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BRIEF REPORT

Standardization of chronic uterine infection of hamsters by *Leptospira santarosai* serovar Guaricura, from serogroup Sejroe

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Abstract

Leptospirosis is an important zoonosis that in cattle is characterized as a reproductive disease. It is well reported that the main agent of bovine leptospirosis worldwide is Sejroe serogroup serovar Hardjo. Reproductive disease in cattle has several gaps in its knowledge and studies with Golden Syrian hamsters, experimentally infected, are limited. Therefore, a protocol that could reproduce the chronic genital disease in hamsters would be extremely valuable for the advance of the knowledge of that syndrome. The aim of this study was to establish an experimental protocol for chronic non-lethal genital infection of female hamsters by *L. santarosai* serovar Guaricura (Sejroe serogroup), strain 2013_VF52. For this, two concentrations (1.0×10^8 leptospires/mL and 1.0×10^4 leptospires/mL) were used intraperitoneally in female hamsters of 06–08 weeks of age. Hamsters that survived for up to forty days after inoculation were euthanized. Uterine and renal tissues were collected to evaluate leptospires' presence by PCR and culture. The protocol demonstrated that 1.0×10^4 leptospires/mL of the strain determined chronic genital leptospirosis in the hamster model. The standardization of a protocol for chronic genital leptospirosis in hamsters can be extremely useful for the understanding of the physiopathology of the infection, as the distribution of leptospires in the uterus and the agent-host interactions.

Keywords Chronic disease · Uterus · hamster · Leptospirosis

Introduction

The Golden Syrian hamster (*Mesocricetus auratus*) is the classic biological model for leptospiral experimental infection, due to its susceptibility to the bacterium and acute clinical presentation (Haake 2006). It has been widely used as a model to study the virulence of various pathogenic strains, mainly those involved in acute severe leptospirosis in humans or animals, such as Icterohaemorrhagiae and Pomona (Zuerner et al. 2012; Miyahara et al. 2014; Ellis 2015; Barbosa et al. 2019a), but not in chronic genital disease. It is known that male hamsters are more susceptible to the infection than females, but it has only been determined for acute renal disease (Gomes et al. 2018).

Traditionally, experimental infection in hamsters is conducted in young animals (3–5 weeks) with a high dose of *Leptospira* inoculum of leptospires (usually 1×10^8 leptospires), in an attempt to reproduce acute disease with a kidney infection. That protocol is particularly useful for studies involving vaccines and determining new strains' virulence (Haake 2006; Miyahara et al. 2014). In contrast, in order to establish a hamster model for chronic infection of the

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reproductive sphere, it is essential to determine a new protocol, including the main variables of inoculum dose and animal age. Herein, we suggest using mature female hamsters, which are hormonally ready to reproduce after five weeks of age (Van Hoosier and Mcpherson 1987).

In contrast to humans and dogs, infection in cattle is usually chronic and silent, being majorly caused by strains of the Sejroe serogroup, such as Hardjobovis and Hardjoprajitno (Ellis 2015). Several strains of the Sejroe serogroup were recovered from natural infection of the bovine genital tract, and are involved in the etiology of Bovine Genital Leptospirosis (Loureiro and Lilenbaum 2020). Besides Hardjo, strains from *L. santarosai* serovar Guaricura have also been frequently reported, mainly in Latin America (Pinto et al. 2016; Loureiro et al. 2016). The 2013_VF52 strain was recovered from bovine vaginal fluid and was identified as *L. santarosai* serovar Guaricura from the Sejroe serogroup (Loureiro et al. 2016). In experimental studies, its virulence was proven, leading to lethality in hamsters in high infecting doses (Barbosa et al. 2019b). Moreover, also its ability to induce chronic genital infection and long-term colonization in sheep was demonstrated (Rocha et al. 2018).

Nevertheless, experimental infections in ruminants are costly, time-consuming, and require facilities that not always are available. Therefore, a protocol that could reproduce the chronic genital disease in hamsters would be extremely valuable for the advance of the knowledge of that syndrome. Apparently, due to the high susceptibility of hamsters to *Leptospira* infection, chronic long-term infection is a difficult goal to achieve, since hamsters usually die a few days after infection. Therefore, the possibility of reproducing chronic genital infection in hamsters and its use for a better understanding of the mechanisms of bovine genital leptospirosis remains a challenge. In this context, the aim of this study was to establish an experimental protocol for chronic non-lethal genital infection of female hamsters by *L. santarosai* serovar Guaricura (Sejroe serogroup).

Materials and methods

In the present study, we aimed to standardize a protocol for chronic infection of female hamsters with *L. santarosai* serovar Guaricura strain 2013_VF52 (strain VF52), from Sejroe serogroup, which was originally isolated from the vaginal fluid of a cow. After reactivation of the virulence (step 1), we proceed to the experimental tests in female hamsters (step 2), as follows:

Bacteria and animals

The studied strain, *L. santarosai* serovar Guaricura strain 2013_VF52 (Sejroe serogroup), herein identified as VF52 strain, was isolated from cattle from a slaughterhouse in the state of Rio de Janeiro/ Brazil (Loureiro et al. 2016). It is maintained in the leptospiral collection of the Laboratory of Veterinary Bacteriology of the University Federal Fluminense – UFF (http://labv.uff.br/ccbvet/). VF52 strain was cultivated for seven days in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium at 29 ºC (Ellis 2015). Bacterial growth was evaluated by dark field microscopy. Bacterial concentration was estimated by counting using a Petroff-Hausser chamber.

This study used nineteen female Golden Syrian hamsters (*Mesocricetus auratus*) of 06–08 weeks of age (CEUA/UFF 4,778,010,519). Animals were acquired from the facility of the Department of Parasitology of the State University of Rio de Janeiro (UERJ) with conventional sanitary standards. The animals were acclimatized for a period of 72 h in the experimental vivarium of the Department of Microbiology and Parasitology of Universidade Federal Fluminense (UFF) in microisolators ventilated racks with *ad libitum* access to rodent food and filtered drinking water, the temperature of 21 ºC (+/-2 ºC) room and photoperiod of 12 h light / 12 h dark.

Experimental infections

Firstly, virulence reactivation in hamsters was performed after thawing and in vitro cultivation of the VF52 strain (which had been frozen immediately after recovery from hamsters). After two passages in EMJH, we inoculated intraperitoneally 1.0×10^8 leptospires in three animals, to constitute the positive control group (PC). Additionally, the other three animals were inoculated with 1.0 mL of EMJH liquid medium without bacterial growth, to constitute the negative control group (NC) (Barbosa et al. 2016, 2019b). After 21 days the animals were euthanized and the inoculated strain was recovered from the kidneys and used as inoculum for the second step.

Secondly, we performed an experiment using a group of hamsters infected with a sublethal dose of VF52. Group A (n = 3) comprised the animals inoculated with the concentration dose of 1.0×10^8 leptospires/mL, herein considered as a positive control for the infection. Group B (n = 7) comprised animals inoculated with the sublethal inoculum dose of 1.0×10^4 leptospires/mL, while hamsters (Group C, n = 3) were used as a negative control and inoculated with liquid EMJH medium. Inoculations were performed intraperitoneally (IP) using a sterile 1 mL syringe, 23-gauge, 3/4-inch needle, in an NB2-class I biological safety cabinet. We

observed animals twice a day for up to 40 days post-inoculation (p.i.) for clinical symptoms, following the parameters of pain according to the Grimace Scale (Mouse Grimace Scale – MGS) (Langford et al. 2010). We performed euthanasia of animals with signals of severe acute leptospirosis. For euthanasia, we used an overdose of ketamine hydrochloride 10% and xylazine hydrochloride 2%, administered intraperitoneally; when the animal stayed unconscious, we performed a cardiac puncture for endpoint and necropsy (Barbosa et al. 2019b).

Sampling and culturing

We performed necropsies inside a containment cabin NB2 - class I for aseptic collection of kidneys and uterus for culture and PCR, and observation of macroscopic lesions in the organs. The right kidney was divided into two longitudinal hemiparts. One hemipart was macerated using a 5.0 mL sterile syringe, deposited in a tube with 5.0 mL of liquid EMJH, homogenized, and left to rest for one hour for sedimentation. After that, we performed a serial dilution, adding 500 µl of the macerated suspension in a second tube with EMJH (dilution of 1: 10), from this to the third tube with EMJH (dilution 1:100), and then to a fourth tube with EMJH (dilution 1:1000). For the other renal hemipart, we also macerated it with the aid of a syringe, stored in Eppendorf tubes, and frozen (-20 ºC) for further performing PCR. We divided the right uterine horn into two longitudinal hemiparts. We cut one part into small fragments using a sterile scalpel blade, deposited them in a tube with 5.0 mL of liquid EMJH, homogenized, and left them to rest for one hour for sedimentation. After, we performed a serial dilution as described above for the kidney. The other hemipart of the right uterine horn was stored in Eppendorf tubes and frozen (-20 ºC) for PCR. Organs were considered positive when positive either on PCR and/or culture. We incubated all culture tubes at 29 ºC for 7 to 30 days and observed weekly for bacterial growth.

Polymerase chain reaction (PCR)

DNA extraction (kidney and uterine samples) was performed using the DNeasy® Blood & Tissue Kit (Qiagen, California, USA), according to the manufacturer's instructions. We used specific primers for the *lipL32* gene, specific for pathogenic leptospires, producing a 240 bp fragment (Hamond et al. 2015). For each test, we used ultrapure water as a negative control, while we used 10 fg of DNA extracted from *L. interrogans* serovar Copenhageni (Fiocruz L1-130) as a positive control. We analyzed the PCR products by electrophoresis in 1.5–2% agarose gel after gel red staining and then visualized them under UV light.

Results and discussion

In the first step, inoculated animals showed inappetence, dehydration, prostration with severe pain, and hematuria on the 4th - p.i. (Table 1); therefore, according to ethical rules, immediate euthanasia was performed. We could identify *Leptospira* by culture and PCR from both uterus and kidneys of those animals. Animals from the negative control group did not show any clinical signs and were negative in all culture/PCR tests (Table 1). Therefore, we confirmed the strain's virulence and ability to establish clinical acute disease in high doses, as expected (Barbosa et al. 2019b).

Secondly, we inoculated hamsters with a sublethal infecting dose of the virulent VF52 strain (Table 2). All animals from the positive control group showed clinical signs of acute disease as described above and were euthanized on the 4th -day p.i. As expected, animals from the negative control group did not show clinical signs and were negative in all culture/PCR tests.

Most importantly, over 40 days, animals from group B, that received a sublethal dose (10^4) of VF52 strain, did not present acute clinical manifestations. Five out of seven animals (71.4%) presented uterine infections in that group. That protocol was therefore considered successful in leading to a chronic non-lethal genital experimental infection in hamsters.

That was certainly the most remarkable result of the study. Female hamsters inoculated with a sublethal dose of the VF52 strain survived for more than the regular period of 21 days and did not show clinical signs that might suggest acute disease. Moreover, uterine infection was demonstrated by culture and/or PCR after 40 days p.i., a time that can be

Table 1 Experimental infection of female hamsters with an acute lethal dose of *L. santarosai* serovar Guaricura strain 2013_VF52.

Hamsters	Dose of inoculum	Euthanasia/day	Clinical Disease	Uterus		Kidney	
				PCR	Culture	PCR	Culture
Control Positive N = 03	1.0ML x 10^8	4th	+	+	+	+	+
	1.0ML x 10^8	4th	+	+	+	+	+
	1.0ML x 10^8	4th	+	+	+	+	+
Control Negative N = 03	1.0 ML EMJH	4th	-	-	-	-	-
	1.0 ML EMJH	4th	-	-	-	-	-
	1.0 ML EMJH	4th	-	-	-	-	-

Table 2 Experimental chronic uterine colonization of mature female hamsters using a sublethal infecting dose of *L. santarosai* serovar Guaricura strain 2013_VF52.

Hamsters	Dose of inoculum	Euthanasia/day	Clinical Disease	Uterus		Kidney	
				PCR	Culture	PCR	Culture
GROUP A N = 03	1.0ML x 10^8	4th	+	+	+	+	+
	1.0ML x 10^8	4th	+	+	+	+	+
	1.0ML x 10^8	4th	+	+	+	+	+
GROUP B N = 07	1.0ML x 10^4	40th	-	-	-	+	+
	1.0ML x 10^4	40th	-	-	-	+	+
	1.0ML x 10^4	40th	-	+	+	+	+
	1.0ML x 10^4	40th	-	+	+	+	+
	1.0ML x 10^4	40th	-	+	+	+	+
	1.0ML x 10^4	40th	-	+	+	+	+
	1.0ML x 10^4	40th	-	+	+	+	+
GROUP C N = 03	1.0 ML EMJH	40th	-	-	-	-	-
	1.0 ML EMJH	40th	-	-	-	-	-
	1.0 ML EMJH	40th	-	-	-	-	-

characterized as a chronic infection in hamsters. Similarly, an experimental infection protocol performed with *L. borgpetersenii* serovar Hardjo strains (strain 203 and JB197) (Zuerner et al. 2012) had an endpoint of 30 days and could establish an asymptomatic chronic disease at concentration 10^9 (strain 203), but without visualization of leptospires in uterine tissue. Still, in the same study, strain JB197 induced acute disease at concentration 10^4 with inconsistent results in uterine tissue (Zuerner et al. 2012). In our study, hamsters inoculated with a high concentration (10^8) showed clinical symptoms of acute severe disease, with the recovery of bacteria from the uterus, but due to the short time after inoculation, septicemia cannot be excluded, and those results may not characterize actual uterine colonization. On the other hand, hamsters inoculated at concentration 10^4 were asymptomatic on physical examination and without macroscopic anatomopathological changes of kidneys for 40 days p.i., and, most importantly, with recovery and detection of leptospires DNA in uterine tissue, what is an indication of chronic uterine colonization.

It is common in the studies of leptospirosis (Santa Rosa et al. 1980; Haake 2006; Zuerner et al. 2012; Ellis 2015) to use young hamsters that are 3 to 4 weeks old with weigh between 50 and 80 g (Charles River Labs 2023), which are animals in the process of developing, both physical and sexual (Van Hoosier and McPherson 1987). Besides, traditionally, Golden Syrian hamsters aren´t used as animal models for chronic leptospirosis (Haake 2006; Miyahara et al. 2014), but the protocol herein described used mature animals with low inoculum doses and was successful in developing the desired chronic genital infection in this model.

Conclusion

In conclusion, we recommend the 1.0×10^4 leptospires/mL protocol as an inoculum dose for virulent strains of genital origin in adult female hamsters. The duration of the infection cycle of 40 days was sufficient to establish uterine long-term chronic colonization. Following that protocol, hamsters can be a valuable biomodel for studying chronic genital leptospirosis, which is the major presentation of the disease in ruminants. The standardization of a protocol for chronic genital leptospirosis in hamsters can be extremely useful for the understanding of the physiopathology of the infection, as the distribution of leptospires in the uterus and the agent-host interactions.

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Declarations

Declaration of competing interest The authors declare that there are no conflicts of interest.

Ethical statement Ethical Committee approved this research for Animal Use at Federal Fluminense University (protocol 4778010519).

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