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Leishmania (*V.*) *braziliensis*: Detection by PCR in biopsies from patients with cutaneous leishmaniasis

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ABSTRACT

Cutaneous leishmaniases present similar clinical appearances, but differing prognosis in the course of infection. Ulcers caused by parasites of the subgenus *Viannia* are more aggressive than ulcers caused by parasites of the subgenus *Leishmania*. Another problem is distinguishing between true *Leishmania* infection and other skin diseases in endemic areas, where cutaneous lesions and a single positive Montenegro intradermal test are enough to submit patients to specific treatment for cutaneous leishmaniasis. This study evaluated the efficacy of PCR in detecting in *Leishmania* in patients with cutaneous lesions. *Leishmania* (*V.*) *braziliensis* complex was determined by a primer pair from the multicopy spliced leader RNA. The results were compared to those of traditional methods. We analyzed biopsies of 109 patients with cutaneous lesions in the second most endemic region of Sao Paulo State, Brazil. Definitive diagnosis was established by clinical and "consensus laboratory criteria" (positive culture, stained tissue smears or PCR). Of 52 patients with cutaneous leishmaniasis, 96% had positive PCR, 69%, positive parasitological tests and 100%, positive Montenegro intradermal tests. Histopathological examination (only in 32 samples) were positive in 14 samples, suggestive in 14 and negative in 4 samples. All 57 patients with other etiologies had negative results in parasitological methods, PCR and histopathological examination (in 39 samples), but Montenegro intradermal tests were positive in 35%. PCR was highly sensitive and specific for *L.* (*V.*) *braziliensis* complex detection compared with other laboratory methods. Despite the specificity of the parasitological tests, the sensitivity was less than 70%. Montenegro intradermal reaction was highly sensitive, but with low specificity, only 65%. As suggestive results in histopathological examinations were shown in 14 samples, it was difficult to determine the true result. PCR applied to biopsies proved to be useful for differential diagnosis of cutaneous lesions of other etiologies in patients living in endemic areas. The advantages are most striking in clinical specimens with scarce amastigotes for which conventional methods have low sensitivity and should be considered for clinical and epidemiological patterns. On the other hand, both Montenegro intradermal test and parasitological methods are only modestly effective in cutaneous leishmaniasis diagnosis.

1. Introduction

Leishmaniases are a group of chronic diseases caused by the genus *Leishmania*, transmitted from animals to humans by the bite of infected female sand flies. There is abroad spectrum of clinical forms, including those that affect skin, mucosa, or internal organs (Grimaldi and Tesh, 1993; Lainson and Shaw, 1998).

Leishmaniases are prevalent on four continents and considered endemic in 88 countries, 72 of which are developing countries. The worldwide prevalence of the disease is estimated at 12 million cases, with 400,000 to 600,000 new cases per year for visceral forms and of 1–1.5 million for the cutaneous forms (WHO, 2007). In Brazil, approximately 2500–5000 cases of visceral forms and 30,000–36,000 cases of cutaneous forms are reported per year. About 3–5% of all patients who develop cutaneous or mucocutaneous lesions have leishmaniasis (MSB, 2007). In Sao Paulo State, where the incidence is low, approximately 600 new cases are reported per year. Another considerable problem is the urbanization of the infection. In recent years, autochthonous cases have been described in rural areas. Presently, the incidence of periurban and urban cases has been increasing. Approximately 10% of the population that live in endemic areas is at risk for acquiring the infection (CVE, 2007). These data are sufficient for developing new control strategies such as facilities for diagnosis, treatment and population information.

Leishmania species in Latin-America belong to two taxonomic subgenera. The first is the subgenus *Leishmania*, composed of

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Leishmania (*L.*) *mexicana* and *Leishmania* (*L.*) *amazonensis*, responsible for localized or diffuse cutaneous disease; and *Leishmania* (*L.*) *infantum chagasi*, the cause of New World viscerotropic leishmaniasis. The second subgenus is *Viannia* cause of New World cutaneous leishmaniasis with cutaneous or mucocutaneous lesions, comprising *Leishmania* (*V.*) *braziliensis*, *Leishmania* (*V.*) *panamensis*, *Leishmania* (*V.*) *guyanesis* and others (Rioux et al., 1990; Lainson and Shaw, 1998). In Brazil, cutaneous leishmaniasis is caused by at least six different *Leishmania* species (Shaw, 1994), but the vast majority of cutaneous lesions are caused by *L.* (*Viannia*) *braziliensis* (Grimaldi and Tesh, 1993; Lainson and Shaw, 1998).

The *Leishmania* species present with a similar clinical appearance, but with different prognosis during the course of the infection. The ulcers caused by parasites of the subgenus *Viannia* are more aggressive and can recur after treatment. The ulcers caused by parasites of subgenus *Leishmania* are less severe and more likely to cure spontaneously or after treatment (Grimaldi and Tesh, 1993; Pirmez et al., 1999; Ramos-e-Silva and Jacques, 2002). As some patients have small numbers of parasites within the lesions, it is difficult to establish a correct diagnosis. Another problem is to distinguish between true *Leishmania* infection and other skin diseases in endemic areas for cutaneous leishmaniasis, where cutaneous lesions and a single positive Montenegro intradermal test are sufficient to submit patients to specific treatment for leishmaniasis.

The traditional diagnosis of New World cutaneous leishmaniasis is performed using clinical and epidemiological features and through parasitological tests, by means of lesion biopsy of the and processing of the specimens for direct examination of smears after Giemsa staining, *in vitro* culture, histopathological techniques, and immunological methods (Grimaldi and Tesh, 1993; Ramos-e-Silva and Jacques,2002). The different *Leishmania* species are not equally easy to culture. Contamination is a constant problem, and variations in efficacy among different growth medium formulations or even batches may be encountered. Likewise, the success of microscopic identification of amastigotes in stained preparations depends on the number of parasites present and/or the experience of the technician examining the slide.

The differentiation of *Leishmania* species is particularly important in regions such as Sao Paulo State where both visceral and cutaneous leishmaniases are co-endemic. Recent findings showed *L.* (*L.*) *chagasi* dispersion in endemic areas for *L.* (*V.*) *braziliensis* (CVE, 2007). The accurate identification of *Leishmania* species has clinical and epidemiological benefits. It allows better orientation of medical treatment and follow-up, since clinical progress and parasite sensitivity to drugs may vary depending on the species of *Leishmania*. Detection of the particular *Leishmania* species from an endemic area is also essential to plan more suitable control activities and to understand the epidemiology of the disease. For this purpose several molecular methods have been developed, such as polymerase chain reaction (PCR);PCR followed by hybridization; and PCR–restriction fragment length polymorphism (PCR–RFLP) (Barker et al., 1991; Rodriguez et al., 1994; Harris et al., 1998; Schallig and Oskam, 2002; Marfurt et al., 2003; Volpini et al., 2003). Our group previously evaluated a PCR for detecting and genotyping *L.* (*L.*) *chagasi* and *L.* (*V.*) *braziliensis* in canine samples, which has been shown to be highly sensitive and specific (Gomes et al., 2007).

This study aimed to evaluate the efficacy of PCR in detecting cutaneous leishmaniasis in patients with cutaneous lesions, and its capacity to distinguish *L.* (*V.*) *braziliensis* from other *Leishmania* species avoiding "in vitro" cultivation. This PCR amplifies a fragment present in the multicopy spliced leader (RNA gene), having been shown to be highly sensitive and specific for *L.* (*V.*) *braziliensis complex* (Harris et al., 1998). The results were compared to those of traditional methods for the laboratory diagnosis of cutaneous leishmaniasis. The patients studied attended public dermatology clinics in Sorocaba, an endemic region of Sao Paulo State. This region is considered the second most endemic region for cutaneous leishmaniasis in Sao Paulo State (about 80 new cases per year) (CVE, 2007), comprising 45 cities (small to medium size) with rural, periurban and urban localities.

2. Materials and methods

2.1. Patients and samples

From June 2005 to November 2006, cutaneous leishmaniasis diagnosis was performed in 109 patients with cutaneous lesions attending public dermatology clinics in 22 cities of Sorocaba region—Sao Paulo State. All patients analyzed in this study also lived in these cities and were selected considering epidemiological risk factors for cutaneous leishmaniasis, such as proximity of other infected patients as well as signs or symptoms of the disease. A complete dermatological examination was performed, and all patients presented cutaneous lesions suggestive of leishmaniasis. None of them had acquired the infection before or had been treated with drugs for leishmaniasis. The lesions were initially cleaned with antiseptics after the administration of a local anesthetic. The borders of the lesion were scraped or smears of material were obtained by punch biopsy of the lesions and immediately added to tubes containing 1–2 ml of a sterile 0.85% NaCl and 200 μg/ml gentamicin solution, and sent to the laboratory within 48 h, where they were immediately processed. Each sample was divided into three or four fragments, two of which were processed for parasitological tests (culture and microscopic examination), and a third for DNA extraction. In 71 specimens, the fourth fragment was used for histopathological examination. The Ethics Committee of Instituto Adolfo Lutz approved this study.

2.2. Traditional diagnosis

2.2.1. Montenegro intradermal test

This test was performed as previously described (Reed et al., 1986). The antigen suspension was supplied by Immunobiological Production and Research Center (Centro de Produção e Pesquisa de Imunobiológicos, PR, Br - purchased by São Paulo State Health Secretariat) and comprised a crude extract of *L.* (*L.*) *amazonensis* dissolved in phenol 0.005 g/ml, at a concentration of 40 μg/ml protein nitrogen. Intradermal injections were made with 0.1 ml of the antigen on the forearm of the patients. The reactions were measured after 48–72 h and were considered positive if indurations were more than 5 mm in diameter.

2.2.2. Parasitological methods

A portion of samples was plated onto a glass slide, fixated with methanol, and stained with Giemsa according to the WHO (WHO, 2007). The presence of amastigotes was observed microscopically (magnification 1000). The second portion was placed in culture. Tissue fragments were homogenized in a sterile plastic pestle and placed into 1.5 ml tubes. The samples were placed into two tubes containing two biphasic media, one of which was 30% rabbit defibrinated blood in Agar Potato Infusion (Ducrey) and the other 5–15% rabbit defibrillated blood in Agar Base pH 6.8 (BAB). Both media were supplemented with 2 ml Brain Heart Infusion medium (BHI), 5% sterile human urine and 200 μg/ml gentamicin (Armstrong and Patterson, 1994). The tubes were incubated at 25 °C for 10–15 days. The presence of promastigotes was carefully observed weekly by microscopy (Grimaldi and Tesh, 1993; WHO, 2007). Parasitological diagnosis was considered positive if parasites were present in at least one of the tests.