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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*. A sequence typical of DTU *T. cruzi* I was found in one howler monkey from the "remote" site, while sequences compatible with DTUs II, V, and VI were found in howlers from the "remote", "rural" and "village" sites. We detected 96% positive samples for RibDNA-PCR, 9 of which were sequenced and displayed 99% identity with *Trypanosoma minasense*, while none showed identity with *T. cruzi*. The results demonstrated the presence of *T. cruzi* and a species closely related to *T. minasense* in blood samples from free-ranging *A. caraya*, belonging to different *T. cruzi* DTUs circulating in these howler monkey populations. The results obtained in this study could help evaluate the role of *A. caraya* as a reservoir of *T. cruzi* in regions where Chagas disease is hyperendemic and where the human-wildlife interface is increasing.

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; Marcili 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; Orozco et al., 2013), 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; Marcili 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; Marcili 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*, trypomastigotes 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).

Black and gold howler monkeys (*Alouatta caraya*) inhabit flooded forests in the island system of the Parana River and in the humid Chaco gallery forests east and west of the Parana River (Zunino and Kowalewski, 2008). Northern Argentina comprises the boundary of the southern distribution of *A. caraya*, which accounts for the largest proportion of biomass of any arboreal mammal (Brown and Zunino, 1994). Like other howlers, *A. caraya* often copes well with moderate deforestation (Bicca-Marques, 2003; Zunino et al., 2007). Habitat fragmentation involves the alteration of habitat, resulting in changes in the interaction between wildlife and humans, which can contribute to the proliferation of emergent zoonotic diseases (Nunn and Altizer, 2006; Gillespie et al., 2008). The available data on *Trypanosoma* infection in *A. caraya* have been scarce (Funayama and Barretto, 1970; Travi et al., 1982; Santa Cruz et al., 2000), and the limited sensitivity and specificity of the diagnostic methods used have hindered the level of detection. Aiming to assess the possible role of howler monkeys in the *T. cruzi* transmission cycle, this study sought to assess and characterize the prevalence of *T. cruzi* DTUs and of other trypanosomatid infections in populations of *A. caraya* living at sites with variable levels of contact with humans and with associated domestic animals.

2. Materials and methods

2.1. Study sites

We collected blood samples from howlers at four sites that differed in their degrees of contact with humans: Isla Brasilera (IB) (27° 20′ S, 58° 40′ W) ("remote"); Estación Biológica Corrientes-Biological Field Station Corrientes (EBCo) (27° 30′ S, 58° 41′ W) ("rural"); Isla del Cerrito (IC) (27° 17′ S, 58° 37′ W) ("village"); and San Cayetano (SC) (27° 34′ S, 58° 42′ W) ("village") (Fig. 1). Long-term studies of the ecology and behavior of *A. caraya* in these sites allowed us to localize and identify the selected studied howler groups. SC and IC are village sites where howler groups live in close proximity to family houses, with tree crowns that are contiguous with trees in the nearby forest fragments. IB is located 20 km north of EBCo and is an island characterized by a continuously flooded forest and little to no human contact. The island has an area of 290 ha, and 32–35 groups of primates have been identified. EBCo is a rural site characterized by a semi-deciduous forest, where at least 34 howler groups are under study in 24 forest fragments covering a total area of 3000 ha (Zunino et al., 2007; Kowalewski et al., 2010).

2.2. Capture of howler monkeys and sample collection

A total of 109 howler monkeys of different ages were captured and examined for trypanosome infections in different study areas (Table 2). Captures were conducted between July and August 2010. The animals were captured and immobilized to obtain blood samples, for which the assistance of at least two veterinarians was necessary. Chemical immobilization of the animals was performed with anesthetic darts (1 cc Pneu-dart type P) shot using an air rifle. Parenteral anesthesia was accomplished with xylazine chlorohydrate (Xilacina 20®, Richmond Vet Pharma, Buenos Aires, Argentina) (20 mg/ml), combined with midazolam (Midazolam®, Richmond Vet Pharma) (5 mg/ml), and ketamine (Ketonal 100®, Richmond Vet Pharma) (100 mg/ml), at the minimum dose appropriate for species and weight (Kreeger and Arnemo, 2007).

The animals were captured from 11:00 h to 13:00 h and from 14:00 h to 16:00 h. During these time intervals, the temperature is warm and howlers are active at relatively low heights in the canopy. A sequence of procedures was applied to each howler: darting; physical examination and health assessment; and collection of blood samples, among others. Individuals in the groups were classified by sex/age categories, according to the classification of Rumiz (1990). We collected 1 ml of peripheral blood per individual