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CONTRIBUIÇÕES PARA A EPIDEMIOLOGIA E CONTROLE DA LEPTOSPIROSE EM RUMINANTES DO NORDESTE DO BRASIL

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Carla Lauise Rodrigues Menezes Pimenta

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Tese submetida ao Programa de Pós-Graduação em Medicina Veterinária, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Doutora em Medicina Veterinária.

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Aos meus pais Roque e Naide, por todo amor
e incentivo durante todos esses anos.

Dedico

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SUMÁRIO

RESUMO

Esta tese é composta por três capítulos, no capítulo I são descritas as estratégias de controle de um surto de infecção por leptospirose em bovinos leiteiros no estado do Maranhão, Nordeste do Brasil. O diagnóstico de leptospirose foi baseado na sorologia, cultura bacteriológica e reação em cadeia da polimerase. De todos os animais da fazenda, 136 (48,6%) foram soropositivos para *Leptospira* sp. Oito dos animais com problemas reprodutivos foram positivos na PCR. O sequenciamento genético de uma amostra PCR positiva de fluido vaginal revelou *Leptospira borgpetersenii*. Um ano após a adoção de medidas de controle, não foram observados problemas reprodutivos. Assim, a leptospirose provavelmente causou falhas reprodutivas no rebanho, e as medidas de controle e prevenção implementadas foram eficientes no controle da doença. No capítulo II é descrita a caracterização sorológica e molecular de *Leptospira* sp. em rebanhos bovinos e ovinos em condições semiáridas do Nordeste brasileiro. Foi realizado diagnóstico sorológico, molecular e tentativa de isolamento de *Leptospira* sp. de 99 fêmeas em idade reprodutiva. Destes 38,4% foram reagentes no teste sorológico, sendo 49% fêmeas bovinas e 27,1% fêmeas ovinas. Os sorogrupos detectados em bovinos foram Sejroe, Hebdomadis, Australis, Djasiman, Balum, Pomona e Cynopteri. Nos ovinos, os sorogrupos reagentes foram Australis, Balum, Djasiman, Tarassovi, Icterohaemorrhagiae e Cynopteri. Na PCR, foi detectado DNA leptospírico em nove amostras de urina. Não foi observado crescimento do agente em meio de cultura em amostras de urina. Em condições semiáridas, a transmissão entre animais da mesma espécie parece ser a principal forma de disseminação de leptospiras nos rebanhos ovinos e bovinos, porém a participação de outros animais domésticos e silvestres não pode ser descartada. Sugere-se ainda que a prática da criação consorciada de bovinos e ovinos e o estreito convívio entre eles propicia a disseminação do agente nas propriedades rurais. O capítulo III determinou a soropositividade para leptospirose e os sorogrupos predominantes nos testes sorológicos realizados no Laboratório de Doenças Transmissíveis (LDT) da Universidade Federal de Campina Grande (UFCG), Patos, Paraíba, Nordeste do Brasil, em bovinos, caprinos, ovinos e bubalinos no período de 2010 a 2017. Foram computados os registros dos exames sorológicos para leptospirose de 5.594 animais, provenientes de quatro estados brasileiros. Foram positivas 662 amostras no teste sorológico, resultando em uma frequência de 11,8%. Sejroe, Autumnalis e Icterohaemorrhagiae foram os sorogrupos mais frequentes para todas as espécies. As frequências individuais de bovinos, caprinos, ovinos e bubalinos foram de 20%, 8,3%, 7,9%, e 27,9%, respectivamente. Com relação aos sorogrupos mais frequentes por espécie animal, o Sejroe predominou em bovinos, Autumnalis foi o mais frequente em caprinos e ovinos e Australis predominou nos bubalinos. infecção por *Leptospira* sp., determinada por sorologia, encontra-se difundida em ruminantes (bovinos, caprinos, ovinos e bubalinos) do Nordeste do Brasil, o que sugere a existência de vias de transmissão alternativas menos dependentes de fatores ambientais, bem como a identificação dos sorogrupos mais frequentes sugere a necessidade de melhoria das condições sanitárias e implementação de medidas de controle eficientes e direcionadas para as principais fontes de infecção.

PALAVRAS-CHAVE: Sorologia; *Leptospira* sp; Controle; Detecção Molecular; Sorogrupos.

ABSTRACT

This thesis consists of three chapters, in Chapter I, the strategies of control of an outbreak of leptospirosis infection in dairy cattle in the state of Maranhão, Northeastern Brazil, are described. The diagnosis of leptospirosis was based on serology, bacteriological culture and polymerase chain reaction. Of all farm animals, 136 (48.6%) were seropositive for *Leptospira* sp. Eight of the animals with reproductive problems were PCR positive. Genetic sequencing of a positive PCR sample of vaginal fluid revealed Leptospira borgpetersenii. One year after the adoption of control measures, no reproductive problems were observed. Thus, leptospirosis probably caused reproductive failures in the herd, and the control and prevention measures implemented were efficient in controlling the disease. In Chapter II the serological and molecular characterization of Leptospira sp. in cattle and sheep in semi-arid conditions of Northeast Brazil. Serological, molecular and and attempt isolation of *Leptospira* sp. of 99 females of reproductive age. Of these 38.4% were reagents in the serological test, being 49% bovine females and 27.1% ovine females. Serogroups detected in cattle were Sejroe, Hebdomadis, Australis, Djasiman, Balum, Pomona and Cynopteri. In sheep, the reactive serogroups were Australis, Balum, Djasiman, Tarassovi, Icterohaemorrhagiae and Cynopteri. In PCR, leptospiral DNA was detected in nine urine samples. Growth of the agent in culture medium was not observed in urine samples. In semi-arid conditions, transmission between animals of the same species seems to be the main form of dissemination of leptospires in sheep and cattle, but the participation of other domestic and wild animals can not be ruled out. It is also suggested that the practice of intercropping cattle and sheep and the close coexistence between them facilitates the dissemination of the agent in the rural properties. Chapter III determined the seropositivity for leptospirosis and the serogroups prevalent in the serological tests carried out at the Transmissible Diseases Laboratory (TDL) of the Federal University of Campina Grande (UFCG), Patos, Paraíba, Northeast Brazil, in cattle, goats, sheep and buffaloes in the period from 2010 to 2017. The records of the serological tests for leptospirosis of 5,594 animals from four Brazilian states were computed. A total of 662 samples were positive in the serological test, resulting in a frequency of 11.8%. Serjoe, Autumnalis and Icterohaemorrhagiae were the most frequent serogroups for all species. The individual frequencies of cattle, goats, sheep and buffaloes were 20%, 8.3%, 7.9%, and 27.9%, respectively. In relation to the most frequent serogroups by animal species, Serjoe predominated in cattle, Autumnalis was the most frequent in goats and sheep and Australis predominated in buffaloes. Leptospira sp. infection, determined by serology, is widespread in ruminants (cattle, goats, sheep and buffalo) in Northeast Brazil, suggesting the existence of alternative transmission routes less dependent on environmental factors, as well as identification of the most frequent serogroups suggests the need to improve sanitary conditions and implement efficient and targeted control measures for the main sources of infection.

KEY-WORDS: Serology; *Leptospira* sp; Control; Molecular Detection; Sorogroups

LISTA DE TABELAS

CAPÍTULO I

LISTA DE TABELAS

CAPÍTULO II

LISTA DE TABELAS

CAPÍTULO III

LISTA DE FIGURAS

CAPÍTULO I

CAPÍTULO II

1. INTRODUÇÃO GERAL

A leptospirose é uma doença bacteriana infecto-contagiosa causada por microrganismos pertencentes ao gênero *Leptospira*, que acometem o homem, os animais domésticos e silvestres, e encontra-se largamente disseminada (ADLER, 2015), sendo sua ocorrência maior em países de clima tropical e subtropical (OLIVEIRA et al., 2010). Nos animais de produção, é uma enfermidade com grande impacto econômico, principalmente pela redução na produção animal e baixa fertilidade dos plantéis, bem como fatores relacionados à saúde pública (ELLIS, 2015).

A infecção e a transmissão de leptospira estão relacionados às exposições aos fatores de risco ambientais, assim como da presença de reservatórios e hospedeiros de manutenção. Nos ruminantes, o sorogupo Sejroe é o mais frequentemente encontrado, sendo os bovinos considerados hospedeiros primários de manutenção deste sorogrupo (CORREIA et al., 2017; HERRMAN et al., 2012). A transmissão ocorre indiretamente pelo contato com água ou solo contaminados ou pelo contato direto com a urina de animais portadores (PICARDEAU, 2013), assm como estudos recentes apontam para importância da transmissão venérea fêmea-macho na disseminação da leptospirose em ruminantes (PIMENTA et al., 2018). Alguns fatores propiciam a disseminação de leptospiras, tais como: o convívio entre as espécies, presença de animais silvestres, medidas de manejo adotadas nas propriedades e das oportunidades de infecção direta e indireta (ESCÓCIO et al., 2010; HASHIMOTO et al., 2012).

O diagnóstico para leptospirose é realizado através do teste de Soroaglutinação Microscópica (SAM) (PINTO et al., 2015; LIBONATI et al., 2017), apesar das suas limitações, um diagnóstico preciso é realizado através do isolamento e tipificação da sorovariedade prevalente, sendo a PCR para este fim, como também para detecção de DNA leptospírico em amostras clínicas (OTAKA et al., 2012).

Diante disso os inquéritos sorológicos são importantes e realizados para conhecimento dos sorogrupos de leptospiras que infectam os animais de determinado local, utilizado para fomentar e aplicar medidas efetivas para controlar a infecção, sendo esse controle da doença em ruminantes baseado em antibióticoterapia, vacinação e outras medidas sanitárias como: controle de roedores, quarentena dos animais, aumento da higiene ambiental, pois reduzem as seqüelas reprodutivas, minimizam a propagação da leptospirose e os riscos econômicos relacionados a infecção (ROLIM et al., 2013; MARTINS e LILENBAUM, 2017).

Esta Tese de Doutorado é composta por três capítulos constituídos por artigos científicos originais. O Capítulo I é referente a um artigo científico pulicado na revista Tropical Animal Health and Production (Qualis B1) e descreve as estratégias de controle de um surto de infecção por leptospirose em bovinos leiteiros no estado do Maranhão, Nordeste do Brasil. O Capítulo II é composto por um artigo submetido à revista Acta Tropica (Qualis A2), e refere a caracterização sorológica e molecular de *Leptospira* sp. em rebanhos bovinos e ovinos em condições semiáridas do Nordeste brasileiro. O Capítulo III compreende um artigo submetido à Revista Semina: Ciências Agrárias (Qualis B1), no qual foi investigada a soropositividade para leptospirose e os sorogrupos predominantes nos testes sorológicos realizados no Laboratório de Doenças Transmissíveis (LDT) da Universidade Federal de Campina Grande (UFCG), Patos, Paraíba, Nordeste do Brasil, em bovinos, caprinos, ovinos e bubalinos no período de 2010 a 2017.

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CAPÍTULO I: Strategies of the control of an outbreak of leptospiral infection in dairy cattle in Northeastern Brazil

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Strategies of the control of an outbreak of leptospiral infection in dairy cattle in Northeastern Brazil

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Abstract The aim of the present study was to describe the strategies of the control of an outbreak of leptospiral infection in dairy cattle in Maranhão state, Northeastern Brazil. In the period from January to July 2015, 18 (17%) out of 106 cows presented abortion, six (5.7%) stillbirth, and 12 (11.3%) repeated estrus, totaling 24 animals with reproductive problems. The diagnosis of leptospirosis was based on serology (microscopic agglutination test - MAT), bacteriological culture, and polymerase chain reaction (PCR). Antibiotic therapy, vaccination protocols, and changes in management practices were suggested as control measures. Of all animals on the farm (n = 280), 136 (48.6%) were seropositive for at least one serovar of *Leptospira* sp. No pure leptospiral culture was obtained. Eight of the animals with reproductive problems yielded positive PCR results (vaginal fluid of seven animals and urine and vaginal fluid of one animal). Genetic sequencing of a vaginal fluid/urine PCR-positive sample revealed *Leptospira borgpetersenii*. One year after the adoption of control measures, no reproductive problems were observed. Thus, leptospirosis probably caused the

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reproductive failures in the herd, and the control and prevention measures implemented were efficient in controlling the disease.

Keywords *Leptospira* sp. ● Control ● Reproductive failures ● Outbreak

Leptospirosis is a zoonotic disease of global importance, caused by pathogenic bacteria belonging to the genus *Leptospira*. The infection has a wide geographical distribution, with higher occurrence in tropical regions, and each serovar is usually associated with a maintenance host. Leptospirosis is important for cattle due to the compromised reproductive performance of the affected herds (Bourhy et al. 2014). In Brazil, investigations of leptospirosis outbreaks in cattle have been reported (Mineiro et al. 2014). In Italy, two outbreaks of reproductive problems caused by *Leptospira borgpetersenii* serovar Hardjo in cattle have been reported (Mughini-Gras et al. 2014).

Cattle become infected mainly with the serogroup Sejroe (Pinto et al. 2016). However, any serovar can infect any animal species, but a limited number of serovars affect livestock species accidentally, leading to outbreaks of abortion, dead fetuses, and repeated estrus. Hardjo and Wolffi serovars are the most prevalently reported in studies on cattle in Brazil (Pimenta et al. 2014).

In outbreak situations, the control strategies used are measures of biosafety, vaccination and selective chemoprophylaxis. Some improved measures have also been implemented, including pest control, extra environmental sanitation programmers, removal of piles of discarded material, closed herd maintenance, limiting access to contaminated water, banning intercropping, and supplying vitamins and mineral supplements (Mughini-Gras et al. 2014).

Thus, the objective of the present study was to describe the strategies of the control of an outbreak of leptospiral infection in dairy cattle in Maranhão state, Northeastern Brazil.

The outbreak occurred from January to June 2015 in a dairy farm in the municipality of Timon, state of Maranhão, Northeastern Brazil. The region has a high annual rainfall (1,383 mm). The herd was composed of 106 pregnant cows, 90 heifers, eight bulls, and 76 calves, totaling 280 animals. The heifers had not yet been covered, and covered cows that did not return to estrus were considered pregnant. The owner reported that 18 cows (17%) aborted in the last trimester of gestation, six (5.7%) had stillbirths, and 12 (11.3%) repeated estrus, totaling 24 animals with reproductive problems. The herd had never been vaccinated for leptospirosis. No clinical signs other than the reproductive problems were observed. The

management system adopted at the farm was semi-intensive, rodent control was performed in the milking and feed storage facilities.

Blood samples were taken from all animals in the farm (n = 280) in July 2015, fifteen days after reproductive problems were noted. Thirty days after this first visit, a new blood collection was performed, and urine and vaginal fluid samples were collected only from the animals that aborted, had stillbirths, or had repeated estrus (n = 24). Blood samples were collected by jugular venipuncture into 10-mL evacuated tubes, followed by serum extraction by centrifugation and storage at -20 °C until serology was performed. Urine was collected using a diuretic (furosemide, 2.5 mL/animal, intramuscularly), and vaginal fluid was collected with sterile swabs directly from the cervical region of the vagina and then stored in sterile 15-mL Falcon tubes with 2 mL of phosphate-buffered saline.

The serological diagnosis of leptospirosis was performed using the microscopic agglutination test (MAT) (OIE 2014). For leptospire isolation, immediately after collection 1 mL of urine and vaginal fluid diluted in phosphate-buffered saline was seeded at the final concentration of 10% in semi-solid EMJH medium (Difco, BD Franklin Lakes, NJ, USA) with amphotericin B, 5-fluorouracil (1 mg/mL), fosfomycin (4 mg/mL), trimethoprim (0.2 mg/mL), and sulfamethoxazole (0.4 mg/mL) for inhibition of the proliferation of contaminating microorganisms (Chakraborty et al. 2011). After 24 hours, 1 mL was seeded in EMJH medium with only 5-fluorouracil (1 mg/mL) added at the proportion of 10%, with subsequent incubation at 30 °C. The tubes were examined weekly for 6 weeks using dark-field microscopy.

DNA from urine and vaginal fluid was extracted using the kit Wizard Genomic SV DNA Purification System (Promega, Madison, USA). PCR and sequencing reactions were performed with the primers corresponding to nucleotides 38 ± 57 5'GGCGGCGCGTCTTAAACATG3' and 348 ± 368 5'TCCCCCCATTGAGCAAGATT3' (Heinemann et al. 2000). The nucleotide sequence alignment was performed in Seaview4. The sequence was aligned with reference *Leptospira* strains obtained from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) (http.//www.ncbi.nlm.nih.gov), using the BLAST tool http://www.ncbi.nlm.nih.gov/BLAST/. A phylogenetic tree was generated using the o software Seaview4. Phylogenetic trees were constructed based on the maximum-likelihood (ML) method with 1,000 bootstraps, model TN 93, using PhyML 3.1. Trees were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/). The phylogenetic reconstruction program included sequences from *Leptospira* sp. for comparison.

Seropositive animals were treated 10 days after the second visit with a single dose (25 mg/kg) of dihydrostreptomycin (Ourofino, Cravinhos, São Paulo, Brazil), and the seronegative animals were vaccinated with the commercial inactivated vaccine CattleMaster® 4 + L5 (Pfizer, Itapevi, São Paulo, Brazil), boosted after 30 days. Simultaneously, environmental measures were implemented, such as preventing the animals from accessing flooded areas and intensifying chemical and physical rodent control.

Of the 280 serum samples analyzed in the first visit, 136 (48.6%) were positive at MAT. The most frequent serogroups were Sejroe (92.7%), Tarassovi (5.1%), Hebdomadis (1.5%), and Australis (0.7%) (Table 1), with titers from 100 to 3200. Of the 24 cows with reproductive problems, eight (33%) were seropositive in the first visit, whereas 23 (95.8%) were seropositive in the second visit (Table 2), with titers from 100 to 400.

No pure leptospiral isolates were obtained; however, the DNA of pathogenic leptospires was detected in the vaginal fluid of eight animals, and in urine of one animal (Table 2). Of the eight PCR-positive samples, a nucleotide sequence obtained in one (295-bp fragment) showed 100% BLAST identity with *Leptospira borgpetersenii* sequences. One year after the adoption of prevention and control measures, reproductive problems were no longer observed in the herd.

The serogroup Sejroe was the most frequent in this study, and it is reported as the most frequent in cattle. Recognized as being adapted to cattle, serovars of this serogroup are generally associated with several reproductive problems such as abortions, stillbirths, weak calves, and infertility. Thus, the reproductive problems observed may be related to the high frequency of these serovars in the herd (Tagliabue et al. 2016).

In conditions of high rainfall, as is the case in the studied region, excellent conditions are available for the survival and spread of leptospires (Robertson et al. 2012). However, infection influenced by environmental factors is more relevant when serogroups not adapted to the species in question are involved (Ellis 2015). Taking into account that the most frequent serovars in this study are adapted to cattle (Wolffi, Hardjobovis and Sejroe), bovine-to-bovine transmission was probably responsible for the outbreak, since in these cases the elimination of leptospires through urine is more constant and the contact with the agent is facilitated (Correia et al. 2017).

A discrepancy was observed between the results of the two serologies in animals with reproductive problems, with eight animals positive (33%) in the first serology and 23 animals (95.8%) in the second. The non-detection of many positive animals in the first

serology may be related to the short-lived immunity produced by infected animals, especially for serovars adapted to cattle (Adler 2015). However, as no control measure was performed in the period between the two collections, the animals may have been re-infected, thus explaining the detection of a higher proportion of positive animals in the second serology.

Host-adapted serovars, mainly serovar Hardjo, are related to reproductive disease in cattle, and in these cases clinical signs such as abortion, return to estrus and birth of weak animals are present (Mughini-Gras et al. 2014; Favero et al. 2017). In cattle, the DNA of *Leptospira* sp. has been identified in samples of vaginal fluid and in samples of cervicovaginal mucus and urine (Santana Oliveira et al. 2016). Positive PCR results from vaginal fluid samples suggesting the possibility of venereal transmission, although the pathogenesis of reproductive impairment in cattle is still not fully elucidated.

The acquisition of pure leptospiral cultures was not possible, and because the bacterium is very fastidious, isolation is not always a sensitive technique for the detection of leptospires, and failure is commonly reported. However, genetic sequencing revealed 100% identity with *L. borgpetersenii*, which belongs to the most frequently observed serogroup (Sejroe) in serology. The *L. borgpetersenii* serovar Hardjo strain Hardjobovis was isolated for the first time in cattle in Brazil and Latin America (Chideroli et al. 2016). In Italy, *L. borgpetersenii* serovar Hardjo strain Hardjobovis was also isolated from urine in two outbreaks of reproductive problems (abortions) in cattle (Mughini-Gras et al. 2014). Therefore, the identification of this species points to the importance of the transmission of leptospires among cattle, acting as the main reservoirs for the bacterium within the herd.

Thus, based on the high frequency of seropositivity and carriers (PCR), leptospirosis can be inferred to be the cause of the reproductive problems, although no other collection of material for bacterial isolation, serology, or PCR was performed in the year after the adoption of control measures. The control of bovine leptospirosis carried out through an integrated program based on immunization, antibiotic therapy and management changes has shown good results (Martins and Lilenbaum 2017). Vaccination is considered the cheapest method and essential measure for control. In cases of adapted strains, the association of immunization with the treatment are efficient measures (Lilenbaum and Martins 2014). Changes in management, also favor the control of the disease. Although serology and molecular analysis were not conducted one year after the application of the control measures, it is possible that a reduction of the disease occurred in the animals, because the sequels of the infection were reduced.

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Compliance with ethical standards All procedures were conducted in accordance with Ethics Committee of the Animal Science Federal Universit of Campina Grande, Brazil

Conflict of interest The authors declare that they have no conflicts of interest

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Figure caption

Fig. 1 The phylogenetic tree based on the fragment 16S rRNA gene sequences from *Leptospira* sp., was constructed Maximum- likelihood phylogenetic tree, model TN93. The analysis included 26 nucleotides sequences. ▲ Sequenced sample

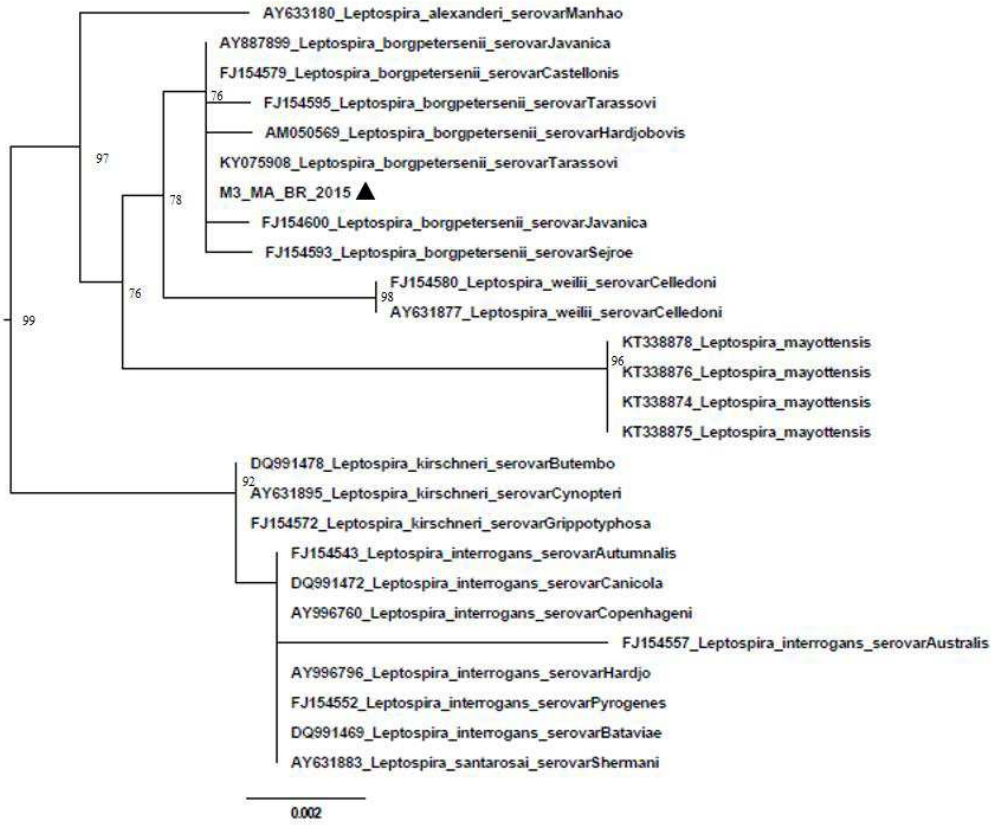
Table 1 Most frequent *Leptospira* sp. serogroups in the first visit and respective titers in a leptospirosis outbreak in cattle in the state of Maranhão, Northeastern Brazil

Serogroup	Titers					Total	Frequency (%)
	100	200	400	800	3200		
Sejroe	45	58	19	3	1	126/136	92.7
Tarassovi	1	2	4			7/136	5.1
Hebdomadis	1	1				2/136	1.5
Australis	1					1/136	0.7
Total	48	71	23	3	1	136/136	100

Table 2 Results of diagnostic tests in 24 cattle with reproductive problems in the second visit in a leptospirosis outbreak in the state of Maranhão, Northeastern Brazil

Animal	Reproductive failures	PCR/vaginal fluid	PCR/urine	1st Serology/serovar/titer	2nd Serology*/serovar/titer	Sequencing
M1	Abortion/repeated estrus	+	-	+/ Wolffi/100	+/ Tarassovi/400	-
M2	Abortion	+	-	+/ Tarassovi/400	+ / Tarassovi/ Wolffi/ 400	-
M3	Abortion	+	+	-	+/ Tarassovi/ Hebdomadis/ Grippotyphosa/ Wolffi/ 100	+
M4	Abortion	-	-	-	+/ Tarassovi/ 200	-
M5	Abortion/repeated estrus	-	-	-	+ / Tarassovi/ 100	-
M6	Abortion/repeated estrus	-	-	+/ Wolffi/ 400	+/ Wolffi/ 400	-
M7	Stillbirth	-	-	-	+/ Tarassovi/ 100	-
M8	Abortion/repeated estrus	-	-	-	+/ Tarassovi/ 100	-
M9	Abortion/repeated estrus	-	-	+/ Hardjoprajitno/ 200	+/ Grippotyphosa/ 400	-
M10	Abortion/repeated estrus	-	-	-	-	-
M11	Abortion	-	-	-	+/ Tarassovi/ 200	-
M12	Abortion/repeated estrus	-	-	+/ Sejroe/400	+/ Hardjoprajitno/ 200	-
M13	Abortion/repeated estrus	-	-	-	+/ Tarassovi/ 100	-
M14	Abortion	-	-	-	+/ Hebdomadis/ Hardjoprajitno/ 200	-
M15	Stillbirth	-	-	-	+/ Hebdomadis/ Hardjoprajitno/ 200	-
M16	Stillbirth	-	-	-	+/ Tarassovi/ 100	-
M17	Stillbirth	-	-	+/ Wolffi/ 100	+/ Tarassovi/ 200	-
M18	Abortion	-	-	-	+/ Tarassovi/ Wolffi/ 200	-
M19	Abortion	+	-	-	+/ Tarassovi/ 400	-
M20	Abortion/repeated estrus	+	-	-	+ /Hardjopratijno/ 200	
M21	Abortion/repeated estrus	+	-	-	+/ Wolffi/ 400	-
M22	Stillbirth	+	-	+/ Wolffi/ 100	+/ Grippotyphosa/ 200	-
M23	Stillbirth/repeated estrus	+	-	+/ Wolffi/ 100	+/ Wolffi/ 400	-
M24	Abortion/repeated estrus	-	-	-	+/ Tarassovi/ 200	

*30 days after first serology


AY633180_Leptospira_alexanderi_serovarManhao
AY887899_Leptospira_borgpetersenii_serovarJavanica
FJ154579_Leptospira_borgpetersenii_serovarCastellonis
FJ154595_Leptospira_borgpetersenii_serovarTarassovi
AM050569_Leptospira_borgpetersenii_serovarHardjobovis
KY075908_Leptospira_borgpetersenii_serovarTarassovi
M3_MA_BR_2015
FJ154600_Leptospira_borgpetersenii_serovarJavanica
FJ154593_Leptospira_borgpetersenii_serovarSejroe
FJ154580_Leptospira_weilii_serovarCelledoni
AY631877_Leptospira_weilii_serovarCelledoni
KT338878_Leptospira_mayottensis
KT338876_Leptospira_mayottensis
KT338874_Leptospira_mayottensis
KT338875_Leptospira_mayottensis
DQ991478_Leptospira_kirschneri_serovarButembo
AY631895_Leptospira_kirschneri_serovarCynopteri
FJ154572_Leptospira_kirschneri_serovarGrippotyphosa
FJ154543_Leptospira_interrogans_serovarAutumnalis
DQ991472_Leptospira_interrogans_serovarCanicola
AY996760_Leptospira_interrogans_serovarCopenhageni
FJ154557_Leptospira_interrogans_serovarAustralis
AY996796_Leptospira_interrogans_serovarHardjo
FJ154552_Leptospira_interrogans_serovarPyrogenes
DQ991469_Leptospira_interrogans_serovarBataviae
AY631883_Leptospira_santarosai_serovarShermani
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CAPÍTULO II: High proportion of cattle and sheep seropositive and renal carriers of Leptospira sp. under semiarid conditions

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High proportion of cattle and sheep seropositive and renal carriers of *Leptospira* sp. under semiarid conditions

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Abstract

This study aimed to serologically and molecularly characterize *Leptospira* sp. in cattle and sheep herds in the semiarid condition of Northeastern Brazil. Based on a preliminary study performed in our research group, we selected six rural properties showing a positivity ≥ 60% for the Sejroe serogroup with a titer ≥ 200 measured on serological tests. Blood samples were collected for serological diagnosis. Urine samples were collected from 99 females of reproductive age (51 bovine, 48 ovine). Molecular diagnostics and the isolation of *Leptospira* sp. were performed of these samples using microscopic agglutination tests (MAT), polymerase chain reaction (PCR), and bacteriological culture. Of the 99 analyzed animals, 38.4% (38/99) were reactive on the serological tests. Of them, 49% (25/51) were bovines and 27.1% (13/48) were ovines. The serogroups detected in cattle were Sejroe (36.8%), Hebdomadis (26.3%), Australis (10.5%), Djasiman (10.5%), Balum (5.3%), Pomona (5.3%), and Cynopteri (5.3%) with titers of 100–800. In sheep, the reactive serogroups were Australis (27.3%), Balum (27.3%), Djasiman (18.1%), Tarassovi (9.1%), Icterohaemorrhagiae (9.1%), and Cynopteri (9.1%) with titers of 100–400. Leptospiral DNA was detected by PCR in nine urine samples, included five cattle and four sheep. Property 1 showed the highest serological positivity frequencies for both cattle (70.6%) and sheep (70.6%). Similarly, the highest frequency was observed by PCR in eight positive samples (89%). In this property, we observed the existence of a consortia of breeding of cattle and sheep. This practice implies that the animals maintained close contact with one another while grazing in the same place. No pathogen growth was observed in the urine samples in the culture medium. In semiarid conditions, transmission between animals of the same species seems to be the main form of *Leptospira* dissemination in sheep and cattle. However, the contribution of other domestic and wild animals cannot be discarded. The practice of consortia breeding of cattle and sheep and their close coexistence might facilitate the spread of the pathogen in rural properties.

Keywords: Animal leptospirosis; Consortia breeding; Ruminants; Molecular detection; Semiarid conditions

1. Introduction

The development of cattle and sheep farming is of paramount importance for Brazilian agriculture and livestock in addition to contributing to the income of rural producers (Campos et al., 2017). Leptospirosis, one of the most important infectious diseases in the cattle and sheep production, is caused by bacteria of the genus *Leptospira* sp. and stands out as causing serious reproductive problems such as abortions, birth of weak animals, stillbirth, and fetal mummification in addition to reduced milk production, which causes substantial economic losses to cattle and sheep industry (Ellis, 2015; Loureiro et al., 2017).

Under natural conditions, any *Leptospira* sp. serovar can affect any animal species. However, some animals might adapt to certain strains. Cattle are recognized as adapted hosts of the Sejroe serogroup, which is reported in >80% of studies in Latin America (Pinto et al., 2016). Hardjo is the most common serovar in cattle worldwide (Hernández-Rodríguez et al., 2011; Pinto et al., 2017). This adaptation favors maintaining the bacteria in the environment since bovines act as sources of infection for their own and other animal species (Mughini-Gras et al., 2014). *Leptospira* sp. infection in sheep has been commonly associated with the serovar Hardjo, also adapted to small ruminants, and the serogroup Autumnalis (Higino et al., 2013; Martins and Lilenbaum, 2014).

Northeastern Brazil is characterized by a semiarid climate with low rainfall and high temperatures. These conditions, associated with the characteristics of caatinga, plant formation in this region, and a unique biome that is exclusively Brazilian and presents a wide diversity of animal species (Pereira Junior et al., 2014), offer unique epidemiological conditions that require consideration in circumstances different from those of other regions of Brazil and the world. Thus, the aim of this study was to perform serological and molecular characterizations of *Leptospira* sp. infection in cattle and sheep under semiarid conditions in Northeastern Brazil.

2. Material and methods

2.1. *Characterization of the study area*

The state of Paraíba, located in the Northeastern region of Brazil, is characterized by warm weather throughout the year. The state is geographically subdivided into the following four major regions, based mostly on vegetation type and rainfall: (i) Zona da Mata (Atlantic forest), (ii) Agreste, (iii) Borborema, and (iv) Sertão. The Zona da Mata and Agreste have

relatively higher rainfall regimes. Both Borborema and Sertão (the semiarid region) are typically within the Caatinga biome, which encompasses an area of 900,000 km² (11% of Brazilian territory) and is the only major biome that occurs exclusively in Brazil. Caatinga is xeric shrubland and thorn forest, which consists primarily of small thorny trees that shed their leaves seasonally. Cacti, thick-stemmed plants, thorny brush and arid-adapted grasses make up the ground layer. However, during the dry periods there is no ground foliage or undergrowth (Andrade-Lima, 1981). The weather is characterized by a hot and semiarid climate, with temperatures averaging 27◦C, and the mean annual rainfall is typically ≈500 mm. There are typically two seasons: a rainy season from February to May, and a long drought period from June to January. However, occurrences of droughts sometimes lasting for longer than one year is also a characteristic of the region (Batista et al., 2007).

2.2. *Study population*

Based on preliminary study performed by our research group (Pimenta et al., 2014), we selected six rural properties presenting ≥ 60% seropositivity for the serogroup Sejroe with antibody titers ≥ 200. These properties were located in the municipalities of Boa Ventura (Property 1), Malta (Property 2), Olho d'Água (Property 3), Piancó (Property 4), Quixaba (Property 5), and Santana dos Garrotes (Property 6) in the mesoregion of Sertão (Figure 1). We selected a total of 99 animals (51 cattle, 48 sheep), all females in reproductive age. None were vaccinated against leptospirosis. Properties 1, 2, 3, and 6 had cattle and sheep, while Properties 4 and 5 had cattle only.

2.3. *Sample collection*

Blood samples were collected by jugular vein puncture in 10-mL vacuum tubes with the aim of subsequently obtaining serum upon centrifugation. The samples were then stored at -20°C until serological testing. In cattle, urine samples were collected using diuretic furosemide (MSD Animal Health, São Paulo, SP, Brazil) at a dose of 2.5 mL/animal intramuscularly. In sheep, urine samples were collected with a urethral probe no. 8 through 10-mL sterile disposable syringes. For the molecular analyses, urine samples (2 mL) were aliquoted into microtubes containing 100 μL of 10× phosphate buffered saline. The samples were immediately refrigerated and transported within a maximum of 2 hours to the laboratory and stored at −20°C until DNA extraction.

2.4. *Serological diagnosis of* Leptospira *sp. infection*

A serological diagnosis of leptospirosis was made using the microscopic agglutination test (MAT), as recommended by the World Organization for Animal Health (OIE, 2014). The serum samples were screened for antibodies against a battery of 24 serogroups. Sera with 50% or more agglutination at the indicated dilution were titrated in several two-fold geometric dilutions. The serum titer was the reciprocal of the highest dilution that presented a positive result.

2.5. *Bacteriological culture*

Immediately after collection, 1 mL of urine was inoculated in a final concentration of 10% in semisolid EMJH medium (Difco, BD Franklin Lakes, NJ, USA) supplemented with 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) to inhibit the proliferation of contaminating microorganisms (Chakraborty et al., 2011). After 24 hours, 1 mL was inoculated in the semisolid EMJH medium supplemented only with 5-fluorouracil (1 mg/mL) in a proportion of 10% and subsequently incubated at 30°C. The tubes were examined weekly by microscopy with the samples in a dark field to evaluate the growth of microorganisms with *Leptospira*-like morphology over a period of at least 6 weeks (Miraglia et al., 2003).

2.6. *Molecular detection of Leptospira sp*.

DNA from *Leptospira* sp. was extracted using the Wizard® Genomic SV DNA Purification System Kit (Promega®, Madison, USA). PCR was performed as previously described (Stoddard et al., 2009). 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 Pomona serogroup strain and ultrapure water were used as positive and negative controls, respectively.

2.7. *Statistical analysis*

The chi-square or Fisher's exact test was used to compare the positivity ratios in MAT and PCR between cattle and sheep. The analyses were performed using the BioEstat 5.03 program (Ayres et al., 2007) considering a significance level of 0.05.

3. Results and discussion

The serogroups identified in cattle were Sejroe (36.8%), Hebdomadis (26.3%), Australis (10.5%), Djasiman (10.5%), Balum (5.3%), Pomona (5.3%) and Cynopteri (5.3%) with titers of 100–800 (Table 1). In sheep, the frequent serogroups were Australis (27.3%), Balum (27.3%), Djasiman (18.1%), Tarassovi (9.1%), Icterohaemorrhagiae (9.1%), and Cynopteri (9.1%) with titers of 100–400 (Table 2).

In Property 1, cattle showed positive reactions against for Hebdomadis, Sejroe, Djasiman, Australis, Balum, and Cynopteri serogroups, while sheep showed positive reactions for Australis, Balum, Djasiman, Cynopteri, Tarassovi, and Hebdomadis serogroups. In Property 2, it was found a reaction for Sejroe and Icterohaemorrhagiae serogroups in cattle and sheep, respectively. In Properties 3 to 6, there were reactions against the Sejroe, Pomona, and Hebdomadis serogroups in cattle (Table 3).

Of the 99 analyzed animals, 38.4% (38/99) were reactive at the serological test, being 49% (25/51) in cattle and 27.1% (13/48) in sheep (P = 0.042). The proportions of PCR-positivity in cattle and sheep were 9.8% (5/51) and 8.3% (4/48), respectively (P = 0.206). Property 1 presented the highest seropositivity for cattle (70.6%) and sheep (70.6%) (Table 4), as well as the highest PCR-positivity for both cattle and sheep (23.5%). It was not possible to isolate viable leptospires from any urine sample.

Despite the animals are under the same climate and management conditions, cattle showed more significant serological results, which possibly suggests a higher resistance of sheep to the infection as indicated in previous study (Costa et al., 2017). Despite this, the seropositivity in sheep was 27.1%, a high percentage considering the results obtained in other studies conducted in this species in Brazil (Amorim et al., 2016; Costa et al., 2016; Higino et al., 2010).

The frequency of *Leptospira* sp. seropositivity was high in both cattle and sheep despite the semiarid region being unfavorable for *Leptospira* survival (Faine et al., 1999;

Hashimoto et al., 2012). This finding points out to the existence of alternative transmission routes in the presence of adverse conditions. Recently, several studies have investigated the possibility of male–female venereal transmission in cattle and sheep (Director et al., 2014; Lilenbaum et al., 2008; Loureiro et al., 2016; Loureiro et al., 2017). Similarly, in a recent study (Silva et al., 2018) by our research group *Leptospira* DNA was detected in 55% (61/111) in tissue samples of genital tract (uterus, ovary, and uterine tubes) collected from sheep slaughtered in the Brazilian semiarid region and in 33.3% (8/24) of vaginal swab samples collected from cattle in a leptospirosis outbreak in Northeastern Brazil (Pimenta et al., 2018). Therefore, this transmission route might favor the leptospirosis to be endemic in herds (Loureiro et al., 2016), justifying the significance of these findings.

The Sejroe serogroup was detected most frequently in the cattle in the present study as reported by other authors (Marques et al., 2010; Martins and Lilenbaum, 2013; Silva et al., 2012). This result is expected since cattle are considered capable of adapting to this serogroup (Martins et al., 2012; Martins and Lilenbaum, 2013). It can be inferred that cattle are acting as important sources of infection within herds, transmitting *Leptospira* by direct contact. These aspects should be considered in the epidemiology and elaboration of control and prevention measures against this serogroup since this transmission is occurring within the same species with low dependence on environmental factors (Martins and Lilenbaum, 2017). The Australis and Balum serogroups were the most frequent in sheep, corroborating the results reported by other authors (Amorim et al., 2016; Azevedo et al., 2004; Benkirane et al., 2014; Carvalho et al., 2011; Costa et al., 2016). Australis is commonly observed in swine (Hamond et al., 2015), while domestic rats are the main reservoirs of the serogroup Balum (Bharti et al., 2003).

In Property 1, Australis, Balum, Hebdomadis, and Djasiman serogroups were identified in both cattle and sheep. The highest serological positivity frequencies were observed in both cattle (70.6%) and sheep (70.6%). It is worth mentioning that among the total number of PCR-positive samples 8 (89%) were from this property. It was verified that there was the practice of consorted rearing of cattle and sheep in Property 1, and it was the only one in which animals were in close contact and grazed in the same place. Thus, it is suggests that, in semiarid conditions, this close contact among species favors the spreading of *Leptospira* as observed by Escócio et al. (2010) and Genovez et al. (2011) when evaluating the transmission of *Leptospira* between cattle and sheep in consortia herds and exclusive sheep herds in the state of São Paulo, Southeastern Brazil.

The non-isolation of leptospires from urine probably occurred due to intermittent *Leptospira* elimination and the possibility that the animals were not eliminating the agent since serology cannot identify animals that are renal carriers (Libonati et al., 2017; Rocha et al., 2017). Other possibilities would be the slow growth rate of the bacteria (Adler and Moctezuma, 2010) and the contamination of cultures by other microorganisms (Rahelinirina et al., 2010).

In conclusion, in semiarid conditions, transmission between animals of the same species seems to be the main form of *Leptospira* dissemination in sheep and cattle, however, the role of other domestic and wild animals cannot be discarded. It is also suggested that the practice of consorted rearing of cattle and sheep and their close coexistence facilitates the spread of the agent in rural properties, as well as the existence of alternative transmission routes in the presence of unfavorable environmental conditions for *Leptospira* survival.

Declaration of conflict of interest

The authors declare no conflicts of interest.

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Figure caption

Figure 1. Map of the state of Paraíba with the municipalities studied. The detail shows the state of Paraíba in Brazil.

Table 1

Serogroups of Leptospira sp. in cattle in semi-arid conditions in Northeast Brazil, with the respective titles.

Sorogroups	Titles				Total (%)
	1:100	1:200	1:400	1:800	
Sejroe	1	4	2	-	7 (36,8)
Australis	-	1	1	-	2 (10,5)
Pomona	-	-	-	1	1 (5,3%)
Balum	1	-	-	-	1 (5,3)
Hebdomadis	2	3	-	-	5 (26,3)
Cynopteri	-	1	-	-	1 (5,3)
Djasiman	2	-	-	-	2 (10,5)

Table 2

Serogroups of Leptospira sp. in semiarid conditions in the Northeast of Brazil, with the respective titers.

Sorogroups	Titers			Total (%)
	1:100	1:200	1:400	
Australis	-	2	1	3 (27,3)
Tarassovi	-	1	-	1 (9,1)
Balum	1	2	-	3 (27,3)
Cynopteri	1	-	-	1 (9,1)
Djasiman	1	1	-	2 (18,1)
Icterohaemorrhagiae	-	1	-	1 (9,1)

Table 3

Frequency of cattle and sheep raised in semi-arid conditions in Northeast Brazil, positive in the serology and molecular detection of Leptospira sp. according to the property of origin.

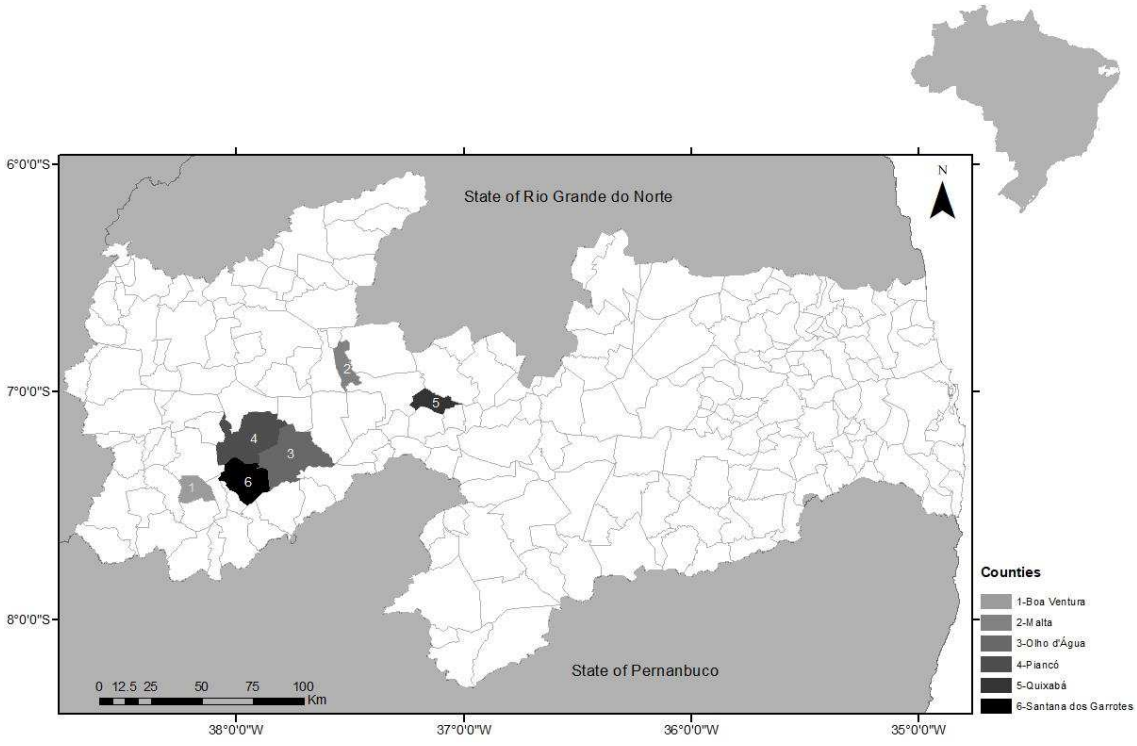
Property	Nº de animals		Seropositivite		PCR positive	
	Catlle	Sheep	Catlle (%)	Sheep (%)	Catlle (%)	Sheep (%)
1	17	17	12 (70,6)	12 (70,6)	4 (23,5)	4 (23,5)
2	7	9	4 (57,1)	1 (11,1)	0 (0)	0 (0)
3	3	17	1 (33)	0 (0)	0 (0)	0 (0)
4	5	...	1 (20)	...	0 (0)	...
5	13	...	4 (30,8)	...	0 (0)	...
6	6	5	3 (50)	0 (0)	1 (16,7)	0 (0)
Total	51	48	25 (49)	13 (27,1)	5 (9,8)	4 (8,3)

Table 4

Serogroups of Leptospira sp. in cattle and sheep raised in semi-arid conditions in Northeast Brazil, according to the original property.

Property	Serogroups found in cattle (%)	Serogroups found in sheep (%)
1	Hebdomadis (25%); Sejroe (25%); Djasiman (16,6%); Australis (16,6%); Balum (8,4%); Cynopteri (8,4%)	Australis (30%); Balum (30%); Djasiman (10%); Cynopteri (10%); Tarassovi (10%); Hebdomadis (10%)
2	Sejroe (100%)	Icterohaemorrhagiae (100%)
3	Serjoe (100%)	Negativo
4	Pomona (100%)	...[a]
5	Sejroe (66,7%); Hebdomadis (33,3%)	...[a]
6	Sejroe (50%); Hebdomadis (50%)	Negativo

[a] Absence of sheep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Km
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Counties
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2-Malta
3-Olho d'Água
4-Piancó
5-Quixabá
6-Santana dos Garrotes

Fig. 1

CAPÍTULO III: Soropositividade e sorogrupos de *Leptospira* sp. predominantes em exames sorológicos de ruminantes do Nordeste do Brasil

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Soropositividade e sorogrupos de *Leptospira* sp. predominantes em exames sorológicos de ruminantes do Nordeste do Brasil

Seropositivity and most frequent *Leptospira* sp. serogroups in serological tests of ruminants from Northeastern Brazil

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Resumo

O objetivo deste estudo foi determinar a soropositividade para leptospirose e os sorogrupos predominantes nos testes sorológicos realizados no Laboratório de Doenças Transmissíveis (LDT) da Universidade Federal de Campina Grande (UFCG), Patos, Paraíba, Nordeste do Brasil, em bovinos, caprinos, ovinos e bubalinos no período de 2010 a 2017**.** Foram computados os registros dos exames sorológicos para leptospirose de 5.594 animais, que incluíram 1.527 bovinos, 1.761 caprinos, 2.170 ovinos e 136 bubalinos, provenientes de quatro estados brasileiros (Paraíba, Pernambuco, Maranhão e Rio Grande do Norte). Das 5.594 amostras de soro de bovinos, caprinos, ovinos e bubalinos, 662 amostras foram positivas no teste sorológico, resultando em uma frequência de 11,8%. Serjoe (30,6%), Autumnalis (13,6%) e Icterohaemorrhagiae (11,3%) foram os sorogrupos mais frequentes para todas as espécies. As frequências individuais de bovinos, caprinos, ovinos e bubalinos foram de 20% (306/1.527), 8,3% (147/1.761), 7,9% (171/2.170), e 27,9% (38/136), respectivamente, com títulos variando de 1:100 a 1:3200. Com relação aos sorogrupos mais

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frequentes por espécie animal, o Serjoe predominou em bovinos (62%), seguido de Icterohaemorrhagiae (12,5%) e Tarassovi (6,6%); Autumnalis foi o mais frequente em caprinos e ovinos (29,4% e 26,9%, respectivamente), seguido de Seramanga (12,5%) em caprinos e Icterohaemorrhagiae (13,5%) em ovinos; Australis predominou nos bubalinos (39,5%), seguido de Pomona (31,6%) e Canicola (21,1%). Conclui-se que a infecção por *Leptospira* sp., determinada por sorologia, encontra-se difundida em ruminantes (bovinos, caprinos, ovinos e bubalinos) do Nordeste do Brasil, o que sugere a existência de vias de transmissão alternativas menos dependentes de fatores ambientais, bem como a identificação dos sorogrupos mais frequentes sugere a necessidade de melhoria das condições sanitárias e implementação de medidas de controle eficientes e direcionadas para as principais fontes de infecção.

Palavras chave: Leptospirose. Sorologia. Ruminantes. Sorogrupos. Controle.

Abstract

The objective of this study was to determine the seropositivity for leptospirosis and the serogroups prevalent in the serological tests performed at the Laboratory of Transmissible Diseases (LDT) of the Federal University of Campina Grande (UFCG), Patos, Paraíba, Northeastern Brazil, in cattle, goats, sheep and buffaloes in the period from 2010 to 2017. The records of the serological tests for leptospirosis of 5,594 animals were used, including 1,527 cattle, 1,761 goats, 2,170 sheep and 136 buffaloes from four Brazilian states (Paraíba, Pernambuco, Maranhão and Rio Grande From north). Of the 5,594 serum samples from cattle, goats, sheep and buffalo, 662 samples were positive in the serological test, resulting in a frequency of 11.8%. Serjoe (30.6%), Autumnalis (13.6%) and Icterohaemorrhagiae (11.3%) were the most frequent serogroups for all species. The individual frequencies of cattle, goats, sheep and buffaloes were 20% (306/1,527), 8.3% (147/1,761), 7.9% (171/2,170) and 27.9% (38/136), respectively, with titers ranging from 1:100 to 1:3200. Serjoe predominated in cattle (62%), followed by Icterohaemorrhagiae (12.5%) and Tarassovi (6.6%); Autumnalis was the most frequent in goats and sheep (29.4% and 26.9%, respectively), followed by Seramanga (12.5%) in goats and Icterohaemorrhagiae (13.5%) in sheep; Australis predominated in buffaloes (39.5%), followed by Pomona (31.6%) and Canicola (21.1%). It is concluded that *Leptospira* sp infection, determined by serology, is widespread in ruminants (cattle, goats,

sheep and buffalo) in Northeastern Brazil, which suggests the existence of alternative transmission routes less dependent on environmental factors, as well as the identification of the most frequent serogroups suggests the need to improve sanitary conditions and implementation of efficient and targeted control measures for the main sources of infection.

Key words: Leptospirosis. Serology. Ruminants. Sorogroups. Control.

Introdução

A leptospirose é uma zoonose causada por espiroquetas do gênero *Leptospira* que afeta muitas espécies de mamíferos, incluindo seres humanos, sendo evidenciada em todo o mundo e particularmente prevalente em países de clima tropical e subtropical, principalmente em períodos de altos índices pluviométricos (AGUIAR et al., 2010; VIEIRA et al., 2018).

Na pecuária, a enfermidade está relacionada à redução do desempenho reprodutivo dos rebanhos acometidos, ocasionando perdas econômicas (ELLIS, 2015). A transmissão da leptospirose ocorre indiretamente pelo contato com água ou solo contaminados ou pelo contato direto com a urina de animais portadores (PICARDEAU, 2013). A bactéria penetra no hospedeiro por lesões na pele e membranas mucosas, invadem a circulação, espalham-se por todo o animal e se alojam nos túbulos renais, sendo eliminadas através da urina, contaminando o meio ambiente e (ADLER, 2014; ELLIS, 2015).

A infecção por leptospiras pode ser incidental ou adaptada. A infecção incidental, altamente dependente de fatores ambientais, é causada por sorogrupos não adaptados transmitidos por outras espécies de animais domésticos ou silvestres. No segundo caso, a infeção é determinada por sorogrupos adaptados, menos dependentes de condições ambientais, no qual o hospedeiro de manutenção age como uma fonte natural de infecção para sua própria espécie, associada a sorogrupos de leptospira específicos (FAINE et al. 1999; LEVETT, 2001; SUEPAUL et al., 2011).

O diagnóstico sorológico através da técnica de soroaglutinação microscópica (SAM) é considerado uma boa alternativa de diagnóstico, principalmente para os animais de produção, onde o diagnóstico é focado no coletivo, ou seja, direcionado para o rebanho (SUEPAUL et al., 2011; PINTO et al., 2015). Neste sentido, os inquéritos soroepidemiológicos são necessários para a realização do monitoramento e controle da leptospirose em uma região, pois eles possibilitam o levantamento de indicadores epidemiológicos e, com base nisso, a

elaboração de estratégias de prevenção e direcionamento de novas políticas públicas, fortalecendo a saúde pública em geral.

Diversos estudos sorológicos para leptospirose foram conduzidos com base em levantamentos conduzidos em laboratórios de diagnóstico. Favero et al. (2002) realizaram um estudo retrospectivo apresentando as variantes sorológicas de leptospiras predominantes em testes sorológicos efetuados em ovinos, caprinos, bubalinos, suínos, cães e equinos de diversos estados brasileiros, no período de 1984 a 1997. Martins e Lilenbaum (2013) avaliaram vários estudos conduzidos no Rio de Janeiro para diagnóstico de leptospirose, em cães, ratos, bovinos, equinos, caprinos, ovinos, suínos e mamíferos silvestres, durante 20 anos. Tagliabue et al. (2016) avaliaram a situação epidemiológica da leptospirose na Itália, com base em dados de soros de bovinos, bubalinos, equinos, ovinos, caprinos, suínos, cães e animais silvestres, provenientes de 10 laboratórios, entre 2010 e 2011. Campos et al. (2017) analisaram amostras de soro de ovinos, caprinos e bovinos do estado do Piauí, entre 2013 e 2015.

O presente trabalho teve por objetivo a determinação da frequência de leptospirose e dos sorogrupos predominantes nos testes sorológicos realizados no Laboratório de Doenças Transmissíveis da Universidade Federal de Campina Grande (UFCG), Patos, Paraíba, Nordeste do Brasil, em bovinos, caprinos, ovinos e bubalinos, durante o período de 2010 a 2017.

Materiais e Métodos

Diagnóstico sorológico de Leptospira sp.

O diagnóstico sorológico de leptospirose foi realizado com a técnica de soroaglutinação microscópica (SAM), de acordo com Galton et al. (1965) e Cole et al. (1973). Utilizou-se coleção com antígenos vivos, representados pelos sorogrupos Icterohaemorrhagiae (sorovares Icterohaemorrhagiae e Copenhageni), Canicola, Pomona, Grippotyphosa, Serjoe (Wolffi, Hardjoprajitno, Hardjobovis e Guaricura), Australis (Australis e Bratislava), Andamana, Autumnalis (Autumnalis e Butembo), Bataviae, Balum (Castellonis), Cynopteri, Djasiman (Sentot), Hebdomadis, Panama, Tarassovi, Javanica, Celledoni (Whitcombi), Pyrogenes, Shermani e Seramanga (Patoc).

Os soros foram triados na diluição de 1:100, e aqueles que apresentaram 50% ou mais de aglutinação foram titulados pelo exame de uma série de diluições geométricas de razão dois. O título do soro foi a recíproca da maior diluição que apresentou resultado positivo. Os antígenos foram examinados ao microscópio de campo escuro, previamente aos testes, com o intuito de verificar a mobilidade e a presença de auto-aglutinação ou de contaminantes. Para o cálculo do sorogrupo mais frequente, os soros que apresentaram duas ou mais variantes sorológicas com título mais alto idêntico foram descartados da análise para a sorogrupo, porém considerados soropositivos para *Leptospira* sp.

Registros

Para a execução deste trabalho realizou-se o levantamento dos resultados da SAM para diagnóstico de leptospirose, e as informações analisadas foram compiladas do banco de dados do Laboratório de Doenças Transmissíveis (LDT) da Universidade Federal de Campina Grande (UFCG), Patos, Paraíba, Nordeste do Brasil. Foram computados os registros dos exames sorológicos para leptospirose de 5.594 animais, que incluíram 2.170 ovinos, 1.761 caprinos, 1.527 bovinos e 136 bubalinos, provenientes de quatro estados brasileiros (Paraíba, Pernambuco, Maranhão e Rio Grande do Norte), durante o período de 2010 a 2017, distribuídos da seguinte maneira: Ovinos - PB (94,5%), PE (5,5%); Caprinos – PB (86,8%), PE (13,2%); Bovinos – PB (53,9%), MA (20,4%), RN (15,9%), PE (9,8%); e Bubalinos - PB (100%).

Resultados e Discussão

Das 5.594 amostras de soro de bovinos, caprinos, ovinos e bubalinos, 662 amostras foram positivas no teste sorológico, resultando em uma frequência de 11,8%. Para o cálculo do sorogrupo mais frequente foram consideradas 601 amostras, sendo o Serjoe (30,6%), Autumnalis (13,6%) e Icterohaemorrhagiae (11,3%) os mais frequentes para todas as espécies (Tabela 1). A frequência de positividade (11,8%) pode ser considerada alta, o que demonstra a ampla difusão da infecção nas espécies estudadas, levando em consideração que a prática de vacinação não é comum na maioria das propriedades rurais do Nordeste brasileiro. Por outro lado, apesar das condições ambientais adversas para a sobrevivência de leptospiras no semiárido brasileiro, este percentual elevado sugere a existência de vias de transmissão

alternativas menos dependentes de fatores ambientais. De fato, trabalhos conduzidos por nosso grupo de pesquisa têm evidenciado a possível importância da transmissão venérea fêmea-macho na disseminação da leptospirose em ruminantes. Pimenta et al. (2018) investigaram um surto de leptospirose em bovinos do Maranhão e verificaram que de 24 vacas com problemas reprodutivos (abortamento, repetição de cio e natimortos) oito (33,3%) foram positivas na reação em cadeia pela polimerase (PCR) de fluido vaginal, com sequenciamento genético positivo para *Leptospira borgpetersenii*. Costa et al. (2018) conduziram infecção experimental em ovinos da raça Santa Inês e mestiços, e referiram que não houve diferença estatística na proporção de amostras de urina e fluido vaginal positivas na PCR. Em outro trabalho (dados não publicados), foi detectado DNA de leptospiras patogênicas em 54,9% (61/111) das amostras de trato genital (útero, fluido vaginal e ovário) de ovinos abatidos na Paraíba.

Em relação ao sorogrupo Serjoe como o mais frequente em ruminantes no presente estudo, este resultado não é inesperado, visto que este sorogrupo é o mais comumente observado em estudos conduzidos em pequenos ruminantes e bovinos (MARTINS; LILENBAUM, 2013; DIRECTOR et al., 2014). As reações aos sorogrupos Australis e Icterohaemorrhagiae estão relacionadas a infecções incidentais por estirpes mantidas por outros animais domésticos e silvestres, sugerindo a necessidade de melhoria das práticas sanitárias, tais como vacinação, terapia antibiótica, gestão ambiental, controle de roedores e aumento da higiene ambiental, objetivando reduzir a propagação da leptospirose (FAINE et al., 1999; ZAKERI et al., 2010).

As soropositividades para bovinos foram de 6,4%, 32,6%, 50,9% e 18,5% nas amostras provenientes dos estados da Paraíba, Pernambuco, Maranhão e Rio Grande do Norte, respectivamente, e para bubalinos foi de 27,9%, no estado da Paraíba, enquanto que nos ovinos e caprinos as frequências foram de 7,5% e 8,5% para amostras da Paraíba e 13,4% e 6,8% para as amostras de Pernambuco (Tabela 2), o que demonstra menores soropositividades dos pequenos ruminantes em relação aos bovinos e bubalinos. Essa discrepância pode ser justificada pela rusticidade e resistência natural à infecção atribuída aos pequenos ruminantes (COSTA et al., 2016). Por outro lado, Costa et al. (2018) referiram maior susceptibilidade de ovinos de raças puras em comparação com animais mestiços, bem como enfatizaram a importância do trato genital como local de infecção extraurinária e destacaram a possibilidade de transmissão venérea nos ovinos.

As frequências individuais de bovinos, caprinos, ovinos e bubalinos foram de 20% (306/1.527), 8,3% (147/1.761), 7,9% (171/2.170), e 27,9% (38/136), respectivamente, com títulos variando de 1:100 a 1:3200. Com relação aos sorogrupos mais frequentes por espécie animal (Tabela 3), o Serjoe predominou em bovinos (62%), seguido de Icterohaemorrhagiae (12,5%) e Tarassovi (6,6%); Autumnalis foi o mais frequente em caprinos e ovinos (29,4% e 26,9%, respectivamente), seguido de Seramanga (12,5%) em caprinos e Icterohaemorrhagiae (13,5%) em ovinos; Australis predominou nos bubalinos (39,5%), seguido de Pomona (31,6%) e Canícola (21,1%).

Em bovinos, a soropositividade obtida foi elevada, assim como resultados observados por Martins e Lilenbaum. (2013) no Rio de Janeiro, com frequência de 23%, Campos et al. (2017) no Piauí com 50%, Pinto et al. (2016) com 44,2% em uma revisão sistemática na América Latina, Pimenta et al. (2014) na Paraíba com 61,1%, e Silva et al. (2012) no Maranhão com 35,9%. Estes resultados, assim como o observado no presente trabalho, indicam que a enfermidade circula nos rebanhos bovinos estudados, determinando a importância que esse agente pode representar na sanidade desses animais e, consequentemente, para a saúde pública.

O sorogrupo predominante nos bovinos foi o Sejroe, apontado com frequência em vários inquéritos epidemiológicos no Brasil e em outras partes do mundo (MARTINS; LILENBAUM, 2013; MENEGAS et al., 2013; PINTO et al., 2015; PINTO et al., 2016; TAGLIABUE et al., 2016; CORREA et al., 2017; CAMPOS et al., 2017). Os bovinos são reconhecidos como hospedeiros de manutenção desse sorogrupo, responsável pelo desenvolvimento da doença crônica, infecção subclínica e persistente do trato reprodutivo, estando sua manutenção no rebanho relacionada à transmissão direta entre os animais por meio de urina e secreção vaginal (MARTINS; LILENBAUM, 2013, 2014). Ocorreram também reações para os sorogrupos Icterohaemorrhagiae e Tarassovi, para os quais os roedores sinantrópicos e os suínos, respectivamente, são considerados hospedeiros de manutenção, o que sugere a existência de roedores nas criações, desempenhando papel fundamental na contaminação ambiental (CAMPOS et al., 2017), bem como a ocorrência de contato entre bovinos e suínos (STRUTZBERG-MINDER; KREIENBROCK, 2011).

As frequências encontradas para caprinos e ovinos foram de 8,3% e 7,9%, respectivamente. Estudos conduzidos por Tagliabue et al. (2016) na Itália e Suwancharoen et al. (2013) na Tailândia observaram frequências próximas às encontradas no presente trabalho (4,7% para ovinos e 7,9% para caprinos), enquanto que outros autores avaliando essas

mesmas espécies, observaram resultados superiores, como é o caso de Aguiar et al. (2010), em Rondônia, que encontraram 33,3% de positividade para ovinos; Salaberry et al. (2011), em Minas Gerais, 22,2% em ovinos; Martins e Lilenbaum et al. (2013), no Rio de Janeiro, 47,4% e 14,95% para ovinos e caprinos, respectivamente; Cortizo et al. (2015), no Espírito Santo, 50% para ambas as espécies; Machado et al. (2016), em Pernambuco, 19,5% em ovinos. A discrepância entre os resultados se deve provavelmente às condições ambientais características de cada região estudada, manejo e medidas de controle adotadas nos rebanhos (COSTA et al., 2016; MACHADO et al., 2016).

O sorogrupo Autumnalis foi o mais frequente nos caprinos e ovinos, resultado que corrobora os achados de pesquisas sorológicas realizadas na Paraíba, Minas Gerais e Pernambuco, nos últimos anos (HIGINO et al., 2010; ALVES et al., 2012; SALABERRY et al., 2011; COSTA et al., 2016; MACHADO et al., 2016), sugerindo a possibilidade de que este sorogrupo esteja adaptado aos pequenos ruminantes. Nos caprinos, o sorogrupo Seramanga foi o segundo mais frequente, considerado não patogênico e associado à presença de roedores e animais de vida livre nas propriedades, tais como gambás (*Didelphis albiventris*), atuando como fontes de infecção (SILVA et al., 2013; PAIXÃO et al., 2016). Nos ovinos, o segundo sorogrupo mais frequente foi o Icterohaemorrhagiae, geralmente relacionado à presença de roedores, responsável por infecções incidentais nos demais hospedeiros (ESCÓCIO et al., 2010; GENOVEZ et al., 2011).

Na espécie bubalina, a soropositividade encontrada foi de 27,9%, resultado inferior aos encontrados por Viana et al. (2009) e Fávero et al. (2002), que avaliando bubalinos no Amazonas e São Paulo, encontraram frequências de 80% e 43,7%, respectivamente. Embora a frequência encontrada seja considerada alta, ela foi menor que nos outros estudos provavelmente pelo fato de que o estado da Paraíba estar localizado no semiárido brasileiro e, consequentemente, apresenta condições climáticas adversas para leptospirose, o que reflete as baixas soropositividades.

O sorogrupo Australis foi o mais frequente nos bubalinos, assim como observado por Viana et al. (2009), avaliando búfalos do Amazonas, embora a maioria dos inquéritos sorológicos citem a presença do sorogrupo Serjoe como mais predominante (FAVERO et al., 2002; SUWANCHAROEN et al., 2013; TAGLIABUE et al., 2016). Os sorogrupos Pomona e Canícola foram o segundo e o terceiro que apresentaram maior frequência, a alta patogenicidade atribuída ao sorogrupo Pomona sugere que os rebanhos bubalinos exibem resposta imunológica ativa contra leptospirose (PAIXÃO et al., 2016), e provavelmente os

suínos possam estar atuando como reservatórios tanto do sorogrupo Australis como Pomona, pois esses animais são reconhecidos como hospedeiros de manutenção desses sorogrupos, sendo sua presença nas propriedades fortemente associada a ocorrência de leptospirose incidental (LILENBAUM; SOUZA, 2003). Em relação ao sorogrupo Canícola, o estreito contato dos bubalinos com cães pode ser a justificativa para a identificação desse sorogrupo como um dos mais frequentes, visto que os cães são considerados adaptados a esse sorogrupo, servindo como reservatórios de leptospiras (MARTINS; LILENBAUM, 2013).

Conclusão

A infecção por *Leptospira* sp., determinada por sorologia, encontra-se difundida em ruminantes (bovinos, caprinos, ovinos e bubalinos) do Nordeste do Brasil, o que sugere a existência de vias de transmissão alternativas menos dependentes de fatores ambientais. A identificação dos sorogrupos mais frequentes permite inferir que os bovinos são responsáveis pela manutenção da bactéria nos rebanhos através do contato direto entre os animais, enquanto que nas outras espécies, outros animais domésticos e sinantrópicos são os responsáveis por manter a doença nos rebanhos, indicando a necessidade de melhoria das condições sanitárias e implementação de medidas de controle eficientes e direcionadas para as principais fontes de infecção.

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Tabela 1. Sorogrupos de *Leptospira* sp. mais frequentes em bovinos, caprinos, ovinos e bubalinos no Nordeste do Brasil, no período de 2010 a 2017.

Sorogrupo	Nº de animais positivos	Frequência (%)
Serjoe	184	30,6
Autumnalis	82	13,6
Icterohaemorrhagiae	68	11,3
Andamana	43	7,2
Australis	31	5,2
Seramanga	30	5,0
Pomona	27	4,5
Djasiman	25	4,2
Tarassovi	24	4,0
Balum	22	3,7
Celledoni	17	2,8
Canicola	16	2,7
Gryppotyphosa	12	2,0
Shermani	8	1,3
Javanica	7	1,2
Hebdomadis	2	0,3
Pyrogenes	1	0,2
Cynopeteri	1	0,2
Shermani	1	0,2

Tabela 2. Frequência de bovinos, caprinos, ovinos e bubalinos do Nordeste do Brasil reagentes no teste de soroaglutinação microscópica para diagnóstico de leptospirose, de acordo com o estado de origem, no período de 2010 e 2017.

Estado	Espécie	Nº de soros testados	Amostras soropositivas	Frequência (%)
PB	Ovino	2.051	155	7,5
	Caprino	1.528	131	8,5
	Bovino	822	53	6,4
	Bubalino	136	38	27,9
PE	Ovino	119	16	13,4
	Caprino	233	16	6,8
	Bovino	150	49	32,6
MA	Bovino	312	159	50,9
RN	Bovino	243	45	18,5

Tabela 3. Sorogrupos de *Leptospira* sp. frequentes em bovinos, caprinos, ovinos e bubalinos do Nordeste do Brasil de acordo com a espécie animal, no período de 2010 a 2017.

Sorogrupo	Bovinos	Caprinos	Ovinos	Bubalinos
	Nº de animais positivos (%)	Nº de animais positivos (%)	Nº de animais positivos (%)	Nº de animais positivos (%)
Andamana	13 (4,8)	11 (8,1)	19 (12,2)	0 (0)
Australis	6 (2,2)	4 (2,9)	6 (3,8)	15 (39,5)
Autumnalis	0 (0)	40 (29,4)	42 (26,9)	0 (0)
Balum	1(0,4)	3 (2,2)	18 (11,5)	0 (0)
Canicola	1 (0,4)	3 (2,2)	4 (2,6)	8 (21,1)
Cynopeteri	0 (0)	0 (0)	0 (0)	1 (2,6)
Gryppotyphosa	8 (3,0)	3 (2,2)	1 (0,6)	0 (0)
Hebdomadis	2 (0,7)	0 (0,0)	0 (0)	0 (0)
Icterohaemorrhagiae	34 (12,5)	13 (9,6)	21 (13,5)	0 (0)
Javanica	0 (0)	0 (0)	7 (4,5)	0 (0)
Seramanga	7 (2,6)	17 (12,5)	5 (3,2)	1 (2,6)
Pomona	7 (2,6)	3 (2,2)	5 (3,2)	12 (31,6)
Pyrogenes	0 (0)	1 (0,7)	0 (0)	0 (0)
Djasiman	2 (0,7)	11 (8,1)	12 (7,7)	0 (0)
Serjoe	168 (62)	5 (3,7)	9 (5,8)	1 (2,6)
Shermani	3 (1,1)	3 (2,2)	3 (1,9)	0 (0)
Tarassovi	18 (6,6)	5 (3,7)	1 (0,6)	0 (0)
Celledoni	0 (0)	14 (10,3)	3 (1,9)	0 (0)
Total	270 (100)	136 (100)	156 (100)	38 (100)

CONCLUSÕES GERAIS

Nas condições metodológicas das pesquisas realizadas e com base nos resultados observados nos três artigos, pode-se concluir que o conhecimento da frequência sorológica de leptospirose, a identificação dos sorogrupos mais frequentes em cada espécie estudada, assim como a detecção molecular do agente em ruminantes da região Nordeste do Brasil, fornecem informações epidemiológicas importantes, que auxiliam na elaboração e implementação de medidas sanitárias adequadas e mudanças no manejo, direcionadas às principais fontes de infecção, permitindo que o controle da enfermidade nestes animais seja realizado de forma mais eficiente, garantindo a sanidade do rebanho. Na investigação do surto de leptospirose em bovinos do Maranhão, foi possível caracterizar a eficácia das medidas de controle recomendadas, apesar das controvérsias atuais acerca de várias medidas de prevenção e controle de leptospirose em ruminantes, bem como foi evidenciada a possível importância do sítio extra-renal genital na transmissão do agente.

APÊNDICE

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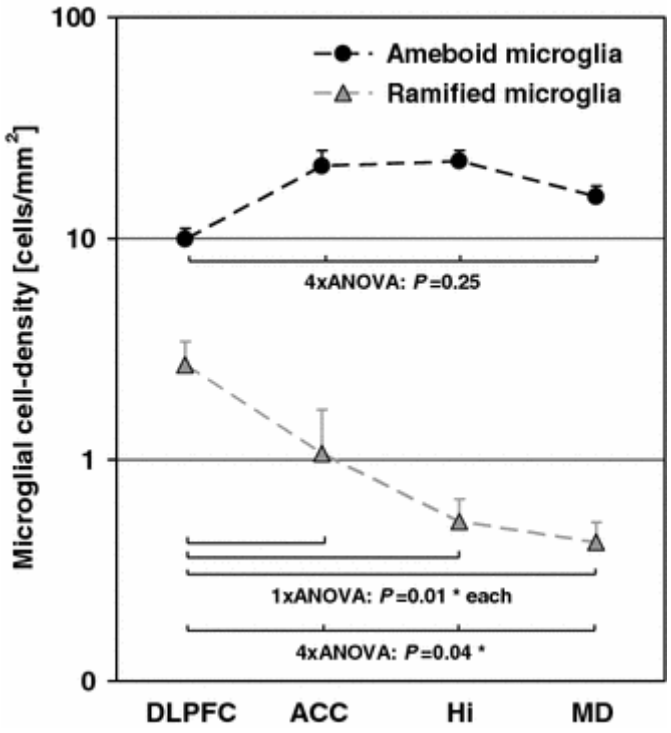

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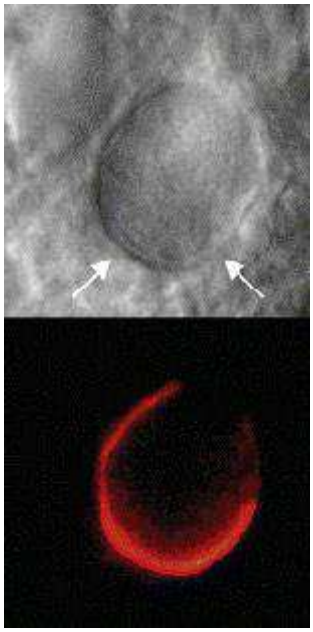

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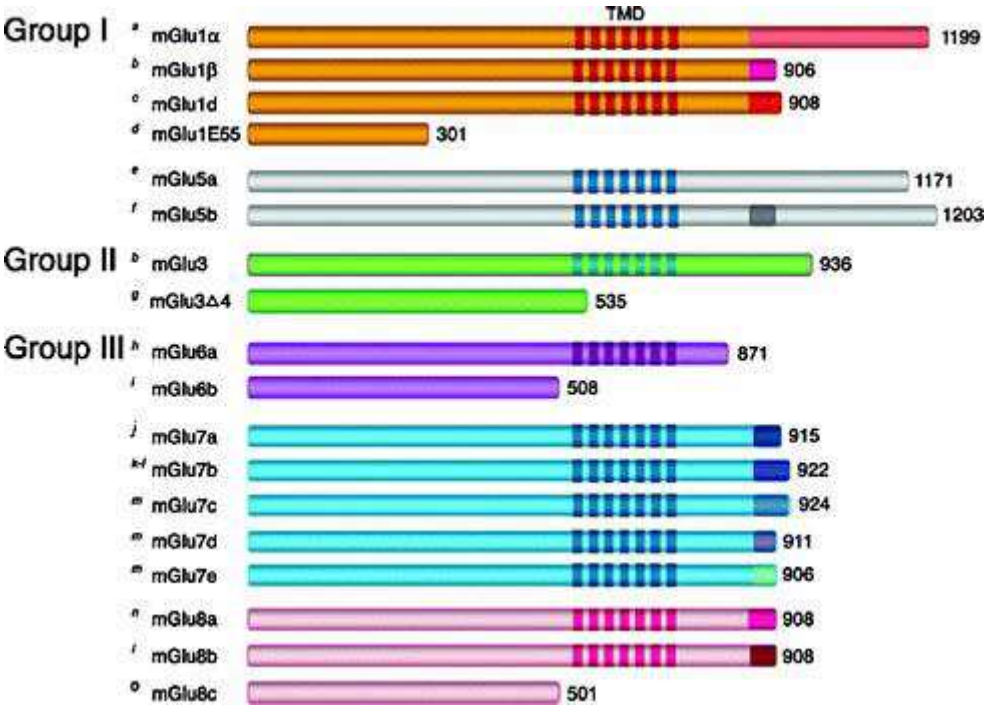

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9. References: The references, according to the standard NBR 6023, Aug. 2000, and reformulation number 14.724 of the Brazilian Technical Standards Association (ABNT), 2011, must be listed in alphabetical order at the end of the manuscript. **All the authors participating in a referenced study must be mentioned, regardless of the number of participants**. The accuracy and adequacy of references for works that have been consulted and mentioned in the text of the article, as well as opinions, concepts, and statements, are entirely the responsibility of the authors.

Note: Consult recently published issues of ***Semina: Ciências Agrárias*** for more details about how to format references in the article.

The remaining categories of works (Scientific Communication, Case Report, and Review) must follow the above-mentioned standards but with the following additional directions for each category:

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Scientific communications must be presented in a concise manner but with a complete description of the term research or ongoing research (Introductory note), with complete bibliographic documentation and methodologies, similar to a regular scientific article. Scientific communications must contain the following sections: Title (in Portuguese and English); Abstract with Keywords in Portuguese; Abstract with Keywords in English; and Body of the text. The body of the text should not be divided into sections but should follow this sequence: introduction, methodology, results and discussion (tables and figures may be included), conclusion, and bibliographic references.

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A review article must contain the following sections: Title (Portuguese and English); Abstract with Keywords in Portuguese; Abstract with Keywords in English; Development of the proposed topic (the text may be divided into sections, but this is not required); Conclusions or Final Considerations; Acknowledgements (if applicable); and Bibliographic References.

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