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Short Communication

Molecular identification of trypanosomatids in wild animals

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ABSTRACT

Diverse wild animal species can be reservoirs of zoonotic flagellate parasites, which can cause pathologic Chagas disease. The present study aimed to detect the natural occurrence of flagellate parasites through direct microscopic examination of the parasites in blood samples and through PCR of whole blood and blood culture (haemoculture) samples from 38 captive and 65 free-living wild animals in the Centre for Conservation of Wild Fauna (CCWF), an area endemic for leishmaniasis. For this study, PCR was accomplished using primers for the ribosomal region (ITS-1) of the flagellate parasites. The amplified fragments were cloned and sequenced to identify DNA of the Trypanosomatid parasite species, observed in blood cultures from 3.9% (04/103) of the animals. Through these techniques, *Trypanosoma cruzi* was identified in haemoculture samples of the following three free-living species: common agouti (*Dasyprocta aguti*), white-eared opossum (*Didelphis albiventris*), and nine-banded armadillo (*Dasypus novemcinctus*). Furthermore, *Trypanosoma minasense* was identified in whole blood samples from 01 (0.9%) captive animal (black howler monkey-*Alouatta caraya*). These results demonstrated the first report of *T. cruzi* isolation in wild species from the CCWF using blood culture, which can be applied in addition to molecular tools for epidemiological studies and to identify trypanosomatids in wild animals.

1. Introduction

Studies have shown that different wild animal species are naturally infected by zoonotic flagellate parasites, which can cause Chagas disease (Yeo et al., 2005; Herrera et al., 2008; Botto-Mahan et al., 2012).This zoonosis is caused by *Trypanosoma cruzi* a protozoan specie of the order Kinetoplastida, family Trypanosomatidae and genus *Trypanosoma* (Brasil, 2003).

Several techniques are used for diagnosing Chagas disease in humans including blood culturing and polymerase chain reaction (PCR) (Portela-Lindoso and Shikanay-Yasuda, 2003). Primers for the internal transcribed spacer1 (ITS-1) of the ribosomal region have been used to amplify DNA fragments from *Leishmania* spp. (El Tai et al., 2000; Schonian et al., 2003; Alam et al., 2009). However, they also amplify sequences from other trypanosomatids (Desquesnes and Dávila, 2002).

Domestic dogs were positive for VL in urban and rural areas of Ilha Solteira (Queiroz et al., 2011; Paulan et al., 2013). At the Centre for Conservation of Wild Fauna (CCWF) in Ilha Solteira, São Paulo State, Brazil, visceral leishmaniasis (VL) had already been detected in many wild canids

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(Jusi et al., 2011; Tenório et al., 2011); however, there was no information about either the occurrence of Chagas disease in humans or the presence of *T. cruzi* in wild animals from the CCWF.

The fact that wild animals are reported to be reservoirs of *T. cruzi* is considered very important in the epidemiological chain of the infection (Souza, 2000). Originally, the parasite was associated only with wild mammals; however, due to ecological imbalance, the natural vectors progressively adapted to different ecotypes, and infected susceptible humans and domestic animals (Barreto, 1965).

The current work aimed to identify tripanosomatid parasites, present in whole blood or blood culture (haemoculture) by means of molecular techniques (PCR, cloning and sequencing) from different species of free-living or captive wild animals living in the CCWF in Ilha Solteira, SP, Brazil.

2. Materials and methods

2.1. Wild animals and blood sample collection

The blood samples were collected from wild animals from the Centre for Conservation of Wild Fauna (CCWF) located in Ilha Solteira, São Paulo, Brazil (20°38′44″ S; 51°06′35″ W). For this study, 103 wild animals were evaluated; 38 animals were captive, and 65 were free-living (Table 1). Two aliquots of blood were taken; one for parasites identification in the blood culture and the other for PCR analysis. The volumes of blood samples varied according to the body size of each animal species.

2.2. Blood culture (haemoculture)

The blood samples were inoculated in LIT (Liver Infusion Tryptose) medium observed weekly using an optical microscope (1000×). Since the flagellate parasites were seen at the first examination in the blood cultures, they were isolated for DNA extraction procedures as described below.

2.3. Preparation for DNA extraction, PCR and amplified product purification

The blood cultures were centrifuged (1400 × *g*) and washed in phosphate buffered saline (PBS), pH 7.4 and the pellet was collected for DNA extraction. Then the DNA was extracted from the blood culture pellets and from whole blood samples using the commercial kit Illustra Blood Genomic Prep Mini Spin Kit (GE Healthcare®). The DNA quality and quantity were assessed by means of a spectrophotometer (Nanodrop 2000c – Thermo Scientific).

The oligonucleotides, MIX-PCR concentration (Platinum® Taq DNA Polymerase, Invitrogen) and amplification conditions were in accordance with El Tai et al. (2000). The *L. major* (MHOM/BR/IOC/2906), *L. infantum* (MHOM/BR/IOC/2821), and *Trypanosoma cruzi* Y strains were maintained in LIT medium and used as positive controls, whereas the negative control was nuclease-free water. Amplified DNA was purified from an agarose gel using the kit Wizard SV Geland PCR Clean-Upsystems (Promega) followed by quantification using the Low DNA Mass Ladder® (Invitrogen).

2.4. Cloning the purified fragments

To generate high levels of quality amplified fragments, the purified products were inserted into the cloning vector pGEM®-T Easy (Promega). The plasmid was inserted into competent *Escherichia coli* cells, lineage JM109 (Promega) and the bacterias were transformed by thermic shock. For propagation, Luria-Bertani (LB) culture medium was used.

After this procedure, those colonies with the inserts were separated and again inoculated in LB culture medium. The plasmid DNA was extracted from the clone by means of the ENZA™ Plasmid Miniprep Kit II (Omega Bio-Teck). The procedures related to cloning and plasmid DNA extraction were carried out according to the manufacturer's instructions.

2.5. Sequencing

The products were sequenced from the plasmid DNA. For the automated sequencing reaction, Big Dye Terminator chemistry (Applied Biosystems) was used. Capillary electrophoresis was performed on the samples submitted to sequencing (Applied Biosystems, AB I3500 model). The electropherograms were analyzed for quality by the program Sequencing Analysis version 5.4 (Applied Biosystems), whereas the sequences were aligned using the software MEGA (Molecular Evolutionary Genetics Analysis) version 5.0 for Windows (Tamura et al., 2011). The sequences were compared with sequences deposited in the NCBI database (BLAST).

3. Results

3.1. Blood culture and PCR

Four (3.88%) of the 103 blood cultures analyzed from wild animals presented flagellate protozoa in LIT culture medium, carried by the following free-living species: common agouti (*D. aguti*, 6.25%), white-eared opossum (*D. albiventris*, 5.88%) and nine-banded armadillo (*D. novemcinctus*, 9.09%). In contrast, few flagellate parasites were observed in the blood culture from a single captive black howler monkey (*A. caraya*, 12.5%) (Table 1).

PCR results showed that products approximately 585 bp long were amplified from the haemocultures of free-living animal, except for that of the captive black howler monkey (*A. caraya*). In this species, a DNA fragment product approximately 650 bp in length was amplified from whole blood even with low levels of protozoan parasites in the blood culture. The results of whole blood and blood cultures without DNA amplification are also shown in Table 1.

3.2. Sequencing

The DNA sequences generated were compared with the BLAST data base and showed 97% similarities with *T. cruzi* from agouti and opossum (accession number: