Acta Scientiae Veterinariae, 2022. 50: 1872.

RESEARCH ARTICLE
Pub. 1872

ISSN 1679-9216

Leptospira spp. of the Urinary Tract of Female Carrier Goats in Semi-Arid Conditions

Laysa Mayara Soares Brito Rocha[1], Pedro Jorge Álvares de Faria[1], Rafael Rodrigues Soares[1], João Pessoa Araújo Júnior[2], Camila Dantas Malossi[2], Leila Sabrina Ullmann[2], Maria Luana Cristiny Rodrigues Silva[1], Severino Silvano dos Santos Higino[1], Sergio Santos de Azevedo[1] & Clebert José Alves[1]

ABSTRACT

***Background*:** Leptospirosis is an important infectious disease in goat farming, with a worldwide distribution. It is usually transmitted by rodents and the genital route, may cause reproductive losses, negatively impacting goat farming. The diagnosis lies on serological, molecular and isolation techniques. Considering the importance of this disease for small ruminants, this work aimed to evaluate the serological, molecular findings and isolation of pathogenic leptospires in the urinary tract (kidney and bladder tissues) of goats.

***Materials, Methods & Results*:** Thirty-four adult goats were used for slaughter. Renal samples (n = 34), bladder (n = 34), were collected for isolation of the agent and molecular detection of *Leptospira* sp. and blood samples (n = 34) for serological testing. The polymerase chain reaction (PCR) was used as a molecular test and the microscopic serum agglutination test (MAT) was used as a serological test. Samples with DNA amplification were subjected to genetic sequencing. The presence of *Leptospira* DNA was found in the tissues of 8 (23.4%) goats, and of these, only 2 were positive in PCR and MAT. There was a slight agreement between the PCR and MAT techniques (k = 0.150; $P = 0.436$). In 6 (17.6%) samples of renal tissue and 2 (5.8%) bladder samples, *Leptospira* DNA was detected. The genes in a kidney tissue sample were sequenced and demonstrated 99% similarity to *Leptospira interrogans*. Anti-*Leptospira* sp. were detected in 6 (17.6%) of the animals tested.

***Discussion*:** Serology identified 3 predominant serogroups: Icterohaemorrhagiae, Tarassovi and Autumnalis, serogroups that are related to the presence of rodents that coexist in rural environments. Autumnalis has been reported in small ruminants, raising the hypothesis that goats are adapted, becoming chronic carriers and possible maintenance hosts. The frequency obtained (17.6%) may be the result of the mixed breed pattern and rustic characteristics inherent to the goat species. Given the characteristics of the semi-arid region, such as low rainfall and high solar incidence, it is essential to use an adapted methodology, with a lower cut-off point (1:50), as the serological titer is an established relationship between the animal species, the level of exposure throughout its evolution and the region studied. Molecular findings and bacterial isolation reveal the agent's ability to colonize the urinary tract of goats. These data show the importance that urine has in the epidemiological chain, being able to transmit the agent through direct contact with this product or through contamination of soil and water. There was no statistical agreement between the diagnostic techniques used in this study, in this case, an association between PCR and MAT is recommended to obtain data with high sensitivity and specificity. A bladder sample was sequenced and showed 99% similarity to *Leptospira interrogans*. In the semiarid region, the most common form of leptospirosis spread is through the sale of animals in business fairs for breeding, rearing or slaughter, as well as sharing the same property with several breeders. The introduction of chronic and asymptomatic carriers on the properties represents a serious risk for the spread of the disease. The results show the presence of *Leptospira* spp. in semi-arid goat herds, having as risk factors the presence of rodents and intercropping. The association of MAT and PCR is necessary for a better diagnosis of the disease.

Keywords: leptospirosis, leptospires, zoonosis, serology, molecular detection, epidemiology, semiarid.

DOI: 10.22456/1679-9216.124079

Received: 8 March 2022 Accepted: 21 May 2022 Published: 6 June 2022

[1]Federal University of Campina Grande (UFCG), Patos, PB, Brazil. [2]Universidade Estadual Paulista (Unesp), Botucatu, SP, Brazil. CORRESPONDENCE: C.J. Alves [clebertja@uol.com.br]. Departamento de Medicina Veterinária Preventiva - UFCG. Av. Universitária s/n. CEP 58708-110 Patos, PB, Brazil.

INTRODUCTION

Leptospirosis is a zoonosis, distributed worldwide and which causes high economic losses. Its main impact is impairment of reproductive performance such as abortions, stillbirths and infertility [2], generally linked to presence of rodents, low venereal transmission, high rainfall and presence of asymptomatic animals [2,22,29,30].

A widely used diagnostic method is microscopic agglutination test (MAT), which uses live cultures of *Leptospira* sp. as antigens [21]. Although with high specificity, it is a deficient test when used alone since absence of detectable levels of serum antibodies in some animal species makes it less sensitive [2].

Thus, isolation and molecular detection of leptospires has a great importance from an epidemiological point of view, as it allows identification of serovars circulating in a given region, in addition to identifying carrier animals that present negative results in other tests, such as MAT [9,30].

In view of relevance of this disease in herds of small ruminants, responsible for significant economic losses, due to its productive and reproductive characteristics and also associated with small number of studies on fundamental aspects of disease in caprine reared in Caatinga biome [9,21] the present work aimed to evaluate serological findings, molecular and bacteriological isolation of pathogenic leptospires from urinary tract of female goats.

MATERIALS AND METHODS

Study area and collection of biological materials

This study was carried out in municipal slaughterhouse of Patos, located in semiarid region of Northeast Brazil (latitude: 07°01'28"S; longitude: 37°16'48"W). From 2012 to 2015, 34 adult female goats were used, destined for slaughter. The blood collected was obtained immediately before slaughter, using sterile 9 mL vacuum tubes properly identified. After being collected, the serum was stored in microtubes at -20°C until serological test was performed. Samples of renal tissue (n = 34) and bladder (n = 34) were collected. One gram (1 g) fragments were used for bacteriological processing, and 5 g fragments used for molecular testing. These were frozen at -20°C until processed.

Serology

Presence of anti-*Leptospira* sp. was determined by MAT [23], using as antigens[1] a collection of 24 serovars belonging to 18 different pathogenic serogroups of 5 species, originating from Pasteur Institute, in France, and supplied by Veterinary Bacteriology Laboratory of Universidade Federal Fluminense: Australis, Autumnalis, Ballum, Canicola, Celledoni, Cynopteri, Djasiman, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Lousiana, Mini, Panama, Pomona, Pyrogenis, Sejroe e Tarassovi. A cut-off point of 1:100 was used, considered positive when presenting agglutination equal to or greater than 50%.

Culture

The kidney and bladder samples were macerated using sterile pistils to form a 10% suspension (weight/volume) in sterile Sorensen buffered saline. From this suspension, 0.5 mL was inoculated into 5 mL of semi-solid EMJH[2], supplemented with amphotericin B[3] (0.05 mg /mL), 5-fluorouracil[4] (1 mg/mL), fosfomycin[5] (4 mg/mL), trimethoprim[3] (0.2 mg/mL) and sulfamethoxazole[5] (0.4 mg/mL) [8]. The tubes inoculated were then stored at 28°C in a BOD incubation chamber. After a period of 24 h, the cultures were serially diluted in Fletcher's semi-solid medium[2] with 5-fluorouracil (1 mg/mL) and incubated at 28ºC and examined weekly for 12 weeks using dark field microscopy.

Molecular detection

DNA extraction from tissues and cultures was performed with Dneasy blood and tissue kit[6], following manufacturer's recommendations. The primers LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3 ') and Lip L32-286R (5'-GAA CTC CCA CCT TTC CAG CGA TT-3') were used to amplify the LipL32 gene [33]. Sequencing reactions were performed according to previous methodology [14], using the LipL32-45F and LipL32-286R primers [33] with the Big Dye Terminator v3.1 sequencing kit[7]. For capillary electrophoresis, a 3130xL gene analyzer and POP-7 polymer were used [27]. The sequence was aligned with a *Leptospira* strain obtained from GenBank[8] (National Biotechnology Information Center) (http://www.ncbi.nlm.nih.gov), using BLAST tool (http://www. ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was generated using Seaview[4] software[9] [13] and built using Bio Neighbor-joining method and Jukes and Cantor initialization model with 1,000 repetitions. This was visualized through FigTree[10] v1.4.4 (http://tree.bio.ed.ac.uk/). Phylogenic reconstruction included sequences of leptospires for comparison.

Statistical analysis

Fischer's exact test was used to compare proportions of positive samples of kidney and bladder tissue, both in PCR and for bacterial growth. To assess the concordance between results of MAT & PCR and MAT & Culture techniques, Kappa indicator was calculated. Analysis were made using BioEstat 5.03[11] [7], considering a significance level of 5%.

RESULTS

Of all 34 evaluated samples by MAT, 6 (17.6%) showed positive reactions (Tables 1 and 2). Title 100 was predominant, with 66.6% (4/6) of sororizations, followed by title 200, with 33.4% (2/6). The serogroups found were Autumnalis (33.3%, 2/6), Icterohaemorrhagiae (33.3%, 2/6) and Tarassovi (33.3%, 2/6)

Table 1. Results obtained from *Leptospira* spp. in goats reared in semi-arid conditions using different diagnostic techniques (MAT, PCR and culture of organs).

	MAT		Kidney	Bladder	Culture	
	100	200			Kidney	Bladder
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	+	-	-
6	Tarassovi	-	-	-	-	-
7	Autumnalis	-	-	-	-	-
8	-	-	-	-	-	-
9	-	Ictero*	-	-	-	-
10	-	-	-	-	-	-
11	-	-	+	-	-	-
12	-	-	-	-	-	-
13	-	-	-	-	-	-
14	-	-	-	-	-	-
15	-	-	-	-	-	-
16	-	-	-	-	-	-
17	-	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	+	-	-	-
20	-	-	-	-	-	-
21	-	-	+	+	-	-
22	-	-	-		-	-
23	-	-	-	-	-	-
24	-	-	-	-	-	-
25	Autumnalis	-	+	-	+	-
26	-	-	-	-	-	-
27	-	-	+	-	-	-
28	-	-	-	-	-	-
29	Tarassovi	-	-	-	-	-
30	-	-	-	-	-	-
31	-	-	-	-	-	-
32	-	-	-	-	-	-
33	-	-	-	-	-	-
34	-	Ictero*	+	-	+	+
Total	3	2	6	2	2	1

*Ictero: Icterohaemorrhagiae

In molecular analysis, 68 samples from 34 animals were tested, and 8 (23.5%) presented leptospiric DNA. Of these animals, 6 had positive samples for renal tissue and 2 for bladder (Table 1). Table 3 shows that 6/34 (17.6%) samples of renal tissue and 2/34 (5.8%) of the bladder had DNA, totaling 8 samples. There was no statistically significant difference between the samples ($P = 0.141$).

One renal tissue showed 99% identity to pathogenic species *L. interrogans* (Figure 1).

According to Table 1, only 5.8% (2/34) of female goats were positive for bacterial growth, and only 1 (2.9%) presented an exclusive sample for renal tissue. In all, growth was observed in 3/68 (4.4%) of samples, with 2/34 (5.8%) renal tissue and 1/34 (2.9%) bladder (Table 2). Comparison between the samples showed no statistically significant difference ($P = 0.488$).

Looking at Table 3, it can be seen that only 2 samples were positive for MAT and PCR, while 3 for MAT and culture. Using Kappa indicator as a base, there was a weak correlation between MAT and PCR ($k = 0.150$; $P = 0.436$) and between MAT and culture ($k = 0.543$; $P = 0.005$).

Table 2. Results of PCR of tissues of the urinary tract and culture of *Leptospira* spp. in goats reared in semi-arid conditions

Tissues	Total	PCR		Culture	
		N Positive	Frequency	N Positive	Frequency
Kidney	34	6	17.6 %	2	5.8 %
Bladder	34	2	5.8 %	1	2.9%

Table 3. Number of samples positives from goats reared in semi-arid conditions according to the method diagnosis.

MAT	PCR			Culture		
	Positive	Negative	Total	Positive	Negative	Total
Positive	2	4	6	3	4	7
Negative	6	22	28	0	27	27
Total	8	26	34	3	31	34

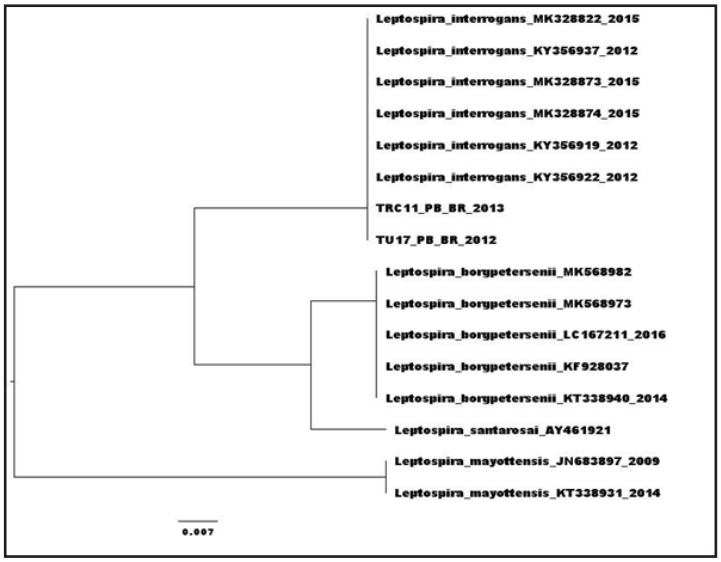

Figure 1. The phylogenetic tree built by the Neighbor-Joining method and Jukes-Cantor model, bootstrap with 1000 repetitions. ▲ Sequenced samples.

DISCUSSION

Serology identified three predominant serogroups, Icterohaemorrhagiae, Autumnalis and Tarassovi, similar to results obtained [6,14,29]. The presence of antibodies against serogroups Icterohaemorrhagiae and Tarassovi, may be related by sharing the same environment among sheep (*Ovis arie*), goats (*Capra hircus*), horses (*Equus caballus*), pigs (*Sus scrofa domesticus*) and rats (*Rattus norvegicus* & *Nectomys squamipes*) [4,5,15,18,24,28,29].

The serogroup Icterohaemorrhagiae was the most frequent in this study, considering participation of rodents in transmission chain, it is necessary to control the reservoir next to feed deposits in the environment, restrict their access where roughage is grown or stored [10]. In relation to the serogroup Autumnalis, numerous reports highlight it as most frequent in small ruminants and raises the hypothesis that these may be possible hosts for the maintenance of these serogroups, with antibody titers of 100 predominating, which may indicate adaptability of this species and possible chronic carriers of disease [9]. A low number of seropositive animals in studies of prevalence in the species in Northeast region, suggests that these goats bred in semiarid region, mostly crossbred and with more rustic characteristics, may be more resistant to leptospirosis [9,29,32].

The cut-off point used (100) is another issue to be considered, since the title presented by certain species is a fact correlated with the level of exposure throughout its evolution [2], giving rise to the hypothesis of considering different interpretations according to degree of adaptation of species and epidemiological reality of studied region [26], with cut-off points below 100 for diagnosis of leptospirosis should be considered to extent that chronic carriers are largely responsible for maintenance of the agent in environment, reinforcing a need for constant serological studies and attempts to isolate agent in this region. Research carried out with sheep [22] revealed the cut-off point 50 in serology was effective in detecting sheep carriers in relation to title 100 in the Brazilian semiarid and, regardless of material used in PCR, the highest sensitivities were obtained considering this cut-off point.

Positive findings of organ PCR and culture revealed colonization of leptospires in urinary tract (kidney and bladder) of goats reinforcing the importance of transmission through direct contact with urine of an infected animal, as well as dissemination in herd through the contamination of soil and water [1,19]. Presence of agent in localization site represented by the bladder demonstrates agent's ability to colonize different organs that until then have been few studied. Although PCR is a more laborious and costly technique, it provides us with a direct, fast and reliable diagnosis. Its use is recommended after MAT screening, making the diagnosis more accurate and the control of the herd more viable and safer [10,11]. A previous study [20] recommended the use of microscopic agglutination as a routine test, with subsequent PCR of urine for the direct detection of leptospiral DNA.

Thus, both methods can be used to control leptospirosis in small ruminants, since the low reactivity identified compared to other regions may be related to low susceptibility to infection and seroreactivity in native animals with high rusticity [9,29,32].

The bladder is a temporary urine reservoir [17] being a possible and important leptospiral colonization site [30]. Studies using this organ for detection and isolation of leptospires are scarce and although the urinary tract is automatically related, it can demonstrate different results in relation of presence DNA [22]. The presence of *Leptospira* sp. in samples taken from kidney and bladder indicates the animal's ability to release leptospires [30].

DNA sequencing directly from PCR products was possible in a bladder sample and there was 99% similarity with *L. interrogans* (Figure 1), a result similar to those found in sheep, according to previous study [22,30]. Molecular characterization (PCR) has been used in several studies with small ruminants in Paraíba. It were found 3 animals positive with PCR in renal tissue [10]; Some authors [30] obtained 44 positive animals, 54.9% in the genital tract (uterus, ovary and fallopian tube) and 4.4% in urinary tract (renal tissue and bladder). A study [22] found results from 69 positive animals, using samples of vaginal fluid, urine, bladder, kidney, uterus, fallopian tube, ovary and placenta.

In Brazil, bacterial isolations and molecular characterization from small ruminants, until now, have only shown that 4 strains of *Leptospira* sp. with 1 in Rio de Janeiro belonging to the Grippotyphosa

serogroup [18]. In Rio Grande do Sul the isolate was identified through molecular diagnosis as belonging to Noguchii serogroup and Autumnalis serovar [31]. In Paraíba, in a study involving 80 sheep slaughtered at Patos, PB Municipal Public Slaughterhouse, isolated *Leptospira* sp. in 2 samples from a placenta and an ampoule of vas deferens [16]. Another study in Paraíba with 104 ovines, managed to isolate *Leptospira* sp. in 3 samples of vaginal fluid [22].

Diagnostic techniques used did not obtain statistical agreement, as evidenced in other studies [9,30] strengthening need to combine, 2 methods for a reliable detection of positive animals, since MAT although it is the most widely used serological test for leptospirosis, having high specificity at level of serogroup and recommended by World Organization for Animal Health [23], it may not have a detectable title in animals that have leptospires present in kidneys and other organs, this due to the low immune response that some serovars cause as well as the adaptation of species to certain serovars [11,12].

The dispersion of leptospirosis in herds can happen through transit of carrier animals, consisting of an important way of introducing the agent into herds. In the semiarid region, the practice of selling small ruminants at animal sales fairs is common, with possibility of introducing animals without any type of control in properties, in addition to the use of shared breeders between properties [3]. The risk of introducing the disease, with asymptomatic chronic animals, increases importance for the knowledge of the serological condition of animals before being introduced into herd, suggesting existence of alternative routes of transmission less dependent on environmental factors, as well as identification of serogroups more frequent suggests need to improve sanitary conditions and implement efficient control measures targeted in main sources of infection [25].

CONCLUSION

It is concluded that in conditions of semi-arid climate, presence of rodents and intercropping breeds are risk factors for maintenance and spread of *Leptospira* sp. in goat herds. The rusticity of animals from semi-arid climate leads to low seroreactivity in MAT, so use of associated MAT and PCR is essential for diagnosis of leptospirosis. Expansion of studies that seek isolation and classification of serogroups circulating in our region are necessary, thus serving as a basis for construction and application of prophylaxis and control strategies for this disease.

MANUFACTURERS

[1]Institut Pasteur. Paris, France.

[2]Difco Laboratories Incorporated. Franklin Lakes, NJ, USA.

[3]Sigma-Aldrich Pty Ltd. Darmstadt, Germany.

[4]Libbs Farmacêutica. São Paulo, Brazil.

[5]Fluka Analytical Sigma - Aldrich. Munich, Germany.

[6]Qiagen GmbH. Hilden, Germany.

[7]Applied Biosystems. Waltham, MA, USA.

[8]National Biotechnology Information Center, Bethesda, MD, USA.

[9]SeaView4. Villeurbanne, France.

[10]FigTree. The University of Edinburgh, Edinburgh, UK.

[11]BioEstat. Instituto Mamirauá. Tefé, AM, Brazil.

***Funding*.** This research is financially supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico/ Ministério da Agricultura Pecuária e Abastecimento by the process N 301029/2018-0.

***Acknowledgments*.** To Coordination for Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior CAPES) for granting the Pos-graduate scholarship. To the Transmissible Diseases Research Group at the Federal University of Campina Grande (UFCG) Patos, PB, Brazil.

***Ethical approval*.** This research was approved by Research Ethics Committee - CEP/CEUA / CSTR Nº 17/2019.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

REFERENCES

1 Adler B. & Moctezuma A.P. 2010. *Leptospira* and leptospirosis. *Veterinary Microbiology*. 140: 287-296. DOI: http://dx.doi.org/10.1016/j.vetmic.2009.03.012.

2 Adler B. 2015. History of leptospirosis and *Leptospira*. *Current Topics in Microbiology and Immunology*. 387: 79-84. DOI: 10.1007 / 978-3-662-45059-8-1.

3 **Alves J.R.A., Lima G.M.S., Silva, J.D., Santos F.A., Higino S.S.S., Azevedo S.S. & Alves C.J. 2017.** Epidemiological characterization and risk factors associated with leptospirosis and brucellosis in small ruminants sold at animal fair in the Sertão Region of Pernambuco State, a semiarid Region of Northeastern Brazil. *Semina: Ciências Agrárias*. 38(4): 1933-1946. DOI: 10.5433/1679-0359.2017v38n4p1933.

4 **Alves C.J., Andrade J.S.L., Vasconcellos S.A., Morais Z.M., Azevedo S.S. & Santos F.A. 2000.** Avaliação dos níveis de aglutininas anti-*Leptospira* spp. em cães no município de Patos-PB, Brasil. *Revista Brasileira de Ciência Veterinária*. 7(2): 17-21. DOI: 10.4322/rbcv.2015.168.

5 **Alves C.J., Alcino J.F., Farias A.E.M., Higino S.S.S., Santos F.A., Azevedo S.S., Costa D.F. & Santos C.S.A.B. 2012.** Caracterização epidemiológica e fatores de risco associados à leptospirose em ovinos deslanados do semiárido brasileiro. *Pesquisa Veterinária Brasileira*. 32(6): 523- 528. DOI: 10.1590/S0100-736X2012000600009.

6 **Araújo Neto J.O., Alves C.J., Azevedo S.S., Silva M.L.C.R. & Batista C.S.A. 2010.** Soroprevalência da leptospirose em caprinos da microrregião do Seridó Oriental, Estado do Rio Grande do Norte, Brasil, e pesquisa de fatores de risco. *Brazilian Journal of Veterinary Research and Animal Science*. 47(2): 150-155. DOI: 10.1590/ S1413-95962010000200007.

7 **Ayres M., Ayres J.R.M., Ayres D.L. & Santos A.D. 2007.** Duas amostras independentes. In: Ayres M. & Ayres J.R.M. (Eds). *Bioestat 5.0: Aplicações Estatísticas nas Ciências Biomédicas*. 5.ed. Tefé: Instituto Mamirauá, pp.125-145.

8 **Chakraborty A., Miyahara S., Villanueva S.Y.A.M., Saito M., Gloriani N.G. & Yoshida S. 2011.** Novel combination of selective agents for isolation of *Leptospira* species. *Microbiology and Immunology*. 55(7): 494-501. Doi: 10.1111/j.1348-0421.2011.00347.x.

9 **Costa D.F., Silva A.F., Farias A.E.M., Brasil A.W.L., Santos F.A., Guilherme R.F., Azevedo S.S. & Alves C.J. 2016.** Serological study of the *Leptospira* spp. infection in sheep and goats slaughtered in the State of Paraíba, semiarid of Northeastern Brazil. *Semina: Ciências Agrárias*. 37(2): 819- 828. DOI: 10.5433/1679-0359.2016v37n2p819.

10 **Costa D.F., Silva A.F., Brasil A.W.L., Loureiro A.P.P., Santos F.A., Azevedo S.S., Lilenbaum W. & Alves C.J. 2017.** Leptospirosis in native mixed-breed sheep slaughtered a semiarid region of Brazil. *Ciência Rural*. 47: 1-6. Doi: 10.1590/0103-8478cr20160563.

11 **Director A., Penna B., Hamond C., Loureiro A.P., Martins G., Medeiros M.A. & Lilenbaum W. 2014.** Isolation of *Leptospira interrogans* Hardjoprajitno from vaginal fluid of a clinically healthy ewe suggests potential for venereal transmission. *Journal of Medical Microbiology*. 63(9): 1234-1236. DOI: 10.1099/jmm.0.065466-0.

12 **Gamage C.D., Koizumi N., Muto M., Nwafor-Okoli C., Kurukurusuriya S., Rajapakse J.R.P.V., Kularatne S.A.M., Kanda K., Lee R.B., Obayashi Y., Watanabe H. & Tamashiro H. 2011.** Prevalence and carrier status of leptospirosis in smallholder dairy cattle and peridomestic rodents in Kandy, Sri Lanka. *Vector-Borne Zoonotic Disease*. 11: 1041-1047. DOI: 10.1089/vbz.2010.0153.

13 **Gouy M., Guindon S. & Gascuel O. 2010.** SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*. 27(2): 221-224. DOI: 10.1093/molbev/msp259.

14 **Hamond C., Martins G., Loureiro A.P., Pestana C., Lawson-Ferreira R., Medeiros M.A. & Lilenbaum W. 2014.** Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock. *Veterinary Research Communications*. 38(1): 81-85. DOI: 10.1007/s11259-013-9582-x.

15 **Hamond C., Pestana C.O., Souza C.M.R., Cunha L.E.R., Brandão F.Z., Medeiros M.A. & Lilenbaum W. 2015.** Presence of leptospires on genital tract of mares with reproductive problems. *Veterinary Microbioly*. 179: 264-269. DOI: 10.1016/j.vetmic.2015.06.014.

16 **Higino S.S.S., Azevedo S.S., Alves C.J., Figueiredo S.M., Silva M.L.C.R. & Batista C.S.A. 2010.** Frequência de leptospirose em ovinos abatidos no Município de Patos, Paraíba. *Arquivos do Instituto Biológico*. 77(3): 525-527. DOI: 10.1590/1808-1657v77p5252010.

17 **Juc R.U., Colombari E. & Sato M.A. 2011.** Importância do sistema nervoso no controle da micção e armazenamento urinário. *Arquivos Brasileiros de Ciências da Saúde*. 36(1): 55-60. DOI: 10.7322/abcs.v36i1.76.

18 **Lilenbaum, W., Souza G.N., Ristow P., Moreira M.C., Fráguas S., Cardoso V.S. & Oelemann W.M.R. 2007.** A serological study on *Brucella abortus*, caprine-arthritis-encephalitis virus and *Leptospira* in dairy goats in Rio de Janeiro, Brazil. *The Veterinary Journal*. 173: 408-412. DOI: 10.1016/j.tvjl.2005.12.003.

19 **Lilenbaum W., Varges R., Medeiros L., Cordeiro A.G., Cavalcanti A., Souza G.N., Richtzenhain L. & Vasconcellos S.A. 2008.** Risk factors associated with leptospirosis in dairy goats under tropical conditions in Brazil. *Research in Veterinary Science*. 84: 14-21. DOI: 10.1016/j.rvsc.2007.03.011.

20 **Lilenbaum W., Varges R., Ristow P., Cortez A., Souza S.O., Richtzenhain L.J. & Vasconcellos S.A. 2009.** Identification of *Leptospira* spp. carriers among seroreactive goats and sheep by polymerase chain reaction. *Research in Veterinary Science*. 87: 16-19. DOI: 10.1016/j.rvsc.2007.03.011.

21 **Martins G. & Lilenbaum W. 2014.** Leptospirosis in sheep and goats under tropical conditions. *Tropical Animal Health and Production*. 46(1): 11-17. DOI: 10.1007/s11250-013-0480-6.

22 **Nogueira D.B., Costa, F.T.R., Bezerra C.S., Silva M.L.C.R., Costa, D.F., Viana, M.P., Silva, J.D., Araújo Júnior J.P., Malossi C.D., Ullmann L.S., Santos C.S.A.B., Alves C.J. & Azevedo S.S. 2020.** Use of serological and molecular techniques for detection of *Leptospira* sp. carrier sheep under semiarid conditions and the importance of genital transmission route. *Acta Tropica*. 207: 1-8. DOI: 10.1016/j.actatropica.2020.105497.

23 **OIE - World Organization for Animal Health. 2014.** *Leptospirosis: Manual of diagnostic tests and vaccines for terrestrial animals*. Paris: World Organization for Animal Health, 598p.

24 **Petrakovsky J., Carpinetti B. & Antonuci A. 2015.** Prevalencia Serológica de *Leptospira* spp. en Cerdos Silvestres (*Sus scrofa*) en Bahía de Samborombón, provincia de Buenos Aires, República Argentina, en el Periodo 2013-2015. *Salud y Tecnología Veterinaria*. 3(1): 23-27. DOI: 10.20453/stv.v3i1.2759.

25 **Pimenta, C.L.R.M., Bezerra C.S., Morais D.A., Silva M.L.C.R., Nogueira D.B., Costa D.F., Santos C.S.A.B., Higino S.S.S., Alves C.J. & Azevedo S.S. 2019.** Seroprevalence and predominant serogroups of *Leptospira* sp. in serological tests of ruminants in northeastern Brazil. *Semina: Ciências Agrárias*. 40: 1513-1522. DOI: 10.5433/1679-0359.2019v40n4p1513.

26 **Picardeau M. 2013.** Diagnosis and epidemiology of leptospirosis. *Médicine et Maladies Infectieuses*. 43(1): 1-9. DOI: 10.1016/j.medmal.2012.

27 **Platt A.R., Woodhall R.W. & George A.L. 2007.** Improved DNA sequencing quality and efficiency using an optimized fast cycle sequencing protocol. *BioTechniques*. 43(1): 58-62. DOI: 10.2144/000112499.

28 **Ramos A.C.F., Souza G.N. & Lilenbaum W. 2006.** Influence of leptospirosis on reproductive performance of sows in Brazil. *Theriogenology*. 66(4): 1021-1025. DOI: 10.1016/j.theriogenology.2005.08.028.

29 **Santos J.P., Lima-Ribeiro A.M.C., Oliveira P.R., Santos M.P., Ferreira Jr. A., Medeiros A.A. & Tavares T.C.F. 2012.** Seroprevalence and risk factors for Leptospirosis in goats in Uberlândia, Minas Gerais, Brazil. *Tropical Animal Health and Production*. 44: 101-106. DOI: 10.1007/s11250-011-9894-1.

30 **Silva A.F., Farias P.J.A., Silva M.L.C.R., Araújo Jr. J.P., Malossi C.D., Ullmann L.S., Costa D.F., Higino S.S.S., Azevedo S.S. & Alves C.J. 2019.** High frequency of genital carriers of *Leptospira* sp. in sheep slaughtered in the semi-arid region of northeastern Brazil. *Tropical Animal Health and Production*. 51(3): 43-47. DOI: 10.1007/s11250-018-1657-9.

31 **Silva E.F., Brod C.S., Cerqueira G.M., Bourscheidt D., Seyffert N., Queiroz A., Santos C.S., Ko A.I. & Dellagostin O.A. 2007.** Isolation of *Leptospira Noguchii* from sheep. *Veterinary Microbiology*. 121: 144-149. DOI: 10.1016/j.vetmic.2006.11.010.

32 **Silva R.C., Costa V.M., Shimabukuro F.H., Richini-Pereira V.B., Menozzi B.D. & Langoni H. 2012.** Frequency of *Leptospira* spp. in sheep from Brazilian slaughterhouses and its association with epidemiological variables. *Pesquisa Veterinária Brasileira*. 32(3): 194-198. DOI: https://doi.org/10.1590/S0100-736X2012000300002.

33 **Stoddard R.A., Gee J.E., Wilkins P.P., McCaustland K & Hoffmaster A.R. 2009.** Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagnostic Microbiology and Infectious Disease*. 64(3): 247-255. DOI: 10.1016/j.diagmicrobio.2009.03.014.