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Short Communication

PCR sensitivity of peripheral blood of dogs co-infected with *Leishmania* spp. and *Ehrlichia* spp. in endemic area of Brazil

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Abstract

Introduction: Peripheral blood of 400 dogs infected with *Leishmania* and *Ehrlichia* were analyzed using polymerase chain reaction (PCR), and clinical signs were characterized. **Methods**: PCR and parasitological tests were conducted. **Results**: PCR was positive for *Leishmania* in 84.75%, and parasitological tests showed that 63.25% and 31.75% were positive for *Leishmania* and *Ehrlichia*, respectively. All animals showed more than three clinical signs. PCR results were negative for *Leishmania* in 15.25% of the samples. **Conclusions**: Conventional PCR of peripheral blood can be used for diagnosing canine visceral leishmaniasis in combination with other techniques, especially in uncertain cases that need species identification.

Keywords: Canine visceral leishmaniasis. PCR. Sensitivity. Peripheral blood.

Leishmaniasis is an endemic protozoonosis found in 97 countries. It accounts for about 20 to 30 thousand deaths, with more than 350 million people at risk of infection. In Brazil, leishmaniasis is endemic in 22 states; visceral leishmaniasis (VL) is mainly caused by *Leishmania infantum* (*Leishmania chagasi*), and American tegumentary leishmaniasis[1] by *Leishmania braziliensis* and *Leishmania amazonensis*.

Visceral canine leishmaniasis (VCL) is a chronic and progressive zoonosis with extreme relevance. It causes high mortality in humans in the endemic regions, which can be attributed to the high number of contagious dogs and intense parasitism. Thus, dogs are the most important reservoirs in the urban areas.

VCL diagnosis and correct identification of the species are important, especially in the regions with different species, in order to know the epidemiological profile and to create strategies for treatment and control. Several techniques are used for this purpose, however the sensitivity or specificity are not enough to distinguish the *Leishmania* species[2].

Molecular biology techniques are increasingly being used to diagnose and identify the *Leishmania* species, and to avoid possible cross-reaction with other diseases in the serological tests. We aimed to evaluate the sensitivity of PCR (polymerase chain reaction) analysis of peripheral blood samples of dogs infected with *Leishmania* and *Ehrlichia* and to characterize the clinical signs of the animals.

We used 400 samples, collected in 2016 by the Control Center of Zoonozes (CCZ) of Campo Grande City (Mato Grosso do Sul State, Brazil). Dogs were referred to CCZ for euthanasia because they were positive for VCL based on the immunochromatographic DPP™ rapid test and subsequent confirmation by ELISA, the serological tests recommended by the Brazilian Ministry of Health. Peripheral blood was collected from the jugular vein in EDTA tubes. For euthanasia, the animals were anesthetized (sodium thiopental), followed by administration of potassium chloride. A questionnaire was completed with information on the clinical signs and epidemiological data. This work was approved by the Ethics Committee for Animal Use, protocol 27/2016.

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From 300 µL of peripheral blood, DNA was extracted according to Araújo et al.[3]. The DNA pellet was hydrated with 50 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and frozen at −20°C.

Initially, LEISH-1 and LEISH-2 primers[4] were used to amplify the kDNA (kinetoplast) of *L. (L.) infantum*. The negative samples were subjected to PCR using 13A and 13B primers[5] to amplify the kDNA of the genus *Leishmania*. The reaction (25 µL) mixture was composed of 0.4 µM of each primer (Sigma), 1.5 mM $MgCl_2$, 0.2 mM dNTP (Invitrogen), 1.5 U Taq DNA Polymerase (Phoneutria), 1× enzyme buffer, and 2 µL of DNA. Amplification was performed in a thermocycler (BIORAD, T100 Thermal Cycler) at 95°C/5 min, followed by 35 cycles each at: 95°C/30 sec, 57.5°C (LEISH-1/LEISH-2 primers) and 61°C (13A/13B primers) for 30 sec each, 72°C/30 sec, and finally 72°C/10 min. Three positive controls [DNA of *L. (V.) braziliensis*, *L. (L.) amazonensis,* and *L. (L.) infantum*] and a negative (water) control were used.

Samples with negative PCR results for *Leishmania* were subjected to PCR with internal canine control. Specific primers for β-actin Forward (5′-CTTCTACAACGAGCTGCGCG-3′) and Reverse (5'-TCATGAGGTAGTCGGTCAGG-3') were used (GenBank: NM_001195845.1). The reaction (15 µL) mixture was composed of 0.4 mM of each primer (Sigma), 1.0 mM $MgCl_2$, 0.2 mM dNTP (Invitrogen), 1.5 U Taq DNA Polymerase (Phoneutria), 1× enzyme buffer, and 1 µL of DNA. Amplification was performed in the thermocycler at 95°C/5 min, followed by 35 cycles each at: 95°C/30 sec, 54.9°C/30 sec, 72°C/1 min, and finally at 72°C/10 min. A negative control (water) was used.

For electrophoresis, 8 µL of the amplified product was loaded on agarose gel stained with ethidium bromide. Presence of bands was identified in a transilluminator (Loccus).

Peripheral blood smears prepared using glass slides were stained with Giemsa for studying *Ehrlichia* and *Leishmania* (parasitological test).

The 400 analyzed dogs (**Table 1**) belonged to different ages, races, sizes, and genders; some of them had been given the anti-rabies vaccine and majority (76.0%) were bred. Some of this information was not found because the animal files were not dated; however, biological samples from such animals were also analyzed.

The PCR results were positive for 84.75% of the samples. Among the samples subjected to parasitological test, 63.25% were positive for *Leishmania* and 31.75% for *Ehrlichia*. Among the former, 38.94% were negative for *Leishmania* and 29.79% were positive for *Ehrlichia,* based on the parasitological test. Among the samples negative for *Leishmania* (15.25%) according to PCR analysis, the parasitological test showed that 75.41% were positivity for *Leishmania* and 42.62% for *Ehrlichia* (**Table 2**).

All the animals showed more than three VCL-compatible clinical signs. Among the mono-infected dogs, the proportions of samples that were positive for *Leishmania* were 87.18% and 56.78% according to the PCR and parasitological test, respectively. Among the co-infected dogs, 79.53% and 77.17% were positive for *Leishmania* based on the PCR and parasitological analyses, respectively (**Table 2**).

Many samples were identified as positive based on PCR analysis as compared to those identified by serological testing. PCR has been used for the diagnosis of diseases, and for epidemiological studies; it is the most sensitive technique.

The analyzed animals had previously been diagnosed as serologically positive for VCL by CCZ using the DPP™ method and ELISA. According to Hirschmann[6], this method showed a sensitivity of 30% and a specificity of 94.8%, and the best results can be obtained by a combination of DPP™/RIFI and standard gold ELISA. Although serological tests are more sensitive than molecular tests, many factors such as quantities of serum antibodies that are higher than the quantity of parasite DNA, may affect the specificity. Therefore, PCR is a reliable methodological alternative, due to high sensitivity and specificity.

Among the studied dogs, 31.75% presented co-infection of *Ehrlichia*; and among the animals that were PCR-negative

TABLE 1: Gender, size, vaccination, and age distributions of the 400 sampled dogs.

Gender	n (%)	Vaccination	n (%)
Male	160 (40,0)	Rabies	248 (62,0)
Female	226 (56,5)	Not rabies	38 (9,5)
NR*	14 (3,5)	NR*	114 (28,5)
Sze	**n (%)**	**Age**	**n (%)**
Small	188 (47,0)	0 to 6 months	10 (2,5)
Medium	147 (36,75)	7 to 12 months	58 (14,5)
Large	61 (15,25)	Over 12 months	321 (80,25)
NR*	4 (1,0)	NR*	11 (2,75)

***NR:** it was not rated (no information).

TABLE 2: Individual breakup of the number of dogs experiencing each of the main clinical symptoms; serological and PCR test results of the peripheral blood samples of dogs; and their correlation.

Clinical symptoms	All dogs (n=400)			Mono-infected dogs (n=273)**			Co-infected dogs (n=127)***		
	Ser*	PCR		Ser*	PCR		Ser*	PCR	
	Pos n (%)	Pos n (%)	Neg n (%)	Pos n (%)	Pos n (%)	Neg n (%)	Pos n (%)	Pos n (%)	Neg n (%)
	400 (100)	339 (84.75)	61 (15.25)	273 (100)	238 (87.72)	35 (12.8)	127 (100)	101 (79.53)	26 (20.47)
Alopecia****	344 (86.0)	292 (86.14)	52 (85.25)	226 (82.76)	198 (83.19)	28 (80.0)	118 (92.91)	94 (93.07)	24 (92.31)
Deep anemia	251 (62.75)	219 (64.6)	32 (52.46)	173 (63.37)	158 (66.39)	15 (42.86)	78 (61.42)	61 (60.4)	17 (65.38)
Skin peeling	329 (82.25)	279 (82.3)	50 (81.97)	216 (79.12)	189 (79.41)	27 (77.14)	113 (88.98)	90 (89.11)	23 (88.46)
Weight loss	272 (68.0)	234 (69.03)	38 (62.3)	187 (68.5)	167 (70.17)	20 (57.14)	85 (66.93)	67 (66.34)	18 (69.23)
Ear tip lesion	273 (68.25)	229 (67.55)	44 (72.13)	185 (67.77)	160 (67.23)	25 (71.43)	88 (69.29)	69 (68.32)	19 (73.08)
Lymphadenopathy	242 (60.5)	211 (62.24)	31 (50.82)	176 (64.47)	156 (65.55)	20 (57.14)	66 (51.97)	55 (54.46)	11 (42.31)
Onychogrifose	295 (73.75)	254 (74.93)	41 (67.21)	196 (71.79)	175 (73.53)	21 (60.0)	99 (77.95)	79 (78.22)	20 (76.92)
Purulent nasal secretion	230 (57.5)	196 (57.82)	34 (55.74)	144 (52.75)	125 (52.52)	19 (54.29)	86 (67.72)	71 (70.3)	15 (57.69)
Purulent ocular secretion	275 (68.75)	232 (68.44)	43 (70.49)	182 (66.67)	156 (65.55)	26 (74.29)	93 (73.23)	76 (75.25)	17 (65.38)
Hepatosplenomegaly*****	67 (16.75)	60 (17.7)	7 (11.48)	56 (20.51)	51 (21.43)	5 (14.29)	11 (8.66)	9 (8.91)	2 (7.69.0)
NR******	6 (1.5)	4 (1.18%)	2 (3.28)	4 (1.47)	2 (0.84)	2 (5.71)	2 (1.57)	2 (1.98)	0 (0.0)

***Ser:** serological tests; ****Mono-infected dogs:** serological positive for *Leishmania* and parasitological negative for *Ehrlichia*; *****Co-infected dogs:** serological positive for *Leishmania* and parasitological positive for *Ehrlichia*; ******Alopecia:** generalize or local; *******Hepatosplenomegaly:** although it was not one of the most frequent signs, it was frequent in VCL; ********NR:** it was not rated (no information); **PCR:** polymerase chain reaction.

for *Leishmania*, 42.62% were positive for *Ehrlichia*. Clinical signs of the dogs that were PCR-negative for leishmaniasis may indicate *Ehrlichia* infection, because both the parasites show similar clinical signs[7]. Failed serological tests have been reported as false-positives due to cross-reactions with other pathologies, and due to chronic and old infections[7,8]. The Bio-Manguinhos ELISA kits use the promastigotes of *Leishmania major*, the species that causes cutaneous leishmaniasis, as the antigen, which generates false results. Although cross-reactivity in animals infected with *Ehrlichia* is a limitation of the Bio-Manguinhos kits, the combination of ELISA and DPP™ significantly improves sensitivity and specificity[9] and overcomes this limitation. Combination of serological methods and molecular tests increases the accuracy of disease detection. Thus, peripheral blood PCR of kDNA can be combined with the serological tests and clinical signs to increase the accuracy of VCL diagnosis.

Based on PCR analysis 15.25% of the animals were negative for *Leishmania*, despite clinical signs of VCL. The sensitivity and specificity PCR can be affected by the choice of primers, methodology used for obtaining the genetic material, type of sample, parasitemia, and the presence of inhibitors[10].

One of the important advantages of using peripheral blood is that the collection is less invasive than from bone marrow, lymph node, and spleen aspirates, and the samples do not require special processing. However, the parasite concentration of the peripheral blood is lower than that of the bone marrow, lymph nodes, and spleen. Another disadvantage of peripheral blood is the presence of inhibitors that affect PCR sensitivity[11].

The choice of the primers is important, because it can influence the sensitivity of the technique, in addition to allowing the differentiation of subgenera and species. The combination of two pairs of primers increased PCR sensitivity and indicated the possibility of the presence of other animal-infecting *Leishmania* species. Recently LEISH1/LEISH2 primers amplified *L. (V.) braziliensis*, contrary to the possibility of identifying only *L. (L.) infantum*; however, infection by the former is low in dogs and thus needs another method of identification[12].

All the control samples were positive for *Leishmania* in the PCR analysis. The DNA extraction method interferes with PCR sensitivity. Extracting DNA from the blood samples was efficient. The PCR analysis showed that all the samples were positive for the canine β-actin gene, representing the absence of PCR inhibitors and excellent DNA integrity.

PCR is essential for the detection and identification of the protozoan involved in the advancement of clinical signs. In addition to monitoring the parasite load, the possibility of analyzing different clinical samples with high sensitivity and specificity makes PCR an undoubtedly advantageous method when compared to the traditional diagnostic methods.

Clinical signs of leishmaniasis can vary because the disease shows several pathological mechanisms. Among the several clinical signs and symptoms, skin lesions, generalized lymphadenomegaly, progressive weight loss, muscular atrophy, decreased appetite, lethargy, splenomegaly, ocular lesions, epistaxis, onychogryphosis, vomiting, and diarrhea are prominent. All the dogs studied showed more than three signs.

Alopecia and skin peeling, the classical dermatological alterations of leishmaniasis, were observed in most of the dogs; the dermatitis varied in extent and severity.

Anemia was observed in 62.75% of the animals. This is commonly caused by chronic kidney disease or because reduction of erythropoiesis in chronic diseases, which is aggravated by blood loss, immunosuppression, or destruction of blood cells. Co-infection with *Ehrlichia* also contributed to anemia.

Weight loss was observed in 68.0% of the animals. This finding is in agreement with other studies[13,14] and can be explained by albuminuria triggered by protein imbalance and gastric mucosal involvement.

Ear tip lesions were observed in 68.25% of the animals. Lesions are more frequently located at the ear tip, muzzle, face, and ears, because they are more exposed.

Lymphadenopathy was detected in 60.5% of the animals, which was also described in other studies[15]. The increase in cell numbers of the phagocytic mononuclear system when the infection is installed, explains this.

Onychogryphosis, the most striking features of VCL, was detected in majority of the animals (73.75%). The parasite can stimulate the nail matrix to grow, and the apathy of animal decreases its movements; therefore, there is no natural nail wear.

Purulent ocular secretion occurred in 68.75% of the animals, which may be due to deposition of immunocomplexes and anti-*Leishmania* antibodies in ocular tissues.

Hepatosplenomegaly, although infrequent in the studied animals, is a common clinical sign, which may occur due to B cell and macrophage production, and proliferation of amastigotes.

Although the analyzed animals had previously been diagnosed with VCL at CCZ, it is important to emphasize that the objective, among other points, is not to question the adopted methods, but to confirm the positive samples through standardization of PCR as a complementary method to conventional tests, especially in uncertain cases, as in the case of asymptomatic animals, which require species identification, and when serological methods are non-resolute. The samples used in this study to evaluate the sensitivity of conventional PCR reflect the reality of CCZ routine. The present work is the first step of a more complex study that aims to validate the complementary molecular test as routine exams.

The clinical picture of mono-infected *Leishmania* cases does not change with co-infection of *Ehrlichia*, although the latter may aggravate the clinical signs. In addition, clinical proximity and endemicity between VCL and ehrlichiosis may make diagnosis difficult. In areas endemic for leishmaniasis and ehrlichiosis, conventional PCR can be used for the diagnosis of VCL in combination with other traditional techniques that identify co-infection of *Ehrlichia* spp., and especially in dubious cases that need species identification.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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