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Avanços no diagnóstico e na epidemiologia da leptospirose bovina em condições semiáridas

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Nathanael Natércio da Costa Barnabé

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Tese submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Doutor em Ciência e Saúde Animal.

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AVANÇOS NO DIAGNÓSTICO E NA EPIDEMIOLOGIA DA LEPTOSPIROSE BOVINA EM CONDIÇÕES SEMIÁRIDAS

Tese apresentada ao Programa de Pós-Graduação em Ciência e Saúde Animal como pré-requisito para obtenção do título de Doutor em Ciência e Saúde Animal.

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RESUMO

Leptospirose é uma doença que causa problemas reprodutivos nos animais de produção, cujo controle evita perdas econômicas. Dessa forma, buscou-se examinar bovinos adultos, embriões e fetos em condições semiáridas, para infecção por *Leptospira* spp. mediante técnicas sorológica, analisando ponto de corte, molecular, avaliando o seu desempenho, e microbiológica. No Capítulo I, foram amostrados 42 fêmeas bovinas, em que 27 (64,29%) sororreagiram para Sejroe, Tarassovi, Australis, Ballum, Djasiman e Hebdomadis. Constatou-se DNA leptospírico em 31 (73,81%) animais, 90/309 (29,13%) amostras positivas e, comparando a positividade de cada material biológico, houve significância estatística ($P < 0,05$) entre urina e rim, útero e placenta, útero e tuba uterina e placenta dos demais. Das 309 culturas, 73 (23,62%) apresentaram crescimento de leptospiras, com 19 confirmadas por PCR. Duas amostras de tecido (bexiga e útero) e duas de cultura (rim e ovário) viabilizaram o sequenciamento, demonstrando 99% de similaridade à *Leptospira borgpetersenii*. A grande frequência de DNA do agente no trato reprodutivo destaca sua importância como sítio extrarrenal, tornando a transmissão venérea um meio fundamental de disseminação. O ponto de corte 50 obteve os maiores valores de sensibilidade quando comparado ao título 100. O Capítulo II reporta 17 (40,48%) bovinos machos sororreativos a Sejroe, Tarassovi, Canicola e Grippotyphosa. A respeito do teste molecular, 26 (61,90%) animais foram positivos, com material genético da bactéria detectado em 86/252 (34,13%) amostras e, comparando a positividade de cada material, houve significância estatística ($P < 0,05$) entre urina e rim e urina e ducto deferente. De 252 culturas, 68 (26,98%) tiveram crescimento de leptospiras, com 16 confirmadas por PCR. Duas amostras de tecido (bexiga e glândula vesicular) e uma de cultura (bexiga) possibilitaram o sequenciamento, precisando 99% de similaridade à *L. borgpetersenii*. A PCR foi eficiente, com melhor desempenho às amostras de glândula vesicular, sensibilidade (100,00%) e especificidade (96,77%). No Capítulo III, dos 15 embriões e fetos bovinos, em diferentes estágios da gestação, sete e cinco eram machos e fêmeas, respectivamente. Três deles, em fase muito prematura, impossibilitaram a distinção do sexo. A despeito do baixo ponto de corte (25) adotado, nenhum soroconverteu. Nove matrizes sororreagiram para Sejroe, Tarassovi e Australis, com títulos variando de 50 a 1600. Um total de 13 (86,67%) indivíduos foram positivos no teste molecular, evidenciando associação ao resultado da PCR do trato reprodutivo das fêmeas adultas, comprovando difusão materno-fetal. Ao todo, 35/131 (26,72%) amostras dos embriões e fetos continham DNA leptospírico, comparando a positividade de cada material biológico, houve significância estatística ($P < 0,05$) entre baço e urina e, rim e urina. Fragmento de sistema nervoso central viabilizou o sequenciamento do patógeno, com 99% de similaridade à *L. borgpetersenii*. Os resultados desta tese mostram que a transmissão venérea e a transplacentária podem representar formas eficientes de disseminação de leptospiras. Também fundamentam o emprego do ponto de corte 50 na sorologia de bovinos criados em condições semiáridas.

PALAVRAS-CHAVE: *Leptospira* spp.; ponto de corte; trato reprodutivo; transmissão venérea; transmissão transplacentária.

ABSTRACT

Leptospirosis is a disease that causes reproductive problems in production animals. Controlling this disease prevents economic losses. Thus, we sought to examine adult cattle (and their embryos and fetuses) that were living under semiarid conditions, for *Leptospira* spp. infection, by means of serological techniques (with analysis of cutoff points), molecular techniques (with assessment of their performance) and microbiological techniques. Chapter I reports on sampling from 42 female cattle, among which 27 (64.29%) were seroreactive for Sejroe, Tarassovi, Australis, Ballum, Djasiman and Hebdomadis. Leptospiral DNA was found in 31 animals (73.81%), and 90/309 samples (29.13%) were positive. Through comparing positivity rates between the different biological materials, there were statistically significant differences ($P < 0.05$) between urine and kidney, uterus and placenta, uterus and fallopian tube, and placenta differed from the other biological materials. Among the 309 cultures, 73 (23.62%) showed *Leptospira* growth, with 19 confirmed via PCR. Sequencing was feasible for two tissue samples (bladder and uterus) and two culture samples (kidney and ovary) and this showed 99% similarity to *Leptospira borgpetersenii*. The high frequency of occurrence of DNA of the agent in the reproductive tract highlights its importance as an extrarenal site. This makes venereal transmission a fundamental means of dissemination. Cutoff point 50 showed higher sensitivity than titer 100. Chapter II reports on 17 male cattle (40.48%) that were seroreactive for Sejroe, Tarassovi, Canicola and Grippotyphosa. Regarding the molecular test, 26 animals (61.90%) were positive. Genetic material of the bacterium was detected in 86/252 samples (34.13%). Through comparing positivity rates between the different materials, there were statistically significant differences ($P < 0.05$) between urine and kidney and between urine and vas deferens. Out of 252 cultures, 68 (26.98%) showed *Leptospira* growth, with 16 confirmed by PCR. Sequencing was possible on two tissue samples (bladder and vesicular gland) and one culture sample (bladder) and this showed 99% similarity to *L. borgpetersenii*. PCR was efficient, with best performance in relation to vesicular gland samples: sensitivity 100.00% and specificity 96.77%. Chapter III reports on 15 bovine embryos and fetuses at different stages of pregnancy, among which seven were male and five were female. The sex could not be determined in the cases of three samples at a very premature stage. Despite the low cutoff point (25) used, no seroconversion was seen. Nine mothers were seroreactive for Sejroe, Tarassovi and Australis, with titers ranging from 50 to 1600. A total of 13 individuals (86.67%) were positive in the molecular test, thus showing an association with the PCR result from the reproductive tract of the adult females and proving maternal-fetal diffusion. In all, 35/131 samples (26.72%) from embryos and fetuses contained leptospiral DNA. Through comparing positivity rates between the different biological materials, there were statistically significant differences ($P < 0.05$) between spleen and urine and between kidney and urine. A fragment of central nervous system material enabled sequencing of the pathogen, and this showed 99% similarity to *L. borgpetersenii*. The results from this thesis show that venereal and transplacental transmission may form efficient means of dissemination of *Leptospira*. They also support use of the cutoff point 50 in serological evaluations on cattle reared under semiarid conditions.

KEYWORDS: *Leptospira* spp.; cutoff point; reproductive tract; venereal transmission; transplacental transmission.

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INTRODUÇÃO GERAL

Mudanças históricas na demanda por produtos de origem animal têm ocorrido no cenário mundial, sendo indiscutivelmente uma das principais preocupações do século XXI. Segundo a Organização das Nações Unidas para Agricultura e Alimentação, para atender ao crescimento exponencial da população humana, a produção de alimentos terá que aumentar 70% até 2050, sendo que os países em desenvolvimento serão os mais pressionados (FAO, 2017). Diante disso, o desafio da agropecuária será suprir a demanda sem degradar o meio ambiente.

No Brasil, dos setores da agropecuária, a bovinocultura assumiu papel de destaque, estando relacionada ao seu crescimento econômico (BRASIL, 2017). O país detém o segundo maior efetivo bovino do mundo, aproximadamente 172 milhões de cabeças, das quais pouco mais de 21 milhões estão concentradas no Nordeste (IBGE, 2017). A maioria dos produtores nordestinos se enquadra na categoria de agricultura familiar, adotando práticas de exploração mista (carne e leite), regime de semiconfinamento, ordenha manual e monta natural (CLEMENTINO *et al*., 2015). A falta de assistência técnica tem como consequência rebanhos de baixa produtividade e problemas reprodutivos. Fatores como a deficiência nutricional e o manejo zoossanitário inadequado comprometem a eficiência reprodutiva do plantel (AONO *et al*., 2013).

Leptospirose desperta interesse da saúde pública devido ao seu caráter zoonótico, enquanto na pecuária o interesse está em evitar as perdas econômicas decorrentes de aumento do intervalo entre partos, queda da taxa de concepção, abortos, natimortos, crias fracas, retardo do crescimento, baixa na produção de leite ou agalactia e morte (MUGHINI-GRAS *et al*., 2014). O agente *Leptospira* spp. é capaz de reduzir o seu genoma e duplicar ou transferir horizontalmente genes no processo de adaptação a novos hospedeiros, formando uma complexa cadeia epidemiológica. Tal dinamismo faz com que essa antropozoonose continue sendo um problema, pois com mais de um século de pesquisa, sua prevenção e seu controle continuam desafiadores (PICARDEAU, 2017). A exposição ao patógeno acontece do contato direto com o portador ou indireto mediante água e solo contaminados pela urina (DE OLIVEIRA *et al*., 2016; COSTA *et al*., 2015). Nos animais a transmissão também pode acontecer por fluido vaginal, restos placentários, durante a cópula e ainda de forma vertical (LILENBAUM *et al*., 2008).

A relação agente-hospedeiro é igualmente complexa. O bovino é reservatório do sorogrupo Sejroe que, desencadeia síndromes como subfertilidade e morte embrionária

precoce; muitas vezes o hospedeiro mantém o microrganismo ao longo da vida, eliminando-o pela urina de maneira intermitente (LOUREIRO; LILENBAUM, 2020). Contudo, a infecção desses animais por cepas não-Sejroe é considerada acidental, podendo cursar com sinais e sintomas clínicos como icterícia, febre, anemia hemolítica, hemoglobinúria e meningite (ELLIS, 2015).

Existe consenso entre cientistas de que o trato urinário, precisamente o rim, funciona como sítio de escape para leptospiras contra a ação imunológica, porém um experimento usando cepas autóctones do sorogrupo Sejroe, demonstrou o trato reprodutor como local de fuga da bactéria, relatando infecção persistente no útero, enquanto a colonização renal foi autolimitada (ROCHA *et al*., 2018). A despeito de nos bovinos ser claramente um problema da esfera reprodutiva, o portador genital é comumente negligenciado (LOUREIRO; LILENBAUM, 2020). Trabalho comprova alta frequência do patógeno em amostras de fluido vaginal, reforçando a ocorrência da transmissão venérea (LOUREIRO *et al*., 2016) e a leptospirose genital bovina é defendida como uma síndrome dissociada da renal (LOUREIRO; LILENBAUM, 2020), entretanto, é necessário haver melhor compreensão de sua fisiopatogênese.

Dos métodos mais conhecidos de diagnóstico, a soroaglutinação microscópica (SAM) é o teste preconizado pela Organização Mundial da Saúde Animal (OIE, 2014) capaz de apontar com segurança o sorogrupo infectante (MARTINS *et al*., 2012); a reação em cadeia da polimerase (PCR) é uma técnica rápida, de altas sensibilidade e especificidade, portanto, muito confiável (VILLANUEVA *et al*., 2016; HAMOND *et al*., 2014), embora o isolamento microbiológico seja considerado padrão ouro (MARTINS; LILENBAUM, 2015; FAINE *et al*., 2000).

A SAM possui baixa sensibilidade no estágio agudo da doença (NILOOFA *et al*., 2015), o que a torna inadequada ao diagnóstico precoce (KOIZUMI, 2020). Apresenta limitações na fase crônica, principalmente na detecção do portador renal ou genital que pode ter título abaixo do amplamente aceito à positividade (100) (ELLIS, 2015). A PCR se adequa à situação de emergência porque é rápida, permitindo o diagnóstico precoce com base na análise do sangue durante a leptospiremia, mas não identifica a cepa infectante (STODDARD *et al*., 2009). O isolamento microbiológico demora até dois meses e se torna inadequado em situação de emergência; o efeito inibitório de algumas amostras e a contaminação por microrganismos secundários comprometem o seu desempenho (ZARANTONELLI *et al*., 2018).

Partindo do princípio de que existem diferenças de incidência relacionadas às condições climáticas (PICARDEAU, 2017) e indivíduos adaptados ao agente que não são detectados pela sorologia, para maior precisão no diagnóstico, faz-se necessário estabelecer um protocolo de teste que melhor se adeque. Ovinos do semiárido evidenciaram resistência à bactéria e curto período de soroconversão (COSTA *et al*., 2018, 2017); investigações nessa espécie revelaram melhor desempenho da SAM adotando o ponto de corte 50 em relação ao título 100, considerando o resultado da PCR (SOARES *et al*., 2021; NOGUEIRA *et al*., 2020) e cultura como padrão ouro (SOARES *et al*., 2021). A seleção de um ponto de corte adequado aumenta a sensibilidade, evitando os falso-negativos, o que justifica esse estudo para bovinos.

Investir na prevenção e controle de doenças que subtraem da produção, como a leptospirose, é uma maneira assertiva de incrementar ganhos à bovinocultura, além de contribuir com a preservação ambiental. As estratégias devem ser baseadas em fatores inerentes ao agente, ao hospedeiro e ao ambiente (CORTESE *et al*., 2014). Identificar estirpes circulantes esclarece a cadeia de transmissão pelo conhecimento do reservatório, direcionando as intervenções. Conforme Martins e Lilenbaum (2017), cada rebanho tem particularidades, necessitando de um programa específico que aborde a identificação dos portadores, o tratamento e a vacinação.

O semiárido oferece condições peculiares na manutenção e disseminação desse patógeno que precisam ser analisadas em um contexto diferente dos outros lugares do mundo. Esta tese objetivou responder questões a respeito do diagnóstico e da epidemiologia da leptospirose bovina em condições semiáridas, pesquisando o trato reprodutivo como sítio extrarrenal e a ocorrência da transmissão transplacentária. Dessa maneira, foi dividida em três capítulos: O primeiro avaliou fêmeas bovinas para infecção por *Leptospira* sp. mediante SAM, analisando ponto de corte, PCR e isolamento microbiológico. O segundo investigou as frequências sorológica, molecular, analisando o desempenho do teste, e microbiológica de *Leptospira* em bovinos machos. E o terceiro verificou a transmissão transplacentária pela detecção de DNA leptospírico em embriões e fetos bovinos, reportando os órgãos colonizados.

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CAPÍTULO I:

Advances in the diagnosis and epidemiology of leptospirosis in female cattle reared under semiarid conditions

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Advances in the diagnosis and epidemiology of leptospirosis in female cattle reared under semiarid conditions

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ABSTRACT

Bovine leptospirosis results in economic losses and arouses public health interest because of its zoonotic nature. There are differences in the incidence of this disease related to climate and to individuals' adaptation to the agent, such that they remain serologically unidentified. In these cases, the test protocol that fits best needs to be established, so as to ensure greater diagnostic accuracy. The aim of this study was to gain better understanding of these dynamics through evaluating female cattle reared in the semiarid region of northeastern Brazil regarding *Leptospira* spp. infection. Blood samples from 42 animals were examined via the microagglutination test (MAT). Urinary tract samples (urine, bladder and kidney) and reproductive tract samples (vaginal fluid, uterus, fallopian tube, ovary and placenta) were examined via the polymerase chain reaction (PCR) and microbiological isolation. Anti-*Leptospira* antibodies were detected in 27 individuals (64.29%; 95% CI 49.17% - 77.01%) at cutoff point 50. The frequent serogroups were Sejroe (55.56%), Tarassovi (22.22%), Australis (7.41%), Ballum (7.41%), Djasiman (3.70%) and Hebdomadis (3.70%). DNA of the pathogen

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was found in 31 females (73.81%; 95% CI 58.93% - 84.70%) and in 90/309 samples (29.13%), especially in the placenta (86.67%), uterus (40.48%) and kidneys (33.33%). Through comparing positivity rates between the different biological materials, there were statistically significant differences ($P < 0.05$) between urine and kidney, uterus and placenta, uterus and fallopian tube, and placenta of the others. In cultures, 29 individuals (69.05%; 95% CI 53.97% - 80.93%) and 73/309 samples (23.62%) samples were positive. Among the materials, the placenta (66.67%), uterus (30.95%), ovary (26.19%) and kidneys (26.19%) were most frequently positive. Regarding positivity, urine differed statistically ($P < 0.05$) from kidney, uterus, ovary and placenta, while the placenta differed from the rest. DNA from the bacterium was identified in 19 cultures. Sequencing of four samples was possible and this showed 99% similarity to *L. borgpetersenii*. PCR was reliable, with high sensitivity (100.00%) and specificity (84.62%). It was seen that the highest sensitivity values for MAT were obtained at the cutoff point 50. The results indicate that, even in a climate that is adverse to survival, the microorganism can spread through alternative routes, as occurs with regard to venereal transmission, which was shown by the high proportion of animals with positive findings from the reproductive tract and vaginal fluid. The cutoff point of titer 50 can be recommended for serological evaluations on cattle in the semiarid region. However, it is essential to refine the technique so as to achieve greater sensitivity and specificity and, when possible, to perform paired PCR tests.

Keywords: *Leptospira* spp., MAT, cutoff point 50, PCR, microbiological isolation, vaginal fluid.

1. Introduction

Leptospirosis is globally widespread and is among the diseases that impair livestock production rates. It arouses public health interest because of its zoonotic nature and through awareness that, even after more than a century of research in this field, the vaccines available do not confer lasting immunity or cross-protection (Picardeau, 2017). The agent *Leptospira* spp. has several hosts and exposure ensues through direct contact with infected animals or indirectly via water and soil contaminated with urine (Costa et al., 2015; De Oliveira et al., 2016). Transmission can also occur through vaginal fluid, placental remains or copulation and also vertically (Lilenbaum et al., 2008). The losses among livestock result from abortions,

stillbirth, weak offspring, diminished growth rate, diminished milk production or agalactia and death (Mughini-Gras et al., 2014). In cattle, this disease is more associated with subfertility and early embryonic death (Loureiro and Lilenbaum, 2020).

Studies conducted in the semiarid region of northeastern Brazil have proven that both domestic animal species (Pimenta et al., 2014; Morais et al., 2019; Silva et al., 2019; Nogueira et al., 2020a, 2020b; Soares et al., 2021) and wild animal species (Fernandes et al., 2020a, 2020b) present high frequencies of *Leptospira*. This occurs despite the conditions of this region, which are adverse to survival of this bacterium in the environment. Investing in disease prevention and control is an assertive way to build up gains for cattle-rearing, ensure safe food and contribute to environmental preservation.

Among the methods for diagnosing leptospirosis, there are indirect methods that investigate the immune response of the host and direct methods that targets the invading microorganism. Among the best known of these methods, the microagglutination test (MAT) has been recommended by the World Organization for Animal Health (OIE, 2014) because of its capacity to safely demonstrate the infecting serogroup (Martins et al., 2012); the polymerase chain reaction (PCR) is a fast technique with high sensitivity and specificity, and therefore very reliable (Hamond et al., 2014; Villanueva et al., 2016), although microbiological isolation is considered to be the gold standard (Faine et al., 2000; Martins and Lilenbaum, 2015).

Starting from the principle that there are differences in incidence related to climatic conditions (Picardeau, 2013) and to individuals' adaptation to the agent, such that they are remain serologically unidentified, the test protocol that fits best needs to be established, so as to ensure greater diagnostic accuracy. Sheep living in the semiarid region have shown resistance to bacteria and short seroconversion periods (Costa et al., 2017, 2018); investigations on this species have shown that the MAT had better performance with a cutoff point of titer 50 than with titer 100, while considering the results from PCR (Nogueira et al., 2020a; Soares et al., 2021) and culturing to be the gold standard (Soares et al., 2021). Selection of an appropriate cutoff point is relevant for increasing sensitivity and avoiding false-negative results. This provided justification for conducting the present study on cattle in this region.

The peculiar epidemiological conditions involved in maintenance and transmission of this pathogen in the Brazilian semiarid region need to be analyzed in a context differing from that of other places around the world. Knowledge of these dynamics is fundamental for improvement of strategies for combating bovine leptospirosis. Therefore, the aim of this study

was to evaluate female cattle regarding *Leptospira* infection through serological techniques, with analysis on molecular and microbiological cutoff points.

2. Material & methods

2.1. Study site and its characteristics

This investigation was developed at the Patos municipal slaughterhouse (latitude: 7°00'19" south; longitude: 37°16'48" west), State of Paraíba, northeastern Brazil, between September and November 2019. This slaughterhouse is used for cattle from this intermediate geographical region and surrounding areas. In this area, the Caatinga biome (an exclusively Brazilian biome) predominates. This biome has a semiarid climate characterized by long periods of water scarcity and stunted vegetation. According to Alvares et al. (2014), the climate is classified as BShw', i.e. hot and dry, with a rainy season in summer/autumn and precipitation concentrated in the months from March to April. However, precipitation may occur at any time between January and May. Batista et al. (2007) and Araújo (2011) added that droughts can last for more than a year, which results in a negative water balance plus high solar radiation. The period during which the present study was conducted corresponded to the dry season, with average precipitation of 0.47 mm and average temperature of 29.28 °C (INMET, 2021).

2.2. Sampling

The minimum sample size was determined using the following formula for proportion analysis (Arango, 2009):

$$n = \frac{p_0 \times q_0 \times \left(z_{1-\beta} + z_{\alpha/2} \times \sqrt{\frac{p_1 \times q_1}{p_0 \times q_0}} \right)^2}{(p_1 - p_0)}$$

Where

n = minimum sample size

$Z_{\alpha/2}$ = 1.96 (Z value for 95% confidence level)

$Z_{1-\beta}$ = 1.64 (Z value for power of 95%)

P_0 = 33% (reference proportion for PCR positivity) (Pimenta et al., 2019)

P_1 = 61.40% (estimate for the experimental proportion of positivity in PCR) (Loureiro et al., 2017)

$q_0 = 1 - p_0$

$q_1 = 1 - p_1$

In line with these parameters, 37 individuals were needed for scientific investigation. However, a total of 42 cattle were used. They were all female, aged greater than or equal to 24 months (≥ 2 years), did not belong to any defined breed and had not been vaccinated against leptospirosis.

2.3. Origin of the animals

According to data from animal traffic forms held by State Veterinary Service of Paraíba, these female cattle came from rural farms in the semiarid region, from municipalities belonging to two federative units: Paraíba (Cacimba de Areia, Condado, Olho D'Água, Patos, Pombal, Santa Terezinha, São José de Espinharas, São José do Bonfim and São Mamede) and Pernambuco (Buíque, Capoeiras and Exu).

2.4. Field activity

During visits to the slaughterhouse, cattle were selected for the study. These were identified using the numbers 01 to 42, according to the chronological order of slaughter. All biological material for analysis was collected in duplicate (except for material destined for culturing) and was obtained *post mortem* in order to avoid pain and any unnecessary discomfort to the animals.

After desensitization, at the time of bleeding, a volume of 8 mL of blood was collected in a sterile tube containing coagulation activator. The tube had previously been identified with corresponding numbering. This blood was used for making an indirect diagnosis through the microagglutination test (MAT).

During evisceration of the carcass, sterile surgical instruments, scalpel blades, syringes and cytological brushes were used to collect fluids and fragments from the urinary tract

(urine, bladder and kidney) and reproductive tract (vaginal fluid, uterus, fallopian tube, ovary and placenta - when present). These were then used to make a direct diagnosis via two techniques: polymerase chain reaction (PCR) and microbiological isolation. Urine was obtained by means of direct puncturing of the bladder; vaginal fluid by means of mechanical action with a brush in the cervical-vaginal region.

The biological material was sent to a specific room in the slaughterhouse, where it was quickly processed as aseptically as possible and under protection using a Bunsen burner. Solid samples were laid out in a sterile Petri dish, while avoiding contact between pieces, thereby reducing them to three pieces of approximately 2 g each. One of these was immediately sown in culture medium specific to *Leptospira*: Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid (Difco; Becton, Dickinson and Company, Franklin, New Jersey, United States). This was supplemented with an antimicrobial cocktail consisting of sulfamethoxazole (0.4 mg/mL) + trimethoprim (0.2 mg/mL) + amphotericin B (0.05 mg/mL) + phosphomycin (0.4 mg/mL) + 5-fluorouracil (0.1 mg/mL) [STAFF cocktail] (Chakraborty et al., 2011). The other two pieces were stored in polypropylene microtubes free of DNA and ribonucleic acid (RNA) for subsequent use in PCR evaluations. Urine samples (100 µl) were sown in culture medium, while 0.5 mL was set aside in polypropylene microtubes for subsequent molecular analysis. In the case of vaginal fluid, one cytological brush was inoculated in EMJH and two other brushes in microtubes containing 0.5 mL of phosphate saline solution.

During transportation to the Communicable Diseases Laboratory of the Center for Health and Rural Technology (CSTR), Federal University of Campina Grande (UFCG), Patos Campus, all content related to serology and molecular testing was conserved at 4 °C. However, the cultures were kept at room temperature.

2.5. Microagglutination test (MAT)

The MAT was performed in accordance with official protocols (Zuerner, 2006; OIE, 2014). Each serological sample was tested for six pathogenic species of *Leptospira*, 18 serogroups and 24 serovars: *L. borgpetersenii* (serovars Ballum, Castellonis, Javanica, Mini and Tarassovi); *L. interrogans* (Autumnalis, Bratislava, Canicola, Copenhageni, Djasiman, Hardjoprajitno, Hebdomadis, Icterohaemorrhagiae, Kennewicki, Pomona, Pyrogenes and Wolffi); *L. kirschneri* (Cynopteri and Grippotyphosa); *L. noguchii* (Louisiana and Panama); *L. santarosai* (Canalzoni and Guaricura); and *L. weilii* (Celledoni).

With dilution levels of 1:50 to 1:3200, titration was based on the highest dilution of serum in Sorensen buffered phosphate saline solution, in which 50% of the *Leptospira* agglutinated, thus indicating the infecting serogroup.

2.6. Polymerase chain reaction (PCR), sequencing and phylogenetic analysis

Leptospiral DNA was extracted from tissues, urine and vaginal fluid using the DNeasy blood and tissue kit (Qiagen, Hilden, Nordrhein-Westfalen, Germany), following the manufacturer's recommendations. Cultures that were found to be positive through microscope readings were also subjected to extraction. The primers LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′), designed by Stoddard et al. (2009), were used for amplification of the *LipL32* gene, which is specific to pathogenic *Leptospira*. *L. interrogans*, Pomona serogroup, Kennewicki serovar served as the positive control; filtered ultrapure water as the negative. The total volume of each sample was analyzed by means of electrophoresis on agarose gel (2%), which was then stained with Evans blue. The DNA bands (≅ 260 bp) were then viewed under ultraviolet light.

The direct and reverse LipL32-45F and LipL32-286R primers, respectively, were used in the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, California, USA) for nucleotide sequencing, as described above (Stoddard et al., 2009). Capillary electrophoresis was performed through a 3130xL genetic analyzer and a POP-7 polymer (Applied Biosystems, Foster City, California, USA) (Platt et al., 2007). The sequence was aligned using BioEdit (Gouy et al., 2010) and was compared with *Leptospira* strains from GenBank (National Biotechnology Information Center, Bethesda, Maryland, USA) (http://www.ncbi.nlm.nih.gov), using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was constructed in the SeaView4 software (Hall, 1999) using the neighbor-joining method, with a bootstrap of 1000 repetitions (http://tree.bio.ed.ac.uk/software/figtree/, and this was viewed through the FigTree software, v1.4.3 (http://tree.bio.ed.ac.uk/)). The phylogenic reconstruction included *Leptospira* sequences for comparison.

2.7. Microbiological isolation/culture

Fractions of urine, bladder, kidney, vaginal fluid, uterus, fallopian tube, ovary and placenta were sown in thread cap-type tubes containing 5 mL of liquid EMJH supplemented with STAFF. Renal tissue was macerated using a sterile disposable syringe (10 mL) for insertion in the medium. After collection, the cultures were packed in a digital incubator of biochemical oxygen demand (BOD) type (CienlaB, Campinas, São Paulo, Brazil) at 29 °C for 24 hours. This was subsequently replicated in semisolid EMJH (Difco, Becton, Dickinson and Company, Franklin, New Jersey, United States), free from antimicrobials, and again incubated. Over a six-month period with monthly repiques, the material was evaluated weekly by means of dark-field microscopy.

2.8. Statistical analysis

The proportions of positive animals and samples were compared by means of the chi-square test with Yates continuity correction or Fisher's exact test, using the BioEstat 5.3 software (Ayres et al., 2007), and the significance level was taken to be 5% ($P \leq 0.05$). The sensitivity and specificity of the MAT at the cutoff points 50, 100, 200 and 400 were calculated using the DAG_Stat software (Mackinnon, 2000) and the results from direct diagnosis (PCR and culture) were taken to be the reference for this.

3. Results

Among the 42 female cattle evaluated, 27 (64.29%; 95% CI 49.17% - 77.01%) presented anti-*Leptospira* antibodies at the cutoff point 50, and Sejroe, Tarassovi, Australis, Ballum, Djasiman and Hebdomadis were the reactive serogroups. At the cutoff point 100, 16 cattle (38.10%; 95% CI 25.00% - 53.19%) agglutinated for Sejroe, Tarassovi and Hebdomadis. At the cutoff point 200, 10 cattle (23.81%; 95% CI 13.48% - 38.53%) were reactive for Sejroe, Tarassovi and Hebdomadis. At the titer 400, eight cattle (19.05%; 95% CI 9.98% - 33.30%) were seroreactive for Sejroe, Tarassovi and Hebdomadis. Overall, the most frequent serogroups were Sejroe, for which 15 cattle (55.56%) were positive; and Tarassovi, for which six cattle (22.22%) were positive, with titers ranging from 50 to 1600 (Table 1).

Leptospiral DNA was found in at least one sample from 31 animals (73.81%; 95% CI 58.93% - 84.70%). Among the 309 samples, PCR detected genetic material of the agent in 90 (29.13%). The materials in which this was found most frequently were the placenta (13;

86.67%), uterus (17; 40.48%) and kidneys (14; 33.33%). Through comparing positivity rates between the different biological materials, there were statistically significant differences ($P < 0.05$) between urine and kidney, uterus and placenta, uterus and fallopian tube, and placenta compared with the others (Table 2). Sequencing of the nucleotides of the bacterium directly from PCR products was possible in two samples, from the bladder and uterus of different females. These showed 99% similarity to *Leptospira borgpetersenii* (Fig. 1). In the cultures, the pathogen was present in at least one sample from 29 individuals (69.05%; 95% CI 53.97% - 80.93%) and 73 cultures (23.62%) were found to be positive through microscope readings, especially the placenta (10; 66.67%), uterus (13; 30.95%), ovary (11; 26.19%) and kidneys (11; 26.19%). Urine differed statistically ($P < 0.05$) from kidney, uterus, ovary and placenta, while placenta differed from the rest (Table 2). DNA of the microorganism was identified in 19 cultures, among which one was from the kidney and another from the ovary, of different females. These enabled sequencing and showed 99% similarity *to L. borgpetersenii* (Fig. 1).

The molecular test showed good performance in diagnosing *Leptospira* sp. infection, with high sensitivity (100.00%) and specificity (84.62%), especially in relation to the fallopian tube samples, with maximum co-positivity and co-negativity (Table 3).

Tables 4 and 5 show the sensitivity and specificity of the MAT for the different cutoff points (50, 100, 200 and 400), based on PCR and culture results, respectively. It was verified that, regardless of the biological material used in making the direct diagnosis, the highest sensitivity values in the MAT were obtained from titer 50.

4. Discussion

The frequency of seroreactivity in the microagglutination test (MAT) among these 27 cattle (64.29%; 95% CI 49.17% - 77.01%) indicates that, even under adverse climatic conditions, *Leptospira* can be present in herds in the semiarid region of Brazil. There was variation in the serogroups found (Sejroe, Tarassovi, Australis, Ballum, Djasiman and Hebdomadis), which suggests that different sources of infection existed, although Sejroe (55.56%) and Tarassovi (22.22%) were more prevalent. In other investigations conducted in semiarid regions, Pimenta et al. (2014) also found that Sejroe (58.17%) was the most prevalent serogroup, followed by Icterohaemorrhagiae (17.32%) and Australis (4.58%); and Pimenta et al. (2020) found the following prevalences: Sejroe (36.8%), Hebdomadis (26.3%), Australis (10.5%), Djasiman (10.5%), Ballum (5.3%) and Pomona (5.3%). Studies conducted in different regions of Brazil have demonstrated that, regardless of the biome involved, Sejroe

was most prevalent: northeastern region (Mineiro et al., 2011; Silva et al., 2012; Pimenta et al., 2014, 2019, 2020); northern region (Guedes et al., 2019a, 2019b); central-western region (Miashiro et al., 2018); southeastern region (Favero et al., 2001; Araújo et al., 2005; Pinto et al., 2015, 2017; Pinna et al., 2018); and southern region (Herrmann et al., 2012; Hashimoto et al., 2017). The other groups of serotypes observed in the present study were also observed in the publications cited above, although with different prevalences. According to Pinto et al. (2017), the plurality of types of *Leptospira* suggests that contact with other animals exists, but there is the possibility that cattle that are free from signs and symptoms may carry and spread other strains within the species (adaptive process).

The contrast between unfavorable climatic condition (average precipitation and temperature of 0.47 mm and 29.28 °C, respectively, in the dry season) and a significant proportion of positive females, especially with regard to the Sejroe serotype) provides evidence that intraspecies transmission takes place. This type of transmission is less dependent on environmental factors (Correia et al., 2017), because cattle are maintenance hosts (Mughini-Gras et al., 2014; Ellis, 2015). The Sejroe serogroup causes leptospirosis worldwide (Loureiro et al., 2016) and has been reported in 80% of the studies in Latin America (Pinto et al., 2016). It triggers reproductive problems, especially abortion (Suepaul et al., 2011). However, according to Loureiro and Lilenbaum (2020), it is more associated with subtle syndromes such as subfertility and early embryonic death. Non-Sejroe strains in cattle are considered to be accidental findings and, when they occur, this is due to interspecies dissemination in the vast majority of cases and is dependent on favorable environmental/climatic conditions (rainy season) (Correia et al., 2017). Nonetheless, according to Pinto et al. (2017), the possibility of intraspecies propagation should not be ruled out; such occurrences induce outbreaks of abortion, dead fetuses and repeated estrus (Pimenta et al., 2019).

Tarassovi is among the main groups of serotypes found in cattle (Soo et al., 2020). Pinto et al. (2015) stated that this type had been little reported in cattle in Brazil. According to Natarajaseenivasan et al. (2011) and Silva et al. (2012), Tarassovi is an agent for clinical leptospirosis. Soto et al. (2007) reported that this serotype also frequently occurred in leptospirosis in pigs, which suggests with regard to our results that interspecies transmission was made possible through rearing cattle and pigs together. In our investigation, Tarassovi was the second most prevalent serotype, with varying titers (50-1600). Australis is associated with pigs and causes reproductive failure, abortion, stillbirth, fetal mummification and weak piglets (Adler and Moctezuma, 2010), and has also been described in mammals kept in zoos

(Lenharo et al., 2012). Ballum is related to reservoir rodents, especially mice (*Mus musculus*). Asymptomatic mice harbor the bacterium in their kidneys, which makes them an important source of infection for humans or animals (Adler and Moctezuma, 2010). Confirmation of presence of this serogroup in cattle indicates that rodents have had access to cattle food, which usually consists of energy and protein concentrate and is stored to ensure supplementation essentially during the period in which there is low production of phytomass; or have had access to the facilities and feeders/water troughs. Hebdomadis has been reported to be among the most common serotypes in cattle (Paixão et al., 2016). Shinya et al. (2021) added that it is maintained in wild pigs and described cases in rabbits, dogs and humans on Amami Oshima Island, Nansei Archipelago, southwestern Japan.

As an indirect method, the MAT detects anti-*Leptospira* antibodies of the immunoglobulin classes M (IgM) and IgG. It has low sensitivity in the acute stage of leptospirosis (Niloofa et al., 2015) and, therefore, is an inadequate test for early diagnosis (Koizumi, 2020). It also presents limitations in the chronic phase, especially with regard to identifying renal or genital carriers, which may present titers below the level widely accepted as denoting positivity (i.e. titer 100) (Ellis, 2015). Inclusion of autochthonous strains as antigens increases the sensitivity of the technique by 9.9% (Pinto et al., 2015). If local isolates are unavailable, evaluation of a cutoff point with better performance is a viable strategy that is not onerous for the process. A study conducted among cattle in the rural zone of Bogotá, Colombia, showed that titer 50 was more appropriate (Hernández-Rodríguez et al., 2011). The principle of the MAT is simple, but technical knowledge is required and a panel of live *Leptospira* spp. (antigens) needs to be maintained. This presents biological risk and thus restricting its use to specialized laboratories (Koizumi, 2020). Its importance lies in its ability to identify the infecting serogroup during an outbreak and clarify the disease transmission chain through knowledge of the reservoir.

The polymerase chain reaction (PCR) found leptospiral DNA in 31 females (73.81%; 95% CI 58.93% - 84.70%) in the present study, which was the highest positivity rate among the tests used. Latosinski et al. (2018) warned that the real frequency may be higher, given that negativity can also be correlated with DNA concentrations below the detectable limit. Through comparing positivity rates between the different biological materials, there were statistically significant differences ($P < 0.05$) that demonstrated that *Leptospira* preferentially accumulated in the placenta, uterus and kidneys, while they were little present in urine. This can be explained by the fact that the agent’s size, morphology (spirochete/helicoid) and translational motility provide it with easy access to these organs, which allow the agent to

escape the immune system, mainly through humoral action due to the physical barrier. On the other hand, elimination through urinary secretion occurs only intermittently. The agent's strong presence in the reproductive tract may be another indication that under adverse climatic conditions, the spread of this bacterium through alternative routes is more constant. In this manner, it does not need to face the environmental stage of its biological cycle, as would occur in relation to venereal transmissions, at the first level, and in relation to vertical transmission, at the second level. The PCR findings confirmed that, for *in vivo* cases, vaginal fluid may be valuable for identifying carriers. It was possible to sequence nucleotides of the pathogen in two samples, bladder and uterus of different animals, and this demonstrated 99% similarity to *Leptospira borgpetersenii*.

In the cultures, *Leptospira* was present in at least one sample from 29 individuals (69.05%; 95% CI 53.97% - 80.93%). Through comparing positivity rates between the different materials, there were statistically significant differences ($P < 0.05$) that demonstrated the tropism of the microorganism for the placenta, uterus, kidney and ovary, while the frequency of occurrence in urine was lower, as explained earlier. The results from the cultures revealed that working on microbiological isolation, using tissue from the placenta, uterus, kidney and ovary can increase the chances of success. However, considering that access to tissue from the uterus, kidney and ovary is difficult, collection of such material is appropriate in *post mortem* cases. Genetic material from the bacterium was identified in 19 cultures, among which one kidney sample and one ovary sample, from different females, enabled sequencing and demonstrated 99% similarity to *L. borgpetersenii*. Although molecular techniques have revolutionized diagnostic response times over recent years, development of complete genome sequencing has renewed interest in isolating *Leptospira* (Goarant et al., 2020). Isolation and characterization of autochthonous strains are important with regard to understanding epidemiology and refining diagnostic tools. Moreover, this gives rise to the possibility of discovering new species and/or variants. If these circulating strains can be incorporated into vaccines, greater protection for animals in the region can be provided (Zarantonelli et al., 2018). On the other hand, microbiological confirmation is laborious, given that culturing may take up to two months, which makes this procedure inadequate in an emergency situation (Merien et al., 2005). In addition, problems such as the inhibitory effect of some samples and contamination by secondary microbes compromise the performance of culturing (Zarantonelli et al., 2018).

L. borgpetersenii belongs to the pathogenic clade and, according to virulence, to subgroup 2, along with the species *L. santarosai*, *mayottensis*, *weilii* and *alexanderi*

(Picardeau, 2017). It causes early embryonic loss and estrus repetition, resulting from uterine inflammation and damage caused by embryo invasion (Loureiro and Lilenbaum, 2020). In its adaptation to cattle, it has a smaller genome compared with other pathogenic and saprobic strains (Picardeau, 2017); the size of the genetic code is related to the ability to adapt to conditions inside and outside the host (Martins and Lilenbaum, 2015). Genomic comparisons have shown coevolution through rearrangement and insertion of DNA sequences (Picardeau, 2017). *Leptospira* shows poor resistance in the environment and it is believed that direct transmission causes loss of genes that are necessary for survival outside the host, which thus impairs indirect dissemination (Martins and Lilenbaum, 2015). In the present study, identification of *L. borgpetersenii* through genetic sequencing provided yet another indication of venereal transmission and perhaps this pathway was responsible for influencing the high frequency of positive findings during the dry period, because any other way that depends on external variables is less likely to succeed.

According to PCR, out of the 31 positive females (73.81%; 95% CI 58.93% - 84.70%), 15 showed positivity in relation to both the reproductive and the urinary tracts, among which three can be highlighted: one with leptospiral DNA in all biological material and the other two in all material except the uterine tube. Another 13 individuals showed positivity only in relation to the reproductive tract; while three showed this only in the urinary tract. Animals with urogenital tract involvement potentiate diffusion of the agent, especially in the rainy season of the semiarid region, due to the possibility of simultaneous transmission via the urinary, venereal and congenital/transplacental routes. The existence of bovine genital leptospirosis is corroborated by the presence of individuals whose reproductive tract alone is affected. This is a dissociated renal/systemic syndrome put forward by Loureiro and Lilenbaum (2020) that is very important in the dry period of the semiarid region, a time when the bacterium has short survival in the environment. Soto et al. (2007) reported that in dry soil *Leptospira* can only survive for 30 minutes. Therefore, at times of drought, the urinary tract may be less relevant for dissemination, except in microclimates, which would have been unlikely in the present study, considering that the animals were living on several different farms.

The molecular test proved to be reliable for diagnosing pathogenic *Leptospira* infection and showed high sensitivity (100.00%) and specificity (84.62%). One explanation for the better performance of PCR than microbiological isolation as a direct method is that cultures are read by viewing intact spirochetes, while the molecular technique identifies free leptospiral DNA (Stoddard et al., 2009). Many factors contribute to the accuracy of PCR, e.g.

the extraction kit, thermostable DNA polymerase (Taq), laboratory equipment, operating procedure and the "gold standard" result. Ahmed et al. (2009) explained about the inhibitors contained in kidney and urine samples and recommended use of 1:10 dilution of the DNA extracted from kidney tissue and extra washing of urine, because the treatment given to biological material influences the detection capacity. Merien et al. (2005) reported that it was easier to find leptospiral DNA in material from individuals that were negative in the MAT, i.e. if there is a high antibody titer, the outcome from PCR tends to be negative. Our results followed this logic of females with high titers: when they were not negative in the molecular evaluation, they presented smaller quantities of positive samples and escape sites (kidney and ovary) showed high prevalence. PCR is very reliable: its cost is the biggest obstacle to large-scale application, but there is consensus among researchers that its use will become increasingly common. According to Stoddard et al. (2009), the refined sensitivity of this test often eliminates the need for isolation as a confirmatory result. It is suitable for emergency situations, given that it is fast and enables early diagnosis based on blood analysis during leptospiremia. Merien et al. (2005) pointed out that it had the limitation of inability to identify the infecting strain at species level, which therefore necessitated use of electrophoresis on non-denaturant polyacrylamide gel or amplicon sequencing.

Analysis on the cutoff points showed that, regardless of the biological material used in making the direct diagnosis, the highest sensitivity values from the MAT were obtained at titer 50, in relation both to PCR (61.29%) and to culturing (62.07%). A similar response was found by Nogueira et al. (2020a) and Soares et al. (2021) through evaluating the cutoff point in the MAT among sheep in the semiarid region of Brazil. Our data show that cutoff point 50 may be more suitable for cattle in this region, due to the low sensitivity of titer 100. In this situation, sensitivity becomes more necessary because this is able to reduce the number of false negatives. However, it needs to be emphasized that the result from making a direct diagnosis will not always be compatible with the indirect diagnosis: this it all depends on the stage of the disease. In the beginning, there are no circulating antibodies (immunological window) and the apex of leptospiremia occurs on the seventh day, which is an ideal time to use techniques that are based on findings of the microorganism in blood (Koizumi, 2020), while the MAT provides a large number of false negatives (Niloofa et al., 2015). On the eighth day, agglutinating antibodies (predominantly IgM) are in the bloodstream, while specific antibodies (IgG) take one or two days longer to appear (Niloofa et al., 2015). During the second week, at the same time as the humoral response, a greater number of bacteria are eliminated in urine (leptospiruria) (Koizumi, 2020). The peak level of antibodies occurs in the

fourth week, with detectable titers that persist for months even in a healing situation (false positive) (Niloofa et al., 2015). The tests are imperfect and use of an appropriate combination of methods and samples for each phase of infection improves diagnostic accuracy (Koizumi, 2020).

This study provides important information that helps in understanding the diagnosis and epidemiological chain of bovine leptospirosis in the semiarid region of Brazil. The notable proportions of positive females found through PCR and culturing indicates that even under adverse climate conditions, the agent can spread through alternative routes, as seen in venereal transmission, with the high proportion of animals with positive findings from the reproductive tract and in vaginal fluid. Titer 50 is recommended as a cutoff point in serological evaluations on cattle in this region. Nevertheless, it needs to be emphasized that refinement of the technique for greater sensitivity and specificity is essential and that, whenever possible, paired PCR tests should be performed.

Ethical approval

The present study was approved by the Ethics Committee for the Use of Animals (*Comissão de Ética no Uso de Animais*; CEUA) of the Federal University of Campina Grande (UFCG), Center for Rural Health and Technology (CSTR), Patos Campus, under protocol CEP/CEUA n 069-2018.

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editing. **Clebert José Alves:** Conceptualization, Funding acquisition, Project administration, Writing - review & editing.

Declaration of competing interest

The authors declare that there are no potential conflicts of interest related to the research, authorship, and/or publication of this article.

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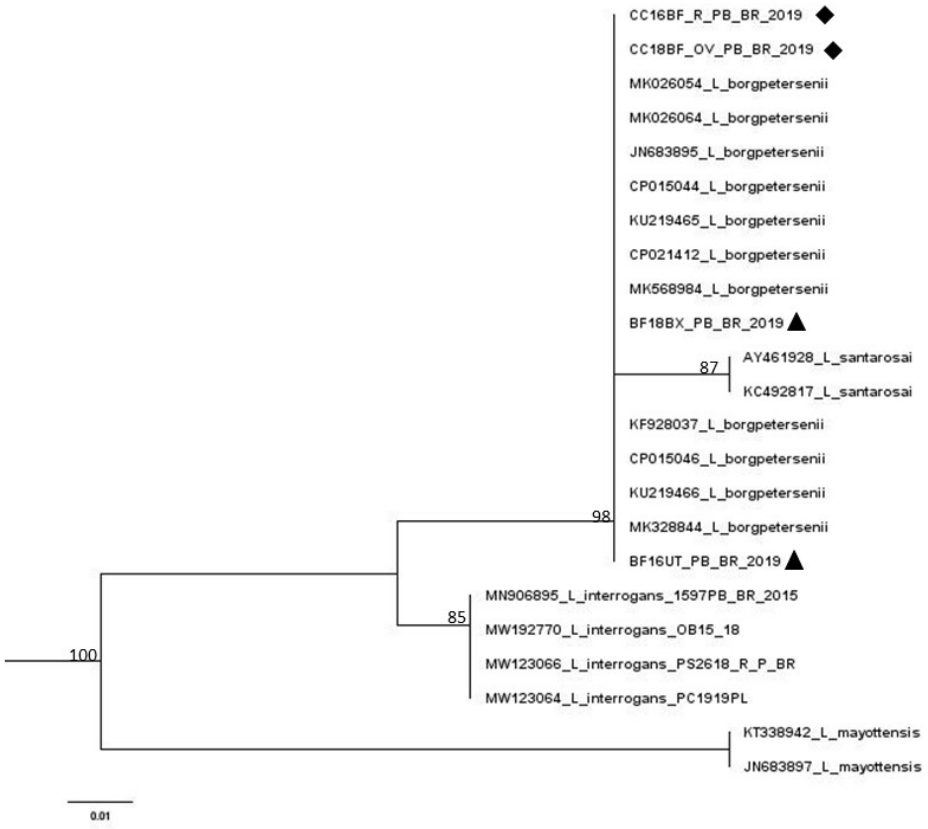

Fig. 1. Phylogenetic tree based on the nucleotide sequence alignment of the *LipL32* gene of *Leptospira* spp. constructed using the neighbor-joining method with 1000 replicates. ♦ Samples sequenced from cultures. ▲ Samples sequenced from tissues.

Table 1
Predominant serogroups and respective antibody titers shown through the MAT technique among female cattle reared under semiarid conditions.

Serogroups	Titers						Total (%)
	50	100	200	400	800	1600	
Sejroe	3	5	1	2	2	2	15 (55.56)
Tarassovi	3	1	1	-	-	1	6 (22.22)
Australis	2	-	-	-	-	-	2 (7.41)
Ballum	2	-	-	-	-	-	2 (7.41)
Djasiman	1	-	-	-	-	-	1 (3.70)
Hebdomadis	-	-	-	1	-	-	1 (3.70)
Total (%)	11 (40.74)	6 (22.22)	2 (7.41)	3 (11.11)	2 (7.41)	3 (11.11)	27 (100.00)

Table 2
Molecular and microbiological diagnoses of *Leptospira* sp. according to different types of biological material from female cattle reared under semiarid conditions.

Sample	Total	PCR		Culture	PCR of Culture	
		31*/42 (73.81%)		29*/42 (69.05%)	13*/42 (30.95%)	
		+ (%)	Sequencing	+ (%)	+ (%)	Sequencing
Urine	42	6 (14.29) [a]	-	4 (9.52) [a]	1 (2.38)	-
Bladder	42	10 (23.81) [ab]	1	8 (19.05) [ab]	1 (2.38)	-
Kidney	42	14 (33.33) [b]	-	11 (26.19) [b]	2 (4.76)	1
Vaginal fluid	42	10 (23.81) [ab]	-	9 (21.43) [ab]	1 (2.38)	-
Uterus	42	17 (40.48) [b]	1	13 (30.95) [b]	8 (19.05)	-
Fallopian tube	42	7 (16.67) [a]	-	7 (16.67) [ab]	2 (4.76)	-
Ovary	42	13 (30.95) [ab]	-	11 (26.19) [b]	2 (4.76)	1
Placenta	15	13 (86.67) [c]	-	10 (66.67) [c]	2 (13.33)	-
Total	309	90 (29.13)	2	73 (23.62)	19 (6.15)	2

* = number of positive individuals; + = positive samples. Different lowercase letters in the same column indicate significant differences in proportion ($P < 0.05$).

Table 3
Performance of molecular tests for diagnosing bovine leptospirosis based on the microbiological result (gold standard) for each material.

Biological material	Culture		PCR			
	29*/42 (69,05%)		31*/42 (73,81%)			
	+	-	+	-	SEN	SPE
Urine	4	25	6	25	100.00	94.74
Bladder	8	21	10	21	100.00	94.12
Kidney	11	18	14	17	100.00	90.32
Vaginal fluid	9	20	10	21	100.00	96.97
Uterus	13	16	17	14	100.00	86.21
Fallopian tube	7	22	7	24	100.00	100.00
Ovary	11	18	13	18	100.00	93.55
Placenta	10	5	13	2	100.00	40.00
Others	29	13	31	11	100.00	84.62

* = number of positive individuals; + = positive samples; - = negative samples; SEN = sensitivity; SPE = specificity.

Table 4
Performance of different serological test cutoffs based on the results of molecular analysis for each biological material.

Biological material	Titers															
	50				100				200				400			
	27*/42 (64.29%)				16*/42 (38.10%)				10*/42 (23.81%)				8*/42 (19.05%)			
	PCR		MAT		PCR		MAT		PCR		MAT		PCR		MAT	
	+	-	SEN	SPE	+	-	SEN	SPE	+	-	SEN	SPE	+	-	SEN	SPE
Urine	4	23	66.67	36.11	3	13	50.00	63.89	2	8	33.33	77.78	2	6	33.33	83.33
Bladder	7	20	70.00	37.50	5	11	50.00	65.63	4	6	40.00	81.25	3	5	30.00	84.38
Kidney	9	18	64.29	35.71	6	10	42.86	64.29	5	5	35.71	82.14	4	4	28.57	85.71
Vaginal fluid	6	21	60.00	34.38	5	11	50.00	65.63	3	7	30.00	78.13	3	5	30.00	84.38
Uterus	8	19	47.06	24.00	6	10	35.29	60.00	4	6	23.53	76.00	4	4	23.53	84.00
Fallopian tube	4	23	57.14	34.29	1	15	14.29	57.14	1	9	14.29	74.29	1	7	14.29	80.00
Ovary	8	19	61.54	34.48	3	13	23.08	55.17	3	7	23.08	75.86	3	5	23.08	82.76
Placenta	7	2	53.85	0.00	5	1	38.46	50.00	3	1	23.08	50.00	3	1	23.08	50.00
Others	19	8	61.29	27.27	10	6	32.26	45.45	8	2	25.81	81.82	7	1	22.58	90.91

* = number of MAT positive individuals; + = positive samples; - = negative samples; SEN = sensitivity; SPE = specificity.

Table 5
Performance of different serological test cutoffs based on the results of microbiological analysis for each material.

Biological material	Titers															
	50				100				200				400			
	27*/42 (64.29%)				16*/42 (38.10%)				10*/42 (23.81%)				8*/42 (19.05%)			
	Culture		MAT		Culture		MAT		Culture		MAT		Culture		MAT	
	+	-	SEN	SPE	+	-	SEN	SPE	+	-	SEN	SPE	+	-	SEN	SPE
Urine	4	23	100.00	39.47	3	13	75.00	65.79	2	8	50.00	78.95	2	6	50.00	84.21
Bladder	6	21	75.00	38.24	4	12	50.00	64.71	3	7	37.50	79.41	3	5	37.50	85.29
Kidney	8	19	72.73	38.71	5	11	45.45	64.52	5	5	45.45	83.87	4	4	36.36	87.10
Vaginal fluid	6	21	66.67	36.36	5	11	55.56	66.67	3	7	33.33	78.79	3	5	33.33	84.85
Uterus	7	20	53.85	31.03	5	11	38.46	62.07	3	7	23.08	75.86	3	5	23.08	82.76
Fallopian tube	4	23	57.14	34.29	1	15	14.29	57.14	1	9	14.29	74.29	1	7	14.29	80.00
Ovary	7	20	63.64	35.48	3	13	27.27	58.06	3	7	27.27	77.42	3	5	27.27	83.87
Placenta	6	3	60.00	40.00	4	2	40.00	60.00	2	2	20.00	60.00	2	2	20.00	60.00
Others	18	9	62.07	30.77	10	6	34.48	53.85	8	2	27.59	84.62	7	1	24.14	92.31

* = number of positive individuals in MAT; + = positive samples; - = negative samples; SEN = sensitivity; SPE = specificity.

CAPÍTULO II:

New perspectives on the diagnosis and epidemiology of leptospirosis in male cattle reared under semiarid conditions

Trabalho submetido à revista **Microbial Pathogenesis**

New perspectives on the diagnosis and epidemiology of leptospirosis in male cattle reared under semiarid conditions

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ABSTRACT

Leptospirosis is the most widespread bacterial zoonosis in the world. The agent *Leptospira* spp. has a complex epidemiological chain. In livestock-rearing, the disease causes economic losses due to reproductive problems. Although in cattle it is clearly a problem within the reproductive sphere, the genital carrier is commonly neglected. The aim of this study was to investigate the serological, molecular and microbiological frequencies of *Leptospira* (with analysis on test performance) in male cattle reared under semiarid conditions, and to investigate the reproductive tract as an extrarenal site. Blood samples from 42 animals were examined by means of the microscopic agglutination test (MAT) and the urinary tract (urine, bladder and kidney) and reproductive tract (cauda epididymidis, vas deferens and vesicular gland) were investigated via the polymerase chain reaction (PCR) and microbiological isolation. Anti-*Leptospira* antibodies were detected in 17 animals (40.48%; 95% CI 27.04% - 55.51%). The serogroups found were Sejroe, Tarassovi, Canicola and Grippotyphosa. Sejroe and Tarassovi were the most frequent. DNA of the bacterium was found in 26 males (61.90%; 95% CI 46.81% - 75.00%), accounting for 86/252 samples (34.13%), especially in the vas

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deferens (45.24%), kidney (40.48%), bladder (35.71%) and cauda epididymidis (35.71%). In comparing the positivity of the different biological materials, there were statistically significant differences ($P < 0.05$) between urine and kidney and between urine and vas deferens. In cultures, 24 animals (57.14%; 95% CI 42.21% - 70.88%) were positive, accounting for 68/252 samples (26.98%). The samples most frequently positive were from the vas deferens (33.33%), cauda epididymidis (30.95%) and kidney (30.95%). The frequency of positivity in urine differed statistically ($P < 0.05$) from that of the vas deferens. DNA of the microorganism was identified in 16 cultures. Three samples enabled genetic sequencing and demonstrated 99% similarity to *L. borgpetersenii*. PCR was shown to be reliable for identifying genital carriers, with best performance in samples from the vesicular gland, with sensitivity of 100.00% and specificity of 96.77%. The results from this study signal that, even under adverse conditions, the pathogen can spread in cattle. This may be explained by venereal transmission.

KEYWORDS: *Leptospira* spp., PCR, genital carrier, vesicular gland, venereal transmission.

1. Introduction

Leptospirosis is the most widespread bacterial zoonosis, both geographically and in terms of the number of host species [1]. Although it was first described in the scientific literature in 1886, ancient Chinese texts reported occurrences of "jaundice of rice fields", thus showing that this disease has been around for thousands of years [2]. The agent *Leptospira* spp. is able to reduce its genome and duplicate or horizontally transfer its genes in the process of adaptation to new hosts, which results in a complex epidemiological chain. Its dynamism means that this anthropozoonosis remains a problem: even after more than a century of research, prevention and control of this disease continues to be challenging [51].

Exposure to the microorganism occurs through direct contact with infected animals or indirectly through water and soil contaminated by urine [16,17]. In animals, transmission can also occur by means of vaginal fluid or placental remains, during copulation and vertically [34]. In livestock-rearing, the disease causes economic losses resulting from longer intervals between deliveries, diminished conception rates, abortions, stillbirths, weak offspring, growth retardation, low milk production or agalactia and death [46].

High frequencies of the pathogen have been reported both in wild animals [21,22] and in domestic animals [45,48,49,62,63], particularly in cattle [9,52], even under adverse

climatic conditions. Although in cattle it is clearly a problem within the reproductive sphere, identification of the genital carrier is commonly neglected [37]. Presence of genital leptospirosis in male cattle has been proven through detection of bacteria in the reproductive tract, especially in seminal vesicles. Thus, venereal transmission has been seen to be a fundamental means of dissemination [34]. Investigations among female cattle reared under semiarid conditions have found that a significant proportion of them are genital carriers [9], which formed the justification for the present study, in which male cattle living under similar conditions were investigated.

Among the best known methods for diagnosing leptospirosis, the microscopic agglutination test (MAT) is recommended by the World Organization for Animal Health [50] given that it is capable of safely pointing out the infecting serogroup [39]. In addition, the polymerase chain reaction (PCR) is a rapid technique with high sensitivity and specificity, and is therefore very reliable [29,68]. Nonetheless, microbiological isolation is considered to be the gold standard [19,40].

Investing in prevention and control is essential for avoiding economic losses. Strategies should be based on factors inherent to the agent, host and environment [15]. If circulating strains can be identified, this clarifies the transmission chain through knowledge of the reservoir, which thus will direct the interventions. According to Martins and Lilenbaum [41], each herd has its own particularities and, therefore, specific programs that address identification of carriers, treatment and vaccination are required.

The Brazilian semiarid region provides peculiar conditions for dissemination of this microorganism. These conditions form a context that needs to be analyzed differently from other places in the world. Knowledge of this dynamic is fundamental for improvement of strategies for combating bovine leptospirosis. The aim of this study was to investigate the serological, molecular and microbiological frequencies of *Leptospira* (with analysis on test performance) in male cattle reared under semiarid conditions, and to investigate the reproductive tract as an extrarenal site.

2. Material and methods

2.1. Study site and its characteristics

This investigation was developed from November 2019 to February 2020 in the municipal slaughterhouse of Patos (latitude: 7°00'19" south; longitude: 37°16'48" west), in the

state of Paraíba, northeastern Brazil, which is used for cattle reared in this intermediate geographic region and surrounding areas. In this region, the Caatinga biome predominates. This exclusively Brazilian biome with a semiarid climate is characterized by long periods of water scarcity that compromise the vegetation. According to Alvares et al. [4], the climate is classified as BShw', i.e. hot and dry, with a rainy season in summer/autumn, such that precipitation is concentrated in the months of March to April but can occur from January to May. Batista et al. [10] and Araújo [7] added that periods of drought can last for more than a year, thus resulting in negative water balance with the addition of intense solar radiation. The period covered by this study corresponded to the dry season, with an average precipitation of 6.70 mm and average temperature of 30.10 ºC [32].

2.2. Sampling

The minimum sample size was determined using the following formula for proportion analysis [5]:

$$n = \frac{p_0 \times q_0 \times \left(z_{1-\beta} + z_{\alpha/2} \times \sqrt{\frac{p_1 \times q_1}{p_0 \times q_0}} \right)^2}{(p_1 - p_0)}$$

Where

n = minimum sample size.

$Z_{\alpha/2}$ = 1.96 (Z value for 95% confidence level).

$Z_{1-\beta}$ = 1.64 (Z value for 95% power).

P_0 = 33% (reference proportion for PCR positivity) [53].

P_1 = 61.40% (estimate of the experimental proportion of positivity in PCR) [36].

q_0 = 1 - p0

q_1 = 1 - p1

In line with these parameters, it was determined that 37 individuals were needed for this scientific investigation. However, in the end, 42 cattle were used. These cattle were male, without any defined breed, greater than or equal to 24 months of age (≥ 2 years) and not vaccinated against leptospirosis.

2.3. Origin of the animals

In accordance with data from the Animal Traffic Guide (GTA) of the State Veterinary Service of Paraíba, the cattle came from farms in the semiarid region, in municipalities belonging to two federative units: Cacimba de Areia, Cacimbas, Condado, Passagem, Patos, Pombal, Quixaba, São José de Espinharas and São Mamede in the state of Paraíba; and São Bento do Una in the state of Pernambuco.

2.4. Field activity

During visits to the slaughterhouse, cattle were selected for this investigation. They were numbered in chronological order of slaughter from 1 to 42. All the biological material was collected *post mortem* and in duplicate (except for material destined for culturing), thus avoiding pain or any unnecessary discomfort for the animals.

After desensitization, at the time of bleeding, a volume of 8 ml of blood was collected from each animal in a sterile tube containing coagulation activator. Each tube had previously been identified with the corresponding number of the animal. These blood samples were used to make indirect diagnoses by means of the microscopic agglutination test (MAT).

During evisceration, using surgical instruments, scalpel blade and sterile syringe, fluid and fragments were collected from the urinary tract (urine, bladder and kidney) and reproductive tract (cauda epididymidis, vas deferent and vesicular gland) in order to make direct diagnoses by means of two techniques: polymerase chain reaction (PCR) and microbiological isolation. Urine was obtained by means of direct puncturing of the bladder.

The biological material was sent to a specific room in the slaughterhouse, where it was quickly processed as aseptically as possible and under protection using a Bunsen burner. Solid samples were placed in sterile Petri dish, while avoiding contact between segments. Thus, these were reduced to three pieces of approximately 2 g each. One of these was immediately sown in a culture medium specific for *Leptospira*: Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid (Difco, Becton, Dickinson and Company, Franklin, New Jersey, United States), supplemented with an antimicrobial cocktail consisting of sulfamethoxazole (0.4 mg/mL) + trimethoprim (0.2 mg/mL) + amphotericin B (0.05 mg/mL) + fosfomycin (0.4 mg/mL) + 5-fluorouracil (0.1 mg/mL) (= STAFF) [12]. The other two were stored in polypropylene microtubes that were free from DNA and ribonucleic acid (RNA) and were

destined for PCR. Urine samples (100 μl) were sown in the culture medium, while 0.5 mL was kept back in polypropylene microtubes for molecular analysis.

During transportation to the Communicable Diseases Laboratory (LDT) of the Center for Health and Rural Technology (CSTR), Federal University of Campina Grande (UFCG), Patos Campus, all material relating to serology and molecular testing was conserved at 4 ºC, but the cultures were kept at room temperature.

2.5. Microscopic agglutination test (MAT)

The MAT was performed in accordance with the official protocols [50,70]. Each serological sample was tested for six pathogenic species of *Leptospira*, 18 serogroups and 24 serovars: *L. borgpetersenii* (serovars Ballum, Castellonis, Javanica, Mini and Tarassovi); *L. interrogans* (Autumnalis, Bratislava, Canicola, Copenhageni, Djasiman, Hardjoprajitno, Hebdomadis, Icterohaemorrhagiae, Kennewicki, Pomona, Pyrogenes and Wolffi); *L. kirschneri* (Cynopteri and Grippotyphosa); *L. noguchii* (Louisiana and Panama); *L. santarosai* (Canalzoni and Guaricura); and *L. weilii* (Celledoni).

With dilution levels from 1:50 to 1:3200, titration was based on the highest dilution of serum in Sorensen's buffered phosphate saline solution in which 50% of the *Leptospira* specimens had agglutinated, thus indicating the infecting serogroup.

2.6. Polymerase chain reaction (PCR), sequencing and phylogenetic analysis

Leptospiral DNA from tissues and urine was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Nordrhein-Westfalen, Germany), following the manufacturer's recommendations. Cultures positive for microscopic readings were also subjected to extraction. The primers LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) and LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′) designed by Stoddard et al. [66] were used to amplify the *LipL32* gene, which is specific for pathogenic *Leptospira* species. *L. interrogans*, Pomona serogroup, Kennewicki serovar, served as the positive control; filtered ultrapure water was the negative control. The total volume of each sample was analyzed by means of electrophoresis on 2% agarose gel, with staining using Evans blue, and the DNA bands (≅ 260 bp) were viewed under ultraviolet light.

The direct and reverse LipL32-45F and LipL32-286R primers, respectively, in the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, California, USA), were used

for nucleotide sequencing, as described earlier [66]. Capillary electrophoresis was performed through a 3130xL genetic analyzer and a POP-7 polymer (Applied Biosystems, Foster City, California, USA) [59]. The sequences were aligned using BioEdit [25] and were compared with *Leptospira* strains held in GenBank (National Biotechnology Information Center, Bethesda, Maryland, USA) (http://www.ncbi.nlm.nih.gov) using the Blast algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was constructed using the SeaView4 software [28] by means of the neighbor-joining method, with 1000 bootstrap replicas (http://tree.bio.ed.ac.uk/software/figtree/, and was viewed using the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/). The phylogenic reconstruction included *Leptospira* sequences for comparison.

2.7. Microbiological isolation/culturing

Urine and material from the bladder, kidney, cauda epididymidis, vas deferens and vesicular gland were sown in screw-cap tubes containing 5 mL of liquid EMJH supplemented with STAFF. Renal tissue was macerated using a sterile disposable syringe (10 mL) for insertion in the culture medium. After the cultures had been set up, they were packed into a biochemical oxygen demand (BOD) digital incubator (CienlaB, Campinas, São Paulo, Brazil), and were kept at 29 ºC for 24 hours. This was then replicated in semisolid EMJH (Difco, Becton, Dickinson and Company, Franklin, New Jersey, United States) that was free from antimicrobials, with incubation again. Over a six-month period, monthly replicates were performed and the material was evaluated weekly by means of dark-field microscopy.

2.8. Statistical analysis

The proportions of animals and samples that were positive were compared by means of the chi-square test with Yates continuity correction or Fisher's exact test, using the BioEstat 5.3 software [8]. The significance level was taken to be 5% ($P \leq 0.05$). The sensitivity and specificity of the PCR were calculated using the DAG_Stat software [38], taking the results from cultures as the reference.

3. Results

Out of the 42 male cattle evaluated, 17 (40.48%; 95% CI 27.04% - 55.51%) presented anti-*Leptospira* antibodies, among which two reached titer 50, one reached titer 100, two reached titer 200, seven reached titer 400, two reached titer 800, two reached titer 1600 and one reached titer 3200. The reactive serogroups were Sejroe, Tarassovi, Canicola and Grippotyphosa. Overall, serogroups most frequently found were Sejroe, with 11 positive animals; and Tarassovi, with four (Table 1).

Leptospiral DNA was found in at least one sample from 26 cattle (61.90%; 95% CI 46.81% - 75.00%). Among the 252 samples, PCR detected genetic material of the agent in 86 samples (34.13%). The materials most frequently found to be positive were the vas deferens (19 samples; 45.24%), kidney (17; 40.48%), bladder (15; 35.71%) and cauda epididymidis (15; 35.71%). In comparing the positivity levels of the different biological materials, there were statistically significant differences ($P < 0.05$) between urine and kidney and between urine and vas deferens (Table 2). Genetic sequencing of the bacterium directly from PCR products was possible in two samples, from the bladder and vesicular gland of two different animals, and these samples reached 99% similarity to *Leptospira borgpetersenii* (Figure 1). In the cultures, the pathogen was present in at least one sample from 24 males (57.14%; 95% CI 42.21% - 70.88%) and 68 cultures (26.98%) were positive in microscopic readings, especially those from the vas deferens (14 samples; 33.33%), cauda epididymidis (13; 30.95%) and kidney (13; 30.95%). The positivity of urine differed statistically ($P < 0.05$) from that of the vas deferens. DNA of the microorganism was identified in 16 cultures (Table 2) and one of these (from the bladder) enabled sequencing, which demonstrated 99% similarity to *L. borgpetersenii* (Figure 1).

Table 3 shows the performance of the molecular test in diagnosing *Leptospira* sp. infection. The test showed high sensitivity (100.00%) and specificity (88.89%), with the best performance from vesicular gland samples, and high co-positivity (100.00%) and co-negativity (96.77%).

4. Discussion

The finding that 17 cattle (40.48%; 95% CI 27.04% - 55.51%) were reactive in the microscopic agglutination test (MAT) indicates that, even under adverse circumstances, *Leptospira* shows the capacity to be present in herds in the Brazilian semiarid region. There was little variation of the serogroups found: Sejroe, Tarassovi, Canicola and Grippotyphosa. Sejroe and Tarassovi predominated, as also observed in previous studies [9]. Other

investigations conducted in semiarid regions have also reported that Sejroe has the most constant presence: 58.17% [52] and 36.8% [54]. Other studies developed in Brazil have shown that regardless of the biome involved, Sejroe is most prevalent: northeastern region [44,53,61], northern [26,27], central-western [43], southeastern [6,20,55,56,58] and southern [30,31].

The contrast between unfavorable climate (dry season, with average precipitation of 6.70 mm and temperature of 30.10 ºC) and a significant proportion of positive males, especially with the Sejroe serogroup, shows that external variables had less influence on transmission, thus emphasizing intraspecies transmission [14]. Cattle are natural carriers of this serogroup [35], but the molecular basis for this specificity is unknown [51]. Sejroe has a leading role among agents responsible for leptospirosis worldwide [35]. In Latin America, it has been reported in 80% of studies [57] and has been found to trigger reproductive problems [67]. It is associated with the subclinical/chronic form of the disease, with subtle syndromes such as subfertility and early embryonic death [37]. Calves usually show clinical signs [69]. Non-Sejroe strains are considered accidental in cattle; when they occur, the disease has acute characteristics and its dissemination is almost always between species [14]. In the semiarid region, production animals come into contact with the wild fauna while grazing in areas of Caatinga, which is dependent on favorable environmental/climatic conditions (i.e. the rainy season). However, according to Pinto et al. [58], it is necessary to consider that the process of agent-host adaptation and vice versa allows intraspecies diffusion.

In the present investigation, Tarassovi was the second most frequent strain, with varying titers (50-1600). This is among the main serogroups found in cattle [64], although it has been little reported in cattle in Brazil [56]. It is an agent for clinical leptospirosis [47,61] and is prevalent in leptospirosis among pigs [65]. Canicola is linked to canids and its main reservoir is domestic dogs (*Canis lupus familiaris*) [24], but its presence has also been reported in the maned wolf (*Chrysocyon brachyurus*) and in the South American cougar (*Puma concolor*) [18]. It is considered common in small wild mammals, but rare in cattle [26]. Caldas et al. [11] reported that the natural hosts for Grippotyphosa were rats (*Rattus norvegicus*) and opossums (*Didelphis marsupialis*).

Through the polymerase chain reaction (PCR), leptospiral DNA was found in 26 males (61.90%; 95% CI 46.81% - 75.00%). This was the highest positivity rate among the tests used. Latosinski et al. [33] signaled that this frequency could even be higher since negativity is also related to DNA concentration below the detectable limit. In comparing the positivity rates of different biological materials, the rate in urine was statistically significantly

lower ($P < 0.05$) than in the kidney and vas deferens, which showed that the bacterium preferentially infected these latter two organs. This can be explained by the fact that because of its size, morphology (spirochete/helical) and translational motility, the microorganism has easy access to the kidney. This allows it to escape from humoral action due to the physical barrier. Moreover, the prominence of infection of the vas deferens reflects release of *Leptospira* via tubular secretions, to colonize the epididymis, testicles and, especially, the seminiferous tubules. On the other hand, elimination through urine takes place only intermittently. The outcome from PCR indicated that, for *in vivo* cases, semen may be valuable for identifying carriers. It was possible to sequence the nucleotides of the pathogen in two samples, from the bladder and vesicular gland of two different individuals, and the sequencing showed that these samples had 99% similarity to *Leptospira borgpetersenii*.

In the cultures, *Leptospira* was detected in at least one sample from 24 animals (57.14%; 95% CI 42.21% - 70.88%). From the positivity rates of the different materials, the agent showed statistically significant higher frequency ($P < 0.05$) in the vas deferens than in urine. The result from the cultures revealed that use of microbiological isolation from the vas deferens, cauda epididymidis and kidney can increase the chances of success. However, because a surgical procedure is required in order to access these materials, collection of such samples is appropriate for *post mortem* cases, which is relevant to slaughterhouse situations. Genetic material belonging to the pathogen was identified in 16 cultures, among which one on a bladder sample enabled sequencing, which showed 99% similarity to *L. borgpetersenii*.

L. borgpetersenii belongs to the pathogenic clade and, in accordance with its virulence, to subgroup 2, together with the species *santarosai*, *mayottensis*, *weilii* and *alexanderi* [51]. It causes early embryonic loss and repetition of estrus, consequent to uterine inflammation and damage from invasion of the embryo [37]. Its adaptation to cattle has led to a smaller genome than that of other pathogenic and saprobic strains [51]. The size of the genetic code is related to the ability to adapt to conditions inside and outside the host [40]. Genomic comparisons showed coevolution through rearrangement and insertion of DNA sequences [51]. Direct transmission is believed to cause loss of the genes that would be necessary for the agent's survival outside the host, thus making it little resistant in the environment and consequently impairing indirect dissemination [40]. In the present study, this species was identified in a vesicular gland sample, which forms another indication of venereal transmission. This means of transmission may have been responsible for influencing the high frequency of positive findings during the dry period, because any other means depending on external variables would have a lower chance of success.

According to PCR, out of the 26 males (61.90%; 95% CI 46.81% - 75.00%), 16 showed positivity in both tracts, especially three animals with leptospiral DNA in all samples; six only in the genital tract and four in the urinary tract. In individuals with urogenital tract involvement, the spread of the microorganism is especially boosted in the rainy season of the semiarid region due to the possibility of transmitting simultaneously by means of urinary and venereal elimination. Rocha et al. [60] reported occurrence of self-limited renal infection, rather than persistence in the reproductive system, thus emphasizing the importance of genital carriers. Just as in female cattle, in which high frequency of the agent has been reported in the uterus, ovary and vaginal fluid [9], it was clearly confirmed through the present study that genital leptospirosis was a syndrome dissociated from renal leptospirosis [37], given that the reproductive tract was affected in some animals without urinary infection. This alternative transmission route seems to have greater importance during the dry period in the semiarid region, in that it allows *Leptospira* to avoid facing the environmental stage of its biological cycle, thus protecting the bacterium from high temperatures, high levels of solar radiation and low air humidity. Soto et al. [65] reported that in dry soil the microorganism can only survive for thirty minutes. Thus, at times of water scarcity, urinary tract transmission is less relevant.

In cattle, genital leptospirosis seems to be more common among females. Barnabé et al. [9] reported that among 31 females (73.81%; 95% CI 58.93% - 84.70%), 15 showed positivity in both tracts, while 13 were positive only in the reproductive tract and a considerably smaller number were positive for leptospiral DNA in the urinary tract (three animals). These findings infer that venereal transmission may be more functional when it occurs from a male to a susceptible female, because the carrier deposits contaminated semen in the vagina and the agent propagates upwards through the new host. The hormonal oscillation of the estrous cycle depresses the immune system, with emphasis on estrus (receptive phase), thus facilitating the entire process of infection. When the scenario is the opposite, i.e. from female carrier to healthy male, it seems that the microorganism faces greater difficulty in entering the urethra. One possible explanation for this relates to the ejaculate itself, or in the post-coverage urination that sweeps it in the opposite direction (regardless of the adhesins). Nonetheless, the penile mucosa can also be invaded. This set of circumstances does not diminish the role of males in the venereal route, because even without infection of the reproductive tract, there is the possibility of the carriage of *Leptospira* through semen, if urinary tract involvement is present. According to Faine et al. [19], in the leptospiruric phase, semen can be contaminated during its passage through the urethra since it shares the excretory duct with urine (a confounding factor in identifying genital carriers

through analysis on the ejaculate). This is important information for the semiarid region where natural mounting predominates and one breeding male is used to cover several females [13], thereby boosting diffusion. Regarding individuals showing urogenital tract positivity, the data available are insufficient to elucidate the primary infection.

The molecular test performed well in diagnosing pathogenic *Leptospira* infection and was shown to have high sensitivity (100.00%) and specificity (88.89%). Many factors contribute to the accuracy of PCR, e.g. the extraction kit, thermostable DNA polymerase (Taq), laboratory equipment, operating procedure and the "gold standard" result. Genovez et al. [23] clarified that the processing needs to be suitable for the tissue, fluid and species. Substances in the biological material interfere in the reaction to act as Taq inhibitors, such as magnesium-free ion chelators, hemoglobin, bile salts and glycoprotein acid polysaccharides. Extreme pH variations and the phenol and chloroform used for DNA extraction and purification are considered critical. During tissue storage, bacteria can be lysed and their genetic material lost with the supernatant after centrifugation. Ahmed et al. [3] highlighted the inhibitors contained in the kidneys and urine, and recommended dilution (1:10) of the DNA extracted from kidney tissue and extra washing of urine. Nevertheless, PCR is very reliable. Its cost remains the biggest obstacle to large-scale application. According to Stoddard et al. [66], the refined sensitivity of this test eliminates the need for agent isolation to confirm the result. It is suitable for emergency situations because it is fast, thus allowing early diagnosis based on blood analysis during leptospiremia. Merien et al. [42] drew attention to the limitation of inability to identify the infecting strain, which therefore necessitated electrophoresis on non-denaturant polyacrylamide gel or amplicon sequencing.

5. Conclusions

A high frequency (61.90%) of pathogenic *Leptospira* was observed in male cattle reared under semiarid conditions. The results from this investigation signal that even under adverse conditions, the agent can spread. The explanation for this may be the existence of venereal transmission. PCR performed well in identifying genital carriers.

Ethical approval

The present study was approved by the Ethics Committee for the Use of Animals (*Comissão de Ética no Uso de Animais*; CEUA) of the Federal University of Campina Grande

(UFCG), Center for Rural Health and Technology (CSTR), Patos Campus, under protocol CEP/CEUA n 069-2018.

Declaration of competing interest

The authors declare that there are no potential conflicts of interest related to the research, authorship, and/or publication of this article.

Credit authorship contribution statement

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Data availability

Data will be made available on request.

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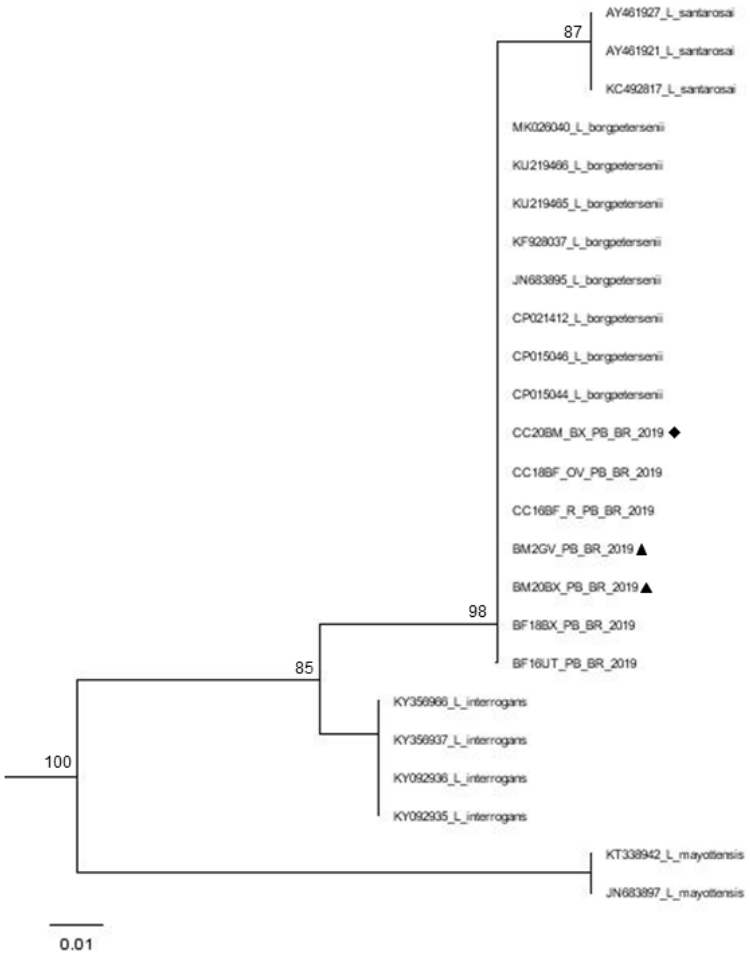

Figure 1. Phylogenetic tree based on the alignment of nucleotide sequences of the *LipL32* gene *Leptospira* sp., constructed using the neighbor-joining model with 1000 replicas. ◆ Sample sequenced from culture. ▲ Sample sequenced from tissue.

Table 1
Results from serological, molecular and microbiological tests for *Leptospira* sp. infection in male cattle reared under semiarid conditions.

Identification of the animal	MAT	PCR						Culture					
	17*/42 (40.48%)	26*/42 (61.90%)						24*/42 (57.14%)					
	Serogroup (titer)	UR	BL	KID	CE	VD	VG	UR	BL	KID	CE	VD	VG
1	-	+	+	+	+	+	+	+	+ +	-	+	+	+ +
2	Sejroe (3200)	-	+	+	+	+	+▲	-	+ +	+ +	+	+	+ +
3	-	-	+	+	+	+	+	-	+ +	-	-	+ +	+
4	Tarassovi (400)	-	+	-	+	+	+	-	+ +	-	+ +	-	-
5	-	+	-	+	-	-	-	+	-	-	-	-	-
6	Sejroe (200)	-	-	-	-	-	+	-	-	-	-	-	+
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	Sejroe (800)	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	+	-	-	-	-	-	-	-
10	Sejroe (100)	-	+	+	+	+	+	-	+	+	+	+	+
11	-	+	+	+	+	+	+	+ +	+	+	+	+	+
12	Grippotyphosa (400)	+	+	+	-	+	-	+	-	+ +	-	-	-
13	-	+	+	+	-	+	-	-	-	+	-	+	-
14	-	+	+	+	-	+	-	-	-	+	-	-	-
15	-	-	-	+	-	-	-	-	-	+	-	-	-
16	-	-	+	-	-	-	-	-	+	-	-	-	-
17	Sejroe (200)	-	+	-	+	+	-	-	+ +	-	+	+	-
18	-	-	-	+	+	-	-	-	-	+	+	-	-
19	-	-	-	-	-	+	-	-	-	-	-	+ +	-
20	-	-	+▲	+	+	+	+	-	+ + ◆	-	-	+	+
21	Canicola (400)	-	-	-	-	-	-	-	-	-	-	-	-
22	Sejroe (1600)	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-
24	Tarassovi (800)	-	-	-	-	-	-	-	-	-	-	-	-
25	Sejroe (400)	-	-	-	+	-	-	-	-	-	+	-	-
26	-	-	-	-	-	-	-	-	-	-	-	-	-

27	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	+	+	+	+	+	+	+	+	+	+	+	+
29	-	-	-	-	-	+	-	-	-	-	-	-	-
30	Sejroe (400)	-	+	+	+	+	+	-	-	+	+	+	+
31	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	+	+	-	-	-	-	+	+	-
33	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-
35	Sejroe (50)	-	-	-	-	-	-	-	-	-	-	-	-
36	Tarassovi (50)	-	-	-	-	-	-	-	-	-	-	-	-
37	-	-	-	-	-	-	-	-	-	-	-	-	-
38	Sejroe (400)	-	-	-	-	-	-	-	-	-	-	-	-
39	Tarassovi (1600)	+	-	+	-	-	-	+	-	+	-	-	-
40	-	-	-	-	-	-	-	-	-	-	-	-	-
41	Sejroe (400)	-	-	+	+	+	+	-	-	+	+	+	+
42	-	-	+	+	+	+	+	-	+ +	+	+	+	+ +
Total (+)	17	8	15	17	15	19	12	6	11	13	13	14	11

* = number of positive individuals; UR = urine; BL = bladder; KID = kidney; CE = cauda epididymidis; VD = vas deferens; VG = vesicular gland; - = negative sample; + = positive sample; + + = positive culture in microscopic reading and PCR; ▲ = tissue sample that enabled sequencing of nucleotides of the agent (*L. borgpetersenii*); ♦ = culture that enabled sequencing of nucleotides of the agent (*L. borgpetersenii*).

Table 2
Molecular and microbiological diagnosis of *Leptospira* sp. according to the biological material from male cattle reared under semiarid conditions.

Sample	Total	PCR		Culture	PCR from culture	
		26*/42 (61.90%)		24*/42 (57.14%)	10*/42 (23.81%)	
		+ (%)	Sequencing	+ (%)	+ (%)	Sequencing
Urine	42	8 (19.05) [a]	-	6 (14.29) [a]	1 (2.38%)	-
Bladder	42	15 (35.71) [ab]	1	11 (26.19) [ab]	7 (16.67%)	1
Kidney	42	17 (40.48) [b]	-	13 (30.95) [ab]	2 (4.76%)	-
Cauda epididymidis	42	15 (35.71) [ab]	-	13 (30.95) [ab]	1 (2.38%)	-
Vas deferens	42	19 (45.24) [b]	-	14 (33.33) [b]	2 (4.76%)	-
Vesicular gland	42	12 (28.57) [ab]	1	11 (26.19) [ab]	3 (7.14%)	-
Total	252	86 (34.13)	2	68 (26.98)	16 (6.35%)	1

* = number of positive individuals; + = positive samples. Different lowercase letters in the same column indicate statistical difference between the proportions ($P < 0.05$).

Table 3
Molecular test performance in diagnosing bovine leptospirosis based on the microbiological result (gold standard) for each material.

Biological material	Culture		PCR			
	24*/42 (57.14%)		26*/42 (61.90%)			
	+	-	+	-	SENS	SPEC
Urine	6	18	8	18	100.00	94.44
Bladder	11	13	15	11	100.00	87.10
Kidney	13	11	17	9	100.00	86.21
Cauda epididymidis	13	11	15	11	100.00	93.10
Vas deferens	14	10	19	7	100.00	82.14
Vesicular gland	11	13	12	14	100.00	96.77
Any material	24	18	26	16	100.00	88.89

* = number of positive individuals; + = positive samples; - = negative samples; SENS = sensitivity; SPEC = specificity.

CAPÍTULO III:

Transmissão transplacentária de *Leptospira borgpetersenii* em bovinos sob condições semiáridas

Trabalho a ser submetido à revista **Animal Reproduction Science**

Transmissão transplacentária de *Leptospira borgpetersenii* em bovinos sob condições semiáridas

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RESUMO

A bovinocultura enfrenta, no semiárido brasileiro, condições climáticas desfavoráveis, e os discretos avanços na tecnificação da cadeia produtiva tornam os animais suscetíveis às doenças infectocontagiosas. Leptospirose é comum nos rebanhos, comprometendo, de maneira silenciosa, a reprodução, refletindo em perdas econômicas. O agente *Leptospira* spp. consegue se propagar mesmo em clima adverso, portanto, compreender essa dinâmica pode contribuir para o aprimoramento das estratégias de controle. O objetivo deste estudo foi investigar bovinos criados em condições semiáridas para a presença de anticorpos anti-*Leptospira* em fetos, bem como verificar a transmissão transplacentária mediante detecção de DNA do patógeno em embriões e fetos, reportando órgãos colonizados. Foram amostrados 15 embriões e fetos de ambos os sexos, idades gestacionais diferentes, coletando-se sangue, órgãos (coroide ovoide, sistema nervoso central, pulmão, fígado, baço, rim, bexiga e aparelho reprodutor) e líquidos (peritoneal, urina e conteúdo abomasal). Comparou-se o resultado da

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soroaglutinação microscópica (SAM) e da reação em cadeia da polimerase (PCR) dos indivíduos ao das matrizes. Nenhum soroconverteu, enquanto nove (60,00%; IC 95% 35,75% - 80,18%) matrizes sororreagiram para Sejroe, Tarassovi e Australis, com títulos variando de 50 a 1600. DNA da bactéria foi detectado em 13 (86,67%; IC 95% 62,12% - 96,26%) dos embriões e fetos, com 35/131 (26,72%) amostras positivas, destacando-se coroide ovoide (1/2; 50,00%), baço (6/13; 46,15%), rim (5/13; 38,46%) e sistema nervoso central (5/15; 33,33%). Não houve amostra positiva de urina. Contrapondo a positividade de cada material biológico, houve significância estatística ($P < 0,05$) entre baço e urina e rim e urina. Fragmento de sistema nervoso central viabilizou o sequenciamento genético do microrganismo, demonstrando 99% de similaridade à *L. borgpetersenii*. Os resultados comprovam ser inadequado o emprego da SAM em fetos e também mostram elevada frequência de embriões e fetos com DNA leptospírico, constatando que a transmissão transplacentária é uma forma eficiente de disseminação do agente em bovinos sob condições semiáridas.

Palavras-chave: Leptospirose, PCR, embriões, fetos, sistema nervoso central.

1. Introdução

A complexidade em desenvolver bovinocultura no semiárido do Brasil se inicia com o clima desafiador. Sua área corresponde a 1 milhão de km² ou 64% de todo Nordeste (Araújo Filho, 2013); o bioma predominante, a Caatinga, caracteriza-se por altas temperaturas, reduzida pluviosidade, intemperismo do solo e baixa produção de fitomassa. Alvares et al. (2014) destacam ambiente quente e seco, com precipitações concentradas no intervalo de março a abril. Araújo (2011) acrescenta que a estiagem pode durar mais de um ano, resultando em balanço hídrico negativo somado à intensa radiação solar. Segundo Clementino et al. (2015), a maioria dos bovinocultores locais desenvolvem atividade sob modelo de agricultura familiar, adotando exploração mista (carne e leite), regime de semiconfinamento, ordenha manual e monta natural. Com discretos avanços na tecnificação da cadeia produtiva, as doenças infectocontagiosas prevalecem. Algumas ocorrem de modo silencioso e, além de prejudicarem a produtividade, comprometem a reprodução do plantel, potencializando as perdas econômicas. É o caso da leptospirose.

Mesmo em condições adversas à sobrevivência de *Leptospira* spp. no ambiente, estudos realizados em animais sob condições semiáridas comprovaram elevada frequência

desse agente (Morais et al., 2019; Silva et al., 2019; Fernandes et al., 2020a, 2020b; Nogueira et al., 2020a; Soares et al., 2021), particularmente em bovinos (Pimenta et al., 2014; Barnabé et al., 2022a, 2022b). A transmissão acontece de forma indireta por contato com água e solo contaminados com a bactéria, de forma direta como resultado do contato com urina e restos placentários do portador ou através das vias venérea e transplacentária (Adler, 2015) que podem ter maior relevância na disseminação do microrganismo durante o período de estiagem. Sabe-se que a transmissão transplacentária intercorre à leptospiremia materna, quando o útero é infectado e o patógeno invade o feto, provocando danos ou interrompendo seu desenvolvimento (Ellis, 2015). Pesquisa constatou que é comum a transmissão vertical na espécie ovina (Nogueira et al., 2020b), o que justifica investigação em bovinos da região.

Na espécie bovina, a gestação dura em média 290 dias (Boligon et al., 2007). Apresenta placenta do tipo cotiledonária, uma estrutura que se integra ao endométrio por meio de interdigitações dos tecidos fetal (cotilédone) e materno (carúncula), originando placentômeros responsáveis por troca de gases e nutrientes (Furukawa et al., 2014). Quanto à interação materno-fetal, é classificada como sinepiteliocorial (Bazer et al., 1994), na qual células binucleadas do trofoblasto se ligam às uninucleadas do epitélio uterino, formando as trinucleadas, cuja função de transferir hormônios e efetores favorece o metabolismo (Wooding e Burton, 2008). A membrana coriônica se funde ao alantoide, resultando na superfície alantocoriônica, densamente vascularizada (Leiser e Kaufmann, 1994); artérias umbilicais se ramificam na superfície irrigando os cotilédones, promovendo o fluxo sanguíneo que se forma o sistema venoso para retorno aos vasos umbilicais após capilarização (Leiser et al., 1997). Colágeno, elastina, fibronectina, laminina, proteoglicanos e glicosaminoglicanos compõem a matriz extracelular (Kakabadze et al., 2019).

A maioria dos trabalhos científicos em fetos com infecção por leptospiras envolve material de aborto (Moore et al., 2003; Atxaerandio et al., 2005). O abate de animais de produção nos estágios gestacionais inicial e intermediário é permitido pela legislação brasileira (Brasil, 2017), viabilizando este estudo. Com isso, objetivou-se investigar bovinos criados em condições semiáridas para a presença de anticorpos anti-*Leptospira* em fetos, bem como verificar a transmissão transplacentária mediante detecção de DNA do agente em embriões e fetos, reportando órgãos colonizados.

2. Material e métodos

2.1. Aprovação ética

O estudo foi aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Campina Grande (UFCG), Centro de Saúde e Tecnologia Rural (CSTR), Campus de Patos, mediante protocolo CEP/CEUA n°069-2018.

2.2. Local do estudo e suas características

A pesquisa foi desenvolvida de setembro a novembro de 2019 no Abatedouro Municipal de Patos (Latitude: 7°00'19" Sul, Longitude: 37°16'48" Oeste), Estado da Paraíba, Nordeste do Brasil, onde ocorre abate de bovinos provenientes desta região geográfica intermediária e áreas circunvizinhas. Nesse território, predomina a Caatinga (bioma exclusivamente brasileiro) de clima semiárido, caracterizada por longos períodos de escassez hídrica com comprometimento da vegetação. O intervalo de realização do trabalho correspondeu ao período de estiagem, com precipitação média de 0,47 mm e 29,28 °C de temperatura média (INMET, 2021).

2.3. Embriões e fetos

Para investigação científica, entre embriões e fetos bovinos de gestação única, foram utilizados 15, sem padrão racial definido, ambos os sexos, idades gestacionais diferentes, originados de matrizes não vacinadas contra leptospirose.

2.4. Origem

De acordo com dados da Guia de Trânsito Animal (GTA) do Serviço Veterinário Estadual da Paraíba, os animais prenhes vieram de propriedades rurais do semiárido, de municípios pertencentes a duas Unidades Federativas: Paraíba (Condado, Olho D'água, Patos, Pombal, Santa Terezinha, São José de Espinharas, São José do Bonfim e São Mamede) e Pernambuco (Buíque e Capoeiras).

2.5. Atividade a campo

Embriões e fetos foram selecionados para estudo recebendo identificação em números arábicos, 01 a 15, seguindo a ordem cronológica do abate das matrizes. Todo material biológico foi coletado *post mortem*, evitando dor ou qualquer incômodo desnecessário aos

animais. No decorrer da evisceração da matriz, utilizando-se de instrumental cirúrgico e lâmina de bisturi estéreis, o embrião ou o feto era retirado do útero para saco plástico zipado estéril, previamente numerado, sendo conservado a 4 °C durante o transporte ao Laboratório de Doenças Transmissíveis (LDT), Centro de Saúde e Tecnologia Rural (CSTR), Universidade Federal de Campina Grande (UFCG), Campus de Patos.

2.6. Atividade laboratorial

Na capela do LDT, sob proteção do bico de Bunsen, dispondo de instrumental cirúrgico, lâmina de bisturi e seringa estéreis, os embriões e fetos foram imediatamente processados de modo mais asséptico possível.

Aferiu-se o peso e as dimensões nos sentidos crânio-caudal e cernelha à extremidade distal do membro torácico; no caso dos embriões, diâmetro do coroide ovoide e comprimento total. A idade foi estimada aplicando metodologia adaptada de Barr et al. (1990), fundamentada no comprimento entre a base da nuca e a base da cauda: 13-21 centímetros (cm) = três meses, 22-31 cm = quatro meses, 32-43 cm = cinco meses, 44-57 cm = seis meses, 58-67 cm = sete meses, 68-85 cm = oito meses e maior que 86 cm = nove meses. Para estágio muito precoce, adotou-se metodologia de Tommasi Junior et al. (2014): 2 cm e 1 g = um mês, 3 cm e 42 g = menos de dois meses e 10 cm e 380 g = menos de três meses.

Após análise macroscópica visando constatar qualquer lesão ou anomalia, cada feto era disposto em decúbito lateral direito para necropsia. Grande maioria possibilitou a distinção do sexo pela visualização da parte externa do órgão sexual, contudo, nos que se encontravam em fase muito prematura, isso foi possível somente com a abertura da cavidade abdominal e reconhecimento do trato reprodutivo da fêmea.

Obteve-se sangue por punção cardíaca (exceto dos indivíduos 3, 6 e 9 em virtude de estágio muito precoce); o sangue foi centrifugado por dez minutos a 1512 g e o soro armazenado em microtubo de polipropileno e conservado a - 20 °C até execução da soroaglutinação microscópica (SAM).

Amostras de órgãos (sistema nervoso central, pulmão, fígado, baço, rim, bexiga e aparelho reprodutor) e líquidos (peritoneal, urina e conteúdo abomasal) foram coletadas em duplicata; dos embriões, coletaram-se sistema nervoso central e coroide ovoide. Amostras sólidas eram dispostas em placa Petri estéril, evitando contato entre os fragmentos para que fossem reduzidas a aproximadamente 1 g. Amostras armazenadas em microtubos de polipropileno livres de ácido desoxirribonucleico (DNA) e ácido ribonucleico (RNA) foram

conservadas a - 20 °C até realização da reação em cadeia da polimerase (PCR). Obtiveram-se líquido peritoneal, urina e conteúdo abomasal por punção direta, reservando-se 100 µl aos microtubos para análise molecular.

2.7. Soroaglutinação microscópica (SAM)

O teste seguiu protocolos oficiais (Zuerner, 2006; OIE, 2014). Cada amostra sorológica foi testada para seis espécies de *Leptospira*, 18 sorogrupos e 24 sorovares: *L. borgpetersenii* (sorovares Ballum, Castellonis, Javanica, Mini e Tarassovi); *L. interrogans* (Autumnalis, Bratislava, Canicola, Copenhageni, Djasiman, Hardjoprajitno, Hebdomadis, Icterohaemorrhagiae, Kennewicki, Pomona, Pyrogenes e Wolffi); *L. kirschneri* (Cynopteri e Grippotyphosa); *L. noguchii* (Louisiana e Panama); *L. santarosai* (Canalzoni e Guaricura); *L. weilii* (Celledoni). Com ponto de corte 25, o resultado baseou-se na aglutinação de 50% das leptospiras à positividade.

2.8. Reação em cadeia da polimerase (PCR), sequenciamento e análise filogenética

A extração de DNA leptospírico dos tecidos, líquido peritoneal, urina e conteúdo abomasal se deu pelo kit DNeasy Blood and Tissue (Qiagen, Hilden, Renânia do Norte-Vestfália, Alemanha), seguindo as recomendações do fabricante. Os primers LipL32-45F (5′-AAG CAT TAC CGC TTG TGG TG-3′) e LipL32-286R (5′-GAA CTC CCA TTT CAG CGA TT-3′) desenhados por Stoddard et al. (2009) foram empregados na amplificação do gene *LipL32*, específico para leptospiras patogênicas. *L. interrogans*, sorogrupo Pomona, sorovar Kennewicki serviu de controle positivo; água ultrapura filtrada como negativo. O volume total de cada amostra foi analisado por eletroforese em gel de agarose (2%), corado com Evans blue e as bandas de DNA (≅ 260 bp) visualizadas sob luz ultravioleta.

Os iniciadores LipL32-45F e LipL32-286R direto e reverso, respectivamente, no kit BigDye Terminator v3.1 (Applied Biosystems, Foster City, Califórnia, Estados Unidos), foram usados para o sequenciamento de nucleotídeos, conforme descrito anteriormente (Stoddard et al., 2009). Realizou-se eletroforese capilar por intermédio de analisador genético 3130xL e um polímero POP-7 (Applied Biosystems, Foster City, Califórnia, Estados Unidos) (Platt et al., 2007). A sequência foi alinhada pelo BioEdit (Gouy et al., 2010) e comparada com cepas de *Leptospira* do GenBank (National Biotechnology Information Center, Bethesda, Maryland, Estados Unidos) (http://www.ncbi.nlm.nih.gov) mediante algoritmo BLAST

(http://www.ncbi.nlm.nih.gov/BLAST/). Construiu-se a árvore filogenética no software SeaView4 (Hall, 1999) por associação entre vizinhos, bootstrap de 1000 repetições (http://tree.bio.ed.ac.uk/software/figtree/, visualizada no software FigTree v1.4.3 (http://tree.bio.ed.ac.uk/)). A reconstrução filogênica incluiu sequências de leptospiras para comparação.

2.9. Análise estatística

A comparação das proporções de embriões e fetos e amostras positivas foi feita pelo teste Qui-quadrado com correção de continuidade de Yates ou teste exato de Fisher no software BioEstat 5.3 (Ayres et al., 2007), considerando nível de significância de 5% ($P \leq 0,05$). Desconsiderou-se coroide ovoide à análise devido à baixa quantidade de amostras.

3. Resultados

Dos 15 embriões e fetos bovinos, sete eram machos, enquanto cinco, fêmeas. A maioria se encontrava no período intermediário da gestação. Três deles, em fase muito prematura, impossibilitaram a distinção do sexo e a coleta do sangue (identificados como 3, 6 e 9). O desfecho da soroaglutinação microscópica (SAM) dos fetos não foi condizente ao resultado das matrizes, visto que enquanto nove (60,00%; IC 95% 35,75% - 80,18%) reprodutoras sororreagiram para Sejroe, Tarassovi e Australis, com títulos variando de 50 a 1600, nenhum feto soroconverteu. DNA leptospírico foi detectado em ao menos uma amostra de 13 (86,67%; IC 95% 62,12% - 96,26%) dos embriões e fetos, atestando forte associação ao desfecho da reação em cadeia da polimerase (PCR) do trato reprodutivo das fêmeas adultas, comprovando a transmissão transplacentária (Tabela 1). O sistema nervoso central do feto 4 viabilizou o sequenciamento genético do agente, demonstrando 99% de similaridade à *Leptospira borgpetersenii* (Figura 1).

De um total de 131 amostras dos embriões e fetos submetidas à PCR, 35 (26,72%) continham DNA do patógeno. As frequências dos materiais biológicos positivos foram coroide ovoide (1/2; 50,00%), baço (6/13; 46,15%), rim (5/13; 38,46%), sistema nervoso central (5/15; 33,33%), pulmão (4/13; 30,77%), fígado (4/13; 30,77%), bexiga (4/13; 30,77%), líquido peritoneal (3/13; 23,08%), conteúdo abomasal (2/13; 15,38%) e aparelho reprodutor (1/13; 07,69%). Não houve amostra positiva de urina. Comparando a positividade

de cada material, houve significância estatística ($P < 0,05$) entre baço e urina e rim e urina (Tabela 2).

4. Discussão

A despeito do baixo ponto de corte (25) adotado na soroaglutinação microscópica (SAM), nem os fetos em estágios mais avançados apresentaram anticorpos anti-*Leptospira*, o que exclui a influência da sensibilidade do teste para o resultado, esclarecendo haver imaturidade do sistema imunológico (Moore et al., 2003). Magajevski et al. (2007) realizaram pesquisa utilizando 213 fetos bovinos entre o terceiro e o sétimo mês da gestação, coletados em abatedouro e, mesmo isolando o agente em duas amostras de rim e uma de fígado, não encontraram imunoglobulinas.

Cruzando o resultado da PCR do material biológico dos embriões e fetos com o desfecho da sorologia e teste molecular das matrizes, constatou-se tendência de que quanto mais alto o título alcançado pelo animal prenhe, menor o número de amostras positivas do feto na PCR. Nos indivíduos oriundos de reprodutoras com títulos entre 400-1600, a média foi de 1,50 amostra com DNA leptospírico. Nesses casos, as fêmeas adultas exibiram positividade no teste molecular somente para o trato reprodutivo. O desvio da regra esteve no feto 7 que revelou DNA do microrganismo em três órgãos (baço, rim e bexiga) e líquido peritoneal, enquanto a matriz soroconverteu a Sejroe (800) e positivou no sistema urogenital. Indivíduos advindos de animais com títulos entre 50-100 tiveram média de 1,80 amostra com DNA da bactéria. Nessa realidade, as reprodutoras positivaram em ambos os tratos. As exceções foram o feto 3 com uma amostra positiva e o embrião 6 sem o patógeno; a fêmea adulta do embrião 6, ainda que sororreativa a Tarassovi (50), mostrou-se negativa na PCR. Indivíduos procedentes de matrizes negativas na SAM obtiveram superior média de amostras positivas no teste molecular (3,33), exceto o feto 13 que apresentou uma amostra positiva (baço). Suas reprodutoras positivaram em maioria para o trato urogenital.

Situações em que os fetos continham pouco DNA de *Leptospira* e suas respectivas fêmeas adultas altos títulos sorológicos indicam proteção por barreira humoral, atenuação na virulência da cepa, ou simplesmente o estádio da infecção do animal prenhe – fase de produção crescente de imunoglobulinas, subsequente à leptospiremia e intercorrente à leptospirúria. Achados do agente isoladamente no sistema reprodutor dessas matrizes reiteram a importância do portador genital na epidemiologia da doença, pois a colonização renal por estirpes do sorogrupo Sejroe é autolimitada, diferentemente de quando ocorre no trato

reprodutivo (Rocha et al., 2018). Casos do embrião e dos fetos com uma quantidade maior de amostras positivas na PCR, provenientes de reprodutoras com baixos títulos, evidenciam inferior proteção dos anticorpos maternos, ou o momento ligeiramente após quadro sistêmico respaldado pelo número de positivas para o trato urogenital. O embrião 6 não detinha material genético do microrganismo, assim como a fêmea adulta correspondente, mesmo soroconvertendo a Tarassovi (50), acontecimento que pode caracterizar cura. Indivíduos com a bactéria disseminada, oriundos de animais negativos na SAM, demonstram a relevância da defesa humoral. Esse cenário pode representar leptospiremia, estágio antecessor à produção de antígenos (janela imunológica). Outras matrizes na mesma circunstância apresentaram DNA do patógeno no sistema urogenital, reforçando estado sistêmico. Proporção significativa positivou somente no trato reprodutor e pode indicar cronicidade, processo de adaptação agente-hospedeiro e vice-versa, corroborando para leptospirose genital bovina como síndrome dissociada da renal (Loureiro e Lilenbaum, 2020).

De acordo com a reação em cadeia da polimerase (PCR), 13 (86,67%; IC 95% 62,12% - 96,26%) dos embriões e fetos estavam em processo de infecção por *Leptospira* spp., demonstrando haver associação ao desfecho da análise molecular das matrizes, comprovando ser comum a transmissão transplacentária nos bovinos criados em condições semiáridas. Do material biológico dos fetos, baço, rim, sistema nervoso central, pulmão, fígado e bexiga podem refletir sítios onde o patógeno se depare com pH ideal (ligeiramente ácido), maior disponibilidade de nutrientes para sua manutenção, crescimento e multiplicação. Na contramão, embora o microrganismo invasor tenha trânsito facilitado na ausência da barreira humoral, líquido peritoneal, conteúdo abomasal, aparelho reprodutor e urina parecem preteridos. Além disso, das quatro menores frequências, três corresponderam a líquidos, nos quais as adesinas não conferem vantagem. Essa conjuntura explica a diferença estatística ($P < 0,05$) entre baço e urina e rim e urina em relação à positividade.

Amostra de sistema nervoso central do feto 4 viabilizou o sequenciamento dos nucleotídeos do agente, precisando 99% de similaridade à *Leptospira borgpetersenii*, também identificada no útero da reprodutora correspondente (Barnabé et al., 2022a) e em fragmentos de glândula vesicular e bexiga de bovinos machos adultos (Barnabé et al., 2022b). Essa espécie pertence ao clado patogênico e, conforme virulência, ao subgrupo 2 junto a *L. santarosai*, *mayottensis*, *weilii* e *alexanderi* (Picardeau, 2017) causa perda embrionária precoce e repetição do estro em consequência dos danos da invasão ao embrião e da inflamação uterina (Loureiro e Lilenbaum, 2020). Adaptada aos bovinos, apresenta curto genoma em comparação a outras cepas patogênicas e sapróbias (Picardeau, 2017). O tamanho

do código genético está relacionado à capacidade de se adaptar às condições dentro e fora do hospedeiro (Martins e Lilenbaum, 2015). Comparações genômicas evidenciaram coevolução mediante rearranjo e inserção de sequências de DNA (Picardeau, 2017). Pouco resistente no meio ambiente, acredita-se que a transmissão direta ocasionou perda de genes necessários à sobrevivência fora do hospedeiro (Martins e Lilenbaum, 2015). A identificação dessa espécie no sistema nervoso central do feto é uma evidência da transmissão transplacentária, as mutações sofridas pelas leptospiras durante o processo evolutivo podem ter facilitado essa via que talvez seja muito importante na realidade do semiárido por representar uma maneira eficiente de propagação durante a estação seca.

5. Conclusão

Mesmo os fetos, que se encontravam em estágio avançado da gestação, demonstraram imaturidade do sistema imunológico, evidenciando ser inadequado o emprego da soroaglutinação microscópica (SAM) para o diagnóstico de leptospirose nessa situação. A elevada frequência (86,67%) de embriões e fetos com DNA leptospírico comprova que a transmissão transplacentária é uma forma eficiente de disseminação do agente em bovinos sob condições semiáridas.

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Declaração de crédito de contribuição de autoria

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Declaração de conflito de interesse

Os autores declaram não haver potenciais conflitos de interesse com relação à pesquisa, autoria e/ou publicação deste artigo.

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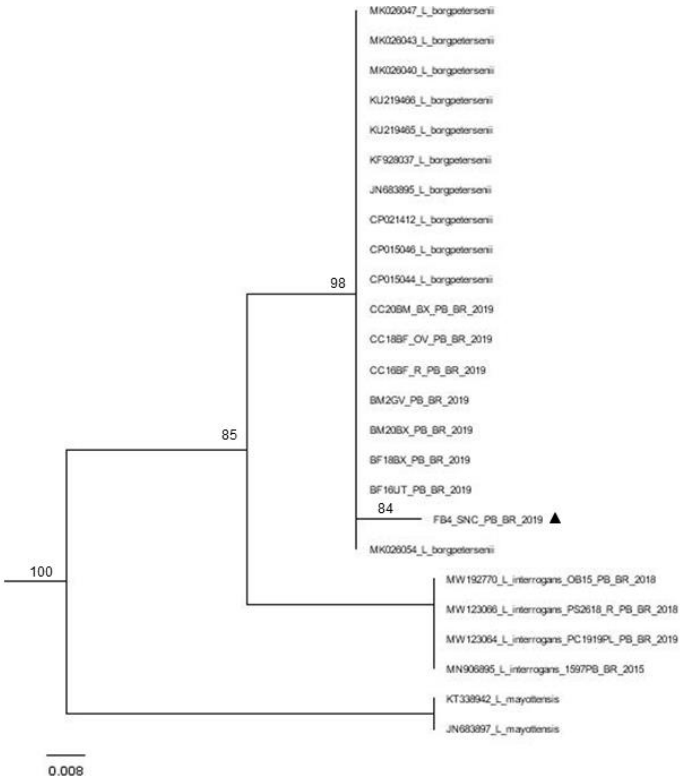

Figura 1. Árvore filogenética baseada no alinhamento da sequência de nucleotídeos do gene *LipL32* de *Leptospira* sp., construída no modelo de associação de vizinhos com 1000 repetições. ▲ Amostra sequenciada a partir de sistema nervoso central de feto.

Tabela 1
Análise de embriões e fetos bovinos para infecção por *Leptospira* sp. e correlação às matrizes.

Embriões/Fetos															Matrizes	
ID	Sexo	Peso (kg)	Dimensão (cm)	Idade (≅)	Testes										Testes	
					SAM	PCR									SAM (título)	PCR (trato)
						CO	SNC	PUL	FIG	BAÇO	RIM	BXG	AR	LP	UR	CA		
1	F	0,531	19 x 27	4 meses	-		+	-	+	-	+	-	-	-	⊅	-	Sejroe (100)	Urogenital
2	M	1,015	20 x 32	5 meses	-		-	-	+	-	-	-	-	-	-	-	Sejroe (1600)	Genital
3	◊	0,040	04 x 09	< 3 meses			-	+	-	-	-	-	-	-	-	-	Sejroe (100)	Urogenital
4	M	1,291	22 x 33	5 meses	-		▲	+	-	+	-	-	+	+	-	-	-	Urogenital
5	F	4,554	33 x 51	6 meses	-		+	-	-	-	+	+	-	-	-	-	Australis (50)	Urogenital
6*	◊	0,005	02 x 05	< 2 meses		-	-										Tarassovi (50)	-
7	F	1,400	12 x 23	4 meses	-		-	-	-	+	+	+	-	+	-	-	Sejroe (800)	Urogenital
8	M	5,510	42 x 63	7 meses	-		-	-	-	-	-	-	-	-	-	-	Tarassovi (1600)	-
9*	◊	0,001	01 x 02	1 mês		+	+										Tarassovi (50)	Urogenital
10	F	0,113	08 x 16	3 meses	-		-	-	-	+	-	-	-	-	-	+	-	Genital
11	M	2,911	29 x 48	6 meses	-		-	+	+	+	+	+	-	+	-	+	-	Genital
12	F	8,227	50 x 69	8 meses	-		+	-	+	-	-	-	-	-	⊅	-	-	Genital
13	M	0,948	20 x 32	5 meses	-		-	-	-	+	-	-	-	-	-	-	-	Urogenital
14	M	0,468	15 x 24	4 meses	-		-	-	-	+	+	+	-	-	⊅	-	-	Urogenital
15	M	0,502	15 x 26	4 meses	-		-	+	-	-	-	-	-	-	-	-	Sejroe (400)	Genital

ID = identificação; * = embrião; F = fêmea; M = macho; ◊ = indefinido; Dimensão = altura x comprimento; CO = coroide ovoide; SNC = sistema nervoso central; PUL = pulmão; FIG = fígado; BXG = bexiga; AR = aparelho reprodutor; LP = líquido peritoneal; UR = urina; CA = conteúdo abomasal; - = resultado negativo; + = resultado positivo; ▲ = amostra com DNA leptospírico sequenciado (*L. borgpetersenii*); ⊅ = não contém.

Tabela 2
Frequência de órgãos e líquidos embrionários e fetais com DNA leptospírico.

Material biológico	Amostras positivas (%)	Amostras totais
Coroide ovoide	1 (50,00)	2
Sistema nervoso central	5 (33,33) [ab]	15
Pulmão	4 (30,77) [ab]	13
Fígado	4 (30,77) [ab]	13
Baço	6 (46,15) [a]	13
Rim	5 (38,46) [a]	13
Bexiga	4 (30,77) [ab]	13
Aparelho reprodutor	1 (07,69) [ab]	13
Líquido peritoneal	3 (23,08) [ab]	13
Urina	0 (00,00) [b]	10
Conteúdo abomasal	2 (15,38) [ab]	13
Total	35 (26,72)	131

Letras minúsculas diferentes na mesma coluna indicam diferença estatística entre as proporções ($P < 0,05$). Ausência de letra = não incluído na análise estatística.

CONCLUSÃO GERAL

Os resultados desta pesquisa mostram que em bovinos as transmissões venérea e transplacentária podem representar formas eficientes de disseminação de leptospiras. Dados do teste molecular e do isolamento microbiológico reforçam o tropismo do agente pelo trato reprodutivo, portanto, o portador genital é peça-chave para o controle da leptospirose. Também foi demonstrado melhor desempenho do teste sorológico na identificação dos positivos quando se adotou o ponto de corte 50, fundamentando o emprego desse protocolo na sorologia de bovinos criados em condições semiáridas.