 2002).

61   The Norway rat, *Rattus norvegicus,* is the primary reservoir species for leptospires in

62   the slums in Brazil (BAROCCHI et al., 2001; DE FARIA et al., 2008) and in many of urban

63   centers in developing and developed countries (ALLAN et al., 2015; EASTERBROOK et al.,

64   2007; HIMSWORTH et al., 2014; THIERMANN, 1981). Despite studies describing the

65   prevalence of *Leptospira* infection among Norway rats in Salvador, Brazil, (COSTA et al.,

66   2014a; DE FARIA et al., 2008), little is known about the maintenance and transmission of

67   this pathogen in these populations or elsewhere (HIMSWORTH et al., 2014).

68   Although environmental transmission is probably the major route for *Leptospira*

69   infection in rodents(COX; TWIGG, 1981). Prevalence studies indicate that young non-

70   reproductive rats, which just left the nest, showed a prevalence of up to 30% of kidney

71   colonization (COSTA et al., 2014a). Infection at a very young age (>2 months) suggests that

72   some transmission may occur through vertical or neonatal infection (in utero or milk) or other

73   routes within the nest, as pre-weaning rats do not venture from the nest. While intrauterine

74 transmission of leptospires has been documented for humans (FAINE et al., 1984; SHAKED

75 et al., 1993), and occurs among horses (POONACHA et al., 1993), there has been a single

76 case report of breastfeeding-associated transmission in humans (BOLIN; KOELLNER, 1988).

77 There also has been a single case report of sexual transmission in humans (HARRISON;

78 FITZGERALD, 1988), and is likely possible among horses, based on findings of leptospiral

79 DNA in semen (HAMOND et al., 2013). While transplacental transmission is well recognize

80 in livestock (and is related to abortions) (HIGGINS et al., 1980; LEON-VIZCAINO;

81 HERMOSO DE MENDOZA; GARRIDO, 1987; POONACHA et al., 1993), there is not clear

82 evidence of this route of transmission in rodent population (LAHIRI, 1941;

83 SCHNURRENBERGER; HANSON; MARTIN, 1970; WARD; TURNER, 1940).

84   Herein we report the presence of *Leptospira* spp. in breast tissue and milk indicating

85 the potential for neonatal transmission in *R. norvegicus*. Our results will help inform

86 predictive models of intra-species maintenance and transmission of leptospires among rodents

87 (HOLT; DAVIS; LEIRS, 2006) to better and generate new hypotheses about the contribution

88 of varied routes of transmission among reservoir populations.

89

90 **METHODS**

91

92 *Study site*

93   *Rattus norvegicus* were trapped on the study area of Pau da Lima (0.16 Km^2) in the

94 coastal city of Salvador, Brazil (FELZEMBURGH et al., 2014; REIS et al., 2008). High

95 human density (>3,700 inhabitants/0,16Km²); low socioeconomic level and lack of basic

96 sanitation and trash collection are features of this area (COSTA et al., 2014b). Prospective

97 cohort studies performed in this setting since 2003 have identified an incidence of leptospiral

98 infection of 36 per 1,000 person-years (FELZEMBURGH et al., 2014; REIS et al., 2008).

99

Trapping of rats and sample collection

100

101 Norway rats were trapped from peridomestic areas in Pau da Lima. Traps containing

102 rats were transported to the Zoonotic Control Center (ZCC) and animals were euthanized

103 following protocols previously described (COSTA et al., 2014a). Only lactating females

104 selected for this study. After euthanasia, the whole body of lactating rats was meticulously

105 cleaned with alcohol 70% and performed trichotomy of all ventral region. Milk was expressed

106 by finger pressure applied to each breasts, generally collected from the two breasts in the

107 upper part, because they had milk. Obtaining 1-2 small drops (about 10 to 20ul) of milk were

108 collected onto poly-L-lysine-coated glass slides for immunofluorescence (IFA) and 1-2 small

109 drops collected in sterile swabs for quantitative PCR (qPCR). After collect the drops of milk

110 onto poly-L-lysine-coated glass slides we made duplicate glass slide pressuring it onto other

111 glass slides. Breasts were cut from the base and the cut surface was pressed on glass slides for

112 IFA testing. Five left breasts were collected and fixed in 10% neutral buffered formalin for

113 immunohistochemistry (IHC).

114 Five breasts were collected in eppendorf tubes and stored at -80 °C until qPCR testing.

115 Breasts from four animals were separated for Scanning Electronic Microscopy (SEM).

116 Additionally, seven breasts were collected to attempt isolation of *Leptospira*. Kidneys were

117 obtained from all animals to identify leptospiral carriage as previously described (COSTA et

118 al., 2015b). Breasts from two healthy and non-infected lactating laboratory rats (*R. norvegicus*

119 Wistar) were used as negative controls. All animal protocols were approved by the

120 Institutional Animal Care and Use Committee (IACUC) committee at the Oswaldo Cruz

121 Foundation, (Salvador, Brazil; 03/2012) and Yale University (New Haven, CT; 2012-11498).

122

123

124   *Indirect immunofluorescence microscopy (IFA)*

125   Slides with imprints of kidney, breast and milk were analyzed as described previously

126   (CHAGAS-JUNIOR et al., 2009). Slides were fixed in acetone for three minutes and then

127   washed with phosphate-buffered saline (PBS). Blocking with 1% bovine serum albumin

128   (BSA) for 40 minutes was followed by a 1 hour incubation with hyperimmune rabbit antisera

129   to whole-*L. interrogans* serovar Icterohaemorrhagiae strain RGA diluted 1:1000 at 30ºC

130   (ATHANAZIO et al., 2008; CHAGAS-JUNIOR et al., 2009). After drying, the samples were

131   fixed in acetone for three minutes and re-washed with PBS. Samples from negative laboratory

132   rats were similarly treated and used as negative controls. Additional controls were generated

133   from kidney-positive wild rats by incubating slides with normal rabbit serum at the same

134   dilution. After washing with PBS, the imprints were incubated with goat anti-IgG rabbit

135   ALEXA488 (Invitrogen) at a dilution of 1:500 for 1 hour. Finally, the imprints were washed

136   3x with PBS, mounted with anti-fading medium (Prolong-Molecular Probes) and examined at

137   400x and 1000x (Olympus BX51 microscope). We estimated the presence of leptospires in

138   milk imprint as the mean number of leptospires per 4 fields of view at a magnification of

139   1000x

140

141
       Immunohistochemistry (IHC)

142   Separate fragments of the breast tissue used for imprints were fixed in 10% formalin,

143   embedded in paraffin according to routine histological procedures and cut into 4 to 5µm

144   sections. Sections were stained with hematoxylin and eosin (HE) for histopathological

145   analyses. Paraffin was removed with xylene and ethanol. Following the methodology

146   described by (CHAGAS-JUNIOR et al., 2009), slides were treated with 3% hydrogen

147   peroxide for 15 min at room temperature, blocked with 1% BSA and then incubated at 37ºC

148   for 1 hour with a 1:2,000 dilution of pathogen-specific rabbit antiserum against LipL32

149 (MURRAY et al., 2009). After three washes with PBS, slides were incubated with

150 biotinylated–conjugated anti-rabbit immunoglobulin and then with horseradish peroxidase

151 conjugated streptavidin (Kit Invitrogen). The chromogen used (3,3-diaminobenzidine) DAB

152 (Invitrogen Kit 00-2114) was visualized as a brown deposit. Finally, the slides were stained

153 with hematoxylin, mounted with balsam and cover slipped. Kidney positive samples were

154 processed identically and used as positive controls. Slides were examined in bright field in

155 microscopy, Olympus BX51, at 1000x.

156

157 *Culture isolation*

158 Breast samples were macerated and inoculated into 5 mL of modified EMJH medium

159 (FAINE; ADLER; PEROLAT, 2000). The cultures were incubated at 28ºC for 24 hours. After

160 this period, 0.5 mL of supernatant was subcultured in another tube with 5 mL EMJH medium.

161 The cultures (including the primary cultures) were examined weekly over three months by

162 dark field microscopy. In case of contamination by other microorganisms, filtering

163 methodologies were attempted using 0.22 uL pore syringe filters (Sterile Millipore).

164

165 *Scanning Electronic Microscopy (SEM)*

166 Breast samples were cut into 1-2 mm pieces and fixed in 2.5% glutaraldehyde and 0.1

167 M sodium cocadylate buffer, pH 7.4, post-fixed with 1% osmium tetroxide for 1hour, washed

168 with 0.1M sodium cocadylate buffer, dehydrated in a series of ethanol and dried using a

169 critical point drying apparatus (Leica EM CPD030). The fragments were mounted on

170 aluminum stubs, sputter coated with gold (DESCK IV, Denton Vacuum) and examined in a

171 scanning electron microscope (JSM6394LV, JEOL) operated at 12 kV.

172

173

174 *Quantitative real-time PCR (qPCR)*

175   Frozen breast tissue from each rat was thawed and cut into small pieces; 500 ul PBS

176 was added and the sample was thoroughly mixed in a blender at maximum speed for 15

177 minutes. The liquid from the blender was collected and the DNA was extracted using

178 Maxwell®16 Tissue DNA Purification Kits (AS1030) by Promega DNA Maxwell® 16 MDx

179 Instrument. Frozen rat milk collected with cotton swabs was thawed and 500 ul PBS was

180 added and thoroughly mixed in blender at maximum speed for 5 minutes. Liquid was

181 collected and DNA was extracted using Maxwell®16 Tissue DNA Purification Kit (AS1030)

182 by Promega DNA Maxwell® 16 MDx Instrument (AS3000).

183   The qPCR was performed using 5' nuclease (TaqMan) assay and primers that

184 amplified a sequence of the pathogen specific *Leptospira lipL32* gene (STODDARD et al.,

185 2009), using the Applied Biosystems® 7500 Fast Real-Time PCR Instrument. For the

186 standard curve, genomic L1-130 DNA obtained from *Leptospira* serovar Copenhageni, the

187 strain infecting Norway rats and humans in Salvador, was quantified using an ND-1000

188 spectrophotometer (Nanodrop Technologies). Eight calibrating dilutions (0 to 10^7 GEq/mL)

189 were prepared and served as reference values. The reaction mix consisted of 12.5μL of PCR

190 SuperMix-UDG (Invitrogen, Carlsbad, CA), 500nM of forward and reverse primers, 100nM

191 of probe, 5μL of DNA extract and ultrapure water (Invitrogen) to a final volume of 25μL. The

192 amplification protocol consisted of 2 minutes at 50°C and 10 minutes at 95°C, followed by 45

193 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 minute. Cycle

194 threshold (CT) < 40 was treated as positive. For quality control, each reaction was duplicated

195 and non- template controls were included in every column of 96 reaction plates. The rodent

196 housekeeping gene glyceraldehyde-3-phophate dehydrogenase (*gapdh*) was used as an

197 internal control to monitor inhibition of PCR amplification and the efficiency of DNA

198 extraction.

199   *Histopathological analyses of breast tissues*

200   For histopathological examination, breast tissue slides were stained with HE,

201   Picrosirius (PIFG) and analyzed by light microscopy. We evaluated the main excretory duct,

202   intralobular excretory duct, acinar tissue/ductal and intralobular stroma. In these structures,

203   we scored and recorded morphological lesions as acute and chronic inflammation, fibrosis,

204   calcification, eosinophilic staining and fat content. The intensity of the lesions was semi-

205   quantitatively rated in each of tissue type on a scale of 0 to 3, where 0 = normal, 1 = mild, 2 =

206   moderate, 3 = severe.

207

208   *Statistical analyses*

209   We performed descriptive analysis evaluating the proportion of positive animals for

210   each sample and laboratory technique. Concordance between different techniques was

211   assessed by the kappa index statistic.

212

213   **RESULTS**

214

215   *Samples*

216   Fifty-five lactating rats were caught during the study period. We obtained all tissues

217   (kidney, breast and milk) from 24 lactating rats, which did not differ in mass/age from other

218   lactating rats captured but from which milk samples could not be obtained.

219

220   **Leptospira** *in kidney*

221   We defined carrier status by evaluating the presence of leptopires in the kidney by IFA

222   (Fig 1), IHC (Fig 2) or qPCR. All 24 rats were positive by one or more tests (Table 1).

223   Control samples from the laboratory Wistar rats and sample incubated with normal rabbit

224   serum were negative.

225

226   **Leptospira *in milk***

227   *Leptospira*, as evaluated by IFA, were observed in 18 (75%) expressed milk samples

228   from the 24 kidney positive females (Fig 1). *Leptospira* were not numerous in milk (Fig 1D)

229   or breast tissue (Fig 1C) by IFA examination and we routinely observed sparsely distributed

230   leptospires. The number of *Leptospira* observed in milk ranged from 1 to 31 leptospires

231   (mean 7.5) per 4 fields of view at a 1000x. Additionally, four samples (17%; 4/24) were

232   positive by qPCR, with a range of 36-1484 GEq/mL.

233

234   **Leptospira *in breast tissue***

235   *Leptospira* were identified in 67% of breast tissues by any test. Considering specifics

236   test, *Leptospira* in breast tissue were observed in 12 (50%), 12 (50%) and 2 (8%) for IFA,

237   IHC and qPCR respectively (Table 1, Fig 1C, Fig 2D-F and Fig 3A-D). The concordance

238   between IFA and IHC findings was fair (kappa=0.61). Two samples were positive to qPCR,

239   and the range of values was similar to those obtained from milk (20-1519 GEq/mg). The

240   positive SEM from one of the four samples examined confirmed the presence of leptospires in

241   breast tissue (Fig 3). Control breast samples from the two Wistar rats were negative by IFA,

242   IHC and qPCR testing. Attempts to isolate leptospires from breast resulted in contaminated

243   cultures and contamination was not controlled by filtering and serial passage.

244   Often sparsely distributed, leptospires were detected by IHC within in the mammary

245   gland 67% (8/12), in the acinar/ductal region and in the connective tissue 33% (4/12). The

246   morphology of leptospires showed well preserved properties with regard to size and helical

247   morphology. We also observed that *Leptospira* helical morphology was more accentuated in

248   breast tissue when compared to kidney tissue. We observed predominantly the leptospires in

249   isolation and with few organisms along the microscopic field. However, visualization of

250   bacteria by SEM showed aggregated leptospires, potentially in the form of biofilms

251

252   *Histopathological examination of the breasts*

253   HE and PIFIG histopathological analysis of the breasts of the 24 rats studied showed

254   that 10 rats (42%) had discrete to moderate lesions in the breast (Table 2). Among the 10 rats

255   that exhibited lesions, 9 rats (37%) had *Leptospira* in breast tissue or milk by one or more

256   tests. Lesions included: a) enlarged main excretory duct, b) enlarged, calcification, and acute

257   or chronic inflammation in the intralobular excretory duct, c) enlarged and acute inflammation

258   of the acinar/ductal tissue comprising eosinophilic and fat secretion and d) fibrosis in the

259   intralobular stroma (Table 2). However, there were no statistical differences in the proportion

260   of females with breast lesions between animals with and without leptospires in the breast

261   (evaluated by IH).

262

263   **DISCUSSION**

264

265   Lack of knowledge regarding the routes of intraspecific transmission of *Leptospira*

266   among natural reservoir-host species, such as the Norway rat, are an obstacle to a complete

267   understanding of the epidemiological factors underlying patterns of acquisition and

268   maintenance of this spirochete. Herein we describe for the first time the presence of

269   pathogenic leptospires in the milk and breast tissue of lactating wild Norway rats

270   demonstrated to be chronically infected by carriage of leptospires within their kidneys.

271   Although the outcomes of various assays varied, leptospires were demonstrated to be

272   present by IFA testing of milk and breast tissue impressions, by IHC staining of breast tissue

273   using pathogen-specific Lip32 antisera and by qPCR. Additionally, confirmation of breast

274   colonization was demonstrated by visualization of clusters of leptospires by SEM.

275   Vertical or neonatal transmission of *Leptospira* has been suggested by findings of

276   prevalence studies stratified by mass/age. Non-reproductive rats, just coming out of the nest,

277   can already be colonized with a prevalence up to 30% (COSTA et al., 2014a). Transmission

278   from mother to offspring could occur via several routes, including *in utero* infection,

279   breastfeeding, via infectious saliva during grooming, or in the nest through contact with urine

280   from an infected mother. During the first 20-30 days of life, contact between offspring and its

281   mother is intense (CALHOUN, 1963) which limits the possibility to identify specific routes of

282   non-placental neonatal transmission. Fetal infection by leptospires has been described

283   (SCHNURRENBERGER; HANSON; MARTIN, 1970; WARD; TURNER, 1940).

284   We found *Leptospira* in 67% (8/12) of lumen of mammary glands sampled by IHC,

285   indicating that leptospires could readily be discharged along with milk during suckling.

286   Preference for lumen compartments is commonly observed in the renal tubules of rat

287   reservoirs (ATHANAZIO et al., 2008). Studies focusing in leptospiral colonization in renal

288   tubules propose that preference for lumen could be explained by the low antibody

289   concentration (ATHANAZIO et al., 2008; FAINE; ADLER; PEROLAT, 2000). In some

290   cases 33% (4/12) leptospires were also observed in the connective tissue matrix, suggesting a

291   capacity to migrate through this tissue.

292   In mammary glands, *Leptospira* were observed predominantly in free form with

293   marked helical morphology but relatively few organisms present in any given microscopic

294   field. However, visualization of bacteria by SEM showed clusters of organisms, or potentially

295   biofilm aggregates, as demonstrated in colonized kidneys (AGUDELO-FLÓREZ et al., 2013;

296   RISTOW et al., 2008). Although SEM showed erythrocytes in one image, suggesting that

297   leptospires could be located in blood vessels, it is likely that the visualized leptospires were in

298   breast tissue. All pregnant rats included in this study were kidney-positive females indicating

299   that a carrier state had been achieved. Leptospires initially circulating in blood during the

300   systemic phase of the disease are cleared by the time of intense renal colonization. Isolation

301   attempts were uniformly contaminated, despite the application of 70% alcohol to the whole

302   body of euthanized rats.

303   The presence of leptospires in milk has been reported on rare occasions from other

304   species. Cows infected with serovar Hardjo shed these bacteria in milk and mount an immune

305   response that can result in mastitis (ELLIS et al., 1976). Besides one report describing a

306   potential breastfeeding transmission of leptospirosis in a human newborn (BOLIN;

307   KOELLNER, 1988), there is no evidence that this is a common mechanism. *Leptospira* have

308   also been isolated from human milk (CHUNG et al., 1963).

309   The number of *Leptospira* in milk (range 1-31 per 1000x field) could only be roughly

310   approximated as milk samples were estimated to be less <20 ul, and concentrations could not

311   be estimated. However, qPCR results indicated similar leptospiral loads of 36-1484 GEq/mL

312   in milk. When milk consumption is taken into account sucking pups could be exposed to high

313   loads of leptospires prior to weaning. An increase in milk uptake occurs during the first 15

314   days of breast feeding then declines when young are supplementing their diet with solid

315   foods-preceding weaning at ~day 27 (GALEF, 1981; THIELS; CRAMER; ALBERTS, 1988).

316   At postpartum- day 15 dams can produce and accumulate up to 14 gms (mls) of milk during

317   each 3-4 hr period prior to feeding pups (THIELS; CRAMER; ALBERTS, 1988). A single

318   pup in a litter of 10 pups (based on median embryo counts from >100 pregnant rats in

319   Salvador, Brazil (Costa, unpublished data) could thus ingest >8 mls of milk per day

320   cumulatively containing hundreds (~300) or many thousands (~12,000) of leptospires based

321   of qPCR results. Additionally the cumulative volume of milk taken over the entire course of

322   breast feeding would increase the exposure over 10-fold.

323   The oral dose for infecting *R. norvegicus* is unknown. However, the intraperitoneal

324   ID_{50} for four-day old pups was 10^2 (the study did not test lower doses) was far lower than the

325   10^4 organisms required to infect adults, suggesting pups are more susceptible as mass alone

326   cannot account for such a large difference (ATHANAZIO et al., 2008; MUSLICH et al.,

327   2015). However a previous study (MUSLICH et al., 2015) indicated it is therefore feasible

328   that an inoculum dose of *Leptospira* in the hundreds or thousands ingested through milk could

329   cause infection.

330   The pathologic consequence of leptospiral infection in breast tissue of Norway rats

331   was minimal and the pathogenic scores of infected tissues were no different from non-infected

332   tissues. Of note, the presence of leptospires was not accompanied by inflammation and there

333   were no significant pathological changes when breasts with presence and absence of

334   leptospires were compared. These negative results suggest that colonization does not affect

335   organ function and dilated acinar ducts and accumulation of hyaline material in the acini,

336   compatible with milk, were present among infected animals. It is unlikely that colonized

337   breasts would in any way inhibit the normal release of milk. In contrast, while the acute phase

338   of clinical leptospirosis caused by serovar Hardjo in cows is usually subclinical, lactating

339   cows show a number of pathologic changes including agalactia ("milk drop syndrome") and

340   small quantities of blood are shed in the milk. Agalactia is associated to rapid drop in milk

341   production, a soft flabby udder, febrile response and milk with appearance of yellow

342   colostrum, thus affecting the quantity and quality of milk available to calves (HIGGINS et al.,

343   1980).

344   It is no surprise that the results from different diagnostic techniques varied, as none of

345   these have been standardized for breast tissue. The presence of inhibitors or other material in

346   milk could have contributed to low frequency of qPCR positives. Additionally, only low

347   volumes of milk were possible to collect from lactating rats. *Leptospira* concentrations and

348 distribution within highly infected kidney tissue are extremely heterogeneous and it is

349 possible the same pattern applies to breast tissue (SANTOS et al., *in press*). These

350 heterogeneities would present a major obstacle to the routine demonstration of leptospires in

351 SEM. However, when present leptospires occurred in clusters similar to SEMs of kidney

352 proximal tubules (ATHANAZIO et al., 2008).

353 Laboratory experiments and refinements to assays could shed light on the variation

354 observed and confirm our findings of *Leptospira* in milk and breast tissue. Irrespective of the

355 limitations mentioned above, our results, when considered in total, confirm the presence of

356 leptospires in the milk and breast tissue of naturally infected Norway rats for the first time.

357 Although presence does not translate into demonstrating *Leptospira* transmission from dams

358 to pups, previous work suggests that up to 30% of young rats leave the nest infected (COSTA

359 et al., 2014a). Characterization of potential transmission pathways is critical to understand

360 how leptospires are acquired and maintained in Norway rat and other reservoir species and are

361 essential for informing parameters used in mechanistic models of leptospirosis in rodent hosts

362 (HOLT; DAVIS; LEIRS, 2006).

363

376

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484

485

486
487 **Fig. 1.** Identification of *Leptospira* by indirect immunofluorescence in milk and breast slide-

488 impressions samples obtained from chronically infected (kidney positive) wild-caught *Rattus*

489 *norvegicus*. (A) negative kidney control ; (B) positive kidney control ; (C) positive breast and

490 (D) positive milk. Magnification of 1000x. Bars: 30μm.

491

492 **Fig. 2.** Identification of *Leptospira* by immunohistochemistry: (A) negative kidney control ;

493 (B) renal tubules positive kidney control (arrow points to a cluster of *Leptospira*); (C)

494 negative mammary gland control; (D) in the mammary gland of a wild-caught chronically

495 infected (kidney positive) Norway rat (arrow points to an individual leptospire; several are

496 visible); (E) negative control in connective tissue of a breast sample; (F) *Leptospira* in

497 connective tissue of a breast sample from a chronically infected (kidney positive) wild-caught

498 Norway rat Zoom highlighting the morphological structure of leptospira, sharp helical

499 structure; bar length 30μ.

500

501 **Fig. 3.** Scanning Electron Microscopy reveals clusters of *Leptospira* in breast tissue from a

502 naturally infected wild-caught *R. norvegicus*. (A) cluster of leptospires, (B) zoom of A

503 showing visible erythrocytes in breast tissue:(C and D) leptospires densely packed side by

504 side.

505

506   Figure 1

507
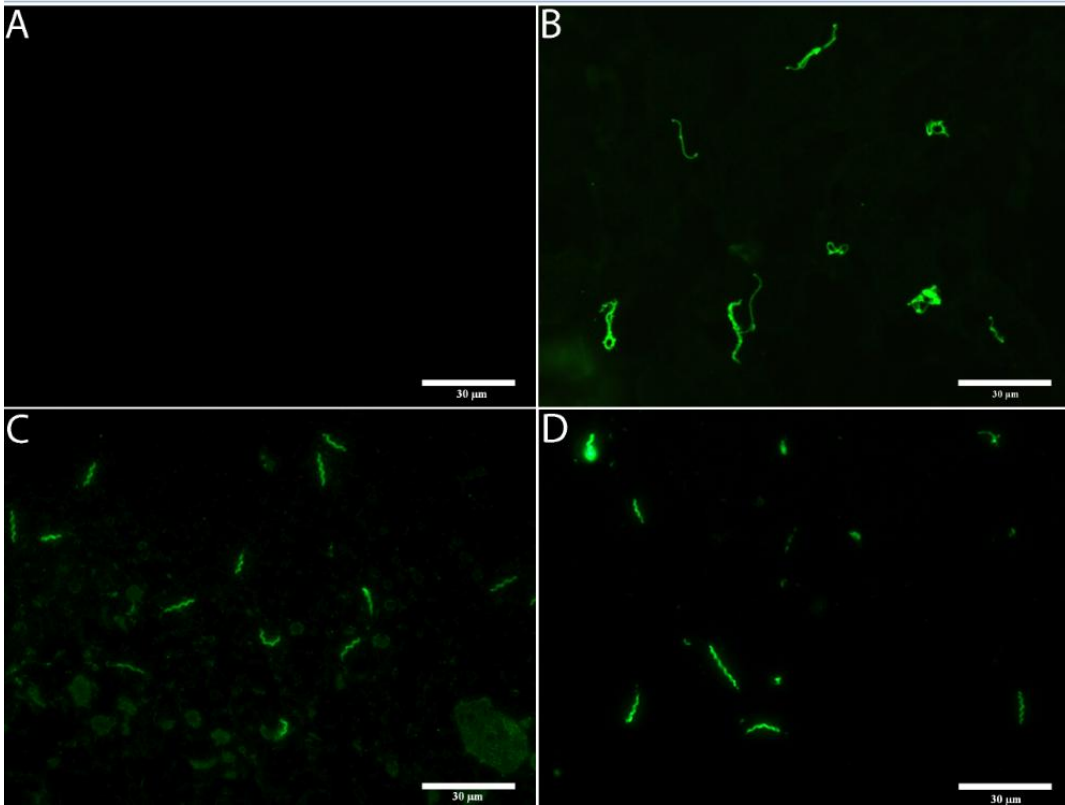
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511   Figure 2
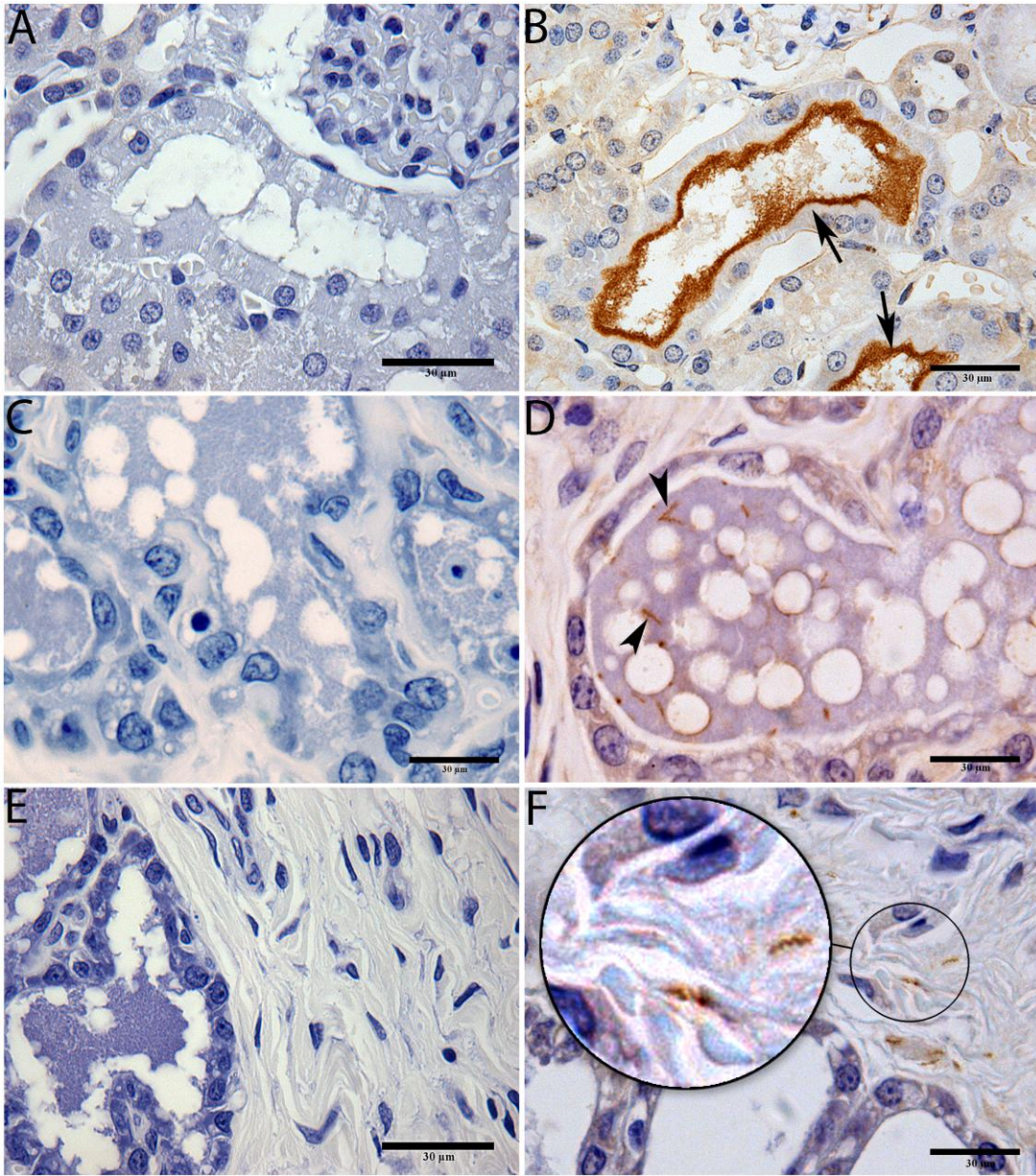
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513   Figure 3
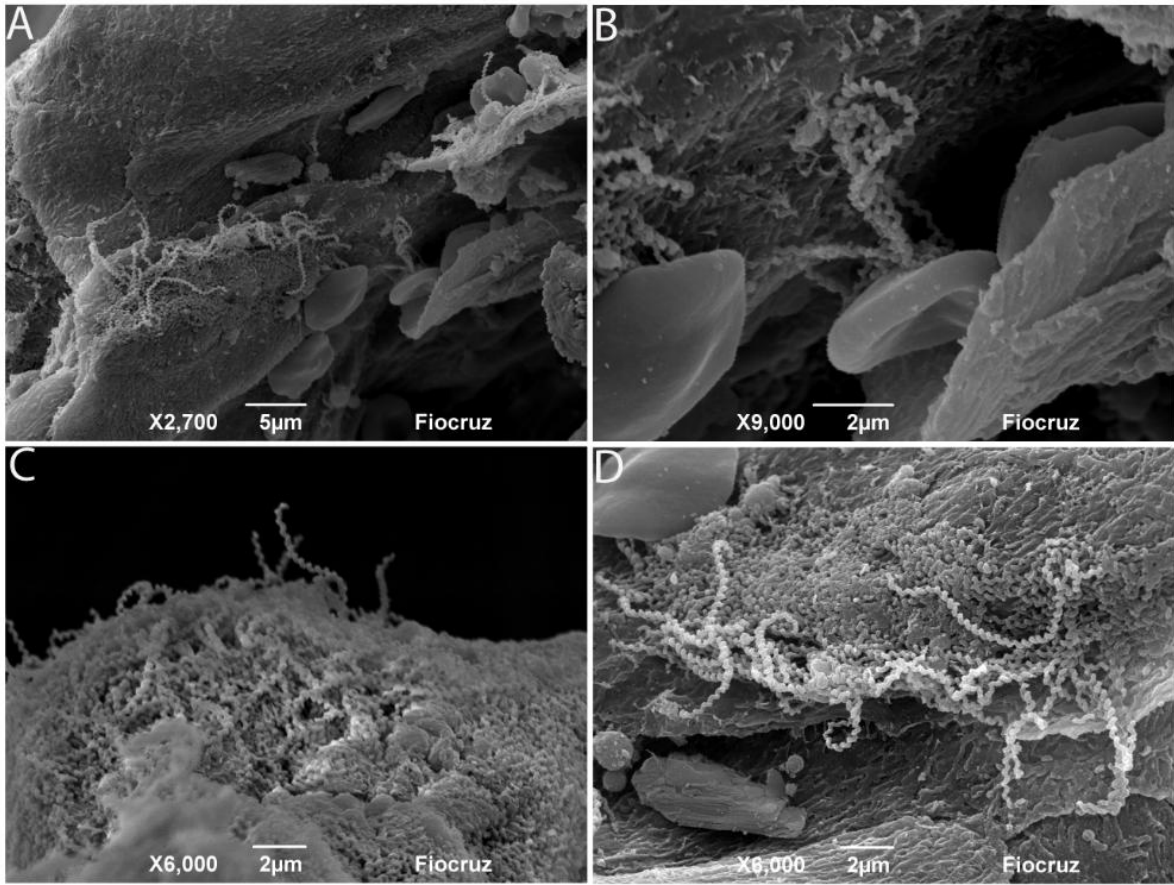
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515

516

517   **Table 1.** Results of laboratory tests for the presence of *Leptospira* in kidney, breast and milk

518   in lactating urban slum *Rattus norvegicus.*

519

	Kidney	Breast	Milk	Breast & Milk
	Nº positive samples/N total samples tested (%)			
Immunofluorscence (IFA)	20/24 (83)	12/24 (50)	16/24 (67)	12/24 (50)
Immunhistochemistry (IHC)	NA	12/24 (50)	NA	NA
qPCR	22/24 (92)	2/24 (8)	4/24 (17)	0 (0)
IFA or IHC or qPCR	24/24 (100)	16/24 (67)	18/24 (75)	14/24 (58)

520

521

522 **Table 2.** Histopathological alterations between breasts positive/negative

Alterations type	Positive breast *n= 16*	Negative breast *n= 8*	Total *n= 24 (100%)*
Main Excretory Duct			
Enlarged	16 (100)	8 (100)	24 (100)
Acute Inflammation	0 (0)	0 (0)	0 (0)
Chronicle Inflammation	0 (0)	0 (0)	0 (0)
Fibrosis	0 (0)	0 (0)	0 (0)
Intralobular Excretor Duct			
Enlarged	16 (100)	8 (100)	24 (100)
Acute Inflammation	3 (19)	1 (13)	4 (17)
Chronicle Inflammation	1 (6)	0 (0)	1 (4.2)
Mixed Inflammation	0 (0)	0 (0)	0 (0)
Fibrosis	0 (0)	0 (0)	0 (0)
Acinar/Ductal Tissue			
Enlarged	16 (100)	8 (100)	24 (100)
Secretion Eosinophilic	16 (100)	6 (75)	22 (92)
Fat Secretion	16 (100)	4 (50)	20 (83)
Acute Inflammation	3 (19)	1 (13)	4 (17)
Chronicle Inflammation	0 (0)	0 (0)	0 (0)
Mixed Inflammation	0 (0)	0 (0)	0 (0)
Calcification	3 (19)	0 (0)	3 (13)
Intralobular Stroma			
Fibrosis	6 (37)	1 (13)	7 (29)
Edema	0 (0)	0 (0)	0 (0)
Acute Inflammation	0 (0)	0 (0)	0 (0)
Chronicle Inflammation	0 (0)	0 (0)	0 (0)
Bleeding	0 (0)	0 (0)	0 (0)
Calcification	0 (0)	0 (0)	0 (0)

523

5 DISCUSSÃO

A falta de conhecimento sobre a transmissão intraespecífica de *Leptospira* em roedores reservatórios tem sido uma importante barreira na compreensão dos fatores epidemiológicos subjacentes aos padrões de aquisição e manutenção da *Leptospira*. A demonstração da presença de *Leptospira* na mama e no leite de *Rattus norvegicus*, neste trabalho, sugere a amamentação como potencial via de transmissão vertical da bactéria nestes animais. É possível, que este mecanismo de transmissão possa contribuir para a manutenção das elevadas taxas de infecção por *Leptospira* observadas em roedores urbanos e consequentemente, na contaminação ambiental e risco aos humanos.

A transmissão vertical ou neonatal de *Leptospira* em roedores foi sugerida em estudos de prevalência estratificados por massa/idade. Ratos muito jovens, que apenas saíram do ninho, apresentaram uma prevalência de até 30% de colonização renal (COSTA et al., 2014a). Transmissão de mãe para filho pode ocorrer através de várias vias, incluindo a transplacentária, leite materno (amamentação), via saliva, ou através do contato com a urina da mãe infectada no ninho. Durante os primeiros 20-30 dias de vida, o contato entre mãe e sua prole é intenso (CALHOUN, 1963) isso dificulta a identificação das rotas neonatais específicas de transmissão.

Neste estudo, encontramos *Leptospira* localizadas no lúmen de glândulas mamárias (33%, 8/24), onde há a produção e armazenamento de leite. Indicando que as leptospiras poderiam ser facilmente liberadas junto com o leite durante a amamentação. Esta constatação sugere que *Leptospira* pode ser eliminada juntamente com o leite sob estímulo da amamentação. Preferência pelo lúmen é comumente observada nos túbulos renais de reservatórios em roedores (ATHANAZIO et al., 2008). Estudos, com foco na colonização de *Leptospira* nos túbulos renais, propõem que a preferência por lúmen poderia ser explicada pela baixa concentração de anticorpo encontrado nesses locais (ATHANAZIO et al., 2008; FAINE; ADLER; PEROLAT, 2000). Também observamos, neste estudo, a presença *Leptospira* na matriz de tecido conjuntivo em 16% das mamas, demonstrando a capacidade dessas bactérias migrarem neste tecido.

A *Leptospira* apresentou morfologia helicoidal acentuada, sendo observada predominantemente de forma isolada e com poucos organismos presentes ao longo do campo microscópico. No entanto, a visualização de bactérias por MEV mostrou grupos de organismos ou agregados, potencialmente na forma de biofilme, seguindo padrão previamente

descrito em rins colonizados (AGUDELO-FLÓREZ et al., 2013; ATHANAZIO et al., 2008). As nossas tentativas de isolar *Leptospira* da mama, não foram bem sucedidas devido ao alto nível de contaminantes nas culturas, mesmo com uma meticulosa higienização em todo o corpo das ratas eutanaziadas. Contudo, os resultados obtidos com a IHQ e MEV indicam a presença de leptospiras na mama, especificamente no lúmen, de onde junto com o leite poderiam ser liberados durante a amamentação para os filhotes.

A presença de *Leptospira* no leite das ratas urbanas foi demonstrada com o auxílio dos testes de IFA e qPCR. Evidência de transmissão pelo leite é rara, embora haja evidencias indiretas de transmissão pela amamentação em recém-nascidos humanos (BOLIN; KOELLNER, 1988). Outros estudos mostraram a presença de *Leptospira*, através de isolamento, no leite humano (CHUNG et al., 1963) e de vacas (ELLIS et al., 1976). Contudo, a presença de leptospiras na glândula mamária e no leite não necessariamente indica que ocorre a transmissão pela amamentação; podem existir mecanismos para que esta via de transmissão seja evitada. Um estudo anterior indicou que o leite tem agentes antileptospiral, lisozima e lactenina, que têm atividade de lise (KIRSCHNER; MAGUIRE; BERTAUD, 1957). Também, já foi demonstrada a presença de anticorpos anti-leptospira no leite de bovinos (DOM et al., 1991). Além disso, Asoh e colaboradores (ASOH et al., 2014) descobriram que a saliva e a mucosa de hamster podem ter ação neutralizante e constituir uma barreira física natural contra estas bactérias.

Embora 75% das ratas foram positivas para *Leptospira*, encontramos uma baixa concentração do patógeno na mama e leite das ratas. Estes achados contrastam com as altas cargas encontradas no rim e urina (COSTA et al., 2015). Estimamos a concentração de *Leptospira* no leite em 1-31 organismos por campo de 100x, porém este valor é aproximado devido ao baixo volume coletado (<20 ul). No entanto, quando consideramos o consumo de leite, os filhotes podem ser expostos a altas cargas de leptospiras antes do desmame. No 15º dia pós-parto uma fêmea pode produzir e acumular até 14 gramas de leite a cada período de 3-4 horas prévias a alimentação dos filhotes (THIELS; CRAMER; ALBERTS, 1988). Um único filhote de uma ninhada de 10 filhotes (Panti et al, não publicado) poderia, assim, ingerir> 8 gramas de leite por dia, cumulativamente, contendo centenas ou milhares de leptospiras.

A dose infectante oral para *Rattus norvegicus* é desconhecida e estudos experimentais prévios apenas descrevem a dose infectante intraperitoneal. Em um estudo experimental com ratos de quatro dias de vida, desafiados com inóculo de 10^2 *Leptospira* por via intraperitoneal,

foi possível observar sintomas relacionados a leptospirose e óbitos. Já ratos com 23 dias de nascidos apresentaram colonização por leptospiras em alguns órgãos, porém não manifestaram quaisquer alterações patológicas e foram capazes de sobreviver à infecção semelhantes aos ratos adultos (MUSLICH et al., 2015). Em estudos com ratos adultos, testados pela via intraperitoneal, tem sido observado que estes precisam de altas doses de *Leptospira* (10^4 ou maiores) para serem infectados (ATHANAZIO et al., 2008). A baixa concentração e o inóculo contínuo ingerido através do leite poderia potencialmente produzir o estado de reservatório em filhotes recém-nascidos. Além disso, o contato prévio com leptospiras ingeridas pelo leite pode ter um papel importante no desenvolvimento de anticorpos de memória o que resultaria na resistência à doença nos ratos jovens (MUSLICH et al., 2015).

As lesões patológicas na mama das ratazanas avaliadas foram mínimas. Não observamos diferença nas frequências de lesões entre animais infectados e não infectados por *Leptospira*. Esses resultados confirmam que, assim como no rim (TUCUNDUVA DE FARIA et al., 2007), as leptospiras estabelecem uma infecção silenciosa na mama o que provavelmente não repercute na funcionalidade do orgão. A presença de ductos acinares dilatados e acúmulo de material hialino nos ácinos, compatível com leite, indicam que a capacidade de produção de leite foi preservada nas ratas colonizadas. Desta forma, o fato das mamas estarem colonizadas com leptospiras não impediria as ratas de amamentarem, pois não haveria estímulos dolorosos provocados por inflamação, o que poderia provocar uma rejeição dos filhotes. Estes achados contrastam com aos observados em vacas lactantes infectadas que evidenciaram um número de alterações patológicas incluindo agalaxia ("síndrome da queda de leite") e pequenas quantidades de sangue eliminadas no leite (HIGGINS et al., 1980).

Foi observada moderada concordância nos resultados obtidos com a utilização das quatros técnicas empregadas no presente estudo. Vale ressaltar que nenhuma delas tinha sido previamente padronizada para a mama. O baixo volume de leite coletado e a distribuição heterogênea de leptospiras na mama (como evidenciada na IHQ) certamente contribuiu na baixa positividade nos testes qPCR. A imunofluorescência foi o teste mais sensível neste estudo e foi capaz de detectar leptospiras intactas com uma morfologia típica, potencialmente indicando o número de leptospiras viáveis. Experimentos de laboratório poderiam contribuir para entender a variação observada entre as amostras testadas por vários métodos. Independentemente das limitações acima mencionadas, os nossos resultados, quando

considerados na sua totalidade, confirmam, pela primeira vez, a presença de *Leptospira* no leite e a mama de ratazanas naturalmente infectadas.

Os resultados deste estudo sugerem que o risco de transmissão pelo leite materno é provavelmente alto e que novos trabalhos no âmbito experimental são necessários para estabelecer uma associação direta entre o aleitamento materno e a infecção pela *Leptospira* nesta espécie. A caracterização das vias de transmissão é importante para compreender como as leptospiras são mantidas nas populações de roedores e abre perspectivas para futuros estudos sobre a história natural da bactéria em populações de reservatórios. Além disso, ajuda a delimitar parâmetros utilizados em modelos mecanicistas de leptospirose em roedores reservatórios (HOLT; DAVIS; LEIRS, 2006).

6 CONCLUSÃO

Com base nos resultados encontrados neste estudo chegamos às seguintes conclusões:

- Identificamos a presença de *Leptospira* na mama e leite das ratas;

- Nas mamas, leptospiras apresentaram-se de formas isoladas e aglomeradas, localizadas na glândula e no tecido conjuntivo;

- As mamas apresentaram poucas alterações histopatológicas e estas parecem não estar associadas com a presença da *Leptospira*;

- A baixa detecção de DNA de *Leptospira* por qPCR em algumas amostras de mama e leite, porém positivas em outras técnicas, apontam para a necessidade da padronização desta técnica na identificação de *Leptospira* em mama e leite de roedores;

- A identificação de *Leptospira* no leite indica que a amamentação pode ser via de transmissão de *Leptospira* de mãe para filho em roedores.

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ANEXO I (Artigo)

PLOS | NEGLECTED TROPICAL DISEASES

Patterns in *Leptospira* Shedding in Norway Rats (*Rattus norvegicus*) from Brazilian Slum Communities at High Risk of Disease Transmission

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Abstract

Background

We address some critical but unknown parameters of individuals and populations of Norway rats (*Rattus norvegicus*) that influence leptospiral infection, maintenance and spirochetal loads shed in urine, which contaminates the environment ultimately leading to human infection.

Methodology/Principal Findings

Our study, conducted in Salvador, Brazil, established the average load of leptospires in positive kidneys to be 5.9×10^6 per mL (range 3.1-8.2×10^6) genome equivalents (GEq), similar to the 6.1×10^6 per ml (range 2.2-9.4×10^6) average obtained from paired urines, with a significant positive correlation ($R^2 = 0.78$) between the two. Based on bivariate and multivariate modeling, we found with both kidney and urine samples that leptospiral loads increased with the age of rats (based on the index of body length to mass), MAT titer and the presence of wounding/scars, and varied with site of capture. Some associations were modified by sex but trends were apparent. Combining with data on the demographic properties and prevalence of leptospiral carriage in rat populations in Salvador, we estimated that daily leptospiral loads shed in the urine of a population of 82 individuals exceeded 9.1×10^{10} leptospires.

Conclusions/Significance

These factors directly influence the risk of leptospiral acquisition among humans and provide essential epidemiological information linking properties of rat populations with risk of human infection.

Author Summary

Leptospirosis is a human disease caused by the bacterium *Leptospira*. It is often acquired through contact with water/soil contaminated with leptospires shed in the urine of rats, the most important reservoirs in urban environment. We evaluated how location, sex, age and other rat characteristics can influence the amount of leptospires shed into the environment. We found that rats from different locations were able to shed different concentrations of leptospires. Rat populations were able to shed more than one billion leptospires per day. The findings of this study provide information linking rat population and the risk of human leptospirosis.

Introduction

Leptospirosis is a human disease caused by a spirochete of the genus *Leptospira* and is most often acquired through contact with environments contaminated with leptospires shed in the urine of infected reservoir mammalian hosts. Leptospirosis is a global public health problem affecting rural and urban populations of both developed and developing nations [1–4]. The estimated annual incidence of leptospirosis exceeds 1 million cases, with a mortality of approximately 10% [5,6]. The increasing recognition of acute renal failure and pulmonary hemorrhagic syndrome accompanying leptospirosis has prompted the WHO to call for increasing surveillance to more accurately determine the global burden of leptospirosis, to increase awareness of the disease in developing countries, and to improve the methods and standards of disease surveillance and control [5]. Pulmonary hemorrhagic syndrome is characterized by massive pulmonary bleeding and acute respiratory distress, and is now reported worldwide and associated with a case fatality of >50% [2,7,8].

In Brazil, as in other tropical developing countries, slum dwellers are at high risk for leptospirosis due to limited access to health care and poor sanitary conditions within neighborhoods [2]. Accumulations of uncollected refuse, the presence of open sewers, and the poor construction of residences create conditions conducive to supporting large populations of rats (the Norway rat, *Rattus norvegicus* and/or the black rat, *Rattus rattus*), which are the primary reservoir hosts of leptospires transmitted to humans in urban locations [9–21]. Direct contact with infected rats, or contact with water and mud contaminated with *Leptospira* spp. shed in the urine of rats, are the primary routes of transmission in these settings. Predictable seasonal spikes in leptospirosis incidence in large urban centers of Brazil are associated with heavy rainfall during the winter months [2,22,23].

Although leptospirosis is caused by many pathogenic species in the genus, the Icterohaemorrhagiae complex causes the most severe disease and is closely associated with both Norway rats and the black rat [20,21,24]. Herein, we restrict our comments to this leptospiral complex and the critical role of the Norway rat in leptospiral transmission to humans, as this species is the primary reservoir host for leptospires within Salvador, Brazil, where our work was carried out [14,15,17].

Norway rats are frequently infected with leptospires in both tropical and temperate cities. For example, five studies published during the period 2003–2014 report the prevalence of leptospiral infection among urban rats to range between 11.1% (N = 592) in Vancouver, Canada [18], 16% (N = 127) in Tokyo, Japan [20], 48% (N = 23) in Santa Fe, Argentina [25], 65.3% (N = 201) in Baltimore, USA [19], and 63% to 83% (N = 226) in Salvador, Brazil [14]. However, the methods used to determine infection varied, including combinations of PCR-based

detection of DNA from rat kidney samples, serum antibody detection, and isolation or antigen detection of leptospires in kidney, so direct comparison of these results is precluded.

Moreover, reports from this geographical range of leptospiral carriage among urban Norway rat populations do not provide information on a critical epidemiological parameters that directly link the load of leptospires shed in urine to the degree of environmental contamination that ultimately determines the risk of transmission to humans. Once infected, Norway rats establish a chronic carriage state with leptospires residing within the proximal tubules of kidneys. Norway rats are considered asymptomatic carriers, as evidence of leptospiral-induced pathology is minimal [26] and experimentally infected rats gain weight at the same rate as non-infected controls [27]. Infected rats have the ability to shed viable leptospires in their urine throughout their lives [26,28,29]. The few studies documenting the load of leptospires shed in the urine infected Norway rats are based on experimental studies; they report loads as high as 10^7 genome equivalents (GEq)/ml, as measured by quantitative PCR (qPCR) or dark field microscopy [27,30], with peak levels of shedding being reached within 28 days.

The presence of serum antibodies does not indicate clearance of leptospires from the kidney [21], but its potential influence on the load of leptospires shed in rat urine is unknown, Once leptospires are shed into the environment, they can survive from days to months [31,32]. However, given the inherent variability of the environment (e.g. soil type, water content, microbiome present, pH etc.), these estimates require validation under conditions representative at field sites.

In this report we examine some of the critical, but unknown, parameters that influence the role of individual rats (and hence rat populations) in maintaining leptospiral infection and that contribute to the urine load of bacteria shed into the environment. Specifically, we describe how the location, sex, age and presence of serum antibody in individual rats influence leptospiral carriage and urine shedding loads and how these parameter estimates, when applied to the demographic characteristics of a previously described [33] rat population in Salvador, determine the degree of environmental contamination potentially influencing the risk of human infection.

Methods

Study sites and data collection

The study sites, data collection methods and types of samples obtained for this study have been described previously [14,33]. Briefly, during June-August of 2010, we captured Norway rats from five urban slum locations in Salvador, Brazil. Sites were selected based on the high annual incidence of severe human leptospirosis reported from these communities in 2010 [14,16]. Study sites were systematically sampled by setting three to five Tomahawk live traps at each of eight contiguous households [33]. Rats were euthanized and sex, weight and the presence of scars (based on a five point wound score) were recorded [14]. Mass has been shown to be excellent proxy for estimating rat age [33–35]. However, we also used the length to mass ratio (L/M) as an additional proxy [36].

Blood was obtained by cardiac puncture using a 5mL syringe, and serum was recovered after centrifugation. Urine was obtained directly from the bladder with a 1mL syringe after which the kidneys were removed. All samples, except kidney smears on slides, were immediately stored at -80°C until tested by qPCR to determine kidney and urine loads of leptospires and by microagglutination tests (MAT) to determine antibody titers. Sera testing positive by MAT at screening were diluted to obtain endpoint titers.

Spiking experiments

We performed two spiking experiments, the first with water and the second using rat urine, to assess the quality of our extraction procedures and their accuracy in quantifying leptospiral

loads. The appropriate amount of leptospires (*Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 [37], hereafter abbreviated as strain L1-130) was spiked into 200μL of water to achieve a final concentration of 1×10^8 leptospires/mL. The same process was performed using rat urine for the second experiment. Rat urine was obtained from an uninfected wild rat (no indication of kidney colonization by culture, and negative IFA and qPCR results for the presence of leptospiral antigen or DNA in kidney samples). After the spiking, serial 10-fold dilutions of 1×10^7 to 1×10^0 leptospires/mL were performed, using water and urine, for the first and second experiments respectively, as diluent. Spiking indicated a near perfect correlation between the number of spiked leptospires and the GEq detected by qPCR assay in urine ($R^2 = 0.9998$) as well as water ($R^2 = 0.9997$) (S1 Fig).

Quantitative real-time PCR of *Leptospira* load in kidney and urine

DNA was prepared from 25mg and 200μL of previously frozen kidney and urine, respectively, with the automated Maxwell 16 System DNA Purification Kits (Promega Corp., Madison, WI). Quantitative real-time PCR (qPCR) for leptospirosis was performed using 5' nuclease (TaqMan) assay and primers that amplified a sequence of *lipL32*, a gene that is exclusively present in pathogenic *Leptospira* [38]. For the calibration curve, genomic DNA obtained from strain L1-130 [37] was quantified using an ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Genomic equivalents were calculated based on a genome size of 4,627Mb [37]. Eight calibrators (10^0 to 10^7 GEq/mL) were prepared upon adjustment of DNA concentration to 10^7 GEq/reaction followed by ten-fold serial dilutions. Quantitative real-time PCR amplifications were performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA). PCR conditions were adapted from a previously described method [38]. The reaction mix consisted of 12.5μL of Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA), 500nM of forward and reverse primers, 100nM of probe, 5μL of DNA extract and ultrapure water to a final volume of 25μL. The amplification protocol consisted of 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min. As an internal control to monitor inhibition of PCR amplification and the efficiency of DNA extraction, we constructed primers to test the presence of a rodent housekeeping gene glyceraldehyde-3-phophate dehydrogenase (*gapdh*). The primer pair and probe were designed using Primer Express version 1.3 (Applied Biosystems). The forward primer of GAPDH_F (5'- GGT GGA GCC AAG AGG GTC AT-3') and GAPDH_R (5'-GGT TCA CAC CCA TCA CAA ACA T-3') were selected to amplify a fragment that was detected by the probe, GAPDH_P (FAM-5'- ATC TCC GCA CCT TCT GCT GAT GCC-3'-BHQ1). Samples were tested in duplicate, and no-template controls (5μL of ultrapure water added instead of DNA) were included in each run. Samples in which replicates were detected within 40 PCR cycles were considered positives. Positive samples were Sanger sequenced to confirm the amplification of the *lipL32* gene from *Leptospira* species.

Microscopic agglutination test (MAT)

Using the Microscopic Agglutination Test (MAT) [39] we tested rat sera against L1-130 strain to determine antibody titers. Positive rat sera at the screening dilutions of 1:50 and 1:100 were titrated using two-fold dilutions to establish endpoint agglutination titers, defined as the highest dilution where 50% or more of the cells where agglutinated [39].

Statistical analyses

We evaluated the associations of *Leptospira* load in both urine and kidney with sex, sexual maturity, mass, L/M ratios, MAT titer and site of capture. Leptospiral loads, as measured by GEq

of leptospiral DNA per mg of kidney and mL of urine, were log transformed for all analyses. We used ANOVA, applying the Bonferroni correction method [40], to evaluate the effects for each variable on the leptospiral loads in kidney and urine. The Shapiro–Wilk test [41] confirmed that log GEq per ml values in both kidney and urine were log-normally distributed. Variables associated with *Leptospira* load in kidney with a p<0.1 where included in multivariate analysis (linear regression), and a backward elimination strategy and the Akaike information criteria (AIC) [42] were used to select the best adjusted model: from amongst models with and AIC of 2 of the lowest value, the simplest model was chosen on grounds of parsimony. The same strategy was used to develop a separate model to predict *Leptospira* load in urine.

We estimated the contribution of a demographically stratified rat population to the degree of environmental contamination through contaminated urine. We used demographic characteristics of the rat population considered for this study, which comprised 82 rats (16 rats <200g, 46 rats weighting between 201–400 and 20 rats >400g). We considered the number of rats (NR) at each weight class, their respective *Leptospira* prevalence as evaluated by qPCR (PREV), volume (in ml) of urine shed per 24h (VOL), and log of GEq/ml of urine (LOAD). So the amount of leptospire shed per day at each weight group was calculated as (NR*PREV*VOL*LOAD). Volume of urine shed per day based on rat mass was obtained from previous studies performed in laboratory conditions [43]. We also combined the results from the three weight groups to obtain the total number (GEq) of leptospires excreted by this population per day. Finally, we divided this estimate by the number of rats captured per m^2 at each site to estimate the number of leptospires shed per day at a given density of rats (NR*PREV*VOL*LOAD) / m^2. We repeated this procedure for each one of the five collection sites.

Ethics statement

Institutional Animal Care and Use Committee (IACUC) from Brazil and United States approved all the protocols used in this study. At Oswaldo Cruz Foundation, Salvador, Brazil; the *Comissão de Ética no Uso de Animais (CEUA) do CPqGM-FIOCRUZ-BA* approved the protocol number 003/2012. At the United States the Yale University's Institutional Animal Care and Use Committee (IACUC), New Haven, Connecticut, approved the protocol number 2012–11498. Protocols adhered to PHS policy, USDA Regulations, the US National Research Council Guide for the Care and Use of Laboratory Animals and all US federal regulations.

Results

A total of 82 *R. norvegicus* were captured from June to September 2010. Forty-five rats were captured from four sites from the Pau da Lima neighborhood (PL1, PL2, PL3 and PL8) and 37 from Sete de Abril neighborhood (7A). The demographic structure and *Leptospira* carriage prevalence of this population were described previously [14,33,44]. Demographic characteristics between sites were not different as described in S1 Table. Kidney and urine samples were obtained from 72 and 55 animals, respectively, of which 88% and 84%, respectively, were qPCR positive (Table 1). Five animals were negative for both kidney and urine and were excluded from further analyses of GEq titers, but were retained in analyses estimating the association of leptospiral loads in kidney and urine.

The average GEq of leptospires in positive kidneys was 5.9 x 10^6, slightly lower than the 6.1 x 10^6 average obtained from positive urines, not quite attaining statistical significance (p = 0.057). The range of GEq for positive kidneys and urines was 3.1–8.2 x10^6 and 2.2–9.4 x10^6, respectively (Table 1), and there was a strong and significant positive correlation (R^2 = 0.78) between the GEq load of leptospires in the paired kidney and urine samples (Fig 1).

Table 1. Bivariate analyses of *Leptospira* load in kidney and urine of wild Norway rats.

Characteristics	Kidney No.	PCR positive samples No. (%)	Mean Log₁₀ PCR positive samples	SD[1]	Urine No.	PCR positive samples No. (%)	Mean Log₁₀ PCR positive samples	SD
Total	72	64 (88)	5.9	1.2	55	46 (84)	6.1	1.5
Sex								
Male	35	31 (88)	6.0	1.2	30	25 (83)	6.1	1.4
Female	37	33 (89)	5.9	1.2	25	21 (84)	5.9	1.7
Weight category								
Juvenile	15	11 (77)	5.6	1.1	7	5 (71)	4.9	1.9
Sub-adult	38	35 (92)	5.9	1.2	30	25 (83)	6.2	1.6
Adult	19	18 (95)	6.0	1.2	18	16 (89)	6.2	1.3
Ratio length/weight category								
I (>1.25)	13	9 (69)	5.6	1.2	6	4 (67)	**4.4**[2]	1.8
II (0.85–1.25)	22	22 (100)	5.9	1.2	16	13 (81)	5.9	1.9
III (˂0.85)	37	33 (89)	6.1	1.2	33	29 (87)	**6.3**	1.2
Wounds/Scars								
0	29	25 (86)	5.6	1.2	20	14 (70)	**5.4**	1.7
1	26	24 (92)	6.1	1.0	18	17 (94)	5.9	1.5
≥2	17	15 (88)	6.4	1.2	17	15 (88)	**6.8**	1.3
Pregnant								
No	19	15 (79)	5.8	1.1	8	5 (62)	6.4	0.7
Yes	18	18 (100)	5.9	1.4	17	16 (94)	5.8	1.9
Vagina								
Closed	5	4 (80)	5.2	NA	2	1 (50)	5.2	NA
Open	32	29 (90)	5.2	2.6	23	20 (87)	6.0	1.7
Lactation								
No	34	30 (88)	5.8	1.2	24	20 (83)	6.1	1.7
Yes	3	3 (100)	6.4	1.2	1	1 (100)	4.2	NA
MAT Titer								
0	40	34 (85)	5.6	1.2	29	21 (72)	5.7	1.5
50	12	12 (100)	6.1	0.8	11	11 (100)	6.1	1.7
100	9	9 (100)	6.9	0.8	10	10 (100)	6.8	1.6
Site								
PL8	6	5 (83)	5.3	1.6	6	4 (67)	**4.5**	2.0
7A	32	26 (81)	5.9	1.2	18	14 (78)	5.6	1.3
PL2	14	14 (100)	5.4	0.9	11	11 (100)	6.1	1.4
PL6	5	5 (100)	6.4	1.3	5	5 (100)	6.0	2.1
PL1	15	14 (93)	6.6	0.9	13	12 (92)	**7.0**	1.0

[1]Standard Deviation

[2]Bold items reflect significant differences (P<0.05 ANOVA adjusted by Bonferroni correction method).

Values given are log10 genome equivalents per mm³ of kidney or per ml of urine.

In bivariate analyses, leptospiral loads in kidney and urine tended to increase with W/L ratio (significant only in urine), increasing number of wounds/scars (significant only in urine), and varied with location of capture (PL1 vs. PL 8, significant only in urine) (Table 1). Male and female rats did not differ in the percentage of qPCR positive kidneys or urines, and their kidney
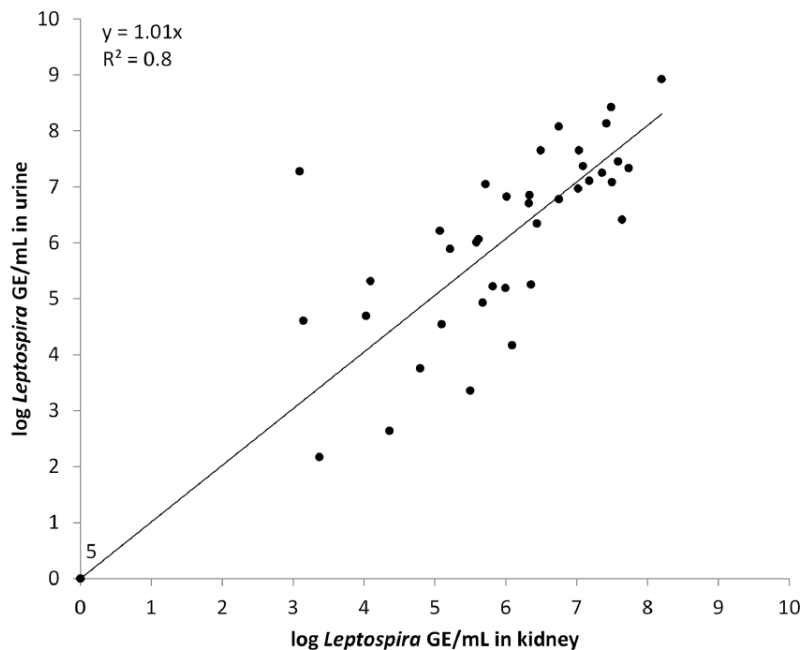
Fig 1. Correlation between the GEq load of leptospires in the paired kidney and urine samples of *Rattus norvegicus* from Brazil, 2010.

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and urine GEqs were indistinguishable. The reproductive status of females did not influence leptospiral loads (Table 1).

Separate multivariate linear regression models of GEq in kidney and urine included all the covariates for which at least one association had $P<0.1$ in bivariate comparisons (Table 1). In addition, any covariate that was retained in either the kidney or urine model was also included in the other model. Overall, the linear regression models from kidney and urine included the same set of variables with highly concordant results. In the kidney model, location of capture, MAT titer and the increasing number of wound/scars per animal were independent risk factors influencing GEq when all rats were pooled, though slight but significant differences in response were found between the sexes (although variables trended the same way; Table 2). In males, increasing W/L ratio was associated with increasing GEq, but not in females. Overall, increasing MAT titer was associated with greater GEq, but only in females. Similarly, increasing number of wounds/scars was associated with higher GEq, but only in females.

In the urine model W/L ratio, location of capture, and increasing number of wound/scars per animal were independent risk factors influencing GEq when all rats were pooled, but differences in response was found between the sexes (Table 2). Of note, including MAT titers increased the fit of the urine model, although not significant in bivariate comparisons. Males with higher W/L ratio had greater GEq in urine, but this association was not significant in females. Overall, three sites of rodent capture (PL2, 7A and PL1) produced animals with

Table 2. Multivariate linear regression models of the relation between Leptospira load in kidneys and urine.

Population AIC	Kidney Total 172.6 Coefficient	Female 90.6 (p value)	Male 79.8[1]	Urine Total 147.3[2] Coefficient	Females 91.6[3] (p value)	Males 72.7[4]
Ratio Length/Weight (continuous)			-2.02 (0.06)	**-1.71 (0.04)**		**-3.43 (0.01)**
Site PL8	**1.0 (ref)[5]**		**1.0 (ref)**	**1.0 (ref)**	**1.0 (ref)**	1.0 (ref)
Site PL2	1.03 (0.12)		1.27 (0.22)	**1.80 (0.04)**	2.74 (0.07)	0.56 (0.60)
Site 7A	0.50 (0.36)		0.39 (0.64)	**1.90 (0.01)**	**3.05 (0.01)**	0.48 (0.59)
Site PL6	0.58 (0.26)		1.25 (0.06)	1.21 (0.10)	**3.12 (0.01)**	0.44 (0.64)
Site PL1	**1.12 (0.05)**		1.81 (0.05)	**2.80 (0.04)**	**3.86 (0.00)**	1.80 (0.07)
MAT 0	**1.0 (ref)**	**1.0 (ref)**	**1.0 (ref)**	**1.0 (ref)**	**1.0 (ref)**	**1.0 (ref)**
MAT 50	0.21 (0.58)	**1.01 (0.04)**	-0.58 (0.32)	0.07 (0.87)	1.55 (0.11)	-0.31 (0.64)
MAT ≥100	**0.86 (0.02)**	**1.61 (0.00)**	0.11 (0.88)	0.59 (0.26)	0.33 (0.65)	1.05 (0.19)
Scars 0	**1.0 (ref)**	**1.0 (ref)**		**1.0 (ref)**		
Scars 1	0.61 (0.06)	**0.91 (0.02)**		0.71 (0.15)		
Scars ≥2	**1.05 (0.01)**	**1.51 (0.01)**		**1.42 (0.01)**		

[1] AIC excluding MAT = 93.5
[2] AIC excluding MAT = 150.7
[3] AIC excluding MAT = 92.4
[4] AIC excluding MAT = 86.4
[5] Bold items reflect significant differences (P<0.05).

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significantly higher urine leptospiral loads than the PL8 site (the reference and lowest value). Increasing number of wounds/scars was associated with higher GEq, but only when males and females were grouped.

Our estimate of the contribution of leptospires shed in the urine of a rat population of 82 individuals, stratified into three age classes, was 9.1×10^{10} per day with a mean density of 5.0×10^{10} per m² of soil around households (Table 3). The independent estimates for rats captured at different sites showing the contribution of demography and leptospire load are shown in S2 Table.

Table 3. Estimates of Leptospira shedding loads in urine from 82 Norway rats based on properties of the demographic structure of the rat population from Salvador, Brazil.

	No. of rats[1]	Prevalence in urine[2]	ml/ day[3]	Log_{10} GEq/ ml[4]	Log_{10} Leptospires shed per day[5]	Log_{10} Leptospires per m² of soil /day
Total	82				9.1	5.0
Juvenile	16	0.77	15	5.6		
Sub-adult	46	0.92	20	5.9		
Adult	20	0.95	20	6		

[1] Number of rats in each mass/age class (NR.)
[2] *Leptospira* prevalence in kidney (PREV: Table 1).
[3] Volume (ml) of urine shed per 24 hours (VOL) as described by Donaldson [43].
[4] Genomic equivalents of *Leptospira* per ml (LOAD: Table 1).
[5] Based on density of rats (DENS) captured around households.

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Discussion

We describe for the first time results that simultaneously investigated how differences between location of capture, sex, age (mass and W/L ratio indices), MAT markers of immune response and the prevalence of wounds/scars influence leptospiral loads of kidney carriage and associated loads shed in urine. Additionally, by extrapolating the values obtained in our analyses, we estimate the daily burden of leptospires shed into the environment by a rat population from an urban slum setting in Brazil [14].

The near perfect correlation between leptospiral loads in urine and kidney indicate that data obtained by measuring GEq in kidney samples alone can serve as a proxy for estimating leptospiral loads shed in urine, and that shedding rates in urine are consistent over time. As obtaining urine is somewhat cumbersome, and some bladders are empty, our findings suggest that qPCR of kidney samples may be sufficient for inferring environmental loads, although these results should be confirmed for other reservoir hosts such as *R. rattus* [45]. The shedding of other species of bacteria, such as *Escherichia coli* [46] and *Coxiella burnetii* [43], has been shown to be highly variable over time in infected hosts in contrast to our results.

The information on urine shedding loads is critical for the construction of mathematical models predicting environmental contamination and consequently human disease risk [47,48]. The average loads in these paired samples ranged between 5.9 and 6.1 x 10^6 GEq, but linear regression indicated that GEq in urine tended to be greater than that in kidney by a factor slightly less than 10. Our results are concordant with those obtained by experimentally infected rats where urine loads as high as 10^7 genome equivalents (GEq) were found, as measured by qPCR and/or dark field microscopy [27,30]. The only other study reporting GEq in wild rats (*R. rattus*) found average loads in the kidney to be higher than what is reported here: 8.27x10^6 (standard deviation of 4.72x10^6). Corresponding urine loads were not evaluated in that study.

Multivariate analysis retained site of capture, L/W ratio (as a continuous variable), MAT titer and severity of wounds/scars as independent variables associated with leptospiral loads in both kidney and urine (Table 2). However, the significance of these variables differed between the sexes as discussed in greater detail below.

Our extrapolation indicates that the heterogeneity in leptospire shedding may be one of the major factors affecting environmental contamination by leptospires. Heterogeneity in the prevalence of leptospiral infection in rats has rarely been reported, almost certainly a reflection of small sample sizes, but was documented in Vancouver, Canada [18]. Geographical differences in leptospiral loads have not been previously reported by any study. The causes of these variations are unknown but could result from differences in the pathogen, environmental load, host genetics [44], or demographic characteristics. Most importantly, the consequences are also not known, but differences in leptospiral shedding and consequently in environmental contamination could be related to the spatial variation in human leptospirosis risk as evidenced in previous studies [49,50].

Leptospiral load in kidneys and urine was identical among male and female rats. When data were pooled, older animals, as determined by L/W ratio, tended to have higher kidney and urine loads. This association was stronger in males than in females indicating that heavier (and older) males had augmented leptospiral shedding (see also [45]). Reproductive status of females based on sexual maturity had no effect on bacterial loads, though limited sample size may be precluded this association.

When male and female data were pooled, increasing MAT titer was positively associated with kidney load. High MAT titers could reflect a high dose inoculum during infection and/or a short period after infection [51] either of which could lead to higher leptospiral kidney

colonization and shedding. Of interest, while females had a similar positive association between MAT titer and kidney load to pooled rats, in males this association was negative for MAT = 50, albeit not significant. A similar pattern for urine load was observed for pooled, male and female rats, but again associations were not significant in males. Further studies are needed to elucidate the reasons why higher agglutination antibodies are related to higher leptospiral colonization in Norway rats.

The association between levels of wounding/scars with pathogen infection is well documented for Seoul virus infection among rats from Baltimore, and the presence of virus in rat saliva supports the potential for transmission via this route [52], but the observation here that increasing levels of wounds/scars were associated with increasing loads of leptospires (urine and kidney) was unexpected. Leptospires have been shown to be transmitted to guinea pigs via rat bite [53]. The load of leptospires in the saliva of wild rats has never been investigated, but as rats are constantly grooming (males spend up to 40% of the time in this activity [54]), it is highly plausible that saliva becomes contaminated with leptospires during oral grooming of the urogenital region-especially given the high bacterial loads in urine. Of note, there are documented instances of leptospiral transmission by rat bite to humans [53,55–57], suggesting that transmission through contaminated saliva occurs. This potential route for horizontal transmission among rats requires further investigation.

Based on our results, it is apparent that the routes of leptospiral transmission among Norway rats, or the inoculum doses required to achieve infection are still unknown, but whatever the route, high prevalence of infection were found among all age classes indicating efficient transmission. In experimental studies, the 50% colonization dose in Wistar rats was determined to be 10^4 leptospires introduced intraperitoneally. However, in Golden Syrian hamsters, the LD_{50} is lower (<50 leptospires)[58]. Additionally, the development of leptospiral loads in the kidneys of another highly susceptible animal varied with the route of exposure [59].

This study was limited to two months in the dry season in a single year, precluding estimates of inter-year and inter-seasonal variations in leptospiral shedding. The demographic characteristics of rat populations are also likely to vary across seasons and years, such that of our example of a model Norway rat population and their contribution to leptospiral loads in the environment should be considered indicative rather than necessarily typical. Insufficient data are available on where rats are most abundant and whether the proximity of rats with higher loads of leptospiral shedding are most likely to lead to human exposure. However, in this study rats were captured close to human residences and our findings are relevant because there is a clear pattern of household clustering of persons infected by leptospires [60], implicating peridomestic acquisition of infection. Sample sizes were low when stratified among the various covariates (eg sex, reproductive status, L/W ratio, site of capture), and therefore some associations may vary when reexamined with additional data. Of major importance, we were not able to distinguish between the differential contribution of live versus dead leptospires as identified by qPCR, possibly causing overestimation of the actual infectious leptospiral load present in kidneys and shed in the urine. We plan to conduct follow-up experiments to elucidate the fraction of live infectious leptospires using dark field microscopy or qPCR techniques that distinguish between living and dead leptospires.

Nonetheless, this study is unique as it is the first to address some of the critical, but unknown, parameters which can influence leptospiral infection, maintenance and shedding leading to environmental contamination. These factors directly influence the risk of leptospiral acquisition among humans and provide essential information on the epidemiological linkage between rats and humans.

Supporting Information

S1 Table. Demographic characteristics of the rat population stratified per geographic site, 2010.
(DOCX)

S2 Table. Estimates of *Leptospira* shedding considering rat demographic structure for the total and individual populations in Salvador, 2010.
(DOCX)

S1 Fig. Spiking experiment showing correlation between the number of spiked leptospires and the GEq detected by qPCR assay in urine and water.
(TIF)

Acknowledgments

Author Contributions

Conceived and designed the experiments: FC MGR AIK MB JEC. Performed the experiments: FC EAW DDO VB GR. Analyzed the data: FC EAW MB JEC. Contributed reagents/materials/analysis tools: FC GR MGR AIK MB. Wrote the paper: FC EAW AIK MB JEC.

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