

Clinical-pathological alterations, serological and molecular diagnosis in dogs with suspected leptospirosis

Alterações clínico-patológicas, diagnóstico sorológico e molecular em cães com suspeita de leptospirose

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Highlights

Combining the MAT and real-time PCR is beneficial in diagnosing leptospirosis.

Canine leptospirosis alters renal parameters.

MAT of paired samples should be performed to diagnose acute canine leptospirosis.

Abstract

Leptospirosis is a globally important zoonosis that causes death in humans and animals, especially unvaccinated dogs. Dogs are infected through contact with urine or water contaminated by pathogenic spirochaete bacteria of the genus *Leptospira*. This study aimed to assess the performance of two diagnostic tests in characterizing dogs with leptospirosis and associate it with the main clinical-pathological alterations in dogs with suspected infection. From September 2019 to September 2020, 24 dogs with suspected canine leptospirosis were treated at the Veterinary Hospital of the Federal University of Uberlândia (UFU). Complementary tests such as a complete blood count (CBC) and serum creatinine, ALT (alanine transaminase) and ALP (alkaline phosphatase) levels were prescribed. In order to confirm the leptospirosis diagnosis, serum and molecular tests were performed using the microscopic agglutination test (MAT) and real-time quantitative polymerase chain reaction (qPCR). Of the 24 suspected cases of canine leptospirosis, six (25%) were positive in the MAT and three (12.5%) in the qPCR, with one (4.17%) returning positive results in both diagnostic tests. Positive results were obtained for 8/24 (33.3%) for combined MAT + qPCR. The predominant reactive serogroups were Icterohaemorrhagiae (33.33%) and

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Djasiman (33.33%), followed by Ballum (16.60%). The sensitivity and specificity of the qPCR in relation to the gold standard test (MAT) were 16.7 and 88.9%, respectively. Changes were observed in serum levels of creatinine, urea and ALP in the group that tested positive (MAT/qPCR); however, only urea and ALP levels were high in the negative group. Comparison of biochemical parameters in the positive (MAT/qPCR) and negative groups showed no statistically significant difference between the elements assessed ($p>0.05$). It was concluded that in order to diagnose acute canine leptospirosis, veterinarians should combine the clinical-pathological findings with paired sample MAT and qPCR urine test results.

Key words: Serum biochemistry. Canine. *Leptospira* spp.. MAT. qPCR. Sensitivity.

Resumo

A leptospirose é uma zoonose de importância mundial que causa mortes tanto em humanos, como em animais, especialmente em cães não vacinados. A infecção em cães ocorre pelo contato com urina ou água contaminada pela bactéria espiroqueta patogênica do gênero *Leptospira*. Objetivou-se avaliar dois testes diagnósticos na caracterização de cães com leptospirose, e associar com as principais alterações clínicas-patológicas de cães suspeitos. Foram atendidos no Hospital Veterinário da Universidade Federal de Uberlândia no período de setembro de 2019 a setembro de 2020, 24 cães com suspeita clínica de leptospirose canina. Exames complementares como hemograma completo, dosagens séricas de creatinina, ureia, ALT (alanina aminotransferase), FA (fosfatase alcalina) foram solicitados. Para o diagnóstico confirmatório de leptospirose foi realizado teste sorológico pelo teste de aglutinação microscópica (MAT) e também teste molecular pela reação em cadeia da polimerase em tempo real (qPCR). Dos 24 casos de cães suspeitos de leptospirose, seis (25%) foram positivos no MAT, e três (12,5%) no qPCR, sendo 1 (um) (4,17%) positivo nos dois testes diagnósticos avaliados. Quando se associou MAT + qPCR a positividade foi para 8/24 (33,3%). Os sorogrupos reativos predominantes foram Icterohaemorrhagiae (33,33%) e Djasiman (33,33%), seguido de Ballum (16,60%). A sensibilidade e especificidade da qPCR em relação teste padrão ouro (MAT) foi 16,7 % e 88,9%, respectivamente. Houve alterações nos níveis séricos de creatinina, ureia e FA no grupo positivo aos testes diagnósticos (MAT/qPCR), porém somente ureia e FA apresentaram valores elevados no grupo negativo. Quando se comparou os parâmetros bioquímicos no grupo positivo aos testes diagnósticos (MAT/qPCR), e negativo, não se observou nenhuma diferença estatística significativa entre os elementos avaliados ($p>0,05$). Conclui-se para o diagnóstico de leptospirose canina aguda, o médico veterinário deve associar os achados clínicos-patológicos aos resultados do MAT de forma pareada e o qPCR de urina.

Palavras-chave: Bioquímica sérica. Canino. *Leptospira* spp.. MAT. qPCR. Sensibilidade.

Introduction

Leptospirosis is a globally important zoonosis that causes death in humans and animals, especially unvaccinated dogs. Dogs are infected through contact with urine or water contaminated by pathogenic

spirochaete bacteria of the genus *Leptospira* (Curtis et al., 2015; Schuller et al., 2015).

Dogs are considered susceptible to infection and may be potential sources of disease transmission in environments contaminated by pathogenic *Leptospira*. Current reports indicate that the clinical

disease has resurfaced in dogs and humans due to the proximity of these species in recent years (Hartskeerl et al., 2011; Schuller et al., 2015; Meny et al., 2019), making it vital to update diagnostic and disease prevention techniques.

In urban areas dogs are a major source of human leptospirosis infection, since they can shed live *Leptospira* in their urine (renal carrier) intermittently and over extended periods, even without exhibiting clinical signs (Paes, 2016). The chronic renal carrier state is not necessarily related to the presence of serum antibodies, which limits the identification of infected and asymptomatic dogs (Greene et al., 2006; Robi, 2020).

Its nonspecific clinical signs make leptospirosis diagnosis a complex process in animals (Mohammed et al., 2011; Robi, 2020), which frequently present with fever, jaundice, anorexia and/or laboratory alterations such as uremia, leukocytosis and thrombocytopenia. The combination of these alterations can manifest itself in everything from a subclinical infection to an acute and fatal disease (Curtis et al., 2015). Since clinical findings are nonspecific, it is important to perform serological tests such as the microscopic agglutination test (MAT) and polymerase chain reaction (PCR) in order to identify the pathogen (Schuller et al., 2015).

The MAT, considered the gold standard, is used worldwide and recommended by the World Health Organization [WHO] (2003), but shows low sensitivity in the early stages of the disease. As such, two samples are recommended: one in the acute stage and the other in convalescence (Harkin et al., 2003; Miotto et al., 2018a), which is not always possible because the animal may die before

this period. Given that anti-*Lepstospira* spp. antibodies only increase to detectable levels about two weeks after infection, it is important to determine the most appropriate sample type and test for each stage of the disease (Reagan & Sykes, 2019).

PCR is a more useful diagnostic test in the early stage because of the large number of bacteria in the blood and urine due to bacteremia, which can last up to 10 days (Greenlee et al., 2005). Bacteria are shed in the urine, since they are capable of invading the kidneys and liver, among other organs (Sykes et al., 2011). Thus, PCR is a potential diagnostic tool for detecting bacterial DNA in urine samples from dogs with leptospirosis (Zakeri et al., 2010). Real-time quantitative PCR (qPCR) improves test sensitivity and is less prone to contamination when compared to conventional PCR (Reagan & Sykes, 2019).

According to Miotto et al. (2018b), the simultaneous use of MAT and PCR improved diagnosis in dogs with clinical suspicion of leptospirosis infection. Waggoner et al. (2015) also demonstrated the strategic importance of combining the two techniques to maximize detection in suspected cases.

This study aimed to assess the performance of two diagnostic tests in characterizing dogs with leptospirosis and associate it with the main clinical-pathological alterations in dogs with suspected infection.

Material and Methods

The study was approved by the Animal Research Ethics Committee of the Federal University of Uberlândia (UFU), under final analysis no. A013/19.

Experimental design

From September 2019 to September 2020, 24 dogs with suspected canine leptospirosis were treated at the UFU Veterinary Hospital.

The inclusion criterion was that the animal exhibited clinical symptoms consistent with leptospirosis (fever, prostration, hyporexia, jaundice). After initial clinical assessment, complementary laboratory tests such as a complete blood count (CBC) and serum creatinine, ALT (alanine transaminase) and ALP (alkaline phosphatase) levels were ordered, in addition to a confirmatory serological test (MAT). However, only 18 suspected cases of leptospirosis were referred for confirmatory testing by the veterinarian. The six remaining samples were obtained from the UFU Clinical Veterinary Laboratory (LCVET), selected based on requests that indicated dogs with a clinical suspicion of leptospirosis.

Blood samples collected in tubes with no anticoagulant were sent to the Infectious Disease Laboratory (LADOC) of the UFU School of Veterinary Medicine for serological testing (MAT). A urine sample was collected from each animal by cystocentesis for subsequent qPCR.

Creatinine (Jaffe's alkaline picrate method), urea (UV kinetic method), ALT - alanine transaminase (kinetic UV IFCC method) and ALP - alkaline phosphatase concentrations (optimized kinetic method) were determined in the serum samples from each animal in a ChemWell multichannel automated analyzer (Awareness Technology Inc., Palm City, FL, USA) at LCVET (using commercial Labtest Diagnostica® kits). The analyzer was previously calibrated with calibra H and qualitol H

universal control serum, both produced by Labtest Diagnostica®.

The blood samples collected from each animal in K₂EDTA tubes were processed in a PochH 100iv diff™ (Sysmex) veterinary blood cell counter. Next, blood smears were prepared for white blood cell differential, stained using the May-Grunwald Giemsa method (Ferreira et al., 1982). For each blood smear, 100 cells were identified in optical microscopy with an immersion objective (100x) and relative and absolute leukocyte count formulas established. Information on clinical signs, age, sex and vaccination history were obtained from the animals' medical charts.

Serological test

Live *Leptospira* spp. strains are stored at LADOC, subcultured weekly in EMJH medium (Ellinghausen-McCullough-Johnson-Harris Difco®), enriched with 10% rabbit serum and placed in an oven at 30°C.

The MAT was used to assess the presence of anti-*Leptospira* spp. antibodies in animal serum, including a panel of 12 serovars (Bratislava, Canicola, Djasiman, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Hardoprajtino, Wolffi, Pomona, Castellonis, Tarassovi, Grippotyphosa), representing 10 serogroups (Australis, Canicola, Djasiman, Hebdomadis, Icterohaemorrhagiae, Sejroe, Pomona, Ballum, Tarassovi, Grippotyphosa) (Table 1). The serum samples were initially diluted at 1:50 (2.45 mL of 85% phosphate buffered saline (PBS) and 50 µL of serum). Next, 50µL of the live culture of each serovar was added with 50µL of the initial serum dilution in a 96-well plate, incubated at 30°C for one hour and then read under a dark field microscope (Carl Zeiss® Axio Scope).

Table 1

Species, serogroups and serovars used in the microscopic agglutination test (MAT) to diagnose canine leptospirosis in the Infectious Diseases Laboratory of the Federal University of Uberlândia, 2020

Species	Serogroup	Serovar
<i>L. interrogans</i>	Australis	Bratislava
	Canicola	Canicola
	Djasiman	Djasiman
	Hebdomadis	Hebdomadis
	Icterohaemorrhagiae	Copenhageni
	Icterohaemorrhagiae	Icterohaemorrhagiae
	Sejroe	Hardoprjino
	Sejroe	Wolffi
<i>L. borgpetersenii</i>	Pomana	Pomana
	Ballum	Castellonis
<i>L. santarosai</i>	Tarassovi	Tarassovi
	Grippotyphosa	Grippotyphosa

Serum with at least 50% agglutination and antibody titers ≥ 100 when compared to a control culture diluted with 85% PBS dilution at 1:2 was considered positive. Reactive serum samples were titrated at decreasing dilutions (1:200; 1:400; 1:800; 1:1600) to determine the reaction endpoint, in accordance with the WHO (2003).

Molecular test

The urine samples were separated into 2 mL aliquots and stored in microtubes at 20° C until they were sent to the Veterinary Molecular Diagnosis Laboratory (LDMVET) for processing.

Real-time quantitative PCR (qPCR) was performed in line with the standardized protocol described by Stoddard et al. (2009), with some laboratory adaptation (LDMVET). Genetic material was extracted using a

DNA extraction kit, in accordance with the manufacturer's recommendations. Positive controls were used in all the reactions, obtained from a naturally infected sample submitted to Sanger sequencing and confirmed with 100% identity. Nuclease-free water was used as negative control. The primers LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3 ') and LipL32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3 ') were used in qPCR to target the LipL32 gene, an abundant outer membrane protein only present in pathogenic *Leptospira* spp. species.

Statistical Analysis

The association and agreement between the two diagnostic tests (MAT and qPCR) were evaluated by Fisher's exact test and Cohen's kappa at 5% significance, respectively, with p-values lower than 0.05 considered statistically significant.

In order to compare serum biochemical results, two groups were considered, namely those that tested positive or negative in diagnostic tests (MAT/qPCR) for dogs with suspected leptospirosis. The biochemical data were submitted to normality testing (Anderson-Darling, 5% significance) using Action 2020 software, but only urea showed normal distribution. As such, the Student's t-test was applied for intergroup comparison. Since creatinine, ALT and ALP exhibited non-normal distribution, a nonparametric test was used (Wilcoxon) at 5% significance.

Statistical analyses were performed using IBM SPSS Statistics for Windows,

version 20.0, and the graphs (box plots) constructed with GraphPad Prism version 8.0.0 for Windows.

Results and Discussion

Of the 24 dogs with suspected leptospirosis, six (25%) were reactive in the MAT, three (12.5%) in qPCR and one (4.17%) was positive in both tests (Table 2), totaling eight animals detected in at least one test. For combined MAT and qPCR, 8/24 (33.3%) cases tested positive, demonstrating an increase in the identification of true positives.

Table 2
Frequency of dogs positive for leptospirosis using combined MAT and qPCR

		MAT		
		Negative (%)	Positive (%)	Total (%)
qPCR	Negative	16 (66.67) ^a	5 (20.83) ^a	21 (87.5)
	Positive	2 (8.33) ^a	1 (4.17) ^a	3 (12.5)
	Total	18 (75)	6 (25)	24 (100)

^aThe amounts in each column do not differ statistically at 5% significance (p-value = 1.00). Fisher's exact test and Cohen's kappa (p<0.05). MAT (microscopic agglutination test) and qPCR (real-time quantitative polymerase chain reaction), with 16.7% sensitivity and 88.8% specificity.

The MAT detected *Leptospira* spp. antibody titers in 25% of dogs with a clinical suspicion of leptospirosis and qPCR 12.5%. Similar results were reported by Martin et al. (2019), who confirmed 7 of 51 suspected cases (13.7%) in dogs using the MAT and six (11.7%) in real-time PCR-*lipL* 32. Troia et al. (2018) also used two diagnostic tools in the same group of animals, with 10.3 % positive in qPCR and 76.2% in the gold standard test (MAT). Although the MAT is still the

most sensitive technique and the standard diagnostic test, these findings demonstrate the importance of complementary serological and molecular techniques, since a combination of these proved to more efficient in diagnosing infectious diseases, especially canine leptospirosis (Martin et al., 2019; Meira et al., 2011; Miotto et al., 2018b), thereby avoiding incorrect diagnosis and facilitating the proper treatment and management of these animals.

Although antibodies can be detected in the bloodstream from around 14 days after infection, *Leptospira* are intermittently shed in the urine of domestic animals from 72 hours after infection over a period of weeks or months (Levett, 2001). PCR is a diagnostic tool used not only to confirm acute leptospirosis in dogs, but also to identify chronic cases with no clinical manifestations. In the present study, two dogs shedding *Leptospiras* spp. in their urine were nonreactive to the MAT, likely due to acute infection, but there was no time for seroconversion, demonstrating the importance of combining diagnostic tools.

The serogroups/serovars most frequently reactive to the MAT and their respective titers are presented in Table 3. The serogroups *Icterohaemorrhagiae*

and *Djasiman* showed 33.33% reactivity, followed by *Ballum* (16.60%). *Djasiman* is not a widely reported serogroup in dogs, but its clinical manifestation is very similar to the symptomology of dogs with infection caused by *Icterohaemorrhagiae*, namely vomiting, diarrhea, anorexia and jaundice (Escarrone et al., 2020). Attempts have been made to correlate the infecting serovar with clinical presentation; however, to date, there is no evidence of a significant correlation. Additionally, clinical signs depend on age, immunological status and the virulence of the infecting serovar (Meira et al., 2011), with more recent research confirming that development of acute disease varies from individual to individual (Di Azevedo et al., 2022).

Table 3

Most frequent serogroups and serovars and their respective titers in the microscopic agglutination test (MAT) in dogs with clinical suspicion of leptospirosis treated at the UFU Veterinary Hospital, 2020

Serogroups/Serovars	Titers			
	100	200	400	Total (%)
<i>Ballum/Castellonis</i>	1	-	-	1(16.60)
<i>Icterohaemorrhagiae/Copenhageni</i>	1	-	1*	2 (33.33)
<i>Icterohaemorrhagiae/Icterohaemorrhagiae</i>	1	1*	-	2 (33.33)
<i>Djasiman/Djasiman</i>	1	1	-	2 (33.33)

*positive for two serovars from the serogroup *Icterohaemorrhagiae*.

Studies indicate that this serovar correlation should be based on serotyping, isolation and genetic tests (Sykes et al., 2011); however, including these tests in the clinical routine is not feasible due to their high cost.

Isolating bacteria in urine samples is complex, challenging and can take months to obtain a result. According to Guedes et al. (2020), isolation is considered a definitive diagnosis for leptospirosis and useful in identifying renal carriers. However, factors such as the short-term viability of *Leptospira*

in urine, their fastidious nature and the lack of standardized protocols make it difficult to obtain successful results. Some authors have reported that urine samples should be processed in up to 2 hours to achieve successful isolation (Zarantonelli et al., 2018).

Miotto et al. (2018a) tested 33 canine urine samples with suspected clinical leptospirosis and obtained 55.5% seroreactive cases, with titers between 100 and 3200. The predominant reactive serogroups were Icterohaemorrhagiae (n=23), Australis (n=7), Pomona (n=4), Butembo (n=4) and Ballum (n=3), once again confirming that Icterohaemorrhagiae is still one of the main causal agents in canine leptospirosis cases reported in Brazil.

Icterohaemorrhagiae is the most important serogroup in terms of public health, since these strains are more related to infections in humans than other serovars (Miotto et al., 2018a; Sevá et al., 2020) and therefore play an important role in the epidemiology of human leptospirosis. Although the sewer rat (*Rattus norvegicus*) is the most relevant host for Icterohaemorrhagiae, dogs are considered accidental hosts and their proximity to humans increases the chances of transmission (Picardeau, 2013). Unlike the present study, Latosinski et al. (2018) conducted an epidemiological serogroup survey in dogs in Botucatu, São Paulo state, and identified Canicola as the most common serogroup (66.6%), followed by Autumnalis (33.3%) and Grippotyphosa (16.6%).

The Kappa test was applied to analyze agreement between the tests and the sensitivity and specificity of qPCR in relation to the gold standard test (MAT) (Table 2.). The

Kappa value obtained was very low (0.067) and the p-value (0.722) non-significant, demonstrating the lack of agreement between tests (MAT/qPCR). Additionally, Fisher's exact test ($p < 0.05$) also found no association or dependence between the two diagnostic tests assessed (p-value=1.00). Although disagreement between the MAT and PCR is common and has been observed in some studies, Latosinski et al. (2018) found that combining these techniques is beneficial because it can contribute to confirming suspected cases. Martin et al. (2019) compared real-time PCR lipL32 and the MAT and observed strong agreement (kappa = 0.76) between the tests.

The sensitivity and specificity of qPCR were 16.7 and 88.9%, respectively. Although several studies have demonstrated high specificity and sensitivity for real-time PCR and found it to be a rapid method for diagnosing acute leptospirosis (Stoddard et al., 2009), in the present study, specificity was high, but sensitivity low. Based on previous research, this can be explained by intermittent urinal *Leptospira* shedding, prior use of antibiotics before analysis, freezing of samples and the pH of urine, which can influence and reduce test sensitivity (Fraune et al., 2013; Rohilla et al., 2020; Schuller et al., 2015). Troia et al. (2018) also observed low diagnostic sensitivity for qPCR, with sensitivity and specificity of 13.5 and 92.0 %, respectively, similar to our findings.

Preanalytical factors may have interfered in this study, generating false negatives. Performing PCR in urine samples at different times, extracting DNA from bacteria without prior freezing and neutralizing urine pH may increase the

detection rate and, consequently, sensitivity (Schuller et al., 2015; Sykes et al., 2011). Further research is needed on using primers better able to detect specific *Leptospira* spp. and controlling preanalytical factors.

The age of animals confirmed as positive (n=8) for canine leptospirosis in at least one diagnostic test (MAT and/or qPCR) ranged from 3 months to 13 years (average of 7 years), and 62.5% were male. Of the 8 positive animals, only one had a clinically recorded vaccination history. The main clinical symptoms present at the

initial consultation were loss of appetite or hyporexia (87.5%), vomiting (62.5%), diarrhea (50%), general weakness or prostration and respiratory difficulties (37.5%), fever and jaundice (25%), with additional symptoms shown in Figure 1. These signs are consistent with most previous studies on leptospirosis in dogs (Miotto et al., 2018a; Rissi & Brown, 2014). Although canine symptoms are similar to the clinical manifestation in humans, the mortality rate is higher in dogs (Major et al., 2014).

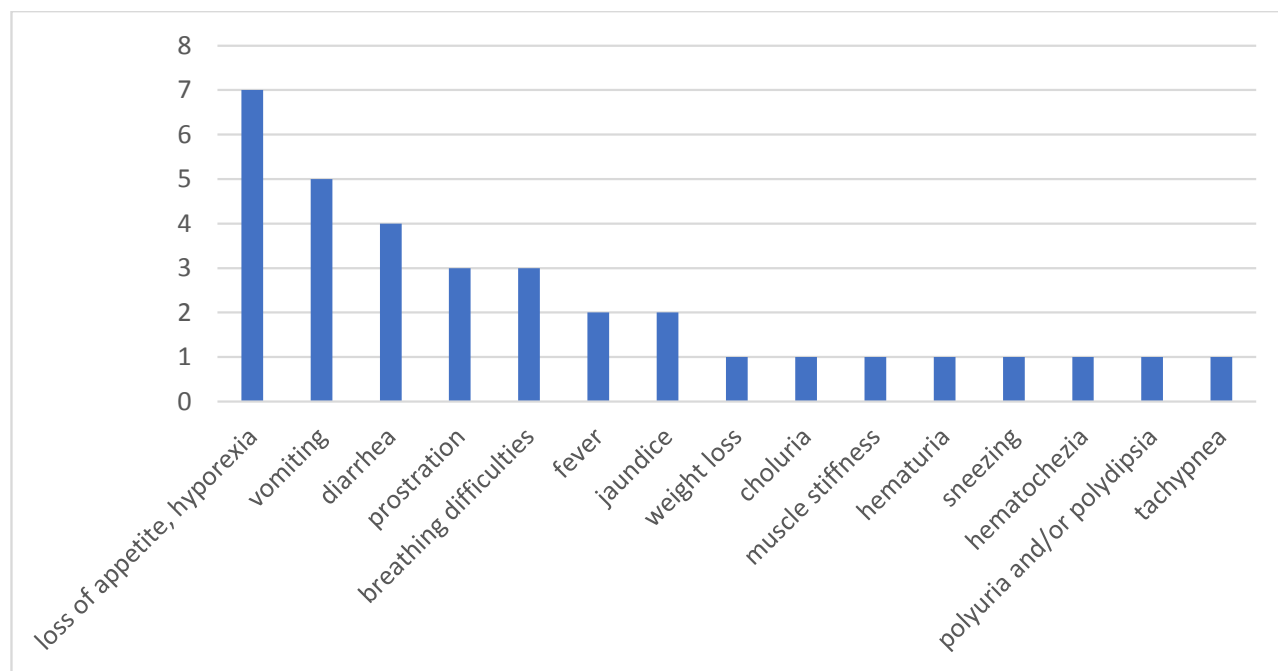


Figure 1. Frequency of clinical signs observed in dogs diagnosed with leptospirosis, treated at the UFU Veterinary Hospital, 2020.

The greater probability and tendency of infection in adult male dogs has also been reported in other studies (Azócar-Aedo & Monti, 2016; Ricardo et al., 2020). The male behavioral characteristics of smelling and licking urine, marking their territory and their reproductive instinct could increase the risk of exposure to pathogenic *Leptospira*. In addition to the greater exposure of male dogs, the probability of infection also increases significantly in adult free-ranging dogs since they may come into contact with contaminated water and garbage (Azócar-Aedo & Monti, 2016; Ricardo et al., 2020).

Vaccination is essential to disease prevention, but commercial vaccines only include serovars such as Canicola, Icterohaemorrhagiae, Grippotyphosa and Pomona, with others also featuring Copenhageni. Only one animal diagnosed with leptospirosis in the present study had a history of vaccination, which highlights the possibility of infection by serovars not incorporated into canine vaccines sold in Brazil. According to Ricardo et al. (2020), there was a resurgence of cases due to accidental infections with serovars not contained in vaccines, whereas Canicola infections have

declined in recent decades precisely due to the rise in canine vaccination.

Another factor to be considered is that vaccines only protect against specific serovars (Oliveira et al., 2012), that is, they do not provide immunity to serovars from other serogroups. In the present study, the fact that one dog was infected by a serovar not included in the vaccines sold in the region indicates the need to incorporate local serovars in order to increase protection in the target population.

The serum biochemical results (creatinine, urea, ALT, ALP) of the animals that tested positive and negative for canine leptospirosis in the diagnostic tests (MAT and/or qPCR) showed high creatinine, urea and ALP levels in the positive group in relation to the reference values established by Kaneko et al. (2008); however, only ALT remained within the normal range for the canine species (Table 4). For the negative group, only high concentrations of urea and ALP were observed. Comparison of the two groups showed no statistically significant difference between the values of the elements assessed ($p > 0.05$).

Table 4
Creatinine, urea, ALT and ALP levels in dogs submitted to diagnostic tests (MAT/qPCR) for canine leptospirosis treated at the UFU Veterinary Hospital, 2020

Biochemistry	Creatinine (mg/dL)	Urea* (mg/dL)	ALT (U/L)	ALP (U/L)
	Md ± standard error	Mean ± SD	Md ± standard error	Md ± standard error
Positive (n=8)	2.35 ± 3.37 ^a	244.43 ± 117.04 ^a	75.5 ± 16.51 ^a	551.00 ± 469.82 ^a
Negative (n=16)	1.07 ± 1.07 ^a	273.20 ± 144.45 ^a	96.00 ± 162.50 ^a	543.00 ± 266.89 ^a
p-value	0.34	0.60	0.21	0.75
Reference values**	(0.5 - 1.5)	(21 - 59.9)	(21 - 102)	(20 - 156)

* Student's t-test, with the remainder submitted to Wilcoxon's test at 5% significance. SD (standard deviation).

**Kaneko et al. (2008). Md= median.

Creatinine and urea levels were above the maximum limit in the positive group, characterizing azotemia, a common sign of canine leptospirosis. High levels of these elements in the blood are caused by the decline in renal perfusion and glomerular filtration rate due to the release of toxins and membrane components by *Leptospira*, which destroy the epithelial cells of the renal tubule (Greene et al., 2006). This biochemical alteration has been reported in dogs diagnosed with leptospirosis (Chideroli et al., 2016; Costa et al., 2013). Geisen et al. (2007) found that 57% of dogs with leptospirosis exhibited azotemia and 81% had high ALP levels.

Among the enzymes that indicate liver damage, only ALP levels were higher than the reference range in the positive group, indicating cholestasis. Since ALT levels in the blood peak within two days of injury (Pinna et al., 2010), this could explain the normal result recorded. Data in the literature indicate that dogs with leptospirosis may have normal levels of this enzyme (Thrall et al., 2015), which is consistent with our findings. Infectious diseases such as leptospirosis can cause hepatocellular dysfunction as a result of the toxins released by the bacteria, thereby raising blood levels of both bilirubin and ALP due to biliary stasis (Chideroli et al., 2016). According to Oliveira (2010), increased serum ALP and total bilirubin levels are more common than high ALT concentrations. The most frequent serum biochemical alterations

in dogs with leptospirosis are caused by kidney damage and include azotemia and hyperphosphatemia, reported in 80 and 100% of cases, respectively. Increased serum liver enzyme activity is rarely observed without renal injury (Geisen et al., 2007).

The hematology profiles of dogs that tested positive for leptospirosis in the MAT and/or qPCR are shown in Table 5. The erythrogram values of positive dogs are lower than the normal range for the species reported by Weiss and Wardrop (2010), characterizing normocytic normochromic anemia. Animals positive for leptospirosis exhibited immune-mediated hemolytic anemia, likely by IgM. Sykes et al. (2011) reported that complete blood counts may include mild to moderate nonregenerative anemia, but severe anemia is rare, as occurs in cases of gastrointestinal or pulmonary hemorrhage.

In the leukogram, the white blood cell count (WBC), segmented neutrophils and band cells were higher than the reference values, characterizing leukocytosis with a left shift. This suggests an inflammatory stimulus, likely due to leptospire multiplication in the bloodstream (leptospiremia). Schuller et al. (2015) reported neutrophilic leukocytosis, at times with a left shift, as the most common hematological alteration, similar to the results of the present study. However, the authors indicated that mild leukopenia may occur during leptospiremia.

Table 5
Mean and standard deviation of the hematological parameters in dogs that tested positive for leptospirosis treated at the UFU Veterinary Hospital, 2020

Parameter	Mean ± SD	Reference values*
RBC (x10 ⁶ / μL)	4.17 ± 1.42	5.5-8.5
Hb (g/dL)	8.63 ± 3.76	12-18
HCT (%)	26.8 ± 8.30	37-55
MVC (fL)	65.11 ± 3.36	60-77
MCHC (g/dL)	33.64 ± 1.39	30-36
RDW (%)	13.51 ± 1.83	11-16
WBC (mm ³)	34,628.57 ± 26,680.87	6,000 - 17,000
Bands (mm ³)	2,283± 2,655.06	0 - 300
Seg. neutro (mm ³)	28,759.71 ± 24,464.52	3,000 - 11,500
Eos. (mm ³)	17.29 ± 45.73	100 - 1,250
Lymphs (mm ³)	2,544.86 ± 1,368.14	1,000 - 4,800
Mon. (mm ³)	1,066.57 ± 425,03	150 - 1,350
Bas. (mm ³)	0.00 ± 0.00	Raros
Platelets (mm ³)	217,285.71 ± 224,847.88	175,000 - 500,000
MPV (fL)	11.68 ± 2.04	6.7 -11.1

RBC (red blood cell count); Hb (hemoglobin); HCT (hematocrit); VCM (mean corpuscular volume); MCHC (mean corpuscular hemoglobin concentration); RDW (red cell distribution width)); WBC (white blood cell count); Bands (band cells); Seg. neutro (segmented neutrophils); Eos (eosinophils); Bas (basophils); Mon (monocytes); Lymphs (lymphocytes); MPV (mean platelet volume). * Weiss and Wardrop (2010).

The platelet count was within the normal range for dogs. Thrombocytopenia was expected, as reported by Andrade et al. (2020), but did not occur. Thrombocytopenia can affect up to 58% of dogs and, when accompanied by evidence of acute kidney damage with or without liver damage, can heighten suspicion of leptospirosis (Sykes et al., 2011). The most frequent hematological alterations observed in the present study in dogs with clinical signs of leptospirosis were also reported by Geisen et al. (2007). The authors found that 45% of dogs displayed anemia with hematocrit results below the normal limit, 81% of the leukogram characterized by neutrophilia with a left shift and 53% exhibited thrombocytopenia.

Complementary tests such as a complete blood count and biochemical blood analyses should be ordered according to the needs of each animal. These tests contribute to early diagnosis of pathologies and help monitor overall health status.

The variety of nonspecific clinical symptoms makes clinical diagnosis of leptospirosis a complex process. Since there is no pathognomonic clinical sign or laboratory test alteration for *Leptospira* spp. infection in dogs, combining diagnostic tools depending on the phase of the disease seems to be the best strategy.

Conclusion

To diagnose acute canine leptospirosis, in addition to investigating the main clinical-pathological alterations, veterinarians should request an MAT of paired serum samples and a qPCR urine test. In this study, qPCR confirmed the true negatives. This complementary diagnostic approach helps guide the management and treatment of dogs with suspected leptospirosis.

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