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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.

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-

Table 1
PCR (*lipL32* gene) positivity on urine and Vaginal Fluid (VF) samples collected from 254 asymptomatic cows from a slaughterhouse, Rio de Janeiro, Brazil.

	VF-PCRpos (%)	VF-PCRneg (%)	Total (%)	P	OR	K	CI (95%)
URINE-PCRpos (%)	53 (20.9)	28 (11.0)	81 (31.9)	0.001	2.5	0.20	1.4–4.3
URINE-PCRneg (%)	75 (29.5)	98 (38.6)	173 (68.1)				
Total (%)	128 (50.4)	126 (49.6)	254 (100.0)				

VF – Vaginal Fluid; P – Significance level; OR – "Odds Ratio"; K – Kappa test; CI – confidence interval.

ables and Kappa test for determine agreement. Analysis was conducted using SPSS statistical software (SPSS Inc., Chicago, USA), and results were considered as significant when $P < 0.05$. Odds ratio (OR) was calculated considering a confidence interval (CI) of 95%.

3. Results

3.1. Bacteriology

Overall, eleven (11/254–4.3%) isolates were recovered from eleven cows, collected on different dates. From one cow, the same leptospiral strain was recovered from both urine and VF (2015_VF237; 2015_U237). From four cows, only VF yielded leptospires (2013_VF52; 2014_VF66; 2014_VF116 and 2014_VF190); and from other six, they were recovered exclusively from urine (2014_U65; 2014_U140; 2014_U213; 2014_U222; 2015_U289 and 2014_U291). All the culture positive samples were positive on *LipL32* PCR.

3.2. PCR lipL32 gene

Leptospiral DNA was detected on 81/254 (31.0%) urine samples, while it was observed in 128/254 (50.4%) samples of VF. From that, 53 (20.9%) cows presented PCR positive both in VF and urine, while 28 (11.3%) presented it exclusively in urine, and 75 (29.5%) only in the VF. Thus, 156 (61.4%) cows presented at least one PCR positive result (Table 1). Positivity on PCR results in urine and VF were different ($P < 0.05$), and cows had two times more chances (OR) to present leptospiral DNA on VF than in urine. PCR results in VF and urine were poorly correlated ($k = 0.20$).

3.3. Genetic and serological characterization of obtained strains

Strains 2013_VF52, 2014_VF66 and 2014_U140 were previously described as belonging to *L. santarosai* genomospecies from serougroup Sejroe (Loureiro et al., 2016) and 2014_U65 was previously classified as *L. noguchii* form sg Australis (Loureiro et al., 2015). In the present study, we report for the first time other five *L. santarosai* isolates: two from sg Sejroe, 2015_VF237 and 2015_U237 (from the same cow); two that were not reactive to all anti-sera tested, 2014_U213 and 2014_U222 and one reactive to sg Grippotyphosa, 2015_U291. The 2015_U289 isolate belongs to *L. noguchii* species, classified as sg Panama. Isolates belonging to the species *L. meyeri* (2014_VF116) and *L. alstonii* (2014_VF190) were not reactive to any of the tested antisera, so the serogroup determination was not possible (Table 2).

Table 2
Genetic and serological classification of leptospiral isolates obtained from urine and vaginal fluid (VF) samples collected from asymptomatic cows from a slaughterhouse, Rio de Janeiro, Brazil.

Sample	Strain	Genomospecies	Serogroup (Titre)
VF	2013_VF52**	*L. santarosai*	Sejroe (12,800)
VF	2014_VF66**	*L. santarosai*	Sejroe (12,800)
VF	2014_VF116	*L. meyeri*	NR
VF	2014_VF237	*L. santarosai*	Sejroe (6,400)
VF	2014_VF190	*L. alstonii*	NR
Urine	2014_U65***	*L. noguchii*	Australis (800)
Urine	2014_U213	*L. santarosai*	NR
Urine	2014_U222	*L. santarosai*	NR
Urine	2014_U140**	*L. santarosai*	Sejroe (3,200)
Urine	2015_U237	*L. santarosai*	Sejroe (6,400)
Urine	2015_U289	*L. noguchii*	Panama (12,800)
Urine	2015_U291	*L. santarosai*	Grippotyphosa (3,200)

*NR – not reactive to any of the tested antisera.
*Loureiro et al., 2016.
***Loureiro et al., 2015.

4. Discussion

A high proportion of urinary carriers (Urine-PCR positive) were detected, demonstrating that leptospiral infection appears to be highly disseminated. That rate was similar to other reports, both in slaughtered (Fang et al., 2015) or live animals (Jafari Dehkordi et al., 2016; Otaka et al., 2013). Since microscopic agglutination test presents limitations on detecting chronic animal as carriers, PCR has been increasingly encouraged as an important tool for the diagnosis of leptospirosis and identification of individual carriers on livestock (Hamond et al., 2014b). Nevertheless, the majority of studies employing that tool refer to urine PCR, and only a few of them was directed to vaginal detection of carriers.

The high rate of vaginal carriers (VF-PCR positive) observed on the present study is highly unexpected. Studied cows presented two times more chances to carry leptospires on vagina than on kidneys, and animals that were exclusively vaginal carriers were significantly more frequent than exclusively renal carriers.

The first study that used VF for detecting infected cows employed direct immunofluorescence, with a small number of positive samples (Dhaliwal et al., 1996). Later, that material was first tested by PCR in small ruminants, reporting 43.7% of positive samples (Lilenbaum et al., 2008). More recently, our group has been testing VF of different species by PCR, reporting remarkable findings on mares (32%, Hamond et al., 2014a; 44.7%, Hamond et al., 2015) and on ewes (33%, Director et al., 2014). VF is simple to collect from live animals and may represent an extremely valuable sample to be tested for detection of vaginal carriers. It has been widely used for diagnosis of other reproduc-

tive diseases, e.g. Campylobacteriosis and Trichomoniasis (Michi et al., 2016) with reliable results, and its usage for leptospiral diagnosis should be encouraged.

One cannot exclude the possibility that leptospires (or its DNA) detected in VF had renal origin, originated by urine's contamination prior to slaughter. Nevertheless, there was only a weak correlation between positivity on VF and urine. Additionally, in order to minimize that bias, all the VF samples were collected from vaginal fornix and after bladder removal. Those points suggest that leptospires (or its DNA) may be present in VF independently of urinary contamination. Similar findings were recently reported in mares, and VF-PCR results were strongly associated to uterine fluid-PCR rather than urine-PCR (Hamond et al., 2015).

It has been suggested that presence of specific immunoglobulins anti-*Leptospira* in the VF of heifers indicates that these organisms may present on the vagina of cattle, for an unknown period of time (Dhaliwal et al., 1996). Moreover, some host-pathogen features such as biofilm production by pathogenic leptospires (Ristow et al., 2008) and low levels of lactobacilli forming cowís vaginal microbiota resulting in pH near-neutral (7.30 ± 0.63) (Beckwith-Cohen et al., 2012; Swartz et al., 2014) could be associated with this presence of leptospires on vaginal mucosa.

Venereal transmission of leptospirosis by contaminated semen is well established. Since bulls are often sub-clinically infected, they are considered as important sources of infection and male-to-female transmission is well recognized in the epidemiology of the infection (Ellis et al., 1986; Heinemann et al., 2000). In that context, the role of vaginal infection and female-to-male transmission has been neglected. In the present study was detected vaginal carriers of pathogenic viable leptospires by culture and a high proportion of carriers by PCR in VF among cows. Those facts, associated to the well-known possibility of active penetration of leptospires via mucous membranes (Ellis, 2015), reinforce the hypothesis that cows could transmit leptospires to bulls during natural mating.

It is well known that bacterial culture is the gold-standard for diagnosis of leptospirosis, and given the fact that those bacteria were live and viable on VF confirms the evidences that were previously suggested by direct immunofluorescence and PCR (Dhaliwal et al., 1996; Lilenbaum et al., 2008). Regarding the five isolates recovered from VF, it is remarkable that three of them belong to serogroup Sejroe, while the other two could not be classified. Members of this serogroup, as Hardjo, are widespread in cattle worldwide, and Hardjo strains are referred as major agents of reproductive failure due to chronic bovine leptospirosis (Ellis, 2015). Besides, the only leptospiral isolate ever recovered from VF (from an asymptomatic ewe) was also a member of that serogroup (Director et al., 2014). In a classical study, a high percent of isolates was obtained from other parts of the genital tract of cows, and Hardjo was the most common strain; thus, the authors suggested a possible tropism of Hardjo to that location (Ellis et al., 1986). It is important to highlight that in the present study the obtained Sejroe strains belong to *L. santarosai* genomospecies, and most probably to serovar Guaricura. Genotypes of Guaricura, a local *L. santarosai* member of serogroup Sejroe, were reported as highly frequent on bovines in Brazil (Loureiro et al., 2016). However, its pathogenicity and role on genital leptospirosis has not yet been established.

5. Conclusion

Detection of leptospiral DNA of a high number of cows and the recovery of viable pathogenic leptospires (mainly of Sejroe serogroup) from VF highlight the extensive 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.

Author contributions

We declare that all authors have made substantial contributions to the research and manuscript as follows: Loureiro and Lilenbaum contributed to the conception and design of the experiments, data interpretation and analysis; Pestana and Medeiros contributed to molecular analysis; Lilenbaum and Medeiros contributed to the final revision of the manuscript.

Conflicts of interest

No conflict of interest declared.

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