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ORIGINAL ARTICLE

TRYPANOSOMA SPP. IN CAPTIVE PRIMATES IN A BRAZILIAN ZOO

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ABSTRACT

Captive animals, despite the constant care provided, are susceptible to infections from different sources. We herein report the natural trypanosome infection of 11 (28.2% positive) out of 39 non-human primates from 13 different species, in a Brazilian zoological park. Immunofluorescent antibody test (IFAT) and conventional polymerase chain reaction (cPCR) ruled out *Trypanosoma cruzi*, the etiological agent of Chagas disease. However, sequencing performed with positive samples employing hsp70 primers revealed similarities from 86% to 88% to diverse trypanosomes, including *T. cruzi, Trypanosoma grayi, Trypanosoma lewisi, Trypanosoma rangeli* and *Trypanosoma vivax*. We believe that the low similarity values obtained by sequencing reflect the difficulties in the molecular identification of trypanosomes, which share a large portion of their genetic material; this similarity may also preclude the diagnosis of co-infection by more than one trypanosome species. Thus, our study demonstrates the presence of diverse trypanosomes in primates, which are susceptible to infection by these parasites. Mechanical devices such as windows and bed nets, etc., are required to avoid vector insects in these environments, in addition to preventive quarantining of animals recently introduced into zoos. Therefore, investigation of the parasites in both the animals already residing in the zoo and those being introduced is of paramount importance, although no easy task.

KEY WORDS: Non-human primates; monkey; diagnosis; trypanosomes.

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INTRODUCTION

Trypanosoma encompasses numerous parasite species that infect practically all vertebrate groups, including humans (Haag et al., 1998). Mammals, in particular, serve as reservoirs for numerous trypanosomatids, such as *Trypanosoma cruzi,* the causative agent of Chagas disease, which is endemic to the Americas and transmitted by many triatomine species (Ministério da Saúde, 2015). Currently, about six to 7 million people worldwide, mostly in Latin America, are estimated to be infected with *T. cruzi* (WHO, 2020). In Brazil, there are approximately three million people still carrying the disease in its chronic form (Ministério da Saúde, 2016). The first work on trypanosomes was performed using primates and involved isolating the parasite from the blood of the animals, after they had been in contact with infected triatomines. Currently it is suggested that these primates (they were Callitrichids) were actually infected by the oral route and not by the contaminative route (Chagas, 1909). Today, primates, both free-living and captive, are a source of infection for several trypanosomes (Jirků et al., 2015; Erkenswick et al., 2017).

Stercorarian trypanosomes include parasites which infect vertebrates utilizing the intestinal route of the vector and are generally eliminated from the invertebrate via feces and urine, as in the case of *T. cruzi*. However, some Salivarian trypanosomes are transmitted by the mouthparts of the vectors to the vertebrate, especially the salivary glands of these insects, as occurs in the transmission of *Trypanosoma brucei*, which causes African Trypanosomiasis, also known as sleeping sickness. The so named African Trypanosomiasis includes several Trypanosoma species. Regarding *Trypanosoma brucei*, besides sleeping sickness in humans, this taxon causes severe cattle, camel and horse diseases.

Laboratory tests to detect trypanosomes in wild animals suffer one of the major bottlenecks in these studies, which is the lack of specific conjugates for serological tests. Therefore, molecular techniques are frequently utilized (Roque et al., 2013, Tenório et al., 2014). However, the polymerase chain reaction (PCR) technique has proved to be an optimal tool due to its high specificity and sensitivity. Despite the advances in detection techniques, many species of trypanosomes have not been correctly identified or are only recorded partially in databases (National Center for Biothechnology Information and U.S. National Library of Medicine, 2016).

Zoos and wildlife conservation centers perform an important role in the recovery of animal victims of illegal trafficking and by enabling the procreation of threatened species (Cuarón, 2005).

Despite action taken over the past decades to eliminate the kissing bug *Triatoma infestan*s, one of the main vectors of Chagas disease in Brazil, cases of acute infection by the protozoan are still being reported in the country (Ministério da Saúde, 2015). This is mainly due to infected triatomines processed together with food, although no longer due to *T. infestans* (Barroso Ferreira et al., 2014).

The understanding of trypanosomes in wild animals is limited, mostly due to difficulties in research in the field (Auty et al., 2012). Zoological parks built in wild environments provide alternatives allowing researchers to study many aspects of wildlife, from the behavior of the animals to their susceptibility to diseases. Non-human primates, both free-living and captive, are naturally susceptible to various trypanosomes (Lisboa et al., 2004; Minuzzi-Souza et al., 2016; Bahia et al., 2017). In this context, we studied the presence of *Trypanosoma* spp. in captive primates living in a Brazilian zoo.

METHODS

Location of the study

The Municipal Zoological Park in Bauru, in the interior of the Brazilian State of São Paulo, opened in 1980 and currently encompasses 484 thousand m² of constructed area located within more than 4.8 million m² of preserved area (Figure 1). The climate at the zoo is hot and humid during the summer and dry during the winter, and the zoo is located in a savannah area presenting a subtropical semi-deciduous forest, in which, part of the natural vegetation sheds its leaves during the dry season (Cavassan, 2013).

Figure 1. Location of the zoo in the study. Map highlighting the location of the Municipal Zoological Park within the city of Bauru, São Paulo, Brazil. Scale refers to the city.

Animals used in the study

Blood samples were collected from 39 primates of 13 different species from September to December 2014 during an odontological intervention previously scheduled by the zoo veterinarian. The primates were anesthetized with a 10 mg/kg dose of ketamine, according to the national guidelines "Surveillance Guide for Epizootic Diseases on Non-Human Primates and Entomology Applied to Yellow Fever Surveillance" and "National Council of Animal Experimentation Control: appendix 1." The study was approved by the Committee for Ethical Use of Animals (CEUA/FMB nº 1104/2015) and by the System for Authorization and Information in Biodiversity (SISBIO/ICMBio/MMA nº 45000-2). All the animals in the study were born in or transferred to the zoo prior to 2013, according to the records supplied by the zoo.

Spatial distribution of the animals

The primates are kept in two locations in the zoo. The New World primates (23 primates of nine species) are located near the visitors' entrance in fenced enclosures encircled by various types of trees also present in the interior of the enclosures, in order to create a diverse environment for these animals. The Old World primates (16 primates of four species) are located in a more remote location. Concrete and glass walls limit the enclosure, and only a fence encloses the top. Despite the native vegetation present inside the enclosure, lianas grow in and around the location where these animals live (Figure 2).

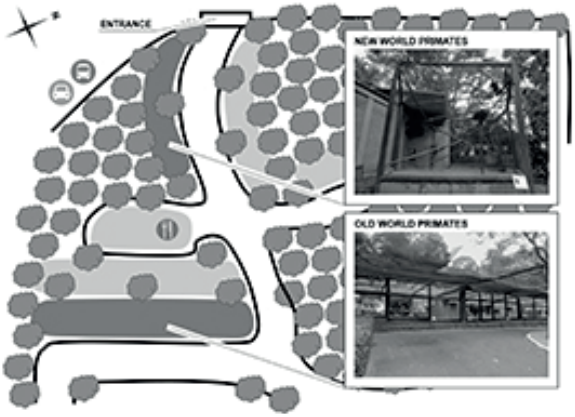

Figure 2. Spatial distribution of the primates in the study within the zoo. The areas in dark gray indicate the location of the primate enclosures, with the location for each group highlighted in the boxes. Map without scale.

Serological evaluation

Immunofluorescent antibody test (IFAT) was used. Conjugated anti-monkey (IgG–γ-chain specific-SAB3700765–Sigma-Aldrich®, USA) was utilized for the reaction. Strain Y of *T. cruzi* was kept in liver infusion tryptose (LIT) medium for 7 days, washed subsequently three times, and suspended in phosphate-buffered saline (PBS), pH 7.2. The final suspension was placed on slides, dried at room temperature, and stored in a freezer at -18 °C until testing (Camargo, 1966).

Molecular evaluation

DNA was extracted from blood samples utilizing the Axy Prep DNA Blood Genomic Miniprep (Axygen Scientific®, USA) commercial kit following the manufacturer's recommendations. The extracted samples were kept in a freezer at -18 °C.

Conventional polymerase chain reaction (cPCR)

The conditions for the reaction were as follows: PCR buffer (50 mM KCl, 20 mM of Tris-HCl), 1.6 mM $MgCl_2$, 0.2 mM dNTPs, 1 U of Taq-polymerase (Platinum® Taq DNA Polymerase, Invitrogen, USA), 0.2 μM each primer, 1 μL of the tested sample, and 8.3 μL of ultra-pure water (MIX-PCR) were added to each 0.2 mL microtube, for a final volume of 11 μL of MIX-PCR and 1 μL of extracted DNA with a minimal concentration of 10 ng/μL. As positive controls, we used 10 ng of DNA extracted from an *in vitro* culture of *Leishmania (L.) infantum* (MHOM/BR/2002/LPC-RPV), *Leishmania (V.) braziliensis* (MHOM/BR/1975/M2903), *Leishmania (L.) major* (MHOM/IL/1980/FRIEDLIN), and *Trypanosoma cruzi* (Y strain). A negative control (nuclease free water) + Mix PCR and a positive control (DNA extracted from culture) + Mix PCR were assigned for each batch of samples undergoing amplification.

For amplification of *T. cruzi* nuclear DNA, the following primers were utilized, according to Virreira et al. (2003): TCZ1: 5′-CGAGCTCTTGCCCACACGGGTGCT-3′ and TCZ2: 5′-CCTCCAAGCAGCGGATAGTTCAGG-3′. The amplification conditions were based on those described by Moser et al. (1989) with modifications: one cycle of initial denaturing at 94 °C for 30 s; 25 cycles of denaturing at 94 °C for 20 s; annealing at 57 °C for 30 s, and extension at 72 °C for 30 s; and a final cycle at 72 °C for 3 min.

For amplification of the gene encoding heat shock protein (hsp70), the following primers were employed, according to Hernández et al. (2014): HSP70F: 5′-AGGTGAAGGCGACGAACG-3′ and HSP70R: 5′-CGCTTGTCCATCTTTGCGTC-3′. The amplification conditions were based on those described by Hernández et al. (2014) with modifications: one initial cycle at 94 °C for 4 min; 40 cycles of denaturing at 94 °C for 30 s, annealing at 59 °C for 30 s, and extension at 72 °C for 30 s; and one final extension cycle at 70 °C for 10 min.

To evaluate the quality of the extracted DNA and the possible presence of PCR inhibitors in the samples, the following primers for amplifying the β-globulin gene were utilized, according to Lee et al. (2001): β1: 5′-ACCACCAACTTCATCCACGTTCACC-3′ and β2: 5′-CTTCTGACACAACTGTGTTCACTAGC-3′. Samples with no amplification products were subjected to reactions with the following primers that amplify the glyceraldehyde 3-phosphate dehydrogenase gene, as described by Kullberg et al. (2006): GAPDH-F: 5′-AGGCTGAGAACGGGAAACTT-3′ and GAPDH-R: 5′-ATTAAGTTGGGGCAGGGACT-3′. The same amplification conditions were used for both primers, consisting of one initial cycle at 95 °C for 5 min; 30 cycles of denaturing at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s; and one final extension at 72 °C for 5 min.

DNA sequencing

Genetic sequencing using the Sanger method was performed in a Genetic Analyzer 3500 automated sequencer with the Big-Dye Terminator v3.1 Cycle Sequencing kit® (Applied Biosystems; Life Technologies, MA, USA). The sense and antisense sequences were visualized using Chromas® v2.1.1 software (Technelysium Pty Ltd., Australia), subjected to global alignment using MEGA7 software (Kumar et al., 2016) and compared with sequences deposited in the GenBank using the nucleotide basic local alignment search tool (BLASTn, www.ncbi.nlm.nih.gov/BLAST).

RESULTS

Serological Results

Five of the samples from the 39 primates were not viable for serological testing. Therefore, the sera from 34 primates were analyzed. All the primates were subjected to IFAT for anti-*T. cruzi* and all sera were non-reactive (Table).

Table. Results of serological and nuclear satellite DNA to *Trypanosoma cruzi* carried out for all non-human primates at the Municipal Zoological Park in Bauru.

Scientific name (Common name)	Nuclear satellite DNA	hsp70	Serological test
New World primates			
Alouatta belzebul (Red-handed howler monkey)- n=2			
01 , 02	-	-	NR
Alouatta caraya (Black howler monkey)- n=5			
03, 04, 05, 06, 07	-	-	NR
Alouatta fusca (Brown howler monkey)- n=1			
08	-	-	NR
Alouatta seniculus (Venezuelan red howler monkey)- n=2			
09	-	+	IS
10	-	-	NR
Ateles chamek (Black-faced black spider monkey)- n=3			
11, 13	-	+	NR
12	-	-	IS
Ateles marginatus (White-cheeked spider monkey) - n=3			
14, 15, 16	-	-	NR
Ateles paniscus (Red-faced spider monkey) - n=2			
17, 18	-	-	NR
Cebus albifrons (White-fronted capuchin monkey) -n=2			
19, 20	-	+	NR
Lagothrix lagotricha (Woolly monkey) - n= 3			
21	-	-	NR
22, 23	-	+	NR
Old World primates			
Erythrocebus patas (Patas monkey) -n=3			
24, 26	-	-	NR
25	-	+	IS
Mandrillus sphinx (Mandrill) - n=4			
27, 28,29,30	-	-	NR
Papio hamadryas (Hamadryas baboon) - n= 8			
31, 34, 35, 36	-	-	NR
32, 33	-	+	NR
37, 38	-	-	IS
Papio (Guinea baboon) - n=1			
39	-	+	NR

NR: non-reactive; R: reactive; IS: insufficient serum for testing; +: positive; -: negative.

Molecular Results

To guarantee the quality of the extracted DNA, all samples were analyzed using the primers β1 and β2 for the β-globulin gene. The gene was amplified in 11 of the 13 species in the study, excluding the *Papio hamadryas* and *Papio* species. GADPH-F and GADPH-R were used to verify DNA quality of the species for which there was no amplification product with the primers β1 and β2. The target gene was amplified with these primers for *P. hamadryas* and *P. papio*, in addition to the species *Erythrocebus patas* and *Alouatta belzebul,* which were used as controls for the amplification.

In the first molecular test utilizing the species-specific primers TCZ1 and TCZ2 for *T. cruzi*, all the primates studied were negative.

Utilizing the hsp70 primers, 11 of the 39 (28.2%) were positive (Table). The samples were submitted to sequencing, revealing 88% similarities for *T. cruzi* (M26595.1 / X67716.1 / KC960011.1), *T. grayi* (XM009317554.1), and *T. lewisi* (KP208748.1) and 86% similarities for *T. rangeli* (KC544896.1 / KC960003.1) and *T. vivax* (KP208747.1).

DISCUSSION

Despite the success of the campaign, the emergence of Chagas disease caused by the ingestion of contaminated food (Nóbrega et al., 2009) demonstrated that the eradication of only one species was not sufficient for prevention, as several other species can participate in the epidemiological cycle of Chagas disease, especially *Panstrongylus megistus, Rhodnius neglectus*, and *Triatoma sordida*. The location of the study is wooded, containing diverse species, and is located in the Brazilian savannah, an environment in which triatomines have become adapted to living in domestic areas. Thus, zoo primates introduced into a savannah environment may be exposed to hematophagous insect vectors of diverse trypanosomes. Furthermore, palm trees (family Arecaceae) are found throughout the zoo, including close to many enclosures, and are part of the local landscaping design. These plants are known habitats of many types of triatomines (Abad-Franch et al., 2015) risking exposure of these animals to the vector as well as to hematophagous flies. Trypanosomiasis by *T. cruzi* is primarily a wild enzooty. In Brazil, wild mammals in every biome are infected by *T. cruzi.* As stated above, only mechanical measures are recommended for controling wild triatomines.

All the primates in the study were negative for *T. cruzi* by cPCR utilizing the primers TCZ1 and TCZ2. However, 11 animals were positive when employing the hsp70 primer, revealing a similarity of up to 88% with *T. cruzi* and differing from the results of cPCR. The molecular method utilizes species-specific primers that amplify known segments of parasites. The wide array of trypanosomes, many of which differ from those already deposited in databases,

contributes to the lack of amplification products for many trypanosomes for which there are already well-established conventional molecular methods (Auty et al., 2012). Owing to the difficulties posed by working with captive animals, additional data collection methods could not be performed at the time of the blood collection, such as cardiological examinations, which should be performed since primates can develop cardiomyopathies similar to those that occur in humans (Jelicks & Tanowitz, 2011).

The gene encoding hsp70 is a conserved region of the genome responsible for regulating activities inside cells, and it is highly expressed during stress, such as changes in temperature upon transit between the vector and host (Burger et al., 2014). Because it is a highly conserved region found in a diverse array of organisms, the sequence is very useful for the identification of a large number of trypanosomes, including some for which primers have not yet been designed for use in cPCR techniques (Adams et al., 2010).

The other trypanosomes identified in the sequenced samples may reflect the large similarity among the sequences of these organisms deposited in databases, considering that some trypanosomes do not infect primates naturally or are only found in other regions of the world. *T. grayi* naturally infects African crocodiles. Although its vector is a tsetse fly (Glossina spp.), *T. grayi* is transmitted through the fecal material of the vector, a mechanism similar to that adopted by many trypanosomes, especially *T. cruzi* (Kelly et al., 2014). Despite its similarity to some trypanosomes that infect alligators in South America, such as *Trypanosoma ralphi* (Fermino et al., 2013), the literature does not indicate whether *T. grayi* can infect mammals, as it lacks membrane proteins that would enable the invasion of mammalian cells, even though *T. grayi* is genetically close to other trypanosomes, such as *T. cruzi* and *T. rangeli* (Martins et al., 2015). This similarity may indicate that another *T. grayi*-like or *T. cruzi*-like trypanosome could infect primates.

However, *T. lewisi* is a trypanosome that commonly infects rats in the wild or in the laboratory. Its principal transmission route is by a bite or the ingestion of fleas infected with the parasite (Desquesnes et al., 2002). Despite infecting and growing only in rats, the infected fleas can interact with other vertebrates, including humans, and transmit the parasite. For example, a newborn child with dengue-like symptoms admitted to a hospital in Thailand. After a blood smear indicated the presence of trypanosomes in peripheral blood, PCR was performed with subsequent sequencing, revealing 98% similarity with *T. lewisi* (Sarataphan et al., 2007).

There have been reports in Brazil, of rats naturally infected by *T. lewisi*, indicating the possibility that the protozoan circulates freely in wild and urban environments, as it is transmitted by many species of fleas (Linardi & Botelho, 2002). As in the case report of the Thai child, the primates in the zoo may have been infected by some unidentified *T. lewisi*-like trypanosome.

The sequencing results also indicated a similarity to *T. vivax*. This parasite was introduced into Latin America by the importation of bovines principally from Africa in the 19th century (Jones & Dávila, 2001) and it is able to infect a large variety of animals of veterinary interest (Cuarón, 2005), including non-human primates, where they are one of the most common findings (Njiokou et al., 2004). In Africa, where it circulates in the wild, *T. vivax* is transmitted by tsetse flies. However, in Brazil, the parasite has adapted to mechanical transmission by stable flies, which comprise multiple hematophagous species of flies, including *Stomoxys calcitrans*, found in many parts of the country.

T. rangeli is a non-pathogenic trypanosome to humans and other mammals (Garcia et al., 2012; Gurtler & Cardinal, 2015). Commonly found in primates in the Amazon rainforest (Ziccardi & Lourenço-de-Oliveira, 1997; Ndao et al., 2000), the parasite is also present in savannah regions (Ramirez et al., 1998), including the environment in the current study, indicating the possibility of triatomines feeding on the blood of primates.

Despite the findings indicating the circulation of trypanosomes among these animals, the primates did not present any clinical signs of infection, as indicated by the zoo data, suggesting that the animals are asymptomatic in the presence of the infection or that the parasites did not demonstrate pathogenicity toward them. However, other laboratory exams such as complete blood counts and biochemical exams could be useful for verifying the clinical alterations.

The similarities found in the sequencing of our samples reflect the sequences deposited at the GenBank™ which returned very close similarity percentages to more than one trypanosome species, which may account for the trypanosome species generally not reported in primates, such as *T. grayi.* Furthermore, these animals can be co-infected with more than one trypanosome species. Co-infection exclusively by trypanosomes is not well documented in the literature as infection is frequently related to other diseases of human interest, as in the case of co-infection by *Leishmania* and HIV (Okwor & Uzonna, 2013). It is known that fish can be co-infected by more than one trypanosome species (Grybchuk-Ieremenko et al., 2014), which may also have occurred with the primates in the present study.

Inhibitors present in blood and a low parasite load are known factors that interfere with PCR (Barea et al., 2004), which can affect the sequencing results, justifying similarities of up to 90%. However, environmental laws protect captive animals and do not allow tissue samples or spinal puncturing for more precise testing, since animal stress and suffering in these procedures are high. Although the use of other molecular techniques such as PCR-RFLP (restriction fragment length polymorphism) can provide better results (Lisboa et al., 2015), the use of primers that amplify conserved regions of these parasites is a viable alternative for identifying trypanosomes in wild animals (Adams et al., 2010).

Some species of primates are included on the list provided by the "Convention on International Trade in Endangered Species of Wild Fauna and Flora" (CITES), which aims to protect many types of animals and plants worldwide and permits the exchange of specimens between locations and reintroduction of animals into the wild. However, there has been no survey of the parasitic state of these animals, which can introduce trypanosomatids into new locations (Roque & Jansen, 2014) and render the local population of animals susceptible to new infections if triatomine vectors or hematophagous flies are present.

Thus, many species of trypanosomes can circulate in the environment where these primates are introduced, indicating that the insect vectors of these parasites are capable of feeding on the blood of these animals. Minuzzi-Souza et al. (2016) identified triatomines infesting primates in a Brazilian zoo, with 17 non-human primates positive for *T. cruzi*, in addition to more than 40 infected triatomines. Unfortunately, the active survey procedures for triatomines are no longer performed in the environment where the zoo in the current study is located, preventing a similar analysis. However, the finding could be common in zoos located in tropical regions containing native vegetation, permitting the movement of wildlife and vectors between animal enclosures. Future actions such as correct maintenance of the primates' enclosures will be enough to detect any invading triatomines coming from the adjacent forest.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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