Etiology of cutaneous leishmaniasis and anthropophilic vectors in Juruti, Pará State, Brazil

Etiologia da leishmaniose cutânea e vetores antropofílicos em Juruti, Estado do Pará, Brasil

Abstract

In a preliminary study in Juruti, a mining municipality in western Pará State, Brazil, 12 out of 21 patients suspected of presenting cutaneous leishmaniasis showed positive PCR (SSUrDNA and G6PD): *Leishmania (Viannia) braziliensis* (9/12; 75%) and *L. (V.)* sp. (3/12; 25%). Entomological studies in the same location revealed the presence of 12 different phlebotomine species (*n* = 105). One of the most common species was *Lutzomyia (Psychodopygus) complexa* (17%) which is both highly anthropophilic and a known vector of *L. (V.) braziliensis* in other regions of Pará. These preliminary findings should serve to guide future epidemiological surveillance in Juruti.

Cutaneous Leishmaniasis; Leishmania; Polymerase Chain Reaction; Entomology

Introduction

In the State of Pará, Brazil, there are seven *Leishmania* species that cause cutaneous leishmaniasis in humans. Two are associated with severe forms of the disease: *Leishmania (Viannia) braziliensis* (mucocutaneous) and *Leishmania (Leishmania) amazonensis* (diffuse cutaneous) 1. Etiological diagnosis of cutaneous leishmaniasis helps clarify local epidemiology and is essential for the clinical management in cases of reactivation 2. Such information, associated with entomological surveillance, is particularly important for controlling the disease in vulnerable areas 1. Thus, during an epidemiological survey in the mining municipality (county) of Juruti in western Pará State, etiological diagnosis was performed for cutaneous leishmaniasis using PCR (polymerase chain reaction) in patients with suspicious lesions treated at the Francisco Barros Municipal Hospital, and anthropophilic species of sylvatic phlebotomines were investigated in a mining area with surviving forest cover.

Material and methods

The study was approved by the Institutional Review Board of the Evandro Chagas Institute (research protocol nº. 0029/2007).
Diagnosis

Skin biopsies were taken from the edge of lesions in 21 individuals with suspicion of cutaneous leishmaniasis at the Francisco Barros Municipal Hospital in Juruti (February to June 2007). The following diagnoses were performed: clinical, parasitological (Giemsa-stained smear/isolation in NNN medium), molecular (PCR), and Montenegro skin test. DNA was extracted from the samples with phenol/chloroform. PCR used oligonucleotides based on the \textit{Leishmania} SSUrDNA and G6PD genes, which jointly discriminate genus, sub-genera \textit{Leishmania} and \textit{Viannia}, and the species \textit{L. (V.) braziliensis}. In PCR-SSUrDNA, the reaction occurred in a thermocycler (Eppendorf Mastercycler Gradient; Eppendorf, Hamburg, Germany) under the following conditions: mix 50µL (Taq DNA polymerase 0.02U/µL; MgCl₂ 2.0mM; dNTPs 0.2mM; KCl Fermentas EP401; primers S4, S12, S17, and S18 0.2mM each; sample 2.0µL); denaturation (94°C/5'); annealing (30 x 94°C/1'); extension (72°C/45'; 72°C/10'). Nested PCR (S17 and S18) was performed with the product under the same conditions. Similar procedures were adopted with the PCR-G6PD, with the added mixture of dimethyl sulfoxide (2.5µL) to a final concentration of 5%. In addition, the first stage was specific for subgenus \textit{Viannia} (ISVA/ISVC), differing the annealing temperature (AT), 60°C, and extension time (30'). The amplified product (1µL) was used in the following stage for the two semi-nested PCR reactions, which identified the non-\textit{L. (V.) braziliensis} subgenus \textit{Viannia} (ISVG/ISVC, AT 68°C) and species \textit{L. (V.) braziliensis} (ISVB/ISVC, AT 67°C). The amplified DNA was separated by agarose gel electrophoresis (1.5% in tris-acetate 40mM/EDTA 1mM) with ethidium bromide (5µL, 10µg/mL) and viewing under ultraviolet light. Controls were used with different species of \textit{Leishmania} (50ng/mL) and distilled water, in addition to the molecular weight marker (100 base pairs, Invitrogen). Polymorphisms that discriminated \textit{L. (L.) amazonensis}, \textit{L. (L.) infantum chagasi}, and \textit{L. (Viannia)} sp. were investigated in negative samples for \textit{L. (V.) braziliensis} after sequencing the products of the nested PCR-USurDNA bound to the TA Cloning 2.1 vector (Invitrogen, Carlsbad, USA) and cloned in DH5α bacteria, according to the protocol for the Big Dye Terminator v. 3.1 kit (Applied Biosystems, Warrington, UK). Sequencing was used to investigate whether the samples' negativity according to ribosomal PCR (PCR-SSUrDNA) was sufficient to confirm positivity for subgenus \textit{Viannia}. The genetic analyzer ABI PRISM (Hitachi) was used, and the sequences were aligned with BioEdit (Tom Hall, North Carolina State University, http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Entomological surveys

Two entomological surveys were conducted in January 2008 in different regions of the municipality, both in the forest around the Capiranga mining base in Juruti, located at S02°30'28.3" W056°11'01.0" and S02°28’32.9” W056°12’45.6”. Shannon light traps were used (at 18:00 and 20:00 hours), and the phlebotomines were identified using the morphological method.

Results

Diagnosis

Of the 21 cases with clinical suspicion of cutaneous leishmaniasis, 12 were diagnosed based on the presence of DNA from genus \textit{Leishmania} (57%), of which 11 (52%) were parasitologically confirmed by direct microscopy. Nine of the 12 (75%) had amplified products for subgenus \textit{Viannia} and species \textit{L. (V.) braziliensis} (G6PD), as shown in Figure 1, and the other three (25%) did not show amplification for subgenus \textit{Viannia}. Sequencing of the products generated from these three samples confirmed that they belonged to subgenus \textit{Viannia}.

In the 9/12 (75%) patients with positive results for \textit{L. (V.) braziliensis}, all confirmed by parasitology, there were delayed hypersensitivity reactions to the Montenegro antigen, with diameters varying from 7mm to 40mm (16.3 ± 3.2). Time since onset of the disease (as reported by the patients themselves) ranged from 1 to 4 months (1.6 ± 0.3).

Entomological survey

A total of 105 sylvatic phlebotomines were captured, belonging to 12 species, as follows: (1) \textit{Lutzomyia} (Psychodopygus) \textit{geniculata} (22%), (2) \textit{Lu. (P.) paraensis} (20%), (3) \textit{Lu. (P.) complexa} (17%), (4) \textit{Lu. (Nyssomyia) flaviscutellata} (13%), (5) \textit{Lu. (P.) davisi} (10%), (6) \textit{Lu. (P.) amazonensis} (6%), (7) \textit{Lu. (P.) chagasi} (5%), (8) \textit{Lu. (N.) richardwardi} (4%), (9) \textit{Lu. (P.) carrerai carrerai} (1%), (10) \textit{Lu. (P.) llanosmartinsi} (1%), (11) \textit{Lu. (P.) longispina} (1%), and (12) \textit{Lu. (T.) ubiquitalis} (1%). None of the specimens were naturally infected with \textit{Leishmania}. 

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Figure 1

PCR-G6PD products developed in agarose gel (1.5%) for etiological diagnosis of cutaneous leishmaniasis.

Pb: marker 100bp; La, Lb, and Lg: L. (L.) amazonensis, L. (V.) braziliensis, and L. (V.) guyanensis; CN: negative control (water).

Note: the analysis used DNA extracted from skin biopsies from the edge of patients’ lesions. The specific band for L. (V.) braziliensis displays 234 base pairs (columns 3, 5, 6, 7, and Lb). Columns 1, 2, and 4 correspond to non-amplified samples (individual without leishmaniasis).

Discussion

Etiological diagnosis by PCR assists in determining the prognosis and appropriate therapy, which vary according to the parasite’s species or strain 8. In this study, the majority of the positive individuals for cutaneous leishmaniasis were infected with L. (V.) braziliensis (9/12, 75%), clearly differentiated from other Amazonian species by PCR-G6PD (Figure 1). This finding was consistent with direct microscopic examination, as well as by the high rate of positive reactions to the Montenegro antigen and the extensive diameter of the skin reactions 9. Despite recent advances in the production of primers 10, new oligonucleotides are still needed to detect polymorphisms that discriminate Amazonian species of non-L. (V.) braziliensis subgenus Viannia using PCR, as well as to optimize PCR-G6PD procedures in order to eliminate the need for cloning.

Entomological surveillance in risk areas for cutaneous leishmaniasis is important for analyzing the epidemiological conditions for transmission, as well as to provide the basis for control measures 1. The forest area around the bauxite mine showed a heavy presence of the vectors for L. (L.) amazonensis, Lu. (N.) flaviscutellata, with low anthropophilia, and for L. (V.) braziliensis, Lu. (P.) complexa, highly anthropophilic. The latter is capable of biting humans avidly in broad daylight and even at high noon, and outside the forest, in addition to its extraordinary population density during the cooler season in the Amazon. Such characteristics, combined with its susceptibility to Leishmania infection, make this species a prime vector for the disease 11. In the 1960s and 70s, when highways were opened for a mining project in Serra dos Carajás, Pará, the incidence of cutaneous leishmaniasis was estimated at one case per kilometer of highway built, with the majority of cases transmitted by Lu. (P.) complexa 12. The daytime habits of this species, rare among Amazonian phlebotomines, increase the risk of human exposure to infection, thus explaining the high number of reported cases due to L. (V.) braziliensis in the sample studied in Juruti.

The knowledge generated by this research will be incorporated into health education activities in Juruti, but further studies are still needed to elucidate the epidemiology of cutaneous leishmaniasis and provide better backing for local control measures.
Resumo

Em um estudo preliminar em Juruti, um município minerário na região oeste do Estado do Pará, Brasil, 12 de 21 pacientes suspeitos de possuírem leishmaniose cutânea tiveram PCRs positivas (SSUrDNA e G6PD): Leishmania (Viannia) braziliensis (9/12; 75%) e Leishmania (Viannia) sp. (3/12; 25%). Estudos entomológicos na mesma localidade revelaram a presença de 12 diferentes espécies de flebotomíneos (n = 105). Uma das espécies mais comuns foi Lutzomyia (Psychodopygus) complexa (17%) que é altamente antropofílica e reconhecida vetora de L. (V.) braziliensis em outras regiões do Estado do Pará. Esses resultados preliminares servem como orientação para futura vigilância epidemiológica em Juruti.

Leishmaniose Cutânea; Leishmania; Reação em Cadeia da Polimerase; Entomologia

Contributors


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