ASYMPTOMATIC HUMAN CARRIERS OF LEISHMANIA CHAGASI


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Abstract. In Brazil, programs based on elimination of infected dogs have not curtailed the spread of visceral leishmaniasis (VL), suggesting that other reservoirs of infection exist. Persons with active VL can infect the sand fly vector, but in endemic areas, persons with asymptomatic infections, whose infectivity to sand flies is unknown, are far more numerous. In this study, a polymerase chain reaction-based assay detected kinetoplast DNA of Leishmania chagasi in the blood of eight of 108 asymptomatic persons living with patients with recently diagnosed VL. These eight persons had low or unmeasurable levels of IgG antibodies to Leishmania, demonstrating the insensitivity of serology for subclinical infection. All eight persons had positive leishmanin skin test results, as did 70% of persons living in households of persons with active VL. Even if a small proportion of such asymptomatic persons are infective to sand flies, they represent a formidable reservoir of infection in endemic areas.

INTRODUCTION

Unlike anthroponotic visceral leishmaniasis (VL) due to Leishmania donovani in India, New World VL, due to L. chagasi, is a zoonosis with reservoirs believed to be wild and domestic canids.1 Because control programs based on elimination of infected dogs have failed to halt or prevent epidemics of urban VL in Brazil, other reservoirs may be important in propagating the infection.1 In 1962, Deane and Deane2 showed that persons with active VL could infect the sand fly vector and hypothesized that these persons were a source of infection for others. Because chronically infected but asymptomatic persons may serve as a reservoir of parasites as well, we used a polymerase chain reaction (PCR)-based assay to document infection in the blood of asymptomatic persons living in Teresina during an urban outbreak of VL.3

MATERIALS AND METHODS

Study population. Participants were residents of Teresina, a city of 700,000 persons and the capital of the state of Piaui in northeastern Brazil. Epidemics of VL have occurred there since 1980, including nearly 1,000 cases in 1993–1994.4 There is no known transmission of American trypanosomiasis or cutaneous leishmaniasis in Teresina, and persons who had resided previously in regions where these diseases are endemic were excluded from the study. All participants or their parent or guardian gave informed consent before entering the study, which was approved by the human subjects committees of the Harvard School of Public Health and the Conselho Regional de Medicina do Piauí.

Three groups of asymptomatic persons were studied: 21 persons who had been treated for VL and cured 158–538 days (median = 316) before enrollment; 27 persons with no history of VL but who had a reactive leishmanin skin test (LST) result documented at least four months before enrollment; and 101 of the 108 persons living in the households of 24 patients who had begun treatment for active VL during the preceding 1–33 days. Five of the 101 household members had themselves received therapy for VL in the past, and 76 of the 96 household members who were tested were LST positive.

Evaluation of participants included detailed questions about symptoms of VL, complete physical examination, and application of the LST. Venous blood was obtained for serologic tests and a PCR-based assay to detect Leishmania DNA. Seven of eight persons who were subsequently identified as asymptomatic carriers were re-examined 18–25 months after the initial examination.

Diagnostic studies. For the LST, 0.1 ml of a suspension of sonicated Leishmania promastigotes (Mayrink antigen3) was injected intradermally. Reactions were measured at 48–72 hr, and induration ≥ 5 mm in the greatest diameter was considered positive. Antigens for an enzyme-linked immunosorbent assay (ELISA) and an indirect immunofluorescent antibody test (IFAT) for IgG were derived from a reference strain of L. chagasi (MHOM/BR/74/PPT75).6,7 Sera from five residents of Teresina with parasitologically confirmed VL served as positive controls. The optical density by ELISA of positive control sera was 10 times greater than that of negative control sera from five residents of Teresina without VL. Serologic tests were considered positive if either the IFAT was reactive at a dilution of 1:80 or the optical density of the ELISA exceeded by two standard deviations that of the negative controls.

PCR-based assay. For isolation of kinetoplast DNA (kDNA), 10 μl of heparinized venous blood previously stored at −20°C were defrosted, loaded on an Isocode® dipstick (Schleicher and Schuell, Keene, NH), dried at room temperature, and then heated to 95°C in 50 μl of distilled sterile water for 30 min. The supernatant was stored at −20°C until used. The PCR was performed as described by Rodgers and others.8 Five microliters of supernatant were added to 50 μl of PCR mixture consisting of 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1.25 units of Taq polymerase (Perkin-Elmer Roche, Branchburg, NJ), 70 mM Tris buffer, 20 mM (NH₄)₂SO₄, 2 mM MgCl₂ and 2 mM of Primers 13A and 13B, which amplify the 120 kb constant region of Leishmania kDNA. Fifty microliters of reaction mixture were subjected to electrophoresis on a Southern alkaline transfer system (GeneScreenPlus®; New
England Nuclear Research Products, Boston, MA) and hybridized with a digoxigenin-labeled probe (PCR Digprobe Synthesis Kit®; Boehringer Mannheim, Indianapolis, IN) made with Primers 13A and 13B. Membranes were prehybridized for 1 hr at 68°C, and after denaturation of probes, hybridized overnight at 68°C. Membrane-linked PCR product was identified by an enzyme-linked immunologic assay for detecting the digoxigenin-labeled probe.

Positive controls consisted of whole DNA of *L. chagasi* (reference strain MHOM/BR/74/PP75) and kDNA from samples of blood of patients with active VL. Negative controls, consisting of heparinized blood from a person who had never lived in a Leishmania-endemic area were processed with each batch of 13–14 samples. To rule out inhibition of the PCR by heparin or the effect of time in storage, 15 blood samples frozen for 26 months were processed by the same PCR procedures to detect a sequence of human *Alu* gene normally present in leukocytes. The primers used to amplify a 244-basepair (bp) sequence of *Alu* from position 15 to 259 were *Alu*-A: 5’-GCTCAGCCCTGTAATCCAGC-3’ and *Alu*-B: 5’-CCAGGCTGGAGTGCACTGG-3’.

**Sequencing.** The PCR products from Subject 6L were reamplified and resolved by agarose gel electrophoresis. To purify the 120-bp band, the corresponding gel slice was melted and centrifuged with glass powder in NaI solution, and the DNA was suspended in TE buffer. Sequencing was performed by a commercial service in an Applied System® model 373A, version 1.2.0 sequencer (Perkin-Elmer Company, Shelton, CT) using the 13A and 13B primers. The sequence was submitted to GenBank-BLAST for comparison with other sequences (GenBank accession number AF027294).

**Comparative hybridization.** Kinetoplast DNA from the blood of Subject 6L and from the blood of Subject 7E obtained two years after the initial examination was extracted and amplified; 2 μL of the product were reamplified, and 8 μL of the second product were resolved by electrophoresis on a 3% agarose gel, transferred to a nylon membrane, and detected by Southern blotting with digoxigenin-labeled probes specific for the kDNA conserved region of *L. chagasi*, *L. mexicana*, and *L. braziliensis*.

**RESULTS**

*Leishmania* kDNA was detected by the PCR-based assay in the blood of eight asymptomatic persons who were living in households of persons with active VL. The results of the assays for kDNA were negative for samples from the 21 persons who had been treated successfully for VL, the 27 asymptomatic persons with a positive LST result of at least four months duration, and the 93 other asymptomatic persons living in households of persons with VL, of whom 71% (62 of 87 persons tested) were LST positive.

The eight PCR-positive persons lived in four different households, including three persons in each of two households. The index person with active VL in each household had been ill for 20–60 days before hospitalization, and members of their households were studied within 20 days of diagnosis. No dogs lived in any of the four households. The age of the asymptomatic PCR-positive persons ranged from four to 28 years. Only two of these persons had elevated levels of anti-*Leishmania* IgG. All seven persons tested had positive

**DISCUSSION**

Our demonstration of asymptomatic carriers of *L. chagasi* by identification of parasite DNA confirms reports of asym-
tomatic carriers of L. donovani in India\textsuperscript{10,11} and eastern Africa,\textsuperscript{12} L. infantum in France,\textsuperscript{13} and recently, L. chagasi in Brazil.\textsuperscript{14} It is unlikely that these are false-positive results because we used primers and probes that have been shown to be highly specific for the genus Leishmania.\textsuperscript{15} Moreover, all samples from the eight asymptomatic carriers were positive in several amplifications, and sequencing of PCR product unequivocally demonstrated the presence of DNA of the genus Leishmania. Comparative hybridization confirmed the identification of L. chagasi DNA at the species level. All positive persons lived in Teresina, where there is no transmission of cutaneous leishmaniasis or Chagas’ disease, and none had a history of cutaneous leishmaniasis or a typical scar on examination. All persons with positive assay results were residents of households in which at least one of the members recently had become ill with VL.

While the diagnosis of asymptomatic infection with L. chagasi typically has been based on documentation of seroconversion, rates of seropositivity in population-based studies are considerably lower than rates of LST reactivity.\textsuperscript{15,16} In the present study, only two asymptomatic carriers were initially seropositive by ELISA, and none was positive by IFAT. Three of the seven persons re-examined two or more years later remained positive for kDNA by PCR; these had the weakest specific IgG responses. These data suggest that asymptomatic infections may occur frequently in the absence of detectable seroconversion. It would appear that detection of asymptomatic infections in follow-up studies is better accomplished by skin testing or even PCR-based assays than by serologic tests.

Persons who had been cured of VL and asymptomatic persons who had been LST positive for at least four months were all negative for kDNA by PCR, whereas all eight asymptomatic carriers had been living in households in which at least one person had active VL. We recently found that a high proportion of symptomatic patients with active VL are infective to sand flies.\textsuperscript{17} Although it cannot be determined whether the asymptomatic carriers acquired infection from the same source that infected the index patient or from the index patients themselves, human-sand fly-humans transmission seems plausible. The index patients remained ill at home for 20–90 days before receiving treatment. None of the asymptomatic carriers lived in households with dogs, and all were known to have been exposed to sand fly bites. Since the amount of kDNA detected in the blood of asymptomatic persons was low compared with that of patients with active VL, they may have been harboring only small numbers of parasites. Also, the presence of amastigotes in the skin may not have been reflected by kDNA circulating in the bloodstream. It is unknown whether persons with subclinical infections of L. chagasi act as reservoirs of American VL. In our sample of asymptomatic persons living in households in which there had been a recent case of active VL, we found an 71\% rate of LST reactivity, which is one of the highest reported prevalences of asymptomatic infection (Costa C, unpublished data). The LST reactivity develops in approximately 90\% of patients after cure of VL,\textsuperscript{15,18} but most persons who are LST positive in endemic areas do not have a history of VL and probably experienced asymptomatic infection.\textsuperscript{15} Even if asymptomatic carriers of L. chagasi are markedly less infective than persons with active VL, their huge numbers in a city such as Teresina would constitute a formidable reservoir of infection.

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REFERENCES
5. Melo MN, Mayrink W, da Costa CA, Magalhaes PA, Dias M, Wil-


