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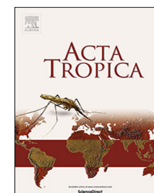
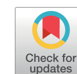


Detection of mixed *Leishmania* infections in dogs from an endemic area in southeastern Brazil



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ABSTRACT

In Brazil, the visceral leishmaniasis (VL) is caused by *Leishmania infantum*, while the tegumentary leishmaniasis (TL) etiological agents are mainly *Leishmania braziliensis* and *Leishmania amazonensis*. The canine visceral leishmaniasis (CVL) diagnosis is an important step of the VL control program in Brazil, which involves the elimination of infected dogs, the main urban VL reservoirs. The current serology-based diagnostic tests have shown cross-reactivity between these three species, whereas molecular diagnosis allows high sensitivity and specie identification. In the present study, 349 dogs of the metropolitan region of Belo Horizonte (Minas Gerais state) were screened by conjunctival swab and the samples analyzed by ITS-1 nested PCR. Thirty dogs (8.5%) tested positive. The RFLP of amplicons using HaeIII demonstrated that 17/30 samples presented a banding pattern compatible with *L. infantum*, 4/30 matched with *L. amazonenis*, 1/30 with *L. braziliensis* and 8/30 showed a mixed infection pattern. The samples that were distinct of *L. infantum* or presented a mixed pattern were submitted to RFPL with HaeIII and RsaI enzymes that confirmed the mixed pattern. Such patterns were also confirmed by Sanger Sequencing. The results pointed eight dogs with mixed infections and the establishment of TL causing species in the Belo Horizonte dog population. These findings highlight the need for more comprehensive epidemiological studies, since the TL transmission profile might be changing. This study also shows the potential of the ITS1-nPCR associated with RFLP for the proper *Leishmania* diagnosis and typing in the dog population.

1. Introduction

Leishmaniasis is a group of diseases with zoonotic and anthroponotic character, caused by species of the *Leishmania* genus (Ross, 1903). The disease is classified as a neglected tropical disease and currently endemic in 98 countries and territories, endangering over 350 million people. In Brazil, the main clinical disease manifestations are the tegumentary leishmaniasis (TL), currently caused by *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*, and the visceral leishmaniasis (VL), also known as Calazar, caused by *Leishmania (Leishmania) infantum*. In the Americas, VL human cases are present in 12 countries and 96% of these cases are reported solely in Brazil (World Health Organization, 2017).

Due to its proximity with the humans, dogs (*Canis familiaris*) are the main VL domestic reservoirs in urban areas (Baneth et al., 2008). Dog infection usually precedes the human infection and for that reason the Brazilian government has instituted the euthanasia of infected dogs as one of the main control policies in endemic areas (Ministério da Saúde, 2019). The euthanasia of infected dogs still generates a lot of controversy since it affects directly the human population. Recent studies have suggested that parasite identification prior to the euthanasia might be useful to avoid unnecessary culling of dogs, since the urban dog population is currently infected with species other than *L. infantum* (Madeira et al., 2006; Ferreira et al., 2015; Sanches et al., 2016). Parasite species identification may be useful as well for prevention of outbreaks due to other leishmaniasis forms.

Canine visceral leishmaniasis (CVL) prevalence in Brazil endemic areas ranges from 5.9 to 29.8% (França-Silva et al., 2003; Malaquias

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et al., 2007; Rondon et al., 2008; Lopes et al., 2010; Leite et al., 2015). In the past few years the number of VL human cases in the Metropolitan Region of Belo Horizonte (Minas Gerais state, Brazil) has increased, suggesting an elevation in the disease transmission rate (dos Reis et al., 2017).

The conjunctival swab (CS) sample is acquired by a non-invasive procedure that uses a sterile swab for sampling the dog conjunctiva and it has been extensively consolidated as a sampling method to obtain *Leishmania* DNA from infected dogs for PCR diagnosis (Strauss-Ayali et al., 2004; Ferreira et al., 2008; Pilatti et al., 2009; Leite et al., 2015). With the objective of identifying the *Leishmania* species infecting the canine population of the Metropolitan Region of Belo Horizonte, CS samples were obtained from dogs and amplified by the internal transcribed spacer-1 nested PCR (ITS-1 nPCR). The *Leishmania* species identification in the positive samples was performed by Restriction Fragment Length Polymorphism (RFLP) and Sanger Sequencing.

2. Materials and methods

2.1. Experiment ethics

The present study was approved by the Committee of Ethics in Animal Experimentation of the Universidade Federal de Minas Gerais (UFMG) (protocol no. 001/2011) and by the City Council of Belo Horizonte (protocol no.0344.0.000.410-11). All procedures were performed in agreement with the guidelines established by the Brazilian Animal Experimental College (COBEA) and by the Brazilian Federal Law 11794 of the 2008. The owners of the dogs enrolled in this project were aware of the research purposes. They were also required to sign an informed consent form before the sample collection.

2.2. Dog sampling

The study was conducted in the North Sanitary District of Belo Horizonte, which covers an area of 34.32 km^2. The North Sanitary District borders the Northeast, Pampulha and Venda Nova districts, besides the cities of Santa Luzia and Vespasiano. The present study was accomplished in collaboration with the Municipality Health Service of Belo Horizonte, during the years of 2012 and 2013. Blood and CS samples were randomly obtained from 349 dogs. The dogs were diagnosed by serological tests (ELISA and IFAT) and CS associated to the ITS1 nested PCR. The collection of peripheral blood on filter paper for ELISA and IFAT assays was performed according to the Brazilian Ministry of Health guidelines (Ministério da Saúde, 2019). The conjunctival swab samples were collected from both conjunctives using sterile cotton swabs manufactured for bacteriological isolation. The samples were stored at 8 °C and processed for DNA extraction in the same day, maximum 8 h after the collection.

2.3. Serological tests

The serological tests were executed by the Laboratory of Zoonotic Disease Control Department (LABZOO) of Belo Horizonte. According to the Brazilian Ministry of Health guidelines (Ministério da Saúde, 2019). Two serological tests were used: enzymelinked immunosorbent assay (ELISA – *EIE* – Canine Visceral Leishmaniasis produced by Bio-Manguinhos/Fiocruz, Brazil) and the immunofluorescence antibody test (IFAT – *IFI* – Canine Visceral Leishmaniasis produced by Bio-Manguinhos/Fiocruz, Brazil). The consolidated serological result was considered positive when ELISA and IFAT were simultaneously reagent ($\geq$1:80).

2.4. Clinical evaluation

The animals were classified in three different groups according to their clinical signs: Asymptomatic (As), dogs without any apparent clinical sign; oligosymptomatic (Os), dogs with up to two clinical signs; polysymptomatic (Ps), dogs that presented three or more typical clinical signs for CVL.

2.5. DNA extraction and purification

The DNA purification from CS was carried out as described by Leite et al. (2015). Swabs from both conjunctives of the same dog were joined and processed as a single sample. The swabs received 300 µL of lysis buffer (50 mMol/L Tris, 50 mMol/L NaCL, and 10 mMol/L EDTA, pH 8.0) containing proteinase K (250 µg/mL) and Triton X-100 (1%). After incubating for 2 h at 56 °C, the solution was eluted from the cotton, transferred into to DNAse-free sterile microtubes (Eppendorf®, Hamburg, Germany) and mixed with 500 µL of 75% Tris-saturated phenol (Sigma, St. Louis, MO), 25% chloroform-isoamyl alcohol. The organic phase was separated from the aqueous phase by centrifugation at 12,000 x g for 5 min which was transferred to a new microtube. The extraction was repeated with 500 µL of 50% phenol, 50% chloroform-isoamyl alcohol and once with 100% chloroform-isoamyl alcohol. The DNA was precipitated with one volume of isopropanol-sodium acetate and washed with 75% ethanol. The DNA pellet was suspended in 50 µL of Tris-EDTA buffer (10 mMol/L Tris and 1mMol/L EDTA, pH 8.0). The DNA was quantified with NanoVue (GE Healthcare, Chicago, IL) and stored at – 20 °C until being used. The DNA concentration found in the samples ranged from 23 µg/µL to 184 µg/µL.

2.6. β-Globin real time PCR

In order to evaluate the DNA sample integrity, a real time PCR for the constitutive gene of the canine β-globin was performed. The primers were used as described previously and should amplify 118 bp (Tm 79 °C) fragment (Greer et al., 1991). The real time PCR was conducted in a total volume of 12.5 µL, containing 3 pmol of each primer 5'-CAA CTT CAT CCA CGT TCA CC-3' and 5'-ACA CAA CTG TGT TCA CTA GC-3', 2.25 µL of 2 x SYBR Green reaction master mix® (Applied Biosystems®, Foster City, CA) and 2 µL of DNA at a final concentration of 20 µg/µL. The reactions were conducted as follow: 95 °C for 10 min, followed by 40 cycles of amplification (95 °C for 15 s, 60 °C for 1 min). The negative samples were excluded from the present study. The reactions were processed and analyzed by the StepOne™ System (Applied Biosystems®)

2.7. Internal transcribed spacer-1 nested PCR (ITS-1 nPCR)

The samples were analyzed by the internal transcribed spacer-1 nested PCR (ITS-1 nPCR). A positive control consisting of genomic DNA from *L. infantum* (strain MHOM/1973/ BH46) was used at 1.0 ng/µL. A negative control without DNA was included in all tests. This PCR protocol was adapted from Schönian et al. (2003). Primers targeting internal transcribed spacer-1 (ITS1) between the genes coding for SSU rRNA and 5.8S rRNA were used. For the first amplification, 10.0 µL of DNA solution (˜20 µg/µL) was added to 40.0 µL of PCR mix containing 15 pmol of the primers 5´- CTGGATCATTTTCCGATG-3´ and 5´.TGAT ACCACTTATCGCACTT-3´ and 0.2 mM of deoxynucleoside triphosphate, 2 mM of MgCl2, 5 mM of KCl, 75 mM of Tris-HCl (pH 9.0), 2.0 mM of (NH4)SO4, and 1.4 U of Taq DNA polymerase (Ludwig Biotec, Porto Alegre, Brazil). The cycling conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The amplification products were revealed on 2% agarose gel stained with ethidium bromide. The PCR product size was between 300 and 350 bp. For the second amplification, 10.0 µL of a 1:40 dilution of the first PCR product was added to 15 µL of PCR mix under the same conditions as the first amplification but with the following primers (15 pmol each): 5´-CATTTTCCGATGATTACACC-3´ and 5´-CGTTCTTC AACGAAATAGG-3´. Positive samples yielded a PCR product of between

280 and 330 bp.

2.8. Restriction fragment length polymorphism (RFLP) analysis of the ITS-1 nPCR amplicons

For the polymorphism analysis, the ITS-1 nPCR products was digested with the enzymes HaeIII (Cupolillo et al., 1995; Dávila and Momen, 2000; Schönian et al., 2003) or RsaI (Promega Corporation, Madison, WI). The digestion with HaeIII was performed using 17 μL of the ITS-1 nPCR amplicons mixed with 2 μL of the restriction enzyme buffer and 5 U of the enzyme. In the digestion with RsaI, 16.5 μL of amplicons were mixed with 2 μL of the restriction enzyme buffer, 0.2 μg of BSA and 5 U of enzyme. All of the restriction mixes were incubated at 37 °C for 4 h and then subjected to electrophoresis in either high resolution 2% agarose gel stained with ethidium bromide 30% or acrylamide gel stained with silver nitrate. Controls of *L. infantum* (MHOM/1973/BH46). *L. amazonensis* (IFLA/BR/67/PH8) and *L. braziliensis* (MHOM/BR/75/M2903) were used. The analysis of the RsaI RFLP gel was established between 100 and 250bp.

2.9. DNA sequencing

The nested PCR targeting the ITS-1 region was performed using the *Pfu* high fidelity polymerase enzyme (Promega Corporation). In this case for the first amplification, to 5.0 μL of DNA solution were added to 20.0 μL of the PCR mix containing 15 pmol of the primers, 0.2 mM of deoxynucleoside triphosphate, 2 mM of MgCl2, 2.5 μL of the *Pfu* DNA Polymerase 10x Buffer and 0.6 U of *Pfu* high fidelity polymerase. The ciclyng was conducted as described above. To perform the second reaction of the nested PCR, 25 μL of the first reaction were diluted in 1 mL of ultrapure sterile water and subjected to amplification only changing the second set of primers. Then, four μL of the amplicons were incubated at room temperature with the cloning mix, composed by 1 μL of Salt Solution and 1 μL of TOPO® Vector, for 30 min. The cloned products were transformed into electrocompetent *Escherichia coli* XL1blue. Colonies were selected and sequenced by the Sanger Sequencing method. The sequences obtained were analyzed with the DNA Baser Sequence Assembly Software and aligned with all the *Leishmania* sequences available in GenBank® using the Blastn tool.

3. Results

The canine housekeeping gene β-globin amplification was used to evaluate the DNA samples integrity. All samples included in the present study were positive for the canine β-globin real time PCR. The ITS-1 nPCR detected the ˜300 bp amplicons in 30 (8.5%) of the 349 dogs evaluated. Fig. 1 shows an ITS-1 nPCR representative gel.

The analysis by RFLP of the amplicons digested with HaeIII demonstrated that 17 out of 30 samples presented a banding pattern compatible with *L. infantum*, 4 samples matched with *L. amazonenis*, 1 sample with *L. braziliensis* and 8 samples showed a banding pattern compatible with mixed infection. Fig. 2 shows the polymorphism patterns displayed by HaeIII RFLP in 15 of the ITS-1 nPCR positive dogs. Six of the eight dogs that presented the mixed patter of infection were polysymptomatic, and four of them were positive in the serology (Table 1).

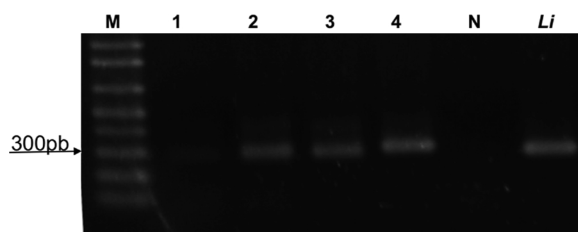

Fig. 1. Agarose gel stained with ethidium bromide containing ITS-1 nPCR amplicons of four samples. (M) Molecular weight standard of 100 bp; (N) negative control, (Li) positive control of *L. infantum* (strain MHOM/1973/ BH46); (2–4) positive CS dog samples.

To confirm the findings related to dog infections due to species other than *L. infantum* and to clarify the mixed pattern presented with HaeIII, it was performed the RFLP analysis using the restriction enzyme RsaI. Fig. 3 shows the polymorphism patterns derived from the digestion with RsaI for these 13 samples.

The samples 1, 6 and 13, identified as *L. amazonensis* by HaeIII RFLP, were confirmed as *L. amazonensis* by the enzyme RsaI. The identification of the sample 11 as *L. braziliensis* by HaeIII was also confirmed using this enzyme. However, RsaI result for the sample 9 (initially identified as *L. amazonensis*) was inconclusive. The mixed infection pattern of samples 2, 3, 4, 5, 7, 8, 10 and 12 was corroborated by Rsal RFLP that exhibited for them digestion fragments related to more than one specie (Fig. 3) (Table 1).

The samples 1, 2, 3, 4 and 14 were sequenced. Sample 1 and 4 were confirmed as *L. amazonensis* and sample 14 *as L. infantum. L. amazonensis* and *L. braziliensis* sequences were obtained for sample 2 and *L. amazonensis*, *L. braziliensis* and *L. infantum* sequences were found in the sample 3 (Table 1), thus confirming the mixed infection. Sample 4 showed a mixed infection profile in the RFLPs but the sequencing found only *L. amazonensis*. This finding probably reflects a limitation of the technique in the presence of a smaller number of *L. infantum* sequences in relation to the sequences of *L. amazonensis*. The sequences may be found in the NCBI GenBank under the access numbers from MH231222 to MH231229. Fig. 4 presents the nucleotide sequences alignment obtained from the ITS-1 region for *L. amazonensis* (MH231223), *L. braziliensis* (MH231225) and *L. infantum* (MH231229), showing that the sequences present enough differences to make the RFLP analysis possible using HaeIII and RsaI enzymes.

Our results point out the presence of 13 dogs infected with other species than *L. infantum* (*L. braziliensis* or *L. amazonensis*) or with mixed infections, including simultaneously two or three different *Leishmania* species.

4. Discussion

Our results found that 8.5% of the evaluated dogs in the Belo Horizonte Metropolitan Region are infected with *Leishmania* species. The *Leishmania* infection in dogs of this region has been extensively reported and the area is classified as endemic for VL. The presence of TL etiologic agents, such as *L. braziliensis* and *L. amazonensis,* infecting dogs in this area has also been previously reported (de Andrade et al., 2006; Ferreira et al., 2015). Domestic dogs were described as the main reservoirs for *L. infantum* but also as hosts for *L. braziliensis* and *L. amazonensis* (Reithinger and Davies, 1999; Madeira et al., 2003; Tolezano et al., 2007; Sanches et al., 2016). However, the dog role in the TL transmission is not entirely understood and therefore, identifying the *Leishmania* species infecting dogs is essential to the proper diagnosis and to the epidemiological understanding and guiding of the control measures. The proximity of the dogs and native rodents might be promoting such infections with TL etiologic agents.

Tegumentary leishmaniasis causing species infecting dogs have been reported in other VL endemic areas in Brazil (Madeira et al., 2006; Ferreira et al., 2015; Sanches et al., 2016). The epidemiologic meaning of such findings is yet to be cleared, since the dog importance as *L. braziliensis* and *L. amazonensis* reservoir remains unknown. Visceral leishmaniasis is the main target of the Brazilian control policies for leishmaniasis and it is mainly caused by *L. infantum* in Brazil. Even though the main reservoirs for *L. amazonensis* are small rodents, in the past few years dog infection with this specie have been observed (Madeira et al., 2006; Ferreira et al., 2015; Sanches et al., 2016). In Minas Gerais state, the *L. amazonensis* presence is a recent finding and it has also been reported as responsible for visceral human cases

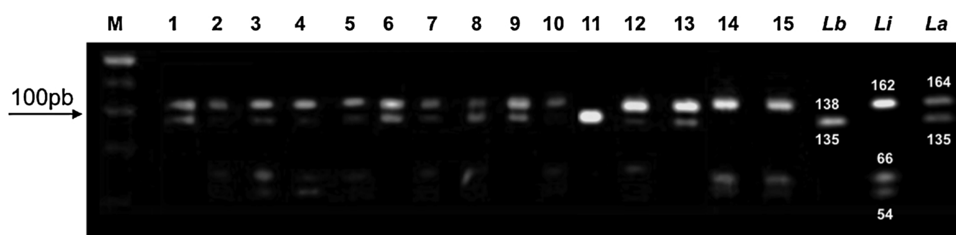

Fig. 2. Agarose gel stained with ethidium bromide containing the HaeIII RFLP fragments from 15 samples. (M) Molecular weight standard of 50 bp; (Lb) *L. braziliensis* (MHOM/BR/75/M2903); (Li) *L. infantum* (MHOM/1973/BH46); (La) *L. amazonensis* (IFLA/BR/67/PH8); (1 to 15) dog samples. Samples 2, 3, 4, 5, 7, 8 and 10 displayed mixed infection patterns. Samples 14 and 15 presented *L. infantum-like* digestion fragments. The sample 11 matches to *L. braziliensis.* Samples 1, 6, 9 and 13 were compatible with *L. amazonensis.*

Table 1
Diagnostic results and *Leishmania* species typing.

Dog	Clinical Evaluation	Serology		PCR		Species Identification		
		ELISA	IFAT	B-globin	ITS-1	HaeIII RFLP	RsaI RFLP	Sequencing
1	As	−	−	+	+	A	A	A
2	Ps	+	+	+	+	M	M	A, B
3	Ps	+	+	+	+	M	M	A, B, I
4	As	−	−	+	+	M	M	A
5	Ps	+	+	+	+	M	M	x
6	As	−	−	+	+	A	A	x
7	Ps	−	−	+	+	M	M	x
8	As	−	−	+	+	M	M	x
9	Ps	−	−	+	+	A	?	x
10	Ps	+	+	+	+	M	M	x
11	As	+	+	+	+	B	B	x
12	Ps	−	−	+	+	M	M	x
13	SII	−	−	+	+	A	A	x
14	As	−	−	+	+	I	x	I
15	Ps	+	+	+	+	I	x	x
16	Ps	+	−	+	+	I	x	x
17	As	−	−	+	+	I	x	x
18	Os	−	−	+	+	I	x	x
19	Ps	−	−	+	+	I	x	x
20	Ps	+	−	+	+	I	x	x
21	Ps	−	−	+	+	I	x	x
22	Ps	+	+	+	+	I	x	x
23	Ps	+	+	+	+	I	x	x
24	As	−	−	+	+	I	x	x
25	Os	−	−	+	+	I	x	x
26	As	−	−	+	+	I	x	x
27	As	−	−	+	+	I	x	x
28	Os	−	−	+	+	I	x	x
29	As	+	+	+	+	I	x	x
30	As	+	+	+	+	I	x	x

As: Asymptomatic; Os: Oligosymptomatic; Ps: Polysymptomatic; +: Positive; −: Negative; A: *L. amazonensis*; B: *L.braziliensis*; I: *L. infantum*; M: Mixed infection profile;? : Inconclusive; x: Not evaluated.

(Tolezano et al., 2007). In the present study, *L. amazonensis* DNA was verified in seven of thirty naturally infected dogs. These results suggest the establishment of *L. amazonensis* in urban areas.

The majority of the TL cases in Brazil are in rural and forest areas, however, there are recent reports in the urban areas as well (Benício et al., 2015). The latest phlebotominae fauna surveys in the Minas Gerais state indicated that the most abundant species in Belo Horizonte Metropolitan Region were *Lutzomyia whitimani*, which is mainly associated with the *L. braziliensis* transmission and secondarily with *L. amazonensis,* and *Lutzomyia longipalpis*, the main vector for *L. infantum* (Margonari et al., 2010; Saraiva et al., 2015). These data support the findings of the present study, once the phebotominae sandflies present in the area are potential vectors for the three *Leishmania* species found in Belo Horizonte.

The current serologic diagnostic methods adopted by the Health Ministry in Brazil were proven to be cross-reactive between *L. amazonensis, L. braziliensis* and *L. infantum* suggesting that these methodologies for targeting VL reservoirs might be overestimating the *L. infantum* prevalence in the serologic positive dogs, due the detection of the other species (Paz et al., 2018). In the present study, for example, the dogs 2 and 11 were positive for ELISA and RIFI. However, the dog 2 was simultaneously infected by *L. amazonensis* and *L. braziliensis*, and the dog 11 was infected with *L. braziliensis.* Considering that the VL control is based on the infected dog's euthanasia, the current control policies might be unnecessarily eliminating dogs. Such findings corroborate the need of improvement in the current diagnostic methods applied.

Only 12 of 30 (40%) positive ITS-1 nPCR dogs presented at least one positive serological test result (Table 1), confirming the high sensitivity of the molecular diagnosis in relation to the serologic diagnosis, as demonstrated in previous studies (Manna et al., 2004; de Assis et al., 2010; Leite et al., 2015). Interestingly, 5 polysymptomatic dogs with negative serology were found (Table 1). This type of result is commonly due to immunosuppressed animals or the presence of co-morbidities with similar symptoms to those of CVL.

The first reports of mixed infection in naturally infected dogs in the Brazil were of Rio de Janeiro state and such dogs were infected with both *L. infantum* and *L. braziliensis* (Madeira et al., 2006; Pires et al., 2019). Recently, in dogs from the São Paulo state a mixed RFLP profile suggested co-infection between *L. infantum* and *L. amazonensis* (Sanches et al., 2016). In a periurban area of the Minas Gerais, state a mixed infection profile involving *L. braziliensis* and *L. infantum* was found in small rodents (Ferreira et al., 2015). We report here, for the first time, the occurrence of dogs simultaneously infected by *L. infantum*, *L. braziliensis* and *L. amazonensis.*

The identification of the TL agents *L. amazonensis* and *L. braziliensis* in dogs from VL urban endemic areas is rising and understanding the meaning of such infections is key to maintaining the epidemiologic approaches updated. The findings of this study point out the need of improvement of the *Leishmania* control policies in Brazil, considering the constant epidemiologic changes. This work also shows that the ITS-1 nPCR associated with HaeIII and RsaI RFLP could contribute to the

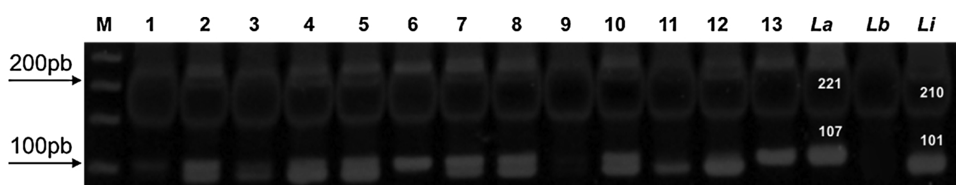

Fig. 3. Acrylamide gel stained with silver nitrate containing the RsaI RFLP fragments from the samples not identified as *L. infantum* or presenting a mixed infection pattern by HaeIII RFLP. (M) Molecular weight standard of 50 bp; (La) *L. amazonensis* (IFLA/BR/1967/PH8); (Lb) *L. braziliensis* (MHOM/BR/1975/M2903); (Li) *L. infantum* (MHOM/1973/BH46); (1 to 13) dog samples. The samples 2, 3, 4, 5, 7, 8, 10 and 12 displayed mixed infection patterns. The sample 11 was similar to *L. braziliensis* and samples 1, 6 and 13 displayed fragments compatible with *L. amazonensis*. The sample 9 pattern was inconclusive. The analysis was established between 100 and 250bp.

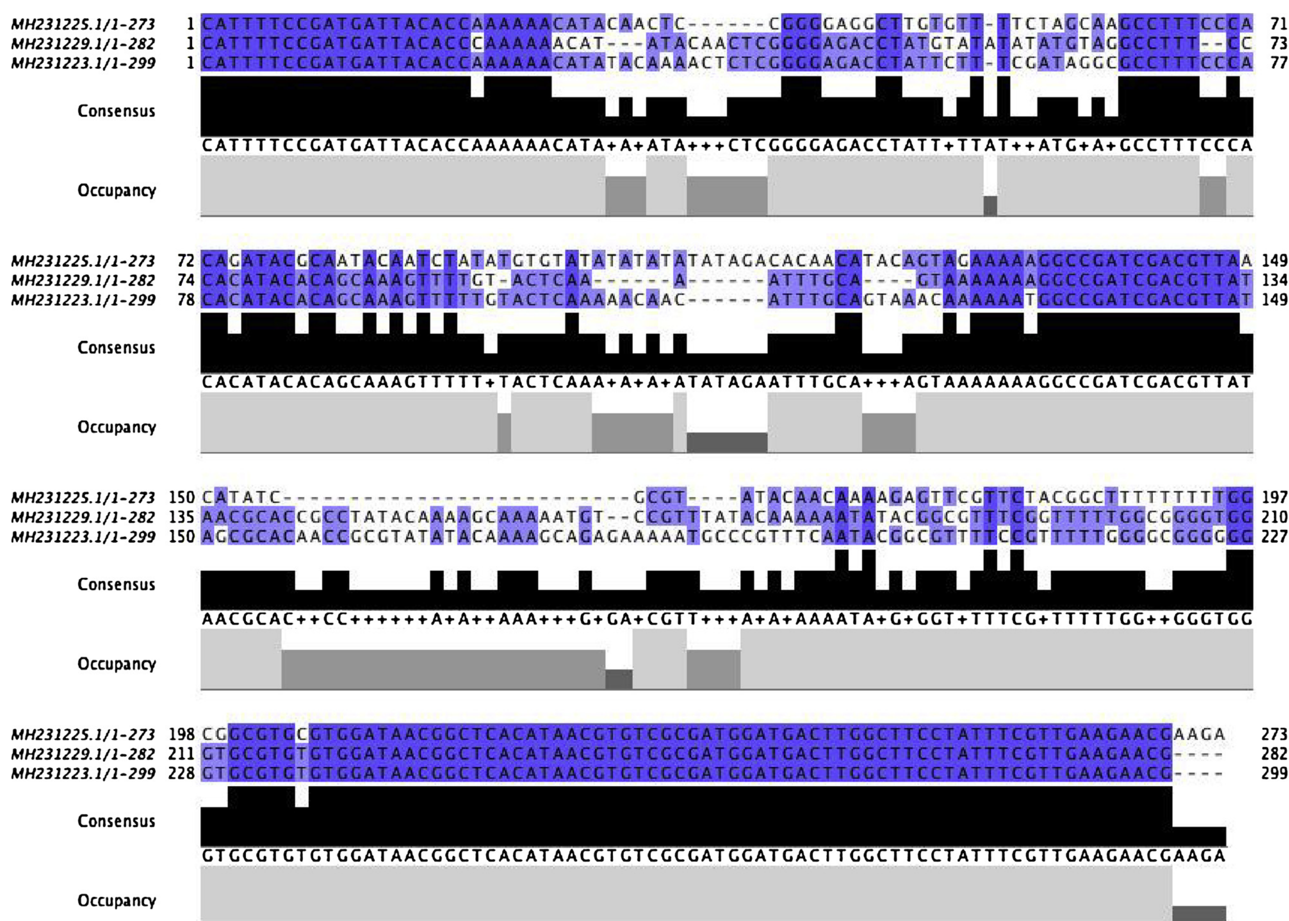

Fig. 4. Alignment of the nucleotide sequences obtained from the ITS-1 region for *L. amazonensis* (MH231223), *L. braziliensis* (MH231225) and *L. infantum* (MH231229). The alignment was generated with ClustalW and Jalview.

proper diagnosis and identification of the *Leishmania* species infecting the dog population.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgements

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