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Leptospirosis in native mixed-breed sheep slaughtered in a semiarid region of Brazil

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ABSTRACT: Sheep breeding is an economic activity of great value for the Brazilian livestock industry, and infectious diseases can significantly compromise its productivity. The aim of this study was to detect *Leptospira* spp. in mixed breed sheep from a semiarid region of the State of Paraíba, Northeast Brazil. In total, 49 blood and renal tissue samples, as well as 25 urine samples, were collected from 49 sheep in the slaughter line. The microscopic agglutination test (MAT) was used to verify the presence of anti-*Leptospira* spp. antibodies in blood samples. Renal tissue and urine samples were analyzed by both PCR and bacterial culture. Sero reactivity was observed in 8.2% (4/49) of animals. Three renal samples were positive for leptospiral DNA by PCR although all urine samples were negative. No leptospires in pure culture were recovered from the samples tested. Low prevalence of *Leptospira* spp. observed by serology and PCR suggested that the rusticity of the mixed-breed sheep may have contributed to the low occurrence of leptospirosis in the semiarid region investigated.

Key words: leptospirosis, small ruminants, PCR, serology, isolation, slaughterhouse.

Leptospirose em ovinos mestiços abatidos em uma região semiárida do Brasil

RESUMO: A criação de ovinos é uma atividade econômica de grande valor para a indústria pecuária brasileira. As doenças infecciosas podem comprometer significativamente a sua produtividade. O objetivo deste estudo foi detectar *Leptospira* spp. em ovinos sem raça definida a partir de uma região semiárida do estado da Paraíba, Nordeste do Brasil. Quarenta e nove amostras de sangue e tecido renal, bem como amostras de urina, de 25 das 49 ovelhas, foram coletados na linha de abate. A presença de anticorpos anti-*Leptospira* spp. foi verificada com o teste de aglutinação microscópica (SAM). As amostras de urina e rins foram processadas por PCR e cultura bacteriológica. Sororreatividade foi observada em 8,2% (4/49) dos animais. Em relação à PCR, três amostras renais foram positivas, no entanto, todas as amostras de urina foram negativas. Nenhuma cultura pura de leptospiros foi recuperada a partir das amostras testadas. Levando em consideração a baixa frequência de positividade na sorologia e PCR, é possível sugerir que a rusticidade das ovelhas de raças mestiças colaborou para a baixa ocorrência de leptospirose na região semiárida investigada.

Palavras-chave: leptospirose, pequenos ruminantes, PCR, sorologia, isolamento, matadouro.

INTRODUCTION

Sheep breeding is an economic activity of great value for the Brazilian livestock industry, mainly for the Northeast region, which is situated in an area possessing more than 56% of semiarid climate (GIRARDI & ROSA, 2011). Since sheep breeding is predominantly developed for families' income generation and subsistence, infectious diseases are an important factor contributing to low productivity (MARTINS et al., 2012). Among these, leptospirosis is relevant as it is widely disseminated and causes abortions, stillbirths or birth of weak offspring, as well as a decrease in milk production (HIGINO & AZEVEDO, 2014; ELLIS, 2015).

Leptospirosis diagnosis in sheep flocks is still a challenge. In livestock, this disease is predominantly subclinical with non-specific symptoms (MARTINS & LILENBAUM, 2014). Although the microscopic agglutination test (MAT) is the serological test recommended by the World Organization for Animal Health (OIE, 2014), it is not a valuable tool in the detection of carriers. It has been suggested that MAT could be employed as a screening test for leptospirosis in herds in combination with polymerase chain reaction (PCR) for carrier detection (MARTINS & LILENBAUM, 2014). Several surveys conducted in the Brazilian semiarid region showed seropositive prevalences

for ovine leptospirosis ranging from 3.5% to 8.2% (AZEVEDO et al., 2004; COSTA et al., 2016).

The occurrence and amplitude of leptospirosis in sheep herds are closely linked to environmental factors such as rainfall index, humidity, topography, presence of rodents, wild animals, and consociated animal rearing (ALVES et al., 1996). In small ruminants, breed has also been found to be a risk factor associated with leptospirosis (SALABERRY et al., 2011; SANTOS et al., 2012; SILVA et al., 2012). Therefore, the purpose of this study was to detect *Leptospira* spp. in mixed-breed sheep from a semiarid region of the state of Paraíba in the Northeast region of Brazil.

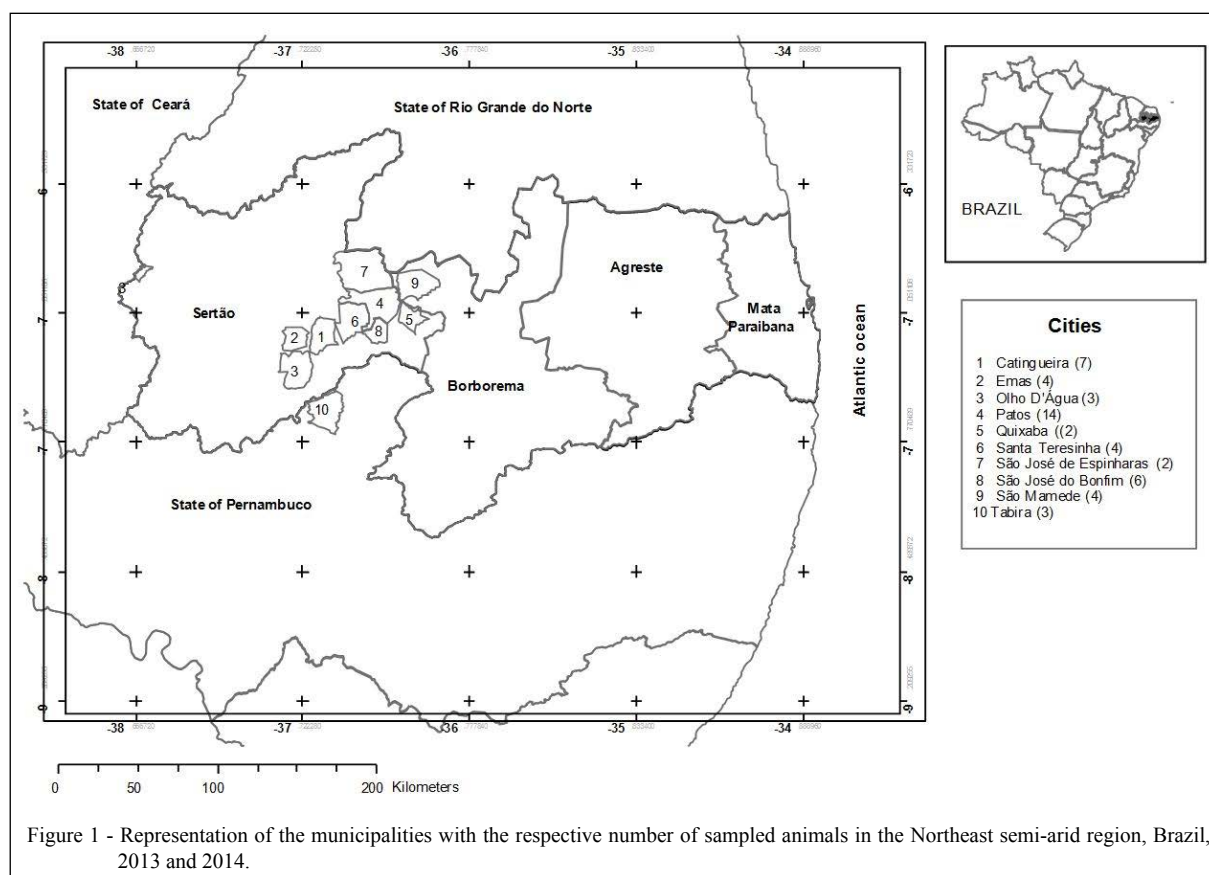
MATERIALS AND METHODS

The study was carried out in a slaughterhouse situated in the municipality of Patos (07°01'28" S; 37°16'48"W), Sertão mesoregion of the state of Paraíba, a semiarid region in northeast Brazil. The study population was constituted of adult female sheep from 10 flocks from 10 municipalities

(one flock per municipality) in the states of Paraíba and Pernambuco (Figure 1).

In order to include both rainy and dry seasons, sample collection was carried out from October 2013 to August 2014. A total of 49 renal and blood samples, as well as 25 urine samples, were collected from 49 sheep. Urine samples could not be collected from the remaining 24 animals because of their empty bladders. Blood samples were collected in bleeding time, using sterile 8mL blood collection tubes, centrifuged, and sera obtained was stored at -20°C to be tested as a batch. One kidney from each sheep was sent to a laboratory, where a section of 5g was stored at -20°C for DNA extraction and another section of 1g was sent for bacterial testing. Urine samples were collected by cystocentesis with sterile syringes during evisceration. Immediately after collection, 1mL of urine was seeded onto culture media and further aliquots of 5mL were stored at -20°C for DNA extraction.

Renal samples were aseptically macerated using sterile syringes and then inoculated in semisolid EMJH medium (Difco, BD Franklin Lakes, NJ,



USA) enriched with Amphotericin B ($0,05\text{mgmL}^{-1}$), 5-fluorouracil (1mgmL^{-1}), fosfomicin (4mgmL^{-1}), trimethoprim ($0,2\text{mgmL}^{-1}$), and sulfamethoxazole ($0,4\text{mgmL}^{-1}$) to inhibit microbial contaminant growth, so that the final concentration of urine and renal inoculated samples was 10%. After inoculation, the tubes were transported to the laboratory at room temperature. After 24 hours, the tubes were submitted to serial dilution (10^{-1} , 10^{-2} , 10^{-3}) in Fletcher semisolid media (Difco, BD, Franklin Lakes, NJ, USA) with the addition of 5-Fluorouracil (1mgmL^{-1}), and incubated at 28°C to 30°C . The tubes were examined weekly using dark field microscopy for a period of six weeks.

Molecular analysis was performed at the Laboratório de Bacteriologia Veterinária (LABV), Universidade Federal Fluminense (UFF), Niterói, Brazil. DNA extraction of *Leptospira* spp. was carried out using the Wizard® Genomic SV DNA Purification System kit (Promega®, Madison, EUA). PCR was performed as described previously by HAMOND et al. (2014). Primers *lip* L32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3') and *Lip* L32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3') (STODDARD et al., 2009), were used to amplify the *lip* L32 gene, which is specific for pathogenic leptospires. *Leptospira interrogans* serovar Copenhageni Fiocruz L1-130 strain was used as a positive control and ultrapure water as a negative control.

MAT was carried out as recommended (OIE, 2012). Blood samples were screened at a 1:100 dilution using a panel of live antigen strains provided by the LABV-UFF. The antigen panel included *L. biflexa* serovars Andamana and Patoc; *L. interrogans* serovars Australis, Copenhageni, Bataviae, Bratislava,

Canicola, Grippotyphosa, Hardjoprajitno, Pomona, Pyrogenes, Ictero haemorrhagiae, Hebdomadis, Wolffi, and Butembo; *L. borgpeterseni* serovars Autumnalis, Castellonis, Hardjobovis, Javanica, and Tarassovi; *L. santarosai* serovars Guaricura and Shermani; *L. kirschneri* serovar Cynopteri; and *L. noguchii* serovar Panama. All samples showing agglutination at a dilution of 1:100 (cut-off point) were considered positive, and were then titrated in 2-fold serial dilutions. The highest titer reached was used to identify the infecting serogroup.

RESULTS

Four animals (8.2%) were sero reactive by MAT. The most common serovars were Javanica (75%) and Autumnalis (25%), with titers from 100 to 800. No pure cultures of *Leptospira* spp. were obtained. The *lip* L32 gene could only be amplified in three renal tissue samples (6.1%). All 24 tested urine samples were negative for leptospiral DNA (Figure 2). Of the three sheep positive by PCR of renal samples, two were also seroreactive by MAT, both for serovar Javanica, with titers of 200 and 800. All PCR positive animals belonged to the same flock from the Tabira municipality (Table 1).

DISCUSSION

Abattoirs are considered an excellent source for collecting samples for surveillance of several infectious diseases, and several studies on leptospiral isolation have previously been conducted there (SILVA et al., 2007; HIGINO et

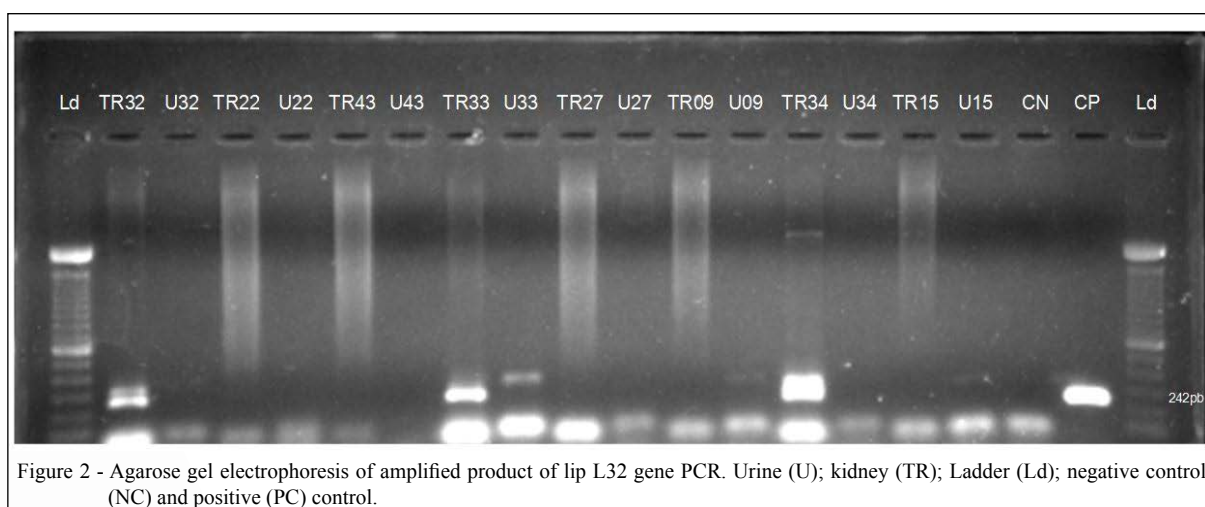


Figure 2 - Agarose gel electrophoresis of amplified product of *lip* L32 gene PCR. Urine (U); kidney (TR); Ladder (Ld); negative control (NC) and positive (PC) control.

Table 1 - Results of serology, polymerase chain reaction (PCR), and bacterial culture of positive animals, according to their origin.

Origin	-----Serology-----		PCR	Bacterial Isolation
	Serovar	Titer		
Patos	Autumnalis	100	–	–
São Mamede	Javanica	100	–	–
Tabira	Javanica	800	+	–
Tabira	Javanica	200	+	–
Tabira	–	–	+	–

al., 2010). *Leptospira* spp. detection from animal clinical samples is still challenging, mainly due to the slow growth of leptospires and overgrowth of co-existing microorganisms from environmental or normal microbiota. Besides, strictly aseptic methods of collection and sample manipulation are rarely possible under field conditions. Given the limitations of bacterial culture in the identification of leptospiral carriers, PCR is considered an important diagnostic tool (DIRECTOR et al., 2014). In the present study, despite sample storage at -20°C that could have reduced the diagnostic sensitivity of PCR, the latter technique proved to be more effective in the detection of the agent than bacterial culture. Additionally, PCR was able to detect a carrier among the seronegative animals. This outcome supports the recommendation of combined use of MAT and PCR for a more efficient diagnosis of leptospirosis (OTAKA et al., 2013).

The present study reported a lower prevalence of seroreactive animals than that observed in other Brazilian regions, such as 34.3% in the South (HERRMANN et al., 2004), 47.4% in the Southeast (MARTINS et al., 2012), and 33.3% in the North (AGUIAR et al., 2010). However, this prevalence was comparable to those reported in studies conducted in the semiarid region of northeast Brazil, which varied from 3.5% in Rio Grande do Norte (AZEVEDO et al., 2004) to 5.4% and 8.2% in Paraíba (ALVES et al., 2012; COSTA et al., 2016). This was not unexpected since the rainfall index in the Northeast region is much lower than that observed in the other cited regions and the studied region possesses semiarid climate conditions (GIRARDI & ROSA, 2011).

Leptospirosis is a waterborne disease and that *Leptospira* spp. are very sensitive to desiccation. The Brazilian northeast has been suffering from low rainfall rates for the past three years. Nevertheless, during 2014, rainfall indices reached about 800mm in the region of Patos (AES, 2014; IPA, 2014), which

is superior to the minimum rainfall index for survival and dissemination of leptospires in the semiarid region, which had previously been established to be 500mm (ALVES et al., 1996). Therefore, drought cannot be the sole factor responsible for the low sero reactivity/positive PCR results detected in sheep in the present study.

All PCR-positive animals belonged to the same flock in the Tabira municipality. These animals were purchased at a fair in the Tabira municipality, which is the second largest animal fair in Pernambuco State. The fair experiences a large movement of animals from different locations, mainly from Paraíba State, where the sanitary conditions of the animals are unknown.

Serovar Javanica, the most frequently detected in the present study with significant titers, has previously been described in a prevalence survey in southern Brazil (SILVA et al., 2007). However, it has never been described in small ruminants in Northeast Brazil, according to recent surveys (HIGINO et al., 2010; ALVES et al., 2012; HIGINO et al., 2012). This serovar is mainly reported in domestic and wild rodents, especially in the *Rattus norvegicus* species, which is considered an important source of infection for sheep (NATARAJASEENIVASAN & RATNAM, 1999). Serovar Javanica has previously been isolated from the urine of a human showing clinical signs of leptospirosis and renal failure in India (SARAVANAN et al., 1998), which shows the potential risk of the serovar for human health.

Breed is a risk factor for leptospirosis in small ruminants. SILVA et al. (2012) reported that purebred sheep presented high seropositivity when compared to mixed-breed sheep, and SANTOS et al. (2012) also stated that purebred sheep were more susceptible to *Leptospira* spp. infection. SALABERRY et al. (2011) reported statistical differences among sheep breeds, and purebred animals were more susceptible to infection than

mixed-breed animals. The majority of the sheep population of northeast Brazil is composed of mixed-breed animals (CEZAR et al., 2004); therefore, the low prevalence reported in the present study may not only be associated with climatic conditions, but also with the differences in susceptibility to *Leptospira* spp. infection among sheep breeds.

CONCLUSION

Taking into consideration the low frequency of positivity at serology and PCR, it is possible to suggest that rusticity of the mixed breed sheep collaborated to the low occurrence of leptospirosis in the semiarid region investigated.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

Authorization to work with domestic animals for this study was granted by the Secretaria da Comissão de Ética o Uso de Animais (CEUA) da UFCG (protocol number: 50-2012 issued on 21/03/2013).

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