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Asymptomatic leptospiral infection is associated with canine chronic kidney disease

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ABSTRACT

Canine leptospirosis is characterized by an acute or chronic disease. Some dogs may act as asymptomatic carriers, keeping the agent in the renal tubules and eliminating it in the urine for an extended period. Chronic kidney disease (CKD) is multifactorial and pathophysiology has been widely discussed. The aim of the study was to investigate whether the occurrence of CKD may possibly be associated with asymptomatic leptospiral infection in dogs in endemic regions. Serology and urine PCR were performed in 16 dogs with CKD and 48 healthy dogs from an endemic area. Dogs with CKD were more frequently shedders (75%) than non-CKD animals (20.8%). Therefore, our results demonstrate that asymptomatic leptospiral infection is associated with canine chronic kidney disease and that differential diagnosis is important for dogs from endemic areas presenting CKD. The early detection of shedders, besides the obvious impact on Public health may also help to improve the animal health and avoid the development of CKD.

1. Introduction

Leptospirosis is a worldwide infection caused by pathogenic spirochetes of the genus *Leptospira*. It affects domestic animals as well as wildlife and is also a major zoonosis [1]. The transmission of leptospirosis occurs mainly by exposure to water or soil contaminated with urine from animal carriers [2].

Canine leptospirosis is a disease characterized by an icteric severe form causing damage to the liver, renal involvement leading to acute renal failure and severe pulmonary form, which can lead to acute respiratory failure and death; and by an anicteric form with sudden onset and milder symptoms [3]. Despite the previous occurrence of the acute course of infection, some dogs may act as asymptomatic carriers, maintaining the agent in their renal tubules and eliminating it in the urine for a prolonged period [4,5]. It has recently been demonstrated that in endemic regions up to 20% of asymptomatic dogs may shed leptospires in urine [6].

Acute leptospirosis is usually diagnosed by serology (Microscopic Agglutination Test - MAT) and is based on the increase in titers of two paired samples [7]. The most frequent serovars in canine populations are Icterohaemorrhagiae and Canicola [8]. In contrast, asymptomatic animals present low titres, and other diagnostic methods, such as PCR, are necessary to detect carriers through detection of leptospiral DNA in the urine [2,9,10].

The chronic kidney disease (CKD) refers to a pathological process involving functional and / or structural damage of renal tissue, with loss of function, usually lasting more than three months [11,12]. CKD is known to be multifactorial and primary diseases (as infectious agents), age, race and genetic load may be related to this affection [11,13]. Among the affections of the urinary system, CKD is the most common in dogs [14] and has high and increasing worldwide prevalence [15].

Although to date there are scarce studies associating asymptomatic leptospiral infection with CKD in dogs, recent research has suggested that in human being asymptomatic renal colonization by leptospires is a neglected risk for renal fibrosis and CKD, especially in endemic areas [10].

Considering these aspects, the objective of this study was to investigate the hypothesis that the occurrence of CKD might be associated with asymptomatic leptospiral infection in dogs in endemic regions (Table 1).

2. Material and methods

This study was approved by the Animal Use Ethics Committee of the Federal Fluminense University under number 709/2015.

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Table 1
Serology and urine PCR of dogs with (A) and without (B) Chronic Kidney Disease from high-prevalence region.

Test\Group	Group A	Group B
Serology (MAT)	12/16 (75%)[a]	12/48 (25%)[b]
Urine PCR	12/16 (75%)[c]	10/48 (20.8%)[d]

*Different letters indicate significantly different results, according to Fisher's exact test.

2.1. Animals and study groups

A total of 64 dogs were studied, divided into two groups, A and B. Group A refers to adult (age 6–16 years) dogs with a confirmed diagnosis of CKD. Group B is a control group of clinically healthy dogs. First, dogs with CKD were identified based on abdominal ultrasonography, hematological (CBC) and biochemical tests. Briefly, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), as well as serum levels of urea (U), creatinine (C), phosphorus (P); calcium (Ca), albumin (A) and total proteins (TP) were performed. Urine samples were tested for Glutamyl Transpeptidase (GGT) and urinalysis. All samples were analyzed in the laboratory within four hours of collection. Then, for each dog defined as CKD (Group A), three healthy dogs of the same age, gender and neighborhood were studied (Group B) and submitted to the same tests of Group A.

The study was conducted on a region known to present high prevalence for leptospirosis (São Gonçalo - RJ, Brazil). Main inclusion criteria for Group A followed the recommendations of the International Renal Interest Society [11], i.e., for CKD it was required U ≥ 60 mg l/dL, C ≥ 1,4 mg/dL, Urine density ≤ 1025 and presence of changes in the medullary cortical relationship and uni or bilateral renal architecture for imaging by abdominal ultrasonography. For dogs of control group (B), the same tests were conducted and dogs with any altered exam were excluded from the study. Population sampling analysis was not performed, since determining the prevalence of CKD or leptospiral infection on the studied regions was not one of the objectives of the study.

For all groups, the exclusion criteria included the recent use (< 30 days) of antimicrobials and the systematic use of angiotensin converting enzyme inhibitors, since this short-term medication causes a decrease in glomerular filtration and consequently an increase in azotemia, which may mask the DRC. Besides, it was excluded dogs immunized against leptospirosis for less than one year, with unknown vaccine history and all dogs that presented seropositive in the screening exams.

2.2. Sampling

Blood and urine samples were collected from all dogs. For blood counts, biochemistry and serology, 5 mL of blood were collected in a single tube and with anticoagulant EDTA (Vacutainer, BD, SP, São Paulo, Brazil), by jugular vein puncture. Blood samples were centrifuged at 3500 rpm for 10 min and the serum was separated in 100 μL aliquots each into microtubes (1.5 mL). Serum samples were conditioned at -20 °C until processing, while whole blood samples were sent immediately after collection to perform the blood count. Urine samples were collected by ultrasound-guided cystocentesis. An amount of 10 mL of urine was collected and a 1 mL aliquot was transferred to microtubes containing 100 μL of 10x PBS. Urine samples were also conditioned at −20 °C until processing.

From all animals, blood and urine samples were collected at the same day of the ultrasonography, and then once a month, performing the same tests in all the collections. Overall, three samplings for a minimum period of three months were conducted.

2.3. Serology

For the detection of anti-*Leptospira* antibodies, microscopic agglutination test (MAT) was conducted according to international recommendations [7] using a panel including eight serovars representing seven serogroups. The antigens used were *Leptospira interrogans* serovars Autumnalis (Akiyami A), Bratislava (Jez-Bratislava), Bataviae (Van Tienen), Canicola (Hond Utrecht IV), Grippotyphosa (Moska V), Icterohaemorrhagiae (RGA), Copenhageni (M 20) and Pomona (Pomona). The reaction titer with 50% of agglutinated leptospires corresponded to the reciprocal of the highest dilution of serum. Since it is an endemic region, titres of 50 a 200 was considered as exposure and > 200 as active infection [16]. Serology was paired with a monthly serological examination for three months of each animal.

2.4. Molecular analysis

PCR was performed as described [17]. Briefly, DNA of the urine samples was extracted using the Promega Wizard SV Genomic DNA Purification System (Promega, Madison, USA). (LipL32_45F-5′AAG CAT TAC TTG CGC TGG TG 3′and LipL32_286R-5′TTT CAG CCA GAA CTC CGA TT 3′), which generated a fragment of 242 bp. The total volume of each sample was analyzed by agarose gel electrophoresis (1.5%), stained with Redgel solution 3% (Biotium, Hayward, USA) and the bands (240 bp) of DNA were visualized under ultraviolet light.As positive control, a strain of *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 was used, and ultrapure water was used as negative control.

To confirm the identity of the amplicons, PCR products from five samples were randomly selected and submitted to nucleotide sequencing of gene *lipL32* in order to confirm the diagnosis of *Leptospira*. Amplicons were purified with GFX PCR DNA Gel Band Purification Kit (GE HealthCare). The sequencing reaction was performed using the Big Dye Terminator v3.1 kit (Applied Biosystems) and capillary electrophoresis was performed in both directions on an ABI 3730 automatic sequencer (Applied Biosystems). The sequences were edited using the BioEdit v.7.0.4 programs [18] and MEGA v.6 [19].

2.5. Statistical analysis

Positivity at PCR and exposition identified by serology (titres of 50–200) were defined as the dependent variables. Thus, Fisher's exact test was used to investigate the association between these variables and dogs with CKD. A confidence interval of 95% ($p < 0.05$) was used in all analyzes.

3. Results

3.1. Serology

In group A, all dogs presented exposure, defined by low titres of 50 (4/16, 25%) or 100 (12/16. 75%). On Group B, 25% were exposed (12/48) and presented titers of 100. For both groups, the observed reactions were directed against serogroups Icterohaemorrhagiae (58.4%) and Canicola (41.6%).

3.2. Molecular analysis (PCR and sequencing)

Regarding urine PCR, in Group A 12/16 animals (75%) were positive, while on Group B, 10/48 animals (20.8%) presented positive, a significant difference ($p = 0.0002$, RR 3.3). Regarding sequencing of amplicons, all the five amplicons that were sequenced were confirmed as *Leptospira* sp.

3.3. Hematologic and biochemical results

All animals in Group A presented their altered hematology tests with results above the reference values proposed in all three samples during three months of medical follow-up confirming the diagnosis of chronic kidney disease. Results of Group A ranged from: CBC 11.4–36.5%, Urea 78–598.4 mg l/dL, Creatinin 2–10.8 mg/dL and Urine density 1005–1020 (Supplementary material Table 1).

3.4. Abdominal ultrasonography

All three ultrasound examinations performed during the three months of follow-up showed both the cortex and the medulla hyperechoic, heterogeneous and poorly delimited images altering the medullary cortical relationship. Kidneys with altered renal architecture, of diminished size and irregular contour. All these changes characterize chronic nephropathy.

4. Discussion

The seroprevalence observed in this study agrees with previous studies in which it is shown that the studied region presents high prevalence of leptospirosis [6]. Similarly, the distribution by serogroups agrees not only to the studied region, but throughout the country [8]. It is noteworthy that the serogroup Icterohaemorrhagiae is composed not only by serovar Icterohaemorrhagiae, but also by serovar Copenhageni. This serovar, particularly the strain FIOCRUZ L1-130, is known as the more prevalent strain in human leptospirosis [20] as well as in dogs [21]. In addition, the Canicola is a serovar cited as one of the most commonly found in dogs [4], being the dog its main natural reservoir [22].

Seroreactivity of dogs affected by CKD in regions of high prevalence was significantly higher than that of dogs of the same age, sex and neighborhood, but without CKD. In addition, most dogs with CKD presented low titers (100), suggesting only exposure [23,16] but no clinical active infection.

The most important outcome of the study regards to the differences on urine PCR positivity on the groups and the association of PCR positivity with CKD. Dogs with CKD were significantly more frequently infected (as detected by urine PCR) than dogs without CKD. In fact, dogs with CKD are three times more likely to be seroreactive and 3.3 times more likely to be infected by leptospires than non-CKD animals of the same area, sex, and age. Although there are scarce studies in dogs referring to the asication of leptospiral infection and CKD, in humans it has recently been shown a high prevalence of CKD in areas endemic to leptospirosis [10].

It is widely known that acute leptospirosis might lead to tubulointerstitial nephritis and interstitial fibrosis [24,25], and when treated improperly or untreated in time can progress to CKD [10,26]. Besides, it is known that animals living in endemic areas can infect and maintain leptospires in the renal tubules and interstices for a long time, developing a silent asymptomatic infection by eliminating leptospires in the urine [6]. Considering the two points, the present study demonstrated that even asymptomatic shedders from endemic regions are more susceptible to develop CKD. Noteworthy that leptospiral infection is treatable with antimicrobials. Considering this, we suggest that animals from endemic regions should be investigated for renal carrier status by urine PCR. The early detection of shedders, besides the obvious impact on Public health [6] may also help to improve the animal health and avoid the development of CKD.

5. Conclusion

Our results demonstrate that in dogs of endemic area there is an association between leptospiral infection and CKD. Acute leptospirosis is not a pre-requisite for the development of CKD, which may also occur due to the long-term silent leptospiral infection of these animals. The early detection of shedders, besides the obvious impact on Public health may also help to improve the animal health and avoid the development of CKD.

Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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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.2018.11.009.

References

[1] B. Adler, *Leptospira* and Leptospirosis, Springer, Berlin, 2015, pp. 1–10.
[2] D.A. Haake, P.N. Levett, Leptospirosis in humans, Curr. Top. Microbiol. Immunol. 387 (2015) 65–97.
[3] W.A. Ellis, Animal leptospirosis, Curr. Top. Microbiol.Immunol. 387 (2015) 99–137.
[4] M. Lelu, C. Muñoz-Zanzi, B. Higgins, R. Galloway, Seroepidemiology of leptospirosis in dogs from rural and slum communities of Los Rios Region, Chile, BMC Vet. Res. 11 (2015) 1–8.
[5] S.E. Grayzel, E.E. De Bess, Characterization of leptospirosis among dogs in Oregon, 2007–2011, J. Am. Vet. Med. Assoc. 248 (2016) 908–915.
[6] R. Sant'anna, A.S. Vieira, J. Grapiglia, W. Lilenbaum, High number of asymptomatic dogs as leptospiral carriers in an endemic area indicates a serious public health concern, Epidemiol. Infect. 145 (2017) 1852–1854.
[7] OIE, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, pp. 1–15 World Organization for Animal Health, Paris, 2014.
[8] P.S. Pinto, H. Libonati, W. Lilenbaum, Systematic review of leptospirosis on dogs, pigs, and horses in Latin America, Trop. Anim. Health Prod. 49 (2017) 231–238.
[9] M.A. Inkelmann, G.D. Kommers, M.E. Trost, C.S.L. Barros, R.A. Fighera, L.F. Irigoyen, I.P. Silveira, Urinary system injuries in 1,063 dogs, Braz. J. Vet. Rec. 32 (2012) 761–771.
[10] C.W. Yang, Leptospirosis renal disease: emerging culprit of chronic kidney disease unknown etiology, Nephron (2018) 129–136.
[11] IRIS International Renal Interest Society, IRIS Guidelines, (2013) (Accessed 15 December 2016), http://www.iris-kidney.com/guidelines/.
[12] L.D. Cowgill, D.J. Polzin, J. Elliott, M.B. Nabity, G. Segev, G.F. Grauer, S. Brown, C. Langston, A.M. Van Dongen, Is progressive chronic kidney disease a slow acute kidney injury? Vet. Clin. North Am. Small Anim. Pract. 46 (2016) 995–1013.
[13] V. Meucci, G. Luci, M. Vanni, G. Guidi, F. Perondi, L. Intorre, Serum levels of ochratoxin A in dogs with chronic kidney disease (CKD): a retrospective study, J. Vet. Med. Sci. 79 (2017) 440–447.
[14] D.G. O'Neill, J. Elliott, D.B. Church, P.D. McGreevy, P.C. Thomson, D.C. Brodbelt, Chronic kidney disease in dogs in UK veterinary practices: prevalence, risk factors, and survival, J. Vet. Intern. Med. 27 (July–August (4)) (2013) 814–821.
[15] H.Y. Yang, C.C. Hung, S.H. Liu, Y.G. Guo, Y.C. Chen, Y.C. Ko, C.T. Huang, L.F. Chou, Y.C. Tian, M.Y. Chang, H.H. Hsu, M.Y. Lin, S.J. Hwang, C.W. Yang, Overlooked risk for chronic kidney disease after leptospiral infection: a population-based survey and epidemiological cohort evidence, PLoS Negl. Trop. Dis. 10 (2015) e0004105.
[16] G. Martins, W. Lilenbaum, The panorama of animal leptospirosis in Rio de Janeiro, Brazil, regarding the seroepidemiology of the infection in tropical regions, BMC Vet. Res. 9 (2013) 237.
[17] C. Hamond, G. Martins, A.P. Loureiro, C. Pestana, R. Lawson-Ferreira, M.A. Medeiros, W. Lilenbaum, Urinary PCR as an increasingly useful tool for an accurate diagnosis of leptospirosis in livestock, Vet. Res. Commun. 38 (2014) 81–85.
[18] T.A. Hall, Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT, Nucleic Acids Symp. 41 (1999) 95–98.
[19] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA 6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol. 30 (2013) 2725–2729.
[20] K.P. Patra, B. Choudhury, M.M. Matthias, S. Baga, K. Bandyopadhya, J.M. Vinetz, Comparative analysis of lipopolysaccharides of pathogenic and intermediately

pathogenic *Leptospira* species, BMC Microbiol. 15 (2015) 244.
[21] E.F. Silva, C.S. Santos, D.A. Athanazio, N. Seiffert, F.K. Seixas, G.M. Cerqueira, M.Q. Fagundes, C.S. Brod, M.G. Reis, O.A. Dellagostin, A.L. Ko, Characterization of virulence of *Leptospira* isolates in a hamster model, Vaccine 26 (2008) 3892–3896.
[22] M. Matsui, L. Roche, S. Geroult, S. Geroult, M.E. Soupé-Gilbert, D. Monchy, M. Huerre, C. Goarant, Cytokine and chemokine expression in kidneys during chronic leptospirosis in reservoir and susceptible animal models, PLoS One 11 (2016) 1–20.
[23] H. Langoni, M.C. Ponte, D. Barbosa, M.P. Manzi, R.C. Silva, B.D. Menozzi, Research antibody and DNA *Leptospira* spp. In canine serum, Vet. Zootec. 22 (2015) 429–436.
[24] E.M. Atasoyu, V. Turhan, S. Unver, T.R. Evrenkaya, S. Yildirim, A case of leptospirosis presenting with end-stage renal failure, Nephrol. Dial. Transplant. 20 (2005) 2290–2292.
[25] H.Y. Yang, P.Y. Hsu, M.J. Pan, M.S. Wu, C.H. Lee, C.C. Yu, C.C. Hung, C.W. Yang, Clinical distinction and evaluation of leptospirosis in Taiwan–a case-control study, J. Nephrol. 18 (2005) 45–53.
[26] N.J. Herath, S.A. Kularatne, K.G. Weerakoon, A. Wazil, N. Subasinghe, V.I.N. Ratnatunga, Long term outcome of acute kidney injury due to leptospirosis? A longitudinal study in Sri Lanka, BMC Res. Notes 7 (2014) 398.