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

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1 Real-time PCR reveals rapid dissemination of *Leptospira interrogans* after intraperitoneal and  
2 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 ( $10^8$  leptospores) or low dose ( $2.5 \times 10^2$  leptospores). We also examined the conjunctival challenge route to mimic the natural history of infection. Quantification of leptospores 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  $10^5$ - $10^8$  leptospores/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

47 **INTRODUCTION**

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

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

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

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

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

82 Animal model studies are essential to understanding the biology, transmission,  
83 colonization, and pathogenesis of *Leptospira* spp. The Golden Syrian Hamster is commonly  
84 employed as a model for acute leptospirosis due to its high susceptibility to leptospiral infection  
85 and because the clinical features mimic those of severe human infection (23). Although  
86 experimental inoculation is typically performed by intraperitoneal inoculation (24, 25), this route  
87 does not reflect natural transmission of the pathogen. Relatively few studies have examined  
88 challenge routes that mimic natural entry of leptospires via skin or mucous membranes (15, 19,  
89 26, 27). Although quantitative PCR assays have been extensively evaluated as a diagnostic tool,  
90 few studies have applied this technique to pathogenesis studies in animal models of leptospirosis.  
91 In this study, we compared the kinetics of leptospiral infection in the hamster after high and low

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

94

95

## MATERIAL AND METHODS

96

97 **Bacterial strains and clones.** Leptospires were cultivated in liquid Ellinghausen-  
98 McCullough-Johnson-Harris (EMJH) medium (28, 29) supplemented with 1% rabbit serum.  
99 Cultures were kept up to 7 days at 30°C, reaching a log phase between 4-5 days of culture.  
100 Bacteria were counted in a Petroff-Hausser counting chamber (Fisher Scientific). *Leptospira*  
101 *interrogans* serovar Copenhageni strain Fiocruz L1-130, a virulent clinical isolate from Brazil  
102 (10, 30) with 4 and 8 passages in vivo and in vitro, respectively, was used for the spiking and  
103 kinetics experiments. Pathogenesis studies involved an attenuated Fiocruz L1-130 strain obtained  
104 after 42 *in vitro* passages (high passage), and *Leptospira interrogans* serovar Canicola strain Kito  
105 attenuated by disruption of the *clpB* gene as previously described (31).

106

107 **Standard curve and spiking experiments.** DNA from *L. interrogans* serovar  
108 Copenhageni strain Fiocruz L1-130 was extracted using the QIAamp DNA minikit (QIAGEN,  
109 Valencia, CA). DNA was quantified using the NanoDrop instrument (Thermo Fisher Scientific  
110 Inc., Waltham, MA). A genome size of 4.6 Mb was used to determine the genomic equivalent  
111 (GEq) concentration per microliter of the purified DNA (30). To generate a standard curve, serial  
112 dilutions of the DNA were made starting at  $1 \times 10^7$  GEq to  $1 \times 10^0$  GEq/5  $\mu$ L. Standard curve  
113 assays were performed in duplicate. The analytic sensitivity of the assay was calculated and the

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

116 Leptospire were added to 1 mL of water or EDTA-anticoagulated whole blood from  
117 uninfected hamsters to achieve a final concentration of  $1 \times 10^7$  leptospire/mL. After spiking,  
118 serial 10-fold dilutions of  $1 \times 10^6$  to  $1 \times 10^0$  leptospire/mL were then performed using blood or  
119 water as diluents, accordingly. For tissues, kidney and liver were acquired from uninfected  
120 hamsters. 50  $\mu$ L of serial 10-fold dilutions of leptospire were spiked into 25 mg of kidney and  
121 liver, before DNA extraction, to achieve concentrations of  $1 \times 10^7$  to  $1 \times 10^0$  leptospire per gram of  
122 tissue. After the spiking, tissues were processed for DNA extraction as described below.  
123 Unspiked water and tissues were used as negative controls.

124

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

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

137 infection, sub-groups of two animals were euthanized. The conjunctival challenge route  
138 experiments were performed by centrifugation of 30 mL culture of leptospire for 10 minutes at  
139 3,000g to obtain an inoculum of 10<sup>8</sup> leptospire in 10 µL of EMJH medium, which was applied  
140 to the conjunctival membrane of the left eye. Animals in groups of four were infected with two  
141 animals euthanized after 7 days of infection for each strain tested. For each experiment, the two  
142 remaining animals were included as positive controls for infection and not euthanized until an  
143 endpoint was achieved.

144 All animals were monitored twice daily for endpoints including signs of disease and  
145 death, up to 30-days post-infection. Surviving animals after 30-days post-infection or moribund  
146 animals presenting with difficulty moving, breathing or signs of bleeding or seizure were  
147 immediately sacrificed by inhalation of CO<sub>2</sub>. Blood was collected by intracardiac puncture. Each  
148 hamster was perfused with 100 mL of saline solution to eliminate blood in the organs that might  
149 contribute to the quantitative PCR signal. After perfusion, the heart, right pulmonary lobe, right  
150 dorsocaudal hepatic lobe, spleen, right kidney and muscle of the right thigh were carefully  
151 removed. The brain was exposed by craniotomy and the right eye was enucleated. All tissues  
152 were collected into cryotubes and immediately placed into liquid nitrogen before being stored at  
153 -80°C until extraction. Sera were obtained by centrifugation of clotted blood at 1,000g for 15min  
154 at room temperature and frozen at -20°C until analysis for the presence of antibodies against  
155 leptospire using the microscopic agglutination test (MAT), performed as previously described  
156 (10, 33). For the pathogenesis and conjunctival route experiments only blood, kidney, liver, lung,  
157 spleen and eye were analyzed. All animal protocols were approved by the Committee for the  
158 Use of Experimental Animals of the Gonçalo Moniz Research Center, Fiocruz, and Yale  
159 University.

160



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

169

170 **Quantitative Real Time PCR analysis.** Concentration of leptospire was quantified by a  
171 TaqMan-based quantitative PCR assay using an ABI 7500 system (Thermo Fisher Scientific,  
172 Inc.) and Platinum Quantitative PCR SuperMix-UDG (Thermo Fisher Scientific, Inc.) (34). The  
173 *lipL32* gene was amplified using the set of primers previously described (34, 35), LipL32-45F  
174 (5'-AAG CAT TAC CGC TTG TGG TG-3') and LipL32-286R (5'-GAA CTC CCA TTT CAG  
175 CGA TT-3') that amplify a fragment of 242bp, which was detected by the probe, LipL32-189P  
176 (FAM-5'-AA AGC CAG GAC AAG CGC CG-3'-BHQ1). The bacterial quantification was  
177 calculated based on the standard curve described above and expressed as the number of  
178 leptospire per milliliter or gram of tissue. For that calculation it was taken in consideration the  
179 amount of sample (200  $\mu$ L of water or blood, 10 mg of the spleen, and 25 mg of other tissues),  
180 the elution volume (100  $\mu$ L) and the volume of template DNA used in the PCR assay (5  $\mu$ L). To  
181 calculate the theoretical limit of the assay we used the following formula: LLOD of the assay X  
182 Elution factor X Volume factor; where elution factor is equal to 20 (100  $\mu$ L / 5  $\mu$ L) and the

183 volume factor for water/blood and liver/kidney is 5 (1000 mL / 200  $\mu$ L) 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  $19 \pm 2$  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  $\mu$ M of each primer, 5  $\mu$ M of  
196 the specific probe and 5  $\mu$ L of DNA in a total volume of 25  $\mu$ L. There was no addition of MgCl<sub>2</sub>  
197 or passive reference dye. The amplification protocol consisted of 2min at 50°C and 10min at  
198 95°C, followed by 45 cycles of amplification (95°C for 15s and 60°C 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 leptospire per gram of tissue or per milliliter of blood or water.

205

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

218 Immunohistochemistry was performed to confirm the presence of *Leptospira* in tissues  
219 section. Paraffin was removed from tissue sections with xylene and ethanol. Slides were blocked  
220 by incubation with PBS/10% milk at room temperature for 20 min, followed by addition of a 1:  
221 1,000 dilution of rabbit polyclonal antiserum to LipL32 or *L. interrogans* serovar  
222 Icterohaemorrhagiae strain RGA or normal rabbit serum (as a negative control) for 1 hour at  
223 room temperature. Slides were treated with 0.3% hydrogen peroxide for 15 min at room  
224 temperature and then incubated at room temperature for 30 min with biotin-conjugated anti-  
225 rabbit secondary antibodies, followed by streptavidin peroxidase (Histostain-Bulk Kit,  
226 Invitrogen). Enzyme reactions were developed using 3.3'- Diaminobenzidine (Sigma-Aldrich, St.  
227 Louis, MO). Slides were examined by optical microscopy (Zeiss Axioskop).

228

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

233

234

## RESULTS

235

236 **Standard curve and spiking experiments.** Three independent quantitative real-time  
237 PCR (qPCR) assays were performed on duplicate serial dilutions of genomic DNA isolated from  
238 *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. In our experiments, the LLOD for  
239 which 95% of the tested samples were positive was 10 GEq with a linear correlation coefficient  
240 ( $R_2$ ) of 0.998 (data not shown). Serial dilutions of the same batch of genomic DNA were used for  
241 subsequent qPCR experiments.

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

251

252           **Kinetics of dissemination of hamsters infected with different dose inoculum.**

253 Consistent with previous results (24), the mean LD<sub>50</sub> for Fiocruz L1-130 strain was 45.9  
254 leptospire in hamster when using IP injection (data not shown). Doses as high as 10<sup>8</sup> leptospire  
255 are normally used for experimental infection and the low dose used here was chosen to be  
256 approximately 5x the LD<sub>50</sub>. Pilot experiments were performed to determine the optimal time  
257 points for sample collection during infection. Time point selection also took into account the  
258 time to death after infection and the first time point to achieve a detectable number of leptospire  
259 per gram of tissue.

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

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

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

287 After a low dose of infection, no bacteria were detected in any tissues 3-days post-  
288 infection, indicating that after low dose inoculation a few days are required before the bacteria  
289 can reach a detectable number of organisms in any tissue. At five days after infection,  $1.3 \times 10^2$   
290 leptospores/mL were detected in blood, with positive detection also in the kidney, liver and  
291 spleen ( $2.74 \times 10^2$ ,  $1.94 \times 10^3$  and  $1.36 \times 10^4$  leptospores/g, respectively; Fig. 1). After 8 days of  
292 infection, all the tissues were positive, with a burden ranging from  $5.4 \times 10^4$  leptospores/g for  
293 muscle to  $6.7 \times 10^7$  leptospores/g in the kidney, with a statistical difference between the day 5 and  
294 day 8 time points in all tissues ( $p < 0.0001$ ) (Fig. 1). On day 11 all analyzed tissues except lung  
295 and brain exhibited a decrease in the number of detected leptospores compared to day 8, which  
296 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 leptospire developed antibodies detectable by MAT.

300

301       **Necropsy and histopathology analysis.** At necropsy, animals inoculated with high and  
302 low doses of leptospire 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<sub>2</sub> 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 *Leptospira* (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

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

325

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

342



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  $2-3 \times 10^7$  per gram, whereas tissues like heart, lung, muscle and  
347 brain showed a threshold of  $1-2 \times 10^5$  leptospores/g. The threshold for blood was  $3 \times 10^6$   
348 leptospores/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 leptospores, 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 leptospores 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  $10^8$  leptospores/g,

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

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

379

380

## DISCUSSION

381

382 Pathogenic leptospire are motile, life-threatening spirochetes that readily disseminate to  
383 all tissues (1, 2). Little is known about the rate of spread to various tissues and its consequence  
384 on the pathogenesis of the disease. Recent studies have shown that different routes of infection  
385 result in changes in dissemination kinetics (15, 19). However, given the use of different animal  
386 models and methods of detection of the agent, major questions remain regarding the effect of  
387 dose on dissemination and the relevance of this approach to understanding pathogenesis. Here  
388 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

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

422         We demonstrated here that the inoculation dose is an important factor contributing to the  
423 kinetics of the infection and disease severity. Intraperitoneal injection using a high dose ( $10^8$ ) of  
424 leptospires resulted in dissemination to all tissues within 1 hour after infection, including eye and  
425 brain, at levels ranging from  $10^2$ - $10^5$  leptospires/g within 1 hour after infection. In contrast,  
426 intraperitoneal injection with a lower inoculum dose ( $2.5 \times 10^2$ ) was associated with longer times  
427 to leptospiral detection in tissues (Fig. 1) and clinical disease development. Animal studies with  
428 the Fiocruz L1-130 strain found an inverse correlation between the dose of infection and the time  
429 to death (24). Similarly, the natural history of the disease in humans varies from an  
430 asymptomatic form to acute and severe disease (1, 8, 10). In addition, factors such as dose,  
431 serovar and genetics of the strain or host may contribute to outcome (8). Our findings suggest  
432 that the inoculum dose is an important factor that contributes to outcome and severity of disease,  
433 particularly in areas where the same serovar is the cause of the majority of the cases identified  
434 (10).

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

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

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

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

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

484

485

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486

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624

625 **Table 1:** Analysis of the major histopathological alterations found in hamsters infected with 10<sup>8</sup> or 2.5  
626 x 10<sup>2</sup> leptospire according to days post-infection for each dose of inoculum. Results are showing the  
627 score for each of 3 randomly chosen animals analyzed individually in all days. For comparison and  
628 analysis, the semi-quantitative ranking was transformed in numbers: negative = 0; discrete = 1; mild =  
629 2; and intense = 3.  
630

Tissue	10 <sup>8</sup> leptospire IP				2.5 x 10 <sup>2</sup> leptospire IP			
	Control	Days post-infection			Control	Days post-infection		
		1	3	4		5	8	12
<b>Kidney</b>								
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
<b>Liver</b>								
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
<b>Lung</b>								
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

631

632 **Table 2:** Lethality and bacterial burden in hamsters. The average burden of leptospire in  
 633 different tissues was measured before the onset of death to calculate the threshold of bacterial  
 634 load at which lethality occurs. The mean average was calculated based on the mean of the  
 635 bacterial load result of three (IP) and two (CJ) independent experiments.  
 636

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

637 pi = Post-infection  
 638 ND = Not determined

639 **FIGURE LEGENDS**

640

641 **Figure 1.** Kinetics of leptospirosis dissemination in tissues taken from hamsters infected IP with  
642  $10^8$  and  $2.5 \times 10^2$  leptospire of the strain Fiocruz L1-130. Bacterial load for each tissue was  
643 calculated based on the mean result of three perfused hamsters for each time point. Each line  
644 represents the mean result of bacterial load (logarithmic scale) of three independent experiments.  
645 Error bars represents the standard deviation. Animals infected with  $10^8$  leptospire (solid line)  
646 were analyzed at 1 hour, 1, 3 and 4-days post-infection. Animals infected with  $2.5 \times 10^2$   
647 leptospire (dotted line) were analyzed at 3, 5, 8 and 11-days post-infection.

648

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

660



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

668

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

676

677 **Figure 5.** Kinetics of the dissemination of leptospires in tissues from hamsters infected  
678 intraperitoneally and conjunctivally with  $10^8$  leptospires. Animals were infected with a virulent  
679 wild-type Copenhageni strain (A) and compared with the attenuated clone of the Copenhageni  
680 strain obtained after 42 *in vitro* passages (B), using the IP route (A and B), and CJ route (C).  
681 Analysis of the tissues was performed 1 and 4-days post IP infection (A and B), and 7-days post  
682 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.