Diagnosis of Cutaneous Leishmaniasis in Colombia: the Sampling Site within Lesions Influences the Sensitivity of Parasitologic Diagnosis

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Parasitologic confirmation of cutaneous leishmaniasis is obligatory before chemotherapy can be considered. Direct microscopic examination of scrapings taken from indurated borders of ulcers has been routinely used as primary method of diagnosis. In this report we compared the sensitivity of examination of dermal scrapings taken from the bottoms of ulcers (BDS) with that of dermal scrapings taken from indurated active margins of lesions (MDS) in a total of 115 patients. The sensitivities of the microscopic examination were 90.4 and 78.3% for BDS and MDS samples, respectively. When the PCR method was used with a group of 40 patients, we also observed a higher sensitivity when BDS samples were examined (80.8% in BDS samples versus 57.7% in MDS samples). The improvement of the diagnostic sensitivity in the BDS samples appears to be related to the higher parasite load and more easily detectable morphology of amastigotes in the centers of the ulcers. Other parasitologic diagnostic methods, such as culture and histopathologic examination of biopsies, are less sensitive (67.5 and 64.3%, respectively). Aspirate culture, however, was shown to be the most sensitive method for the diagnosis of patients with chronic ulcers. When microscopic examinations of both BDS and MDS samples are combined, the sensitivity of diagnosis may rise up to 94%. We therefore recommend this method as a primary routine procedure for diagnosis of cutaneous leishmaniasis.

Fourteen New World-specific Leishmania species have been reported to cause leishmaniasis in the Americas. Typically, a wide spectrum of clinical forms of the disease can be observed in this area (22, 27). In Colombia, the most common clinical presentation is the cutaneous form, representing more than 90% of symptomatic infections (5). The Leishmania species most frequently isolated from cutaneous leishmaniasis (CL) lesions are Leishmania (Viannia) panamensis and Leishmania (Viannia) braziliensis, both belonging to the Viannia subgenus (6, 23). In areas of endemicity without sufficient laboratory infrastructure, CL is often diagnosed on the basis of the clinical characteristics of the lesions. Parasitologic confirmation of a Leishmania infection is absolutely critical in order to exclude an erroneous diagnosis, which may easily occur due to (i) the wide spectrum of cutaneous presentations caused by Leishmania (12, 16) and (ii) confusion with other dermal lesions which mimic the presentation of CL, such as sporotrichosis and bacterial ulcers, both of which are frequent in regions where leishmaniasis is endemic (1). In addition, treatment of leishmaniasis is expensive, toxic, and difficult to administer (4), and cutaneous lesions caused by Leishmania species of the Viannia subgenus may reactivate and produce the progressive and defiguring mucosal form if not adequately treated (11, 18).

Parasitologic diagnosis of CL relies on two major methods: (i) visualization of amastigotes by direct microscopic examination of tissue samples and (ii) isolation of parasites (16, 20). Novel methods, including the PCR technique, have gained increasing importance over the last few years and have been successfully applied in order to detect parasite DNA (3, 10, 13, 19).

Several studies in which different conventional parasitologic methods were evaluated showed heterogenous and sometimes conflicting results (2, 7, 8, 14, 17, 21, 26). However, due to the low cost, ease of performance, speed, and lack of a need for sophisticated laboratory equipment, the direct microscopic examination of Giemsa-stained scrapings of lesions still represents the most suitable method for the definitive diagnosis of leishmaniasis. It has usually been recommended that scrapings should be taken from the indurated margins of the lesions (28). In work on the optimization and improvement of this simple method of diagnosis, we performed a detailed study aimed to compare the sensitivities of parasite detection by microscopic examination and PCR in samples collected from two different sites within lesions. Our results provide evidence that the sensitivity of the dermal scraping technique may significantly increase when a different site of sample extraction is used. We found that samples taken from the bottom of the lesion allow parasitologic confirmation with higher sensitivity than the routinely used technique of sample extraction from the margin of the lesion and therefore recommend that both sample extraction sites be considered for the routine diagnosis of leishmaniasis.

MATERIALS AND METHODS

Patients. A total of 115 patients with skin lesions compatible with CL were included in this study. All patients live in or have visited areas in different regions of Colombia where CL is endemic. They were attended in the outpatient service of the Programa de Estudio y Control de Enfermedades Tropicales. The patients’ diagnosis and treatment were supervised by medically qualified persons. A total of 14.8% (17 of 115) of patients were 0 to 17 years old, 59.1% (68 of 115) were between 18 and 35 years old, and 26.1% (30 of 115) were older than 35 years; 81.7% were male. Seventy-four patients (64.3%) had one lesion, 15 patients (13%) had two lesions, and 24 patients (20.8%) had three or more lesions. Additionally, two patients showed symptoms of disseminated CL, with at least 30 lesions per patient. Lesions were localized as follows: 59.1% (68 of 115) of patients had at least one lesion in the superior extremities, 34.8% (40 of 115) had at least one lesion in the inferior extremities, and the remaining 7 patients (6.1%) had lesions in other areas.
had lesions distributed on different parts of the body, such as the head, neck, face, and trunk. The majority of lesions were typical indurated ulcers with well-defined margins. Patients with nodule or papules (plaques, nodules, verrucous, and papular lesions) were excluded from this study.

Montenegro skin test (MST). A 0.1-ml portion of Montenegro antigen (leishmanin) was injected intraocularly into the right fornix of the patients, and 48 h later, the diameter of induration was read by the ballpoint pen method (28). A diameter of 5 mm or larger was considered positive. The preparation of Montenegro antigen was performed as follows: after large-scale culturing of L. panamensis (MHOM/CO/85/UA140) in NNN medium, the promastigotes were washed three times with phosphate-buffered saline and resuspended at 2 x 10^6 parasites/ml in autoclaved solution of Coca (NaCl, 5 gl; NaHCO_3, 2.75 g; phenol, 4.0 g/liter). The antigen was subsequently tested for sterility and infectivity and refrigerated until use.

Samples and diagnostic procedures. For patients exhibiting more than one lesion, a detailed examination of each lesion was performed in order to choose the site of sample extraction. Generally, samples were obtained only from those sites which showed the most indurated margin. The lesion was cleaned of debris with saline solution. Purulent or necrotic ulcers were treated with particular care, and debris was removed. None of the patients had received any antimicrobial chemotherapy treatment prior to diagnostic examination. In two cases where bacterial infection presents in the ulcers complicated adequate collection of the samples and led to painful open sores, patients were treated with antibiotics during a period of 5 days before sample extraction. Once CL had been diagnosed by one of the parasitologic methods used, patients received treatment with Glucantime at the dosage internationally recommended (28). Samples for parasitologic diagnosis included dermal scrapings of the active indurated margins of lesions (margin dermal scrapings [MDS]) (25) (Fig. 1), dermal scrapings of the bottoms of the ulcers (bottom dermal scrapings [BDS]) (Fig. 1), fine-needle aspirates of the ulcers (aspirates), and biopsies. All BDS and MDS samples were taken by the same person in order to avoid individual variation. For the MDS, a thorough cleaning of the indurated active margin of the lesion with 70% alcohol was performed. The selected site of the margin was then subjected to pressure with the hemostat or the scissors in order to achieve a maximal volume of 25 ml. The following protocol for PCR cycling in the GeneAmp PCR System 9700 (Perkin-Elmer) was used: initial heat activation of the enzyme at 95°C for 5 min, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, and polymerization for 1 min at 72°C. A final extension step at 72°C for 10 min ended at the end of the reaction. Fifteen microliters of the PCR product was run in 1% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. The intensity of the amplification product of 750 bp was quantitatively determined by one-dimensional image analysis (Digital Science System DC 120; Kodak, Rochester, N.Y.), calculated on the basis of the known amounts of DNA fragments produced by the DNA marker. According to the image analysis results, we assigned ++ + + to a DNA amount of > 250 ng, ++ + + + to an amount in the range of 100 to 250 ng, ++ + + + + to an amount in the range of 50 to 100 ng, and + + + + + + + to an amount of < 50 ng. In cases where no amplification product was observed, we assigned a negative result (−).

Statistics. Due to the lack of a “gold standard” test for the parasitologic diagnosis of leishmaniasis, different diagnostic approaches are difficult to compare. For our studies, we defined as the reference diagnostic method the positivity obtained by at least one of the applied methods. The sensitivity of each method was then calculated based on this criterion. To compare the MDS and BDS methods, the Cohen’s kappa (κ) and weighted kappa coefficients were calculated based on the percentage of positivity of the total analyzed samples. Fisher’s exact test was performed in order to determine the association between positivity of methods and duration of lesions. All of these tests were calculated by using the Stata/IC 3.0 computer program.

RESULTS

Table 1 summarizes the results of the parasitologic diagnosis for a total of 115 patients with symptoms of CL. The MST was applied for 92 patients; 76 individuals (82.6%) were MST positive. Leishmaniasis was confirmed in 72.2% of the initial patient group (83 of 115) and in 85.5% of the MST-positive individuals (65 of 76) by at least one of the methods used for parasitologic diagnosis. Only one patient from the group of 16 MST-negative patients gave a positive result in the parasitologic diagnosis.

Both the MDS- and BDS-based microscopic examinations as well as culture aspirates were performed for a total of 115 patients. PCR was performed on the samples collected from the MDS and BDS sites of 40 patients (34.5%). From this group of patients, leishmaniasis in 65% (26 of 40) was parasitologically confirmed by at least one of the parasitologic methods. Histopathologic examination of biopsies was performed for 37 patients. Among the 19 negative biopsies, 4 showed a granulomatous reaction compatible with leishmaniasis, but amastigote forms were not observed and the patients were therefore registered as negative. However, these four patients were positively diagnosed by other methods. Bacterial or fungal contamination of NNN cultures was observed in three patients.
patients; one had a negative MST and negative parasitologic diagnosis, and two were positive for both.

As shown in Table 1, the most sensitive method of parasitologic diagnosis is the microscopic examination of BDS samples (90.4% of confirmed patients). Applying the classical MDS-based microscopic examination, a significant drop in sensitivity, down to 78.3%, could be observed. Applying the PCR technique, we detected infection in 80.8 or 57.7% of the demonstrated cases in BDS or MDS samples, respectively. One patient diagnosed as positive by BDS-based PCR was negative by all other methods, including MDS-based PCR. The biopsy was positive in 64.3% of confirmed cases. One positive sample as diagnosed by this method remained negative in microscopic examination and culture. Aspirate culture was positive in 67.5% of the confirmed cases. A total of 56 Leishmania isolates were obtained. From these, 54 were identified as L. braziliensis and 2 were identified as L. panamensis.

Subsequently, we performed a detailed semiquantitative analysis in order to compare the level of positivity of the microscopic examination in samples collected either from the margins or the bottoms of the lesions in a group of 75 patients. The results are shown in Table 2. Results for 66.7% of the cases (50 patients) were concordant between the two methods. Discordance of results was observed in 25 samples. In this group 22 samples (88%) appear to be better diagnosed by the BDS-based microscopic method due to the larger amount of parasites observed, while only 3 samples (12%) were better diagnosed by the MDS-based microscopic examination. A parasite load equivalent to more than the + level was observed in 21 patients when samples were obtained from BDS, compared with only 9 patients in the MDS group (Table 2). In Giemsa-stained BDS samples we also observed consistently sharper and larger amastigotes with nuclei and kinetoplastids which could be more easily distinguished than those typically obtained from the MDS samples. In addition, mitotic figures and intracellular forms are more frequently observed in the BDS samples (not shown).

The tendency to discover parasites in larger amounts in the BDS compared with the MDS samples was also evident in the group of 40 patients for whom the PCR method was used. DNA was extracted from MDS and BDS samples from the 40 patients analyzed, and PCR amplification was performed on all 80 samples. Figure 2 shows a typical result of the PCR amplification. The intensities of the diagnostic 750-bp band corresponding to the full-length minicircle sequence (9) were comparable in approximately 50% of the MDS and BDS sample pairs. However, in the rest of the patients a reproducible stronger signal was generally observed in BDS compared with MDS samples (Fig. 2). The intensity of the 750-bp band in each patient’s sample was analyzed by a digital image system and semiquantitatively interpreted using a grading scale described in Materials and Methods. In a similar way as for the microscopic examination, PCR results for both sample sites were compared (Table 2). Equal amounts of the PCR product in both the BDS and MDS samples were obtained with 57.5% of patients (23 of 40), while 42.5% of samples showed discordance; 94.1% (16 of 17) of these discordant samples produced larger amounts of PCR amplification products when DNA was extracted from the BDS site, compared with 5.9% (1 of 17) when DNA was obtained from the MDS site. In 14 patients the intensity of the 750-bp diagnostic band was equivalent to more than the + level in BDS samples, compared with 7 patients in the MDS group (Table 2). This stronger signal reflects a major

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of BDS samples with the following result a</th>
<th>Total</th>
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<tbody>
<tr>
<td></td>
<td>− (+) + +++ +++++</td>
<td></td>
</tr>
<tr>
<td>Microscopic examination</td>
<td>21 7 0 0 0</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>+ 1 24 7 5 1</td>
<td>38</td>
</tr>
<tr>
<td>MDS samples</td>
<td>0 0 3 2 0 5</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>+++ 0 1 1 0 3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>++++ 0 0 0 0 1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>22 32 11 8 2 75</td>
<td></td>
</tr>
</tbody>
</table>

a Boldface indicates concordant results.

b $\kappa = 0.64$ ($P < 0.001$).

c $\kappa = 0.70$ ($P < 0.001$).

FIG. 2. PCR amplification of the diagnostic 750-bp DNA fragment representing kinetoplastid minicircle DNA. Lanes 1, molecular size marker (200-bp ladder); 2, positive control with 50 ng of purified L. panamensis DNA; 3, negative control (no DNA template); 4 to 15, samples from six CL patients. BDS samples (even-numbered lanes) and MDS samples (odd-numbered lanes) of each patient are directly compared.
TABLE 3. Sensitivities of methods for parasitologic diagnosis of CL as related to chronicity of lesions

<table>
<thead>
<tr>
<th>Time of lesion evolution (mo)</th>
<th>No. positive/total (% positive) as determined by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microscopy</td>
</tr>
<tr>
<td>BDS samples</td>
<td>MDS samples</td>
</tr>
<tr>
<td>≤3</td>
<td>66/70 (94.3)</td>
</tr>
<tr>
<td>&gt;3</td>
<td>9/13 (69.2)</td>
</tr>
</tbody>
</table>

* Calculated for the parasitologically confirmed patients (n = 83 for microscopy and culture; n = 26 for PCR).

* P < 0.05 (Fisher’s exact test).

FIG. 3. Comparison of the sensitivities of different methods for parasitologic diagnosis of CL. The relative positivity of each analyzed method with respect to the total number of parasitologically diagnosed patients is indicated. Bars: A, combined microscopic examination of BDS and MDS samples; B, PCR detection of parasite DNA by combining BDS and MDS samples; C, aspirate cultures; D, biopsy.

Ulcerated skin lesions account for more than 90% of clinical manifestations of CL (5, 15, 24). However, the relatively wide range of morphological variations of the skin lesions, which are particularly frequent in New World leishmaniasis, as well as the prevalence of other microbial infections in areas where leishmaniasis is endemic which may mimic the symptoms of a Leishmania infection, often complicate the diagnosis of leishmaniasis. Parasitologic confirmation of Leishmania infection is therefore necessary before the relatively toxic chemotherapy should be applied (5, 28). Among the diagnostic methods available at present, the fine-needle aspirate culture has been reported to be the most sensitive method (17, 26), although the direct microscopic examination of lesion scrapings still continues to be the diagnostic method most widely applied due to the ease of performance, low cost, and speed of this technique.

In this report we provide evidence that variations in the technique of direct microscopic examination, with special emphasis on the site of sample collection, influence the sensitivity of this method. Our results suggest that sample extraction from the central region of the bottom of the ulcer significantly increases the sensitivity of direct microscopic examination compared to the routinely recommended extraction of samples from the margin of the lesion (28). This also holds true when applying the PCR-based diagnostic method. When we analyzed the parasite loads in both sites of sample collection using microscopy and PCR, it could be observed that around 90% of discordant cases were due to a major quantity of amastigotes in BDS samples, confirming that the larger amount of parasites present in the bottoms of lesions accounts for this increased sensitivity. In addition to this overall higher number of parasites found in BDS samples, by microscopy we observed that a better morphology of amastigotes allowed easier and faster identification of Leishmania. We therefore believe that these observations may have major implications for routine laboratory diagnosis. For example, in areas of endemicity, where health care personnel are not always sufficiently well trained and experienced to take adequate MDS samples and to identify the parasite by microscopy, the abundant and easily detectable amastigotes in BDS samples could significantly improve the diagnosis.

A previous study that aimed to compare the sensitivities of the microscopic examination technique in samples obtained from Guatemalan CL patients did not show significant differences which correlated with different sampling sites (17). However, we think that the discrepancy between their and our results can be explained by the larger group of subjects in our study and the technical details of sample extraction, as described in Materials and Methods.
The diagnostic sensitivity of the microscopic examination observed in our study is the highest reported so far. Weigle et al. (26) reported an extremely low sensitivity (32.7% of confirmed patients) with conventional microscopic MDS. In other reports, however, the diagnostic sensitivity was significantly higher (2, 21), and in one case it reached 84% of confirmed patients (17). Different modifications of sample extraction have been recommended. Navin et al. (17) found a substantial increase in the sensitivity of this method, from 40 up to 80%, when the number of samples collected from each lesion was increased from one to four. In our study, in contrast, only a single sample was taken for each applied method of parasite diagnosis, and the sensitivity was 90.4% when the BDS-based technique was used.

The aspirate culture of lesions is an easy and nontraumatic method of parasitologic diagnosis. However, it requires special equipment, it is time-consuming, and contamination is often observed under field work conditions. In contrast to our studies, this method was previously reported to be the most sensitive for the parasitologic diagnosis (17, 26). In this study we found a significant number of patients (25 of 78) who were diagnosed by microscopy but had a negative result by aspirate culture. The low sensitivity of the microscopic method observed previously could significantly underestimate the total number of patients suffering from CL and therefore overestimate the sensitivity of other methods, such as culture. We think that this is the most plausible explanation for this discrepancy.

Consistent with other studies, the least sensitive method reported for the diagnosis of CL was the histopathologic analysis. However, in one case this test was the only method which allowed diagnosis of a Leishmania infection, while it failed in 10 of 28 patients (36%) parasitologically confirmed by any other method. We therefore agree with other authors that histopathologic examination is more helpful in the diagnosis of pathologies which are not related to leishmaniasis and that it is not recommended as the primary method (26).

PCR is at present the most widely used molecular method for the study of clinical and epidemiological aspects of infectious diseases, due to its high sensitivity. In leishmaniasis, although many target sequences for PCR amplification have been characterized over the last few years, this technique is more routinely used in studies related to epidemiological aspects than in clinical tests. This may be partly due to the prerequisite of a specialized laboratory infrastructure and to the relatively high cost in developing countries. In the present study the PCR technique detected Leishmania parasite DNA in 81% of the confirmed cases. In three patients where PCR failed, direct microscopic examination demonstrated the presence of only a single amastigote on the whole slide, thus indicating that the overall sensitivity of the PCR technique may be less than that of the traditional microscopic method at least under the conditions used in our study. So far, most of the PCR studies have been performed with biopsy material from lesions, as they were shown to allow parasite detection with highest sensitivity (unpublished observation). Since we observed a similar or even superior sensitivity of parasite DNA amplification in BDS-extracted samples compared with biopsy material (unpublished observation), we think that BDS-based PCR could be a noninvasive alternative option for PCR diagnosis.

Our observations that (i) most of the parasitologic methods analyzed in this study are significantly less sensitive in lesions with more than 3 months of evolution time and (ii) culture appears to be the most sensitive method in this group of chronic patients are not completely novel and have already been demonstrated elsewhere (26). However, this study addi-