Continuous Excretion of *Leptospira borgpetersenii* Ballum in Mice Assessed by Viability Quantitative Polymerase Chain Reaction

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Abstract. Rodents are the main reservoir animals of leptospirosis. In this study, we characterized and quantified the urinary excretion dynamics of *Leptospira* by *Mus musculus* infected with 2×10^8 virulent *Leptospira borgpetersenii* serogroup Ballum. Each micturition was collected separately in metabolic cages, at 12 time points from 7 to 117 days post-infection (dpi). We detected *Leptospira* in all urine samples collected (up to 8 per time point per mouse) proving that *Leptospira* excretion is continuous with ca. 90% live *L. borgpetersenii* Ballum, revealed by viability quantitative polymerase chain reaction. Microscopic visualization by Live/Dead fluorescence confirmed this high proportion of live bacteria and demonstrated that *L. borgpetersenii* Ballum are excreted, at least partly, as bacterial aggregates. We observed two distinct phases in the excretion dynamics, first an increase in *Leptospira* concentration shed in the urine between 7 and 63 dpi followed by a plateau phase from 63 dpi onward, with up to 3×10^7 *Leptospira* per mL of urine. These two phases seem to correspond to progressive colonization of renal tubules first, then to stable cell survival and maintenance in kidneys. Therefore, chronically infected adult mice are able to contaminate the environment via urine at each micturition event throughout their lifetime. Because *Leptospira* excretion reached its maximum 2 months after infection, older rodents have a greater risk of contaminating their surrounding environment.

INTRODUCTION

Leptospirosis is a bacterial zoonosis,¹ which mostly impacts rural or peri-urban populations in tropical and subtropical regions worldwide.² Humans get infected via the contact of wounded or abraded skin or mucosae with the urine of chronically infected reservoir mammals, directly or most frequently indirectly through a contaminated freshwater environment. Virtually any mammal can act as a reservoir of an adapted pathogenic *Leptospira*.³ However, rodents were identified as reservoirs of leptospirosis almost one century ago,⁴ and their prominent role as a source of human infections has been recognized for more than 50 years.⁵ In New Caledonia, a French Pacific island, where this study took place, rodents are regarded as the major animal reservoir associated with human leptospirosis.⁶

Yet, despite this recognition of the prominent role of rodents in the epidemiology of human leptospirosis and the availability of modern molecular techniques allowing the quantification of leptospires in virtually any type of specimen, little is known about the quantitative excretion of leptospires by reservoir rodents. Notably, the amount of leptospires shed in the urine of reservoir rodents or whether this excretion is continuous or intermittent is still poorly understood.⁷ The urinary shedding of *Leptospira interrogans* serovar Copenhageni by its *Rattus norvegicus* reservoir was determined in laboratory studies addressing *Leptospira* changes during chronic renal carriage.^{8,9} These studies showed the excretion of 10^5 – 10^7 *Leptospira* per mL of urine. Urine specimens from experimentally infected *R. norvegicus* were also collected over 6 weeks and used to validate a real-time polymerase chain reaction (PCR)

assay,¹⁰ showing a rapid increase in excretion reaching 5×10^4 to 5×10^5 *Leptospira* per mL of urine.

Recent field research in Brazil¹¹ has focused on the quantification of leptospires in the urine and kidneys of wild-caught *R. norvegicus*, providing quantitative data about *Leptospira* environmental contamination. In this study, a single urine sample was collected from the bladder for every individual animal upon euthanasia, showing that almost 90% of rats were carriers and excreted 6×10^6 leptospires per mL of urine.

The co-adaptation of specific *Leptospira* strains (serogroup Icterohaemorrhagiae) to the brown rat (*R. norvegicus*) has notably been recognized,¹² as well as other mammal host-*Leptospira* associations. Among rodents, mice are recognized as a reservoir host for *Leptospira borgpetersenii* from serogroup Ballum in most settings worldwide, including in New Caledonia¹³. A recent literature review aimed at a meta-analysis identified only one published study, providing quantitative excretion data for mice.⁷ However, in this study, mice were infected with a *L. interrogans* serovar Copenhageni, a strain for which mice are not a natural reservoir.¹⁴ To our knowledge, *Leptospira* quantitative excretion by domestic mice infected with a naturally co-adapted *L. borgpetersenii* from serogroup Ballum was not reported.

Serogroup Ballum has recently been reported as possibly emerging in some leptospirosis-endemic regions.^{15,16} In this study, we infected outbred domestic mice *Mus musculus* intraperitoneally with an isolate of *L. borgpetersenii* from serogroup Ballum. At various time points after infection, we collected urine samples from individual micturition events to characterize and quantify the dynamics of the urinary excretion of *Leptospira*. We notably evaluated if *Leptospira* excretion was continuous or intermittent, in a typical reservoir model. We used both real-time quantitative PCR (qPCR) and molecular as well as microscopy techniques to assess the viability of the bacteria recovered in the urine collected from spontaneous micturition over a period of 117 days after infection.

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MATERIALS AND METHODS

Leptospira strain. Virulent *L. borgpetersenii* serogroup Ballum isolate B3-13S was obtained from an Ellinghausen–McCullough–Johnson–Harris (EMJH) culture from the kidneys of a wild mouse (*M. musculus*) caught in 2009 in New Caledonia.¹³ The isolate was passaged monthly in hamsters to maintain virulence.¹⁷ A Petroff–Hausser counting chamber (Hausser Scientific, Horsham, PA) was used to determine bacterial cell concentration.

Animals and ethics statement. For this study, nine outbred OF1 mice (*M. musculus*) (five males and four females) were used from progenitors originating from Charles River Laboratories and bred at Institut Pasteur in New Caledonia. Intraperitoneal injection was administered to nine adult mice by the age of 9 months, with 2×10^8 *L. borgpetersenii* serogroup Ballum B3-13S in EMJH (380, 620, and 570 μ L per mouse for the three independent infections using three independent cultures) to reproduce chronic reservoir-like infection. Mice were then held in individual cages, where they were given food and water ad libitum. The experiment was performed as three biological replications with different cultures and at different dates, infecting three mice each time, leading to a total of nine mice. Animal manipulations were conducted according to the guidelines of the Animal Care and Use Committees of the Institut Pasteur and followed European Recommendation 2007/526/EC.

Experiment. Tecniplast® Metabolic Cages were used (Tecniplast S.p.A, Maggio, Italy) to collect urine samples, as described by Bonilla-Santiago and Nally¹⁸ with the following changes: 1) each mouse was placed in a metabolic cage for a 4-hour duration from 8:00 AM to noon before infection and then at 7, 14, 21, 28, 35, 42, 49, 63, 64, 65, 91, and 117 days post-infection (dpi); 2) during this period, the urine from each individual micturition event was immediately collected in a 1.5-mL Eppendorf tube, gently homogenized by pipetting, and its volume measured. Subsamples of 25 μ L (for each micturition) were aliquoted and used to quantify *Leptospira* excretion and assess their viability using quantitative viability PCR.

At the end of each collection period, mice were put back in their individual housing cages and the metabolic cages were cleaned and disinfected.

Mice were killed by carbon dioxide inhalation and atlanto-occipital dislocation at 117 dpi. Kidneys were collected immediately after death to quantify leptospires by qPCR after DNA extraction. Three small pieces (~25 mg) of both right and left kidneys were collected in the cortical areas of the kidneys to precisely quantify *Leptospira* kidney concentrations.

Purification of DNA and quantification of *Leptospira*. QIAamp DNA Mini kit (Qiagen, Doncaster, Victoria, Australia) was used for total DNA extraction. For each urine sample collected, 25 μ L of urine were incubated with 175 μ L phosphate-buffered saline (PBS), 200 μ L AL lysis buffer, and 20 μ L proteinase K at 56°C for 30 minutes. Kidney fragments were placed into 50 μ L PBS with 180 μ L ATL lysis buffer (QIAamp DNA Mini Kit; Qiagen, Doncaster, Victoria, Australia) and total DNA was extracted as per the manufacturer's instructions for tissues.

For both urine and kidney samples, *Leptospira* were quantified using a real-time quantitative PCR technique¹⁹ using a standard curve created from the analysis of specimens spiked with known numbers of *Leptospira*. This PCR targets the

lipL32 gene using TaqMan technology with primers and probe purchased from Eurogentec (Liège, Belgium). Reactions were performed on a LightCycler480 using the LightCycler480® probe Master kit (Roche Applied Science, Auckland, New Zealand). Results are expressed as the number of *Lep-tospira* per mL of urine or per μ g of kidney tissue DNA.

A TaqMan qPCR targeting the Mammal β -actin gene^{20,21} was performed on *Leptospira*-negative samples to check for the absence of PCR inhibitors and validate the negative results. This β -actin PCR was previously validated in our laboratory using the same reaction mix and amplification conditions as the *lipL32* qPCR.

Leptospira viability. For each mouse, for each time point, *Leptospira* viability was assessed on the first micturition where at least 80 μ L of urine was collected (first or second micturition of the day). BLU-V Viability PMA Kit²² (Qiagen, Doncaster, Victoria, Australia) was used to evaluate *Leptospira* viability in urine specimens. In preliminary experiments, we used EMJH-cultured leptospires to optimize the protocol for our model leptospire. These preliminary experiments led us to avoid as many mechanical stresses as possible, for example, to exclude vortexing or centrifugation, which both resulted in very low viability estimates from viability PCR (data not shown), probably as a result of high sensitivity to mechanical stress and cell surface damage.²³ To avoid degradation of urine^{24–26} and impairment of *Leptospira* viability, quickly after excretion (within 20 minutes), three tubes each containing 25 μ L of urine and 75 μ L of PBS were treated. Compared with the manufacturer's instructions, initial steps from the protocol were modified into gently pipetting and slight agitation, whereas vortexing and centrifugation were systematically omitted. Propidium monoazide (PMA, 2 μ L of a 50 μ M stock solution) was added in one tube directly after collection, corresponding to "live bacteria" and in a second tube after a 10-minute incubation at 80°C, corresponding to "dead bacteria control," which controls the effective capacity of PMA masking (because PMA only penetrates cells which wall/membrane are compromised where it irreversibly binds DNA through photoactivation). The third tube did not receive PMA, representing "total bacteria."

Mixtures were incubated for 10 minutes in the dark at room temperature then photoactivated for 10 minutes under blue 3-watt LED lights (460–470 nm) with slow homogenization every 2 minutes. PBS was added (100 μ L) to each tube before DNA extraction with QIAamp DNA Mini kit (Qiagen, Doncaster, Victoria, Australia).

Live/Dead microscopy assay. Visualization of *Leptospira* in urine was performed with Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes, Eugene, OR) as described in previous studies.²⁷ As for viability PCR, minimal handling stress was sought and the urine was neither vortexed nor centrifuged. The observation was made under a Leica DM4000 fluorescence microscope. ImageJ processing software (<http://rsbweb.nih.gov/ij/>) version 1.50i was used to merge images visualized by fluorescein isothiocyanate and tetramethylrhodamine filters and to adjust contrast, luminosity, and color balance.

Statistical analysis. All statistical analyses and graphics were performed using R software version 3.1.0.²⁸ All *Leptospira* concentrations measured were Log₁₀-transformed prior to analysis. For the analysis of leptospires excretion over time and leptospires concentrations in kidneys, only data from the four mice for which urinary excretion could be monitored until 117 dpi

were used. For the analysis of leptospires excretion over time, graphical exploration showed that the concentrations measured on the same animal at a given time point were only moderately variable from one micturition to another (see Supplemental Figure 1). Hence, for each mouse and each time point, data were averaged across all micturitions before modeling.

Data were analyzed using linear (analysis of viability and kidney data) or nonlinear (analysis of leptospires concentrations over time) mixed-effect models. Mixed-effect modeling is a statistical framework suitable for the analysis of data with repeated measurements performed on the same individual.²⁹ Validation of all the models presented was performed by graphically checking normality of residuals (for all levels of random effects) with a normal qq-plot. Details on the models developed can be found in Supplemental Material.

RESULTS

Urinary excretion. All urine samples collected before infection tested negative for *Leptospira*, in agreement with the routine surveillance of our animal facility. We then collected 244 urine samples between 7 and 117 dpi, with 1–8 micturition events per time point per mouse. Of these, 34 samples from one mouse were excluded from further analyses, because this mouse cleared *Leptospira* from its kidneys; after initial excretion, all urine samples at days 63, 64, and 65 were negative.

In all 210 urine samples from the other eight mice, *Leptospira* qPCR were positive, suggesting that excretion of *Leptospira* in urine is continuous. Though qualitatively continuous, the excretion was not quantitatively constant: first, a colonization phase was observed, followed by a plateau phase (Figure 1). *Leptospira* concentrations increased sharply from 0 to 3×10^6 *Leptospira* per mL of urine at 49 dpi, then remained quite stable tending to a maximum of 3×10^7 at 117 dpi.

Because of ongoing lesions caused by mites (*Myobia* sp.), three mice had to be humanely euthanized after 30 (two individuals) or 40 dpi (one mouse). Another mouse died of an

unknown cause at 115 dpi, just before the end of the experiment. Four mice provided urine specimens at all time points from 7 to 117 dpi. For the four mice for which excretion could be monitored until 117 dpi, we performed paired *t*-test to compare excretion at different dpi. For each of the four mice, concentrations were averaged across all urine samples collected on the same day. A significant increase in *Leptospira* excretion could be detected between 49 and 117 dpi ($N = 4$, $P = 0.047$), whereas no significant increase could be detected between 63 (and subsequent time points) and 117 dpi ($N = 4$, $P = 0.074$), meaning that the plateau phase was probably reached between 49 and 63 dpi.

Models were fitted on a total of 51 measurements performed on four mice at the 13 time points (from day 0 before infection to day 117). Models where the excretion was allowed to vary from one mouse to another fitted the data better than the model where the Michaelis–Menten parameters were held the same for all four mice³⁰ (akaike information criterion [AIC] of 117 for the model with fixed parameters, 82 for the model with random $C_{\max}$, 77 for the model with random T_{50} , 78 for the model with one random parameter, and 277 for the model with both parameters varying randomly), suggesting significant quantitative variability of urinary excretion between individual mice. Among the three random models, the one where only T_{50} , the time to reach half the maximal concentration of *Leptospira*, is allowed to vary randomly performed the best. In this model, y_{ij} , the (log-transformed) *Leptospira* concentration measured on mouse i at dpi j is modeled by:

$$y_{ij} = \frac{C_{\max} \cdot t_{ij}}{T_{50i} + t_{ij}} + \varepsilon_{ij}$$

where t_{ij} denotes the number of dpi, $C_{\max}$ is (the log of the) average maximal *Leptospira* concentration at infinite time, and $\varepsilon_{ij} \sim \mathcal{N}(0, \sigma_{\varepsilon}^2)$. T_{50i} , the time needed to reach half the maximal *Leptospira* concentration for mouse i , varies randomly from one mouse to another:

$$T_{50i} = T_{50} + u_i, \text{ with } u_i \sim \mathcal{N}(0, \sigma_u^2)$$

where T_{50} is the average, over all mice, of the time needed to reach half the maximal *Leptospira* concentration and σ_u represents the standard deviation of this time among the different mice.

Figure 1 shows the average urinary excretion of *Leptospira* through time as expected by the model. Estimation of the parameters showed that, on average, urinary excretion could reach a maximal concentration of 2×10^8 *Leptospira* per mL of urine ($C_{\max} = 8.3 \pm 0.4$), and the average time over all mice needed to reach half this concentration is $T_{50} = 14$ dpi. For each individual mouse, this time can vary from T_{50} by 5 days on average ($\sigma_u = 5$ days). The average concentration at 117 dpi as estimated by the model is 2×10^7 *Leptospira* per mL of urine and the time needed to reach 90% of this concentration is 58 days (T_{90} on Figure 1).

***Leptospira* viability.** Viability qPCR was performed for 57 urine samples (one urine for each mouse at each time point). The average viability of *Leptospira* was 91% (95% confidence interval = 76–100%), with no notable temporal pattern (P value of the effect of time since infection on viability = 0.933) and no variability between individual mice. This high proportion of viable cells is also confirmed by Live/Dead fluorescent microscopy, where green cells represent live and red cells represent dead or membrane-compromised leptospires (Figure 2).

FIGURE 1. Evolution in time of the urinary excretion of *Leptospira borgpetersenii* Ballum in *Mus musculus*. Dotted curves represent *Leptospira* concentrations (log scale) measured for each mouse at each time point. When several micturitions were collected on the same mouse at a given time point, (log-transformed) concentrations were averaged across all micturitions. The plain line represents the average of the best model for all four mice that could be followed up until 117 days post-infection. The equation is $y = \frac{C_{\max} t}{T_{50} + t}$ (see Supplemental Methods).

FIGURE 2. Photography of virulent *Leptospira borgpetersenii* B3-13S in urine (Mouse #S08 day101 post infection), made under a Leica DM4000 fluorescence microscope using Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes). Magnification $\times 1,000$. Scale bar = 10 μ m. The image shows clustering of *Leptospira* with a high proportion of live cells. This figure appears in color at www.ajtmh.org.

Leptospira load in kidney. We tested a piece of both kidneys from the mouse that cleared the infection by qPCR, which gave negative results, confirming that the infection was cleared. All other 32 kidney samples were positive for *Leptospira*.

Figure 3 shows *Leptospira* concentrations in the four mice for which six kidney samples could be obtained at 117 dpi. The analysis of the six distinct pieces of kidneys taken from each mouse upon euthanasia at 117 dpi shows that the variability of *Leptospira* concentration between samples taken on the same mouse is lower than the variability between different mice (Figure 3). Results from the model show that the side on which the kidney sample was taken has no significant effect on *Leptospira* concentrations ($P = 0.132$). The mean concentration across all mice is 4×10^3 *Leptospira* per μ g of kidney tissue DNA (*Leptospira*/ μ g DNA). The standard deviation of the random effect of the model is 0.512, representing the variability of the logarithm of leptospires concentrations from one mouse to another. The residual standard deviation of the model is 0.349. It represents the average variability of the logarithm of leptospires concentrations from one sample to another within a mouse. It can thus be interpreted as an estimate of the average error

FIGURE 3. Load of *Leptospira borgpetersenii* Ballum in kidneys of *Mus musculus*. Concentrations of *Leptospira* are in log-scale. Horizontal bars represent, for each mouse, the mean of the (log-transformed) concentration across all six samples.

committed when measuring the logarithm of *Leptospira* concentration on only one sample taken from one random kidney.

DISCUSSION

In the mice with an established chronic infection, we detected *Leptospira* in all the urine samples collected, clearly demonstrating that *Leptospira* excretion is continuous in the *M. musculus*–*L. borgpetersenii* Ballum reservoir association. In addition, we observed a variability over time with two phases, an initial 2-month period probably corresponding to a progressive colonization of renal tubules, then a plateau phase corresponding to chronic infection with excretion of around 10^7 *Leptospira* per mL of urine. This interpretation of a two-phase excretion pattern with an initial colonization of renal tubules was also proposed by other authors.^{31,32} Actually, a similar temporal pattern was observed in mice infected with a *L. interrogans* serovar Manilae, with *Leptospira* concentrations first increasing in kidneys, then remaining stable for more than 1 year.³² In our model, the plateau phase was reached between 49 and 63 days after infection, whereas that was suggested to happen earlier in a recent review of leptospirosis studies in mice³¹ or in independent studies in *R. norvegicus* infected with a *L. interrogans* Copenhageni.^{8,10} The concentration observed at the plateau phase is in the range observed by Monahan and others, by dark field microscopic counting⁸ or by Nally and others⁹ (from 10^5 to 10^7 *L. interrogans* serovar Copenhageni on *R. norvegicus* during chronic infection). Not surprisingly, the *Leptospira* cells in urine were mostly live as assessed by both viability qPCR and Live/Dead double fluorescence methods. The viability values measured were as low as 21% and as high as 168%. The most probable explanation for extreme values of viability, notably values above 100%, is an uneven distribution of leptospires in urine samples, which might be explained by bacteria remaining clustered upon micturition and collection (which excluded vortexing). This variability in subsamples of individual urine samples from the same mouse led us to hypothesize that leptospires were excreted in clusters rather than as individual cells. The double staining fluorescence microscopy assay confirmed this hypothesis (Figure 2). Though viability PCR was recently used in *Leptospira* in a source-tracking study,³³ our study reports (to the best of our knowledge) the first use of Live/Dead double fluorescence to study leptospirosis. The clusters observed might represent *Leptospira* aggregates released from the kidney tubules into the urine as described in previous studies.³⁴ These aggregates suggest a biofilm lifestyle in kidney tubules, a multicellular organization thought to provide protection, notably against urine acidity and which might later allow a better survival in the environment.^{35–37} The microscopic visualization of bacterial aggregates from the urine (Figure 2) supports this hypothesis, being one of the four criteria defining a biofilm etiology of an infection.³⁸

The chronic *Leptospira* carriage is confirmed by the detection of *Leptospira* in all kidney and urine samples marked with relatively high concentrations (1.14×10^4 *Leptospira*/ μ g DNA and 3.6×10^7 *Leptospira*/mL of urine). It has been suggested that renal colonization is responsible for an uneven distribution of *Leptospira* in kidneys.^{3,32} Based on our findings in this mouse model, probably because of the small size of the kidneys and the high loads of *Leptospira*, a single sample taken randomly from either kidney allows a relatively precise

quantification of *Leptospira* concentration, with a standard deviation of roughly half a Log₁₀.

One mouse cleared the infection in less than 2 months, despite initial colonization of the kidneys and transient urinary excretion. Interestingly, this clearance of an adapted *Leptospira* by its typical reservoir host was also reported in *R. norvegicus* experimentally infected with *L. interrogans* serovar Copenhageni.⁹ These individuals probably reflect the inter-individual variability in susceptibility of outbred rodents and probably also occurs in wild populations. Our study shows no association between the concentration of leptospires found in kidney and urine (data not shown). The low number of animals in our study is, however, an important limitation in the statistical interpretation of this result and might explain why we could not confirm this correlation. Indeed, other studies, with a larger sample size, have shown a significant correlation.¹¹ It could be interesting to carry out additional studies with larger numbers to study this possible correlation in mice.

To collect the highest volume of urine, we used 9-month-old adult mice, a relatively old age for experimental infections. In wild populations, infection would probably happen in younger animals, but the life expectancy would be significantly shorter due to competition and predation. According to Drickamer,³⁹ an adult (more than 3.5 month old) mouse's daily urine output is about 2.1 mL for a female and 4.3 mL for a male. We have estimated that an adult mouse that has reached the excretion plateau phase excretes on average 2×10^7 *Leptospira* per mL of urine. Furthermore, our study estimates that ca. 90% of *Leptospira* are live, therefore probably infectious and susceptible to contaminate other mammalian hosts, in theory corresponding to 3.78×10^7 and 7.74×10^7 viable *L. borgpetersenii* Ballum per day for female and male, respectively. In situ wild mice are known to have a shorter lifespan than laboratory animals. Thus, depending on the longevity of the animals in the wild, the maximum excretion at the plateau phase may not be reached by all wild individuals. In addition, the evidence of an initial colonization phase before reaching maximal excretion at the plateau phase suggests that the longevity of rodents in a given environment correlates with the risk of environmental contamination.

This study was performed with virulent *L. borgpetersenii* serogroup Ballum in a mouse reservoir model using the outbred OF1 mice. Although it was increasingly recognized in human leptospirosis in several regions, the serogroup Ballum has rarely been studied in animal models.^{16,40,41} However, *L. borgpetersenii* (notably serovar Hardjo) is regarded as more sensitive to environmental conditions and nutrient deprivation, and is considered to be more frequently associated with direct transmission between hosts.⁴² Therefore, similar experiments could be carried out with other *Leptospira* strains and their respective animal reservoir. Under adequate biosafety conditions,¹⁸ it could notably be interesting to capture and directly investigate wild animals after natural or experimental infection. The methodology and modeling approaches presented in our study provide a possible framework for future studies.

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