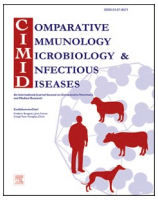
Comparative Immunology, Microbiology and Infectious Diseases 77 (2021) 101664

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid



Multidisciplinary approach in the diagnosis of acute leptospirosis in dogs naturally infected by *Leptospira interrogans* serogroup Icterohaemorrhagiae: A prospective study

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ARTICLE INFO

Keywords:
Azotemia
Canine
Leptospirosis
Nephritis

ABSTRACT

Leptospirosis, a zoonotic disease with worldwide distribution, is caused by spirochetes of the genus *Leptospira*. In dogs, this disease is frequently misdiagnosed. Few studies have attempted to associate the detection of *Leptospira* spp. infection with clinicopathological and renal histopathological findings using a multidisciplinary approach. The present study isolated and characterized *Leptospira* spp. obtained from naturally infected dogs and described relevant clinical and histopathological findings. Blood and urine were collected from 57 dogs with clinical symptomatology suggestive of leptospirosis; 38 cases were confirmed by PCR in urine or by culture or microscopic agglutination testing (titers ≥800). A total of 12 strains of pathogenic *Leptospira* were isolated from the studied dogs (seven in blood, four in urine and one in both blood and urine samples). All isolates were characterized as *Leptospira interrogans* serogroup Icterohaemorrhagiae. Of the confirmed cases, almost one-third of the animals had been vaccinated. Our analysis of laboratory testing revealed that azotemia and proteinuria were statistically significant predictors of infection. The main histopathological findings seen in kidney tissues were necrosis, degeneration, tubular regeneration, mononuclear inflammatory infiltrate and congestion. A multidisciplinary approach involving clinicopathological and histopathological characterization of renal involvement can aid in the identification of acute leptospirosis infection.

1. Introduction

Leptospirosis, a neglected tropical disease caused by spirochetes of the genus *Leptospira* [1], can greatly impact the health of both humans and animals. Dogs face significant exposure to leptospires, due to high contact with rodents and contaminated environments. The clinical diagnosis of canine leptospirosis is often hampered by non-specific signs of infection, and the severity of disease is directly linked to individual immune response [2]. The most frequent clinical signs of leptospirosis include fever, prostration, jaundice, abdominal pain, vomiting, polyuria/polydipsia and anuria/oliguria [3–5].

Infective serovars vary among canine populations in accordance with exposure to infected animals (wild or domestic), reservoir species and geographic region [6,7]. The determination of this information is

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https://doi.org/10.1016/j.cimid.2021.101664
Received 14 December 2020; Received in revised form 5 April 2021; Accepted 14 April 2021
Available online 7 May 2021

essential to designing prophylactic measures and enabling disease control [8]. One strategy for leptospirosis control is vaccination; however, the vaccines available for veterinary use comprise a limited panel of inactivated leptospires serovars (heat-killed or formalin-killed). Bacterins must not only be formulated with as few serovars as possible, but also employ local isolates [6,9]. The commercially available *Leptospira* vaccines in Brazil are generally based on foreign epidemiological profiles. Imported and locally produced vaccines are both primarily focused on the Canicola and Icterohaemorrhagiae serovars, while others target Pomona and Grippotyphosa [4,10,11].

Multidisciplinary approaches allow for a more robust characterization of infection, yet the isolation of *Leptospira* spp. in conjunction with the correlation of clinical and pathological findings is infrequently found in the literature [12]. The present study aimed to characterize isolates of *Leptospira* spp. in naturally infected dogs, as well as describe relevant clinicopathological and histopathological findings.

2. Material and methods

2.1. Animals

The present prospective hospital-based study involved a population consisting of 57 dogs from the city of Salvador and metropolitan area who were seen at the veterinary hospital of the Federal University of Bahia (HOSPMEV-UFBA). When the animals were first seen by a veterinarian, the presentation of fever, prostration or jaundice provoked a suspicion of leptospirosis. The dogs were then physically examined and their owners answered a questionnaire to obtain epidemiological information (age, sex, breed), vaccination status (vaccinated <12 months prior), and information regarding pertinent clinical signs and their duration. Clinical signs and biochemical analysis specifically associated with renal involvement included: polydipsia, polyuria, anuria, oliguria, physical and chemical analyses of urine, serum urea and creatinine.

2.2. Sample collection

Blood (5 mL) was collected by venipuncture of the cephalic vein in vacuum tubes (Vacutainer, BD Diagnostics; BD EDTA K2, BD Diagnostics) for serology, culturing, and the evaluation of hematological and biochemical parameters. Urine samples were collected aseptically by cystocentesis and immediately placed in culture medium. Additional aliquots were transported to a clinical pathology laboratory for urinalysis.

2.3. Serology

The detection of anti-*Leptospira* antibodies was performed by microscopic agglutination testing (MAT), as recommended by the World Organization for Animal Health [13]. The testing panel consisted of 24 serovars, representing 20 serogroups maintained in the collection of the Bacterial Disease Laboratory of the Federal University of Bahia (LABAC-UFBA) (Supplementary Table 1). The antigen presenting the highest titer was considered the presumptive infective serogroup. Samples were characterized as reactive when titers $\geq$ 100 [13].

2.4. Molecular diagnosis using lipL32 PCR in urine samples

DNA extraction was performed in urine samples using a commercial QIAamp DNA Mini Kit (Qiagen) in accordance with the manufacturer's recommendations. LipL32-PCR assays were conducted following a previously described method [14].

2.5. Bacteriological cultures

Following collection, three drops of urine or blood from each animal were immediately inoculated into sterile tubes containing 5 mL of liquid Ellinghausen-McCullough Johnson-Harris (EMJH) culture medium (Difco Laboratories) and 5 mL of Fletcher semisolid medium (Difco Laboratories). All tubes were incubated at 28 °C and examined weekly under dark-field microscopy for a four-month period to detect the presence of morphology and motility suggestive of leptospires [15,16].

2.6. Serological and molecular characterization of isolates

All isolates were serotyped by MAT using a panel of 19 polyclonal rabbit antisera of the following serogroups: Icterohaemorrhagiae, Canicola, Grippothyphosa, Pomona, Australis, Bataviae, Ballum, Cynopteri, Javanica, Panama, Pyrogenes, Sejroe, Tarassovi, Autumnalis and Hebdomadis. A panel of three monoclonal antibodies (mAb) against the Icterohaemorrhagiae serogroup were also employed: mAb F70C14 (serovar Icterohaemorrhagiae), mAb F70C24 (serovar Copenhageni) and mAb F8C12 (all serovars of Icterohaemorrhagiae serogroup except for serovar Copenhageni) [17].

DNA was extracted from the isolates using a QIAamp DNA minikit (Qiagen) in accordance with the manufacturer's instructions. Primers LA 5′-GGCGGCGCGTCTITAAACATG-3′ and LB 5′-TTCCCCCCATTGAGCAAGATT-3′ were used to amplify a region of the *rrs* gene (16S rRNA) [18], while primers secYF (5′-ATGCCGATCATTTTTGCTTC-3′) and secYR (5′-CCGTCCCTTAATTTTAGACTTCTTC-3′) were used to amplify a region of the *secY* gene [19]. Following amplification, the PCR products were purified and the amplicons were sequenced in both directions using a Big Dye Terminator 3.1 cycle sequencing kit (Applied Biosystems). All data were analyzed using the Staden Software Package v2.0b9. Genotyping of *Leptospira* isolates was performed by multi-locus variable-number tandem-repeat analysis (MLVA) targeting three discriminatory loci (VNTR4, VNTR7 and VNTR10), following a previously described protocol [20].

2.7. Clinical pathological analysis

2.7.1. Hematology and serum biochemistry

Complete blood counts (CBC) were performed on a pocH-100iV DIFF hematology analyzer (Sysmex Corporation), while serum concentrations of biochemical parameters creatinine (reference range: 0.5–1.5 mg/dL), urea (ref: 15−40 mg/dL), alanine aminotransferase (ALAT, ref: 21–102 U/L) and alkaline phosphatase (ALP, 20–156 U/L) were quantified using a BIOPLUS 200 semiautomatic biochemical analyzer (Bioplus Produtos para Laboratórios) All biochemical analyses were performed using commercial kits (Doles) in accordance with the manufacturer's recommendations.

2.7.2. Urinalysis

Urine samples were divided into two aliquots. Specific gravity (reference range: 1.015–1.040) was measured using a refractometer and chemical analyses were performed using dipstick tests (Combur10 Test, Roche Diagnostics). One aliquot of each sample was centrifuged at 4000 × g for 5 min; the supernatant was then removed and stored at −20 °C until the time of urine protein analysis. After centrifuging, pellets were used to identify and quantify the presence of cells, cylinders, crystals and bacteria. Other aliquots were used for microscopic examination (10 fields, 400x magnification).

2.8. Histopathology

Eight fragments of renal parenchyma (from dogs that died or were humanely euthanized) measuring 2.0 × 2.0 × 0.5 cm were obtained by necropsy and stored in flasks containing neutral 10 % buffered formalin for fixation. Subsequently, samples were processed following a routine paraffin inclusion technique. Sections measuring 2 μm were stained with hematoxylin-eosin (H&E) [21]. The presence of renal tissue injury was noted, and interstitial nephritis (IN) was quantified according to infiltrate intensity: mild, moderate and severe.

2.9. Statistical analysis

To perform statistical analysis, the dogs were grouped according to infection status. Animals were considered infected if positive results were obtained for at least one of the following tests: bacterial isolation, PCR in urine or MAT titers ≥800. For dogs that tested positive exclusively on MAT, all of the following additional criteria were applied: the presence of clinical symptoms, abnormalities in clinical pathology profile and negative vaccination status in the 12 months prior to sample collection. Animals that returned negative tests results were considered uninfected. Results were analyzed using Epi info v7 software. Variables were expressed as means and standard deviation (SD) or frequencies. Odds ratio (OR) and 95 % confidence intervals (95 %CI) were calculated. Associations between the evaluated variables and confirmed cases were investigate using the chi-square test. *P*-values <0.05 were considered statistically significant.

3. Results

Of the 57 sera tested by MAT, 70.18 % (40/57) presented seroreactivity based on a cutoff point of 100 titers. The titers of 200 predominated, representing 30 % (12/40) of the positive animals, followed by 800 (22.50 %, 9/40), 400 (17.50 %, 7/40), 100 (15 %, 6/40). A titer level of 1600 was observed in five samples (12.50 %, 5/40), while one sample (2.50 %, 1/40) presented 6400 titers. Seven different presumptive infecting serogroups were identified: Icterohaemorrhagiae was the most prevalent (65 %, 26/40), followed by Australis (12.50 %, 5/40) and Canicola (7.50 %, 3/40) (Supplementary Table 2).

Regarding leptospiral DNA detection by lipL32-PCR, a total of 32/57 (56.14 %) urine samples tested positive. Of these PCR-positive samples, 9/32 (28.13 %) presented MAT titers ≥ 800, while 23/32 (71.87 %) had titers < 800.

Leptospira spp. was successfully isolated in 12/57 dogs (21.05 %): 4/12 (33.34 %) isolates were obtained from urine, 7/12 (58.33 %) from blood, and the bacterium was isolated from both urine and blood in one (8.33 %) dog.

Serological characterization by MAT revealed that all isolates belonged to the serogroup Icterohaemorrhagiae. Additionally, monoclonal testing was presumptive for serovar Copenhageni in eight isolates. Two isolates were considered inconclusive and showed reactivity for the mAb F89C12 (C41 F89C12: 5.120, C51 F89C12: 2.560). The C29 isolate was negative for all mAb's (F89C12, F70C24, F70C14), this may indicate the possibility of a new serovar of the Icterohaemorrhagiae serogroup. Due to insufficient amount of monoclonal serum, testing was not performed on the C72 isolate (Table 1). Genotyping based on *rrs* and *secY* gene sequences identified all 12 isolates as *L. interrogans* (sequence data submitted to GenBank, accession numbers available in Supplementary Table 3). MLVA analysis confirmed all isolates as *L. interrogans* serovar Icterohaemorrhagiae/Copenhageni. VNTR profiles were found to be identical among the isolates and indistinguishable from the Copenhageni and Icterohaemorrhagiae serovars (see Supplementary Table 3).

A total of 38 cases were confirmed by culture and/or PCR: 32 were positive by lipL32-PCR, 12 were positive in culture and nine animals tested positive under both techniques. While positive serology is not a definitive indication of disease, three unvaccinated animals with clinical symptoms presented MAT titers ≥800 (one dog, C50, died). Of the 14 vaccinated animals, isolates were successfully obtained in three cases (individual results available in Supplementary Table 1). Recent contact with rodents was reported by 75.93 % (41/54) of the owners; 11/12 (91.66 %) of these were cases in which isolates were obtained (Table 2).

Renal function was assessed by serum urea and creatinine levels; 14/30 (46.67 %) of the confirmed animals presented azotemia (OR 5.68 [95 %CI 1.08–29.68] ($P = 0.02$). With regard to abnormalities on urinalysis 32.43 % confirmed animals indicated decreased specific gravity. Proteinuria (OR 0.11 [95 %CI 0.02−0.64]) ($P = 0.007$) was found to be strongly associated with leptospirosis (Table 2). No significant associations were identified between clinical signs and leptospirosis infection.

Kidney samples were obtained from 8/57 animals that spontaneously died or were euthanized at HOSPMEV-UFBA following owner consent. Microscopy revealed renal congestion, the presence of mononuclear inflammatory infiltrate (interstitial nephritis – Figs. 1a, 1b and

Table 1
Characterization of isolates of *Leptospira interrogans,* serogroup Icterohaemorrhagiae, serovar Copenhageni, obtained from naturally infected dogs.

Animal ID	Isolate Origen	MAT (cut-off: 100 titers) Titration/Serogroup	Serological Characterization Serogroup/Serovar	Molecular Characterization Species
C1	Whole Blood	Titers – 400 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C3	Whole Blood	Titer - 400 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C7	Urine	Titer – 800 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C20	Whole Blood	Titer - 400 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C25	Urine	Titer - 800 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C29	Urine	Titer – 200 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Inconclusive	*L. interrogans*
C41	Whole Blood	Titer – 1600 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Inconclusive	*L. interrogans*
C51	Whole Blood	Titer - 100 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Inconclusive	*L. interrogans*
C52	Whole Blood	Titer – 800	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C52	Urine	Sg. Australis	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C53	Whole Blood	Titer – 1600 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C56	Urine	Titer – 800 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. Copenhageni	*L. interrogans*
C72	Whole Blood	Titer – 400 Sg. Icterohaemorrhagiae	Sg. Icterohaemorrhagiae Sv. ND	*L. interrogans*

ND - Not Determined; Sg. – Serogroup; Sv. – Serovar.

Table 2
Characterization of dogs with clinical symptomatology suggestive of leptospirosis.

	n	Confirmed (n = 38) Mean (SD) or % of group	n	Unconfirmed (n = 19) Mean (SD) or % of group	n	Total cases (n = 57) Mean (SD) or % of group	n	Total cases (n = 57) OR (95 %CI)
Epidemiological data								
Age (Months)	37	46.78 (34.17)	19	80.63 (55.02)	56	58.26 (44.91)	56	ND
Male	38	55.26%	19	68.42%	57	59.65 %	57	1.75 (0.55–5.59)
Female	38	44.74%	19	31.58%	57	40.35%	57	ND
Purebred	38	50.00%	19	52.63%	57	50.88%	57	0.95 (0.29–2.71)
Crossbred	38	50.00%	19	47.37%	57	49.12%	57	ND
Recent exposure to rodents	37	75.68%	17	76.74%	54	75.93 %	54	0.95 (0.24–3.68)
Contact with other dogs	35	60.00%	17	41.18%	52	53.85%	52	2.14 (0.65–6.96)
Vaccination	32	31.25 %	15	26.67%	47	29.79%	47	1.25 (0.31–4.90)
Dogs with no street access	31	29.03%	16	25.00 %	47	27.66%	47	ND
Free outdoor access	31	70.97%	16	75.00 %	47	72.34%	47	0.81 (0.20–3.21)
Presentation								
Clinical Evolution	36	8.10 (8.91)	19	8.15 (6.91)	55	8.14 (8.21)	55	ND
Fever	30	36.67%	19	47.37%	49	40.82%	49	0.64 (0.20–2.06)
Renal involvement	27	40.74%	15	26.67%	42	35.71%	42	1.89 (0.47–7.50)
Hemoptysis	33	6.06%	16	12.50 %	49	8.16%	49	0.45 (0.05–3.54)
Lethargy	37	86.49%	19	73.68%	56	82.14%	56	2.28 (0.56–9.17)
Jaundice	35	77.14%	17	76.47%	52	76.92%	52	1.03 (0.26–4.08)
Melena	34	35.29%	15	26.67%	49	32.65 %	49	1.50 (0.39–5.74)
Abdominal pain	28	42.86%	14	28.57%	42	38.10 %	42	1.87 (0.47–7.45)
Laboratory analysis								
Complete blood count								
Anemia	38	50.00%	19	68.42%	57	56.14 %	57	0.46 (0.14–1.46)
White cell count								
Leukocytosis (x 10^9/L)	38	26.58 (21.05)	19	29.84 (17.52)	57	26.33 (19.79)	57	1.12 (0.35–3.53)
Neutrophilia (x 10^9/L)	38	18.92 (11.58)	19	18.32 (13.46)	57	18.72 (12.12)	57	1.43 (0.44–4.59)
Monocytosis (x 10^9/L)	38	1.42 (1.58)	19	1.37 (1.43)	57	1.40 (1.52)	57	1.12 (0.34–3.65)
Eosinophilia (x 10^9/L)	38	0.27 (0.41)	19	0.62 (0.95)	57	0.38 (0.65)	57	0.29 (0.04–1.94)
Lymphocytosis (x 10^9/L)	38	3.02 (9.38)	19	1.72 (2.19)	57	2.59 (7.75)	57	0.47 (0.06–3.64)
Thrombocytopenia (x 10^9/L)	37	197.37 (104.00)	17	167.11 (129.37)	54	187.85 (112.25)	54	0.56 (0.14–2.11)
Liver function biochemistry								
Elevated (ALP / ALAT) (U/L) [a]	31	64.52%	15	80.00%	46	69.57%	46	0.45 (0.10–1.96)
ALAT (U/L)	36	212.66 (210.14)	18	483.71 (730.29)	53	304.71 (469.97)	53	0.54 (0.14–2.03)
ALP (U/L)	30	1174.38 (876.54)	15	1531.48 (1992.71)	45	1293.41 (1341.21)	45	4.46 (0.37–53.70)
Renal function biochemistry								
Azotemia (U/C) (x 10^2/L)	30	46.67 %	15	13.33%	45	35.56%	45	**5.68 (1.08–29.68)**
Urea (x 10^2/L)	30	212.19 (184.57)	16	151.60 (167.92)	46	191.12 (179.46)	46	3.16 (0.91–10.95)
Creatinine (x 10^2/L)	34	4.09 (4.46)	17	1.91 (2.16)	51	3.36 (3.96)	51	3.65 (0.98–13.51)
Urine Specific Gravity (USG)								
Normal range	22	68.18%	15	66.67%	37	67.57%	37	0.93 (023 – 3.78)
Decreased specific gravity	22	31.82%	15	33.33%	37	32.43 %	37	ND
Urine chemical evaluation								
Protein	22	40.91%	14	85.71%	36	58.33 %	36	**0.11 (0.02 – 0.64)**
Bilirubin	22	68.18%	15	86.67%	37	75.68%	37	0.32 (0.05–1.87)
Blood	23	34.78%	15	46.67 %	38	39.47%	38	0.60 (0.16–2.30)

ND -not determined; UN - undefined; [a] - Values calculated in relation to two variables; Bolded text indicates odds ratio values predictive of infection.

1c) and necrosis/degeneration (Fig. 1c) in all eight (100.00 %) samples. Tubular regeneration (megalocytosis - Fig. 1d) was observed in 6/8 samples (75.00 %), while hyaline/granular cylinders (Fig. 1a) and hemorrhage were observed in 3/8 (37,50 %). Bile pigment and tubular ectasia were present in 2/8 (25 %) samples (Table 3). For more accurate characterization, interstitial nephritis (IN) was scored according to infiltrate intensity: mild (1/8–12.50%), moderate (5/8–62.50%) or severe (2/8–25.00%).

4. Discussion

The multidisciplinary approach allowed the successful characterization of confirmed leptospirosis cases. We were able to accompany the animal throughout the case evolution, from admission to recovery or death.

With respect to the serological investigation of leptospirosis, reports in the literature employ variable interpretations of cutoff points using single serum MAT titers to classify reactive samples [4,22]. Considering the guidelines on leptospirosis in animals published by the OIE [13], and American [4] and European [22] consensuses, results are suggestive of leptospirosis when serum titer levels are equal to or greater than 800 on a single test. However, half of the animals evaluated herein that were positive on culture presented titers <800, the lowest of these being 100. Any of the studied animals with titers ≥100 were considered reactive for leptospirosis [23], while those with titers ≥800, positivity on PCR and/or culture were considered confirmed cases. The literature contains reports of positivity for *Leptospira* spp. in cultures from seronegative dogs, as well as in animals with titers between 200–400 [2,24], highlighting the need for veterinarians carefully evaluate results indicating <800 titers. Thus, we consider the data presented fundamental for application in the local scenario, given that many animals may not be classified as sick due to inadequate serological screening and/or omission in the request of paired tests. Moreover, the use of a multidisciplinary approach could contribute more accurately confirming a diagnosis leptospirosis in clinically suspected patients.

Reports indicate that the Icterohaemorrhagiae serogroup is more frequently found in tropical regions [25,26], which is consistent with our findings. Synanthropic rodents are widely described as chronic carriers well-adapted to this serogroup, likely implicating these animals in the maintenance of this serogroup in the region studied; moreover, rodents are considered the main source of infection in other animals and humans [27–30]. The occurrence of serovar Icterohaemorrhagiae/Copenhageni has also been reported in other studies in tropical regions [9,31]. It is important to emphasize that almost all of the owners

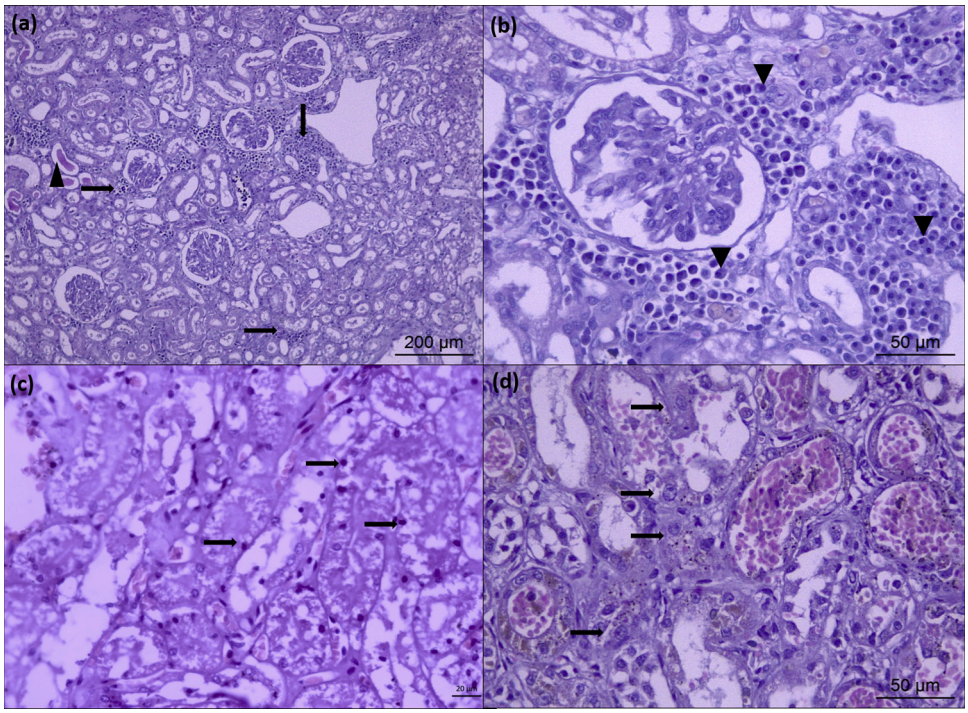

Fig. 1. Photomicrography of histological changes in the renal parenchyma of dogs naturally infected with *Leptospira* spp. **1a.** Multifocal interstitial mononuclear inflammatory infiltrate (arrows) and hyaline cylinder inside renal tubule (arrowhead). **1b.** Predominantly plasmacytic mononuclear inflammatory infiltrate, (arrowheads). **1c**. Marked necrosis of epithelial cells, evidenced by nuclei pycnotization (arrows). **1d.** Tubular regeneration: megalocytosis of tubular epithelial cells (arrows). **Detail:** Staining (H&E); Magnification (1a:10x, 1b–1d: 40x).

Table 3
Description of primary lesions found in the renal parenchyma of dogs suspected of leptospirosis.

	Kidney							
Histopathological finding	C20	C22	C29	C43	C46	C50	C51	C62
Congestion	+	+	+	+	+	+	+	+
Mononuclear inflammatory infiltrate	+	+	+	+	+	+	+	+
Necrosis and degeneration	+	+	+	+	+	+	+	+
Tubular regeneration	+	–	+	+	+	+	+	–
Hyaline / granular cylinders	–	+	+	+	–	–	–	–
Hemorrhage	+	–	+	–	–	–	+	–
Bile pigment	–	–	–	+	–	+	–	–
Tubular ectasia	–	–	+	–	–	–	+	–

(+) present; (-) absent.

in whose dogs *Leptospira* was isolated reported previous contact with rodents in the household environment. Accordingly, we speculate that infection may have occurred by direct or indirect contact among dogs and rodents, particularly brown rats (*Rattus norvegicus*), in a peridomestic environment [4,32,33].

According to Bourhy et al. [34], immunity to infection is restricted to the antigenically responsible serovar. Our findings corroborate previous studies indicating the circulation of serovar Copenhageni in both dogs and humans in Salvador and other regions of Brazil [28,35]. We suggest that the isolates described herein may belong to the serovar Copenhageni, since previous studies have shown the isolation of this serovar in humans and rodents in the same epidemiological scenario [28]. The high circulation of this serovar may be attributable to the extensive use of multivalent vaccines lacking this serovar [23], as well as by the fact that rodents (especially *Rattus norvegicus*) are considered reservoir hosts of serovars Icterohaemorrhagiae/Copenhageni [23,36]. In Brazil, the vaccines mainly commercialized (national or imported) include serovars Canicola and Icterohaemorrhagiae in their composition; frequently, serovars Pomona and Grippotyphosa are also included [11]. It is important to note that bacteria-based vaccines generate limited immunity, and most vaccinated dogs present negativity for antibodies 15 weeks after vaccination. However, a fraction may present reactivity for up to 12 months [22,37]. The fact that almost one-third of the confirmed cases were in vaccinated animals could indicate deficient prophylaxis, and/or that the vaccines in use do not contain the serovar most prevalently circulating in the region.

Studies describing *Leptospira* spp. isolation in association with a multidisciplinary diagnostic approach are generally lacking in the literature [2,24,38–40]. High rates of *Leptospira* detection on culture in urine samples from dogs with acute presentations highlights the risk of exposure to communicants, including humans. The data reported herein contributes to a more accurate understanding of local epidemiology, can be used in evaluations of diagnostic tests (to increase MAT sensitivity), as well as in the development of novel vaccines and public health policies aimed at controlling disease [8,17,34].

While the clinical presentation of leptospirosis is characterized by non-specific signs, making diagnosis difficult, some clinicopathological findings, such as jaundice, lethargy and renal involvement, strengthen the suspicion of disease [4,22]. Statistical analysis revealed a strong association between the clinicopathological finding of azotemia in confirmed cases of leptospirosis (Table 2). A strong association was also observed between proteinuria and *Leptospira* infection, which further reinforces renal involvement. In canines with leptospirosis, studies have reported that azotemia and the presence of protein in urine lead to reduced renal perfusion (presence of ischemic lesions) and decreased glomerular filtration rates in association with the destruction of renal

epithelial cells by toxins and components present in the membrane of *Leptospira* [4,41].

The fact that most of the confirmed cases herein presented titers below the cutoff recommended by the OIE, we therefore strongly suggest the need for studies employing noninvasive methods for the early detection of kidney injury, which would greatly aid in achieving the clinical diagnosis of canine leptospirosis. This need is further bolstered by the fact that clinical and pathological investigations are not capable of detecting early tubular lesions, since the parameters currently used, i.e. elevated serum levels of creatinine, are seen in later stages of disease and are considered non-specific indicators of renal impairment.

5. Conclusion

Investigations aimed at the identification of autochthonous strains are critically important to epidemiological studies and the reformulation of prophylactic methods. Although most of the clinical signs of canine leptospirosis observed in our study were similar to those reported in the literature, the presently adopted multidisciplinary approach enabled the identification of acute infection via culture and/or PCR in tandem with the clinicopathological and histopathological characterization of renal involvement. Urinary shedding of pathogenic leptospires in dogs is not only of great relevance to animals, but also to public health.

Funding

This work was supported by the National Council for Scientific and Technological Development (CNPq—grant no. 248110/2016-9).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Dr. Gabriela Covizzi (Metropolitan Union of Education and Culture) for providing some of the samples used in this study. We wish to thank Dr. Marta Vasconcelos Bittencourt and Dr. Sabrina Mota Lambert for their scientific insights and contributions that improved the quality of the manuscript. The authors are profoundly grateful to the Postgraduate Program in Animal Science in Tropics (PPGCAT) and the Coordination for the Improvement of Higher Education Personnel (CAPES) for scholarship assistance via the National Postdoctoral Program.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2021.101664.

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