DETECTION OF *Leishmania (Viannia)* IN *Nyssomyia neivai* AND *Nyssomyia whitmani* BY MULTIPLEX POLYMERASE CHAIN REACTION, IN SOUTHERN BRAZIL

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SUMMARY

Sandflies transmit pathogens of leishmaniasis. The natural infection of sandflies by *Leishmania (Viannia)* was assessed in municipalities, in the state of Paraná, in Southern Brazil. Sandflies were collected with Falcão and Shannon traps. After dissection in search of flagellates in digestive tubes and identification of the species, female sandflies were submitted to the Multiplex Polymerase Chain Reaction (multiplex PCR) for detection of the fragment of the kDNA of *Leishmania (Viannia)* and the fragment from the IVS6 cacophony gene region of the phlebotomine insects. The analysis was performed in pools containing seven to 12 guts from females of the same species. A total of 510 female sandflies were analyzed, including nine *Migonemyia migonei*, 17 *Pintomyia fischeri*, 216 *Nyssomyia neivai*, and 268 *Nyssomyia whitmani*. Although none of the females was found naturally infected by flagellates through dissection, the fragment of DNA from *Leishmania (Viannia)* was shown by multiplex PCR in one sample of *Ny. neivai* (0.46%) and three samples of *Ny. whitmani* (1.12%). It was concluded that *Ny. neivai* and *Ny. whitmani* are susceptible to *Leishmania* infection, and that multiplex PCR can be used in epidemiological studies to detect the natural infection of the sandfly vector, because of its sensitivity, specificity and feasibility.

KEYWORDS: Cutaneous leishmaniasis; Sandfly; PCR; *Leishmania*; *Nyssomyia whitmani*; *Nyssomyia neivai*.

INTRODUCTION

Knowledge of the fauna composition, behavior, rates of natural infection of the sandfly, *Leishmania* species identification, and environmental characteristics of endemic areas are essential for the public-health services responsible for protecting populations that live in areas in which leishmaniasis is endemic. Leishmaniasis has worldwide propagation. In Brazil, cutaneous leishmaniasis (CL) has been reported in all states.

Cases of CL have increased significantly since the 1980s, and are appearing over wider areas in the State of Paraná, in the South of Brazil. This disease has been recorded in occupied areas for more than a century, even in urban areas, contrary to expectations that human pressure would eliminate natural foci and reduce the incidence of this endemic disease. The organization of rural areas in the Brazilian colonial period created environmental conditions that clearly favor CL transmission.

There are several reports of *Leishmania* detection in sandflies in endemic CL areas in Brazil and in the world. However, considering the wide geographical distribution of this dermatosis in the Americas, knowledge of the natural infection rate of sandflies is still insufficient to estimate the risk of *Leishmania* infection in many endemic areas.

Given the occurrence of autochthonous CL cases in several municipalities, in the state of Paraná, natural infection rates of sandflies by *Leishmania (Viannia)* were investigated, in order to identify the species of *Leishmania* present in locations where cases of this disease had been reported.

MATERIAL AND METHODS

**Sandfly collection:** Sandflies were collected in the localities of Recanto Marista, Água Azul Farm and Flor de Maio Grange, in the municipalities of Doutor Camargo, Fênix and Mandaguari, respectively, in the state of Paraná, where CL cases had been reported. Sandflies were collected from January through September 2006, from 6 p.m. to 6 a.m., with Falcão light traps and a Shannon trap, installed in woods, domiciles, peridomicile and domestic-animal shelters (cattle shelter and pigsty). The collected insects were kept alive for further dissection and observation of the natural infection by flagellates.

**Dissection and identification of sandflies:** The dissection was carried out in an insecticide-free environment, eliminating the risk of infection due to the multiplication of the parasites. The dissection was performed in pools containing seven to 12 guts from females of the same species. A total of 510 female sandflies were analyzed, including nine *Migonemyia migonei*, 17 *Pintomyia fischeri*, 216 *Nyssomyia neivai*, and 268 *Nyssomyia whitmani*. Although none of the females was found naturally infected by flagellates through dissection, the fragment of DNA from *Leishmania (Viannia)* was shown by multiplex PCR in one sample of *Ny. neivai* (0.46%) and three samples of *Ny. whitmani* (1.12%). It was concluded that *Ny. neivai* and *Ny. whitmani* are susceptible to *Leishmania* infection, and that multiplex PCR can be used in epidemiological studies to detect the natural infection of the sandfly vector, because of its sensitivity, specificity and feasibility.

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Sandflies transmit pathogens of leishmaniasis. The natural infection of sandflies by *Leishmania (Viannia)* was assessed in municipalities, in the state of Paraná, in Southern Brazil. Sandflies were collected with Falcão and Shannon traps. After dissection in search of flagellates in digestive tubes and identification of the species, female sandflies were submitted to the Multiplex Polymerase Chain Reaction (multiplex PCR) for detection of the fragment of the kDNA of *Leishmania (Viannia)* and the fragment from the IVS6 cacophony gene region of the phlebotomine insects. The analysis was performed in pools containing seven to 12 guts from females of the same species. A total of 510 female sandflies were analyzed, including nine *Migonemyia migonei*, 17 *Pintomyia fischeri*, 216 *Nyssomyia neivai*, and 268 *Nyssomyia whitmani*. Although none of the females was found naturally infected by flagellates through dissection, the fragment of DNA from *Leishmania (Viannia)* was shown by multiplex PCR in one sample of *Ny. neivai* (0.46%) and three samples of *Ny. whitmani* (1.12%). It was concluded that *Ny. neivai* and *Ny. whitmani* are susceptible to *Leishmania* infection, and that multiplex PCR can be used in epidemiological studies to detect the natural infection of the sandfly vector, because of its sensitivity, specificity and feasibility.

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out under a stereoscope; the legs and wings were removed and the dissection was carried out by making two incisions in the distal portion of the abdomen and, with zigzag movements, the digestive tubes were removed and examined under an optical microscope (400 x) in the search for flagellates and the identification of the species of the sandfly\textsuperscript{26}. The nomenclature of the species follows GALATI\textsuperscript{13}.

After dissection and identification\textsuperscript{26} of species, digestive tubes were stored at -18 °C in tubes containing 150 µL STE buffer (0.1 M NaCl, 10 mM Tris-base; Na\textsubscript{2}EDTA-2H\textsubscript{2}O 1 mM, pH 8.0), each containing seven to 12 guts from females of the same species.

**Extraction of DNA:** The samples were macerated, and DNA was extracted with a solution of guanidine isothiocyanate and phenol, and hydrated in 20 µL of ultra-pure H\textsubscript{2}O\textsubscript{15}. For each group of 22 samples submitted for DNA extraction, one negative control (male sandflies) and one positive control [male sandflies plus 10\textsuperscript{5} L. (V.) braziliensis promastigotes] were used.

**Multiplex PCR:** Two pairs of primers were used: MP3H (5’-GAA CGG GGT TTC TGT ATG C-3’) and MP1L (5’-TAC TCC CGC ACA TGC CTC TG-3’)\textsuperscript{10} to amplify a fragment of 70-bp from the mini circle region of the kinetoplast (kDNA) of the *Leishmania* (Viannia), and 5Llca (5’-TGG CCG AAC ATA ATG TTA G-3’) and 3Llca (5’-CCA CGA ACA AGT TCA ACA TC-3’)\textsuperscript{10} to amplify a fragment of 220-bp from the *IVS6* cactophagy gene region of the phlebotomine insects.

The PCR reaction mixture (final volume 25 µL) was composed of 0.5 µM of each of the primers (Invitrogen Life Technologies, São Paulo, Brazil), 0.2 mM dNTP (Invitrogen, Carlsbad, CA, USA), 1U Platinum Taq DNA Polymerase, (Invitrogen, Carlsbad, CA, USA), 1.5 mM MgCl\textsubscript{2}, 1X enzyme buffer, and 2 µL DNA template. The amplification was carried out in a PC Thermocycler (Biometra, Germany) at 94 °C for seven min to activate the enzyme, followed by 30 cycles, each divided into three stages, of denaturation (1.5 min at 95 °C), annealing (1.5 min at 57 °C), and polymerization (two min at 72 °C). After this, the extension was continued for a further 10 min at 72 °C, and the tubes were then kept at 4 °C until analysis\textsuperscript{14}. The amplification products were submitted to electrophoresis in 2% of agarose gel (Invitrogen, Paisley, Scotland, UK) stained with 0.1 µg/mL ethidium bromide, at 10-15 V/cm. The presence of bands was observed in a transilluminator (Macro Vue\textsuperscript{23} UV-20, Hoefer). For every five samples, one positive control [reaction mixture plus L. (V.) braziliensis DNA] and one negative control (reaction mixture plus water) were added.

### RESULTS

In total, 510 (52 pools) female sandflies were analyzed by dissection and multiplex PCR, including nine *Migonemys migonei* (one pool), 17 *Pintomyia fischeri* (two pools), 216 *Nyssomyia neivai* (22 pools), and 268 *Nyssomyia whitmani* (27 pools) (Table 1). A total of 244 female sandflies were collected at Recanto Marista, 107 at Água Azul Farm, and 159 at Flor de Maio Grange (Table 1).

All 52 sandfly pools contained the 220-bp fragment from the *IVS6* cactophagy gene region of the sandflies, and four pools (7.7%) showed the 70-bp fragment from the mini circle kDNA of *Leishmania* (Viannia) (Fig. 1). The minimal infection rate of *Ny. neivai* was 0.46% (1/216), and of *Ny. whitmani* was 1.12% (3/268). At Recanto Marista one *Ny. neivai* pool with *Leishmania* infection was detected on the porch of a domicile; at Água Azul Farm, one *Ny. whitmani* pool was detected in the peridomicle (near the woods); and at Flor de Maio Grange, two *Ny. whitmani* pools were detected, one on the porch of a domicile and another from a domestic-animal shelter. The minimal infection rate of Recanto Marista was 0.41% (1/244), of Flor de Maio Grange was 1.26% (2/159), and at Água Azul Farm was 0.93% (1/107).

### DISCUSSION

*M. migonei*, *Pi. fischeri*, *Ny. neivai*, and *Ny. whitmani* are frequently found in Brazil\textsuperscript{2,4,11,34,35,44}. Both species detected with *Leishmania* infection are widely propagated in Brazil\textsuperscript{4,5,6,9,20,21} and in the state of

### Table 1

| Specimens/Localities (Municipalities) | Recanto Marista (Doutor Camargo) | Água Azul Farm (Fênix) | Flor de Maio Grange (Mandaguari) | Total |
|-------------------------------------|---------------------------------|------------------------|----------------------------------|-------|
| **Migonemyia migonei** N\textsuperscript{a} | 9 | 0 | 0 | 9 |
| Positive pools / Pools\textsuperscript{a} | 0/1 | 0/0 | 0/0 | 0/1 |
| **Nyssomyia whitmani** N\textsuperscript{a} | 19 | 97 | 152 | 268 |
| Positive pools / Pools\textsuperscript{a} | 0/2 | 1/10 | 2/15 | 3/27 |
| **Nyssomyia neivai** N\textsuperscript{a} | 216 | 0 | 0 | 216 |
| Positive pools / Pools\textsuperscript{a} | 1/22 | 0/0 | 0/0 | 1/22 |
| **Pintomyia fischeri** N\textsuperscript{a} | 0 | 10 | 7 | 17 |
| Positive pools / Pools\textsuperscript{a} | 0/0 | 0/1 | 0/1 | 0/2 |
| **Total** N\textsuperscript{a} | 244 | 107 | 159 | 510 |
| Positive pools / Pools\textsuperscript{a} | 1/25 | 1/11 | 2/16 | 4/52 |

\textsuperscript{a}Number of Specimens; \textsuperscript{b}Number of positive pools/number of pools composed. Each pool contained seven to 12 guts from females of the same species.
NEITZKE-ABREU, H.C.; REINHOLD-CASTRO, K.R.; VENAZZI, M.S.; SCODRO, R.B.L.; DIAS, A.C.; SILVEIRA, T.G.V.; TEODORO, U. & LONARDONI, M.V.C. - Detection of Leishmania (Viannia) in Nyssomyia neivai and Nyssomyia whitmani by Multiplex Polymerase Chain Reaction, in Southern Brazil. Rev. Inst. Med. Trop. Sao Paulo, 56(5): 391-5, 2014.

The advantage of employing the multiplex PCR technique is that, in addition to the primers for detection of Leishmania (Viannia), the pair of primers used for internal control can assess the presence of probable interference from digestive contents of insects, which can inhibit the detection of Leishmania.31,34

The results show the susceptibility of sandflies to Leishmania strains. The minimum infection rates in Ny. neivai (0.46%, 1/216) and Ny. whitmani (1.12%, 3/268) are low, and might explain the low CL endemicity in the municipalities in question. Multiplex PCR, because of its sensitivity, specificity and feasibility, can be used in epidemiological studies to detect the natural infection of the sandfly vector.

RESUMO

Deteccão de Leishmania (Viannia) em Nyssomyia neivai e Nyssomyia whitmani por Multiplex Reação em Cadeia da Polimerase, no sul do Brasil

Flebotomíneos transmitem os patógenos das leishmanioses. Foi avaliada a infecção natural de flebotomíneos por Leishmania (Viannia) em municípios do Estado do Paraná, sul do Brasil. Os flebotomíneos foram coletados com armadilhas de Falcão e Shannon. Após dissecação para pesquisa de flagelados no tubo digestório e identificação das espécies, as fêmeas de flebotomíneos foram submetidas a Multiplex Reação em Cadeia da Polimerase (multiplex PCR) para a detecção do fragmento do DNA de Leishmania (Viannia) e do fragmento do gene IVS6 da cacofonia de flebotomíneos. A análise foi realizada em pools contendo sete a 12 tubos digestórios de fêmeas da mesma espécie. Um total de 510 fêmeas foram analisadas, incluindo nove M. migonei migonii, 17 P. ficheri, 216 N. neivai e 268 N. whitmani. Embora nenhuma fêmea tenha sido encontrada naturalmente infectada com flagelados pela dissecação, o fragmento de DNA de Leishmania (Viannia) foi mostrado por multiplex PCR em uma amostra de Ny. neivai (0.46%) e três amostras de Ny. whitmani (1,12%). Conclui-se que Ny. neivai e Ny. whitmani são suscetíveis à infecção por Leishmania, e que multiplex PCR, devido à sua sensibilidade, especificidade e viabilidade, pode ser utilizada em estudos epidemiológicos para a detecção da infecção natural do inseto vetor.

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393
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