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Short communication

Detection of natural infection in *Lutzomyia cruzi* and *Lutzomyia forattinii* (Diptera: Psychodidae: Phlebotominae) by *Leishmania infantum chagasi* in an endemic area of visceral leishmaniasis in Brazil using a PCR multiplex assay

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ABSTRACT

In order to identify *Lutzomyia* spp. naturally infected by *Leishmania* parasites a PCR multiplex assay coupled to non-isotopic hybridization was used for the analysis of insect samples collected by CDC light traps in an endemic area of visceral leishmaniasis (VL) in the municipality of Corumbá, Mato Grosso do Sul State, Brazil in May/June 2006. Wild sand flies were identified and grouped into pools of 10 female specimens and 27 groups in total were collected. Positive results were obtained from *Lutzomyia cruzi* (2 out of 13 pools) and *Lutzomyia forattinii* (1 out of 14 pools). The positive pools were confirmed as being infected by *Leishmania infantum chagasi* after hybridizing the PCR products with a species-specific biotinylated probe derived from the kinetoplast minicircle conserved sequence. Given that we detected infection in 3 out of 27 groups and that there was at least 1 infected insect in each, it was possible to infer an infection rate of 1.5% for *Lu. cruzi* and 0.7% for *Lu. forattinii* in the analyzed samples. These results confirm the vectorial role of *Lu. cruzi* in transmitting *L. infantum chagasi* and suggest *Lu. forattinii* as a potential VL vector in the municipality of Corumbá, where notifications of the disease in humans and dogs have increased over the last two decades.

1. Introduction

Visceral leishmaniasis (VL) is endemic in several Latin American countries and the disease was classically observed in the northeast of Brazil (Deane and Deane, 1962). In the past two decades the re-emergence and urbanization of VL in Brazil has led the disease to become one of the major health problems in the country (Arias et al., 1996; Lainson and Rangel, 2005), where *Leishmania infantum chagasi* Cunha & Chagas, 1937 is the etiologic agent and *Lutzomyia longipalpis* (Lutz & Neiva, 1912) acts as primary vector (Deane and Deane, 1962; Lainson and Rangel, 2005).

In the State of Mato Grosso do Sul, VL was reported as early as 1911 with sporadic human cases. Since then, there has been an increase in the number of VL notifications, and spread to new areas so that it can actually be considered as being endemic in several regions within Mato Grosso do Sul (Arruda et al., 1949; Arias et al., 1996; Oliveira et al., 2006). The municipality of Corumbá in the northwest of the State represents one of these endemic areas with active transmission of canine and human visceral leishmaniasis. Although three males of *Lu. longipalpis* were found in 2001 in Corumbá (Santos et al., 2003), other entomological studies have not demonstrated its presence in the area (Galati et al., 1985, 1997; Brazil et al., 2002). The predominance of *Lutzomyia cruzi* (Mangabeira, 1938) and *Lutzomyia forattinii* (Galati et al., 1985) and their anthropophily suggested a possible participation of both sand fly species in the transmission of the disease in Corumbá (Galati et al., 1997). Moreover, the finding of natural *Leishmania* infection in *Lu. cruzi* strongly supported its identification as a VL vector in this region (Santos et al., 1998).

In this study, a PCR multiplex non-isotopic hybridization assay was used to evaluate natural infection rates of sand flies captured in a peri-domiciliary area in the municipality of Corumbá by *Leishmania* spp. The data presented here confirm previous findings regarding the participation of *Lu. cruzi* in the transmission of *L. infantum chagasi*, and describe for the first time the occurrence of natural infection by this parasite in *Lu. forattinii*, bringing a

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better understanding of the eco-epidemiology of leishmaniasis in the State of Mato Grosso do Sul, Brazil.

2. Materials and methods

2.1. Sand fly capture and taxonomic identification

Sand flies were collected in May/June 2006 with CDC light traps (Sudia and Chamberlain, 1962) placed in a pigpen of a peridomicile (19°01′75″S, 57°08′33″W), in Cristo Redentor district of Corumbá, Mato Grosso do Sul State. Alive insects were transferred to labelled containers, taken to the laboratory and killed in the freezer. Using a stereoscopic microscope, pools of 11 individuals were usually performed based on their external coloration (dark in *Lu. cruzi* and completely pale in *Lu. forattinii*). Afterwards, one specimen from each pool was randomly taken and slide-mounted in Canada Balsam to confirm the identification using Young and Duncan's keys (1994).

Females of *Lu. longipalpis* and *Lu. cruzi* are morphologically similar. However, in the present study the absence of *Lu. longipalpis* males and the capture of 180 males of *Lu. cruzi* has led us to consider the females as belonging to this latter species.

2.2. DNA extraction and PCR multiplex-dot blot hybridization

Pools of 10 specimens belonging to the same species were conditioned in 1.5 mL tubes containing 100 μL of TE buffer (pH 8.0) and stored at −20 °C until DNA extraction. DNA was extracted as previously described (Pita-Pereira et al., 2005) and rigorous procedures were assumed in order to control potential contamination, e.g. we included negative control groups (male sand flies) in the DNA extraction step and decontaminated instruments and working areas with diluted chloride solution and ultraviolet light.

For the PCR multiplex assay, two primer pairs were used. The primers [5′-GGCCCACTATATTACACCAACCCC-3′ and 5′-GGGGTAGGGGCGTTCTGCGAA-3′] were employed to amplify the conserved region of kinetoplast minicircle DNA from *Leishmania* spp. (Passos et al., 1996). The second primer set amplifies the *cacophony* gene IVS6 region in sand flies of the Neotropical genus *Lutzomyia*: 5Llcac [5′-GTGGCCGAACATAATGTTAG-3′] and 3Llcac [5′-CCACGAACAAGTTCAACATC-3′] (Lins et al., 2002). The reactions were carried out in a final volume of 100 μL containing 1× *Taq* polymerase buffer (Applied Biosystems, Foster City, CA, USA), 4.5 mM $MgCl_2$, 200 μM dNTPs (Invitrogen), 0.2 μM of each primer, 1.25 U *Taq Gold* DNA polymerase (Applied Biosystems) and 10 μL DNA sample. The mixture was incubated in a PerkinElmer thermocycler (GeneAmp PCR System 9600, Applied Biosystems) at 94 °C for 12 min for enzyme activation (hot-start PCR), followed by 35 cycles, each consisting of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and a final extension step for 10 min at 72 °C. Ten microliters of the amplification product were resolved on 2% agarose gel electrophoresis and visualized under ultraviolet transillumination following ethidium bromide staining.

The amplified products were also analyzed by dot blot hybridization using a *L. infantum*-specific biotinylated probe [5′-AAAAATGGGTGCAGAAAT-3′] designed to an internal sequence of the kDNA minicircle conserved region (Francino et al., 2006). The amplicons (10 μL) were denatured in 0.4N NaOH before application to nylon membranes (Boehringer Mannheim GmbH, Mannheim, Germany), using a manifold vacuum device (Bio-Dot™; BioRad, Richmond, CA, USA). Pre-hybridization was performed at 60 °C for 3 h in BLOTTO solution (1.5× saline–sodium-citrate buffer containing 1% sodium dodecylsulfate, 0.5% skimmed milk). After addition of the biotinylated probe (5 ng/mL), hybridization reactions were performed overnight at 46 °C in the same BLOTTO solution. Filters were washed five times using 0.05% Tween-20 (BioRad) in PBS, at 25 °C for 5 min each, before incubation with a streptavidin–peroxidase conjugate diluted 1:100 (Sigma, St. Louis, MO, USA) in the same buffer at 25 °C for 1 h. The membranes were then washed five times with PBS–Tween-20 solution at 25 °C for 5 min. Hybridization reactions were revealed by using Luminol reagent (Santa Cruz Biotechnology, CA, USA).

3. Results and discussion

One important step towards the incrimination of *Leishmania* vectors is the report of the occurrence of naturally infected sand flies (Killick-Kendrick and Ward, 1981). In most epidemiological studies, accurate knowledge of the *Leishmania* parasite and its vector species can result in an appropriate intervention when transmission is taking place in a given area. Although sand fly digestive tract dissection is the method most commonly used to reveal the rate of natural infections in endemic areas, it is laborious and time-consuming. Another limiting factor is the difficulty of processing the large number of samples that would be required for epidemiological investigations (Aransay et al., 2000; Perez et al., 2007). Alternatively, molecular techniques allow for DNA detection of a single *Leishmania* parasite (Pita-Pereira et al., 2005) and probably represent a more sensitive tool than manual dissection and microscopic examination (Nascimento et al., 2007), which in cases of low parasitaemia, may give underestimated scores of natural sand fly infection rates. Moreover, in putative positive cases revealed by sand fly gut dissection, the infection has to be confirmed by in vitro culture of parasites (often susceptible to contamination), or by inoculation into laboratory animals, as other non-identified flagellates are commonly found in the insect midgut (Ryan and Brazil, 1984; Freitas et al., 2002).

The use of PCR for detection of *Leishmania* DNA in wild sand flies is a useful technique to identify putative leishmaniasis vectors in different geographic areas (Silva and Grunewald, 1999; Aransay et al., 2000; Miranda et al., 2002; Pita-Pereira et al., 2005; Perez et al., 2007). The main advantages of molecular methods are their sensitivity and specificity, independent of the number, stage and localization of the parasite in the insect midgut (Perez et al., 1994). In a previous study we developed a PCR multiplex assay coupled to dot blot non-isotopic hybridization, yielding a highly specific procedure for the identification of *Leishmania* (*V.*) *braziliensis* infection in wild *Lutzomyia* from the municipality of Rio de Janeiro, Brazil (Pita-Pereira et al., 2005). The same methodology was applied in this study.

A total of 450 specimens (180 male and 270 female insects) were collected from the end of May to June 2006, in the district of Cristo Redentor in the municipality of Corumbá, where recent cases of human and canine visceral leishmaniasis have been reported. Two species of sand flies were identified, the most frequent being *Lu. cruzi* (55.6%) followed by *Lu. forattinii* (44.4%).

These insects were analyzed in groups of 10 sand flies, from the same species; in total 18 male and 27 female groups. The male insect groups were used only as negative controls for the DNA extraction step and as a way to verify eventual contamination in the PCR analysis (Pita-Pereira et al., 2005). Positive female groups were confirmed as infected by *L. infantum chagasi* when the PCR products were subjected to hybridization with a specific probe (Fig. 1). Three out of 27 (11%) female groups were positive, 2 of them being *Lu. cruzi* and the other of *Lu. forattinii*. Thus, if one infected insect occurred in each of these three groups, the minimal infection rate for *Lu. cruzi* and *Lu. forattinii* was 1.5 and 0.7%, respectively. Interestingly, one positive group from each *Lutzomyia* spp. could be detected prior to