Real-Time PCR Reveals Rapid Dissemination of Leptospira interrogans after Intraperitoneal and Conjunctival Inoculation of Hamsters

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ABSTRACT

The pathogen *Leptospira interrogans* is a highly motile spirochete that causes acute and fulminant infections in humans and other accidental hosts. Hematogenous dissemination is important for infection by this pathogen, but remains poorly understood because few animal model studies have used sensitive tools to quantify bacteria. We evaluated the kinetics of leptospiral infection in Golden Syrian hamsters by a sensitive quantitative real time PCR (TaqMan) with *lipl32* as the target gene. The dissemination and bacterial burden was measured after intraperitoneal infection with a high dose (10^8 leptospires) or low dose (2.5×10^2 leptospires). We also examined the conjunctival challenge route to mimic the natural history of infection. Quantification of leptospires in perfused animals revealed that pathogens were detected in all organs of intraperitoneally infected hamsters including the eye and brain within 1 hour after inoculation of 10^8 virulent *L. interrogans*. Peaks of 10^5-10^8 leptospires/g or /mL were achieved in blood and all tissues between day 4 and day 8 after intraperitoneal inoculation of a high and low dose challenge, respectively, coinciding with macroscopic and histological changes. The conjunctival route resulted in a delay in the time to peak organ burden in comparison to intraperitoneal infection, indicating that although infection can be established, penetration efficiency was low across this epithelial barrier. Surprisingly, infection with a high inoculum of a high passage attenuated *L. interrogans* strains resulted in dissemination in all organs in the first 4-days post-infection, albeit at a lower burden, followed by clearance from the blood and organs 7-days post-infection and survival of all animals. These results demonstrate that leptospiral dissemination and tissue invasion occur. In contrast, development of a critical level of tissue burden and pathology are dependent on the virulence of the infecting strain.

Keywords: *Leptospira*, Real Time PCR, dissemination kinetics, hamster, pathogenesis

INTRODUCTION

Leptospirosis is a neglected life-threatening disease occurring in a diverse range of epidemiological settings with a higher incidence in low-income, tropical countries (1-3). The causative agents are a unique group of spirochetes divided into ten pathogenic *Leptospira* species and >200 serovars (1, 2, 4, 5). Leptospires are able to establish acute disease in susceptible hosts, chronic carriage in the proximal kidney tubules of reservoir hosts, and persist for weeks to months in the environment after excretion (1, 2, 6, 7). Leptospirosis is among the most important bacterial zoonoses worldwide. It causes substantial morbidity and mortality in diverse human populations exposed to the wide range of wild and domestic reservoir hosts living in close proximity to anthropogenically modified environments (3, 8).

Human infection most often results from contact with an environment contaminated with the urine of animals with chronic or acute infection. A broad spectrum of clinical manifestations may result, ranging from a self-limited febrile illness to potentially fatal infection characterized by liver dysfunction, bleeding, kidney failure, shock and pulmonary hemorrhage (8, 9). In endemic areas, acute leptospirosis can account for more than 10% of hospitalizations for acute febrile illness (2), with leptospirosis outbreaks predictably occurring after periods of heavy rainfall and flooding (10). The major public health burden of leptospirosis involves its ability to cause severe clinical outcomes, with mortality rates varying from 10-70% among recognized cases (2, 3, 8).

Leptospires are highly motile spirochetes that penetrate abraded skin and mucous membranes and cross tissue barriers to disseminate hematogenously resulting in a systemic infection (2, 5, 8, 11, 12). After dissemination, leptospiremia persists until the onset of the host immune response from the host, which occurs within two weeks after exposure (13). Leptospires

can be detected in the bloodstream within minutes after intraperitoneal inoculation (14) and are present in visceral organs two days later (13, 15-19) reaching a range of 10^6-10^7 organisms per milliliter (mL) of blood or per gram (g) of tissue of patients and animals (16, 19, 20). In 1957 and 1964, Faine (17) and Green et al. (18), respectively, studied the kinetics of the leptospiral infection using dark field microscopy and culture. The advent of molecular biology techniques, such as real-time PCR, enables a new level of sensitivity and precision for these studies.

The development of leptospirosis and disease progression are influenced by the susceptibility of the host, the virulence of the infecting strain, and the initial inoculum dose of infection (8). In animal model studies, higher inoculum doses have resulted in shorter incubation periods and decreased survival in a dose-dependent manner (13, 21). Nonetheless, many questions remain regarding the correlation of the bacterial burden in tissues with the natural history of the disease (16, 20, 22).

Animal model studies are essential to understanding the biology, transmission, colonization, and pathogenesis of *Leptospira* spp. The Golden Syrian Hamster is commonly employed as a model for acute leptospirosis due to its high susceptibility to leptospiral infection and because the clinical features mimic those of severe human infection (23). Although experimental inoculation is typically performed by intraperitoneal inoculation (24, 25), this route does not reflect natural transmission of the pathogen. Relatively few studies have examined challenge routes that mimic natural entry of leptospires via skin or mucous membranes (15, 19, 26, 27). Although quantitative PCR assays have been extensively evaluated as a diagnostic tool, few studies have applied this technique to pathogenesis studies in animal models of leptospirosis. In this study, we compared the kinetics of leptospiral infection in the hamster after high and low

inoculum dose of distinct *L. interrogans* strains by intraperitoneal and ocular infection using a sensitive TaqMan quantitative real time PCR assay.

MATERIAL AND METHODS

Bacterial strains and clones. Leptospires were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (28, 29) supplemented with 1% rabbit serum. Cultures were kept up to 7 days at 30°C, reaching a log phase between 4-5 days of culture. Bacteria were counted in a Petroff-Hausser counting chamber (Fisher Scientific). *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, a virulent clinical isolate from Brazil (10, 30) with 4 and 8 passages in vivo and in vitro, respectively, was used for the spiking and kinetics experiments. Pathogenesis studies involved an attenuated Fiocruz L1-130 strain obtained after 42 *in vitro* passages (high passage), and *Leptospira interrogans* serovar Canicola strain Kito attenuated by disruption of the *clpB* gene as previously described (31).

Standard curve and spiking experiments. DNA from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 was extracted using the QIAamp DNA minikit (QIAGEN, Valencia, CA). DNA was quantified using the NanoDrop instrument (Thermo Fisher Scientific Inc., Waltham, MA). A genome size of 4.6 Mb was used to determine the genomic equivalent (GEq) concentration per microliter of the purified DNA (30). To generate a standard curve, serial dilutions of the DNA were made starting at $1x10^7$ GEq to $1x10^0$ GEq/5 µL. Standard curve assays were performed in duplicate. The analytic sensitivity of the assay was calculated and the

LLOD (Lower Limit of Detection) was determined to be the concentration at which 95% or more of the replicated reactions yielded a positive reaction.

Leptospires were added to 1 mL of water or EDTA-anticoagulated whole blood from uninfected hamsters to achieve a final concentration of $1x10^7$ leptospires/mL. After spiking, serial 10-fold dilutions of $1x10^6$ to $1x10^0$ leptospires/mL were then performed using blood or water as diluents, accordingly. For tissues, kidney and liver were acquired from uninfected hamsters. 50 µL of serial 10-fold dilutions of leptospires were spiked into 25 mg of kidney and liver, before DNA extraction, to achieve concentrations of $1x10^7$ to $1x10^0$ leptospires per gram of tissue. After the spiking, tissues were processed for DNA extraction as described below. Unspiked water and tissues were used as negative controls.

Hamster infection and necropsy. All experiments were performed using 5-8 week-old male Syrian Golden hamsters. For the kinetics experiments with the Copenhageni strain, two groups of fifteen animals each were inoculated intraperitoneally (IP) with either a high-dose (10^8 leptospires) or a low-dose (2.5×10^2 leptospires) in 1 mL of EMJH medium. For animals infected with a high-dose inoculum, three animals were euthanized at 1 hour, 1 day, 3 days and 4 days after infection. For the animals infected with a low-dose inoculum, three animals were euthanized at 3 days, 5 days, 8 days and 11 days after infection. For each dose, one group of 3 animals remained as a positive control. As negative controls, two groups of 3 animals were injected intraperitoneally with 1 mL of EMJH medium and euthanized at either 4 or 11 days after injection for the high- and low-dose inoculum experiments, respectively.

For the pathogenesis experiments, one group of six animals for each strain was inoculated intraperitoneally with 10^8 leptospires in 1 mL of EMJH medium. After 1 hour and 4-days post-

infection, sub-groups of two animals were euthanized. The conjunctival challenge route experiments were performed by centrifugation of 30 mL culture of leptospires for 10 minutes at 3,000g to obtain an inoculum of 10_8 leptospires in 10 µL of EMJH medium, which was applied to the conjunctival membrane of the left eye. Animals in groups of four were infected with two animals euthanized after 7 days of infection for each strain tested. For each experiment, the two remaining animals were included as positive controls for infection and not euthanized until an endpoint was achieved.

All animals were monitored twice daily for endpoints including signs of disease and death, up to 30-days post-infection. Surviving animals after 30-days post-infection or moribund animals presenting with difficulty moving, breathing or signs of bleeding or seizure were immediately sacrificed by inhalation of CO_2. Blood was collected by intracardiac puncture. Each hamster was perfused with 100 mL of saline solution to eliminate blood in the organs that might contribute to the quantitative PCR signal. After perfusion, the heart, right pulmonary lobe, right dorsocaudal hepatic lobe, spleen, right kidney and muscle of the right thigh were carefully removed. The brain was exposed by craniotomy and the right eye was enucleated. All tissues were collected into cryotubes and immediately placed into liquid nitrogen before being stored at -80ºC until extraction. Sera were obtained by centrifugation of clotted blood at 1,000g for 15min at room temperature and frozen at -20ºC until analysis for the presence of antibodies against leptospires using the microscopic agglutination test (MAT), performed as previously described (10, 33). For the pathogenesis and conjunctival route experiments only blood, kidney, liver, lung, spleen and eye were analyzed. All animal protocols were approved by the Committee for the Use of Experimental Animals of the Gonçalo Moniz Research Center, Fiocruz, and Yale University.

DNA extraction. Using scissors and scalpels, 200 µL of water or blood, 25 mg of heart, lung, liver, kidney cortex, muscle, brain and eye and 10 mg of the spleen were aseptically collected. DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions but using only 100 µL of elution volume. Previous results showed that there was inhibition of the amplification of the DNA from the blood of the hamster obtained with the Qiagen kit (data not shown). For that reason, blood DNA was extracted from 200 µL using the Maxwell 16 Tissue DNA purification Kit (Promega Corporation, Madison, WI), following the manufacturer's instructions and using 200 µL of elution volume.

Quantitative Real Time PCR analysis. Concentration of leptospires was quantified by a TaqMan-based quantitative PCR assay using an ABI 7500 system (Thermo Fisher Scientific, Inc.) and Platinum Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Inc.) (34). The *lipL32* gene was amplified using the set of primers previously described (34, 35), LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3') and LipL32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3') that amplify a fragment of 242bp, which was detected by the probe, LipL32-189P (FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1). The bacterial quantification was calculated based on the standard curve described above and expressed as the number of leptospires per milliliter or gram of tissue. For that calculation it was taken in consideration the amount of sample (200 µL of water or blood, 10 mg of the spleen, and 25 mg of other tissues), the elution volume (100 µL) and the volume of template DNA used in the PCR assay (5 µL). To calculate the theoretical limit of the assay we used the following formula: LLOD of the assay X Elution factor X Volume factor; where elution factor is equal to 20 (100 µL / 5 µL) and the

183 volume factor for water/blood and liver/kidney is 5 (1000 mL / 200 µL) and 40 (1000g / 25 mg),
184 respectively.

185 As a control for PCR inhibitors and to monitor nucleic acid extraction efficiency, spiked
186 and experimental specimens were tested for the presence of a hamster housekeeping gene
187 glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer pair and probe were designed
188 using Primer Express version 1.3 (Thermo Fisher Scientific, Inc.). The forward primer of
189 GAPDH_F (5'- GGT GGA GCC AAG AGG GTC AT-3') and GAPDH_R (5'-GGT TCA CAC
190 CCA TCA CAA ACA T-3') were selected to amplify a fragment that was detected by the probe,
191 GAPDH_P (FAM-5'- ATC TCC GCA CCT TCT GCT GAT GCC-3'-BHQ1). A sample with a
192 threshold cycle (Ct) value between 19 ± 2 was considered as positive and further analyzed by
193 real-time PCR targeting *lipL32*. In case of a negative sample, a new DNA extraction was
194 performed.

195 Final PCR reaction for both set of primers and probe was 10 µM of each primer, 5 µM of
196 the specific probe and 5 µL of DNA in a total volume of 25 µL. There was no addition of $MgCl_2$
197 or passive reference dye. The amplification protocol consisted of 2min at 50ºC and 10min at
198 95ºC, followed by 45 cycles of amplification (95ºC for 15s and 60ºC for 1min). A negative result
199 was assigned where no amplification occurred or if the Ct was greater than 40 (35). A no
200 template control (NTC) that contained all the reagents described above but no DNA was also
201 included to detect the presence of contaminating DNA. For each organ, the DNA was extracted
202 from one sample and the Real Time PCR was performed in duplicate. Considering the amount of
203 tissue that was used for DNA extraction, an equation was applied to express the results as the
204 number of leptospires per gram of tissue or per milliliter of blood or water.
205

Histopathology and immunohistochemistry studies. Kidney, liver and lung tissue sections were embedded in paraffin. 3 to 5μm sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy (Zeiss Axioskop), using previous standardized protocols for each organ. A semi-quantitative analysis was made, ranking the presence of each parameter as negative (0), discrete (1), mild (2) or intense (3). The pathologist viewed the histopathological preparations without knowing the infection status of the animals. The following parameters were analyzed: hemorrhage, calcification, edema, atrophy, dilatation, necrosis, inflammation, hyaline degeneration, hydropic degeneration and presence of cylinders for kidney; hydropic degeneration, septation, apoptosis, fibrosis, hyaline degeneration, infiltrating mononuclear or polymorphonuclear cells, necrosis, steatosis and loss of parenchymal architecture for liver and; alveolar and bronchial hemorrhage, bronchial hemorrhage, necrosis, edema, fibrosis, pneumonia, alveolar congestion and septal thickening for lung.

Immunohistochemistry was performed to confirm the presence of *Leptospira* in tissues section. Paraffin was removed from tissue sections with xylene and ethanol. Slides were blocked by incubation with PBS/10% milk at room temperature for 20 min, followed by addition of a 1: 1,000 dilution of rabbit polyclonal antiserum to LipL32 or *L. interrogans* serovar Icterohaemorrhagiae strain RGA or normal rabbit serum (as a negative control) for 1 hour at room temperature. Slides were treated with 0.3% hydrogen peroxide for 15 min at room temperature and then incubated at room temperature for 30 min with biotin-conjugated anti-rabbit secondary antibodies, followed by streptavidin peroxidase (Histostain-Bulk Kit, Invitrogen). Enzyme reactions were developed using 3.3'- Diaminobenzidine (Sigma-Aldrich, St. Louis, MO). Slides were examined by optical microscopy (Zeiss Axioskop).

Statistical analysis. GraphPad Prism 5.0c (GraphPad Software, San Diego, CA) was used to perform all the statistical analysis. Fisher's exact test and analysis of variance (ANOVA) were applied to assess statistical difference between pairs of groups and multiple groups, respectively. A *P* value of <0.05 was considered significant.

RESULTS

Standard curve and spiking experiments. Three independent quantitative real-time PCR (qPCR) assays were performed on duplicate serial dilutions of genomic DNA isolated from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. In our experiments, the LLOD for which 95% of the tested samples were positive was 10 GEq with a linear correlation coefficient (R^2) of 0.998 (data not shown). Serial dilutions of the same batch of genomic DNA were used for subsequent qPCR experiments.

We were able to determine the theoretical limit of detection of our assay. With a LLOD of 10 GEq leptospires (in 5 µL), water and blood would have a theoretical limit of $1x10^3$ leptospires/mL, and kidney and liver would have a theoretical limit of 8×10^3 leptospires/g. Water and blood spiked with leptospires showed a LLOD of $1x10^2$ leptospires/mL, whereas kidney and liver had a LLOD of 1×10^4 leptospires/g (Fig. S1). These data indicate that the assay has a better performance with liquids than with solid tissues (Fig. S1). Water and tissues from control animals that were used as donors for the spiking experiments were negative by qPCR (data not shown). The *gapdh* real-time PCR results showed amplification in all samples tested and all the NTC (Non Template Control) samples were negative.

Kinetics of dissemination of hamsters infected with different dose inoculum. Consistent with previous results (24), the mean LD_{50} for Fiocruz L1-130 strain was 45.9 leptospires in hamster when using IP injection (data not shown). Doses as high as 10^8 leptospires are normally used for experimental infection and the low dose used here was chosen to be approximately 5x the LD_{50}. Pilot experiments were performed to determine the optimal time points for sample collection during infection. Time point selection also took into account the time to death after infection and the first time point to achieve a detectable number of leptospires per gram of tissue.

To control for the possibility of discrepant quantification results when collecting tissue samples from different parts of a same specimen, tissues were sampled from three different sites in the same organ (kidney, liver and lung) from animals infected with either high or low doses. No site-specific differences were observed in any tissue tested (Table S1). These data indicate that leptospires are distributed equally throughout these organs, indicating that a single DNA extraction yields quantification results applicable to the entire organ. Furthermore, there were no differences in results between the right and left kidneys (data not shown).

Hamsters infected IP with 10^8 leptospires (high dose) were euthanized 1 hour, 1, 3 and 4 days after infection. Positive control animals died in 5 to 6 days post-infection. Hamster infected IP with 2.5 x 10^2 leptospires (low dose) were euthanized at time points 3, 5, 8 and 11 days after infection while positive control animals died in 9 to 11 days post-infection. Although there was a delay in the detection of leptospires in tissues after a low dose of infection compared to a high dose of infection, organisms increased exponentially in tissues after 8 days, reaching equivalent high levels of tissue burden observed in animals infected with a high dose (Fig. 1).

After high dose infection, all tissues analyzed were positive after 1 hour for the presence of *Leptospira*, with a range of 1.2 x 10^2 - 7.6 x 10^2 leptospires/g for brain, heart, eye, muscle, lung (Fig. 1) and 5.8 x 10^3 – 2.4 x 10^5 leptospires/g for blood, liver and kidney (Fig. 1). Spleen was the organ that showed the higher number of leptospires per gram after 1 hour of infection with 3.5 x 10^5 leptospires/g (Fig. 1). We observed a statistically significant difference in the bacterial burden in all tissues between the first and the third day post-infection ($p<0.005$). The bacterial burden continued to increase in vivo, as demonstrated by the observation that after 4 days, kidney, liver and spleen reached levels of 1.65 x 10^8, 8.5 x 10^7 and 7.72 x 10^7 leptospires/g respectively, with a difference of 4 logs between the first and the last time point (Fig. 1). The eye had the lowest tissue load, 2 x 10^4 leptospires/g and also the lowest differences among time points. Blood was found to contain 1.6 x 10^7 leptospires/mL and the burden for the other tissues on the last day sampled was between 1.4 x 10^5 and 9.3 x 10^5 leptospires/g, with no decrease in burden.

After a low dose of infection, no bacteria were detected in any tissues 3-days post-infection, indicating that after low dose inoculation a few days are required before the bacteria can reach a detectable number of organisms in any tissue. At five days after infection, 1.3 x 10^2 leptospires/mL were detected in blood, with positive detection also in the kidney, liver and spleen (2.74 x 10^2, 1.94 x 10^3 and 1.36 x 10^4 leptospires/g, respectively; Fig. 1). After 8 days of infection, all the tissues were positive, with a burden ranging from 5.4 x 10^4 leptospires/g for muscle to 6.7 x 10^7 leptospires/g in the kidney, with a statistical difference between the day 5 and day 8 time points in all tissues ($p<0.0001$) (Fig. 1). On day 11 all analyzed tissues except lung and brain exhibited a decrease in the number of detected leptospires compared to day 8, which was not a statistically significant difference ($p=0.084$) (Fig. 1). In tissues such as blood, liver and

297 spleen, the marked decrease in burden could be associated with the appearance of agglutinating
298 antibodies, as 50% of the animals had serum MAT titers ranging from 1:25 to 1:100. No animals
299 infected with a high dose of leptospires developed antibodies detectable by MAT.
300
301 **Necropsy and histopathology analysis.** At necropsy, animals inoculated with high and
302 low doses of leptospires showed clinical signs and macroscopic lesions 3 and 8-days post-
303 infection, respectively, culminating in death 2 to 3-days later. The most common clinical signs
304 observed were anorexia, depression and hemorrhage just before the onset of death. In terms of
305 gross pathology observed at necropsy, animals showed a slight amount of jaundice in the liver
306 and abdominal muscles, enlargement of the spleen and hemorrhage of the kidneys. Localized
307 hemorrhage of the lungs was the most prominent feature observed at necropsy (Fig. 2), and was
308 most likely related to infection rather than CO_2 inhalation considering that negative control
309 animals didn't have these lesions (data not shown). Kidney, liver and lung histopathology
310 revealed no differences between animals at 1 and 3-days post-infection with high inoculum and 5
311 and 8-days post-infection with lower inoculum.
312 Analysis of haematoxylin and eosin stained sections of the kidney, liver and lung
313 revealed similar histopathological features of leptospirosis at the time of maximal tissue damage
314 in animals infected with high or low dose of *Leptospir*a (Fig. 2). In the kidney, discrete
315 hemorrhage, inflammation, and mild hyaline degeneration were observed in animals at the last
316 two time points for both doses. In the liver, animals infected with lower dose showed discrete
317 hydropic degeneration and infiltrating mononuclear cells. In animals infected with either high or
318 low inoculum, we also observed discrete hepatic necrosis, mild steatosis and intense loss of
319 parenchymal architecture. In the lungs, mild to intense alveolar hemorrhage and discrete

bronchial hemorrhage were observed in all animals at later stages of infection with a high or low dose (Fig. 2 and Table 1). The localization of leptospires in tissues, as determined by immunohistochemistry, was found to be intercellular in the liver, in the kidney interstitium and tubules and in the alveolar septa of the lungs (Fig. 3). 70% of the lung sections analyzed were negative for leptospires.

Mimicking the natural route of infection by conjunctival instillation. Infection kinetics in animals challenged with 10^8 leptospires by the conjunctival (CJ) and IP routes were compared (Fig. 4). Blood, kidney, liver, spleen, lung and eye tissues were analyzed. Both infection routes caused death of the animals, but the time to death in animals challenged by the CJ route was 3-4 days longer. All tissues of animals challenged by the IP route were positive at the 1-day time point with a burden ranging from 3×10^1 to 3.5×10^5 leptospires/g. These numbers increased exponentially for all tissues by 4-days after infection, reaching 4.2×10^5 to 3.3×10^7 leptospires/g (Fig. 4). All these results are similar to the previous experiments. By contrast, leptospires were undetectable in all tissues from animals at the 1-day time point after infection by CJ route. However, tissue burden at the 4 and 7-day time point were as high as 1.4×10^4 leptospires/g and 1×10^7 leptospires/g, respectively. This results indicates that although there was a delay in the time to death of the animals when comparing IP and CJ route, the bacterial load in the tissues analyzed is similar on the days previous to the onset of death for both routes. Furthermore, the leptospiral burden in the eye of animals infected by the CJ route was lower when compared to IP-challenged animals, and detection was possible only after seven days of infection, reaching 8.3×10^2 leptospires/g (Fig. 4).

343 **Bacterial burden as a marker for lethality.** The mean bacterial burden observed in each tissue
344 for the different routes of infection (infected with low or high dose) was used to calculate the
345 threshold of leptospiral burden that could be related to lethality (Table 2). Kidney, liver and
346 spleen showed a threshold of 2-3 x 10^7 per gram, whereas tissues like heart, lung, muscle and
347 brain showed a threshold of 1-2 x 10^5 leptospires/g. The threshold for blood was 3 x 10^6
348 leptospires/mL (Table 2).
349
350 **Use of Real Time PCR for pathogenesis studies.** The infection kinetics of two different
351 attenuated strains were analyzed. One of these attenuated strains was a clone of a Copenhageni
352 strain passaged 42 times *in vitro* and the other was a *clpB* mutant obtained by transposon
353 mutagenesis from a virulent strain of serovar Canicola (31). In both cases, the inoculum was 10^8
354 leptospires, and samples were collected 1 and 4 days after challenge. The bacterial burden one
355 day after intraperitoneal infection for the culture-attenuated Copenhageni strain was similar to
356 that of the virulent strain (Fig. 5A and B). However, 4 days after challenge, the burden of
357 leptospires was statistically different between hamsters infected with the two strains ($p=0.014$),
358 with the attenuated strain showing a lower burden in all analyzed tissues (Fig. 5A and B). The
359 result was similar for the *clpB*- mutant and wild-type strains; after 1 day of infection the burden
360 in the tissues were similar (Fig. S2A and B), while the leptospiral burden in all tissues after 4
361 days was significantly lower for the mutant in comparison to the wild-type strain (Fig. S2A and
362 B). All control animals infected with the virulent Copenhageni and Canicola strains died after 5-
363 6 days of infection while animals infected with the attenuated strains survived. Analysis of
364 tissues from animals that survived after 30 days revealed that only animals infected with *clpB*-
365 mutant were chronically infected in their kidney with a bacterial burden of 10^8 leptospires/g,

with the ability to be re-isolated from the kidney and retain the attenuated status upon reinfection (data not shown). Of note, for the non-virulent clones, leptospires were not detected in the eye of infected animals at any time point.

When the attenuated Copenhageni (Fig. 5C) and Canicola (Fig. S2C) strains were inoculated via the conjunctival route, no leptospires were detected in any tissues at 7 days after challenge, whereas the parental strains were detected in every tissue. The leptospiral burden in tissues was similar to that found in previous experiments involving the conjunctival challenge route for the virulent Copenhageni (Fig. 4) strain, with the control animals dying after 8 and 9-days post-infection. All controls animals infected with the attenuated Canicola and Copenhageni strains survived and tissues were negative 30 days after challenge. These data indicate that attenuation resulting either from passage *in vitro* or *clpB* gene disruption affected the overall ability of the cells to cause disease, either by increasing their susceptibility to the innate immune system or, most likely, by disrupting their ability to actively penetrate mucous membranes.

DISCUSSION

Pathogenic leptospires are motile, life-threatening spirochetes that readily disseminate to all tissues (1, 2). Little is known about the rate of spread to various tissues and its consequence on the pathogenesis of the disease. Recent studies have shown that different routes of infection result in changes in dissemination kinetics (15, 19). However, given the use of different animal models and methods of detection of the agent, major questions remain regarding the effect of dose on dissemination and the relevance of this approach to understanding pathogenesis. Here we demonstrate that maximum tissue burden was independent of challenge route and dose. On

389 the other hand, the time to maximum tissue burden was dependent on both the challenge route
390 and dose. These findings are of great importance to our understanding of the natural history of
391 leptospirosis and its pathogenesis, as well as to leptospirosis vaccine development and testing.

392 No specific tissue tropism was observed during the dissemination and multiplication
393 phase of leptospiral infection. Our results indicate that pathogenic leptospires rapidly invade the
394 bloodstream and are subsequently distributed to all body tissues even before multiplication (Fig.
395 1), a finding in accordance with previous studies (36, 37). As noted in previous studies, the
396 burden is higher in highly vascularized tissues such as the spleen, liver, and kidney which
397 receive greater blood flow than other organs (18, 38). After IP challenge, leptospires were
398 detected in brain and eye of animals infected with high and low doses by one hour and eight-
399 days, respectively (Fig. 1). The ability of leptospires to cross the brain-blood barrier, indicated
400 by the presence of leptospires in the brain, and other endothelial barriers formed by tight
401 junctions is consistent with previous animal and human studies (18, 39, 40).

402 The intraperitoneal and conjunctival challenge routes have fundamental differences.
403 Intraperitoneal infection bypasses tissue barriers and host immune defenses, particularly after
404 high challenge doses (41), by providing direct access of the peritoneal cavity to the bloodstream
405 via lymphatic and thoracic duct. In contrast, the conjunctival challenge route requires bacteria to
406 adhere to and penetrate mucous membranes, a more natural route of leptospiral infection (42).
407 The conjunctival route is of interest in identifying mechanisms of tissue penetration that might be
408 targeted by leptospiral vaccines. Although the dose required to cause disease in naturally
409 occurring infections is unknown, it is likely to be lower than the 10^8 dose used in this study. This
410 difference may be explained by the expression of virulence factors occurring only in the
411 reservoir host or in the environment that do not occur *in vitro*. Nevertheless, our results are

similar to previous studies with animal models (19, 26) in which higher leptospiral numbers were needed to cause disease by conjunctival inoculation. While both the intraperitoneal (higher and lower dose) and conjunctival inoculation routes ultimately lead to overwhelming infection, dissemination, multiplication and spread of leptospires are delayed under conjunctival inoculation (Fig. 4). Our findings are consistent with those of a previous study (19), which showed that leptospires inoculated by the conjunctival route caused systemic infection detectable within 4 days after challenge, with a bacterial burden similar to but occurring later than animals infected by the intraperitoneal route. Additional investigations are needed to examine different mucous membrane routes of infection, the ability of leptospires to penetrate intact and abraded skin, and environmental factors contributing to mucous membrane penetration.

We demonstrated here that the inoculation dose is an important factor contributing to the kinetics of the infection and disease severity. Intraperitoneal injection using a high dose (10^8) of leptospires resulted in dissemination to all tissues within 1 hour after infection, including eye and brain, at levels ranging from 10^2-10^5 leptospires/g within 1 hour after infection. In contrast, intraperitoneal injection with a lower inoculum dose (2.5×10^2) was associated with longer times to leptospiral detection in tissues (Fig. 1) and clinical disease development. Animal studies with the Fiocruz L1-130 strain found an inverse correlation between the dose of infection and the time to death (24). Similarly, the natural history of the disease in humans varies from an asymptomatic form to acute and severe disease (1, 8, 10). In addition, factors such as dose, serovar and genetics of the strain or host may contribute to outcome (8). Our findings suggest that the inoculum dose is an important factor that contributes to outcome and severity of disease, particularly in areas where the same serovar is the cause of the majority of the cases identified (10).

Manifestations of disease are related to bacterial tissue burden. The relationship between leptospiral tissue burden, lesion formation and death has previously been described (8), in which the first appearance of macroscopic hemorrhage in the animals coincided with a defined leptospiral burden (8). Another study found that the time of death coincided with a higher leptospiral tissue burden (43). However, regardless of the inoculum size and the length of the course of infection, we found that the threshold burden of leptospires in all tissues was comparable, above which macroscopic lesions appeared and animals were near death. In both cases, this disease threshold of leptospiral burden was over 10^7 leptospires/g in tissues as kidney, liver, spleen and blood (Fig. 1). Similar to what was found in other studies (15, 19), our final bacterial burden before the onset of death (Fig. 1 and 4; Table 2) was similar among the different doses and routes of infection. Furthermore, there was no major differences between the dose and route of infections when comparing the macroscopic and histopathological changes in different tissues (Fig. 2). Although our calculated threshold for lethality was as high as 10^6 leptospires/mL in blood and 10^7 leptospires/g in kidney, liver and spleen, our findings corroborate previous studies in human patients with clinical leptospirosis (16, 20, 44), that showed that a threshold burden of 10^4 - 10^6 leptospires/mL in the blood was associated with poor outcomes.

Attenuated mutants of pathogenic *L. interrogans* can disseminate but not multiply in tissues or cause death of infected animals after a high inoculum dose. An important finding of our study was that after one-day of infection with a high intraperitoneal dose of two different attenuated strains there were no statistical differences between the abilities of virulent and attenuated organisms to penetrate and spread from the peritoneal cavity to the bloodstream. In contrast, attenuated organisms were found at lower levels in multiple organs by day four after injection and had been almost completely cleared from blood and organs by day 30. A previous

study (19) in guinea pigs showed no dissemination of the same in-vitro attenuated strain of Fiocruz L1-130 in guinea pigs, indicating that the assay used in our study had greater sensitivity. Dissemination and rapid clearance of attenuated organisms had been noted by Faine in studies published in 1957 (13). Our findings corroborate these results suggesting that a critical threshold number of leptospires in tissues is required to cause lesions and death in experimental animals. Moreover, that threshold has to be reached before the host immune system can prevent the establishment of severe disease. Interestingly, while both mutants were unable to actively penetrate mucous membranes or cause death, only the *clpB*- mutant was able to establish renal colonization, suggesting a fundamental difference between the mutants. Our data validate the use of this assay for the identification and characterization of mutants that have lost the ability to penetrate, disseminate and/or multiply in tissues. Furthermore, our data suggest that it is possible to avoid high numbers of animals to study the kinetics of dissemination, because blood kinetics alone can serve as a surrogate for the entire dissemination process which can be determined with relatively few animals per group.

In summary, our results demonstrate that both pathogenic and attenuated leptospires rapidly disseminate within 1-hour after challenge and this early dissemination is independent of virulence. Regardless of the inoculum dose and the challenge route, the burden in target tissues is more important for pathogenesis than the ability of the pathogen to disseminate. However, the burden of infection is correlated with disease outcomes, as has been shown in humans (16, 20, 44). The quantitative Real Time PCR assay, which we have standardized and described here, may be useful in tracking mutant strains during the infectious process to identify genes involved in penetration, dissemination, and multiplication in the host. Furthermore, this method can be used to verify the effectiveness of vaccines in preventing dissemination and colonization.

Regardless of the application, our results and findings indicate that this approach is an important tool for the study of leptospirosis, an important yet neglected disease, the biology and pathogenesis of which remains to be fully unveiled and understood.

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Table 1: Analysis of the major histopathological alterations found in hamsters infected with 10^8 or 2.5 x 10^2 leptospires according to days post-infection for each dose of inoculum. Results are showing the score for each of 3 randomly chosen animals analyzed individually in all days. For comparison and analysis, the semi-quantitative ranking was transformed in numbers: negative = 0; discrete = 1; mild = 2; and intense = 3.

Tissue	10^8 leptospires IP				2.5 x 10^2 leptospires IP			
	Control	Days post-infection			Control	Days post-infection		
		1	3	4		5	8	12
Kidney								
Hemorrhage	0,0,0	0,0,0	1,1,0	0,0,0	1,1,1	1,0,2	1,0,1	0,0,0
Inflammation	0,0,0	0,0,0	0,0,0	1,0,0	0,0,0	0,0,0	0,0,1	0,0,1
Hyaline Degeneration	0,2,1	1,1,0	0,1,1	1,1,1	1,1,1	2,2,2	2,3,3	2,2,1
Hydropic Degeneration	0,0,0	0,0,1	0,0,0	1,1,1	0,0,0	0,0,0	0,1,1	3,1,3
Cylinders	1,1,1	0,0,0	0,1,1	1,1,0	1,1,0	1,1,0	2,1,2	1,0,1
Liver								
Hydropic Degeneration	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	2,2,0	0,0,0
Infiltrating Mononuclear Cells	0,0,0	0,0,0	0,0,0	1,0,0	1,0,0	0,0,0	1,0,0	1,0,0
Necrosis	0,0,0	0,0,0	0,0,1	0,0,1	0,0,0	0,0,0	0,0,1	0,0,0
Steatosis	0,0,0	0,0,0	2,1,0	2,1,1	0,0,0	0,0,0	0,1,0	2,1,1
Loss of Parenchymal Architecture	0,0,0	2,2,1	0,0,0	0,0,0	0,0,1	2,2,2	2,3,3	1,1,1
Lung								
Alveolar Hemorrhage	0,0,0	0,0,0	1,2,2	1,2,1	0,0,0	0,0,0	0,2,1	3,2,2
Bronchial Hemorrhage	0,0,0	0,0,0	1,1,0	1,1,1	0,0,0	1,0,0	1,1,1	1,1,1
Alveolar Congestion	0,0,0	0,0,0	1,1,0	2,1,1	0,0,0	0,0,1	1,1,1	2,2,1
Septal Thickening	0,0,0	1,1,1	1,1,0	1,1,1	0,0,0	1,1,1	1,1,1	1,1,0

Table 2: Lethality and bacterial burden in hamsters. The average burden of leptospires in different tissues was measured before the onset of death to calculate the threshold of bacterial load at which lethality occurs. The mean average was calculated based on the mean of the bacterial load result of three (IP) and two (CJ) independent experiments.

	Bacterial burden average (GEq)			
Tissue	**Intraperitoneal 10^8 (day 4 pi)**	**Intraperitoneal 2.5 x 10^2 (day 8 pi)**	**Conjunctival 10^8 (day 7 pi)**	**Total average (log ± SD)**
Blood	1.6 x 10^7	5.0 x 10^6	3.16 x 10^5	3.2 x 10^6 (6.5 ± 0.87)
Spleen	7.9 x 10^7	4.0 x 10^7	5.01 x 10^6	2.5 x 10^7 (7.4 ± 0.62)
Kidney	1.6 x 10^8	6.3 x 10^7	3.16 x 10^6	3.2 x 10^7 (7.5 ± 0.88)
Liver	7.9 x 10^7	5.0 x 10^7	1.00 x 10^7	3.2 x 10^7 (7.5 ± 0.47)
Lung	1.0 x 10^6	1.6 x 10^5	5.01 x 10^4	2.0 x 10^5 (5.3 ± 0.65)
Brain	1.6 x 10^5	1.6 x 10^5	ND	1.6 x 10^5 (5.2 ± 0.00)
Heart	1.6 x 10^5	1.6 x 10^5	ND	1.6 x 10^5 (5.2 ± 0.00)
Muscle	4.0 x 10^5	5.0 x 10^4	ND	1.4 x 10^5 (5.1 ± 0.63)
Eye	5.0 x 10^4	7.9 x 10^4	7.94 x 10^2	1.6 x 10^4 (4.2 ± 1.10)

pi = Post-infection
ND = Not determined

FIGURE LEGENDS

Figure 1. Kinetics of leptospirosis dissemination in tissues taken from hamsters infected IP with 10^8 and 2.5 x 10^2 leptospires of the strain Fiocruz L1-130. Bacterial load for each tissue was calculated based on the mean result of three perfused hamsters for each time point. Each line represents the mean result of bacterial load (logarithmic scale) of three independent experiments. Error bars represents the standard deviation. Animals infected with 10^8 leptospires (solid line) were analyzed at 1 hour, 1, 3 and 4-days post-infection. Animals infected with 2.5 x 10^2 leptospires (dotted line) were analyzed at 3, 5, 8 and 11-days post-infection.

Figure 2. Pathology of hamsters infected intraperitoneally with the L1-130 Fiocruz strain. (A) Representative photographs of gross examination of negative control hamster, and hamsters infected with 10^8 leptospires (high dose) or 2.5 x 10^2 leptospires (low dose), at day 4 and 11 post-challenge, respectively. Infected hamsters had evident localized hemorrhage of the lungs (B) Histopathology analysis showing representative photomicrographs of haematoxylin and eosin (HE) stained sections of kidney, liver and lung of negative control animal (i), and animals infected with high dose (ii) or low dose (iii) of leptospires, at day 4 and 11 post-challenge, respectively. Histopathology photomicrographs were taken at a magnification of ×400. Infected animals had similar histopathological features, with kidney showing mild hyaline degeneration and hemorrhage, liver with mild loss of parenchymal architecture and steatosis and lungs with hemorrhage.

Figure 3. Representative photomicrographs of immunohistochemically stained sectioned of kidney, liver and lung from hamsters infected intraperitoneally with L1-130 Fiocruz strain. Stained tissues of an animal infected with 10^8 leptospires (high dose) at day 4 post-challenge, and an animal infected with 2.5 x 10^2 leptospires at day 11 post-challenge are displayed on columns A and B, respectively. Detection was performed with monoclonal antiserum specific to LipL32. Photomicrographs were taken at a magnification of ×1,000, and are showing whole leptospires and degraded cells in kidney tubules, interstitium of liver and alveolar septa of lung.

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

Figure 5. Kinetics of the dissemination of leptospires in tissues from hamsters infected intraperitoneally and conjunctivally with 10^8 leptospires. Animals were infected with a virulent wild-type Copenhageni strain (A) and compared with the attenuated clone of the Copenhageni strain obtained after 42 *in vitro* passages (B), using the IP route (A and B), and CJ route (C). Analysis of the tissues was performed 1 and 4-days post IP infection (A and B), and 7-days post CJ infection (C) by the mean result of two perfused hamsters for all the strains. Each column

683 represents the mean (logarithmic scale) of two independent experiments. Error bars represent the
684 standard deviation.