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Evaluation of PCR for Diagnosis of Visceral Leishmaniasis

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An evaluation of *Leishmania* PCR was performed with bone marrow, lymph node, and blood samples from 492 patients, 60 positive controls, and 90 negative controls. Results were compared with microscopy results for Giemsa-stained smears. PCR and microscopy of lymph node and bone marrow aspirates from patients with microscopically confirmed visceral leishmaniasis (VL) were equally sensitive. However, in patients clinically suspected of having VL and in whom parasites could not be demonstrated by microscopy, PCR was positive for 12 of 23 (52.2%) lymph node aspirates and 8 of 12 (66.7%) bone marrow aspirates, thus confirming the clinical diagnosis of VL. With PCR on filter paper, *Leishmania* DNA was detected in the blood of 33 of 47 (70%) patients with confirmed VL and in 2 of 11 (19%) patients suspected of having VL. Positive PCR results were more frequently found for blood samples on filter paper than for samples stored in EDTA. In conclusion, PCR is a more sensitive method than microscopy for the detection of *Leishmania* in lymph node and bone marrow aspirates, being especially useful for the confirmation of cases of suspected VL. Blood from a finger prick may be used for the initial PCR screening of people suspected of having VL. If the PCR of blood is negative, one should perform PCR with lymph node and/or bone marrow material, because PCR with these materials is more often positive.

The leishmaniases are parasitic diseases which are endemic in many countries in the tropics and subtropics. Approximately 350 million people are considered to be at risk of contracting the disease. Visceral leishmaniasis (VL), also known as kala-azar, accounts for an estimated 75,000 deaths annually, with more than half of these occurring in Sudan and India (2, 26). Sudan is one of the areas where VL is the most highly endemic, and epidemics also occur from time to time (7, 20).

At present the routine diagnosis of VL is done by direct microscopy of patient material or by culture. The microscopic detection of *Leishmania* amastigotes in Giemsa-stained spleen, bone marrow, or lymph node aspirates is relatively simple and cheap, but performance of spleen aspiration may be dangerous under field conditions, bone marrow and lymph node aspirates are of limited sensitivity (21, 29), and retrieval of such samples is inconvenient for the patient. Isolation of parasites by culturing is time-consuming, expensive, and difficult (25). Because of these drawbacks, there is a need for the development and evaluation of more sensitive methods.

Because of the limitations of the direct diagnostic methods mentioned above, a number of indirect immunological methods such as the indirect immunofluorescent-antibody test, enzyme-linked immunosorbent assay, and direct agglutination test (DAT) have been developed over the years (for a review, see reference 13).

In recent years, PCR has proved to be a rapid, sensitive, and specific method for the detection of a variety of parasites in a number of different clinical materials (22). Several groups have developed PCR assays for the detection of *Leishmania* infection (8, 18, 19, 23, 24). Even though it was shown that PCR for the detection of *Leishmania* infections is applicable to a wide variety of clinical materials (15), the value of different materials for the molecular biological diagnosis of VL remains to be established.

We report here the results of an extensive comparison of *Leishmania* PCR and Giemsa-stained smear microscopy of lymph node and bone marrow aspirates obtained from patients proven to have VL, patients suspected of having VL (VL suspects), patients who previously had VL (ex-VL patients), patients with post-kala-azar dermal leishmaniasis (PKDL), and healthy controls during an epidemiological study in an area where VL is endemic. Since the performance of lymph node or bone marrow aspiration is inconvenient for the patient, we also examined whether the use of PCR with blood from a finger prick may be suitable for the detection of VL.

MATERIALS AND METHODS

Study area and patients. This study was carried out in eastern Sudan in the villages of Um Salala and Moshra Koka from April 1994 to November 1995. The total population (2,300 persons) was examined twice yearly. People from other villages who presented during these trips were investigated and managed along the same lines. Demographic and clinical data were recorded for each inhabitant. During each field visit DAT was performed with blood samples from all the inhabitants of the villages as described previously (9). The leishmanin skin test (LST) with *Leishmania infantum* antigen was performed for those people who had tested negative in the previous visit. In patients who had fever, hepatosplenomegaly, and lymphadenopathy, a lymph node aspiration was done. When the lymph node aspirate did not show parasites, a bone marrow aspirate was examined microscopically. Lymph node and bone marrow aspirations were performed as described previously (28). Splenic aspiration and culture were not performed.

Definitions. Patients with confirmed cases of VL were those patients in whom *Leishmania* amastigotes were demonstrated by microscopy of either lymph node or bone marrow specimens. VL suspects were those patients in whom parasites were not demonstrated but who had clinical symptoms and signs suggestive of VL and a positive DAT titer (≥1:6,400). VL suspects were treated for VL. The development of VL or PKDL between one field visit and the next field visit is referred to as a "clinical change." Immunological changes are defined as the conversion to a positive DAT titer (≥1:6,400) or a positive LST result (≥5 mm).

Healthy persons were those villagers who had no history of VL or PKDL, who had no current clinical signs or symptoms of VL or PKDL, who had not gone through any recent immunological changes, and who were generally in good health at the time of sampling.

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TABLE 1. Comparison between PCR and microscopy for the detection of *Leishmania* in lymph node aspirates[a]

PCR result	No. of specimens with the following microscopy result:		
	Positive	Negative	Total
Positive	29	48	77
Negative	3	139	142
Total	32	187	219

[a] $\chi^2 = 37.96$; $P < 0.001$.

Collection of material for PCR. Finger-prick blood from patients confirmed to have VL, VL suspects, ex-VL patients, individuals with PKDL, and healthy persons from the two villages was collected on Whatman no. 3 filter paper. In addition, 0.5 ml of peripheral blood was collected in tubes containing EDTA, and the tubes were stored at −20°C until use. About 25 μl of lymph node or bone marrow aspirate was collected on Whatman no. 3 filter paper. Each filter paper sample was stored in a separate plastic bag at −20°C.

As a negative control we used blood from healthy Dutch volunteers. Part of this blood was spiked with 10^5 *L. donovani* promastigotes ml^{-1} for use as a positive control in the PCR.

DNA isolation. DNA was isolated as described previously (15). Briefly, whole EDTA-treated blood was incubated overnight at 60°C in an equal volume of lysis buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.4], 10 mM EDTA; 1% [vol/vol] Triton X-100, 200 μg of proteinase K per ml). The samples were then subjected to phenol-chloroform extraction, precipitated with ethanol, and redissolved in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.5]).

Blood spots, lymph node aspirates, and bone marrow aspirates on filter papers were placed between two sheets of clean paper and holes were punched out with a paper puncher. After each sample was obtained a clean sheet of paper was punched 10 to 12 times in order to prevent DNA contamination from one sample to the next. Two punched out holes (containing approximately 15 μl of aspirate or blood) were placed in 250 μl of lysis buffer. The DNA was extracted as described above.

In every batch of 34 patient samples, 4 positive and 10 negative controls were randomly included to check for contamination and inhibition.

PCR amplification. The PCR amplification method described previously by Meredith et al. (15) was used, with minor modifications. Five microliters of isolated DNA was added to 45 μl of a PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 250 μM (each) deoxynucleoside triphosphate, 500 μM dUTP, 0.5 U of *Taq* polymerase, 0.5 U of uracil nucleotide glycosylase, 100 pmol of primer 174 (5′-GGTTCCTTTCCTGATTTACG-3′), and 100 pmol of primer 798 (5′-GGCCGGTAAAGGCCGAATAG-3′).

Samples were preincubated at 50°C for 5 min, followed by initial denaturation at 94°C for 10 min and 38 cycles consisting of denaturation at 94°C for 75 s, annealing at 60°C for 1 min, and extension at 72°C for 2 min.

Amplification reactions were visualized on a 2% agarose gel, and a 100-bp DNA ladder (Pharmacia, Uppsala, Sweden) was used as a marker. Samples were scored as positive when a PCR product of 560 bp could be detected.

Statistical analysis. McNemar's chi-square test (contingency tables) was used for statistical analysis.

RESULTS

Positive and negative controls. We have tested 492 clinical samples, 60 positive controls, and 90 negative controls. All negative controls were negative by PCR, whereas all positive controls were positive. One hundred three randomly chosen clinical samples which were negative by the *Leishmania* PCR were tested by using the human β-globin primers (4). All samples showed a 150-bp band specific for β-globin, indicating that none contained factors inhibitory to the PCR.

Lymph node aspirates: microscopy versus PCR. Lymph node aspirates were subjected to PCR and microscopy. A comparison of the results is given in Table 1. PCR detected *Leishmania* isolates in significantly more lymph node aspirates than microscopy ($P < 0.001$). Of the 48 lymph node samples with a positive PCR result but a negative microscopy result, 5 were from patients with confirmed VL on the basis of microscopy of a bone marrow aspirate, 12 were from VL suspects, 7 were from people who had been treated for VL in the past, and 10 were from persons diagnosed as having PKDL. Of the 14 remaining positive patients without immunological or clinical indications of *Leishmania* infection at the time of sampling, 11 were available for follow-up. Five showed conversion by LST and/or DAT within 12 months after the date of sampling indicating *Leishmania* infection. PCR was negative for three samples that were positive by microscopy. Two of these were from people who had received VL treatment in the past. The other one was from a person who showed no clinical or immunological changes at follow-up. For 139 individuals with clinical signs compatible with VL (fever and splenomegaly), both microscopy and PCR were negative. The vast majority of these patients proved to have malaria due to chronic *Plasmodium falciparum* infection.

TABLE 2. Comparison between PCR and microscopy with materials obtained from different patient groups

Patient group and clinical material	No. of samples	No. (%) of samples with the following result:	
		Microscopy positive	PCR positive
Confirmed VL			
Peripheral blood	47	Not done	33 (70)
Lymph node aspirate	38	31 (81.6)	33 (86.8)
Bone marrow aspirate	13	12 (92.3)	13 (100)
VL suspects[a]			
Peripheral blood	11		2 (18.8)
Lymph node aspirate	23		12 (52.2)
Bone marrow aspirate	12		8 (66.7)

[a] Lymph node and bone marrow microscopy negative.

As Table 2 indicates, PCR and microscopy did not differ significantly ($P > 0.05$) in the detection of *Leishmania* in lymph node aspirates from patients with confirmed VL. However, Table 2 also indicates that by PCR of lymph node material, we could demonstrate *Leishmania* DNA in 12 of 23 individuals with suspected VL who were, by definition, all negative by microscopy.

Bone marrow aspirates: microscopy versus PCR. Bone marrow aspirates were subjected to PCR and microscopy. A comparison of the results is given in Table 3. PCR detected *Leishmania* in significantly more bone marrow aspirates than microscopy ($P < 0.001$). PCR was positive for 20 patients who were negative by microscopy. Of these 20, 5 were diagnosed as having confirmed VL on the basis of microscopy positivity after repeated testing of lymph node aspirates, 14 were VL suspects, and 1 had PKDL.

By PCR, *Leishmania* DNA was demonstrated in bone marrow material from all 13 patients with confirmed cases of VL

TABLE 3. Comparison between PCR and microscopy for the detection of *Leishmania* in bone marrow aspirates[a]

PCR result	No. of specimens with the following microscopy result:		
	Positive	Negative	Total
Positive	12	20	32
Negative	0	28	28
Total	12	48	60

[a] $\chi^2 = 18.05$; $P < 0.001$.

TABLE 4. Comparison between *Leishmania* PCR results with peripheral blood collected on Whatman no. 3 filter paper and in tubes containing EDTA[a]

Result for blood in EDTA	No. of specimens of blood on filter paper with the following result:		
	Positive	Negative	Total
Positive	17	4	21
Negative	16	55	71
Total	33	59	92

[a] $\chi^2 = 6.05$; $P < 0.02$.

(Table 2) and 8 of 12 patients suspected of having VL (Table 2).

PCR of peripheral blood: comparison between blood in EDTA and blood on filter paper. It was previously described that either blood in EDTA or dried blood spots may be used in the PCR (16, 17). We first compared the influence of these two methods of storage on PCR. Table 4 indicates that *Leishmania* DNA was detected significantly more often in blood samples collected on Whatman no. 3 filter paper than in blood samples in EDTA ($P < 0.02$). Sixteen blood samples in EDTA were negative by PCR, even though the corresponding filter paper samples were found to be positive by PCR. To exclude false-negative PCR results due to a high concentration of inhibitory factors, these 16 samples were subjected to a second PCR at a higher dilution. Only 1 of 16 samples in EDTA was positive in the second PCR.

By PCR of blood on filter paper (Table 2), *Leishmania* DNA was detected in 33 of 47 (70%) patients with confirmed VL and in blood samples from 2 of 11 VL suspects. PCR of blood on filter paper was negative for 49 samples from healthy villagers.

DISCUSSION

Lymph node aspiration is a safe procedure; the sensitivity of microscopic examination of smears varies from 78% (21) to 58.3% (29), while the sensitivities of microscopy of splenic and bone marrow aspirates were 96.4 and 70.2%, respectively (29). The present study indicates that PCR of lymph node and bone marrow aspirates is more often positive than microscopy (Tables 1 and 3). For patients with confirmed cases of VL, there was no significant difference between microscopy and PCR (Table 2).

The main advantage of using PCR is for VL suspects. *Leishmania* DNA was demonstrated in more than 50% of these patients, thereby confirming the clinical diagnosis. The finding of *Leishmania* DNA in samples from VL suspects, ex-VL patients, PKDL patients, and persons with high DAT titers and enlarged spleens, both of which are indicators of present or past *Leishmania* infection, indicates that PCR is a very sensitive technique that is capable of detecting low levels of *Leishmania*. It has previously been shown that *Leishmania* PCR with the 18S rRNA gene as a target is highly specific (24), which makes the occurrence of false-positive results very unlikely.

We also found positive PCR signals in lymph node aspirates from six persons who had no symptoms of *Leishmania* infection either at the time of sampling or during follow-up. It may be that these people went through a transient infection with *Leishmania*, analogous to the transient infections found in leprosy (11), and that the patient's immune system was able to eliminate the parasite. Another factor to keep in mind is that most people who get *Leishmania* infection go through a subclinical infection and do not get active VL (3). In three microscopically positive lymph node samples, PCR gave false-negative results. All of the observations presented above indicate that the significance of positive or negative PCR results should always be judged carefully against clinical data.

Dried blood spots are easy to store and to handle and were previously shown to be suitable for the detection of *P. falciparum* (14) and human immunodeficiency virus (27) by PCR. Positive PCR results were more frequently found with blood samples on filter paper than samples stored in EDTA.

Even though with microscopy the chances of finding *Leishmania* in peripheral blood from Sudanese patients are small (12), we found that with PCR it was possible to detect *Leishmania* DNA in the peripheral blood of 70% of all patients confirmed to have VL. Only 19% of the VL suspects were found to be positive. In a study of peripheral blood samples from a group of Indian VL patients and VL suspects, 93% of the VL patients and 72% of the VL suspects were found to be positive by PCR (1). These percentages are substantially higher than the ones that we found. However, we used only target DNA equivalent to 1.5 μl of blood in our PCR, whereas Adhya et al. (1) used the equivalent of 200 μl of blood. Alternatively or additionally, the different results either may be due to the fact that in Indian VL the parasites are more frequently found in the blood than in Sudanese VL (5) or may be due to the fact that the Indian samples were taken at a later stage of the disease, when the level of parasitemia may be higher.

In a recent study, PCR was positive for all 12 blood samples obtained from patients with human immunodeficiency virus and *Leishmania* coinfections (6). In contrast, when Hassan et al. (10) performed PCR with peripheral blood from immunocompromised VL patients, they found only 45% of all samples to be positive. However, most of these patients had been treated in the past, which may explain the lower percentage of positive samples.

In conclusion, PCR is a more sensitive method than microscopy for the detection of *Leishmania* in lymph node and bone marrow aspirates. PCR is especially useful for the confirmation of cases of suspected VL. Because lymph node, bone marrow, and splenic aspiration is inconvenient or even dangerous for the patient, finger-prick blood, which is easy to obtain, may be used for the initial PCR screening of people suspected of having VL. If the PCR of blood is negative, one should perform PCR of lymph node and/or bone marrow material, because PCR on these materials is more often positive.

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