Original Article

First report of *Sapajus cay* naturally infected by *Trypanosoma cruzi* in San Pedro Department, Paraguay

Primeiro relato de infecção natural em *Sapajus cay* por *Trypanosoma cruzi* em Departamento San Pedro, Paraguai

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Abstract

To verify the occurrence of natural *Trypanosoma cruzi* infection in non-human primates from a rural endemic area of the east region of Paraguay, xenodiagnosis was performed in 35 animals belonging to two species. For genotyping and *T. cruzi* discrete typing unit (DTU) assignment, a combination of four markers was used, including amplification products of the small (18S) and large (24Sα) subunits of ribosomal ribonucleic acid gene, the intergenic region of mini-exon gene and the heat shock protein 60 *Eco*-RV polymerase chain reaction-restriction fragment length polymorphism (*HSP60*/*Eco*RV-PCR-RFLP). One specimen of *Sapajus cay* was found positive and infected by the DTU TcII. This result constitutes the first record of natural *T. cruzi* infection in a sylvatic monkey in Paraguay, harbouring a DTU associated with severe Chagas disease in humans.

Keywords: *Trypanosoma cruzi*, primates, Paraguay.

Resumo

Com o objetivo de verificar a infecção natural por *Trypanosoma cruzi* em primatas não-humanos de uma área endêmica rural da região leste do Paraguai, foi realizado o xenodiagnóstico em 35 animais pertencentes a duas espécies. Para a genotipagem foi utilizada a unidade discreta de tipagem (UDT) do *T. cruzi*, em uma combinação de quatro marcadores, incluindo amplificação de produtos de pequena (18S) e grande (24Sα) subunidades do gene do ácido ribonucleico ribossômico, da região intergênica de miniéxon e do gene da proteína de choque térmico 60 (*HSP60*/*Eco*RV-PCR-RFLP), pela reação em cadeia da Polimerase. Um espécime de *Sapajus cay* se mostrou positivo pelo UDT TcII. Este resultado constitui o primeiro relato da infecção natural pelo *T. cruzi* em um macaco silvestre no Paraguai, abrigando um UDT associado com a doença de Chagas grave em humanos.

Palavras-chave: *Trypanosoma cruzi*, primatas, Paraguai.

Introduction

Trypanosoma cruzi is the causal agent of Chagas disease, a neglected parasitic disease that is estimated to affect approximately six millions people living in 21 countries of the Americas. It is estimated that 60 to 80 million are under risk of infection in endemic areas (WHO, 2015).

The infection for this parasite is considered primarily a zoonosis. Several species of sylvatic mammals were found naturally infected with this parasite, including over 73 genera and members of nine orders (JANSEN et al., 2015). Despite this wide range of hosts, the epidemiological importance of *T. cruzi* reservoirs varies according to the geographic region, the biology and ecology of these mammals

and their interaction with triatomine vectors and humans. Studies on *T. cruzi* mammal infected species that have been performed in Paraguay include that one of Canese (1978), which examining a total of 17 wild animals found two specimens of *Didelphis azarae* positive for *Trypanosoma* spp. The remaining animals consisting of two armadillo species *Dasypus novemcinctus* and *Tolypeutes matacus* were negative as were two specimens of the fox *Cerdocyon thous*. A further study (FUJITA et al., 1994) encompassing 112 domestic and 4 sylvatic animals, including opossum, yellow armadillos and long-nosed armadillo from San Pedro Department, revealed 24 domestic animals seropositive for *T. cruzi*, of which 16 were dogs. Yeo et al. (2005) analyzed 146 animals in the area of San Pedro and Central Chaco, between 2000 and 2005, from ten different species, including *D. novemcinctus*, *Euphractus sexcinctus*, *T. matacus*, *Chaetophractus* spp., *Galea musteloides*, *Calomys laucha*,

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Graomys griseoflavus, *Monodelphis domestica*, *Didelphis albiventris* and *Conepatus chinga*. Twenty three animals being positive, which were *D. novemcinctus*, *E. sexcinctus*, *Chaetophractus* spp. and *M. domestica*. Monkey species were not included in these surveys. In general, primates act as reservoirs of parasitic diseases very related to human. Thus, parasitological studies about these species provide important information for a better understanding of the epidemiology and risk for emergence of several antropozoonosis.

Trypanosoma cruzi shows a phenotypic and genotypic substantial heterogeneity. Currently and according to the last consensus *T. cruzi* is divided into six discrete typing units or DTUs (ZINGALES et al., 2012), referred as TcI, TcII (ex TcIIb), TcIII (ex TcIIc), TcIV (ex TcIIa), TcV (ex TcIId) and TcVI (ex TcIIe). Additionally, a seventh DTU was described associated with bats (MARCILI et al., 2009a). These DTUs circulate in transmission cycles occurring in domestic and sylvatic habitats. The domestic cycle involves humans, domestic or synanthropic mammals, and domiciliated triatomines, whereas the sylvatic cycle includes multiple sylvatic triatomine species and several species of wild mammals. These transmission cycles can occur separately or overlapped, with the potential risk of parasite introduction into the domestic environment from the sylvatic area. A better understanding of the eco-epidemiology of *T. cruzi* in a given area can help in the development of improved disease control and surveillance strategies. Previous studies have been performed in Paraguay about the characteristics of strains of the domestic cycle, isolated from patients and domiciliary triatomines (CHAPMAN et al., 1984; MIMORI et al., 1992; ACOSTA et al., 1995, 2001), but very little is known about strains circulating in the sylvatic environment and their interrelations with the domestic cycle. Earlier studies in the San Pedro Department reported 14.3% of human seroprevalence for Chagas disease (CANESE, 1978). Likewise, *Triatoma infestans* and *T. sordida* specimens were found infesting houses (SENEPA, 2014) and currently is under epidemiological surveillance. This study was carried out in order to verify the occurrence of natural *T. cruzi* infection in non-human primates from a rural endemic area of the east region of Paraguay.

Materials and Methods

Animals were captured between February and April 2008 in ten rural localities of San Pedro Department (Table 1). This region corresponds to tropical and subtropical grassland, savanna and shrubland ecoregion, according to Olson et al. (2001). It is formed by wide plains with topography of valleys and small hills. It has abundant vegetation, humid climate with an annual rainfall from 800 to 1000 mm, relative humidity of 63% and 83% and an average temperature of 23°C. Approximately 30% of the population from this area still lives in precarious dwellings built with walls of wattle and brick, adobe, palm trunk, soil floor and straw, palm or tin roof (CENSO PARAGUAY, 2014).

The study included specimens kept as pets in the houses, and free-living ones, captured alive with the help of local hunters in wooded zones located at more than 3 kilometers from the dwellings. The specimens were identified by sex and species (NERIS et al., 2002), conforming to the taxonomy of capuchins lately proposed, in which the genus *Cebus* split into two genera: *Cebus* for gracile capuchins from the Amazon and *Sapajus* for robust capuchins from the Atlantic Forest (ALFARO et al., 2011, 2012; WALLACE, 2015). Then they were examined in the capture site; the free-living ones were released after sample collection. Animal handling procedures were according to the Guidelines of the American Society of Mammalogists (SIKES & GANNON, 2011). This work was performed within the project: "Study of Yellow Fever in primates of outbreak areas of the San Pedro and Central Departments of Paraguay". The authorization of the Secretariat of Environment of Paraguay (SEAM), Certificate DVS No. 02/08 and the approval of the Scientific and Ethical Committees of the Instituto de Investigaciones en Ciencias de la Salud, Universidad Nacional de Asunción were obtained (code P03/08).

Table 1. Species of non-human primates tested for infection by *Trypanosoma cruzi* in localities from San Pedro Department, Paraguay.

Locality	Latitude	Longitude	Species	Total captured	Habitat	Positive
Yrybu cua	24°31'27,50"S	56°05'16,73"W	*Sapajus cay*	1	pet	0
Vy'a renda	24°29'11,52"S	56°07'12,28"W	*Sapajus cay*	9	pet (1) free-living (8)	0
			Alouatta caraya	1	pet	0
Guayaibi	24°31'46,65"S	56°24'20,24"W	*Sapajus cay*	1	pet	0
Arroyo guazú	23°47'57,76"S	56°06'47,49"W	*Sapajus cay*	1	pet	0
Coronel Mongelós	24°40'39,62"S	56°26'39,12"W	*Sapajus cay*	2	pet	0
Calle 8000 Bertoni	24°39'49,89"S	56°25'30,77"W	*Sapajus cay*	1	pet	0
Compañía General Cáceres	24°32'42,39"S	56°37'41,086"W	*Sapajus cay*	11	free-living	1
Colonia Friesland	24°36'58,75"S	56°46'55,34"W	*Sapajus cay*	3	pet	0
			Alouatta caraya	1	pet	0
Compañía Tuyango	24°34'41,34"S	56°38'8,49"W	*Sapajus cay*	1	free-living	0
Compañía San Fernando	24°49'42,94"S	56°46'7,80"W	*Sapajus cay*	3	free-living	0
Total captured				**35**		**1**

Infection with trypanosomes was determined by xenodiagnosis (SCHENONE, 1999) using 20 fourth to fifth instar nymphs of *Triatoma infestans* reared in laboratory and free of trypanosome infection. Bugs for xenodiagnosis were provided by the Insectary of the Medicine Tropical Department of the Instituto de Investigaciones en Ciencias de la Salud. The primates were anesthetized with ketamine (Holliday-Scott°, 50-80 mg/kg) by intramuscular via to be submitted to xenodiagnosis. After two weeks, the feces of the insects were examined by optical microscopy to verify the presence of flagellates. Controls were performed until 60 days after feeding. The positive insects were dissected and the intestinal content was cultured in LIT (liver infusion tryptose) medium, (CAMARGO, 1964), supplemented with 10% foetal calf serum and incubated at 28°C. DNA extraction was performed from parasite pellets using a DNeasy kit (QIAGEN™) following the manufacturer instructions. For genotyping and DTU assignment, a combination of four markers were used, including amplification products of the small (18S) and large (24Sα) subunits of ribosomal ribonucleic acid (rRNA) gene, the intergenic region of mini-exon gene and the polymerase chain reaction-restriction fragment length polymorphism of the heat shock protein 60 gene (*HSP60/Eco*RV-PCR-RFLP) profiles, as previously reported (YEO et al., 2005; LEWIS et al., 2009) (see Table 2). Amplified products were separated by electrophoresis in agarose gels (Sigma Chemical Co., St Louis, Mo) with 0.5X TBE buffer, stained with ethidium bromide, and visualized under ultraviolet light. X10 Clone I (TcI), Esmeraldo-Cl3 (TcII), ARMA 13 (TcIII), CAN III (TcIV), SC43 (TcV) and CL Brener (TcVI) were used as reference strains.

Results

Thirty five animals were analyzed in total, twenty males and fifteen females. Thirty three animals showed characteristics of the genus *Cebus*, which according to the new division belonging to *Sapajus cay* (Azaras's capuchin) species and the remains two were *Alouatta caraya* (howler monkey) (Table 1). Twelve of them corresponded to pets and 23 were free-living specimens. One sylvatic *S. cay* male specimen from Compañía General Cáceres was positive for *T. cruzi* infection giving a 2.85% of overall infection. Genotyping techniques performed in the *T. cruzi* isolate gave profiles expected for DTU TcII, according to Yeo et al. (2005) and Lewis et al. (2009), with amplification products at 125 bp for the 24Sα rRNA PCR, at 165 bp for the variable domain of the 18S rRNA gene, at 300 bp for the non-transcribed spacer region of the mini-exon gene and one band at 462 bp for the PCR-RFLP analysis of the

HSP60 gene. Figure 1 shows the result of this last marker, which allows the discrimination between TcII and TcVI DTUs. .

Discussion

This study constitutes the first report of *T. cruzi* infection in a monkey from Paraguay. *T. cruzi*-like trypanosomes were early reported infecting *Cebus apella* by Carvalheiro & Barretto (1966) in woodland from the São Paulo Estate, Brazil. Bar et al. (1999) found 3.8% of infection in this primate species in Corrientes, Argentine Chaco. Jansen et al. (2015) also described specimens of *Cebus* spp. with natural infection in distinct Brazilian ecotopes especially in the Amazon and Atlantic forest. Primates from other species have been also found infected with this parasite, including *Leontopithecus rosalia*, *Leontopithecus chrysomelas*, *Saimiri sciureus*, in different regions of Brazil (ZICCARDI & LOURENÇO-DE-OLIVEIRA, 1997; FERNANDES et al., 1999; LISBOA et al., 2004, 2006; MONTEIRO et al., 2007; JANSEN et al., 2015); *Macaca silenus*, *Varecia variegata variegata*, *Lemur catta* in the United States, (PUNG et al., 1998; HALL et al., 2007) among others. Reported infection rates ranged from 4% to 88% and infections of *T. cruzi* combined with *T. rangeli* in the Brazilian Amazon rainforest were described (ZICCARDI & LOURENÇO-DE-OLIVEIRA, 1997; SILVA et al., 2008; JANSEN et al., 2015).
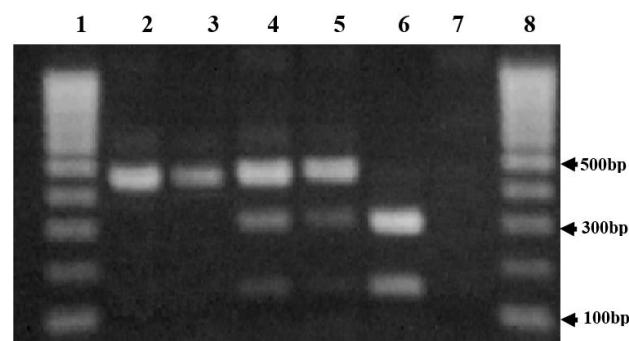

Figure 1. Agarose gel electrophoresis of PCR-RFLP products from *HSP60/Eco*RV analysis of *Trypanosoma cruzi* isolates. Lanes: 1 and 8- molecular weight markers; 2- TcII reference strain Esmeraldo-Cl3 (462 bp); 3- *Sapajus cay* isolate (462 bp); 4- TcVI reference strain CL Brener (462/314/148 bp); 5- TcV reference strain SC43 (462/314/148 bp); 6- TcIII reference strain ARMA 13 (314/148 bp); 7- Negative control.

Table 2. Amplification products in base pairs (bp) of reference strains from different PCR reactions*.

PCR reaction	TcI	TcII	TcIII	TcIV	TcV	TcVI	DTU identification
24Sα rRNA	110	125	110	120	110/125	125	TcIV, TcV
18S rRNA	160	165	165	155	165	165	TcI, TcIV
Mini-exon	350	300	250 or none	400 or none	300	300	TcI, TcIV, TcIII
RFLP-PCR (*HSP60/Eco*RV)	462	462	314/148	462	462/314/148	462/314/148	TcIII**

* According to Yeo et al. (2005) and Lewis et al. (2009). ** Allows the differentiation between TcII and TcVI DTUs.

The two *A. caraya* of the present study were negative. However, species from the genus *Alouatta* were reported infected in Brazil (JANSEN et al., 2015) and in a recent survey in the northeastern of Argentina (MARTINEZ et al., 2016), therefore it is plausible that there are also positive specimens in this region of Paraguay.

C. apella is highly susceptible to *T. cruzi* infection and has been used as a model for experimental infections, being able to maintain parasitemia during long-term infection, with low level of severe symptoms that characterized Chagas disease in humans and without mortality (ALMEIDA et al., 1992; RIARTE et al., 1995). Long-lasting natural infection was also observed in *L. rosalia* (golden lion Tamarin) specimens, which after ten-year follow-up were able to maintain high parasitemias (LISBOA et al., 2015). This feature observed in primates can represent a risk factor for *T. cruzi* introduction and maintenance in the domestic environment, taking into account that they very often are kept as pets in this rural area of Paraguay, where *T. infestans* and *T. sordida* specimens were found infesting houses (SENEPA, 2014).

One positive specimen was found harbouring *T. cruzi* DTU TcII. This finding is interesting taking into account that this DTU was previously observed in a human case from San Pedro Department (ACOSTA et al., 2001) and that a different DTU (TcIII) was found circulating among sylvatic armadillos and in the marsupial *M. domestica* in the same area (YEO et al., 2005). In surveys performed in Brazilian Amazonia, several non-human primate species were reported infected with TcI and TcIV, the DTU TcI being the most common, associated with *Rhodnius* species (MARCILI et al., 2009b). In the Brazilian Atlantic forest, several monkey species were found infected with TcI and 'TcII' (without determination of TcII subgroup, according to the TcIIa-e *T. cruzi* nomenclature at the time) separately and in mixed infections (LISBOA et al., 2006). Fernandes et al. (1999) also reported specimens of *L. rosalia* infected with 'TcII' (without determination of TcIIa-e subgroup) in Rio de Janeiro state (Brazil). Recently, among captive Neotropical primates in a Brazilian zoo, TcI was reported associated with *Panstrongylus* species transmission (MINUZZI-SOUZA et al., 2016). *T. cruzi* isolates from lemurs in the United States were assigned to TcIV (HALL et al., 2007; ROELLIG & YABSLEY, 2010) as well as isolates from wild primates in Bolivia and Venezuela (WESTENBERGER et al., 2006). TcII is found in the domestic area in Southern Cone countries, associated with severe Chagas disease in humans and its dynamics of transmission in sylvatic reservoirs is still poorly understood, despite that this DTU is considered ancestral (WESTENBERGER et al., 2005; FREITAS et al., 2006). Therefore, this study contributes with a new finding of DTU TcII infecting a sylvatic mammal in this zone. Further surveys are necessary to determine the transmission mechanism, maintenance and distribution of *T. cruzi* among these animals in this region.

Despite the low rate of infection observed in primates in this research, it expands our knowledge of the spectrum of wild reservoir species that could be infected by *T. cruzi* in a given area, especially when the same DTU that circulates in domestic area was found in the sylvatic monkey. Thus, primates from this region can constitute a source of parasites, being a potential threat for human health. This understanding should be taken into account when designing control measures against the parasite.

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