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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA E SAÚDE ANIMAL

Juciê Jales Fernandes

Isolamento e caracterização molecular de *Leptospira* sp. em animais silvestres do Semiárido da Paraíba, Brasil

Patos/PB
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Dissertação submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do título de Mestre em Ciência e Saúde Animal.

Prof. Dr. Severino Silvano dos Santos Higino
Orientador

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Mestrando

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RESUMO

A leptospirose é uma doença bacteriana zoonótica que está entre as mais difundidas globalmente, acometendo animais domésticos, silvestres e ser humano. Essa infecção é causada por espécies patogênicas de *Leptospira* sp., que podem penetrar no hospedeiro por meio da pele íntegra ou lesionada, por contato direto com a urina de um animal infectado ou através da água contaminada. A ocorrência de leptospirose está ligada a fatores ambientais e a presença de animais reservatórios, sendo que para o controle da doença um passo crucial é a identificação desses hospedeiros que são fontes de infecção, os quais são responsáveis por disseminar o agente no ambiente. Uma série de estudos desenvolvidos no Semiárido do Nordeste permitem o entendimento sobre a ocorrência da leptospirose em diversas espécies de animais domésticos, no entanto existe uma lacuna sobre o conhecimento nas espécies silvestres, bem como o papel destes no ciclo de transmissão da doença. Diante disso objetivou-se investigar a ocorrência de *Leptospira* sp. nas espécies teiú (*Tupinambis merianae*) e gambá-de-orelha-branca (*Didelphis albiventris)* do semiárido paraibano. Para isso o Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) autorizou a captura de 12 teiús e 12 gambás (protocolo Nº57190-1). Amostras de tecido do trato reprodutivo, rim, fígado e bexiga, além da urina, foram coletados para a detecção molecular (nos gambás ainda foi coletado tecido do sistema nervoso central) e tentativa de isolamento, e o soro sanguíneo para o teste sorológico. O teste molecular realizado foi a reação em cadeia da polimerase (PCR) e o sorológico foi a prova de soroaglutinação microscópica (SAM), na tentativa de isolamento foram utilizados os meios de cultura Fletcher, EMJH e EMJH enriquecido com antibióticos, além disso as amostras com amplificação de DNA foram submetidas ao sequenciamento genético. Nos teiús foram encontrados seis (50%) animais com anticorpos anti-*Leptospira* sp., sendo a maioria das reações para o sorogrupo Tarassovi (50%) e Pyrogenes (33,3%), o DNA leptospiral foi detectado em nove (75%) animais, e os genes de duas amostras (fígado e urina) foram sequenciados e apresentaram identidade com *Leptospira interrogans*. Nos gambás não foi encontrado anticorpos anti-*Leptospira* sp., em nenhuma das amostras sorológicas, pela PCR foi detectado o DNA leptospírico no sistema nervoso central (SNC) de cinco animais (41,7%), o gene de uma dessas amostras foi sequenciado e apresentou identidade com *Leptospira interrogans*. Em nenhuma das espécies houve o crescimento microbiano nos tecidos cultivados. Os resultados mostram que os *Tupinambis merianae* participam do ciclo epidemiológico da leptospirose no Semiárido, sendo uma fonte de infecção para os animais domésticos e os seres humanos. E a presença de *Leptospira* sp. no SNC de *Didelphis albiventris* não permite ainda caracterizar esta essa espécie como reservatório com potencial de transmissão do agente na região, no entanto representa um sítio de localização que precisa ser melhor investigado. Isso evidencia a dificuldade no controle da doença em regiões como o Nordeste brasileiro, onde existe uma diversidade de espécies silvestres, que através do contato indireto acabam disseminando o agente para outros animais.

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PALAVRAS-CHAVE: leptospiras; reservatórios; animais silvestres; semiárido.

ABSTRACT

Leptospirosis is a zoonotic bacterial disease that is among the most widespread globally, affecting domestic animals, wild and humans. This infection is caused by pathogenic species of *Leptospira* sp., Which can penetrate the host through intact or damaged skin, by direct contact with the urine of an infected animal or through contaminated water. The occurrence of leptospirosis is linked to environmental factors and the presence of reservoir animals, and for the control of the disease a crucial step is the identification of these hosts that are sources of infection, which are responsible for spreading the agent in the environment. A series of studies carried out in the Semi-arid region of the Northeast allow for an understanding of the occurrence of leptospirosis in several species of domestic animals, however there is a gap about knowledge in wild species, as well as their role in the disease transmission cycle. Therefore, the objective was to investigate the occurrence of *Leptospira* sp. in the species teiú (*Tupinambis merianae*) and gambá-de-orelha-branca (*Didelphis albiventris*) from the semi-arid region of Paraíba. For this, the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) authorized the capture of 12 teiús and 12 possums (protocol Nº57190-1). Samples of tissue from the reproductive tract, kidney, liver and bladder, in addition to urine, were collected for molecular detection (tissue from the central nervous system was still collected) and attempted isolation, and blood serum for serological testing. The molecular test performed was the polymerase chain reaction (PCR) and the serological test was the microscopic agglutination test (SAM), in an attempt to isolate were used the medium culture Fletcher, EMJH and EMJH enriched with antibiotics, in addition to the samples with amplification of DNA were subjected to genetic sequencing. In the teiús, six (50%) animals were found with anti-*Leptospira* sp. antibodies, the majority of the reactions for the serogroup Tarassovi (50%) and Pyrogenes (33,3%), the leptospiral DNA was detected in nine (75%) animals, and the genes from two samples (liver and urine) were sequenced and showed identity to *Leptospira interrogans*. In opossums, no anti-*Leptospira* sp. antibodies were found. In none of the serological samples, the leptospiric DNA was detected in the central nervous system (SNC) of five animals (41.7%), the gene from one of these samples was sequenced. and presented identity with *Leptospira interrogans*. In none of the species there was microbial growth in the cultured tissues. The results show that the *Tupinambis merianae* participate in the epidemiological cycle of leptospirosis in the Semiarid region, being a source of infection for domestic animals and humans. And the presence of *Leptospira* sp. no SNC of *Didelphis albiventris* does not yet allow characterizing this species as a reservoir with potential for agent transmission in the region, however it represents a location that needs to be further investigated. This shows a difficulty in controlling the disease in regions such as Northeastern Brazil, where there is a diversity of wild species, which through indirect contact has just disseminated the agent to other animals.

KEY-WORDS: leptospires; reservoirs; wild animals; semiarid.

INTRODUÇÃO GERAL

A leptospirose é uma doença bacteriana zoonótica que está entre as mais difundidas globalmente (ADLER, 2014; GOARANT, 2016), acomete animais domésticos, espécies silvestres, além do ser humano (ADLER; MOCTEZUMA, 2010). É uma infecção causada por espécies patogênicas de *Leptospira* sp., pertencentes a família *Leptospiraceae* e ordem *Espirochaetales* (FAINE et al., 1999), que foi descrita pela primeira vez em 1886 por Weil (LEVETT, 2001).

Esse gênero foi por muito tempo dividido em 2 grupos considerando apenas a virulência (LEVETT, 2001; VICENT et al., 2019), posteriormente foi possível distinguir 19 genoespécies com base no genoma (DJELOUADJI et al., 2012), e as leptospiras passaram a compreender 13 espécies patogênicas: *L. alexanderi, L. alstonii, L. borgpetersenii, L. inadai, L. interrogans, L. fainei, L. kirschneri, L. licerasiae, L. noguchi, L. santarosai, L. terpstrae, L. weilii* e *L. wolffi,* com mais de 260 sorovares e 6 espécies saprófitas: *L. biflexa, L. meyeri, L. yanagawae, L. kmetyi, L. vanthielii e L. wolbachii*, com mais de 60 sorovares (ADLER; MOCTEZUMA, 2010). Vicent et al., (2019) propõem um novo sistema esquemático de classificação, com 64 espécies classificadas filogenaticamente, que compreendem 37 genoespécies patógenas e 27 genoespécies saprófitas que incluem espécies ambientais para o qual o status de virulência não foi comprovada.

Com base na antigenicidade o agente é diferenciado em sorovares (JORGE et al., 2012), tendo como base a expressão de epítopos expostos na superfície do mosaico de lipopolissacarídeos (LPS) (ADLER; MOCTEZUMA, 2010). Esses sorovares relacionados antigenicamente formam sorogrupos, e estes apresentam grande importância clínica e epidemiológica (JORGE et al., 2012).

As bactérias penetram no hospedeiro através da pele íntegra ou lesionada e mucosas, invadindo a circulação, onde se espalham para vários órgãos (ADLER, 2014; ELLIS, 2015). Ao colonizar os rins, as leptospiras são excretadas na urina e podem então contaminar todo o ambiente (LOUREIRO; LILENBAUM, 2020), além de poder serem eliminadas pelo fluido vaginal (LILENBAUM et al., 2008). A transmissão se dá pelo contato com água ou solo contaminado, ou devido o contato direto com a urina de animal portador (PICARDEAU, 2013).

A doença pode ser diagnosticada por métodos indiretos, como a soroaglutinação microscópia (SAM), que é o teste sorológico de referência recomendado pela Organização

Mundial de Saúde Animal (OIE, 2014). Por métodos diretos, por meio das ferramentas moleculares como a reação em cadeia de polimerase (PCR), que detecta o DNA bacteriano em diferentes amostras, como urina, sangue, sêmen, líquido vaginal e outros tecidos (LILENBAUM et al., 2008; GAMAGE et al., 2011; HAMOND et al., 2012; LATOSINSK et al., 2018).

Os animais são classificados como hospedeiros suscetíveis, os quais geralmente desenvolvem uma doença de gravidade variável podendo levar a morte, mas que podem se recuperar e eliminar totalmente a bactéria do organismo em questão de semanas; ou hospedeiros de manutenção, que não desenvolvem quadro clínico da doença, mas persistem com as leptospiras principalmente nos túbulos renais (GOARANT, 2016).

A ocorrência de leptospirose está ligada a fatores ambientais e a presença de reservatórios (ADLER; MOCTEZUMA, 2010), sendo os portadores considerados como chave no ciclo de transmissão (LILENBAUM et al., 2008). Assim, para qualquer estratégia de controle, um passo crucial é a identificação de reservatórios, pois estes são responsáveis por disseminar o agente (HAAKE; LEVETT, 2015).

A leptospirose é um excelente exemplo para essa nova abordagem chamada "*One Health*", na qual se deve analisar a relação entre seres humanos, animais e ecossistemas (SCHNEIDER et al., 2015). Nos últimos anos tem sido observada uma maior aproximação entre os seres humanos e animais domésticos com espécies de animais silvestres, essa interação pode aumentar a exposição a diversos agentes infecciosos (SOUZA, 2011). Além disso, fatores associados a ação do homem na natureza podem levar a desequilíbrios ambientais, contribuindo para uma maior disseminação de zoonoses, sendo muito importante o monitoramento de reservatórios silvestres para algumas dessas doenças (WOLFE et al., 2005, CHOMEL; BELOTTO; MESLIN, 2007).

Nesse contexto, o conhecimento sobre a epidemiologia da leptospirose em animais silvestres ainda não está bem elucidado, assim também como o papel destes no ciclo dessa doença (VIEIRA; PINTO; LILENBAUM, 2018). No Brasil os estudos nessas espécies ainda são escassos, muitas vezes limitados a investigações em populações cativas, centros de conservação ou animais de zoológicos (SILVA et al., 2010). Esse fato impede a compreensão da doença em regiões onde existe uma grande diversidade biológica, como o semiárido nordestino, existindo assim uma lacuna no conhecimento da cadeia epidemiológica da doença nessa região.

As pesquisas têm levantado a possibilidade de várias espécies silvestres atuarem como reservatórios de *Leptospira* sp. (PIMENTEL et al., 2009; MONTE et al., 2013; OLIVEIRA et

al., 2013; OLIVEIRA et al., 2014), assim são necessários estudos que determinem o real papel desses animais no ciclo epidemiológico da leptospirose, bem como sua capacidade de transmissão (JORGE et al. 2010).

Em várias regiões do mundo tem sido investigada a ocorrência de leptospiras nas diversas espécies de animais silvestres (LENHARO; SANTIAGO; LUCHEIS, 2012; RICARDO et al. 2018). Na região semiárida do Nordeste uma ampla gama de estudos desenvolvidos sobre as espécies de animais domésticos, trouxeram o conhecimento sobre a leptospirose em bovinos (PIMENTA et al., 2014; OLIVEIRA et al., 2016; PIMENTA et al., 2019), caprinos (ARAÚJO-NETO et al., 2010; COSTA et al., 2016; PIMENTA et al., 2019), ovinos (HIGINO et al., 2010; ALVES et al., 2012; DIRECTOR et al., 2014; COSTA et al., 2017; SILVA et al., 2019), suínos (AZEVEDO et al., 2008; FIGUEIREDO et al., 2013; LEITE et al., 2018) e cães (ALVES et al., 2004; BATISTA et al., 2004; FERNANDES et al., 2018). Já sobre as espécies silvestres do semiárido, poucos estudos podem ser citados, como o de Brasil et al., (2013) em animais silvestres de cativeiro e Silva et al., (2016) nas espécies *Cavia aperea*, *Cerdocyon thous* e *Euphractus sexcinctus*. Por essa limitação de estudos, pouco se sabe sobre o real papel dos animais silvestres no ciclo da doença na região. Diante disso essa Dissertação foi elaborada em dois capítulos, no capítulo I foi investigado a ocorrência de *Leptospira* sp. em teiús (*Tupinambis merianae*) no semiárido paraibano, enquanto no capítulo II foi pesquisado na espécie gambá-de-orelha-branca (*Didelphis albiventris).*

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CAPÍTULO I:

Tupinambis merianae carreadores de *Leptospira* sp. na região Semiárida do Nordeste do Brasil

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Tupinambis merianae carriers of *Leptospira* sp. in the Semi-arid region of Northeast Brazil

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ABSTRACT

Several species of wild animals have been identified as reservoirs for leptospirosis. The etiology of multiple species, the role of reservoirs and the Brazilian biological diversity encourage research on these animals. The species *Tupinambis merianae* is abundant in the northeastern semiarid region and its role in the disease cycle has been little investigated, so this study aimed to verify the presence of the bacteria and anti-*Leptospira* sp. antibodies in these animals. Twelve animals (Brazilian Institute of the Environment and Renewable Natural Resources - protocol No. 57190) were used. Samples of tissue from the reproductive tract, kidney, liver and bladder were collected for molecular detection and blood serum for serological testing. The polymerase chain reaction (PCR) and the microscopic agglutination test (MAT) were performed. Samples with DNA amplification were subjected to genetic sequencing. In six animals (50%) anti-*Leptospira* sp. antibodies were detected, with the majority of reactions for the Tarassovi (50%) and Pyrogenes (33.3%) serogroups, and the leptospiral DNA was detected in nine (75%) animals. The genes from two samples (liver and urine) were sequenced and showed similarity to *Leptospira interrogans*. The results show a high rate of infected *Tupinambis merianae*, indicating their participation in the transmission cycle of leptospirosis in the semiarid region.

KEYWORDS: leptospirosis, wild animals, reservoirs, semiarid.

INTRODUCTION

Wild animals are considered reservoirs of several agents that cause emerging infectious diseases [1,2], which can have a major impact on public health and economic losses [3,4]. In recent years, factors related to increasing urbanization, industrialization and advances in agriculture and livestock have contributed to a closer relationship between humans and domestic animals with the populations of wild animals [5,6]. This interaction results in an increased risk of exposure to infectious agents that can compromise the conservation of these animals, besides agricultural production and public health. [6].

Factors associated with human action, such as rampant deforestation, aggressive agricultural development and even ecotourism, may cause imbalances in the environment and contribute to the greater spread of zoonoses [3,7]. Thus, for surveilling some emerging diseases, the monitoring of the wildlife reservoirs is essential [3].

Leptospirosis is a zoonotic and emerging infectious disease that is distributed worldwide [8]. It is caused by spirochetes of the genus *Leptospira* sp. [9], which can penetrate the hosts through mucous membranes or skin lesions [10]. In addition to man and domestic animals, wild species can also be affected [11]. After infection, some animals may recover and become asymptomatic carriers, remaining with the microorganism colonizing their renal tubules, which allows the agent the dissemination in the environment for a long time through urine [10, 12].

The etiology of multiple species of *Leptospiras* sp., the role of reservoirs, the environment and the Brazilian biological diversity are factors that influence the occurrence of the disease, and therefore encourage studies in wild animals, since little is known about the function of these animals in the epidemiology of the disease [13]. Studies have detected the possibility of several wild species acting as reservoirs of *Leptospira* sp., such as the *Cebus libinosus*, the *Cebus xanthosternus*, the *Cerdocyon thous*, the *Procyon cancrivorus*, the *Hidrochoerus hidrochaeris, Ctenomys lami,* the *Cavia aperea* [14,15,16,17], so that research is needed to determine how these animals act in the transmission of leptospirosis, evaluating their real role in the epidemiological cycle of the disease [18].

The occurrence of leptospires has been investigated in several species of wild animals [19,20], in the northeastern Semiarid the agent was identified in the *Cavia aperea*, *Euphractus sexcinctus* and in the *Cerdocyon thous* [21]. The *Tupinambis merianae* species is abundant in the Northeastern semi-arid region and, so far, studies on this species have been mainly with captive animals in zoological parks, evaluating only the presence of antibodies as in the work

of [22]. Given the possibility of this species functioning as a reservoir of the pathogen, the objective of this study was to identify *Tupinambis merianae* carriers of leptospires in the Semiarid region of Northeastern Brazil.

MATERIALS AND METHODS

This project was approved by the Ethics and Research Committee (CEP) of the Rural Health and Technology Center (CSTR) of the Federal University of Campina Grande (UFCG) under protocol number CEP 023.2017 and by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) under Protocol No. 57190.

Study area and capture of animals

To meet the requirements of the inspection agencies (IBAMA), 12 *Tupinambis merianae* (6 males and 6 females) were used, 10 animals from the municipality of Patos and 2 from Brejo do Cruz, both located in the Semiarid of the State of Paraíba, Northeastern Brazil. The animals were captured using non-lethal traps made up of plastic pipes and cages (Fig. 1), between September 2018 and March 2019. After identifying the presence of animals of this species in a certain area, the traps were installed, they consisted of 80 cm diameter pipes with an occluded end, buried under a 45° inclination and a specific wire cage for the species. Baits made of fruits, meat and eggs were placed inside the traps. When entering in search of food, the animals were prevented from escaping. The traps were checked three times a day.

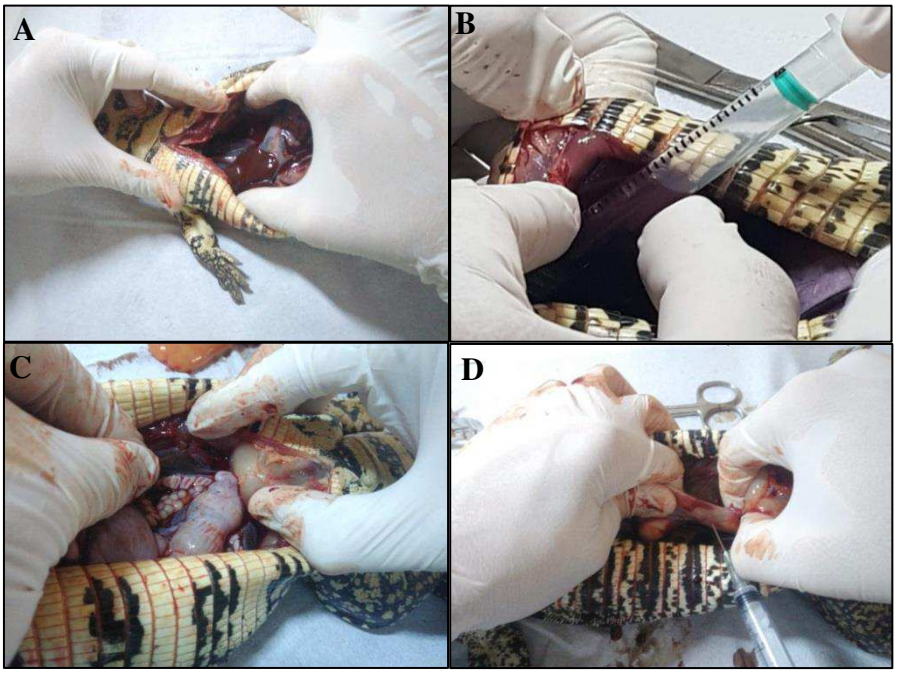
Figure 1 Traps used to catch *Tupinambis merianae*. Trap made of plastic pipe (A) and Cage (B)

Euthanasia and organ collection

After capture, the animals were taken in transport boxes for dogs (height: 38 cm; width: 43 cm; length: 59 cm) and in custom-made polyvinyl chloride (PVC) boxes, to the Communicable Diseases Laboratory (LDT) of the Rural Health and Technology Center (CSTR) of the Federal University of Campina Grande (UFCG), in Patos - PB. The animals were weighed and, following chemical containment, with xylazine 2% (Syntec, Xilazin, Santana do Parnaíba-SP, Brazil) and ketamine 10% (Ceva, Dopalen, Paulínia-SP, Brazil) in doses of 5mg/kg and 100mg/kg, respectively, intramuscularly in the posterior region of the thigh, complying with Annex IV of the Ordinance of the Federal Council of Biology No. 148/2012 (Annex I). For euthanasia, 2 mL of 2% lidocaine (Bravet, Lidovet, Rio de Janeiro, Brazil) was applied intrathecally to the atlanto-occipital joint (Fig. 2), followed by cardiac exsanguination as a method of confirmation. 2 ml of blood were collected and after centrifugation, the serum was stored at -20° C until the serological test was performed. After opening the abdominal and thoracic cavities, fragments of liver, kidney, bladder and reproductive tract were collected (females: uterus with the oviduct together; males: vas deferens and epididymis together), using surgical scissors, anatomical forceps and sterile scalpel blade no. 15 (Fig. 3). The organs of the reproductive tract of each animal were collected together due to their small size, which made their individual locating difficult. Urine was collected with a sterile disposable syringe of 3 mL by cystocentesis.

Figure 2 Euthanasia of *Tupinambis merianae*, identification of the atlanto-occipital joint (A) and application of intrathecal lidocaine (B)

Figure 3 Collection of organs. Liver (A), blood (B), reproductive tract (C) and Urine (D)



Serological diagnosis of infection by *Leptospira* sp.

To detect the presence of anti-*Leptospira* sp. antibodies the microscopic serum agglutination test (MAT) was used, as recommended by the World Organization for Animal Health [23]. A collection of live antigens containing 22 pathogenic serovars and two saprophytes was used: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Javanica, Panamá, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Tarassovi, Andamana, Patoc and Sentot. Samples with agglutinating activity at a 1:50 dilution were considered positive and, shortly after, the reagent samples were serially titrated for a ratio of two (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200), in which the highest titer obtained determined the infectious serogroup. The readings were performed in an optical microscope (ZEISS®, Oberkochen, Germany) with a dry dark field condenser, with a 10x/0.20 objective lens and an ocular 10 (100X) lens, observing the formation of agglutinations.

Isolation

For isolation, a 1g fragment of each organ (liver, kidney, bladder, reproductive tract) and 2 mL of urine were sown in culture medium. The culture media used were produced at the Communicable Diseases Laboratory (LDT), the Ellinghausen-McCullough-Johnson-Harris (EMJH, Difco®-USA) medium supplemented with 10% rabbit serum and enriched with 1% pyruvate sodium, 1% calcium chloride, 1% magnesium chloride and 3% L-asparagine. This one was prepared in two formulations, one with the addition of 5-fluorouracil (400 mg / L; Sigma®-USA) and the other with the addition of a cocktail of antibiotics (enriched EMJH). The enriched EMJH medium contained the following antibiotics, in quantities for 500 ml of Solution, 200 mg of Sulfametaxazole, 100 mg of Trimetropim, 25 mg of Amphotericin B, 2000 mg of Phosfomycin and 500 mg of 5-Fluoracil, according to the recommendations by [24]. Immediately after collection, the organs were macerated with the aid of sterile syringes and spread directly in the enriched EMJH medium, after 24 hours 1 mL of the culture was spread in EMJH and spread again every 15 days, all the spread media were incubated in the greenhouse in a temperature of 28° C. To check for the presence of microorganisms, weekly readings were performed for at least twelve weeks, using an optical microscope with a dry dark field condenser, with 10x/0.20 objective lens and a 10 (100X) eyepiece.

Molecular detection and phylogenetic analysis

For DNA extraction from kidney, liver, bladder, reproductive tract and urine samples, the Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany) was used, following the manufacturer's recommendations. The polymerase chain reaction (PCR) was performed with primers LipL32-45F (5′-AAGCATTACCGCTGGTG-3′) and LipL32-286R (5′-GAACTCCCATTTCAGCGATT-3′) designed by [25], to amplify the LipL32 gene, which is specific for pathogenic leptospires. To perform the PCR, a mix of the following reagents was calculated for each sample to be tested: DNA template extracted (5µL), DEPC water (32,8µL), Taq DNA polymerase buffer (5µL), dNTP (1,0µL), solution of $MgCl_2$ (3µL), Primer *LipL32*-45F (1,5µL), Primer *LipL32*-286R (1,5µL), Taq DNA Polymerase (0,2µL), totaling 50µL in microtubes [26]. The serogroup of *L. interrogans* Pomona sorovar Kennewicki was used as a positive control and ultrapure water as a negative control.

Sample sequencing was performed with primers LipL32-45F e LipL32-286R described by [25] and with the sequencing kit Big Dye Terminator v3.1 (Applied Biosystems, Foster City, CA, EUA). For capillary electrophoresis, a 3130 gene analyzer was used and the polymer POP-7 [27].

Sequence alignment was performed with the software Seaview4. The dataset strings were obtained from the GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) (http.//www.ncbi.nlm.nih.gov) using the BLAST tool http://www.ncbi.nlm.nih.gov/BLAST/. Phylogenetic analysis was generated using the Seaview4 software built with Neighbor-Joining method and Jukes-Cantor model, bootstrap with 1,000 repetitions [28]. The phylogenetic tree was visualized using FigTree v1.4.3. The phylogenetic reconstruction was performed using the *Leptospira* sp. Genbank for comparison.

RESULTS

The antibody anti-*Leptospira* sp. was detected in six (50%) of the 12 animals examined by MAT, three of these reactions (50%) were for the serogroup Tarassovi, two (33.3%) for Pyrogenes and one (16.7%) for Grippothiphosa (Table 1).

Table 1 Titers of anti-*Leptospira* sp. antibodies and serogroups found in *Tupinambis merianae* from the Semi-arid of Paraíba, Brazil, in 2019

Sorovares	**Titers**				**Total (%)**
	50	100	200	400	
Tarassovi	-	1	2	-	3 (50%)
Pyrogenes	1	-	-	1	2 (33,3%)
Grippothiphosa	-	-	1	-	1 (16,7%)
Total (%)	1(16,7%)	1(16,7%)	3(50%)	1 (16,7%)	6 (100%)

It was not possible to isolate any of the spread samples. In the molecular analysis, the DNA of *Leptospira* sp. was found in nine (75%) of the 12 animals analyzed, of which, only two were positive in MAT, and the only three that were negative in the molecular diagnosis were positive in serology (Table 2).

Table 2 Positive reactions in MAT and PCR techniques in *Tupinambis merianae* from the Semi-arid of Paraíba, Brazil, in 2019

MAT	**PCR**		**Total (%)**
	Positive	Negative	
Positive	3	3	6 (50%)
Negative	6	0	6 (50%)
Total (%)	9 (75%)	3 (25%)	12 (100%)

From the organs analyzed by PCR, leptospiral DNA was detected in six (50%) samples of the bladder, three (25%) of the liver, two (16.7%) of the reproductive tract, one (8.3%) of the kidney and one (12.5%) of urine, in relation to the total samples of each tissue (Table 3). Noting that from the animals tested positive in the bladder, only one was also positive in the urine sample. The PCR results for each material, as well as the tests performed per animal, can be seen in Table 4.

Table 3 Frequency of positive samples by PCR according to the organ evaluated in *Tupinambis merianae* from the Semi-arid of Paraíba, Brazil, in 2019

Organ used for PCR	Total number of samples	Number of positive reactions	Frequency (%)	Sequencing
Liver	12	3	25 %	1
Kidney	12	1	8,3 %	-
Bladder	12	6	50 %	1
Reproductive Tract	12	2	16,7 %	-
Urine	8	1	12,5%	-

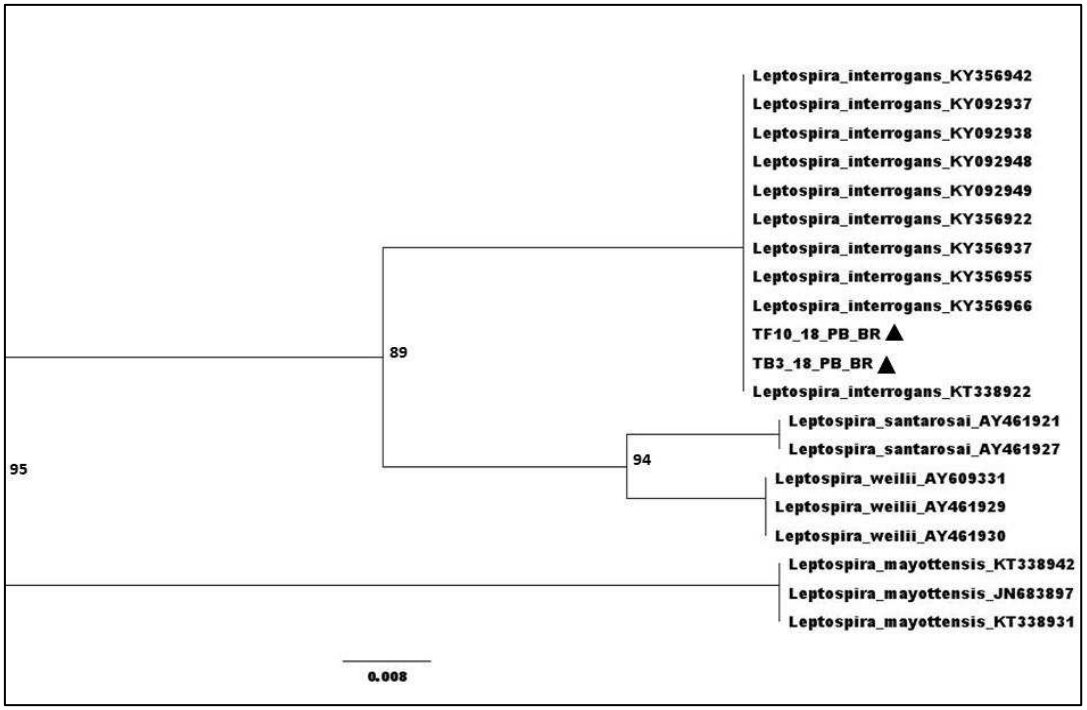
Table 4 Result of the serological and molecular analyzes in *Tupinambis merianae* from the Semi-arid of Paraíba, Brazil, in 2019

ANIMAL	SEX	MAT	PCR				
			Liver	Kidney	Bladder	Reprod. Tract	Urine
T. merianae 01	M	+	-	+	-	-	NC
T. merianae 02	M	+	-	-	+	-	NC
T. merianae 03	F	-	+	-	+*	-	-
T. merianae 04	M	+	-	-	-	-	-
T. merianae 05	F	-	-	-	+	-	-
T. merianae 06	M	-	-	-	-	+	-
T. merianae 07	M	-	-	-	+	-	-
T. merianae 08	M	-	-	-	+	+	-
T. merianae 09	F	-	+	-	-	-	-
T. merianae 10	F	-	+*	-	+	-	+
T. merianae 11	F	+	-	-	-	-	NC
T. merianae 12	F	+	-	-	-	-	NC

(+) positive, (-) negative, (*) sequenced samples, (NC) Not collected.

A positive sample of liver and another of bladder were submitted to sequencing, where the fragment of 260 nucleotides in the phylogenetic analysis showed identity with sequences of *Leptospira interrogans* (Fig. 4).

Figure 4 Phylogenetic tree built by the Neighbor-Joining method and Jukes-Cantor model, bootstrap with 1000 repetitions. ▲ Sequenced samples



DISCUSSION

The study provides relevant information, since little has been investigated about leptospirosis in wild animals in the northeastern semiarid of Brazil. For [29] the laws that protect these animals pose obstacles to access and capture, which often makes it difficult to carry out detailed investigations. Despite studies pointing wild species as possible key subjects in the transmission of the disease, wild fauna of free life has been little investigated, being observed that a large part of the research is restricted to surveys in ecological parks, as the studies developed by [30], [31], [32], [13] e [22].

The distribution of leptospirosis depends on environmental conditions which allow the agent to survive [10], highlighting that it is transmitted indirectly through water and that leptospires are very sensitive to desiccation [33]. The semi-arid region has experienced periods of below-average rainfall in recent years, between 2015 and 2018 the region had rainfall averages below 600 mm [34], a fact that hampers inter-species transmission [35]. Even so, the studies carried out show that the disease is still present in various animal species in the region, this fact being observed in some studies such as the ones from [36], [29], [35] and [37], which the identified through of MAT 61.1% in cows, 5.2% in goats, 19.3% in sheep

and 78.8% in swine respectively. In this context, when there are adverse environmental factors, maintenance hosts become even more important, since they influence the epidemiology of the disease, guaranteeing the perpetuation of the pathogen. [38].

In a study at the Ribeirão Preto Zoo, captive reptiles were responsible for many of the reagent samples in serology [39]. [40] Identified 92.8% of positive reactions in free-living *Tupinambis merianae* in the state of São Paulo, but it should be noted that his study was carried out in a conservation center in which the various species in captivity were also reactive, in which case they may be the source for the free-living animals. In the current study, a frequency of (50%) by MAT was found, which is considered high, since it represents contact with the agent through natural environmental interactions in the diversity of the species present, including domestic animals.

According to [31] the most frequent serogroup in reptiles is Pomona, however in the studied species (*Tupinambis merianae*), no reaction was found for it. The Pyrogenes serogroup was the second most detected, and it has wild animals themselves as natural hosts. [41]. The most frequent serogroup was Tarassovi, which was detected, but in small proportions (4.85%) in other species of wild animals [39]. The literature indicates pigs as maintenance hosts for the Tarassovi serogroup [42], which persist with the bacteria colonizing their renal tubules and releasing them into the environment [10]. In the semiarid region, this serogroup was responsible for 50% of the reactions found in pigs in Paraíba [43] and 6.3% in Rio Grande do Norte [37]. [44] found the Tarassovi serogroup in 19.2% of goats tested positive in Minas Gerais and suggested this high frequency is due to the presence of pigs on the properties. Although this serogroup is directly linked to the swine species, we can consider other reservoirs for it, such as the *Tupinambis merianae* detected with high frequency in this study.

The isolation of the agent from urine or other tissues represents an important way of identifying carriers [45]. In the current study, no bacterial growth was obtained in any sample, this has also been observed in other studies [33,35]. Considering that the culture is hampered by the slow growth of some strains of *Leptospira* sp., due to contamination problems and the long incubation period that occurs before obtaining an isolate [12,46]. Due to all these limitations found in the culture, PCR starts to gain an even greater importance in the detection of leptospirosis reservoirs [47].

The detection of 9/12 (75%) of positive animals by means of PCR is something that should be highlighted, since there are still no records on the colonization of the agent in the species in the region, and this is fundamental in the characterization of new carriers for the

disease. The molecular technique identified carriers that were not detected in the MAT, as well as [48] that also detected a greater number of positives through PCR, confirming a greater sensitivity of the technique. According to [42] and [45] this method of direct diagnosis can find leptospiric DNA even though microorganisms are no longer viable, which has made this tool a valuable tool in epidemiological investigations. In contrast, three positive animals were found in the MAT which the PCR was unable to capture, this can be justified by the pathogenesis itself, which, in this case, may be a response with the production of anti-*Leptospira* sp. antibodies and the agent has not yet colonized the examined organs, or even the infection might not have occurred in these animals. This corroborates what was described by [49], who reported that the stage of the disease influences the choice of the test and the most suitable sample for diagnosis, which reinforces the recommendation of [50] on the combined use of MAT and PCR for a more efficient diagnosis of leptospirosis, given the low agreement found in several studies [35,51].

The high proportion of animals tested positive in PCR and negative in the MAT (50%) raises the possibility that these animals are in the stage of chronic carriers, since with the post-infection time there may be a reduction in antibodies to levels undetectable by the MAT [52]. The lack of data on the titration of antibodies and the maintenance of titers in wild animals prevents a more accurate discussion [13], as well as raising consideration on the cutoff point to be used in wild species**.** In addition, it should be considered that environmental conditions can give very specific characteristics to each region, what makes it necessary to rethink about the most appropriate cutoff point. The work carried out on domestic species in the Semi-Arid region has used the cut-off point of 1:100 [33,35,43,53], but in this context [54] observed a reduction in false negatives in cattle in Colombia, considering as positive from 1:50 dilution instead of 1:100. Some studies with wild animals used a cut-off point of 1:100 [14,22,40,55] and other studies considered animals with lower titers, such as 1:40, as positive [39]. In the present study, reactive animals were considered positive from 1:50 dilution, and it is worth mentioning that a positive animal was found that would not have been detected with a cut-off point of 1:100.

The urinary tract was an important location of the bacterium, in most animals the leptospires were present only in the bladder, however with few reactions in the molecular examination of the kidney and urine. The detection of pathogens in the urine of reservoir hosts may not be possible in all cases due to the intermittent elimination of the pathogen, and the concentration of the bacteria may be at low levels at the time of collection [52], considering that the threshold detection is generally of 10^3 leptospires/ml of blood or urine

[56]. The non-detection in the renal tissue cannot always exclude the presence of the agent in that location, considering the size of the fragment that is collected [43]. Studies in other species have also observed few reactions from the renal tissue in relation to other sites in the genitourinary tract [35,43], this reinforces that the agent may also have tropism for other tissues [42]. The detection of leptospiral DNA in the reproductive tract of these animals indicates these organs as an important alternative pathway in the maintenance of the agent.

The detection of *L. interrogans* through sequencing was also observed in other species in the region, such as [35] in sheep from the semi-arid region of Paraíba. In addition to sequencing from isolates in *Cavia aperea*, *Euphractus sexcinctus* and *Cerdocyon thous* in the semi-arid in Ceará by [21]. This shows that *L. interrogans* may be circulating in several animal species in the semiarid, a relevant fact, since *L. interrogans* has genetic characteristics that allow greater resistance outside the host, and the ability to form biofilms favoring transport by reservoir animals, in addition to to be the most frequent and serious species in infections in humans [12,57].

CONCLUSION

The results of this study demonstrate a high rate of *Tupinambis merianae* infected by *Leptospira* sp., indicating the participation of these animals as reservoirs in the disease transmission cycle in the Semiarid Region. With the possibility of accidental transmission to human beings, but mainly to domestic animals, considering the indirect contact and the environmental sharing between these species, since the lack of rain leads to the formation of few natural water reservoirs, that would enable the contact of these animals in these available aquatic environments.

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CAPÍTULO II:

Didelphis albiventris carreando *Leptospira* sp. em tecido nervoso no semiárido do Nordeste do Brasil

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Didelphis albiventris as a carrier of *Leptospira* sp. in nervous tissue in the semiarid region of Northeast, Brazil

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ABSTRACT

Leptospirosis is a zoonotic and emerging bacterial disease, which has been investigated in several species of wild animals. The white-eared opossum (*Didelphis albiventris*) is a common wild species in the Northeast region of Brazil, so, given the need to understand the role of this species in the disease cycle, this study aimed to investigate the occurrence of *Leptospira* sp. in *Didelphis albiventris* in the semiarid region of Northeast Brazil. 12 animals were used, from which samples of liver, kidney, bladder and reproductive tract were collected for the attempt of isolation, these same tissues along with nervous tissue and urine were used for molecular detection, while blood serum was used for serological examination. The serological test performed was the microscopic serum agglutination test (MAT) and the molecular one was the Polymerase Chain Reaction (PCR), positive samples in the PCR could be amplified and subjected to genetic sequencing. There was no microbial growth in any of the tissues, nor were any anti-*Leptospira* sp. antibodies found in any of the serological samples, while the PCR detected leptospiric DNA in the central nervous system (CNS) of five animals (41.7 %). The gene in one of the samples of the nervous tissue was sequenced and showed identity with *Leptospira interrogans*. The presence of *Leptospira* sp. in the CNS of *Didelphis albiventris* does not allow the characterization of the studied animals as reservoirs with potential for transmission of the pathogen in the region, however it represents a site that needs to be further investigated.

KEY WORDS: leptospirosis, opossum, reservoirs, nervous tissue.

INTRODUCTION

Leptospirosis is an emerging and worldwide bacterial disease, caused by several pathogenic serovars of *Leptospira* sp. (Goarant 2016; Gomes-Soleck et al. 2017). Domestic and wild animals, as well as humans, are affected by this infection. (Vieira et al. 2018), what might be considered an excellent example for the *One Health* approach, in which the relation between humans, animals and ecosystems must be analyzed (Schneider et al. 2015). The disease has great zoonotic potential, and it is estimated that more than one million cases of human leptospirosis occur annually in the world, causing more than 58 thousand deaths (Costa et al. 2015).

Leptospirosis can be acquired through direct contact with secretions from an infected animal, especially urine, or indirectly through contaminated water (Fornazari et al. 2018). These spirochetes invade the host through the mucous membranes or injured skin, reaching the circulatory system and they are distributed throughout the organism, after this phase they start to lodge in tissues such as the renal tubules, which allows the release of the agent for long periods in the environment. (Adler 2014; Ellis 2015). Animals that recover from the disease can become asymptomatic carriers, excreting the bacteria in their urine, thus contributing to the infection of other individuals (Adler and Moctezuma 2010). These maintenance hosts guarantee the permanence of the microorganism, directly influencing the epidemiology of the disease (Levett 2001).

The control of leptospirosis should be focused on interrupting direct or indirect transmission (Ellis 2015), regardless of the strategy used, the identification of reservoir animals is crucial (Haake; Levett, 2015), considering that leptospires can be carried by a wide variety of animals (Adler and Moctezuma 2010). Understanding the ecology of these reservoirs and their interactions with the etiological agent is also of fundamental importance (Cosson et al. 2014).

Many wild species have been identified as reservoirs of *Leptospira* sp. in recent studies such as *Streptipeli capicola*, *Mungos mungo*, *Phacochoerus africanus*, *Chlorocebus pygerythrus*, *Lepus microtis*, *Tenrec ecaudatus*, *Akodon* spp., *Oligoryzomys* spp. (Jobbins et al., 2015; Lagadec et al. 2016; Vieira et al. 2019a). Considering the high biological diversity found in Brazil and the need to understand the disease cycle in regions such as the Semi-Arid, there is a need for further investigations in other species.

The white-eared opossum (*Didelphis albiventris*) is a species highly adaptable to environmental variations, being present in the great Brazilian biomes, including the Caatinga (Jorge et al. 2012a; Paglia et al. 2012). Its eating habits (frugivorous-omnivorous) (Eisenberg;

Redford, 2000) can facilitate the approximation of these animals with other species and even with humans (Jorge et al. 2012a). Given the scarcity of studies and the possibility of this wild species participating in the cycle of leptospirosis in the Semiarid region, this study aimed to investigate the occurrence of *Leptospira* sp. in *Didelphis albiventris* in the semiarid region of northeastern Brazil.

MATERIALS AND METHODS

The project was approved by the Ethics and Research Committee (CEP) of the Rural Health and Technology Center (CSTR) of the Federal University of Campina Grande (UFCG) under protocol number CEP 023.2017 and by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) under Protocol No. 57190-1.

Animals studied

The species studied was the marsupial *Didelphis albiventris*. Twelve animals were captured by means of non-lethal traps (buckets buried with baits and a styrofoam platforms to prevent drowning), between August 2017 and May 2019 (Figure 1-A). After their capture, the animals were taken in transport boxes (height: 38 cm; width: 43 cm; length: 59 cm) (Figure 1-B) for the Vaccines and Diagnosis Laboratory (LAVADI) of the Health and Technology Center Of the Federal University of Campina Grande, in Patos- PB.

Figure 1 Capture of *Didelphis albiventris* using plastic bucket traps (A) and transport in boxes (B)

Euthanasia and material collection

Upon arrival at the laboratory, the animals were identified and weighed. Euthanasia was performed in accordance with Annex IV of the Ordinance of the Federal Council of Biology No. 148/2002 (Annex I), with tranquilization performed by the use of 2% xylazine (Ceva, Anesadan, Paulínia-SP, Brazil) and 10% ketamine (Ceva, Dopalen, Paulínia-SP, Brazil) in doses of 5mg/kg and 100mg/kg, respectively, applied intramuscularly in the posterior thigh. For euthanasia, an overdose was administered with 2 mL of the same associated anesthetics, in the subarachnoid space directly in the foramen magnum. After the complete interruption of the physiological signs, exsanguination by cardiac puncture was performed, as a method of confirmation.

The blood collected by the cardiac puncture (3 mL) was subjected to centrifugation and the serum was placed in microtubes and then stored at -20° C until the serological test was performed. Then, the abdominal and thoracic cavities were opened, from where fragments of the liver, kidney, bladder, reproductive tract were collected (females: oviduct along with the uterus; males: vas deferens along with the epididymis) and the central nervous system, using sterile surgical scissors, anatomical forceps and scalpel blade n°. 15. About 3 mL of urine was collected by cystocentesis, divided into two samples, one used for inoculation in culture dishes and the other stored equally to the serum for molecular diagnosis.

Serological diagnosis

In the serological examination, the presence of anti-*Leptospira* sp. antibodies was researched through the microscopic agglutination test (MAT), according to the World Organization for Animal Health (OIE, 2014). To perform the technique, a battery of live antigens containing 22 pathogenic serovars and 2 saprophytes was used: Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Javanica, Panamá, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Tarassovi, Andamana, Patoc and Sentot. Each culture was examined for purity and absence of autoagglutination under dark field microscopy. Each serological sample was tested against an antigen battery with 24 serovars, being considered positive when they presented agglutination (≥ 50%) in the initial dilution of 1:100, and in a second stage, the positive samples were titrated in the dilutions of (1:200, 1:400, 1:800,

1:1600, 1:3200), being the highest obtained titer used as determinant for the infectious serogroup. The readings were performed in an optical microscope (ZEISS®, Oberkochen, Germany) with a dry dark field condenser, with a 10x/0.20 objective lens and an ocular 10 (100X) lens, observing the formation of agglutinations.

Polymerase Chain Reaction Diagnosis (PCR)

DNA extraction from tissue samples (kidney, liver, bladder, reproductive tract, urine and nervous system) was performed using the Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations. The polymerase chain reaction (PCR) was performed with the primers LipL32-45F (5′-AAGCATTACCGCTGGTG-3′) and LipL32-286R (5′-GAACTCCCATTTCAGCGATT-3′) designed by Stoddard et al. (2009), which aims to amplify the gene LipL32 (specific for pathogenic leptospires). To perform the PCR, a mix of the following reagents was calculated for each sample to be tested: DNA template extracted (5µL), DEPC water (32,8µL), Taq DNA polymerase buffer (5µL), dNTP (1,0µL), solution of $MgCl_2$ (3µL), Primer LipL32-45F (1,5µL), Primer LipL32-286R (1,5µL), Taq DNA Polymerase (0,2µL), totaling 50µL in microtubes (Hamond et al. 2014). The serogroup of *L. interrogans* Pomona serovar Kennewicki was used as a positive control and ultrapure water as a negative control.

All reactions occurred in the thermal cycler Gene Amp PCR System 9700® (Applied Biosystems, Foster City, USA). After an initial denaturation at 95° C for 5 min, the PCR profile was defined as follows: 30 seconds of denaturation at 94° C, 30 seconds of primer ringing at 53° C and 1 min of primer extension at 72° C, with a total of 35 cycles, with a final extension at 72° C for 5 min. The total volume of each sample was analyzed by agarose gel electrophoresis (2%), stained with Blue Green Loading Dye I and the DNA bands were visualized under ultraviolet light. The expected size of the amplicon was around 242 bp (base pairs), varying slightly between the different species of *Leptospira* sp. (Hamond et al. 2014).

Phylogenetic sequencing and analysis

Sample sequencing was performed with primers LipL32-45F and LipL32-286R described by Stoddard et al. (2009) and with the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA). For capillary electrophoresis, a 3130 gene analyzer and the POP-7 polymer were used (Platt et al. 2007).

Sequence alignment was performed with the Seaview4 software. The dataset strings were obtained from GenBank (*National Center for Biotechnology Information, Bethesda, MD, USA*) (http.//www.ncbi.nlm.nih.gov) using the BLAST tool http://www.ncbi.nlm.nih.gov/BLAST/. Phylogenetic analysis was generated using the Seaview4 software built with Neighbor-Joining method and Jukes-Cantor model, bootstrap with 1,000 repetitions (Gouy et al. 2010). The phylogenetic tree was visualized using FigTree v1.4.3. Phylogenetic reconstruction was performed using sequences *Leptospira* sp. from Genbank for comparison.

Bacterial culture

The culture media used were produced in the CSTR/UFCG Laboratory of Communicable Diseases (LDT), with Ellinghausen-McCullough-Johnson-Harris (EMJH, Difco®-USA) supplemented with 10% rabbit serum and enriched with 1% sodium pyruvate, 1% calcium chloride, 1% magnesium chloride and 3% L-asparagine. It was prepared in two formulations, one with the addition of 5-fluorouracil (400 mg/L; Sigma®-USA) (EMJH) and another (EMJH Enriquecido) who received a cocktail containing the following antibiotics: in quantities for 500 ml of Solution: 200 mg of Sulfametaxazole, 100 mg of Trimetropim, 25 mg of Amphotericin B, 2000 mg of Fospomycin and 500 mg of 5-Fluoracil, according to the recommendations by Chakraborty et al. (2011). The Fletcher (DIFCO-USA) was prepared with the same antibiotic formulations as EMJH. A fragment with about 1g of internal organs (liver, kidney, bladder and reproductive tract) and 1 ml of urine were spread. Right after the collection, the organs were macerated with sterile syringes and spread directly in the EMJH Enriched medium (after 24 hours 1 mL of this medium was spread again in EMJH and FLETCHER), all the spread media were incubated in the greenhouse at 28° C and this was repeated for a new medium every 15 days. To check for the presence of microorganisms, readings were performed weekly for at least twelve weeks, using an optical microscope (ZEISS®, Oberkochen, Germany) with a dry dark field condenser, and a 10x / 0.20 objective lens and an ocular 10 (100X) lens.

RESULTS

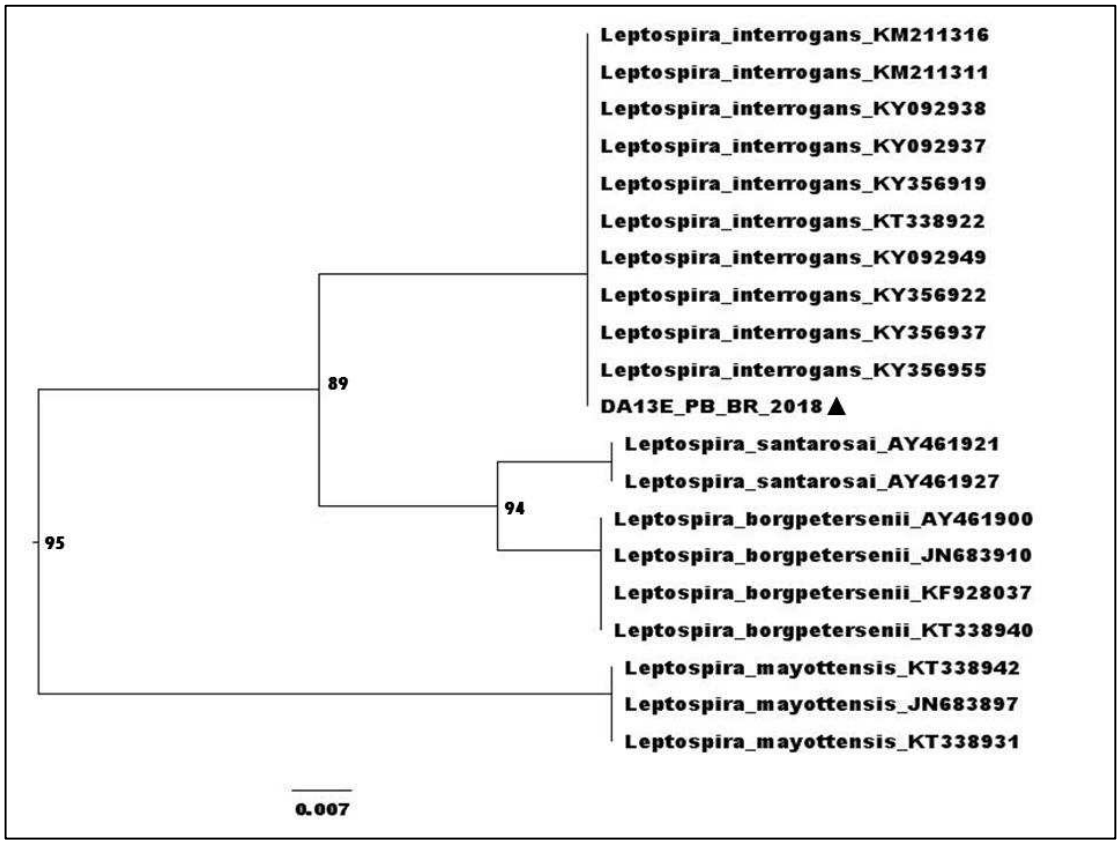
The serological test did not detect the presence of anti-*Leptospira* sp. antibodies in any of the animals, as well as it was not possible to observe microbial growth in any of the samples. However, based on molecular diagnosis, bacterial DNA was detected in the tissue of the central nervous system of five animals (41.7%) (Table 1), it should be noted that these animals were part of the same brooding and that the mother and all puppies were positive.

Table 1 Results of serological, molecular and bacterial examination of tissue samples of *Didelphis Albiventris* from the semiarid region of Northeast Brazil, in 2019

ANIMALS	EXAMINATIONS PERFORMED		
	MAT	PCR	Isolation
D. A. 1	-	-	-
D.A. 2	-	-	-
D.A. 3	-	-	-
D.A. 4	-	-	-
D.A. 5	-	-	-
D.A. 6	-	-	-
D.A. 7	-	-	-
D.A. 8	-	+	-
D.A. 9	-	+	-
D.A. 10	-	+	-
D.A. 11	-	+	-
D.A. 12	-	+	-
TOTAL (%)	0 (0%)	5(41,7%)	0(0%)

One of the positive PCR samples was subjected to sequencing, and the 260 nucleotide fragment in the phylogenetic analysis showed identity with *Leptospira interrogans* sequences (Figure 2).

Figure 2 The phylogenetic tree built by the Neighbor-Joining method and Jukes-Cantor model, bootstrap with 1000 repetitions. ▲ Sequenced sample



DISCUSSION

Serological studies on these animals in different ecosystems are essential for understanding the presence of predominant *Leptospira* sp. serogroups (Jorge et al. 2012a), this information being of great epidemiological importance (Cerqueira; Picardeau, 2009; Ko et al. 2009). Some works developed in Brazil have detected seroreactivity in this species, as Silva CS et al. (2010) in the state of São Paulo who found two seropositive free-living animals at the Ribeirão Preto zoo; while Silva FJ et al. (2010) detected 44% of seroreagent opossums, most of them for serovars Patoc and Autumnalis, based on the analysis of the serum of 25 opossums present at the UNESP campus in Jaboticabal; and Horta et al. (2016) identified 3.5% of seropositive animals from 345 possums of the species *Didelphis albiventris* and *Didelphis aurita* in São Paulo. Vieira et al. (2019b) carried out a study with animals captured in conservation units of the Atlantic Forest biome in Rio de Janeiro, where they verified the presence of a seroreactive opossum. However, it should be considered that these

studies were developed in environments other than the Caatinga biome present in the Semiarid.

Investigations into these wild species are still limited, both by the difficulties in capturing the animals and also by the laws that protect them (COSTA et al. 2016), which are of paramount importance to protect the country's biodiversity, but end up bringing some obstacles about authorization of studies on these animals. What, in a way, limits the knowledge about leptospirosis in wild animals in the various Brazilian biomes.

In the present study, all animals were negative by the MAT. However, serology and carrier status may not be correlated, although serological testing is useful in the diagnosis of leptospirosis in herds, it is not suitable for the individual identification of reservoirs, and it is also necessary to use other methods that directly detect the organism or its DNA (Gamage et al. 2011; Fornazari et al. 2012; Otaka et al. 2012). For Vieira et al. (2018) the inclusion of these methods of direct diagnosis is necessary, because through serology alone it is not possible to have an understanding of strains that are circulating among animals in a region.

The molecular detection of the agent in the tissues of *Didelphis albiventris* is little reported, Vieira et al. (2019b) found bacterial DNA in renal samples from two animals in Rio de Janeiro, however in the present study it was possible to detect five animals with leptospiral genetic material, although all of them had the DNA of the bacteria only in the central nervous system (CNS). This non-relationship between the results of the serological and molecular diagnosis in these animals corroborates with several studies in which animals negative in the MAT were positive by PCR (Director et al. 2014; Hamond et al. 2014; Costa et al. 2017; Latosinski et al. 2018), emphasizing the importance of associating the two techniques for a safer diagnosis, as suggested by Otaka et al. (2013).

These results reinforce the importance of using molecular testing to detect positive animals, considering the sensitivity and specificity of the technique, as well as the ability to identify *Leptospira* sp. in various types of samples (Subharat et al. 2011; Hamond et al. 2014; Latosinski et al. 2018). The feasibility of a fast and safe direct diagnosis should intensify the use of this tool as an important part of disease control programs in field conditions (Director et al. 2014).

Something that should be highlighted in the molecular diagnosis is the fact that bacterial DNA was detected only in the tissue of the central nervous system of these animals. Although this type of colonization is not well understood, there are several reports of acute leptospirosis with neurological involvement in humans. (Lelis et al. 2009; Romero et al. 2010; Singh et al. 2016; Muthaiah et al. 2016; Moestrup et al. 2019). The events involved in the

nerve form and the mechanisms by which spirochetes interact with cellular defenses are unknown, but it is known that the bacterium crosses the brain barrier migrating from the bloodstream to the cerebrospinal fluid (CSF) (Mahesh et al. 2015). The simple colonization of the agent in the central nervous system does not allow configuring a manifestation of neurological pathology in the species studied, however it suggests a possible extrarenal colonization that needs to be investigated.

It was not possible to establish whether the animals developed the infection, or whether they were in an acute or chronic phase. According to Latosinski et al. (2018) with the evolution of the infection there is a gradual reduction of antibodies to very low and undetectable levels. This can be well evidenced with serogroups adapted to the host (Sakoda et al. 2012). In this context, it could be thought that specimens of *Didelphis albiventris* tested positive in PCR were in a chronic stage, due to the non-detection of antibodies in the MAT. But the presence of leptospires in the central nervous system also suggests a more acute picture, since the neurological problems caused by leptospirosis in humans are associated with meningitis in a phase of leptospiremia (Romero et al. 2010).

In the pathogenesis of leptospirosis, as the infection progresses, circulating antibodies appear that remove the leptospires from the circulatory system (Adler and Moctezuma 2010), and they may find refuge in places with a minimal concentration of antibodies, such as the anterior chamber of the eyeball, reproductive system and renal tubule light (Faine et al. 1999). The migration of antibodies to the brain is limited by the blood-brain barrier (Muldoon et al. 2012), which could explain the presence of leptospires in the CNS as a way of refuge from the humoral response, however the literature does not show nervous tissue as an agent hosting site.

The crucial point of this study is the fact that the positive animals belong to an offspring, where the mother and all the pups were detected with leptospiric DNA in the CNS. This information raises the possibility of vertical transmission, considering that this type of transmission has been mentioned in domestic animals (Pescador et al. 2004; Otaka et al. 2013) and even in humans (Shaked et al. 1993). The analyzed *Didelphis albiventris* were apparently healthy, while studies that indicated infection of the fetus via uterine in domestic animals, also recorded fetal death and abortion (Otaka et al. 2013; Mori et al. 2017), however Soto et al. (2006) detected leptospires in clinically healthy piglets born from experimentally infected sows. The absence of anti-Leptospira sp. agglutinins could also suggest an immunological immaturity, and thus raise a reflection on the need to use a lower dilution in the MAT. However, it is worth mentioning that these low cut-off points are used by studies

developed with fetuses at various stages of development, such as Magajevski et al. (2007), very different from the current study.

In addition to the herd diagnosis, the identification of animals that are spreading the bacteria in the environment is important for disease control. PCR is able to directly identify this release status by the carrier (Subharat et al. 2011; Hamond et al. 2014), detecting bacterial DNA in the kidney (Costa et al. 2017; Vieira et al. 2019a), in the urine (Director et al. 2014), and in vaginal fluid (Lilenbaum et al. 2008) of several animals. In the present study, there was no positive sample for liver, reproductive tract, kidney, bladder or urine, which does not signal bacterial release by the animals analyzed. However, there is still the possibility that leptospires are colonizing these tissues, but in concentrations below the detection threshold (Picardeau 2013; Latosinski et al. 2018), since the ideal limit of perception for PCR is of at least 10^3 bacteria per milliliter of sample (Subharat et al. 2011; Limmathurotsakul et al. 2012).

The etiology of leptospirosis is constituted by a diversity of species (Jorge et al. 2012a). According to Lilenbaum et al. (2008) PCR still presents an obstacle for not being able to define the species of the infective leptospira, as it is a method specific to the genus, but this can be achieved through the sequencing of the PCR product (Subharat et al. 2011). In the present study, a DNA fragment was sequenced that showed identity with the species *L. interrogans.*, however, this finding does not signal the animals in this study as reservoirs with potential for transmission, because the agent was found only in the CNS. On the other hand, Jorge et al. (2012a) sequenced *L. borgpetersenii* from an isolate obtained from the urine of a *Didelphis albiventris*, indicating the participation of this animal as a reservoir. The species *L. interrogans* was also sequenced in southern Brazil from the *Cavia aperea* (Monte et al. 2013) and *Hydrochoerus hydrochaeris* (Jorge et al. 2012b), and in the semi-arid region of Paraíba in sheep samples (Silva et al 2019). These works demonstrate that *L. interrogans*. can be carried by domestic and wild animals, which promotes the circulation of the agent and the infection of other hosts.

The isolation of the agent represents an important advance, since it allows the exact knowledge about the species that circulate in a region (Freitas et al. 2004), in addition the inclusion of an autochthonous strain in the MAT increases the sensitivity of this exam. In this context, Jorge et al. (2012a) obtained an isolate from *Didelphis albiventris* and included it in the MAT, verifying an increase in the sensitivity of the technique when testing samples from dogs and from the opossums previously examined. The culture is complex due to slow growth and contamination (Adler; Moctezuma, 2010; Subharat et al. 2011) that can occur anytime in the process. In the current work, there was no microbial growth in any of the samples spread,

in this case justified even by the absence of leptospires in the tissues used, since they were not detected by the molecular test either.

CONCLUSION

It was possible to detect the presence of *Leptospira* sp. in the central nervous system of *Didelphis albiventris*. However, it is not yet possible to characterize the species as a reservoir agent in the region. Despite the fact that the CNS does not represent a location capable of releasing the pathogen into the environment, this situation needs to be further investigated, as there may be another site for the extrarenal location of *Leptospira* sp.. In addition, the study reinforces the need for the use of molecular techniques in epidemiological studies of leptospirosis.

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CONCLUSÃO GERAL

Os resultados encontrados mostram que *Tupinambis merianae* participam do ciclo epidemiológico da leptospirose no semiárido, representando uma importante fonte de infecção para as espécies domésticas e também para os seres humanos. E a presença de *Leptospira* sp. no SNC de *Didelphis albiventris* não permite ainda caracterizar esta essa espécie como reservatório com potencial de transmissão do agente na região, no entanto representa um sítio de localização que precisa ser melhor investigado. Isso evidencia a dificuldade no controle da doença em regiões como o Nordeste brasileiro, onde existe uma diversidade de espécies silvestres, que através do contato indireto acabam disseminando o agente para outros animais. Além disso, se vê a necessidade de mais investigações em outras espécies silvestres, assim como pesquisar a bactéria em outros sítios de localização, inclusive nos animais domésticos. Deve-se evidenciar ainda a dificuldade em diagnosticar a doença e identificar hospedeiros que estão eliminando o patógeno, enfatizando a importância de se utilizar várias técnicas de diagnóstico nos estudos epidemiológicos com a leptospirose.

ANEXO I

PORTARIA CFBio Nº 148/2012

"Regulamenta os procedimentos de captura, contenção, marcação e coleta de animais vertebrados previstos nos Artigos, 4º, 5º, 6º e 8º da Resolução CFBio nº 301/2012".

O CONSELHO FEDERAL DE BIOLOGIA - CFBio, Autarquia Federal, com personalidade jurídica de direito público, criado p ela Lei nº 6.684, de 03 de setembro de 1979, alterada pela Lei nº 7.017, de 30 de agosto d e 1982 e regulamentada pelo Decreto nº 88.438, de 28 de junho de 1983, no uso de suas a tribuições legais e regimentais, e

considerando a Resolução CFBio nº 301/2012 que dispõe sobre os procedimentos de captura, contenção, marcação, soltura e coleta de animais vertebrados *in situ* e *ex situ*, e dá outras providências;
considerando o Parecer do GT-Fauna constituído pela Portaria CFBio nº 140/2012; e considerando o deliberado e aprovado na 266ª Sessão Plenária Ordinária, realizada em 8 de dezembro de 2012;

RESOLVE:

Art. 1º A captura pode ser realizada de forma manual, com equipamentos ou por armadilhas, seguindo as particularidades das espéci es ou comunidades alvo do estudo devendo ser posicionadas em locais e horários de acordo com a biologia da espécie ou comunidade, e sua revisão deve ser efetuada no menor tempo possível, considerando a temperatura e insolação local, buscando reduzir o e stresse e sofrimento do animal, devendo-se observar os tempos para revisão das arma dilhas de acordo com o anexo I.
Art. 2º A contenção física e química deve ser indicada primariamente para as atividades de captura e marcação, assim como ferramenta no processo para coleta de espécime animal ou material biológico com base em literatura específica sobre a dosagem de anestésicos segundo a espécie do animal envolvido d e acordo com o anexo II.
Art. 3º O uso de marcação é permitido nos estudos, pesquisas e serviços nas áreas de inventário, resgate, soltura, manejo, criação, vigilância zoonótica e conservação da fauna silvestre nativa e exótica, desde que cause dor ou aflição apenas momentânea ou dano

passageiro e considerados os princípios de biossegurança e de assepsia de acordo com o anexo III.

Art. 4º A coleta de espécime animal ou de material biológico acompanhada de morte, quando for imprescindível ao alcance dos objetivos dos estudos, pesquisas, atividades de ensino e serviços em geral, deve ser realizada com minimização do sofrimento por meio de métodos que produzam inconsciência rápida e subsequente morte sem evidência de dor ou agonia, ou utilizando drogas anestésicas em doses suficientes para produzir a perda indolor da consciência, seguida de parada cárdio-respiratória de acordo com o anexo IV.

Art. 5º O exercício das atividades previstas nesta portaria devem seguir os protocolos e técnicas consagradas pela literatura (anexo V) para as espécies de cada grupo, constantes nos anexos de I a IV.

Art. 6º Esta Portaria poderá ser atualizada sempre que inovações tecnológicas e metodológicas possibilitem eliminar ou reduzir o sofrimento dos animais da fauna silvestre nativa e exótica.

Art. 7º Esta Portaria entra em vigor na data da pub licação no Diário Oficial da União, da Resolução CFBio nº 301/2012. Brasília/DF, 8 de dezembro de 2012.

Anexo IV

Grupo	Métodos de coleta aceitos	Métodos de uso restrito
Peixes	Pesca elétrica; resfriamento, inalação ou imersão na forma gasosa ou química: (Tricaino-metano-sulfonato, MS-222, TMS), Benzocaína (etil-p-aminobenzoal), 2-Fenoxietanol, Sulfato de Quinaldina, Dióxido de Carbono (CO2), Óleo de Cravo e injeção de Barbitúricos (Pentobarbital Sódico) e outros permitidos.	Arpão, restrito para grupos onde não há outro método o viável de coleta. Para o congelamento rápido é necessária anestesia p rofunda. Decapitação.
Anfíbios	Anestésicos inaláveis: Dióxido de Carbono (CO2), Monóxido de Carbono (CO), Halotano, Isofluorano. Anestésicos injetáveis: Lidocaina, Barbitúricos (Pentobarbital, Tiopentato de Sódio), Tricaino-metano-sulfonato (MS-222); Imersão em MS-222, Hidrocloreto de Benzocaína, Benzocaína e outros permitidos.	Secção de medula, exclusivamente para procedimentos de laboratório e condicionado a prévia anestesia antes do procedimento. Resfriamento. Para o congelamento rápido é necessária anestesia profunda. Imersão em álcool até 20%.
Répteis	Arma de fogo e pressão, estilingue, resfriamento. Anestésicos inaláveis: Dióxido de Carbono (CO2), Monóxido de Carbono (CO), Halotano, Isofluorano. Anestésicos injetáveis: Lidocaina, Barbitúricos (Pentobarbital, Tiopentato de	Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no.5.197/1967 e aquelas pertinentes ao porte de armas de fogo). Arma de pressão (utilizar o

	Sódio), Propofol e outros permitidos.	grão compatível com a espécie a ser coletada). Para o congelamento rápido é necessária anestesia profunda.
Aves	Anestésicos inaláveis: Dióxido de Carbono (CO2), Monóxido de Carbono (CO),Halotano, Sevoflurano, Isofluorano; Anestésicos injetáveis: Barbitúricos (Pentobarbital, Tiopentato de Sódio), Butorfanol e outros permitidos.	Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no.5.197/1967 e aquelas pertinentes ao porte de armas de fogo). Arma de pressão (utilizar o grão compatível com a espécie a ser coletada) Deslocamento cervical (utilizar prévia anestesia se o coletor não tiver prévia experiência). Não aceitável para espécimes com mais de 3 kg.Compressão torácica, restrito para aves de pequeno e médio porte (não aceitável para aves mergulhadoras e de grande porte) e quando técnicas alternativas não sejam viáveis, não podendo ser usa do como técnica de anestesia.
Mamíferos Chirópteros	Arma de fogo e pressão. Anestésicos inaláveis: Halotano, Isoflorano, Óxido Nitroso, Dióxido de Carbono (CO^2), Monóxido de Carbono (CO); Anestésicos injetáveis: Barbitúricos (pentobarbital, Tiopentato de sódio), Propofol, Ketamina e outros permitidos.	Deslocamento cervical (apenas com prévia anestesia) .Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no. 5.197/1967 e aquelas pertinentes ao porte de armas de fogo). Arma de pressão (utilizar o grão compatível com a espécie a ser coletada).
Mamíferos de pequeno porte	Anestésicos inaláveis: Halotano, Isofluorano, Óxido Nitroso, Dióxido de Carbono (CO^2); Monóxido de Carbono CO); Anestésicos injetáveis: Barbitúricos (pentobarbital, Tiopentato de sódio), propofol, Ketamina e Benzodiasepinas e outros permitidos.	Deslocamento Cervical apenas para animais com peso inferior a 200g (roedores) Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no.5.197/1967 e aquelas pertinentes ao porte de armas de fogo). Arma de pressão (utilizar o grão compatível com a espécie a ser coletada).
Primatas não humanos	Arma de fogo e outros permitidos. Anestésicos inaláveis: Halotano, metofani, Isoflurano, Sevoflurano, N2, Ar, Dióxido de Carbono (C02), e Monóxido de Carbono (CO). Anestésicos injetáveis: Propofol Ketamina, Ketamina associada Zolazepam ou Xilasina, Tiletamina associa da ao Zolazepam, Etomidato, Benzodiasepinas, Barbitúricos (Pentobarbital-Sódico).	Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no. 5.197/1967 e aquelas pertinentes ao porte de armas de fogo).
Outros mamíferos de médio e grande porte	Anestésicos inaláveis: Halotano, metofani, Isoflurano, Sevoflurano, N2, Ar, Dióxido de Carbono (C02), Monóxido de Carbono (CO) e arma de fogo e outros permitidos. Anestésicos injetáveis: Propofol Ketamina,	Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no. 5.197/1967 e aquelas pertinentes ao porte de armas de

	Ketamina associada Zolazepam ou Xilasina, Tiletamina associa da ao Zolazepam, Etomidato, Benzodiasepinas, Barbitúricos (Pentobarbital-Sódico).	fogo).
Mamíferos aquáticos	Anestésicos injetáveis: Aplicação de barbitúricos, hidrocloreto etorfina (narcótico) e outros permitidos.	Arma de fogo (utilizar o calibre e o grão compatíveis com a espécie a ser coletada; observar as restrições da Lei no. 5.197/1967 e aquelas pertinentes ao porte de armas de fogo).