

## First encounter of subclinical human *Leishmania (Viannia)* infection in the state of Rio Grande do Sul, Brazil

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*The objective of the present study was to evaluate the specificity of the Montenegro skin test (MST) in an area in Brazil, state of Grande do Sul State (RS), which was considered to be non-endemic for leishmaniasis. Sixty subjects presented a positive MST and were reevaluated by clinical examination, serology and polymerase chain reaction (PCR) of peripheral blood for the detection of subclinical Leishmania infection. None of the subjects presented clinical signs or symptoms of current leishmaniasis or a history of the disease. Leishmania (Viannia) DNA was detected in blood by PCR and hybridization in one subject. The PCR skin test-positive individual remained asymptomatic throughout the study. Clinical examination showed no scars suggestive of past cutaneous leishmaniasis. Human subclinical infection with Leishmania (Viannia) in RS was confirmed by PCR. This is the first report of subclinical infection with this parasite in the human population of this area.*

Key words: *Leishmania* - PCR - Montenegro skin test - subclinical infection - Rio Grande do Sul - Brazil

*Leishmania (Viannia) braziliensis* infection occurs in most Brazilian states, and some authors believe that the dispersal of this parasite from the western Amazon is associated with anthroponotic action (Marzochi & Marzochi 1994). The traditional clinical manifestations are single or multiple cutaneous lesions, but *L. (V.) braziliensis* has also been associated with mucosal or mucocutaneous leishmaniasis (Marzochi & Marzochi 1994). Nevertheless, most infected individuals remain asymptomatic indefinitely, and show a cell-mediated or humoral immune response to *Leishmania* antigens or parasites in peripheral blood detected by polymerase chain reaction (PCR) (Follador et al. 2002, de Oliveira Camera et al. 2006).

The Montenegro skin test (MST) is the most widely used complementary test for the presumptive diagnosis of *Leishmania* infection. Classically, a positive MST is an indicator of previous contact with the parasite through natural inoculation after the bite of the sandfly. Alternatively, MST positivity can be the result of immunity acquired by vaccination (Mayrink et al. 1979, Marzochi et al. 1998), or nonspecific reactions to the merthiolate or phenol used in the MST as a preservative (Marzochi et

al. 1998, Fagundes et al. 2003, 2007). In some cases, the nonspecific reaction was found to be morphologically identical to a classical positive MST (Marzochi et al. 1998, Fagundes et al. 2007), and it was not possible to discriminate between a false-positive MST due to merthiolate hypersensitivity and a true positive MST result.

In view of these nonspecific reactions, we performed a study in an area non-endemic for leishmaniasis in order to compare the MST response using antigens preserved in merthiolate or phenol. The study protocol has been described in detail by Fagundes et al. (2007). The study population consisted of 151 healthy male military volunteers from the state of Rio Grande do Sul (RS), Southern Brazil, where no case of leishmaniasis has been described so far. Informed consent was obtained from all volunteers and the study was approved by the Institutional Ethics Committee (Fiocruz) and the Brazilian Defense Ministry.

The Biomanguinhos<sup>®</sup> MST antigen (40 mg/ml *Leishmania amazonensis* protein antigen, IFLA/BR/1967/PH8 strain) (Fiocruz, Rio de Janeiro, Brazil) preserved in 1:10,000 thimerosal or 0.4% phenol was used. All MST-positive and thimerosal- or phenol-allergic volunteers were reevaluated by clinical examination, serology and PCR. Venous blood samples (3 ml) were collected into Vacutainer<sup>®</sup> tubes containing EDTA for DNA extraction. One-hundred microliter of buffy coat was used for DNA extraction with DNAzol<sup>®</sup> reagent (Gibco BRL), followed by washing in 95% ethanol, according to manufacturer instructions. Extraction was performed in a DNA workstation (Airclean System<sup>®</sup>, Raleigh, NC, USA), with no more than six samples being processed simultaneously to avoid cross-contamination. Addition-

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ally, another blood sample was collected and serum was separated for the detection of *Leishmania* antibodies by indirect immunofluorescence (IFI) and ELISA (Madeira et al. 2000) and *Trypanosoma cruzi* by enzyme immunoassay and IFI (Silva et al. 2002). For PCR amplification, 1 ml of a 1:20 dilution of the DNA extracted from each sample was submitted to hot-start PCR with primers that amplify the conserved region of the minicircle molecules present in all *Leishmania* species (Degraeve et al. 1994). The reaction mixture contained 100 ng of each primer (5'-(G/C)(G/C)(C/G)CC(A/C)-CTAT(A/T)TTACACCAACCCC and 5'-GGGGAGGGG-CGTTCTGCGAA), 200 mM of each dNTP (GE Healthcare Life Sciences, São Paulo), 2.5 units of Taq polymerase (Ampliaq Gold, Perkin-Elmer, Norwalk) in the buffer supplied by the manufacturer, and 1.5 mM MgCl<sub>2</sub>. Amplification was carried out in a Perkin-Elmer 900 thermocycler under the following conditions: 94°C for 10 min, 30 cycles of 94°C/30 s, 50°C/30 s and 72°C/30 s, and a final cycle at 72°C/10 min. A negative control tube containing no DNA was included in each amplification. A positive control, consisting of 100 pg DNA extracted from an axenic culture of *Leishmania braziliensis*, was also performed. The DNA from the agarose gels was transferred to nylon membranes by capillary blotting using 0.4 N NaOH and hybridized against *Leishmania (Viannia)*-specific probes as described elsewhere (Schubach et al. 1998, Pirmez et al. 1999).

The population sample for clinical and laboratory reevaluation consisted of 60 originally MST-positive individuals, 14 subjects with nonspecific reactions to thimerosal or phenol, and 77 MST-negative individuals as controls. None of these 151 individuals presented anti-*Leishmania* antibodies. Anti-*T. cruzi* antibodies were detected by ELISA in three volunteers (one of this MST-positive), and by IFI in two (one of this MST-positive). None of the volunteers presented anti-*T. cruzi* antibodies simultaneously detected by the two techniques. Of the 151 PCRs for the detection of *Leishmania* DNA, one was positive and hybridization showed that the product corresponded to *Leishmania (Viannia)* DNA. Hybridization did not enhance the sensitivity of the ethidium bromide-stained products, demonstrating that the other 150 samples were really negative. The positive PCR corresponded to an 18 year-old healthy male volunteer who tested positive in the MST with merthiolate antigen (11 mm induration diameter), and negative in the preservative test. The subject was asymptomatic, born in RS (Santa Maria city) and had never lived in a different place. The man did not present any scar or history of allergy or blood transfusion and was seronegative for Chagas' disease.

The MST is the most widely used presumptive diagnostic method for cutaneous leishmaniasis. It is a simple and reliable test, but nonspecific results may occur due to the presence of allergenic reagents in the preparation that are not related to *Leishmania* antigens (Pineda et al. 2001, Fagundes et al. 2007). Moreover, the test is unable to distinguish between active, inactive or past infection and may become positive in individuals vaccinated

against leishmaniasis (Mayrink et al. 1979), thus indicating an immune response to *Leishmania* antigens in the absence of infection.

In clinical practice, methods for the etiological diagnosis of leishmaniasis (imprint, histopathology, in vitro culture and PCR) are used to confirm leishmaniasis infection in an MST-positive individual. These methods are generally applied in the presence of clinical disease. In our setting, since we studied asymptomatic MST-positive individuals, the most appropriate technique was PCR of peripheral blood because of its high sensitivity and specificity (Pirmez et al. 1999, de Oliveira Camera et al. 2006) which revealed one positive case. In addition to MST-positive subjects, we performed PCR on 91 other individuals (allergic or MST-negative) who tested negative.

In visceral leishmaniasis, PCR has been used as a tool for the evaluation of subclinical infection (Costa et al. 2000, 2002). Some studies have shown that the persistence of *Leishmania* DNA in blood or bone marrow of treated visceral leishmaniasis patients is associated with disease relapse or the appearance of post-kala azar dermal leishmaniasis (Osman et al. 1997). Furthermore, PCR permitted the detection of *Leishmania (Viannia)* DNA in scars of clinically cured cutaneous leishmaniasis patients, demonstrating the persistence of the parasite (Schubach et al. 1998). Likewise, the scar of one of these patients was also positive by culture (Schubach et al. 1998). Assuming that a positive PCR corresponds to the presence of viable parasites, it might be considered to be an indicator of active infection (Tarleton et al. 1999).

The hypothesis of occurrence of persistent subclinical infection detected by a positive MST has been suggested since 1940 in Brazil (Pessoa & Pestana 1940), and was later supported by PCR (Schubach et al. 1998, Costa et al. 2002, de Oliveira Camera et al. 2006). Whereas the MST does not discriminate among the infectious *Leishmania* species, the PCR-hybridization approach permits the identification of the infectious agent. In our case, the amplified product corresponded to DNA of the *Viannia* subgenus.

In conclusion, the finding of this MST-positive individual who presented *Leishmania (Viannia)* DNA detected by PCR, confirms the first case of subclinical human *Leishmania* infection in RS, Brazil. In contrast, the prevalence of positive responses to the MST is very high, excluding thimerosal- or phenol-allergic individuals. Other studies are in progress to identify *Leishmania* DNA in other populations, including domestic animals. The finding of MST positivity and *Leishmania* infection in individuals without symptoms and in an area where no human cases have been detected so far suggests the circulation of *Leishmania* in the study area, and the establishment of subclinical infection as the base of the "iceberg", whose peak is represented by the clinical confirmed cases. Besides, PCR might be a useful tool for surveillance in order to identify areas that are at risk for acquisition of the disease, and the circulating parasites, starting out from MST positive results. In fact, after the preparation of this manuscript, some cases of cutaneous leishmaniasis were notified (Rio Grande do Sul 2006) in another site in RS far from the study area.

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