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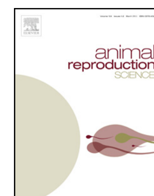

High frequency of leptospiral vaginal carriers among slaughtered cows

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ABSTRACT

Bovine leptospirosis is one of the most important reproductive diseases that compromise the productivity of cattle farming. However, the presence of the agent on vaginal environment is still poorly understood in cattle. Considering this context, the present study aimed to detect the presence of pathogenic *Leptospira* sp. in vaginal fluid (VF) of cows. VF and urine were collected from 254 cows from a slaughterhouse for bacteriological culture and PCR (*lipL32* gene). Overall, eleven pure culture (4.3%) of leptospiral isolates were obtained. Leptospiral DNA was detected in 128 (50.4%) of VF samples and 81 (31.0%) of urine samples, while on 75 (29.5%) it was exclusively in VF and 28 (11.3%) only in the urine. Detection of leptospiral DNA and the recovery of viable leptospires from VF of a high number of cows without apparent symptoms highlight the role of vaginal carriers and indicate that venereal transmission (female-to-male) could occur in that species. Moreover, VF should be encouraged as a valuable sample for diagnosis of bovine genital leptospirosis.

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1. Introduction

Leptospirosis is a worldwide zoonosis determined by pathogenic spirochaetes that belong to the genus *Leptospira* (Adler, 2015). Among many diseases related to reproduction problems, leptospirosis is one of the most common, and compromises the productivity of cattle farming (Martins et al., 2012; Sanhueza et al., 2013).

Bovine leptospirosis is mainly characterized by reproductive disorders, such as infertility, increasing the number of services per conception and prolonged calving intervals, abortion, occurrence of stillbirths and weak offspring (Ellis, 2015). Kidneys are the main site of colonization, what leads to urinary shedding of live leptospires by infected animals, namely carriers (Adler, 2015). However, in relation to the reproductive consequences, even though its pathogenesis is not fully understood, it is believed that after bacteremia, spirochetes can also persist in the genital tract, interfering with embryo implantation (Arent et al., 2013; Ellis et al., 1986; Lilenbaum et al., 2008). Eventually, the agent could cross the placenta invading the fetus, leading it to death and consequent abortion (Plunkett et al., 2013; Subharat et al., 2010).

The presence of leptospires in the genital tract of livestock was first reported in the 1980's (Ellis et al., 1986), in an attempt to establish its association with reproductive losses. Since then, only few studies have addressed this point. In small ruminants, leptospires were isolated from the uterus (Arent et al., 2013) and vaginal fluid (VF) of ewes (Director et al., 2014). Additionally, our group detected leptospiral DNA in vaginal fluid from goats, ewes

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and mares (Director et al., 2014; Hamond et al., 2014a; Lilenbaum et al., 2008), and also demonstrated its presence by immunofluorescence in uterus of mares (Hamond et al., 2015).

Regarding cattle, leptospires were isolated from the oviduct and uterus of cows (Ellis et al., 1986; Ellis and Thiermann 1986) and aborted fetuses (Monte et al., 2015). Moreover, leptospiral antigen was detected by immunofluorescence in cervico-vaginal mucus (Dhaliwal et al., 1996) and placenta (Smyth et al., 1999), and by molecular methods in aborted fetuses (Richtzenhain et al., 2002). Since then, genital tract has been recognized as an extra-urinary site of *Leptospira* infection (Ellis, 2015), but its real role on the epidemiology of the disease and relationship with reproductive losses remains to be better understood.

In light of these studies, it has been suggested that venereal transmission could play an important role on the epidemiology of leptospirosis in small ruminants (Arent et al., 2013; Director et al., 2014; Lilenbaum et al., 2008) as well as in horses (Hamond et al., 2014a). However, the real meaning of the presence of the agent on vaginal environment and its potential transmission is still poorly understood. Considering this context, the present study aimed to detect the presence of pathogenic *Leptospira* sp. in vaginal fluid of cows at slaughter.

2. Material and methods

2.1. Sampling

This study was part of a large project in slaughterhouses conducted in Rio de Janeiro state that aims to evaluate leptospirosis impact on cattle production. From November 2013 to June 2015, samples of vaginal fluid (VF) and urine were collected from 254 cows selected by convenience from a slaughterhouse located 130 Km from Rio de Janeiro, Brazil. Cattle origin and individual history were not provided. All cattle were considered healthy on *ante mortem* clinical examination and none had any apparent clinical signs of disease.

On the slaughter line, prior to evisceration, urine was obtained by direct puncture of bladder. After that, bladder was removed, and a section of vagina (including the cervix) was collected and conserved into labelled sterile plastic bags. From each vagina, three samples of VF were obtained using cytology brushes (Kolplast, Itupeva, SP, Brazil). In order to avoid urine contamination, samples were collected from the vaginal fornix. For molecular analysis, one brush was dipped into a sterile tube containing 3 mL of sodium phosphate buffer 1X (PBS), homogenized, and aliquoted into microtubes, while the two others were reserved for bacteriology. Urine aliquots (2 mL) were distributed into microtubes containing 100 μL of PBS 10X. All the samples were immediately chilled and transported (within approximately three hours) to the laboratory, where they were stored at −20 °C to be tested as a batch.

2.2. Bacteriology

Immediately after collection, few drops of urine were seeded into one tube containing 5 mL of EMJH (BD Difco, Franklin Lakes, NJ, USA), and one tube with 5 mL of EMJH supplemented with antimicrobial cocktail STAFF (EMJH-STAFF; Chakraborty et al., 2011). Each vaginal brush was dipped into one tube of each medium. Tubes were maintained at room temperature and transported to the laboratory. Cultures were incubated at 28 °C and evaluated weekly (dark-field microscopy) for up to 16 wk. Filtration (0.22 μm sterile syringe filter; Millipore Corporation, Billerica, MA, USA) to a new or a subculture in EMJH-STAFF was made if there was apparent contamination by other microorganisms.

2.3. Polymerase chain reaction (PCR) of lipL32 gene

DNA was extracted from VF and urine using the Promega Wizard SV Genomic DNA Purification System® (Promega, Madison, WI, USA). PCR methodology was performed as described by Hamond et al. (2014b). Employed primers were designed by Stoddard (2013) and targeted the *lipL32* gene, which is referred to be present only in pathogenic leptospires. In all reactions, we included negative controls for extraction and reaction to validate the assay. We considered as positive those samples that presented in electrophoresis gel the 240 bp band.

2.4. Serological classification

Microscopic agglutination test (MAT) was performed to determine the serogroup of leptospiral isolates. Isolates were tested against a panel of polyclonal rabbit antisera of 32 reference serovars representing 24 serogroups (provided by Royal Tropical Institute – KIT, Amsterdam). Additionally, rabbit antisera against the local serovar Guaricura (kindly provided by Prof. Silvio Vasconcellos, São Paulo – USP, Brazil), was added to test isolates that presented reactivity against the serogroup Sejroe. High agglutination titres of the tested isolates to particular serum were used to identify the presumptive serogroup of the infective bacterium (Bourhy et al., 2010).

2.5. Genetic characterization

Obtained isolates were classified into genomospecies based on *rrs* sequence comparison. DNA was extracted from recovery isolates by the same method described for PCR of *LipL32* gene. For amplification of partial *rrs* gene the primers LA (5′-GGCGGCGCGTCTTAAACATG-3′) and LB (5′-TTCCCCCCATTGAGCAAGATT-3′) (Mérien et al., 1992) were used. The amplicons were sequenced in both directions using Big Dye Terminator 3.1 cycle sequencing Kit (Life Technologies, Foster City, USA) on ABI 3730XL Genetic Analyzer (Life Technologies, Carlsbad, CA, USA) in DNA sequencing platform RPT01A (Laboratório de Genômica Funcional e Bioinformática IOC/FIOCRUZ). All molecular epidemiological data were stored and analyzed with DNA Star Software Laser gene (Version 5.05; DNASTAR®).

2.6. Statistics

Non-parametric data were analyzed by the Pearson's Chi-square test for evaluating independence between vari-

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