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REGULAR ARTICLES

High frequency of genital carriers of *Leptospira* sp. in sheep slaughtered in the semi-arid region of northeastern Brazil

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Abstract
Although some studies in sheep have indicated leptospire colonization of the genital tract, further studies are needed to clarify the role of genital carriers in this species. Thus, this study aimed to evaluate the colonization of pathogenic leptospires in the genital and urinary tract of slaughtered sheep. Fifty-seven adult, female woolless sheep destined for slaughter were used. Renal ($n = 57$), bladder ($n = 57$), ovary ($n = 34$), uterine tube ($n = 44$), and uterus ($n = 33$) samples were collected for molecular detection of *Leptospira* sp. DNA, and blood samples ($n = 57$) for serological testing. The molecular testing was performed using polymerase chain reaction (PCR), and the serological testing was performed using microscopic serum agglutination test (MAT). Samples with amplifying DNA were subjected to genetic sequencing. In total, leptospiral DNA was found in the tissues of 44 (77.2%) sheep, whereas only nine animals were positive on both PCR and MAT; there was slight agreement between PCR and MAT techniques ($k = 0.0268$; $p = 0.684$). In 61 (54.9%) genital tract and in five (4.4%) urinary tract samples, the leptospiral DNA was detected, with significant difference ($p < 0.001$). The genes of one sample from the uterine tube and another from the bladder were sequenced and demonstrated 99% similarity to *Leptospira interrogans*. Anti-*Leptospira* antibodies were detected in 11 (19.3%) of the tested animals. The results reinforce the importance of the genital tract as an extra-renal site of colonization, suggesting the possibility of venereal transmission in sheep.

Keywords Sheep · Leptospirosis · Genital tract · Venereal transmission

Introduction

Leptospirosis is a zoonotic disease distributed worldwide (Adler 2015; Ellis 2015) and is considered one of the main pathologies that cause economic losses in small ruminants (Suepaul et al. 2011). The disease is usually associated with clinical cases of abortion, birth of weak offspring, recurrence of estrus, and decrease in milk production (Ellis 2015). Its dissemination is related to environmental factors and the presence of rodents and wild animals (Adler and Moctezuma 2010). Sheep may manifest leptospirosis in acute, subacute, and chronic forms. The chronic form is the most described, since it is responsible for high economic losses (Ellis 2015).

The diagnosis of leptospirosis is usually performed by the microscopic agglutination test (MAT), a serological test that uses live cultures of *Leptospira* sp. as antigens and are preferably obtained from local isolates (Martins and Lilenbaum 2014). Although widely used as a diagnostic method at the herd level, the absence of detectable levels of serum antibodies in some animal species makes indirect methods less sensitive (Ellis 2015). Thus, the principle of identification of carrier animals is performed by isolating the agent or detecting its DNA in urine and other tissues (Lilenbaum et al. 2008).

Although studies have described the colonization of *Leptospira* sp. in the urinary tract, information on the colonization of the bacteria in the genital tract of sheep is scarce. However, some studies have indicated the presence of leptospiral DNA in samples of vaginal fluid and uterine tissue, emphasizing the importance of this tract as an extra-renal site of infection (Lilenbaum et al. 2008; Arent et al. 2013; Director et al. 2014a). Thus, in order to obtain more information, the

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present study aimed to evaluate the colonization of *Leptospira* sp. in the genital and urinary tracts of sheep destined for slaughter.

Material and methods

This research was approved by the Research Ethics Committee (REC) of the Universidade Federal de Campina Grande (UFCG) under number 58/2012.

Study area and sample collection

The study was carried out at the municipal slaughterhouse of Patos, Sertão mesoregion of the state of Paraíba, located in the semi-arid region of northeastern Brazil (latitude: 07° 01′ 28″ S; longitude: 37° 16′ 48″ W). From 2012 to 2015, 57 adult, female woolless sheep, destined for slaughter, were used. Blood samples were obtained from the external jugular vein, just before the slaughter, using properly identified 9-mL sterile vacuum tubes. After the serum was collected, it was stored in microtubes at − 20 °C until the serological testing was performed. Samples of renal tissue ($n = 57$), bladder ($n = 57$), ovary ($n = 34$), uterine tube ($n = 44$), and uterus ($n = 33$) were collected. One-gram fragments were used for the bacteriological processing, and 5-g fragments were used for the molecular testing. The samples were frozen at − 20 °C until processing.

Bacterial isolation

The samples of renal tissue, bladder, ovary, uterine tube, and uterus were macerated using sterile pistils to form a 10% (weight/volume) suspension in sterile Sorensen buffered saline solution. From this suspension, 0.5 mL was inoculated in 5 mL of semi-solid EMJH (Difco, BD, Franklin Lakes, NJ, USA), supplemented with the antibiotics amphotericin B (0.05 mg/mL), 5-fluorouracil (1 mg/mL), fosfomycin (4 mg/mL), trimethoprim (0.2 mg/mL), and sulfamethoxazole (0.4 mg/mL) (Chakraborty et al. 2011). The inoculated tubes were then stored at 28 °C in a BOD incubator chamber. After a 24-h period in the antibiotic-enriched medium, the cultures were serially diluted (10^{-1}, 10^{-2}, 10^{-3}) in Fletcher semi-solid medium (Difco, BD, Franklin Lakes, NJ, USA), with 5-fluorouracil (1 mg/mL), and incubated at 28 °C. The cultures were examined weekly for the presence of microorganisms with similar morphology to *Leptospira* sp. for 12 weeks.

Serological testing

The presence of anti-*Leptospira* antibodies was determined by MAT, using a collection of 24 pathogenic serogroups, as recommended by the World Organization for Animal Health (OIE 2014). All samples with agglutinating activity at the 1:100 dilution were considered positive and then serially titrated by a ratio of two. The highest titer obtained was used to identify the infecting serogroup.

Molecular detection

DNA was extracted from the tissues using the Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. The polymerase chain reaction (PCR) was performed as previously described (Hamond et al. 2014). The primers *LipL* 32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and *Lip L* 32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′), which were designed by Stoddard et al. (2009), were used to amplify the LipL32 gene, which is specific for pathogenic leptospires. The *L. interrogans* serogroup Pomona serovar Kennewicki was used as the positive control, and ultrapure water was used as the negative control.

Nucleotide sequencing and phylogenetic analysis

Sequencing reactions were performed using the LipL32-45F and LipL32-286R primers (Stoddard et al. 2009) with the Big Dye Terminator v3.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For capillary electrophoresis, a 3130xl Genetic Analyzer and POP-7 polymer (Platt et al. 2007) were used. Sequence alignment was performed using BioEdit (Gouy et al. 2010), while dataset sequences were obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) (http://www.ncbi.nlm.nih.gov), using BLAST tool http://www.ncbi.nlm.nih.gov/BLAST/. Phylogenetic analysis was performed using the Seaview4 software package (Hall 1999), and the phylogenetic tree was constructed using the Neighbor-Joining model, with a bootstrap value of 1000 replicates (http://tree.bio.ed.ac.uk/software/figtree/, visualized through FigTree v1.4.3 (http://tree.bio.ed.ac.uk/)). The phylogenetic reconstruction included *Leptospira* sequences for comparison.

Table 1 Percentage of positive animals according to the diagnostic method used in sheep slaughtered in northeastern Brazil

MAT	PCR		Total
	Positive	Negative	
Positive	9 (15.8%)	2 (3.5%)	11 (19.3%)
Negative	35 (61.4%)	11 (19.3%)	46 (80.7%)
Total	44 (77.2%)	13 (22.8%)	57 (100.0%)

Table 2 Samples in which leptospiral DNA was amplified in the molecular evaluation and sequencing in sheep slaughtered in northeastern Brazil

Tissue	Total number of samples	Number of positive animals	Frequency (%)	Sequencing
Uterus	33	20	60.3	–
Ovary	34	19	55.9	1
Uterine tube	44	22	50.0	–
Kidney tissue	57	3	5.3	1
Bladder	57	2	3.5	–

Statistical analysis

Chi-square test with Yates continuity correction was used to compare the proportions of positive samples in the PCR of the genital and urinary tracts. To evaluate the agreement between the results of the MAT and PCR techniques, the Kappa indicator was calculated. The analyses were done using BioEstat 5.03 (Ayres et al. 2007), considering a significance level of 0.01.

Results

On PCR, leptospiral DNA was found in the tissues of 44 (77.2%) sheep, whereas only nine animals were positive on both PCR and MAT, as illustrated in Table 1. According to the Kappa indicator, there was slight agreement between PCR and MAT techniques ($k = 0.0268$; $p = 0.684$). As shown in Table 2, leptospiral DNA was found in 61 (54.9%) samples of the genital tract (uterus, ovary, and uterine tube) and five (4.4%) urinary tract samples (bladder and kidney tissue), with a significant difference between proportions ($p < 0.001$). In the genital tract, leptospiral DNA was found in 22 (50%) uterine tubes, 19 (55.9%) ovaries, and 20 (60.6%) uteri. In the urinary tract, leptospiral DNA was found in three (5.3%) of the renal tissue samples and two (3.5%) bladder samples. The genes from one sample of uterine tube and another of bladder were sequenced and were 99% similar to *L. interrogans* in BLAST (Fig. 1). As shown in Table 3, anti-*Leptospira* antibodies were detected in 11 (19.3%) of the tested animals, and *Icterohaemorrhagiae* (54.5%) was the most frequent serogroup, followed by *Javanica* (18.2%), *Grippotyphosa* (9.1%), *Australis* (9.1%), and *Hardjo* (9.1%), with titers ranging from 1:100 to 1:800.

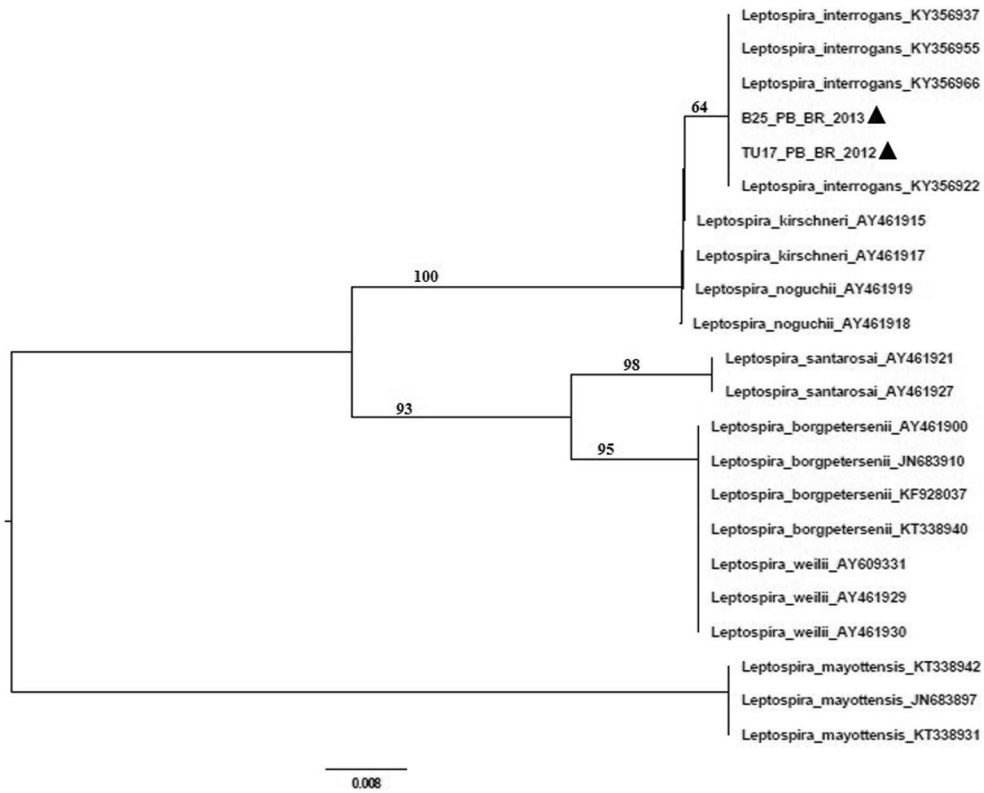

Fig. 1 The phylogenetic tree was constructed by the Neighbor-Joining test, using the Kimura 2-parameter method. The analysis included 231 nucleotide sequences, with 1000 replicates in the bootstrap test. Triangle, sequenced samples

Table 3 The most prevalent serogroups and their respective antibody titers in sheep slaughtered in northeastern Brazil

Serogroup	Antibody titer		Total (%)
	1:100	1:200	
Icterohaemorrhagiae	4	2	6 (54.5)
Javanica	2	–	2 (18.2)
Hardjo	1	–	1 (9.1)
Grippotyphosa	1	–	1 (9.1)
Australis	–	1	1 (9.1)
Total (%)	8 (72.8)	3 (27.2)	11 (100)

Discussion

This study demonstrated that leptospires colonized all tissues tested, especially the genital tract. Thus, we must consider this tract as an important extra-renal site of infection, as well as the possibility of venereal transmission, as previously described (Lilenbaum et al. 2008; Arent et al. 2013; Director et al. 2014a). In ruminants, colonization of the genital tract interferes with implantation of the embryo and other gestational events (Ellis 2015), compromising the sustainability of the production chain. Moreover, the bacteria present in the genital tract may suggest venereal transmission in the species, which may allow the disease to be endemic in herds (Lilenbaum et al. 2008).

Despite the natural resistance to infection attributed to sheep (Martins and Lilenbaum 2014), leptospiral DNA was detected in 77.2% of the animals, which is a higher percentage than that found in studies with sheep in other Brazilian regions (Lilenbaum et al. 2008; Arent et al. 2013; Martins and Lilenbaum 2014), as well as previous studies in the semi-arid region (Alves et al. 2012; Costa et al. 2016). The semi-arid region of Brazil has experienced intense drought in recent years (INSA 2015), hindering indirect transmission of the bacteria by contaminated water. Thus, carrier sheep may be responsible for maintaining the disease in the herds.

Statistically, there was no agreement between the techniques used for the detection of positive animals. Other authors found similar results in comparing serological and molecular techniques (Director et al. 2014b; Costa et al. 2017), thus emphasizing the need for the association of direct and indirect diagnostic methods for the reliable detection of positive animals.

Amplified leptospiral DNA detected *L. interrogans*. Serology, in turn, revealed that most of the animals were reactive for the *Icterohaemorrhagiae* serogroup, belonging to *L. interrogans* (Adler and Moctezuma 2010). Previous serological investigations carried out in the semi-arid region showed agglutinating reactions for the *Icterohaemorrhagiae* serogroup (Higino et al. 2010; Costa et al. 2016). This finding is quite relevant, given that *L. interrogans* consists of various serogroups that cause acute disease in sheep (Adler and Moctezuma 2010; Martins and Lilenbaum 2014). These results, in addition to the results obtained in previous study detecting *L. interrogans* in ovine uterus culture (Arent et al. 2013), support the hypothesis that *L. interrogans* can infect uterine tissues in sheep, as already demonstrated for cows (Pires et al. 2017).

Conclusion

The results revealed that pathogenic leptospires affect the reproductive tract of sheep. This finding emphasizes the importance of this tract as an extra-renal site of colonization and suggests the possibility of venereal transmission in sheep.

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Compliance with ethical standards

This research was approved by the Research Ethics Committee (REC) of the Universidade Federal de Campina Grande (UFCG) under number 58/2012.

Competing interests The authors declare that they have no conflict of interest.

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