Short Report: Highly Virulent *Leptospira borgpetersenii* Strain Characterized in the Hamster Model

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Abstract. A recent study by our group reported the isolation and partial serological and molecular characterization of four *Leptospira borgpetersenii* serogroup Ballum strains. Here, we reproduced experimental leptospirosis in golden Syrian hamsters (*Mesocricetus auratus*) and carried out standardization of lethal dose 50% (LD50) of one of these strains (4E). Clinical disease features and histopathologic analyses of tissue lesions were also observed. As results, strain 4E induced lethality in the hamster model with inocula lower than 10 leptospirae, and histopathological examination of animals showed typical lesions found in severe leptospirosis. Gross pathological findings were peculiar; animals that died early had more chance of presenting severe jaundice and less chance of presenting pulmonary hemorrhages ($P < 0.01$). *L. borgpetersenii* serogroup Ballum has had a considerable growth in human leptospirosis cases in recent years. This strain has now been thoroughly characterized and can be used in more studies, especially evaluations of vaccine candidates.

Leptospirosis occurs throughout the world, especially in tropical settings and developing countries.¹ Both man and animal become accidental hosts through exposure to chronically infected animals, mostly rodents, that shed leptospirae in their urine.² This zoonosis is characterized by a broad spectrum of clinical manifestations, ranging from subclinical infection to Weil's syndrome³ and severe pulmonary hemorrhage syndrome.⁴

Similarly to human disease, leptospirosis in hamsters is acute, with lesions prominent in kidney and liver.⁵ The interstitial nephritis, which results in both acute and chronic kidney damage and loss of renal function as well as pulmonary hemorrhage, has been reported in hamsters, including infections with serovar Icterohaemorrhagiae and Canicola,⁶ serovar Pomona,⁷ and serovar Hardjo.⁸

Experimental infection with *Leptospira interrogans*, *L. noguchii*, and *L. kirschneri* in the hamster model usually runs a fatal course.^{6,9} Our group used hamsters as an experimental model for the study of leptospirosis because of the susceptibility to a broad range of pathogenic strains and reproduction of severe forms of human and animal leptospirosis.⁶ These results made available a panel of five virulent strains for future studies.

Reports on experimental investigation of the course of disease and histopathological findings by *L. borgpetersenii* serogroup Ballum in hamsters are sparse, despite the emergence of this serogroup as an important cause of disease worldwide.^{10–13} In this study, we carried out the additional virulence characterization in the hamster model of one strain of *L. borgpetersenii* serogroup Ballum, and our group reported the isolation, serogrouping, and 16S rRNA gene sequencing.¹⁴ Furthermore, this strain may now be applied in vaccine challenge experiments, comparative virulence studies, and diagnosis.

Leptospirae were cultivated in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Difco, Sparks, MD) at 29°C. Density of the leptospiral challenge strain was determined by dark-field microscopy using a Petroff–Hausser

counting chamber (Fisher Scientific) as described previously.¹⁵ A low passage of *L. borgpetersenii* serogroup Ballum strain 4E, which is a *Mus musculus*-derived isolate,¹⁴ was used as the leptospiral challenge strain in the mid-log phase of growth with density of $\sim 10^8$ cells/mL. This strain was passed two times in hamsters and stored at -70°C . The aliquots were thawed and passed in liquid medium five times before they were used as low-passage isolates in the infection experiments. Serial 10-fold dilutions in liquid EMJH medium were prepared to yield concentrations ranging from 10^8 to 10^0 cells/mL.

Male and female Golden Syrian hamsters (Biotério Central/UFPe) were used. Nine-week-old hamsters were infected with 10-fold serial dilutions ranging from 10^4 to 10^0 organisms. The number of animals for each inocula was eight according to the availability of animal facilities and the recommendation of the Committee for Animal Care and Use (UFPe). Hamsters were inoculated intraperitoneally with 1.0 mL using a 1.0-mL syringe and 22-gauge needle. All animals were monitored daily after infection. Moribund animals were euthanized and necropsied, gross lesions were observed and noted, and kidney tissue was cultured at 29°C in liquid EMJH. Furthermore, during necropsy, tissue samples from liver, kidneys, and lungs were collected and stored in neutral-buffered formalin for posterior histopathological assessment. Animals that survived infection were euthanized on the 25th day after challenge to collect tissues and determine sublethal infection. These animals were submitted to the same post-mortem assays.

Histopathology was carried out by fixing the tissue samples in paraffin and staining with hematoxylin and eosin (HE). The samples were analyzed by a pathologist that was not aware of the tissues precedence, including samples from two non-infected hamsters of the same age euthanized 9 days after inoculation with 1 mL sterile EMJH media. Fisher exact test method and GraphPad Prism 4 software systems (GraphPad Software) were used to perform the statistical analyses and generate the survival curves, respectively. Lethal dose 50% (LD50) was calculated by the recommended method.^{6,16}

Initial clinical symptoms, such as dehydration, piloerection, tremors, and prostration, appeared around the first 4 days post-inoculation (dpi), and the mean period of death was ~ 10 dpi. However, females died between 9 and 14 dpi, whereas

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FIGURE 1. Kaplan-Meier analysis of the results from LD50 experiments. Groups of four hamsters were inoculated with 10⁴, 10³, 10², 10¹, and 10⁰ leptospirae. Survivors were followed for up to 25 days post-infection.

males died 8–18 dpi. The mean LD50 was 5.18 leptospirae, with values of 6.81 and 4.22 for females and males, respectively. Survival curve and days to death data are shown in Figure 1.

In this study, *L. borgpetersenii* 4E produced lethal infection in a high percentage of male and female hamsters. The LD50 given by intraperitoneally inoculation in this experiment was less than 10 leptospirae. Doses of 10⁴ to 10¹ were uniformly lethal to male hamsters, whereas one female hamster that received a dose of 10¹ leptospirae survived. All animals in the 10⁰ group survived to 25 dpi (Figure 1) and did not show any clinical abnormalities.

The gross findings at necropsy were that hamsters infected with *L. borgpetersenii* showed jaundice, pulmonary hemorrhages, and a dark congestive kidney process with varied

intensity according to the inoculum group (full macroscopic analyses not shown). Histopathological lesions detected in the inoculated hamsters also depended on the inoculum group. Table 1 summarizes the main findings in the target organs (kidney, liver, and lung). Animals that sustained high-challenge inoculations (10³ and 10⁴) had less chances of developing extensive macroscopically observable pulmonary hemorrhage than the animals that sustained low-challenge doses (10¹ and 10²; $P < 0.01$). Likewise, animals that were inoculated with high doses seemed more subjected to severe jaundice than others ($P < 0.01$). When microscopic lesions were compared, no statistical difference was observed.

Virulence characterization assays and LD50 determination are important to further study leptospirosis, especially with vaccine trials. There is a great need to develop cross-protective leptospirosis vaccines^{17,18} that may retire current bacterins. To assess cross-protection, standardized strains of different species and serogroups of *Leptospira* are needed, and few are available.^{19–23} This work attempts to fill this void by describing the pathogenesis and LD50 of a *Leptospira* isolate, which is not only highly virulent but also belongs to a serogroup of growing importance in recent years.^{10–13}

Experimental reproduction of leptospirosis was successfully achieved in the hamster model using strain 4E. Furthermore, we describe a thorough standardization of the LD50 and pathological inflections. In 2008, Silva and others⁶ published a similar study using different strains that enabled the execution of several assays, mainly vaccine trials²⁴ and pathogenicity studies.²⁵ The current work, associated with the complete genome sequence of *L. borgpetersenii* serovar Hardjo,²⁶ should allow for the development of studies related to heterologous vaccine trials as well as compare pathogenesis with virulence studies.

The mean LD50 was of 5.18 leptospirae. Compared with previous studies, this strain is similarly virulent to *L. interrogans*

TABLE 1
Macroscopically and microscopically observable findings in necropsied hamsters

Outcomes/inoculums	10 ⁰		10 ¹		10 ²		10 ³		10 ⁴	
	M	F	M	F	M	F	M	F	M	F
Deaths	0/4	0/4	4/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4
Interval of days until death	–	–	11–18	12–14	10–11	10–13	9–10	9–12	8–10	9–11
Macroscopic findings										
Pulmonary hemorrhages	0/4	0/4	4/4	3/4	4/4	3/4	3/4	1/4	3/4	2/4
Jaundice	0/4	0/4	4/4	3/4	4/4	3/4	4/4	4/4	4/4	4/4
Dark congestive kidneys	0/4	0/4	2/4	1/4	4/4	4/4	4/4	4/4	4/4	4/4
Microscopic findings										
Kidney										
Congestion and hemorrhage										
Moderate	3/4	0/4	3/4	4/4	3/4	0/4	1/4	1/4	1/4	1/4
Severe	0/4	0/4	1/4	0/4	1/4	4/4	3/4	3/4	3/4	2/4
Tubular degeneration and necrosis										
Moderate	0/4	0/4	1/4	3/4	3/4	3/4	0/4	1/4	1/4	1/4
Severe	0/4	0/4	2/4	0/4	1/4	1/4	4/4	3/4	3/4	2/4
Glomerular atrophy	0/4	0/4	1/4	1/4	4/4	3/4	4/4	3/4	3/4	3/4
Liver										
Congestion and hemorrhage										
Mild	3/4	3/4	3/4	2/4	2/4	1/4	0/4	0/4	4/4	2/4
Moderate	1/4	0/4	1/4	1/4	2/4	3/4	4/4	4/4	0/4	2/4
Severe	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4
Hepatocyte degeneration and necrosis	0/4	0/4	4/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4
Lung										
Congestion and hemorrhage										
Mild	0/4	1/4	1/4	2/4	1/4	0/4	1/4	0/4	2/4	3/4
Moderate	3/4	0/4	2/4	2/4	2/4	2/4	1/4	3/4	1/4	0/4
Severe	0/4	0/4	1/4	0/4	1/4	2/4	1/4	1/4	1/4	1/4

serovar Canicola strain Kito (2.7 leptospire) and *L. noguchii* serovar Autumnalis strain Bonito (3 leptospire).⁶ It is substantially more virulent than *L. interrogans* strain Fiocruz L1-130 (~80 leptospire), which was widely used because of the availability of the complete genome sequence.²⁷ Highly virulent strains with low LD50 are desirable for simulating the disease in several studies, because it has been argued that high-challenge doses make poor experimental models.²

Strain 4E produced most of the classical clinical and pathological signs of leptospirosis, including but not limited to pulmonary hemorrhages, jaundice, and dark congestive kidneys. Microscopically, it produced mostly classical lesions like those described in naturally infected humans and animals.^{2,28} Furthermore, it was observed in the previous study¹⁴ that strain 4E produced pulmonary hemorrhage, which was associated with leptospirosis acute pulmonary syndrome, the most severe form of leptospirosis.⁴

Another interesting result was the statistically different pathological findings in the comparison among high- and low-dose challenge subjects. Animals that died early (high-challenge dose) had more chance of presenting severe jaundice and less chance of presenting pulmonary hemorrhages ($P < 0.01$) than animals that died late (low-challenge doses) in the experiment. This may indicate that the lungs are compromised later than the kidneys and/or liver when the disease occurs, but high-dose challenge kills before such lesions occur, adding to the claim that high-challenge doses make poor experimental models.² The strain was deposited in the Fiocruz/BA collection under the responsibility of Albert Ko, and it is available to the scientific community.

In conclusion, this work successfully describes the LD50 of *L. borgpetersenii* serogroup Ballum strain 4E. Furthermore, pathological findings revealed additional insights into the pathology of the disease. This strain is now available to be used in additional studies, especially vaccine trials and cross-protection assays.

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