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TESA-blot for the diagnosis of Chagas disease in dogs from co-endemic regions for *Trypanosoma cruzi*, *Trypanosoma evansi* and *Leishmania chagasi*

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ABSTRACT

We standardized serodiagnosis of dogs infected with *Trypanosoma cruzi* using TESA (trypomastigote excreted-secreted antigen)-blot developed for human Chagas disease. TESA-blot showed 100% sensitivity and specificity. In contrast, ELISA using TESA (TESA-ELISA) or epimastigotes (epi-ELISA) as antigen yielded 100% sensitivity but specificity of 94.1% and 49.4%, respectively. When used in field studies in an endemic region for Chagas disease, visceral leishmaniasis and *Trypanosoma evansi* (Mato Grosso do Sul state, Central Brazil), positivities were 9.3% for TESA-blot, 10.7% for TESA-ELISA and 32% for epi-ELISA. Dogs from a non-endemic region for these infections (Rondonia state, western Amazonia) where *T. cruzi* is enzootic showed positivity of 4.5% for TESA-blot and epi-ELISA and 6.8% for TESA-ELISA. Sera from urban dogs from Santos, São Paulo, where these diseases are absent, yielded negative results. TESA-blot was the only method that distinguished dogs infected with *T. cruzi* from those infected with *Leishmania chagasi* and/or *Trypanosoma evansi.*

1. Introduction

Dogs are important reservoirs of *Trypanosoma cruzi* and *Leishmania chagasi* and play an important role in the transmission of Chagas disease and visceral leishmaniasis to humans in rural areas of Central and South America. *T. cruzi* infection, which is primarily a zoonosis, is widespread and has a high prevalence in the region extending from the southern half of the USA to the southern countries of South America (Miles et al., 2003). In regions where human Chagas disease is endemic, *T. cruzi* circulates between humans and domestic animals and is transmitted by home-dwelling blood-sucking triatomine bugs. Several mammals and triatomine species sustain domestic and sylvatic transmission cycles, while domestic mammals (dogs and cats) and peridomestic mammals (rodents and marsupials) are responsible for the interaction between these two cycles, playing an important role in the transmission of *T. cruzi* from sylvatic to domestic cycles. Dogs from rural areas frequently enter wild environments where *T. cruzi* enzootic populations circulate between wild mammals and triatomines (Gürtler et al., 1998, 2007; Crisante et al., 2006; Estrada-Franco et al., 2006; Cardinal et al., 2007, 2008).

Domestic dogs are a risk factor for Chagas disease because they can be a source of *T. cruzi* infection in humans, and close contact between humans and dogs, particularly when the latter are kept inside a house overnight, can significantly enhance transmission of this disease to humans. Because of their persistent parasitemia, dogs have a greater capacity for infecting triatomine bugs than do humans and have been used as efficient natural sentinels to assess *T. cruzi* reinfections in vector surveillances (Gürtler et al., 1993; Castanera et al., 1998; Estrada-Franco et al., 2006; Cardinal et al., 2006a).

The importance of domestic dogs as reservoirs and as a risk factor for the transmission of *T. cruzi* to humans has been extensively studied in Argentina. The prevalence of infected *T. infestans* in households was found to increase about 3–4 times with the number of infected dogs, and the seroprevalence of infected humans doubled in households with 1–2 infected dogs (Gürtler et al., 1993, 1996, 2007; Diosque et al., 2004; Cardinal et al., 2007). In Mexico, dogs are known to provide frequent blood meals for *Triatoma barberi* and *T. pallidipennis,* and *T. cruzi* infected triatomines and have been associated with human infections (Ramsey et al., 2005; Estrada-Franco et al., 2006). Finding of a direct correlation between

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seropositivity in humans and dogs suggest that the diagnosis of *T. cruzi* infected domestic dogs can be of help in assessing the risk of transmission of human Chagas disease.

In Brazil, studies conducted in rural communities in Northeast Brazil found high prevalence of *T. cruzi* infected domestic animals, with high importance of dogs and cats in the domestic transmission cycles (Barrett et al., 1979). The human seropositivity was five times greater in houses inhabited by infected dogs and cats than in houses with non-infected domestic animals (Mott et al., 1978).

Surveys of canine Chagas disease have also been carried out in rural areas of regions that are endemic for the human disease in Chile (Burchard et al., 1996), Venezuela (Crisante et al., 2006); Paraguay (Fujita et al., 1994), and Mexico (Ramsey et al., 2005; Estrada-Franco et al., 2006; Jimenez-Coello et al., 2008). Prevalence rates of *T. cruzi* in dogs from these countries varied widely. The highest rates (65–83%) were reported in Northwest Argentina before the spraying of residual insecticides (Castanera et al., 1998) and were followed by a rate of 67% infection for dogs in Venezuela (Crisante et al., 2006), 38% in Paraguay (Chapman et al., 1984), 21% in Mexico (Estrada-Franco et al., 2006), 20.3% in Texas, USA (Kjos et al., 2008), and 19% in Brazil (Barrett et al., 1979). There have been several reports of *T. cruzi*-infected dogs in the southern USA, where human Chagas disease is not endemic but dogs infected with *T. cruzi* have been associated with domestic transmission of human infection (Barr et al., 1991; Shadomy et al., 2004; Duprey et al., 2006; Kjos et al., 2008).

Diagnosis of canine Chagas disease is usually made by serological methods developed for humans (Barr et al., 1991; Lauricella et al., 1998; Shadomy et al., 2004; Cardinal et al., 2006b; Estrada-Franco et al., 2006). Although several conventional serological tests based on crude antigens from epimastigotes, such as ELISA (enzyme-linked immunosorbent assay), IFA (indirect immunofluorescence assay) and IHA (indirect hemagglutination assay), showed good sensitivity, their specificity decreased when sera from hosts with visceral leishmaniasis were tested (Frank et al., 2003; Caballero et al., 2007; Ferreira et al., 2007). Nevertheless, most serological surveys of dogs infected with *T. cruzi* have used methods that have not been evaluated in terms of cross-reaction with *Leishmania* spp. In addition, these methods also ignored cross-reaction with other important canine pathogens, including *Trypanosoma evansi* that is endemic in overlapping areas of *T. cruzi* in Brazil, Venezuela, Colombia and Bolivia (Ventura et al., 2002; Herrera et al., 2004), and that shares antigens with *T. cruzi* (Desquesnes et al., 2007).

TESA-blot, previously described for the diagnosis of human Chagas disease, is a Western blotting technique that uses TESA (trypomastigote excreted–secreted antigen) as antigen. In this method no cross-reactivity with sera from humans infected with *Leishmania* spp., *T. rangeli* or other pathogens is observed (Umezawa et al., 1996, 2001; Caballero et al., 2007). In the present study, whose objective was to standardize a highly specific and sensitive serological assay for canine Chagas disease, we evaluated the diagnostic performance of TESA-blot and compared this method with TESA-ELISA and the conventional epi-ELISA. These methods were further evaluated for use in field epidemiology of canine Chagas disease in areas of Brazil that are endemic or non-endemic for Chagas disease, visceral leishmaniasis and *T. evansi* infection.

2. Materials and methods

2.1. Antigens

TESAs (trypomastigote excreted–secreted antigens) from the Y strain of *T. cruzi* were obtained as previously described (Umezawa et al., 1996, 2001). Briefly, the supernatants of LLC-MK2 cell cultures (in serum-free medium or with 2% FCS) infected with *T. cruzi* were collected when the concentration of trypomastigotes reached about $10–20 \times 10^6$/mL. After being centrifuged at $1800 \times g$ for 15 min at 4 °C, the supernatant containing TESA was then re-submitted to a second centrifugation ($7000 \times g$ for 5 min at 4 °C) and used directly without any further treatment or stored at −80 °C in small aliquots.

The extract of epimastigotes of *T. cruzi*, isolate José-IMT, was prepared with fresh parasites cultivated in LIT medium as described previously (Umezawa et al., 2001). Briefly, 500 μg of epimastigote forms were incubated with 0.3 N NaOH (500 μL) and, after 18 h at 4 °C, neutralized with 0.3 N HCl, centrifuged at 12,000 *g* for 1 min at 4 °C and used after determining the protein content (Micro-BCA protein reagent kit, Pierce Co.).

2.2. ELISA using TESA or epimastigote forms of T. cruzi as antigen

ELISAs using TESA (TESA-ELISA) and epimastigotes (epi-ELISA) were performed as previously described (Umezawa et al., 2001). For this study, the optimal antigen concentration and dilution of dog sera were previously determined by checkerboard titration. The sera were diluted 1:200 for TESA-ELISA and 1:100 for epi-ELISA. Horseradish peroxidase-labeled anti-dog immunoglobulin G (Sigma Co.) was used to detect antigen-specific antibodies, the mixture was incubated with hydrogen peroxide and O-phenylenediamine dihydrochloride (OPD-tablets, Sigma Co.), and the reaction stopped by adding 4 N HCl. The absorbance ($A_{492\,nm}$) was measured in an ELISA reader (Labsystems Multiskan MS). All the experiments were carried out in duplicate and repeated at least twice on different days. The cutoff values for ELISAs were calculated as the mean plus three standard deviations of the absorbance ($A_{492\,nm}$) values for 30 true negative dog sera.

2.3. TESA-blot

Proteins from TESA were separated by SDS-PAGE, transferred to nitrocellulose sheets, and blocked with PBS containing 5% fat-free milk for 1 h at room temperature. Membrane strips (5 mm) were incubated with canine sera (1:100) diluted in PBS with 1% milk for 2 h or overnight at room temperature, washed, and the bound antibodies were detected with horseradish peroxidase-labeled anti-dog IgG (Sigma Co.). The color of detected bands was developed by addition of 0.05% hydrogen peroxide and 4-chloro-1-naphthol or 3, 3′ Diaminobenzidine (DAB). Samples were considered positive when a large 150–160 kDa band and/or five bands between 130 and 200 kDa were observed (Umezawa et al., 1996).

2.4. Serum sampling and T. cruzi isolates

Domestic canine sera were obtained from blood samples collected by femoral puncture from domestic dogs living in rural areas of Brazil ($n = 297$) between 2004 and 2006, diluted in glycerine (v/v) and stored at −20 °C. TESA-blot was compared with TESA-ELISA and epi-ELISA using 10 positive control sera from dogs chronically infected with *T. cruzi*, eight from dogs experimentally infected intraperitoneally with culture trypomastigotes of Esmeraldo strain, which showed high parasitemia in the acute infection, and two sera from dogs naturally infected with *T. cruzi*. These two dogs were from Furnas de Dionísio, Mato Grosso do Sul State, and were diagnosed by xenodiagnosis followed by isolation in culture of parasites from the guts of the triatomines (xenocultures). Molecular diagnosis of cultured isolates was performed using the PCR method described by Fernandes et al. (2001). Lineage genotyping was carried out as described previously (Martins et al., 2008; Marcili et al., 2009).

Fifty negative control serum samples were obtained from healthy domestic dogs (without any clinical symptoms) from Santos, a coastal city in São Paulo State, Southern Brazil that is non-endemic for Chagas disease, visceral leishmaniasis and *T. evansi*.