Acta Tropica 120 (2011) 273–275

Contents lists available at SciVerse ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Short communication

Lutzomyia (*Pintomyia*) *fischeri* (Diptera: Psychodidae: Phlebotominae), a probable vector of American Cutaneous Leishmaniasis: Detection of natural infection by *Leishmania* (*Viannia*) DNA in specimens from the municipality of Porto Alegre (RS), Brazil, using multiplex PCR assay

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ARTICLE INFO

Article history:
Received 14 February 2011
Received in revised form 4 August 2011
Accepted 9 September 2011
Available online 16 September 2011

Keywords:
Leishmania (*Viannia*)
Lutzomyia (*P.*) *fischeri*
PCR multiplex
Natural infection
Porto Alegre, Brazil

ABSTRACT

In order to determine natural *Leishmania* (*Viannia*) infection in *Lutzomyia* (*Pintomyia*) *fischeri*, a multiplex PCR methodology coupled to non-isotopic hybridization was adopted for the analysis of sand fly samples collected by CDC light traps in an endemic area of American Cutaneous Leishmaniasis (ACL) in the periurban region of the municipality of Porto Alegre, Rio Grande do Sul State, Brazil. We analyzed by PCR methodology 560 specimens of *Lutzomyia* (*Pintomyia*) *fischeri* (520 females and 40 males). The wild sand flies were grouped into 56 pools (52 females and 4 males) of 10 each, and positive results were detected in 2 of the 52 female pools, representing a minimum infection rate of 0.38% based on the presence of at least 1 infected insect in the pool. This result associated with some local evidence such as anthopophily, spatial distribution in accordance with the transmission area and human case incidence, suggests that *L.* (*P.*) *fischeri* may be considered as a secondary vector of ACL in the studied locality.

1. Introduction

In Brazil, American Cutaneous Leishmaniasis (ACL) is widely distributed, cases having been reported throughout the country. *Leishmania* (*V.*) *braziliensis* is the most broadly distributed causative agent of ACL in Brazil, and its transmission is associated with a variety of sand fly vectors in different geographic regions (Ministério da and Saúde Secretaria de Vigilância em Saúde, 2007; Rangel and Lainson, 2009), such as *Lutzomyia* (*Ps.*) *wellcomei*, in the sylvatic cycle and *L.* (*N.*) *whitmani*, *L.* (*N.*) *intermedia*, *L.* (*N.*) *neivai* and *L. migonei* in domiciliary transmission areas in both periurban regions and those suffering environmental alterations imposed by man (Rangel and Lainson, 2009).

The first three cases of ACL in the State of Rio Grande do Sul were diagnosed in 2001, two from the city of Santo Antonio das Missiões and the third from the municipality of Viamão. In 2002, two new cases were diagnosed from the municipality of the Porto Alegre State capital (Santos et al., 2002). According to the Rio Grande do Sul Health Secretary during the period from 2002 to 2010 a total of twenty three new human cases of ACL were confirmed in Porto Alegre (Souza, G.D., personal communication). As reported in entomologic and molecular biology-based studies, *Lu.* (*N.*) *neivai* was the most frequent species encountered in the transmission area and was considered as a potential ACL vector (Gonçalves, 2003; Pita-Pereira et al., 2009; Souza et al., 2008; Rangel and Lainson, 2009). According to Souza et al. (2008), *Lutzomyia* (*P.*) *fischeri* was the second most abundant sand fly species in the same region, accounting for 25.2% of the total specimens collected with CDC light and Shannon traps, in great numbers around domiciles, especially in rabbit and quail sheds.

In this study, a PCR multiplex non-isotopic hybridization assay was adopted to evaluate natural infection rates of *L.* (*P.*) *fischeri* by *Leishmania* (*Viannia*) spp. The insects were collected in periurban Porto Alegre, where a natural infection in *Lutzomyia* (*Nyssomyia*)

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0001-706X
doi:10.1016/j.actatropica.2011.09.004

neivai by *Leishmania* (*Viannia*) had previously been detected (Pita-Pereira et al., 2009). This (Quinta do Portal) is an important tourist area subjected to environmental changes where human cases of ACL have been registered since 2002, increasing in the number of cases in recent years. The presented data may contribute to a better understanding of the eco-epidemiology of ACL in Rio Grande do Sul State.

2. Materials and methods

2.1. Sand fly capture and taxonomy of specimens

Sand flies were collected monthly (one night) from October/2006 to May/2007 in the locality of Quinta do Portal, with CDC light traps (one for each site) in 10 monitoring stations. Sand flies were captured inside houses, in the peridomiciles (close to domestic animal shelters) and in the forest. All sand flies were transported to the Entomology Laboratory at the Center for Monitoring of Rodent and Vector, the Health Secretary of Porto Alegre and subsequently identified based on morphological nomenclature and taxonomy proposal according to Young and Duncan (1994).

2.2. DNA extraction and multiplex PCR non-isotopic hybridization assays

Five hundred sixty *L.* (*P.*) *fischeri* phlebotomine (520 females and 40 males) were sent to the laboratory for PCR examination. The insects were grouped into 56 pools of 10 specimens (52 unfed females and 4 males for contamination control) and submitted to molecular analysis for *Leishmania* infection. DNA was extracted as previously described (Pita-Pereira et al., 2005). Multiplex PCR was designed to simultaneously amplify the cacophony gene IVS6 region in sand flies of the neotropical genus *Lutzomyia* (as an internal control for the polymerase enzyme activity and DNA extraction) and the conserved kinetoplast DNA minicircle region from *Leishmania* spp. The amplified products further underwent dot blot hybridization with a *Leishmania* (*Viannia*)-specific biotinylated probe (Pita-Pereira et al., 2005). Notably, 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 as well as working areas with diluted chloride solution and ultraviolet light. We also added artificially infected females as positive controls.

3. Results and discussion

PCR technology represents an alternative method for the detection and identification of *Leishmania* spp. in field studies, presenting many advantages over the other techniques available due to its enhanced sensitivity, enabling an accurate identification at the level of genus, subgenus or even species. The amplified product containing *Leishmania* DNA may be use in the procedures for molecular genotyping of parasites, such as the analysis of fragment length polymorphisms (RFLPs), hybridization with probes specific for subgenus/complex/species and sequencing (Azizi et al., 2006; Garcia et al., 2007; Jorquera et al., 2005; Martín-Sánchez et al., 2006; Pita-Pereira et al., 2005). Many studies have been employing this technique to evaluate natural sand fly infection, and distinct values of sensitivity as well as specificity have been observed, depending upon the assay conditions (Aransay et al., 2000; Córdoba-Lanús et al., 2006; Marcondes et al., 2009; Paiva et al., 2006; Pita-Pereira et al., 2005, 2008, 2009; Rodriguez et al., 1999; Silva and Grunewald, 1999). The natural infection molecular diagnosis in sand flies only determines the presence of the parasites in the insect digestive tract. In order for us to be able to suggest a potential vector of leishmaniasis, some precautions must be taken, such as not using blood gorged females in the diagnostic assays and correlating the positive results of natural infection with ecological and epidemiological studies of the area. In this study, the results obtained by molecular diagnosis were associated with epidemiological evidence having already been discussed in literature concerning the participation of *Lu.* (*P.*) *fischeri* in the transmission of ACL and compared with local information.

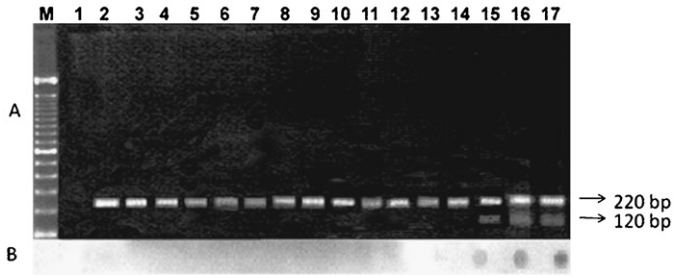

Fig. 1. PCR multiplex followed by hybridization for the diagnosis of *Leishmania* (*Viannia*) infection in *Lutzomyia fischeri* collected in the municipality of Porto Alegre, Rio Grande do Sul State, Brazil. Phlebotomines were grouped into pools of 10 female specimens and PCR was performed with total DNA extracted from these pools. (A) Ethidium bromide-stained 2% agarose gel revealing the 220 bp product from the cacophony gene amplification (*Lutzomyia* genus) and the 120 bp fragment corresponding to the conserved region of kinetoplast minicircles from *Leishmania* spp. M, molecular weight marker (100 bp DNA ladder). Lane 1, amplification reaction without added DNA (PCR negative control); lanes 2–5, negative controls for the DNA extraction step (male insect pools); lanes 6–16, female sand fly pools (lanes 15 and 16, *Lu. fischeri* positive pools); lane 17, PCR positive controls (DNA extracted from artificially infected females). (B) Dot hybridization using a biotinylated probe specific for parasites from the *Viannia* subgenus.

The PCR analyses proved positive in 2 out of the 52 (3.8%) female pools submitted to the test, subsequent hybridization confirming the infection by parasites of the *Viannia* subgenus. Considering that 3.8% of the pools were positive, each containing at least one infected insect, the minimum rate of infection of *L.* (*P.*) *fisheri* was estimated at 0.38%. All analyzed samples yielded the 220 bp amplified product corresponding to a constitutive gene (cacophony) from *Lutzomyia* spp., thus confirming the integrity of the insect DNA preparations as well as the absence of eventual PCR inhibitors. The male control samples only amplified a product of 220 bp, demonstrating that there was no contamination in our molecular assay, while the samples of artificially infected females, serving as positive controls for our methodology, amplified products with 220 bp and 120 bp confirming the efficiency of our molecular diagnostics (Fig. 1).

Lutzomyia (*P.*) *fischeri* has been registered in the Brazilian States of Central-east, Northeast, Southeast and South regions of the country. It was always considered as an essentially sylvatic species but has been adapting to peridomestic rural habitats since 1953, reported to inhabit proximities of domestic animal shelters (Aguiar and Medeiros, 2003; Rangel and Lainson, 2009). Lainson (1983), considering the abundance of this sand fly in deforested ACL areas, suggested that this species must be adapted to environmental changes, maintaining the transmission of *L.* (*V.*) *braziliensis* among wild animals in the secondary forested areas. Studies in the Brazilian Southeast indicated that in more recent years (1986–1995), this sand fly was present in the domiciliary habitat in 53.6% of the municipalities of the State of São Paulo, in association with cases of ACL (Camargo-Neves et al., 2002). Recently, *L.* (*P.*) *fischeri* has been found naturally infected by *L.* (*V.*) *braziliensis* in sand flies collected in the State of Espírito Santo, but the authors commented the need for additional evidence in order to incriminate this sand fly species as a local vector of ACL (Rocha et al., 2010).

There are some reports of *L.* (*P.*) *fischeri* presenting an anthropophilic behavior. However there is some discussion concerning its preference for feeding on dogs and chickens (Souza et al., 2002;

Rangel and Lainson, 2009). Evidence related to *L.* (*P.*) *fischeri* feeding on humans was established during the mid-morning (10:00 am), in the native forest District Lami, Porto Alegre, during an investigation of disease in monkeys in November 2008 (Souza, G.D., personal communication).

Since 2002, when the first human cases of ACL (by *Leishmania* (*V.*) *brazileinsis* according to Department of State Health, RS) were registered in the periphery of Porto Alegre, some studies have been undertaken with the purpose of understanding the local transmission in this area (Quinta do Portal) where environmental changes have been occurring for tourist activities and human cases of ACL registered. Previous studies in this area revealed great abundance of *L.* (*N.*) *neivai* in domiciliary habitats while *L.* (*P.*) *fischeri* abounded more in peridomiciliary environments, both species feeding on man (Souza et al., 2008). Considering the prevalence of *L.* (*N.*) *neivai* and the need to identify the potential ACL vector, later studies accused the natural infection of this sand fly by *Leishmania* (*Viannia*), probably *L.* (*V.*) *braziliensis*, implying this insect species as the ACL vector in this area (Pita-Pereira et al., 2009).

The natural infection of *L.* (*P.*) *fischeri* by *Leishmania* (*Viannia*) spp. obtained in the present investigation associated with some local evidence such as anthropophilic behavior, spatial distribution in accordance with the transmission area, occurrence of human cases and presence in lower frequency than *L.* (*N.*) *neivai* suggest that *L.* (*P.*) *fischeri* may be considered as a secondary vector in this endemic area of American Cutaneous Leishmaniasis (ACL) of Porto Alegre, Rio Grande do Sul State, Brazil. Nevertheless, further investigation and surveillance are warranted to insure unequivocal certainty.

Acknowledgements

This investigation was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa no Estado do Rio de Janeiro (FAPERJ). Daniela Pita-Pereira is a fellow post doctorate from CNPq; Elizabeth Rangel and Constança Britto are fellows of the CNPq Institution.

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