Molecular characterization of trypanosomatid infections in wild howler monkeys (Alouatta caraya) in northeastern Argentina

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ABSTRACT

The transmission of Trypanosoma cruzi by vectors is confined to the Americas, and the infection circulates in at least two broadly defined transmission cycles occurring in domestic and sylvatic habitats. This study sought to detect and characterize infection by T. cruzi and other trypanosomes using PCR strategies in blood samples from free-ranging howler monkeys, Alouatta caraya, in the northeastern Argentina. Blood samples were collected at four sites with variable levels of habitat modification by human activity. PCR was conducted using primers for kinetoplast DNA, satellite DNA and ribosomal DNA of the trypanosomatid parasites. Ribosomal and satellite DNA fragments were sequenced to identify the trypanosomatid species and to characterize the discrete typing units (DTUs) of T. cruzi. Overall, 46% (50/109) of the howlers were positive according to the kDNA-PCR assay, but only 7 of the howlers were positive according to the SatDNA-PCR protocol. We sequenced the amplicons of the satellite DNA obtained from five specimens, and the sequences were 99% and 100% similar to T. cruzi and a species closely related to T. minasense.

1. Introduction

Chagas disease is the most important parasitic disease in Latin America, and as a result of infection of humans by the parasite Trypanosoma cruzi (Kinetoplastida: Trypanosomatidae), approximately eight million people are infected worldwide (World Health Organization, 2010). The transmission of this protozoan parasite by vectors is confined to the Americas and circulates in at least two broadly defined transmission cycles, occurring in domestic and sylvatic habitats. Infection with T. cruzi is a complex zoonosis, transmitted to vertebrate hosts by the feces of multiple blood-sucking triatomine species (Reduviidae: Triatominae) and sustained by more than 160 species of mammals belonging to 25 families in the Americas, with marsupials, edentates, and rodents being the most frequent sylvatic hosts. Chagas disease has emerged in regions previously considered to be relatively free of the disease, such as the Amazon basin, where mainly sylvatic, rather than domestic, vectors transmit the parasite, and local micro-epidemics of orally transmitted disease have been observed (World Health Organization, 2010). Infection in humans can also occur via blood

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transfusion, organ transplant, and vertical transmission from mother to offspring in endemic and non-endemic areas. Oral transmission has been observed in humans exposed to contaminated food (Nóbrega et al., 2009; Souza-Lima et al., 2013), which seems to be the main infection mechanism in wild mammals (Roque et al., 2008; Marcial et al., 2009; Rocha et al., 2013).

*T. cruzi* is currently classified into six discrete typing units (DTUs). *T. cruzi I* – *T. cruzi VI* (Tibayrenc, 2003; Zingales et al., 2009), defined as sets of stocks that are genetically more related to each other than to any other stock and that are identifiable by common genetic, molecular or immunological markers (Tibayrenc, 1998). The DTUs of *T. cruzi* are distributed differentially among triatomine insects, vertebrate host species and habitats in different geographical areas (Higo et al., 2004; Maffey et al., 2012). *T. cruzi I* occurs in many domestic cycles and mainly in sylvatic cycles throughout the Americas, involving opossums (genus Didelphis), which live in both arboreal and terrestrial sylvatic and peridomestic ecotopes (Bernabé et al., 2000; Yeo et al., 2005; Orozco et al., 2013). *T. cruzi II*, *V* and *VI* occur primarily in domestic cycles in Brazil and in the southern cone countries of South America (Yeo et al., 2005; Noireau et al., 2009); *T. cruzi III* has been detected in armadillos (Dasypus novemcinctus) throughout the Americas (Yeo et al., 2005; Lewis et al., 2009; Lisboa et al., 2008), whereas *T. cruzi IV* has been isolated mostly from sylvatic mammals in the northern Amazon basin and in the United States (Bernabé et al., 2000; Yeo et al., 2005; Llewellyn et al., 2009; Marcoli et al., 2009). Wild non-human primates appear to be associated naturally with *T. cruzi I*, *T. cruzi II* and *T. cruzi IV* in Atlantic and Amazonian forests (Lisboa et al., 2007; Da Silva et al., 2008; Marcoli et al., 2009; Araújo et al., 2011; Lisboa et al., 2015).

In addition to two zoonotic trypanosome species, *T. cruzi* and *T. rangeli*, neotropical non-human primates, primarily species of the Cebidae family, can also be infected with *T. (Megatrypanum) minasense* (Chagas, 1908; Dunn et al., 1963; Hoare, 1972; Deane et al., 1974; De Resende et al., 1994; Ziccardi et al., 2000; Sato et al., 2008; Tenório et al., 2014). Regarding the genus *Alouatta*, trypanostigotes of *T. minasense* have been recorded by morphologic techniques in wild individuals of *Alouatta palliata* from Costa Rica (Chinchilla et al., 2005) and by molecular diagnosis in one captive *A. caraya* in the Centre for Wild Fauna (CCWF) from Brazil (Tenório et al., 2014). The highly pleomorphic nature of *T. (Megatrypanum) minasense* in the bloodstream requires accurate identification of the species based on not only morphology. Sato et al. (2008) detected *T. minasense* infection by molecular diagnosis in *Saguinus midas* exported to Japan as experimental laboratory animals. The first molecular phylogenetic characterization of *T. minasense*, showed that it is closely related to trypanosomes with *T. theileri*-like morphology (Sato et al., 2008).

The “Gran Chaco” ecoregion includes northern Argentina, Bolivia, Paraguay and southwestern Brazil, and it is a hyperendemic region for Chagas disease. In the southeastern limit of the “Gran Chaco”, known as humid Chaco, and in the neighboring savannas and gallery forests in northeastern Argentina, studies of the dynamics of *T. cruzi* transmission and infection characterization of *T. cruzi* have been scarce (Bar et al., 1999, 2010). In this region, some wild triatomine insects that are potential vectors of Chagas disease, such as *Triatoma sordida* and *Psammolestes coreodes*, colonize wild biotopes, such as palms, tree hollows and bird nests (Bar and Wisnivesky-Colli, 2001; Damborsky et al., 2001; Bar et al., 2010). Additionally, *T. cruzi* has been detected in *T. sordida*, suggesting that this triatomine species could play a role in the maintenance of the wild *T. cruzi* transmission cycle in northeastern Argentina (Bar and Wisnivesky-Colli, 2001).
from the femoral vein. After blood collection, we placed the blood samples in EDTA 0.5 M-containing tubes (1:1), which were stored at 4 °C for up to 72 h or at −20 °C until processing for molecular studies. After collecting the samples, the individuals were placed in an open cloth bag for recovery from anesthesia. Once the animals fully recovered, they were released at the point of capture and were monitored for several hours to be certain that they were unharmed and capable of a full range of locomotion behavior. On subsequent days, the animals were monitored to follow up their normal recuperation.

These procedures were successfully used in other research studies (Schmidt et al., 2007; Oklander et al., 2007). This study complied with the laws of Argentina and the University of Illinois guidelines for the ethical treatment of primates (IACUC protocol 09267).

2.3. Molecular detection of trypanosomatid infections

2.3.1. DNA extraction

DNA was isolated from 200 µl of blood using the High Pure PCR Template Preparation Kit (Roche Diagnostic Corp., Indiana, USA), following the instructions of the manufacturer. Prior to DNA extraction, 200 pg of linearized p-ZErO plasmid containing a sequence of Arabidopsis thaliana was added to each sample as an external control for DNA extraction and polymerase chain reaction (PCR) (Duffy et al., 2009).

2.3.2. DNA amplification

Different PCR strategies were applied, as follows. 1) Detection of T. cruzi infections: 1a) kDNA-PCR, amplification of the 330 bp fragment from the minicircle DNA of the kinetoplastid genome of T. cruzi using the primers “121” (5’-AAATAATGTACGGG(T/G) GAGATGCATGA-3’) and “122” (5’-GGTCGAATTGGGTGGTGATTATAA-3’) and the cycling conditions reported by Burgos et al. (2007). 1b) Real-time SatDNA-PCR amplification of 166 bp fragments from specific satellite sequences of T. cruzi using the primers “cruzi 1” (5’-ASTCGGCTGATCGTTTTCGA-3’) and “cruzi 2” (5’-AATTCCCTCAAGCAGCGGATA-3’) and the probe “cruzi 3” (5’-CACACACTGAGCACCAAG-3’); the probe was labeled with 5_FAM (6-carboxyfluorescein) and 3_MGB (minor groove binder), and the cycling conditions were as reported by Duffy et al. (2013). PCR was performed using a Rotor Gene 3000 (Corbett Research, Sydney, Australia) Real Time thermocycler. Only positive kinetoplastid DNA-PCR (kDNA-PCR) samples were analyzed by this protocol. 2) Detection of trypanosomatid infections other than T. cruzi: RibDNA-PCR amplification was performed from the polymorphic D7 domain of the 24S a rRNA genes using the primers “D75” (5’-GCA-GATCTTGGTTGGCGTAG-3’) and “D76” (5’-GGTTCTCTGTTGCCCCTTTT-3’) and the cycling conditions reported in Schijman et al. (2006) (Table 1).

Each PCR run included an amplification reaction without DNA as a negative PCR mixture control and an amplification reaction with total DNA from the T. cruzi reference strain (T. cruzi VI CL-Brener) and/or from T. rangeli stock from Brazil (Cuba Cuba C. personal communication; Gurgel-Gonçalves et al., 2012) as positive controls. Up to 10 µl of the amplified product by conventional PCR (kDNA-PCR and RibDNA-PCR) was analyzed by 2% agarose gel electrophoresis in TBE buffer containing ethidium bromide (0.5 µg/ml), which was added previously to the gel.

2.3.3. Satellite and ribosomal DNA sequencing

PCR products were loaded on 2% low melting point agarose gels.
alignment of satellite sequences with those from representative cases except for S-61, which had 100% sequence identity. The database reference sequences was obtained in all of the T. cruzi (Table 3 and Fig. 2). There was no statistically significant difference among positive kDNA (X² = 0.15, df = 3, p-value = 0.99), SatDNA (X² = 0.23, df = 3, p-value = 0.97), and RibDNA-PCR (X² = 0.89, df = 3, p-value = 0.83) samples among the four study sites.

3. Results

Fifty (46%) howlers were PCR positive according to kDNA-PCR (Table 3 and Fig. 2). There were no statistically significant differences between sexes (chi-square test: X² = 0.44, degrees of freedom (df) = 1, p-value = 0.8) or between age categories (X² = 0.46, df = 2, p-value = 0.5; respectively) for positive kDNA-PCR samples. In addition, we identified kDNA amplicons with slightly variable molecular weights between 300 and 330 bp (Fig. 3), which were very similar to the size of the T. cruzi DNA fragment used as a reference and slightly smaller than the T. rangeli amplicon used as a reference.

Using the Real Time SatDNA-PCR protocol, we detected infection with T. cruzi in seven howler monkeys (Table 3 and Fig. 2), which yielded kDNA fragments of 330 bp (six samples) and 300 bp (one sample). Sequencing was performed on five of seven SatDNA PCR-positive samples (S-15 and S-52 [EBCo], S-40 [SC], S-61 and S-81 [IB], all yielding kDNA fragments of 330 bp). The two remaining SatDNA PCR-positive samples did not yield sufficient material for purification and sequencing. A nucleotide identity of 99% with T. cruzi database reference sequences was obtained in all of the cases except for S-61, which had 100% sequence identity. The alignment of satellite sequences with those from representative strains of the different T. cruzi DTUs allowed for the caracterization of T. cruzi in the SC and IB samples (Fig. 4). These sequence data are available in the GenBank database under the accession numbers KT369011 to KT369015.

The RibDNA-PCR protocol detected 96% (105/109) of the positive samples (see Table 3). Identical fragments with sizes of 240 bp were obtained in all of the RibDNA-positive samples (Fig. 5). This fragment size was expected for T. rangeli. However, RibDNA sequences from 9 samples (5 from EBCo and 4 from IB) (GenBank accession number: KT369016), of which 3 were kDNA PCR-positive, displayed maximum nucleotide identity with the homologous gene of T. minasense when a BLAST search was performed against a reference sequence published in GenBank (Max. Ident.: 99.5%, accession number: AB362411, Sato et al., 2008). This finding suggested the presence of T. minasense or a very closely related species in the nine specimens, which yielded identical sequences. In addition, 49 of 105 specimens positive for RibDNA were also kDNA PCR-positive; therefore, these animals might have been co-infected with T. minasense and with T. cruzi or other trypanosomatid. It was not possible to obtain a culture of T. minasense to be used as a positive control to compare the sizes of DNA fragments obtained by RibDNA-PCR.

There was no statistically significant difference among positive kDNA (X² = 0.15, df = 3, p-value = 0.99), SatDNA (X² = 0.23, df = 3, p-value = 0.97), and RibDNA-PCR (X² = 0.89, df = 3, p-value = 0.83) samples among the four study sites.

4. Discussion

This study was the first to describe the molecular detection of T. cruzi infection in free-ranging howler monkeys from Argentina.
confirming previous results based on morphological and serological techniques (Travi et al., 1982; Santa Cruz et al., 2000). We detected 46% of positive samples by amplification of the minicircle variable region of Trypanosoma sp. in all of the study sites, whereas only 6% (n = 7) of these samples were also positive according to SatDNA-PCR, confirming T. cruzi infection. This result was expected because SatDNA-PCR is less sensitive than kDNA-PCR, especially for infections with T. cruzi I or T. cruzi IV (Duffy et al., 2009; Schijman et al., 2011; Ramírez et al., 2015). Therefore, the low prevalence of T. cruzi infection obtained with SatDNA-PCR might have been due to a low parasite burden. Alternatively, several samples that yielded positive results for kDNA-PCR might have come from individuals infected by trypanosomatids other than T. cruzi, which were not distinguishable by the protocols implemented here. This possibility would be compatible with the variability of fragment sizes obtained by kDNA-PCR.

The prevalence of positive samples for the three diagnostic protocols (kDNA, SatDNA and RibDNA-PCR) were similar among the four study sites. However, we identified howler monkeys infected with different DTUs, as revealed by the SatDNA sequences. For instance, we recorded the occurrence of T. cruzi I in one monkey from IB (our “remote” site), consistent with the association of this DTU with the wild transmission cycle (Bernabé et al., 2000; Yeo et al., 2005; Orozco et al., 2013). We also found sequences from IB that shared 99–100% identity with those previously reported in DTUs II, V, and VI. These DTUs seem to be primarily associated with the domestic transmission cycle of T. cruzi (Yeo et al., 2005), although they have also been found in the wild (Noireau et al., 2009). Therefore, the presence of these DTUs in IB was not so unexpected. Monkeys from SC and EBCo were infected by strains that shared 99.58% identity with the reference strain of T. minasense (Yeo et al., 2005), from the first time by Sato et al. (2008). The per-

mambo burrows were also seen at the bases of these sleeping site trees. Psammolestes coreodes associated with bird nests was found within of the home range of the studied howler groups (Martínez, unpublished data). Birds are refractory to T. cruzi infection but can be important sources of blood meals for triatomines. These triatomines, which are potential vectors of trypanosomases (Cruz-Guzmán et al., 2014), possibly feed from birds (Rabinovich et al., 2011) or small mammals that live in these nests, but the possibility that they fed on howler monkeys cannot be discounted. In non-human primates, it is presumed that transmission of trypanosomes in enzootic cycles occurs mainly through the ingestion of infected triatomines (Da Silva et al., 2008; Roque et al., 2008; Marcili et al., 2009). The diet of howler monkeys primarily consists of leaves, fruits and flowers (Fernández, 2014; Dias and Rangel-Negrín, 2015), but they can ingest insects accidentally when eating fruits and leaves or when removing ectoparasites (grooming). More rarely, they might eat ants from old trees (Ránio, personal communication). Therefore, howler monkeys could be infected in a number of different manners. Nevertheless, if the studied monkey population was already chronically infected, congenital (vertical) transmission from mother to offspring is possible (Eberhard and D’Alessandro, 1982).

Trypomastigotes of T. minasense were originally described by Chagas (1908) in the blood of a marmoset, Callithrix penicillata, from southeastern Brazil. This trypanosome is a widely distributed species detected in several neotropical non-human primates, such as A. palliata, Saimiri sciureus, Saginus midas, and Callithrix penicillata (Dunn et al., 1963; Souza and Dawson, 1976; Ziccardi and Lourenço de Oliveira, 1997; Ziccardi et al., 2000; Chinchilla et al., 2005; Sato et al., 2008). The transmission route of T. minasense is unknown. Because most of the recorded primate hosts are primarily arboreal, Dunn et al. (1963) suggested that the vectors for T. minasense are probably arboreal or partially arboreal insects.

Our results showed identical rDNA amplicons that displayed nucleotide identity with the reference strain of T. minasense, which was determined for the first time by Sato et al. (2008). The percentage of identity (99.5%) was similar to that obtained between available 245 ribosomal sequences of two T. rangeli strains (“San Agustín” and “SC58”: 99.58%; GenBank Accession Nos.: U73612, and KJ742907, respectively) and was greater than that obtained between T. cruzi stocks belonging to the same DTU (i.e., stocks “DM28” and “La Cruz” [TC I: 96.4%] [Souto et al. (1999) and Accession No.: L22334, respectively] and stocks “TC Ilib” and “V” [TC

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### Table 3

Infection prevalence determined by kDNA-PCR, RibDNA-PCR and SatDNA-PCR in wild howlers from Estación Biológica Corrientes (EBCo), Isla Brasilera (IB), San Cayetano (SC), and Isla del Cerrito (IC). Data presented as percentage, with 95% confidence intervals.

| Study site – PCR protocol | No. positive groups/No. examined groups | No. positive monkeys/No. examined monkeys | % Prevalence (IC 95%) |
|---------------------------|-----------------------------------------|-------------------------------------------|-----------------------|
| EBCo (rural) kDNA         | 8/11                                    | 19/42                                     | 45.2 (29.84–61.33)    |
| RibDNA                   | 11/11                                   | 40/42                                     | 95.2 (83.83–99.42)    |
| SatDNA                   | 3/8                                     | 3/19                                      | 15.8 (3.38–39.58)     |
| IB (remote) kDNA          | 13/15                                   | 22/49                                     | 44.9 (30.66–59.77)    |
| RibDNA                   | 15/15                                   | 48/49                                     | 98 (89.14–99.95)      |
| SatDNA                   | 3/13                                    | 3/22                                      | 13.6 (2.90–34.92)     |
| SC (village) kDNA        | 4/5                                     | 8/16                                      | 50 (24.65–75.35)      |
| RibDNA                   | 5/5                                     | 15/16                                     | 93.8 (69.76–99.85)    |
| SatDNA                   | 1/4                                     | 1/8                                       | 12.5 (0.31–52.66)     |
| IC (village) kDNA        | 1/1                                     | 1/2                                       | 4             |
| RibDNA                   | 1/1                                     | 2/2                                       | 4             |
| SatDNA                   | 0/1                                     | 0/1                                       | 4             |

*a Prevalence was not estimated due to the very limited sample size.

*b Sat-DNA PCR was carried out only in kDNA-PCR-positive samples.
The samples that were kDNA-positive (46%) and their corresponding ribosomal sequences showed that identity with *T. minasense* was likely to indicate mixed infection with *T. minasense*/*T. cruzi* or with other Trypanosomatids that were not identified using our methods. *T. minasense* could have resulted in a 330 bp kDNA fragment; however, only 46% of the samples were positive according to kDNA-PCR. This finding suggests that the primers used for this protocol do not recognize the kDNA target in *T. minasense*. However, the lack of reference DNA from *T. minasense* precluded the confirmation of this possibility. In addition, in mixed infections, RibDNA-PCR yielded only *T. minasense* fragments but not *T. cruzi*-specific fragments (Schijman et al., 2006), perhaps because of a higher burden of *T. minasense* in mixed infection, favoring the amplification of the parasite DNA in a larger amount.

This study was the first to show the presence of *T. minasense* or a...
species closely related to T. minasense in wild howler monkeys. Tenório et al. (2014) found T. minasense in one captive individual of A. caraya that had 90% similarity with the homologous sequence referenced by Sato et al. (2008). The prevalence found in our study (96%) was much higher than that recorded in the updated bibliography of non-human primates; however, these reports were based on blood smear analyses, hemoculture and/or xenodiagnosis (De Resende et al., 1994; Ziccardi and Lourenço de Oliveira, 1997; Saguinus midas individuals by PCR amplification. These high prevalence values detected were likely due to the greater sensitivity of molecular diagnostic methods.

Our current knowledge regarding the transmission process of T. minasense, the effects of the infection on non-human primates, and how it or other non-zoonotic trypanosomes interact with T. cruzi in a mixed infection is very scarce. The results of the present study enabled us to survey the biology of T. minasense in free-ranging non-human primate populations, as well as how it interacts with T. cruzi infection dynamically.

Parasitological surveys of non-human primates provide an important opportunity to better understand the epidemiology, transmission dynamics and emergence risk of various anthropozoonoses such as Chagas disease, which affect the human population.
populations in most locations in the semiarid and humid Argentine Chaco region. The results obtained in this study will help evaluate the role of A. caraya as a reservoir of human trypanosomiasis in regions where Chagas disease is hyper-endemic and where the human-wildlife interface is increasing. Massive changes in land use, among other effects, usually result in an increasing probability of parasite cross-transmission as the contact between howlers and humans and associated domestic animals increases. Our current knowledge regarding the impact of T. cruzi on the health of free-ranging A. caraya populations remains minimal. Consequently, we believe that research into the epidemiology, transmission ecology, and clinical consequences of T. cruzi infections of A. caraya and other non-human primate populations will provide more accurate information on the dynamics of this disease in the wild-domestic interface.

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