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# Chronic experimental genital leptospirosis with autochthonous *Leptospira* santarosai strains of serogroup Sejroe



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#### ABSTRACT

Although ruminants may be experimental infected by different leptospiral strains, the majority of them determine acute infection. In contrast, members of serogroup Sejroe seems to be prevalent on chronic infection. Despite the importance of naturally occurring chronic genital infection, the parasite-host long-term relationship in genital tract remains to be elucidated. Few studies succeeded on reproducing the chronic genital infection it on experimental conditions, in this context, this paper aimed to assess the chronic experimental genital leptospirosis with autochthonous *Leptospira santarosai* strains of serogroup Sejroe. The animals were randomly divided into three experimental groups. Each group was inoculated with a different strain of the serogroup Sejroe (FV237, FV52 and U81). On the follow-up of the infection, blood, urine and vaginal fluid collection were performed, as well as the daily follow-up of the animals clinic, and on 30, 60 and 90 days post-infection (p.i.) was performed to collect uterine tissue, uterine lavage and follicular aspirate. There were no clinical signs or haematological alterations in any of the animals, and positive MAT was detected from day 4 p.i. The group in oculated with the FV237 strain showed the highest number of positive samples in the genital tract. The chronic model of leptospiral genital infection could be successfully established, using autochthonous strains that are not often studied. It is of extreme value in order to a better understanding on the pathophysiology of the genital leptospiral infection on ruminants and consequent reproductive disease.

#### 1. Introduction

Leptospirosis is an infectious disease caused by pathogenic spirochetes of the genus *Leptospira*. It affects domestic and wild animals, being also considered a zoonotic disease (Ridzuan et al., 2016). In order to understand the different aspects of leptospirosis, several experimental trials have been performed since the beginning of the XX century (Adler, 2015). Noteworthy to remark that the dawn of the leptospirosis' understanding was only possible after experimental infections in guinea pigs (Inada et al., 1916). After that, several studies focused on the replication of the lethal and acute form of the infection in high susceptible animal models, mainly hamsters (Suepaul et al., 2010; Evangelista et al., 2017).

In contrast to the acute clinical disease of human beings and dogs, leptospirosis in livestock presents as a chronic infection, that leads to reproductive problems such as abortions, early embryonic death and stillbirths, with important economic hazards (Ellis, 2015). Nevertheless,

for a better understanding of that reproductive disease, experimental acute infection on hamsters is of limited value, and experimental chronic infection would be much valuable.

Throughout the 1970s and 1980s, several studies were devoted to investigating the effects of *Leptospira* infection on the genital tract of cattle. In this context, pregnant cows were inoculated by different vias and with different strains. These studies revealed that abortion did not occur in all infected animals, but that leptospires were present in all abortions. Stillbirths and weak calves have also been reported in addition to metritis (Ellis and Michina, 1977; Thiermann, 1982). Besides, most of the time the clinical signs were mild (Nervig et al., 1978; Aycardi et al., 1982).

Although ruminants may be infected by different leptospiral strains, the majority of them determine acute infection. In contrast, members of serogroup Sejroe seems to be prevalent on chronic infection (da S. Pinto et al., 2016). Serovars of this serogroup such as *L. interrogans* Hardjoprajitno and *L. borgpetersenii* Hardjobovis are the most prevalent in

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cattle worldwide, colonizing and persisting in the genital tract of infected cows (Ellis, 2015). Additionally, *L. santarosai* serovar Guaricura, another member of this serogroup, are prevalent on cattle in Latin America (Santa Rosa et al., 1980; Loureiro et al., 2016).

Recent studies have demonstrated the importance of serovar Guaricura in the scenario of the reproductive disease caused by leptospirosis, being this serovar emergent in Latin America, particularly in Brazil (Sarmento et al., 2012). A recent study have reported the dissemination not only of serovar Hardjo but also of other members of the Sejroe serogroup, such as *L. santarosai* serovar Guaricura genotypes. Strains of this serovar were commonly found in urine and vaginal fluid samples from asymptomatic cows in the Rio de Janeiro, Brazil (Loureiro et al., 2016), highlighting that similar silent disease caused by members of serogroup Sejroe in ruminants.

Despite the importance of naturally occurring chronic genital infection, the parasite-host long-term relationship in genital tract remains to be elucidated. Few studies succeeded on reproducing the chronic genital infection it on experimental conditions (Dozsa and Sahu, 1970; Aycardi et al., 1982), and non-Hardjo members of serogroup Sejroe have never been studied. In this context, this paper aimed to assess the chronic experimental genital leptospirosis with autochthonous *Leptospira santarosai* strains of serogroup Sejroe.

#### 2. Material and methods

This research was approved by the Ethical Committee for Animal Use of the Universidade Federal Fluminense (protocol 814/2016) and conducted under the ethical principles of the Sociedade Brasileira de Ciência em Animais de Laboratório.

#### 2.1. Animals and experimental groups

In this experimental trial, six Santa Inês lamb ewes (eight months old) were used. Animals were randomly allocated into three groups (A, B, and C) of two animals each. Sheep had never been vaccinated against leptospirosis, and they were all tested seronegative (titres < 50) and also negative on urine PCR. They were healthy and without hematobiochemical alterations. Trials were carried out at the Experimental Research Unit in Goats and Sheep (UniPECO-UFF), a BSL-2facility designed for leptospiral experimental infections. All measures were taken in order to avoid environmental contamination or cross-infection between groups.

### 2.2. Strains

Three strains (FV52, FV237 and U81) were used on this experiment. All of them belong to the Collection of Bacterial Cultures of Veterinary Interest of the Laboratory of Animal Microbiology (labv.uff.br) and were originally isolated from cows, being FV52 and FV237 isolated from vaginal fluid and U81 from urine. Strains were characterized by molecular methods as *Leptospira santarosai* and by serology as members of serogroup Sejroe (Loureiro et al., 2017). Strains were kept on liquid nitrogen and thawed as recommended (Narduche et al., 2016). In order to reactivate the virulence of strains, *Leptospira* were inoculated in hamsters and recovered from renal macerate in EMJH medium (7–10 days of incubation) after death.

#### 2.3. Experimental infection

All sheep were inoculated by intraperitoneal via with  $1\times10^8$  leptospires (1 mL), as recommended (Nervig et al., 1978). Group A was inoculated with strain FV237, Group B with FV52 and Group C with U81. Throughout the trials, clinical signs (pyrexia, prostration, jaundice, hematuria, dyspnea, polypnea, dehydration, and color of mucosae) were monitored. In addition, serological, molecular, and hematobiochemical tests were performed. The trials occurred during 90 days.

Blood samples were collected daily for 28 days, and urine and vaginal fluid (VF) were collected at D0, D7, D16, D22 p.i. Due to ethics on animal experiments regulation, laparotomy was performed only on days D30, D60 and D90 p.i., after infection was confirmed. On those days, all blood, urine and VF samples were collected, in addition to uterus fragment, uterine fluid and follicular aspirate samples.

Blood was collected by puncture of the jugular vein using sterile needles ( $40 \times 12 \, \text{mm}$ ) and was stored in two vacuum tubes (Vacutainer\*, BD, São Paulo, SP, Brazil). One tube (without anticoagulant) was used for serology and biochemistry, while the other (with EDTA) was used to assess the complete blood count (CBC) and PCR. Serum aliquots were stored in duplicates in 1.5 mL micro tubes at a temperature of  $-20\,^{\circ}\text{C}$  until processing.

Urine samples were collected by natural urination after the intravenous administration of furosemide 5 mg/kg (MSD, São Paulo, SP, Brazil) in conic sterile tubes ( $\sim 15$  mL). In addition, VF was collected by the introduction of sterile swabs (Vi-pak, Copan Diagnostics, Murrieta, CA, USA) into the vaginal fornix and then stored in microtubes (1.5 mL) added with 100  $\mu$ L PBS 1  $\times$  solution (DMPBS, Biodux, São Paulo, SP, Brazil). Importantly, VF samples were always collected before urination in order to avoid contamination. All samples were stored at  $-4\,^{\circ}\text{C}$  until processing.

For laparotomy, sheep were deprived of food for 24 h and water for 12 h. Sedation was induced using acepromazine maleate IV (0.1 mg/kg, Acepran 1%, Vetnil, São Paulo, Brazil), diazepam IV (0.3 mg/kg, Uni-Diazepax, União Química, São Paulo, Brazil) and morphine IM (0.4 mg/ kg, Dimorf, Cristália, São Paulo, Brazil). General anesthesia was induced and maintained using ketamine hydrochloride IV (6 mg/kg Cetamin, Syntec, São Paulo, Brazil). The reproductive tract (uterus, uterine coupons and ovaries) was exposed through longitudinal ventral laparotomy with animals in recumbent dorsal position. Then, a 18gauge IV catheter (BD, New Jersey, USA) was inserted near the uterotubal junction and 40 mL of heated (37 °C) buffered phosphate solution (DMPBS, Biodux, S. Paulo, Brazil) was injected into uterine lumen. This lavage was recovered using a Foley catheter (size 16 Fr) previously inserted into the external bifurcation of the uterine horns and stored in 50 mL sterile conic tubes. The follicular fluid was aspirated using syringes (1 mL) attached to 26 G needles. For the biopsy, a small portion of the uterine body was fixed using forceps and removed by a scalpel blade (n° 15). After each surgical process, all animals were treated with meloxicam IM s.i.d. (0.5 mg/Kg, Maxican, Ouro Fino, São Paulo, Brazil)) for five days.

Antimicrobial therapy with streptomycin IM  $(25\,\text{mg/kg};$  Estreptomax, Ouro Fino, São Paulo, Brazil) for three consecutive days was applied after the last surgery (at D90 p.i.). After treatment, the animals were released to the flock only after exhibiting three consecutive daily negative urinary PCR results.

#### 2.4. Laboratorial procedures

# 2.4.1. Serology

To detect anti-Leptospira antibodies, Microscopic Agglutination Test (MAT) was performed using an antigen panel represented by 28 reference strains (originated from Institut Pasteur, Paris) in accordance with international standards (OIE, 2014). Sera displaying at least 50% agglutinating activity at a 1:100 dilution were considered positive.

## 2.4.2. Polymerase chain reaction (PCR)

Leptospiral DNA from the urine, vaginal fluid, uterine lavage and follicular aspirate samples was extracted by the Wizard SV Genomic DNA Purification System (Promega, Madison, USA). For blood and uterus samples, DNA was extracted by the Qiagen DNeasy Blood & Tissue kit. A PCR targeting the *lip*L32 gene (referred as specific for pathogenic leptospires) was performed as described (Hamond et al., 2014).

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