Revista da Sociedade Brasileira de Medicina Tropical 47(2):243-246, Mar-Apr, 2014
http://dx.doi.org/10.1590/0037-8682-0007-2013

Short Communication

Cutaneous and visceral leishmaniasis co-infection in dogs from Rio de Janeiro, Brazil: evaluation by specific PCR and RFLP-PCR assays

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ABSTRACT

Introduction: During a diagnostic evaluation of canine visceral leishmaniasis (VL), two of seventeen dogs were found to be co-infected by *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) chagasi.* **Methods:** Specific polymerase chain reaction (PCR) and restriction fragment length polymorphism-PCR (RFLP-PCR) assays were performed. **Results:** PCR assays for *Leishmania* subgenus identification followed by RFLP-PCR analysis in biopsies from cutaneous lesions and the spleen confirmed the presence of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) chagasi* in those fragments. **Conclusions:** This report reinforces the importance of using serological and molecular techniques in the epidemiological surveillance of canine populations in endemic areas in which both diseases are known to co-exist. In such cases, a reassessment of the control measures is required.

Keywords: Visceral leishmaniasis. Tegumentary leishmaniasis.
Restriction fragment length polymorphism-polymerase chain reaction.

Visceral leishmaniasis (VL) and tegumentary leishmaniasis (TL) are zoonoses of great importance for public health. In the State of Rio de Janeiro, *Leishmania (Viannia) braziliensis* is the most prevalent species implicated in the epidemiological cycle of TL. Its transmission occurs in periurban areas in which primitive rain forest vegetation is being depredated due to disorderly human occupation[1], and infections in man, dogs and horses have been reported[2,3]. Canine VL, which is caused by *Leishmania (Leishmania) chagasi,* is endemic in the Municipality of Rio de Janeiro. Dogs represent one of the main reservoirs in urban areas in which the disease has been observed due to several factors that influence the natural epidemiological scenario[4]. The overlapping transmission of TL caused by *L.* (*V.*) *braziliensis* and VL caused by *L.* (*L.*) *chagasi* has been reported in certain areas of the Municipality of Rio de Janeiro. Mixed infection with both parasites has already been reported in a patient[5] and in a dog[6]. Fortunately, according to Marzochi et al.[7], no new human cases of co-infection have been registered since, but there is concern about the persistence of canine seroprevalence. Control measures are based on interrupting the transmission cycle, which involves the diagnosis and treatment of human cases and vector control through insecticides and serological screening, with the subsequent culling of dogs found to be seropositive[4].

The present article discusses the detection of mixed TL and VL infections in two of seventeen dogs from endemic areas of Rio de Janeiro, Brazil, which tested seropositive by indirect immunofluorescence (IIF) analysis of serum samples. The occurrence of canine cases with both diseases in the same geographic area impairs the diagnosis and implementation of control measures.

The animals included in this study were referred to the Zoonosis Service of the *Instituto de Pesquisa Clínica Evandro Chagas-Fundação Oswaldo Cruz* (IPEC-FIOCRUZ) with an indication for euthanasia according to the recommendations of the Brazilian Program for the Control of Leishmaniasis[4] after serological tests by IIF on serum samples, which were performed by the Epidemiology Service of the Municipality of Rio de Janeiro. This study was approved by the Ethics Committee on Animal Experimentation of the *Fundação Oswaldo Cruz* (CEUA/FIOCRUZ; program nº L-023/06).

All studied dogs were from urban and periurban areas of the Municipality of Rio de Janeiro and presented IIF titers ranging from 1:80 to 1:1,280. In certain animals, clinical symptoms of VL were evident, whereas others were asymptomatic. Cutaneous lesions were frequent **(Table 1)**.

Fragments of the cutaneous lesions, intact skin from the scapular region, cervical lymph nodes and spleen from

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Received 29 January 2013
Accepted 25 March 2013

TABLE 1 - The serological titers, clinical status and PCR results of the 17 dogs from Rio de Janeiro.

			PCR with specific primers for *Leishmania donovani*				
Dog	Serology	Clinical status	cutaneous lesion	skin	spleen	cervical lymph node	popliteal lymph node
A28	1:160	asymptomatic	A	-	+	+	+
A29	1:80	symptomatic	A	+	+	-	+
A30	1:160	symptomatic	+	+	+	+	+
A33	1:640	symptomatic	A	+	-	+	+
A34	1:80	symptomatic	A	+	+	+	+
A36	1:1,280	asymptomatic	A	+	+	+	+
A37	1:1,280	asymptomatic	A	-	-	+	+
A39	1:640	symptomatic	A	+	+	+	+
A40	1:640	symptomatic	A	-	+	+	+
A41	1:640	asymptomatic	+	+	-	+	+
A42	1:320	asymptomatic	A	-	+	+	+
A43*	1:640	symptomatic	-	+	+	+	+
A44	1:320	symptomatic	A	+	+	+	+
A53	1:640	asymptomatic	A	+	+	+	+
A61	1:1,280	symptomatic	A	+	+	+	+
A62	1:1,280	asymptomatic	A	-	+	+	+
A63*	1:1,280	asymptomatic	-	+	+	+	+

PCR: polymerase chain reaction; A: absent; *mixed infection.

all animals were collected after thiopental-overdose euthanasia and submitted for polymerase chain reaction (PCR) analyses using primers for the variable regions of kinetoplast DNA (kDNA) minicircles. Specific primers for the *L. braziliensis* complex (5'-GGGGTTGGTGTAATATAGTGG-3' and 5'-CTAATTGTGCACGGGGAGG-3')[8] and for the *Leishmania donovani* complex (5'-CCAGTTTCCCGCCCCG-3' and 5'-GGGGTTGGTGTAAAATAG-3')[9] were adopted, as previously described[10,11]. Then, the amplified PCR products were visualized on agarose gels. Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) analyses were performed with a panel of four restriction enzymes (Msp I, Rsa I, Hinf I and Mbo I) to confirm the specificity of the amplified kDNA minicircle products.

All of the canine biopsy fragments, except for two from cutaneous lesions, produced the expected 800bp diagnostic bands after PCR with primers D1/D2. Those fragments of cutaneous lesions that tested negative were submitted to PCR assays with the primers B1/B2, and the expected 750bp diagnostic bands were observed **(Figure 1A)**.

PCR was performed in combination with RFLP-PCR to confirm the presence of *L.* (*V.*) *braziliensis* deoxyribonucleic acid (DNA) in the cutaneous lesion biopsies and *L.* (*L.*) *chagasi* DNA in the spleen and lymph node fragments from two dogs (dogs A43 and A63, **Table 1**). In **Figures 1B, 1C, 1D** and **1E**, the PCR/RFLP results confirming the mixed infection are observed. Because previous results from our group[12,13] have demonstrated that the restriction enzymes Msp I, Rsa I, Hinf I and Mbo I are the most appropriate for typing *Leishmania* species from the subgenera *Viannia* and *Leishmania*, these enzymes were adopted in the present study. The Msp I restriction enzyme linearizes the kDNA minicircles, displaying a major band of approximately 750bp in the case of *Leishmania (V.)* species. In contrast, a polymorphic restriction profile is always observed in *L (L.) chagasi*[12].

Herein, the Hinf I restriction patterns of the amplified parasitic DNA from cutaneous lesions were similar to the patterns of the *L. (V.) braziliensis* (MHOM/BR/75/M2903) reference strain kDNA. The Rsa I restriction patterns obtained with the amplified products from the spleen and lymph node fragments were also similar compared with the patterns of the *L. (L.) chagasi* (MHOM/BR/74/PP75) reference strain kDNA **(Figure 1C)**. Both subgenera were confirmed after digestion with Msp I **(Figure 1E)**. RFLP-PCR analysis with the restriction enzymes Msp I, Rsa I and Mbo I corroborated the PCR results, justifying the use of such a technique in *Leishmania* species identification.

In clinical-epidemiological surveillance, emphasizing the need to perform extensive sampling in dogs with cutaneous lesions is important, particularly in endemic areas in which

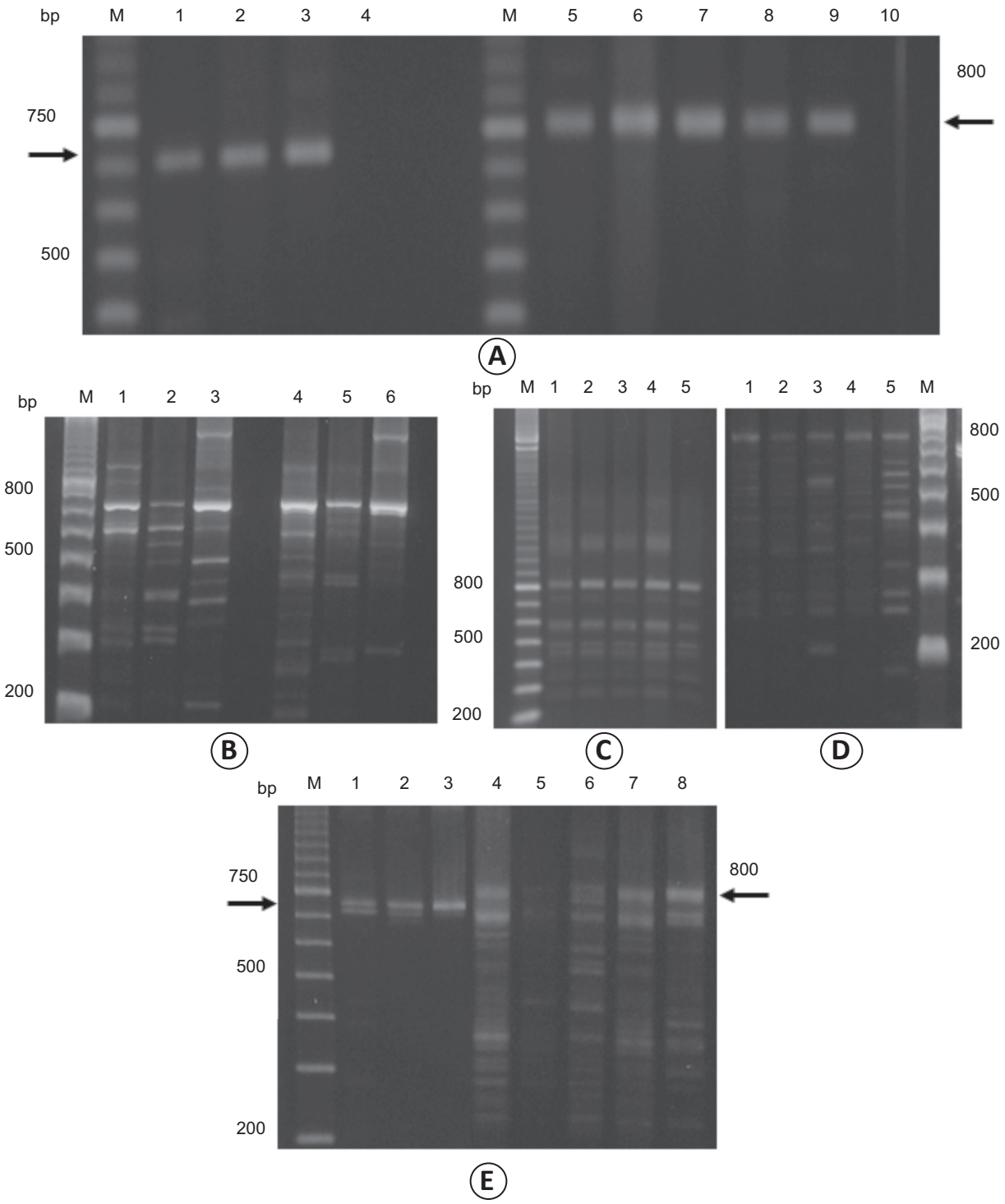

FIGURE 1 - A: Specific PCR products in 1.5% agarose gel: line M: DNA ladder, 100bp; primers B1/B2: line 1: dog A43, cutaneous lesion; line 2: dog A63, cutaneous lesion; line 3: *Leishmania (Viannia) braziliensis* reference strain; lines 4 and 10: negative controls; primers D1/D2: lines 5-6: dog A43, spleen fragment and cervical lymph node, respectively; lines 7-8: dog A63, spleen fragment and cervical lymph node, respectively; line 9: *Leishmania (Leishmania) chagasi* reference strain. B: RFLP analyses in high-resolution 1.8% agarose gel after digestion with the enzymes Rsa I and Hinf I: lines 1 and 4: dog A43, cutaneous lesion; lines 2 and 5: dog A63, cutaneous lesion; lines 3 and 6: *Leishmania (Viannia) braziliensis* reference strain. C and D: RFLP analyses with Rsa I and Mbo I: lines 1-2: dog A43, spleen and cervical lymph node, respectively; lines 3-4: dog A63, spleen and cervical lymph node, respectively; line 5: *Leishmania (Leishmania) chagasi* reference strain. E: RFLP analyses with Msp I: line 1: dog A43, cutaneous lesion; line 2: dog A63, cutaneous lesion; line 3: *Leishmania (Viannia) braziliensis* reference strain; lines 4-5: dog A43, spleen and cervical lymph node, respectively; line 6-7: dog A63, spleen and cervical lymph node, respectively; line 8: *Leishmania (Leishmania) chagasi* reference strain. PCR: polymerase chain reaction; DNA: deoxyribonucleic acid; RFLP: restriction fragment length polymorphisms.

both diseases overlap, such as certain rural areas in Rio de Janeiro. In these areas, where closely related etiological groups are present, the interpretation of serological data is a limiting aspect due to possible serological cross-reactions. Although *Trypanosoma cruzi* infection is unknown in the Municipality of Rio de Janeiro, a new species, *Trypanosoma caninum*, was recently described in dogs[14]. In this scenario, a reassessment of control measures is required.

The control of leishmaniasis is relatively complex, particularly in areas where both the tegumentary and visceral forms of the disease co-exist. As a control measure, the Brazilian government usually culls seropositive dogs[4]. However, according to a recent review[15], the strategy of killing dogs is hampered for several reasons, including the low accuracy of the methods used to assess the infectivity of dogs and the high replacement rate of these animals. In this scenario, a search for sensitive and specific molecular tools is needed to distinguish dogs infected with *L. (V.) braziliensis*, thus preventing unnecessary sacrifice. The results presented here show the usefulness of specific PCR assays and the RFLP technique for differentiating between *L. (V.) braziliensis* and *L. (L.) chagasi* and may contribute to providing support for control programs. Conversely, dogs with TL and VL co-infection would be subjected to euthanasia according to the guidelines of the Ministry of Health. Concerning control measures, including the detection and treatment of human cases, the disposal of dogs with VL, the efforts to control vectors with systematic indoor and outdoor spraying and the use of collars and mosquito nets impregnated with insecticides, the latter measure alone would likely be more efficient than the first two measures together. Finally, the development of human vaccines should also be considered as a high priority.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work received financial support from *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Fundação Carlos Chagas de Apoio a Pesquisa do Estado do Rio de Janeiro* (FAPERJ) and *Instituto Kinder do Brasil* (IKB). RS Pacheco is a Brazilian CNPq investigator.

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