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Short communication

Leishmania (*Viannia*) DNA detection by PCR-RFLP and sequencing in free-ranging owl monkeys (*Aotus azarai azarai*) from Formosa, Argentina

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ABSTRACT

American Cutaneous Leishmaniasis (ACL) is caused by protozoan parasites of the *Leishmania* genus, and transmitted by females of the Phlebotominae family.

The role of wild and domestic hosts in the cycle of *Leishmania* is still unknown.

ACL is endemic in the province of Formosa where *Nyssomyia neivai* was the most abundant species in several captures and 31 cumulative ACL human cases were reported between 2005 and 2011 in the province.

The present report describes the detection, by PCR-RFLP and confirmed by sequencing, of subgenus *Leishmania* (*Viannia*) DNA in four free-ranging owl monkeys (*Aotus azarai azarai*) from Formosa Province. The sequence amplified was the mini-exon gene present in tandem repeats in all species of the *Leishmania* genus from buffy coat samples. The absence of inhibitors in the samples was checked by a β-globin protocol originally designed to amplify the human β-globin gene.

However, other free-ranging primates were found with natural infections of *L.* (*V*) *braziliensis* complex and *Leishmania* (*Viannia*) subgenus by parasitological means in America. To the best of our knowledge, there are no published reports on detection of subgenus *Leishmania* (*Viannia*) DNA by PCR-RFLP in argentinean free-ranging primates.

Additional eco-epidemiological and parasitological studies are necessary to confirm owl monkeys, or any other natural infected mammal species detected by PCR, as a reservoir, incidental host or to propose it as an animal model for research on this topic.

1. Introduction

American Cutaneous Leishmaniasis (ACL) is caused by protozoan parasites of the *Leishmania* genus, and transmitted by females of the Phlebotominae family. In Argentina, this zoonosis is mainly produced by *L.* (*Viannia*) *braziliensis* and transmitted by *Nyssomyia neivai* in peridomestic areas, and by *Nyssomyia whitmani* in forest environments (Salomón et al., 2009). The role of wild and domestic hosts in the cycle of *Leishmania* is still unknown in Argentina (Programa Nacional de Leishmaniasis, 2004; WHO, 2010).

ACL is endemic in the Argentinean province of Formosa, located at the South American Chaco biogeographical region. Cases of ACL were reported from Formosa since

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1955, but in the Argentinean Chaco region the first cases were recorded in 1916 (Salomón et al., 2006). In 2001, *Ny. neivai* was the most abundant species of Phlebotominae (97%) captured around the city of Las Lomitas, Formosa. A year later, 96 ACL human cases were reported in that locality, and *Ny. neivai* was again the most abundant species (91%) (Salomón et al., 2006). After this outbreak, between 2005 and 2011, 31 cumulative ACL cases were reported in the province, four of them in the capital district. Further, *Ny. neivai* and *Migonemyia migonei* were also captured only 15 kilometers from the capital city, where there is a recall among the health system agents of an unrecorded outbreak during the early 60 s (Sistema Nacional de Vigilancia de la Salud, Ministerio de Salud de la Nación, 2011; Salomón et al., 2011). Despite these numerous cases of ACL, the actual reservoir of *L. braziliensis* in Argentina remains unknown.

In order to evaluate the involvement of potential hosts in the *Leishmania* cycle, the proper identification of natural infections for the detection and identification of parasite species is required. The sensitivity and specificity of molecular techniques such as Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP) and automated sequencing of amplicons, provide a useful alternative to determine parasites in clinical samples, accidental hosts, potential reservoirs and Phlebotomine insects (Marfurt et al., 2003; Pita-Pereira et al., 2009; Acardi et al., 2010; Cordeiro Malta et al., 2010). Despite the sensitivity of these molecular techniques, standard parasitological procedures such as blood smears and parasite culture should also be performed for definitive diagnosis as they constitute direct biological evidence of infection (Programa Nacional de Leishmaniasis, 2004).

We report here the detection, by PCR-RFLP confirmed by sequencing, of subgenus *Leishmania Viannia* DNA in free-ranging owl monkeys (*Aotus azarai azarai*) from Formosa Province. Owl monkeys (*Aotus* spp.), are small (698–1230 g) (Smith and Jungers, 1997) arboreal, sexually monomorphic primates inhabiting the forests of Central and South America. They live in groups generally consisting of an adult heterosexual pair, one infant and one or two juveniles and subadults (Fernandez-Duque, 2012). The possible role of owl monkeys in the *L.* (*V.*) *braziliensis* cycle and the need for additional research to confirm its presence are discussed.

2. Methods

Samples analyzed in the present study were provided from a broad project that evaluates monkey population dynamics and health status in the eastern Chaco region of Argentina; the capture area comprises a semi-deciduous gallery forest along the banks of the Pilagá River (58°11W, 25°58S) on lands belonging to the cattle ranch Estancia Guaycolec, 30 km away from Formosa City, Argentina. Between February and April 2011, eight adult owl monkeys (four males and four females) belonging to different groups were captured and immobilized in order to take blood samples (Fernandez-Duque and Rotundo, 2003). Captures were performed in a 1.2 km^2 area and individuals did not show clinical signs for Leishmaniasis. It was also obtained a spleen necropsy from a male adult owl monkey that was found dead in the study area.

Whole blood samples (5 mL) were collected by venipuncture of the femoral vein in syringes with EDTA, and centrifuged at 4000 rpm (TLD-4, Arcano®) within 4 h of collection. One milliliter of buffy coat (BC) was stored in cryovials in liquid nitrogen until sent to the laboratory. The spleen necropsy was stored in sterile water and also preserved in liquid nitrogen.

DNA extraction from BC was performed with 500 μL lysis buffer (10 mM Tris-HCl, pH 8; 100 mM NaCl; 0.45% Tween 20; 0.25% SDS; and 100 μg/sample Proteinase K) and placed over night at 58 °C. DNA was purified by phenol-chloroform-isoamilic alcohol (25:24:1) procedure, precipitated with ethanol 100% and resuspended in 50 μL DNAse/RNAse-free distilled water. The spleen necropsy was processed similarly, with the exception of 200 μg Proteinase K/sample final concentration.

The absence of inhibitors in the samples was checked by a β-globin PCR which has an expected 267 bp amplicon (sense primer GH20 5′-GAA GAG CCA AGG ACA GGT AC-3′, antisense primer PC04 5′-CAA CTT CAT CCA CGT TCA CC-3′) (Saiki et al., 1992; Prychitko et al., 2005). Since this protocol was originally designed to amplify the human β-globin gene, we previously tested it by bioinformatic analysis against the sequence of β-globin gene of *Aotus azarai* (AY279113). Both primers GH20 and PC04 aligned at positions 755 and 1022 of the gene generating the expected fragment, encouraging us to proceed empirically with the samples. We obtained a 267 bp fragment from all samples analyzed; in order to confirm these products, three of the fragments were sequenced (Genbank Accessions numbers: JQ043342, JQ043343, JQ043344) giving maximum identity with *A. azarai* beta globin gene when BLAST was performed against Genbank.

The PCR-RFLP assay for *Leishmania* detection was done under conditions previously suggested by Marfurt et al. (2003) which has an expected 226 bp product (Fme 5′-TAT TGGTAT GCG AAA CTT CCG-3′ and Rme 5′-ACA GAA ACT GAT ACT TAT ATA GCG-3′ primers), and targets the mini-exon gene present in tandem repeats in all species of the *Leishmania Viannia* subgenus. The reaction was carried out with 5 μL of extracted DNA in a final volume of 50 μL containing 1× PCR Buffer (200 mM Tris-HCl, pH 8), 0.1 mM EDTA, 1 mM DTT, 50% glycerol (v/v) (Invitrogen), 1 mM $MgCl_2$ (Invitrogen), 10% DMSO (SIGMA), 0.2 mM dNTP Mix, 0.5 μM of each primer and 1.4 U Taq polymerase (Invitrogen). Up to 10 μL of the amplified product was analyzed by 2% agarose gel electrophoresis in TBE buffer containing ethidium bromide (0.5 μg/mL) added previously to the gel. *L.* (*V.*) *braziliensis* reference strain (MHOM/BR/1975/2903) was employed as positive control. The RFLP assay was carried out with *Hae III* restriction enzyme (5′-GGCC-3′ Promega) by digesting 10 μL of PCR reaction. The two fragments generated were resolved by 2.5% agarose gel electrophoresis. The 226 bp product was confirmed by automated cycle sequencing of both DNA chains according to the fluorescent ddNTP terminator system (MACROGEN Inc., Korea).

DNA chromatograms were analyzed with Codon Code Align™ software and sequences were identified by

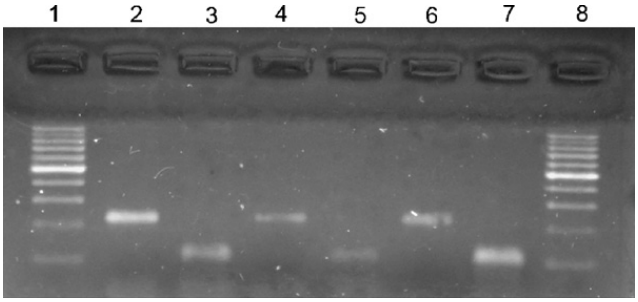

Fig. 1. Agarose gel 2,5% showing expected product of *Leishmania Viannia* and the restriction fragment length polymorfism pattern obtained with *HaeIII*. Lanes 1 and 8: 100 MW 100 bp DNA ladder (Biodynamics); lanes 1 and 2: PCR product from *Aotus a. azarai* buffy coat sample (uncut) and digested PCR product; lanes 3 and 4 PCR product from *Aotus a. azarai* buffy coat sample (uncut) and digested PCR product; lane 5 and 6 PCR product from promastigotes DNA (*L. Viannia braziliensis* MHOM/BR/75/2903 reference strain) (uncut) and digested PCR product.

BLAST search against Genbank. For homology sequence analysis, *L. braziliensis* sequences (JF720019, JF720020, JF720021) obtained from LTA human cases at Wanda and Puerto Libertad cities (Misiones Province, Argentina), MHOM/BR/1975/2904 and MHOM/BR/75/2903 *L. braziliensis* reference strains (FR798976, X69441) were employed using Clustal X 2.1 (Larkin et al., 2007).

3. Results and discussion

Four samples were positive by PCR for *Leishmania Viannia*, giving the 226 bp expected fragment; when RFLP assay was performed a 118 bp fragment expect to *L.*(*V.*) *braziliensis* was obtained (Fig. 1). Although the proportion of PCR positivity samples observed (4 over 9 samples) may seem unexpected, it should be noted that all monkeys were captured in the same relatively small geographic area, although they belong to different groups. This spatial proximity among groups might explain the results obtained, supported by the strictly controlled PCR conditions employed.

The sequences (JN661691, JN661692, JN661693, JN661694) showed different identities with *L.* (*V.*) *braziliensis* by BLAST against Genbank database. Sequence JN661691 had an identity of 98% and 97% with JF720019 and JF720020 Argentinean *L. braziliensis* sequences and an identity of 97% and 96% with MHOM/BR/75/2904 (FR798976) and MHOM/BR/75/2903 (X69441) *L. braziliensis* reference strains. Sequence JN661692 had an identity of 97% with JF720021, and 95% with JF720019 and JF720020 Argentinean *L. braziliensis* sequences. It also showed a 99% and 98% identity with FR798976 and X69441 *L. braziliensis* reference strains. Referred to sequence JN661693, the identity was 98%, 97% and 96% with JF720021, JF720020 and JF720019 local isolated sequences, and 99% and 97% with FR798976 and X69441 reference sequences, respectively. Finally, JN661694 sequence had 100% identity with JF720020, 99% with FR798976, 98% with X69441, 97% with JF720021 and 96% with JF720019.

To the best of our knowledge, there are no published reports on detection of *Leishmania* (*Viannia*) DNA by PCR in free-ranging primates. However, free-ranging *Aotus trivirgatus* and the tamarin *Saguinus geoffroyi* of Panama were found with natural infections of *L. braziliensis* complex by parasitological means in the 70s (Herrer and Christensen, 1976). Additionally, capuchin monkeys (*Cebus apella*) and bearded sakis (*Chiropotes satanas*) of the Amazon basin were found infected by *L.* (*V.*) *shawi* (Lainson et al., 1989). Moreover the owl monkey species *A. trivirgatus* was used as an experimental model for demonstrating susceptibility to infections by *L.* (*V.*) *panamensis* (Lujan et al., 1986a, 1986b, 1987), *L.* (*L.*) *mexicana* (Christensen and de Vazquez, 1981), and *L.* (*L.*) *donovani* (Broderson et al., 1986).

With regards to *L.* (*L.*) *donovani* complex, the parasite DNA was amplified by PCR from a dead *Callicebus nigrifrons* kept in captivity at the zoo of Belo Horizonte, Brazil, where one third (12/36) of frozen peripheral blood samples stored from captive monkeys were also positive by PCR: one *Aotus nigriceps*, one *Callicebus nigrifrons*, one *Alouatta guariba*, three *Cebus xanthosternos*, one *Leontopithecus chrysomela*, two *Pithecia irrorata* and three *Saguinus imperator* (Cordeiro Malta et al., 2010).

In America, Leishmaniases are zoonoses with reservoirs established in wild or domestic animals (Chaves et al., 2007). Usually, there is a main reservoir of each *Leishmania* species in each area. Besides the existence of a primary reservoir, it is possible that other animals can become infected as secondary or incidental hosts. Secondary hosts might play a role in disease transmission bringing the parasitic source closer to humans, while incidental hosts do not have any role in the maintenance of the parasite in nature (Chaves et al., 2007). The primary reservoir for *L.* (*L.*) *braziliensis* has not been well established, and a dynamic reservoir community involving different species has been proposed (Haydon et al., 2002; Salomón et al., 2006). Although we provide the first report of *Leishmania Viannia* in free-ranging owl monkeys, additional eco-epidemiological and parasitological studies are necessary to confirm owl monkeys or any natural infected mammal species as a reservoir, incidental host, or to propose it as an animal model for research on this topic. Besides, molecular detection of *Leishmania* spp. should be performed by other PCR protocol with a better discriminatory capacity, and supported by parasite isolation. Future studies should examine the distribution in time and space of *Leishmania* infection incidence, prevalence, persistence of living parasites, and clinical signs within each population, including eventual vertical and horizontal transmission.

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