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The Use of Radionuclide DNA Probe Technology for Epidemiological Studies of Tegumentary Leishmaniasis in Mato Grosso State

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ABSTRACT

DNA hybridisation, using probes labelled with ^{32}P, was used to type Leishmania samples isolated from patients living in endemic areas of Mato Grosso State (Brazil), and clinically diagnosed as having tegumentary leishmaniasis. kDNA cloned mini-circle probes specific for the Leishmania mexicana and Leishmania braziliensis complexes were used. The results showed that L. braziliensis is the predominant group infecting human patients in the state. Sixty-eight samples were typed, 64 samples (94.1%) belonging to the L. braziliensis complex and only four (5.9%) belonging to the L. mexicana complex. Accurate identification of the Leishmania permits better orientation of the medical follow-up, since clinical manifestations may vary depending on the complex to which the parasite belongs. The epidemiological information furnished by the identification of the Leishmania in given endemic area is also essential for the design of appropriate control measures

Key words: *Leishmania*, leishmaniasis, DNA hybridisation, Mato Grosso state

INTRODUCTION

New World leishmaniasis occurs from the southern United States to northern Argentina and is caused by 13 species grouped into three complexes: *Leishmania braziliensis*, *Leishmania mexicana*, and *Leishmania donovani.* Members of the *L. braziliensis* complex cause cutaneous or mucocutaneous lesions, those of the *L. mexicana* complex cause localised or diffuse cutaneous involvement, and *L. donovani* complex species produce visceral disease (Grimaldi, and Tesh, 1993).

The tegumentary (cutaneous and mucocutaneous) forms of leishmaniasis occur throughout Brazil. Prevalence of the disease has increased in all 26 states during the last years, from 1985 to 2003, 523,975 cases of tegumentary leishmaniasis were recorded in Brazil (Basano and Camargo, 2003). Current diagnosis of cutaneous leishmaniasis is usually made on a clinical basis, but parasitological confirmation is important because of the high cost and toxicity of the treatment. Furthermore, accurate identification of the

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Leishmania species involved permits better orientation of the medical follow-up, since clinical manifestations may vary (cutaneous or mucocutaneous), depending largely on the species to which the parasite belongs (Grimaldi, and Tesh, 1993). Cutaneous leishmaniasis ulcers may persist for months to years, but they eventually heal. Mucocutaneous leishmaniasis, however, can result in destructive lesions on the nose, oral pharyns, lips or face, and is an important cause of disfigurement and morbidity in endemic areas of the country.

Detection of the particular *Leishmania* species involved is also essential to plan more suitable control activities and to understand the epidemiology of the disease.

The incidence of tegumentary leishmaniasis in the Mato Grosso state has increased in the last years. The annual rate has surpassed 200 cases/100,000 inhabitants (FUNASA, 2004). The disease is associated with the colonization of new areas, and the consequent ecological unbalance. The state presents three different ecosystems: Amazonian forest, swampland and and savanna. The patients studied come from rural regions, especially areas of deforestation and disordered occupation from the north of the state. They had occupations linked to the forest or associated with forest areas, showing clear association with recent colonization. The occupational disease profile seems to be very evident in the region. Most of the people infected work in agricultural activities (FUNASA, 2004). The National Health Foundation has characterized the distribution and the frequency of the leishmaniasis in the state, but it has no information about the dynamics of transmission, the clinical behavior of the disease and, mainly, the species involved.

Leishmania are members of the order Kinetoplastida, which have a distinguishing feature: the kinetoplast organelle, a unique mitochondrial structure containing concatenated DNA. Wirth and Pratt (1982) demonstrated that the kinetoplatid DNA (kDNA) of the members of the *L. mexicana* complex did not display any homology with the kDNA of the *L. braziliensis* complex in hybridization experiments. Thus, this feature can be exploited to identify *Leishmania*, as it discriminates between the two complexes.

In the present work, kDNA cloned minicircle probes, labeled with ^{32}P, was used to type *Leishmania* isolates obtained from human patients, clinically diagnosed as having tegumentary leishmaniasis and living in endemic areas of the Mato Grosso state.

MATERIAL AND METHODS

Sample Collection. Biopsies were taken from lesions of patients suspected of having leishmaniasis. Besides the presence of the typical lesion, they also presented responses to the Montenegro skin test and indirect fluorescent antibody test (IFAT) (titre > 1:45). All presented exclusively cutaneous lesions, the majority ulcerated with three to four months of evolution The patients were from rural areas and were attended at Júlio Muller Hospital in Cuiaba. The cutaneous lesions were prepared in the normal manner for biopsy that included disinfectant and anesthesia The biopsies were taken with a sterile punch from the raised border of the lesion and inoculated in Evans biphasic medium. The positive isolates were cultured in minimum essential medium supplemented with 10% foetal bovine serum. The cells were counted, and between 5×10^3 and 10^4 parasites were spotted on nitrocellulose filters. Positive and negative controls used the WHO reference strains IFLA/BR/68/PH8 (*Leishmana (Leishmania) amazonensis*) and MHOM/BR/75/M2903 (*Leishmania (Viannia) braziliensis*).

Treatment of the Filters for DNA Hybridisation. Before filters were processed, they were treated to reduce background. The filters were incubated at 37 ^oC for 2 h in ETT buffer (0.01 M Tris, 0.01 M EDTA, 0.1% Triton X-100, pH 10.0) containing 200 μg/ml of proteinase K, then treated for DNA denaturation for 10 min at room temperature in 0.5 M NaOH, 1.5 M NaCl solution, using just enough liquid to moisten the filter. The filters were neutralised by incubating twice in 1.0 M Tris-HCl, pH 8.0 solution for 10 min each, and baked at 50 ^oC for 1 h.

DNA Labelling with ^{32}P. Cloned kDNA minicircles from *L. (V.) panamensis* IPAN V and *L.(L.) amazonensis* IFLA/BR/67/PH8 (Fernandes et al., 1996) were used as probes. These minicircles were labelled with ^{32}P [α]dCTP using the random priming DNA labelling system procedure (Gibco BRL).

The labeled kDNA probe was separated from unincorporated nucleotides by chromatography in Sephadex G-50 fine. The column was equilibrated and eluted with 10 mM Tris-HCl (pH 8.0), 50 mM NaCl and 0.1 mM EDTA solution

Hybridisation. The filters were pre-soaked at 58 ^{0}C for 30 min in 0.5% non-fat milk, 1% SDS and 2 x SSC (0.3 M NaCl, 0.3 mM sodium citrate) solution. The solution was changed using just enough liquid to cover the membrane. The kDNA probe was added to the solution after being heated for 3 min in a boiling water bath. The filters were incubated for 14 h at 58 ^{o}C, with shaking, then placed in 2 x SSC for 20 min at room temperature and washed in 0.5 x SSC (75 mM NaCl, 0.075 mM sodium citrate), 0.5% SDS, at 65 ^{o}C for 30 min (Andrade et al., 2001). Finally, they were dried and exposed to autoradiography at –70 ^{o}C, using a cassette with intensifying screens.

RESULTS AND CONCLUSIONS

Sixty-eight samples were typed, 64 samples (94.1%) belonging to the *L. braziliensis* complex and only four samples (5,9%) belonging to the *L. mexicana* complex. The specificity of the procedure was 100%, since no cross hybridisation between the two kDNA minicircles probes occurred for any sample. The typical autoradiogram result for 10 samples collected in the Mato Grosso state is presented in Fig.1.

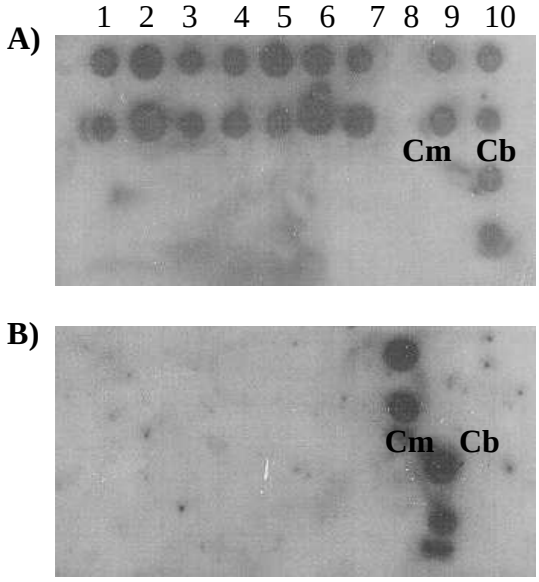

Figure 1 - Autoradiogram of Blotted Parasites Hybridised with kDNA Radioactive Probes.
Two nitrocellulose membranes with the same 10 samples, in duplicates, were prepared and hybridized with the specific kDNA probes. The membrane A was hybridized with the kDNA probe for the *L. braziliensis* complex and the membrane B with the kDNA probe for the *L. mexicana* complex. Sample 8 showed a positive reaction for the *L. mexicana* and the remaining samples were positive for *L. braziliensis*. Cm, control for *L. mexicana*. Cb, control for *L. braziliensis.*

The positivity of the analysis of the cultured parasites blotted in nitro-cellulose was assured by using a number of parasites comfortably superior to the threshold detection level of the analysis (Degrave et al., 1994). This methodology also has the advantage that many samples can be applied to the membrane and processed simultaneously, considerably reducing the time required. The results of this study show that the use of kDNA probes is a useful method for the detection and typing of *Leishmania* isolates to species complex level. The mucocutaneous clinical manifestation of leishmaniasis involves infection by *L. braziliensis* complex species, mainly *L. (V.) braziliensis sensu*

stricto in Brazil (Desjeux, 1996). The lesions caused by species of the *L. braziliensis* complex are also generally more aggressive and can recur after treatment and cure. The prevalence of mucocutaneous involvement varies among different areas of the country, values of 5% of the cases having been recorded for the Rio Doce Valley in Minas Gerais [Genaro et al., 1993), 12.5% in Três Braços and Corte da Pedra in Bahia State [Costa et al., 1988)], and 26.9 % in Mato Grosso State (Hueb, 1997). In this study using the radionuclide DNA probe methodology, *L. braziliensis* complex was shown to be the prevalent group causing tegumentary leishmaniasis in the Mato Grosso state.

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RESUMO

Hibridização, utilizando sondas de DNA marcadas com ^{32}P, foi utilizada para a tipagem de amostras de *Leishmania* isoladas de pacientes do estado do Mato Grosso (Brasil), diagnosticados clinicamente como portadores de leishmaniose tegumentar. Sondas de minicírculos clonados de kDNA, específicas para os complexos *Leishmania mexicana* e *Leishmania braziliensis*, foram utilizadas. Os resultados demonstraram que o complexo *L. brasiliensis* é o grupo predominante infectando pacientes humanos no estado do Mato Grosso. Foram tipadas 68 amostras: 64 (94,1%) foram identificadas como pertencentes ao complexo *L. brasiliensis* e somente 4 (5,9%) como pertencentes ao complexo *L. mexicana*.

A tipagem de *Leishmania* é importante para um melhor acompanhamento médico, uma vez que as manifestações clínicas podem variar em função do complexo ao qual o parasita pertence. A informação fornecida pela identificação também é essencial para a definição das medidas de controle mais adequadas e compreensão da epidemiologia da doença.

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