Infectivity and virulence of leptospiral strains of serogroup Sejroe other than Hardjo on experimentally infected hamsters

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Abstract

To assess the virulence of leptospires from the serogroup Sejroe (from ruminants), hamsters were tested against 12 strains. Three Guaricura strains induced severe lethal disease, in contrast to the Hardjo strains. Although with the preliminary outcomes, this finding may be useful for the control of bovine leptospirosis in the Americas, where Guaricura is prevalent.

Keywords *Leptospira* spp. · Experimental infection · Bovine leptospirosis · Chronic infection

Bovine leptospirosis is a disease of great economic importance as it can lead to infertility, estrus repetition, and abortions [1]. Cattle are common renal carriers of leptospires, shedding viable bacteria in the urine and thereby contaminating the environment. Although cattle may harbor and shed many leptospiral strains from different serogroups [2], it is well known that the major agents of bovine leptospirosis worldwide are members of the serogroup Sejroe [3, 4]. Among the Sejroe strains, Hardjo seems to be the most common serovar in the two genotypes Hardjoprajitno (*Leptospira interrogans*) and Hardjobovis (*Leptospira borgpetersenii*) [2, 5]. More recently, another serovar of the same serogroup, named Guaricura (*Leptospira santarosai*), has emerged as a major agent of bovine leptospirosis in the Americas [6].

The physiopathology of reproductive disease first needs to be elucidated. There are many gaps in the knowledge about the host-agent relationship, and studies on experimentally infected animals are limited due to the low virulence of Hardjo strains on biomodels. The most common biomodel for leptospirosis studies is the golden Syrian hamster [7], and Hardjo strains are usually not virulent to that animal [8]. This impairs a variety of studies and compromises a full understanding of the physiopathology of the infection.

In this context, little is known about the virulence of Sejroe strains other than Hardjo. Although apparently predominant, Hardjo strains are not the only ones to infect cattle, and to our knowledge, no other strain of the serogroup Sejroe has been tested for infectivity and virulence on hamsters. The aim of this study is to evaluate the infectivity and virulence on the hamster model of *Leptospira* strains from the serogroup Sejroe obtained from ruminants (mainly bovine) in Brazil.

Leptospira strains ($n = 12$) were isolated from cattle (10), sheep (1), and buffalo (1) from 1967 to 2016 in Brazil. The used strains were classified through the sequencing of *rrs* and *secY* genes and by serogrouping (MAT) using specific polyclonal antisera [6, 9]. The descriptions of the Variable Number Tandem Repeat (VNTR) patterns of the inoculated strains were characterized [10]. These belong to the Collection of Bacterial Cultures of Veterinary Interest (Universidade Federal Fluminense, Brazil, www.labv.uff.br). A virulent *L. interrogans* serovar Copenhageni strain Fiocruz L1-130, provided by Prof. Odir Dellagostin, Federal University of Pelotas in Rio Grande do Sul, Brazil, was used in all experiments as the reference pathogen [11]. All *Leptospira* strains were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco Laboratories) at 29 °C and stored in liquid nitrogen until use. Nine strains presented a maximum of six passages in vitro before the first freezing and three strains (BovG, M04-98, 2012_OV5) presented an unknown number of subcultures, as described in Table 1.

The trials were performed after approval from the animal ethics committee of the Universidade Federal Fluminense (611/2015). Female golden Syrian hamsters (*Mesocricetus auratus*) 6–8 weeks of age and 80–120 g in weight were

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Table 1 Leptospiral strains from the Sejroe serogroup used in the experimental infection in hamsters

Strain	Source	Origin	Species	Serovar	Infectivity		Virulence	Reference
					Blood culture at 4th day	Renal tissue PCR*		
L1-130**	Human	Urine	<i>L. interrogans</i>	Icterohaemorrhagiae		NT	+	11
2015_U349	Cattle	Urine	<i>L. interrogans</i>	Hardjo	–	+	–	2
2014_U140	Cattle	Urine	<i>L. santarosai</i>	Guaricura	–	+	–	6
2014_VF66	Cattle	Vaginal fluid	<i>L. santarosai</i>	Guaricura	+	NT	–	6
U214/2013	Cattle	Urine	<i>L. santarosai</i>	Guaricura	+	NT	–	12
2014_U81	Cattle	Urine	<i>L. santarosai</i>	Guaricura	+	NT	+	6
2014_VF237	Cattle	Vaginal fluid	<i>L. santarosai</i>	Guaricura	+	NT	+	9
2013_VF52	Cattle	Vaginal fluid	<i>L. santarosai</i>	Guaricura	+	NT	+	13
BovG	Cattle	Kidney	<i>L. santarosai</i>	Guaricura	–	–	–	14
M04-98	Buffalo	Urine	<i>L. santarosai</i>	Guaricura	–	–	–	15
2014_U76	Cattle	Urine	<i>L. santarosai</i>	Guaricura	–	–	–	6
2015_U376	Cattle	Urine	<i>L. interrogans</i>	Hardjo	–	–	–	2
2012_OV5	Ewe	Vaginal fluid	<i>L. interrogans</i>	Hardjo	–	–	–	16

* Only conducted on strains that were not recovered from the blood; NT not tested

** Reference strain, used as positive control

purchased from breeding facilities, housed in ventilated racks, and submitted to an adaptation period of 72 h prior to the tests at a temperature of 20–22 °C, a light/dark photoperiod, access to feed and water ad libitum, and an enriched environment. All procedures occurred under a biosafety cabinet [17].

The strains were passed up to four times in hamsters [18, 19] with adaptations. One hamster was used per passage [20] for each strain, with a total of 52 animals. For this purpose, strains were thawed and cultured in liquid EMJH for 7 to 15 days, depending on the isolate. Each strain was inoculated in hamsters only once the culture had reached 10^8 leptospires per milliliter concentration. Each hamster was inoculated intraperitoneally with 1.0 mL of leptospiral culture (10^8 leptospires) using a sterile tuberculin syringe and a 23 3/4 in. gauge needle. Each animal was monitored daily for signs of infection. Four days after inoculation (day 4 post-infection [p.i.]), blood (0.3 mL) was collected by gingival puncture [20] for the recovery of that isolate in EMJH and for confirmation of the infection. Re-isolated strains were serogrouped to confirm their identity [6]. The virulence of the strains in hamsters was estimated qualitatively for up to 21 days p.i., evaluating the severity of pain based on the Mouse Grimace Scale (MGS) [21] and external clinical signs (presence of epistaxis, hematochezia, and hematuria). Euthanasia occurred once pain was considered severe with irreversible clinical signs of acute leptospirosis or on the 21st day p.i. regardless of the animal's clinical condition, according to the Brazilian guidelines of the Council of Veterinary Medicine. For euthanasia, an overdose of anesthetics (100 mg/kg of 10% ketamine and 10 mg/kg of

2% xylazine) administered intraperitoneally was used [19]. When the animal was unconscious with a loss of pedal reflexes, cardiac puncture exsanguination was performed for endpoint and necropsy. At this moment, the kidneys were macerated and used for leptospires culturing for 4 weeks and then examined using dark-field microscopy.

The strains were assessed (i) by their infectivity, given by the recovery of the inoculation strain in the blood of the animal at day 4 p.i. or by PCR positivity of the renal tissue samples, and (ii) by their virulence, given by the ability to determine acute disease and eventually death in the animal inoculated up to 21 days p.i.

The results are presented in Table 1. Out of the 12 tested strains, seven (2013_VF52, 2014_VF237, 2014_VF66, 2014_U81, 2014_U140, U214/2013, and 2015_U349) were recovered by culturing from blood on day 4 p.i. or were detected by renal tissue PCR, and were considered infective to hamsters. The other five strains (two *L. interrogans* serovar Hardjo and three *L. santarosai* serovar Guaricura) were neither recovered by culture nor detected by PCR, and were thus considered non-infective and avirulent.

Among the seven infective strains, four did not lead to acute clinical disease. In contrast, three (2013_VF52, 2014_VF237, and 2014_U81) led to a lethal infection in the hamsters and were considered virulent. The 2013_VF52 strain was fatal since the first passage in vivo and killed the hamsters on day 4 p.i. in all four passages. This strain presented severe clinical signs (ataxia, back arching, tachypnea, and deepening of the abdominal region), and on the fourth passage,

hemorrhage was detected. Necropsy findings include pulmonary petechiae, mild jaundice, hyperemia and enlargement of uterus, and blackened bile and urine.

The virulence of the 2014_VF237 strains increased throughout the passages, killing the hamster only at the fourth passage, presenting similar clinical signs as 2013_VF52, although visible hemorrhage did not occur. The necropsy findings included hemorrhage in the lungs, kidney, and intestines and a friable spleen.

The most interesting outcomes of the present study were the important differences in infectivity and virulence observed among the strains, particularly for those belonged to the same species (*L. santarosai*) and serogroup (Sejroe). Regarding the genotypic characterization, it has been recently reported (Jaeger et al., 2019) that those studied *L. santarosai* strains fit to the same clonal complex, despite the high intraspecific variability observed by MLST. It might be related to the diversity observed also in infectivity and virulence, despite the lack of information enrolled specifically to virulence-associated genes in those strains. These findings reinforce that experimental infections on animal models are mandatory for the determination of virulence of strains.

Hardjo strains are known to be usually not lethal, determining a chronic infection [22], and that finding was confirmed in the present study. In contrast, strains of the serovar Guaricura determined both chronic and lethal infections. Golden Syrian hamsters have been reported as good models for acute lethal infection by several *Leptospira* serovars. The high virulence of three Guaricura strains is a remarkable result, and lesions on hamsters could be comparable with exemplary virulent strains of *L. interrogans*, such as Icterohaemorrhagiae strain L1-130 [23] or Canicola strain LO4 [24]. It is remarkable that despite that difference of the infectivity and virulence among studied strains in hamster model, all original hosts of the virulent strains were cows apparently asymptomatic. In this context, we suggest that the virulence degree observed in hamsters is not related to the disease or renal carrier status in the original host.

Although with the preliminary outcomes and small sampling, the results were consistent during the in vivo passages. Thus, when an alteration of the virulence profile was observed, it was always progressive (increase of the virulence). In this context, we performed a virulence test with significantly reduced animal numbers, considering the 3Rs (replacement, reduction, and refinement). Infectivity and virulence were evaluated from the same animal, when bacterium was recovered from the blood on the 4th day p.i., by evaluation of clinical symptoms up to 21 days of infection, and after euthanasia by renal tissue cultures and urine. In contrast, previous studies have reported [18, 19] the usage of two hamsters per passage during four passages, totaling eight hamsters per strain, twice the number of animals used in our study.

In conclusion, it was observed that leptospiral strains of serogroup Sejroe other than Hardjo, particularly strains of

the serovar Guaricura, may be infective and virulent to the hamster model, determining both chronic and lethal infections. This finding may be useful for experimental studies using hamsters as biomodels and increases the knowledge on the physiopathology of infection by strains of the serogroup Sejroe on livestock.

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Author's contributions CB was involved in the original methodological design of the study. GM was involved in the supervision of the project. CB and GM led the writing of the research article. WL was in charge of supervising the study, and read and approved the manuscript's final version.

References

- Martins G, Lilenbaum W (2017) Control of bovine leptospirosis: aspects for consideration in a tropical environment. *Res Vet Sci* 112: 156–160
- Pinto PS, Pestana C, Medeiros MA, Lilenbaum W (2017) Plurality of *Leptospira* strains on slaughtered animals suggest a broader concept of adaptability of leptospires to cattle. *Acta Trop* 172:156–159
- Pinto PS, Libonati H, Penna B, Lilenbaum W (2016) A systematic review on the microscopic agglutination test seroepidemiology of bovine leptospirosis in Latin America. *Trop Anim Health Prod* 48: 239–248
- Ellis WA (2015) Animal leptospirosis. *Curr Top Microbiol Immunol* 387:99–137
- Chideroli RT, Pereira UP, Gonçalves DD, Nakamura AY et al (2016) Isolation and molecular characterization of *Leptospira borgpetersenii* serovar Hardjo strain Hardjobovis in the urine of naturally infected cattle in Brazil. *Genet Mol Res* 15(1): gmr.15018473
- Loureiro AP, Hamond C, Pinto P, Bremont S, Bourhy P, Lilenbaum W (2016) Molecular analysis of leptospires from serogroup Sejroe obtained from asymptomatic cattle in Rio de Janeiro - Brazil reveals genetic proximity to serovar Guaricura. *Res Vet Sci* 105:249–253
- Haake DA (2006) Hamster model of leptospirosis. *Curr Protoc Microbiol* Chapter 12, Unit 12E.13. doi: <https://doi.org/10.1002/9780471729259.mcl2e02s02>
- Alt DP, Wilson-Welder J (2013) Expansion of the *in vitro* assay for *Leptospira* potency testing to other serovars: case study with *Leptospira* Hardjo. *Biologicals* 41:323–324
- Loureiro AP, Pestana C, Medeiros MA, Lilenbaum W (2017) High frequency of leptospiral vaginal carriers among slaughtered cows. *Anim Reprod Sci* 178:50–54
- Jaeger LH, Loureiro AP, Lilenbaum W (2018) VNTR analysis demonstrates new patterns and high genetic diversity of *Leptospira* sp. of animal origin in Brazil. *Lett Appl Microbiol* 67: 183–189
- Ko AI, Galvão RM, Ribeiro DCM, Johnson WD et al (1999) Urban epidemic of severe leptospirosis in Brazil. *Salvador Leptospirosis Study Group. Lancet* 354:820–825

12. Hamond C, Martins G, Lilenbaum W, Pinna M, Medeiros MA (2015) Infection by *Leptospira* spp. in cattle in a tropical region, Rio de Janeiro, Brazil. *Am J Trop Med Hyg* 92:210
13. Pinto PS, Loureiro AP, Penna B, Lilenbaum W (2015) Usage of *Leptospira* spp. local strains as antigens increases the sensitivity of the serodiagnosis of bovine leptospirosis. *Acta Trop* 149:163–167
14. Santa Rosa CA, Sulzer CR, Castro AF, Yanaguita RM et al (1980) Two new leptospiral serovars in the Hebdomadis group isolated from cattle in Brazil. *Int J Zoonoses* 7:158–163
15. Vasconcellos SA, Oliveira CFJ, Morais ZM, Baruselli PS (2001) Isolation of *Leptospira santarosai*, serovar Guaricura from buffaloes (*Bubalus bubalis*) in Vale do Ribeira, São Paulo, Brazil. *Braz J Microbiol* 32:298–300
16. Director A, Penna B, Hamond C, Loureiro AP, Martins G, Medeiros MA, Lilenbaum W (2014) Isolation of *Leptospira interrogans* Hardjoprajitno from vaginal fluid of a clinically healthy ewe suggests potential for venereal transmission. *J Med Microbiol* 63:1234–1236
17. Barbosa C, Martins G, Lilenbaum W (2018) Atypical virulence of *Leptospira kirschneri* serogroup Icterohaemorrhagiae isolated from capybaras (*Hydrochoerus hydrochaeris*) in hamster model. *Microb Pathog* 126, 134–137
18. Silva EF, Santos CS, Athanazio DA (2008) Characterization of virulence of *Leptospira* isolates in a hamster model. *Vaccine* 26: 3892–3896
19. Suepaul SM, Carrington CV, Campbell M, Borde G, Adesiyun AA (2010) Study on the efficacy of *Leptospira* vaccines developed from serovars isolated from Trinidad and comparison with commercial vaccines using a hamster model. *Vaccine* 28:5421–5426
20. Barbosa CS, Martins G, Lilenbaum W (2016) Blood collection by gingival puncture on hamsters reduces animal number in leptospirosis virulence tests. *ALTEX* 33:322–323
21. Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, LaCroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AMJM, Ferrari MD, Craig KD, Mogil JS (2010) Coding of facial expressions of pain in the laboratory mouse. *Nat Methods* 7:447–449
22. Zuerner RL, Alt DP, Palmer MV (2012) Development of chronic and acute golden Syrian hamster infection models with *Leptospira borgpetersenii* serovar Hardjo. *Vet Pathol* 49:403–411
23. Zilber AL, Belli P, Grezel D, Artois M, Kodjo A, Djelouadji Z (2016) Comparison of mucosal, subcutaneous and intraperitoneal routes of rat *Leptospira* infection. *PLoS Negl Trop Dis* 10: 3e0004569
24. Miraglia F, Morais ZM, Dellagostin OA, Seixas FK et al (2013) Molecular and serological characterization of *Leptospira interrogans* serovar Canicola isolated from dogs, swine, and bovine in Brazil. *Trop Anim Health Prod* 45:117–121

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