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TRYPANOSOMA CANINUM, A NEW PARASITE DESCRIBED IN DOGS IN BRAZIL: ASPECTS OF NATURAL INFECTION

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ABSTRACT: *Trypanosoma caninum* constitutes the most recent trypanosomatid species infecting dogs in Brazil. Due to the limited data available about this parasite, this study aims to disclose clinical and laboratory findings from 14 dogs naturally infected. The dogs were diagnosed during a cross-sectional survey in Cuiabá (Mato Grosso, Brazil) and followed up at an interval of 3, 6, and 12 mo in order to evaluate the clinical evolution and to investigate the parasite, the DNA, or both in different biological samples (intact skin, cutaneous scar, blood, bone marrow, and lymph node aspirate) by parasitological (culture and smear exam) and molecular (DNA-based tests) methods. Specific anti-*T. caninum* and anti-*Leishmania* antibody production was also evaluated. Ten of 14 dogs infected by *T. caninum* showed a good general state at the time of diagnosis, and this status did not vary during the follow-up. Anti-*T. caninum* and anti-*Leishmania* IgG antibodies were detected by IFAT in 10 and 2 animals, respectively. Concomitant infection by *Leishmania chagasi* was confirmed in 2 dogs, indicating an overlap of endemic areas in Cuiabá. *Trypanosoma caninum* (parasite or DNA) was found only in the intact skin in all animals examined. Our results suggest that *T. caninum* infection can be manifested as an asymptomatic case with low humoral immune response.

Trypanosoma caninum was initially described in the state of Rio de Janeiro (Madeira et al., 2009; Pinto et al., 2010) and later in other states in Brazil. Although it is only a recently identified species, 53 cases of natural infection in dogs were already reported in areas where canine visceral leishmaniasis (CanL) is endemic (Barros et al., 2012). Data related to the pathogenesis of this parasite in dogs and the epidemiological aspects are still unknown. *Trypanosoma caninum* is isolated exclusively from intact skin fragments, an unusual feature of the *Trypanosoma* genus; it also does not infect triatomine insects and it grows very well in axenic cultures, with a predominance of epimastigote forms (Madeira et al., 2009). Molecular analyses of partial SSU ribosomal DNA sequences have shown that all isolates from *T. caninum* described so far are genetically identical or closely similar (Barros et al., 2012). Apparently, this parasite is not very immunogenic for dogs, even though it is able to stimulate the production of specific antibodies (Alves et al., 2012).

Visceral leishmaniasis (VL) is endemic in many Brazilian regions, and the dog is the main domestic reservoir, playing an important role in the epidemiological cycle, and is considered to be an important target in the control of this parasite (Ministério da Saúde, 2006). The phenomenon of *T. caninum* parasitizing domestic dogs in overlapping areas with VL is an important consideration, mainly for its potential impact on the VL control strategies that are being implemented in Brazil.

The acquisition of 14 dogs naturally infected by *T. caninum* allowed a rare opportunity for clinical and laboratory study of this disease.

MATERIALS AND METHODS

Animals and collection of biological samples

Fourteen dogs naturally infected by *T. caninum* were studied. The animals were identified during the cross-sectional survey for CanL conducted in Cuiabá city, Mato Grosso, in 2009 (Almeida et al., 2011). Infection was diagnosed by parasite isolation and posterior identification by PCR (18S rDNA), followed by amplicon sequencing for which results

have already been deposited in GenBank (Barros et al., 2012). For the physical examination and collection of biological samples, the animals were sedated with ketamine associated with acepromazine, as approved by the Ethics Committee on the Use of Animals, Oswaldo Cruz Foundation (CEUA, license number LW-01/10). The physical examination was based on clinical signs compatible with CanL (e.g., weight loss, lymphadenopathy, conjunctivitis, alopecia, splenomegaly, and presence of ulcers or cutaneous scars). Then the following biological samples were collected: (1) Blood: obtained from the jugular vein with syringe and placed into 2 tubes, one for obtaining serum for the serological tests and the other containing anticoagulant (EDTA) for culture and molecular assays. Also, part of this material was smeared onto slides for cytological exam. (2) Bone marrow: about 0.5–1.0 ml were collected from the sternum and placed into a tube containing EDTA and processed in the same way as the blood. Slides with bone marrow smears were also prepared. (3) Lymph node: this material was obtained by puncture of the most-enlarged popliteal lymph node with a Valery aspirator. Two slides with smears were prepared with the punctured material and the remainder were used for molecular analysis. (4) Tissue fragments: intact skin fragments were collected from the scapular region after trichotomy, antiseptic, and local anesthesia with lidocaine 2%. Two fragments were placed in physiological solution for culture and 1 fragment was stored at –20 °C for molecular tests. Cutaneous scar fragments were processed in the same way as the skin.

The animals were followed up at intervals of 3, 6, and 12 mo after the initial diagnosis in order to evaluate the clinical evolution of the infection by *T. caninum* and investigate the parasite or the DNA in different biological samples by parasitological and molecular tools.

Serological tests

Anti-*T. caninum* and anti-*Leishmania* sp. IgG antibodies were assessed in serum of all the animals by indirect fluorescence antibody test (IFAT), immunoenzymatic assay (an ELISA), and an immunochromatographic test DPP® (Dual Path Platform).

Anti-*T. caninum* IgG antibodies were researched using in-house tests (IFAT and ELISA) with homologue antigens following protocols already standardized by our group (Alves et al., 2012). For the tests to detect anti-*Leishmania* IgG antibodies, IFAT (IFI-leishmaniose visceral canina), ELISA (EIE-leishmaniose visceral canina), and the rapid immunochromatographic test (DPP®) kits were used. These commercial tests are manufactured by Bio-Manguinhos/FIOCRUZ/MS and distributed to public service offices for CanL diagnosis in Brazil (Ministério da Saúde, 2006). The kits were used following the manufacturer's instructions.

Culture procedure and smear examination

The culture of intact skin and scar fragments, blood, and bone marrow was done according to the following steps. Tissue fragments were initially immersed in saline solutions containing 1,000 U penicillin, 200 µg

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streptomycin, and 50 µg 5' fluorocytosine per milliliter and stored at 4 C for 24 hr. Thereafter, each fragment was seeded into screw-cap tubes containing blood-agar slants (NNN—Novy, MacNeal, and Nicolle) overlaid with 1.5 ml of Schneider's *Drosophila* medium supplemented with 10% fetal calf serum. The blood and bone marrow (about 0.2–0.3 ml) were seeded immediately after the collection in tubes containing the same culture medium. All cultures were duplicated, processed, and kept at 27 C (±0.4 C) and examined weekly under optical microscopy for 40–50 days. The isolated parasites were identified by PCR assay (epimastigote forms) or by an isoenzyme electrophoresis technique (promastigote forms) as previously described (Almeida et al., 2011; Barros et al., 2012).

The smears of blood, bone marrow, and lymph node aspirates were fixed in methyl alcohol and later stained by Giemsa. The slides were examined through optical microscopy scanning at least 2,000 microscopic fields (×1,000) looking for the presence of parasites.

Molecular assays

The blood, bone marrow, and tissue (intact skin and scar fragments) samples were processed by 3 different PCR assays. Initially, the DNA of all samples was extracted in accordance with the phenol-chloroform method (Gomes et al., 2007). After this procedure the DNA was stored at –20 C until PCR assays.

DNA detection with targets directed to 18S gene (rDNA): The nested-PCR protocol was used (Smith et al., 2008), and this same approach had been used to identify all *T. caninum* isolates until then. External primers TRY927F (5'-GAAACAAGAAACACGGGAG-3') and TRY927R (5'-CTACTGGGCAGCTTGGGA-3') were applied for the first round and internal primers SSU561F (5'-TGGGATAACAAAGGAGCA-3') and SSU561R (5'-CTGAGACTGTAACCTCAAAGC-3') were applied for the second round. Each PCR run included *T. caninum* DNA (MCAN/BR/2003/A27) and *L. chagasi* DNA (MHOM/BR/1974/PP75) as positive controls.

DNA detection with targets directed to kDNA-minicircle: This followed a protocol previously described using the primers 5'-(G/C)(G/C)(C/G)CC(A/C)CTAT(A/T)TTACACAACCCC-3' and 5'-GGGGAGGGGCGT-TTCTGCGAA-3' (Degraeve et al., 1994). *Leishmania chagasi* DNA was used as a positive control.

DNA detection of the β-globin gene: This assay was used as an internal control of PCR reactions in order to confirm the absence of inhibitors in the samples. The primers 5'-CAA CTT CAT CCA CGT TCA CC-3' and 5'-ACA CAA CTG TGT TCA CTA GC-3', which amplify a product of 118 bp, were used following the protocol according to Quaresma et al. (2009).

All PCR products were run on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.

RESULTS

Among the 14 dogs studied, 11 were male, 12 had non-defined breed, and the ages ranged between 8 mo and 8 yr (median = 2 yr). The follow-up was carried out at 3, 6, and 12 mo after the *T. caninum* infection diagnosis in 8, 3, and 1 dogs, respectively. The other dogs could not be found or were taken to other regions. At the moment of the initial diagnosis, 10 dogs were in good general state and 4 showed 1 or 2 clinical signs such as weight loss (n = 2), lymphadenomegaly (1), and splenomegaly (2). One animal (no. 799) displayed a cutaneous scar on the scrotum. Only 1 dog (no. 732) had its clinical status changed at 6 mo after it developed lymphadenopathy, splenomegaly, conjunctivitis, and hair loss, compatible with the clinical signs of CanL, and infection by *L. chagasi* was later confirmed by culture methods and isoenzyme electrophoresis.

Anti-*T. caninum* antibodies were detected in 10 animals by IFAT, with titers varying from 1:40 to 1:640, and in 8 animals by ELISA. Employing the specific kits for CanL diagnosis, 2 animals (732 and 799) were positive to IFAT, with titers of 1:40 and 1:80, respectively. All animals were negative in EIE and only animal no. 732 showed a positive result in DPP.

Trypanosoma caninum infection was established through culture of skin fragments. By this technique, cutaneous scar (only dog 799), blood, and bone marrow collected at the same time were negative for the presence of this parasite in all cases. Three months later, *T. caninum* was re-isolated from skin fragments of all the 8 dogs reexamined. The attempt to re-isolate *T. caninum* from the skin in 6 mo (3 cases) and 12 mo (1 case) did not succeed. Blood and bone marrow samples collected for culture at 3, 6, and 12 mo were negative in 3, 3, and 1 animal, respectively, except in 1 dog (732) in which *L. chagasi* was isolated from bone marrow at 6 mo.

The smear examinations of the blood, bone marrow, and lymph node samples collected upon the occasion of initial diagnosis and during the follow-up were negative except for dog no. 732 from which amastigote forms were viewed in the bone marrow sample in the 6th mo.

The PCR assay (18S rDNA) performed with skin fragments of the 14 dogs, collected at the moment of the initial diagnosis, showed positive results with amplification products similar to *T. caninum* in 12 cases, and 2 animals showed negative results. The blood, bone marrow, and lymph node samples collected at that time were negative for *T. caninum* amplification pattern in all cases. However, by this assay 1 animal (732) showed an amplification product similar to the *L. chagasi* pattern in the bone marrow sample. When we performed research of *Leishmania*-specific DNA in these samples, only the bone marrow collected from animal 732 was positive.

Of the skin fragments collected from 8 dogs after 3 mo, 4 were PCR positive for *T. caninum* (nos. 527, 798, 799, and 808), whereas the research of *Leishmania* DNA was negative in all 8 cases. Both *T. caninum* and *Leishmania* DNA were negative in blood, bone marrow, and lymph node samples of the 3 dogs examined in this interval. After 6 mo, *T. caninum* DNA was not detected in skin, blood, bone marrow, and lymph node samples of the 3 animals reevaluated. Nevertheless, a pattern of amplification similar to *L. chagasi* was detected in the bone marrow and lymph node samples of animal 732 and in the cutaneous scar and lymph node samples of animal 799. This result was later confirmed by PCR using specific primers for the *Leishmania donovani* complex, and the expected 800-bp product was obtained in the lymph node sample (data not shown). By PCR specific assay, *Leishmania* DNA was detected in these samples, and the other samples showed negative results. After 12 mo, only 1 animal (604) evaluated showed negative results for both PCR assays in all the samples analyzed.

In the PCR assay for β-globin, all samples were positive, showing an expected product of about 118 bp.

All the results can be found in detail in Table I, and the pattern of amplification of *T. caninum* and *Leishmania* by PCR assays can be observed in Figure 1.

DISCUSSION

Trypanosoma caninum is a recently described parasite, and many aspects of this agent are unknown—among them is accurate diagnosis. In all cases reported so far, *T. caninum* infection has been established from the culture of dog skin fragments; however, in all of these cases the aim was the CanL diagnosis (Madeira et al., 2009; Silva et al., 2011). Using culturing success as a diagnostic tool, a prevalence of 3.19% and 5.23% of *T. caninum*

TABLE I. Serological, parasitological, and molecular assays performed with different biological samples obtained from dogs naturally infected by *Trypanosoma caninum*. Tc (*Trypanosoma caninum*), Lc (*Leishmania chagasi*), (–) negative results, (+) positive results; nd (not done).

Dog no.	IFAT titers (<i>T. caninum</i>)	Parasitological tests						Molecular assays—PCR							
		Culture			Smear exam			18S rDNA				<i>Leishmania</i> -kDNA			
		Blood	Bone marrow	Skin	Blood	Bone marrow	Lymph node	Blood	Bone marrow	Skin	Lymph node	Blood	Bone marrow	Skin	Lymph node
527*	1:640	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
527†	nd	nd	nd	+ (Tc)	nd	nd	nd	nd	nd	+ (Tc)	nd	nd	nd	–	nd
531*	–	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
531†	nd	nd	nd	+ (Tc)	nd	nd	nd	nd	nd	–	nd	nd	nd	–	nd
534*	1:80	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
534†	nd	nd	nd	+ (Tc)	nd	nd	nd	nd	nd	–	nd	nd	nd	–	nd
576*	–	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
577*	1:160	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
604*	1:80	–	–	+ (Tc)	–	–	–	–	–	–	–	–	–	–	–
604§	nd	–	–	–	–	–	–	–	–	–	–	–	–	–	–
627*	–	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
669*	1:80	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
669†	nd	nd	nd	+ (Tc)	nd	nd	nd	nd	nd	–	nd	nd	nd	–	nd
732*	1:80	–	–	+ (Tc)	–	–	–	–	+ (Lc)	–	–	–	+ (Lc)	–	–
732‡	nd	–	+ (Lc)	–	–	+	–	–	+ (Lc)	–	+ (Lc)	–	+ (Lc)	–	+ (Lc)
769*	–	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
769†	nd	nd	nd	+ (Tc)	nd	nd	nd	nd	nd	–	nd	nd	nd	–	nd
784*	1:160	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
798*	1:40	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
798†	nd	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
798‡	nd	–	–	–	–	–	–	–	–	–	–	–	–	–	–
799*	1:160	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
799†	nd	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
799‡	nd	–	–	–	–	–	–	–	–	–	+ (Lc)	–	–	–	+ (Lc)
808*	1:160	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–
808†	nd	–	–	+ (Tc)	–	–	–	–	–	+ (Tc)	–	–	–	–	–

* Biological samples collected at the same occasion as the *T. caninum* diagnosis.

† After 3 mo.

‡ After 6 mo.

§ After 12 mo.

infection has been reported (Pinto et al., 2010). Using the same test in Cuiabá city, Almeida et al. (2011) found a prevalence of 3.25%. These data suggest that the prevalence of *T. caninum* may be low or that such rates are influenced by other factors. In fact, both the test and the biological sample analyzed can have an impact on the sensitivity and specificity of the diagnostic test and, consequently, on the infection prevalence rates.

Of the 14 animals studied, 71% were in good general state, even during the follow-up. Relevant clinical signs were found in only 1 animal that was co-infected by *L. chagasi* and then attributed to CanL. The results obtained herein, associated to previous observations, suggest that *T. caninum* infection can be asymptomatic, without specific clinical signs, and occurs with low parasitemia, making its detection difficult by the methods used up to the moment. The lack of knowledge about the biological cycle of *T. caninum* restricts the choice of biological samples to be analyzed and of more appropriate tools for its diagnosis. Considering the molecular tools applied in this study, we confirmed that this parasite is found exclusively in intact skin.

It is interesting that the first case of *T. caninum* infection was described in a dog co-infected by *Leishmania braziliensis* (Madeira et al., 2009), and now we report the co-infection by *T. caninum*

and *L. chagasi*, demonstrating that *T. caninum* and *Leishmania* parasites can co-exist in concomitant infections.

Species-specific molecular targets are not available for *T. caninum*. Here, the use of targets for the region of the ribosomal gene (18S rDNA), despite being general, allowed the detection of *Leishmania* DNA in 2 dogs, and these results were confirmed by other tests. This approach shows that a combination of methods may be useful when the parasites are rarely studied, such as in co-infections with *T. caninum*.

The humoral immune response in the infection by *T. caninum* has been low or absent (Alves et al., 2012), and the results obtained herein corroborate this observation, showing that of 14 dogs naturally infected by this parasite 43% were seronegative, even when the homologous antigen was used in the tests. This may be related to the characteristic of *T. caninum* in which its presence appears to be limited and restricted to skin. Unlike CanL, the presence of *L. chagasi* in different organs and tissues is considered as 1 of the factors responsible for the high humoral immune response. The low humoral response observed in the *T. caninum* infection may on one hand limit the variety of options for its diagnosis but, on the other hand, is a positive issue considering that serology constitutes the recommendation for CanL diagnosis

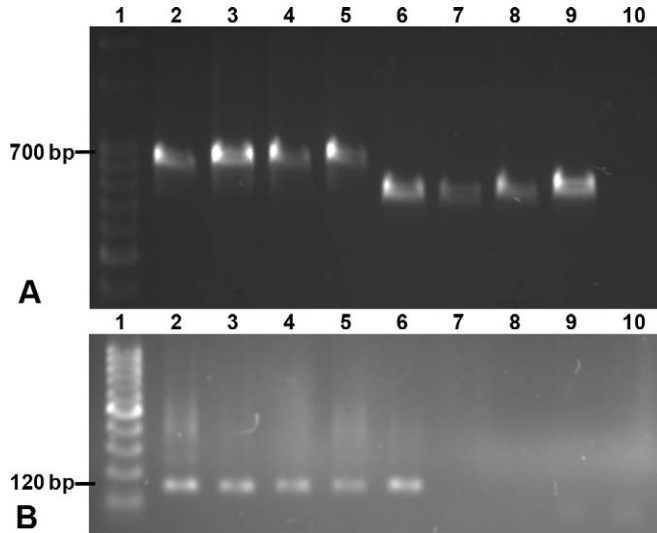


FIGURE 1. Agarose gel electrophoresis showing the amplified products with representative samples of the study. (A) PCR assay that targeted a partial sequence of the 18S rDNA gene. Lane 1: DNA ladder, 100 bp; Lane 2: DNA control of *Trypanosoma caninum* (MCAN/BR/2003/A27); Lanes 3–5 (positive samples with *T. caninum* pattern): skin of dogs 527, 531, and 534, respectively; Lanes 6–8 (positive samples with *L. chagasi* pattern): bone marrow of dog 732, cutaneous scar of dog 799, and lymph node of dog 799, respectively; Lane 9: DNA control of *Leishmania chagasi* (MHOM/BR/1974/PP75); Lane 10: negative control. (B) PCR assay that the conserved region of *Leishmania* kDNA minicircles. Lane 1: DNA ladder, 100 bp; Lane 2: DNA control of *Leishmania chagasi* (MHOM/BR/1974/PP75); Lane 3: bone marrow sample of dog 732; Lane 4: cutaneous scar sample of dog 799; Lane 5: lymph node sample of dog 799; Lane 6: lymph node sample of dog 732; Lanes 7–9: skin of dogs 527, 531, and 534, respectively; Lane 10: negative control.

in Brazil. Among the dogs studied, only the animals co-infected by *Leishmania* reacted to specific tests for CanL.

The follow-up of dogs in endemic areas is a hard task. Dogs are usually taken from 1 area to another, which constitutes 1 of the reasons for the geographic expansion of some zoonoses that have the dog as a dispersion element; for instance with visceral leishmaniasis (Marzochi et al., 2009). Although *T. caninum* is apparently not pathogenic to dogs, it may represent another agent in this complicated context. Thus, it is important that the infection dynamics in this host be understood so that specific diagnostic tools can be applied.

In conclusion, this study supports a biological feature of *T. caninum* and suggests that this parasite is not pathogenic or not very virulent to dogs. Moreover, the infection course is asymptomatic and transitory with low humoral immune response.

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