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ARTIGOS

EVALUATION OF A DIRECT IMMUNOFLUORESCENT ANTIBODY (DIFMA) TEST USING LEISHMANIA GENUS-SPECIFIC MONOCLONAL ANTIBODY IN THE ROUTINE DIAGNOSIS OF CUTANEOUS LEISHMANIASIS

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A direct immunofluorescent antibody (DIFMA) test using a Leishmania genus-specific monoclonal antibody was evaluated in the routine diagnosis of cutaneous leishmaniasis (CL) in Ecuador. This test was compared with the standard diagnostic techniques of scrapings, culture and histology. Diagnostic samples were taken from a total of 90 active dermal ulcers from patients from areas of Ecuador known to be endemic for cutaneous leishmaniasis. DIFMA was positive in all lesions. It was shown to be significantly superior to standard diagnostic methods either alone or in combination. The sensitivity of DIFMA did not diminish with chronicity of lesions. This test proved to be extremely useful in the routine diagnosis of CL because it is highly sensitive, is easy to use and produces rapid results.

Key-words: Cutaneous leishmaniasis. Diagnosis. Monoclonal antibody.

Cutaneous leishmaniasis is endemic in Ecuador[6]. Detailed clinical and parasitological studies have implicated 5 different species (*Leishmania braziliensis, L. panamensis, L.guyanensis, L. mexicana and L.amazonensis*) as causative agents[4,14]. These species vary geographically between the different tropical and subtropical regions of the country[3].

Diagnosis in developing countries usually relies on clinical assessment. However, where facilities permits diagnosis may be made by parasite detection in tissue isolates and/or by the leishmanin (Montenegro) skin test. The low sensitivity of standard parasite detection methods[5], the poor specificity of the leishmanin test in endemic areas[12], and the need to distinguish cutaneous leishmaniasis (CL) from lesions of different aetiology, have stimulated the search for other diagnostic assays. To date serodiagnostic assays have proved disappointing in the diagnosis of New World CL[1,19]. The availability of novel techniques using monoclonal antibodies or kinetoplast DNA probes offers rapid diagnosis with high sensitivity and specificity[1]. The usefulness of such assays in the routine diagnosis of CL remains largely untested.

In this study, the direct immunofluorescent monoclonal antibody (DIFMA) test using a genus-specific monoclonal antibody[7] was compared with standard parasite detection methods.

MATERIAL AND METHODS

Study population

Dermatological clinics were held in Quito and Santo Domingo de los Colorados in the province of Pichincha, Ecuador, over a 1 year period. Patients with active skin lesions suggestive of CL were selected for the study. These patients came from the following regions of the country: the tropical and subtropical areas of the Pacific Coast, the high semi-arid plains of the Andes, and the tropical eastern Amazon regions.

Each patient was examined and documented regarding age, sex, race, occupation, time in endemic areas, lymphatic involvement, and the number, anatomical site, size and duration of lesions.

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Sample collection and examination

Before samples were taken, necrotic areas were debrided and the lesions cleaned thoroughly with savlon to avoid secondary contamination. From each patient one sample of each of the following were taken: scraping, needle aspirate and punch biopsy. In patients with multiple lesions additional samples were taken.

DIFMA

Impression smears of the biopsy were made on glass slides and air dried. The touch preparations were processed by the DIFMA technique using the genus-specific anti-leishmanial monoclonal antibody (Mab) 83-J3D2. This method has been described elsewhere[7]. The preparations were examined under oil immersion using an ultraviolet microscope (Zeiss) for the presence of amastigotes (x43, 100 fields).

Scrapings

Scrapings were taken from the active borders of lesions using a size 20 scalpel blade. They were smeared onto glass slides to make a thin preparation, air dried and fixed in methanol. After Giemsa staining, the preparations were examined under oil immersion (x100 magnification, 100 fields).

Culture

Samples for culture were taken by needle aspirate and biopsy punches. Needle aspirates were obtained with a 3ml syringe and 23 gauge needle. The syringe was filled with 0.1ml sterile phosphate buffered saline (pH 7.4). The needle was inserted into the outer border of the lesions, the syringe rotated and the tissue fluid aspirated on withdrawal.

Full thickness biopsies were obtained with a 4mm punch from the active borders of the ulcers, after anaesthetising with 2% lidocaine and adrenaline. The biopsies were placed in saline supplemented with 100,000 units of benzylpenicillin for 24 hours at 4°C. The biopsies were then homogenised in saline before culturing.

Needle aspirates and biopsy homogenates were each cultured in all the following media: defribinated rabbit blood agar-NNN medium, Schneiders Drosophila medium (GIBCO, Green Island, NY) supplemented with 20% heat inactivated foetal calf serum, and diphasic blood agar meduim with 0.5ml Dulbeccos phosphate buffered saline as the liquid overlay (DBA-PBS). Cultures were maintained at 25°C for at least 30 days before reporting negative.

Histology

Punch biopsy fragments were obtained as described above. These were fixed immediately in 10% buffered formaldehyde and later stained with haematoxylin and eosin, embedded in paraffin, and sectioned. At least 100 fields were examined before being reported negative.

Consent

Informed consent for this study was obtained from all subjects and procedures were explained in the local language. The study was carried out under protocols approved by the Ministry of Public Health of Ecuador and the Ethical Committee of Hospital Vozandes, Quito.

Stastistical analysis

McNemar's test for comparison of two paired samples was used[3].

RESULTS

A total of 90 active skin ulcers from 88 patients were assessed. The results of the different diagnostic methods are shown in Table 1. DIFMA was superior to all other methods employed and was positive in 100% of lesions. Histology (74.4%) was more senstive than either scrapings (51.1%) or culture (51.1%).

Table 1 - Positive lestons by the four diagnostic methods.

Diagnostic method	Postive	
	Nº.	%
DIFMA	90	100
Histology	67	74.4
Culture	46	51.1
Scrapings	46	51.1

Thirteen (14.4%) lesions were positive by DIFMA alone. Positive results in all tests were obtained in 29 (32.2%) lesions. When DIFMA is excluded from the analysis none of the other detection methods either alone or in combination were positive in more than 85.6% of lesions (Table 2).

Table 2 - Comparison of detection methods for diagnosis of cutaneous leishmaniasis.

Method compared	Nº.	%		Nº.	%	P
DIFMA vs culture	90	100	vs	46	51.1	p<0.001
DIFMA vs scrapings	90	100	vs	46	51.1	p<0.001
DIFMA vs histology	90	100	vs	67	74.4	p<0.001
DIFMA vs 3 methods	90	100	vs	77	85.6	p<0.005
Histology vs scrapings	67	74.4	vs	46	51.1	P<0.001
Histology vs culture	67	74.4	vs	46	51.1	p<0.005
Cutlure vs scrapings	46	51.1	vs	46	51.1	NS

A direct comparison of the 4 methods is shown in Table 2. DIFMA was far more sensitive than any of the other detection methods either alone or in combination. Histology was superior to scrapings and culture. There was no difference in sensitivity between culture and scrapings.

The sensitivity of standard detection methods decreased with duration of the lesion (Table 3). After 6 months duration a small proportion of lesions were positive by histology (40.0%), culture (30.0%) or scrapings (30.0%). On the other hand all lesions were positive by DIFMA irrespective of duration.

Table 3 - Rates of positivity by the four diagnostic tests in relation to duration of lesion.

Duration of lesion	Diagnostic method			
	scrapings	culture	histology	DIFMA
	%	%	%	%
< 3 months (n=43)	58.1	54.1	67.4	100
3-6 months (n=37)	32.4	37.6	81.1	100
> 6 months (n=10)	30.0	30.0	40.0	100

DISCUSSION

Our results show that in the routine diagnosis of cutaneous leishmaniasis, the DIFMA method is not only a useful technique with high sensitivity, but is greatly superior to standard diagnostic methods. The high sensitivity and specificity of this monoclonal antibody (Mab) for *Leishmania* has been demonstrated in other studies[1,7]. This Mab (83-J3D2) recognizes a dominant antigen common to promastigotes and amastigotes of isolates from 3 major species and 5 subspecies of New World CL[1].

Definitive diagnosis of CL requires the demonstration of amastigotes in lesions. Isolation of amastigotes in tissue samples is fraught with difficulties. In cutaneous lesions success at parasite isolation is inversely proportional to the duration of the lesions[20]. This is also reflected in our findings. However, DIFMA was positive (100%) irrespective of duration of the lesions. Furthermore, in patients receiving antileishmanial chemotherapy, DIFMA was shown to be useful in quantitatively assessing treatment efficacy (Chico M, Guderian RH, unpublished data).

The findings in this study of a low sensitivity of culture and scrapings, either alone or in combination, mirrors those of other workers[5]. In this study, histology was also superior to culture and scrapings, which is inconsistent with the findings of other workers[8,18]. The clarity with which amastigotes can be identified by DIFMA far exceeds that which is seen on conventional staining with haematoxylinn and eosin. DIFMA also identified a high proportion of cases that were not detected by the other mehods. The observation that all these lesions resolved following adequate course of leishmanial chemotherapy is indirect evidence for a correct diagnosis.

Immunological methods have proven of limited use in the diagnosis of New World CL. Patients rarely have detectable antibodies. Usually the delayed hypersensitivity response (leishmanin test) has not developed at the time of clinical presentation[19]. Similarily such methods cannot distinguish current from past infection. Recent improvements in ELISA have improved the sensitivity of such assays[5,17], but most assays still suffer from poor specificity. False positive results have been reported in patients with malaria, toxoplasmosis, amoebiasis[15], and Chagas diseases[2]. Differences between locations with regard to specificity and sensitivity have also been reported[20]. The leishmanin test remains a useful epidemiological tool, but it's value as a diagnostic test is limited in endemic areas where a large proportion of the population may test positive[9,16].

A genus-specific anti-leishmanial probe, as used in this study, is advantageous for several reasons. Firstly, the primary requirement of a diagnostic test for CL is to distinguish genuine cases from the multitude of similarly presenting skin lesions such as sporotrichosis, yaws, cutaneous mycoses and tropical ulcers. Furthermore, because of the heterogeneity of

species causing CL in Ecuador[4,14], diagnosis by species-specific probes would require at least 5 different probes before a diagosis of CL could be excluded. Though species-specific Mab probes are available[13], this would clearly be impractical in routine diagnosis. There is, however, a need to distinguish more benign species from *L.braziliensis* which has a tendency to cause destructive mucocutaneous leishmaniasis. This could be achieved by screening suspected lesions with a genus-specific probe and then testing all positive cases with a *L.braziliensis* specific Mab. Though species identification may influence therapeutic options, in practice in most developing countries, such choice is limited to one or two drugs, rendering speciation irrevelant.

The advantages of novel diagnostic tests using Mab or kinetoplast DNA (kDNA) probes over immunological or parasite isolation methods are clear. Such assays are rapid and highly sensitive and specific[1,7,11]. For example, positive culture may take weeks, delaying diagnosis, while DIFMA can be performed in one hour. However, the sensitivity of Mabs and kDNA probes may detect differences so fine as to render their significance impossible to evaluate[10]. While this is not so important in diagnosis in the presence of characterisitic lesions, their usefulness in epidemiological studies would be questionable.

There remains a need for a highly specific and sensitive diagnostic test that is inexpensive, rapid and simple to perform without the need for sophisticated equipment. The DIFMA test employed in this study satisfies most of these criteria, and is superior to culture, scrapings and histology. DIFMA has a number of advantages over indirect immunofluorescence using Mabs, and *in situ* hybridization using kDNA probes, due to its simplicity, rapidity and relatively low cost. In addition the availability of inexpensive ultraviolet systems (FW Kirk Ltd, Cambridge, UK) that can be used with any conventional microscope makes this technology accessible to everyone in developing countries.

SUMMARY

O método de imunofluorescência direta (DIFMA), com anticorpos monoclonais gênero-específicos para Leishmania, foi avaliado na rotina diagnóstica da leishmaniose cutânea no Equador. O método foi comparado com técnicas diagnósticas de rotina: o esfregaço, a cultura e o exame histopatológico. As amostras para o diagnóstico foram obtidas de um total de 90 lesões cutâneas ativas, de doentes das áreas do Equador, endêmicas para leishmaniose cutânea. O DIFMA foi positivo em todas as lesões, com resultados significativamente superior aos métodos diagnósticos de rotina, isolado ou em combinação. A sensibilidade do DFIMA não diminui em lesões crônicas. O método mostra-se muito útil no diagnóstico de leishmaniose cutânea, pela sua sensibilidade, rapidez e facilidade de execução.

Palavras-chaves: Leishmaniose cutânea. Diagnóstico. Anticorpos monoclonais.

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