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Occurrence of *Trypanosoma caninum* in areas overlapping with leishmaniasis in Brazil: what is the real impact of canine leishmaniasis control?

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ABSTRACT

Trypanosoma caninum is a parasite of the *Trypanosoma* genus recently described in the natural infection of dogs in the municipality of Rio de Janeiro, Brazil. Suspecting the existence of a natural cycle and the circulation of this new species, the objective of this study was the taxonomic identification of samples of *Trypanosoma* spp. isolated from dogs in different Brazilian regions. Parasites were solely obtained from skin fragments culture and characterized by nested-PCR targeting the partial sequence of 18S rRNA gene and PCR products were sequenced. Thirty-three samples, obtained in São Paulo, Minas Gerais, Goiás, Mato Grosso and Rio de Janeiro states were analyzed. PCR and sequencing showed that the isolates were genetically identical or closely similar and confirmed *T. caninum* identity. This report broadens the geographical distribution of *T. caninum* in Brazil and discusses the impact of the presence of this parasite in areas of canine leishmaniasis occurrence.

1. Introduction

The genus *Trypanosoma* includes a complex group of parasites that infect a broad range of hosts worldwide. According to the species, they may infect blood or other tissues, under the trypomastigote or amastigote forms, respectively.[1] Recently, a new species, *Trypanosoma caninum*, was described in domestic dogs in the state of Rio de Janeiro, Brazil, isolated into culture from intact skin fragments; attempts to isolate it from blood or other tissues from naturally infected dogs did not succeed.[2,3] Aspects related to the biological forms present in the vertebrate host and its natural cycle are still unknown. However, molecular characterisation shows it is not related to *T. cruzi* or *T. rangeli*, and analysis of partial SSU ribosomal DNA sequences give *T. pestanai* and a wombat trypanosome as the closest matches.

In Brazil, visceral leishmaniasis (VL) is a serious public health problem and the domestic dog is one of the targets for control actions because it is considered a major reservoir of *Leishmania* (*L.*) *chagasi* (= *L. infantum*), the VL etiological agent.[4] The Brazilian leishmaniasis control program recommends diagnosis and euthanasia of sera reactive dogs as control measures for VL.[5] In this context, the presence of other trypanosomatids, besides *Leishmania* parasites, infecting domestic dogs in overlapping areas,

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may constitute a confounding factor in canine diagnosis, since surveys are based on serological tools for the identification of positive animals.

Our team has monitored *Leishmania* species and the genetic variants that may be infecting domestic dogs,[6] and samples of *Trypanosoma* spp. were isolated during these studies from different Brazilian endemic areas. Morphological and biological aspects of these isolates show patterns very similar to *T. caninum*, however, the correct identification of these isolates is fundamental, since they come from VL endemic areas and evaluating the impact that *T. caninum* has in those areas is still a great challenge. Thus, the objective of this study was to characterize 33 samples of *Trypanosoma* spp. isolated from dogs and report the presence of *T. caninum* in different Brazilian regions, where cases of canine VL occur.

2. Materials and methods

2.1. Samples

The samples for this study were obtained during surveys of domestic dogs, the objective of which was the diagnosis of canine VL conducted in the states of Rio de Janeiro, comprising the municipalities of Rio de Janeiro, Niterói and Maricá; São Paulo (Bauru); Minas Gerais (Belo Horizonte); Mato Grosso (Cuiabá) and Goiás (Brasília). In each of these regions dogs were randomly evaluated, dogs of both sexes and aged six months or more being sampled. Only housed dogs participated in the study, and after clinical examination intact skin fragments were collected for parasite isolation in culture and molecular analysis by PCR.

2.2. Isolation of parasites from dogs

Three approximately 3 mm sized skin fragments were collected from each animal; two were culture processed and one was frozen at −20 °C for molecular studies. For isolation in culture the skin fragment was immersed in saline containing 100 μg of 5′fluorocytocine, 1000 IU of penicillin and 200 μg of streptomycin per milliliter and stored at 4 °C for 24 h according to the protocol described by Madeira et al.[7] After this period, each fragment was transferred aseptically to a biphasic culture medium NNN (Novy-Neal-Nicolle) and Schneider's *Drosophila* medium (Sigma, St. Louis, MO, USA) containing 10% of fetal calf serum, incubated at 26–28 °C and examined weekly for a maximum of 50 days. The parasites isolated were maintained in the same culture medium, weekly subcultured and expanded in Schneider's medium until reaching about 10^9 parasites/mL. The parasites were harvested and twice washed in PBS pH 7.2 and the pellets stored in liquid nitrogen to be used in molecular assays.

2.3. Molecular analysis

Genomic DNA of cultured trypanosomes was extracted using DNAzol (Invitrogen, Carlsbad, CA, USA) and for skin samples, the DNA was extracted using Wizard® Genomic DNA purification kit (Promega, Madison, Wisconsin, USA). The manufacturer's instructions were followed in both procedures. The PCR amplifications were perfomed in a nested-PCR that targeted a partial sequence of the 18S rRNA gene using oligonucleotides and reaction conditions as described by Smith et al.[8] External primers TRY927F (5′ GAAACAAGAAACACGGGAG 3′) and TRY927R (5′ CTACTGGGCAGCTTGGA 3′) were used in the first round and internal primers SSU561F (5′ TGGGATAACAAAGGAGCA 3′) and SSU561R (5′ CTGAGACTGTAACCTCAAAGC 3′) were used in the second round. The PCR products were run on a 1.5% agarose gel, stained with ethidium bromide, and visualized under UV light. After PCR, the amplified products obtained in the second round with cultured forms or skin fragments were purified using the QIAquick Purification Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions and the nucleotide sequences were determined with an automatic sequencer (3730 DNA Analyzer, Applied Biosystems, Carlsbad, CA, USA). All nucleotide sequences obtained in this study were edited by the Bioedit program (BioEdit Sequence Alignment Editor version 7.0.5.2, Ibis Therapeutics; Carlsbad, CA, USA)) and analyzed by the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The similarity percents were obtained comparing the 33 isolates with the sample of *T. caninum* (stock A27) already deposited in the GenBank (Accession no. GU385824).[3] Using the ClustalX program,[9] all sequences studied were aligned and compared each other and with sequences of *T. caninum* and other trypanosomatids available in GenBank.

3. Results

3.1. Samples

Thirty-three samples of *Trypanosoma* spp. were isolated from dogs in the states of São Paulo (Bauru, n = 6), Minas Gerais (Belo Horizonte, n = 3), Goiás (Brasília, n = 3), Mato Grosso (Cuiabá, n = 12) and Rio de Janeiro (Rio de Janeiro, n = 7, Niterói, n = 1 and Maricá, n = 1) during the surveys conducted between 2005 and 2011. All samples were obtained from intact skin cultures, and the geographical location of the animals is shown in Figure 1.

All isolates showed similar morphological aspects in culture, with exuberant growth in NNN/Schneider's medium, although seven samples were lost due to secondary contamination or because of reduced growth through the subcultures of the initial isolate. For this reason, samples of the skin of the animals whose cultures were lost were processed by PCR, using the same protocol as the cultured forms.

3.2. PCR and data analysis

The results of PCR assays showed the same amplification pattern for all 33 samples (26 from culture and 7 from skin), with product size of approximately 900 bp for the first round and 700 bp for the second round. The sequencing of the partial 18S rDNA showed that the isolates were genetically identical or closely similar and confirmed *T. caninum* identify. The similarity percents of the isolates and the access number in GenBank are shown in Table 1.

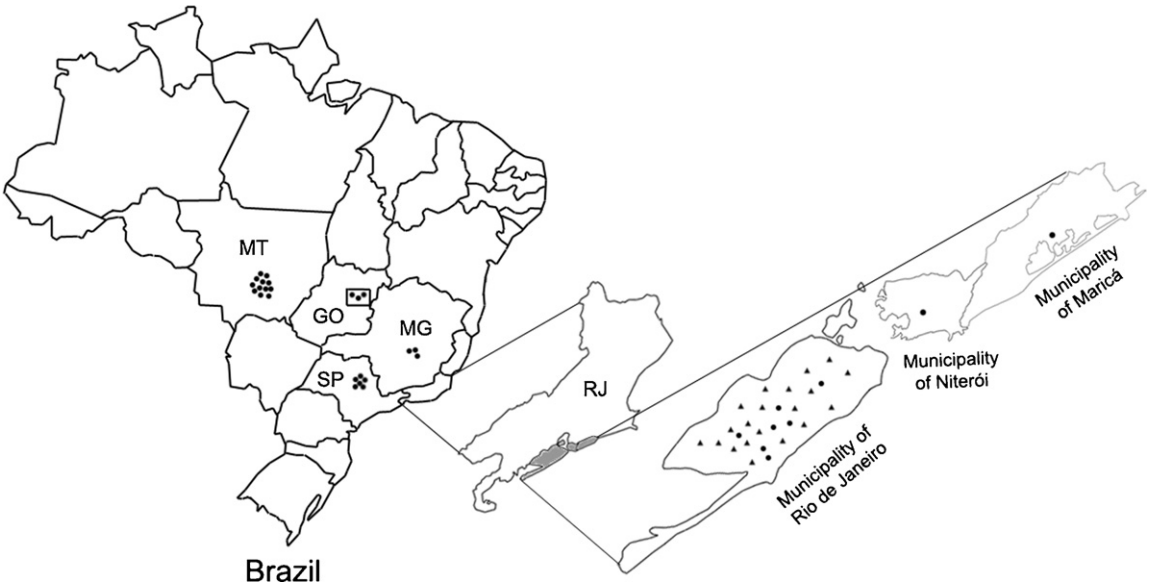

Figure 1. Geographical origin of *Trypanosoma caninum* stocks described to date in Brazilian states: MT (Mato Grosso), GO (Goiás), MG (Minas Gerais), SP (São Paulo) and RJ (Rio de Janeiro). Cases described in this study (●) and cases already published by Madeira et al.[2] and Pinto et al.[3] (▲).

Table 1
Data of the stocks of *Trypanosoma caninum* analyzed in this study

Sample code	Year of isolation	Localization (State/municipality)	Sample used for characterisation	Accession number GenBank	Similarity[a] (%)
RJ8150	2011	RJ/Maricá	Culture	JF951431	99
RJ002	2008	RJ/Rio de Janeiro	Culture	JF907507	99
RJ016	2008	RJ/Rio de Janeiro	Culture	JF907508	99
RJ058	2008	RJ/Rio de Janeiro	Culture	JF907509	98
RJ066	2008	RJ/Rio de Janeiro	Culture	JF907510	99
RJ071	2008	RJ/Rio de Janeiro	Culture	JF907531	97
RJ134	2008	RJ/Rio de Janeiro	Culture	JF907523	97
RJ4814	2007	RJ/Rio de Janeiro	Culture	JF907517	97
RJ4052	2005	RJ/Niterói	Culture	JF907516	96
SP137	2008	SP/Bauru	Culture	JF907518	99
SP269	2008	SP/Bauru	Culture	JF907519	99
SP350	2008	SP/Bauru	Skin	JF907525	99
SP301	2008	SP/Bauru	Skin	JF907526	98
SP385	2008	SP/Bauru	Skin	JF907527	99
SP393	2008	SP/Bauru	Skin	JF907528	99
GO533	2008	GO/Brasília	Skin	JF907529	99
GO705	2008	GO/Brasília	Skin	JF907530	99
GO718	2008	GO/Brasília	Culture	JF907522	99
MG610	2007	MG/Belo Horizonte	Skin	JF907524	99
MG764	2007	MG/Belo Horizonte	Culture	JF907520	99
MG771	2007	MG/Belo Horizonte	Culture	JF907521	99
MT769	2011	MT/Cuiabá	Culture	JF907535	98
MT798	2011	MT/Cuiabá	Culture	JF907536	98
MT799	2011	MT/Cuiabá	Culture	JF907537	99
MT808	2011	MT/Cuiabá	Culture	JF907538	98
MT527	2009	MT/Cuiabá	Culture	JF907511	99
MT531	2009	MT/Cuiabá	Culture	JF907513	99
MT534	2009	MT/Cuiabá	Culture	JF907532	98
MT576	2009	MT/Cuiabá	Culture	JF907514	97
MT577	2009	MT/Cuiabá	Culture	JF907515	96
MT604	2009	MT/Cuiabá	Culture	JF907512	98
MT669	2009	MT/Cuiabá	Culture	JF907533	99
MT732	2009	MT/Cuiabá	Culture	JF907534	98

[a] The similarity percents were obtained comparing the 33 isolates with the sample of *T. caninum* stock A27 (accession no. GU385824) by Blast program.
GO: Goiás, MG: Minas Gerais, MT: Mato Grosso, RJ: Rio de Janeiro, SP: São Paulo.

4. Discussion

The *Trypanosoma* genus comprises numerous species already described and associated to different hosts. This study gives information about *Trypanosoma caninum*, a new species of this genus, about which little is known, identified in canine natural infection. This parasite was described in the municipality of Rio de Janeiro in 2003[2] and now, for the first time, we report the presence of this parasite in the states of São Paulo, Minas Gerais, Mato Grosso and Goiás, which added to the cases already described in the state of Rio de Janeiro[2,3] gives a total of 53 cases of canine natural infection by this parasite. These data clearly show the circulation and the existence of a natural cycle of *T. caninum* and calls attention to an epidemiological scenario newly described in Brazil.

Most of the time *T. caninum* was isolated from healthy dogs, suggesting that this parasite might be non-pathogenic in dogs, although this fact has not been investigated since one dog presented clinical signs compatible with canine VL. It is a matter of concern that *T. caninum* might stimulate the humoral immune system of infected dogs, producing specific antibodies that might cross-react with *Leishmania* in serological tests. This hypothesis is based on the reports of Pinto et al.[3] and Silva et al.[10] who isolated *T. caninum* from seroreactive dogs which were euthanized by leishmaniasis control programs in the municipality of Rio de Janeiro although *Leishmania* parasites were not isolated from these animals. The cross reactivity between different trypanosomatids, e.g., *Leishmania* and *T. cruzi* in canine infection has already been reported.[11] Considering that VL is a growing disease in Brazil[5] and that the domestic dog has a relevant role in the transmission cycle, this hypothesis, if true, will deeply impact VL control and will initiate discussions on canine euthanasia based solely on serological tests.

The fact of *T. caninum* being recently described may be related to the anatomical site where this parasite has been isolated, since the parasites of *Trypanosoma* genus are not usually searched for in the skin. In addition, few studies use intact skin in the diagnosis of canine VL. Fortunately, our team has used this site for canine VL diagnosis for many years,[12] which allowed isolation and identification of this new *Trypanosoma* species. All the samples of *T. caninum* obtained up to this moment were isolated exclusively from this site; all the attempts of isolation from hemoculture failed.

In this study, we demonstrate that the analysis of partial SSU ribosomal DNA sequence was enough to taxonomically identify the 33 samples isolated from different Brazilian regions and grouped all stocks reported to date in a single clade (data not shown). Although the *T. pestanai* and the wombat trypanosome appear to be the most similar trypanosomes, only part of the partial 18S sequences can be aligned with some reliability, and the *T. caninum* sequence is substantially different from others within this group. Biological aspects had already revealed similar patterns between the samples described in this study and the sample of *T. caninum* (stock A27) previously described by Madeira et al.[2] It is important to mention that the molecular target selected in this study to characterize the samples isolated in culture, was also able to amplify the DNA of *T. caninum* in skin fragments, showing that PCR directed to a selected target can be a useful tool for the molecular tracking of this parasite directly in different clinical samples of dogs from endemic areas. Therefore, with regards to this point and considering the limited sensitivity of culture, further study, using molecular approach on blood samples and other anatomical sites in dogs naturally infected by *T. caninum* is in progress.

Numerous aspects of *T. caninum* biology and epidemiology need to be investigated, e.g., the morphology of the forms present in the vertebrate host; the transmission vector; the possible pathogenic potential for dogs; the prevalence of this parasite; and the impact that its presence may cause in areas where other trypanosomatid species occur, especially those of the *Leishmania* genus.

The observation of autochthonous cases of infection by *T. caninum* in different VL areas in Brazil is an alert for epidemiological surveillance. An overlapping geographical distribution of both *T. caninum* and *Leishmania* parasites indicates the need for techniques to ensure the identification and differentiation of these agents in canine infection.

Considering the lack of information that we have about this new parasite, and the importance of such knowledge in the context of public health, studies to evaluate the serological cross reactivity between *T. caninum* and *L. chagasi* and description of the genetic polymorphism among *T. caninum* stocks are in progress and aim to clarify more aspects of this new parasite.

Authors' contributions: JHSB and MFM conceived and designed the study. JHSB drafted the manuscript. MFM critically revised the manuscript for intellectual content. ABPFA, FBF and VRFS conducted fieldwork and analysed the data. AF, AGSP and CB participated in the laboratory tests and interpreted the data. All authors read and approved the final version. JHSB and MFM are the guarantors of the paper.

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Competing interests: None declared.

Ethical approval: The collection of biological samples of dogs was approved by the Ethics Committee on the Use of Animals of FIOCRUZ (CEUA/FIOCRUZ), license numbers L-023/06 and LW-1/10.

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