JOURNAL OF CLINICAL MICROBIOLOGY, June 1998, p. 1621–1624 Vol. 36, No. 6
0095-1137/98/$04.00+0

Use of PCR for Diagnosis of Post-Kala-Azar Dermal Leishmaniasis

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Received 13 November 1997/Returned for modification 8 February 1998/Accepted 15 March 1998

Microscopy and PCR were compared for use in the diagnosis of post-kala-azar dermal leishmaniasis (PKDL) in 63 patients. Aspirates of lymph nodes (samples from 52 patients), skin (23 samples), and bone marrow (18 samples) were used. For 11 patients lymph node aspiration could be repeated 6 months after they recovered from PKDL. During active PKDL, PCR was positive for 42 of 52 (80.8%) lymph node aspirates and 19 of 23 (82.7%) skin aspirates, whereas microscopy was positive for only 9 of 52 (17.3%) lymph node aspirates and 7 of 23 (30.4%) skin aspirates. PCR was always positive when parasites were seen by microscopy. When the results obtained with lymph node and skin aspirates from the same patient ($n = 16$) were compared, there was complete agreement. Bone marrow samples were negative by microscopy and PCR for 16 patients and positive by both methods for 1 patient; for one sample only the PCR was positive. PCR confirmed the co-occurrence of visceral leishmaniasis and PKDL in one patient and confirmed the suspicion of this co-occurrence in the other patient. After recovery, no parasites were found by microscopy, but 2 of 11 (18.2%) samples were still positive by PCR. Thirty negative controls were all found to be PCR negative, and 15 positive controls were all PCR positive. Cross-reactions with *Mycobacterium leprae* could be ruled out. In conclusion, PCR with inguinal lymph node or skin aspirates is suitable for confirming the clinical diagnosis of PKDL. In some patients, lymph node aspirates are probably preferred because aspiration of material from the skin may leave scars.

Post-kala-azar dermal leishmaniasis (PKDL) is a dermatropic form of leishmaniasis. It is found in areas where *Leishmania donovani* is the causative agent of visceral leishmaniasis (VL) (19). PKDL develops after the apparent cure of VL and is characterized by the occurrence of macules, papules, or nodules on the skin of the face, limbs, or trunk (4). Although it was reported in the past that PKDL is far less common in Africa than on the Indian subcontinent (16), Zijlstra et al. (23) found that PKDL developed in more than half of the cured VL patients in one study in eastern Sudan.

Methods for the diagnosis of VL often lack sensitivity or specificity for the diagnosis of PKDL: (i) the number of parasites in skin smears and biopsy specimens is often low or zero, thus requiring prolonged searches by routine microscopy (4, 5); (ii) serological tests are often positive due to the past occurrence of VL (6, 7); (iii) the leishmanin skin test (LST) may or may not be positive (16); and (iv) cultures are often not positive and, in the rural region where VL is endemic, cultures are prone to contamination and often remain negative (12).

PKDL, in particular its nodular form, may easily be confused with a number of dermatological conditions, of which leprosy is the most important (4).

PKDL patients may be an important source of infection in the transmission of VL (1, 18, 21).

In view of the low parasite load in clinical samples from PKDL patients in combination with the possibility of confusion with other skin disorders, particularly leprosy, there is an urgent need for more sensitive diagnostic techniques. An increased sensitivity of microscopy was reported when an immunoperoxidase technique with an anti-*L. donovani* monoclonal antibody were used for the detection of the parasite in skin biopsy specimens from PKDL patients (8). Recently, several groups have shown that PCR is both a sensitive and a specific method for the detection of *Leishmania* DNA in a variety of clinical samples (2, 10, 13, 15, 19, 20).

Here we describe the application of PCR as a tool for use in the diagnosis of PKDL in comparison with the use of microscopy and clinical data in a longitudinal study in eastern Sudan, where PKDL is seen in 56% of all patients cured of VL (23). Leprosy also occurs in this area.

MATERIALS AND METHODS

Study area. This investigation was carried out in eastern Sudan in the villages of Um-Salala and Moshra Koka between April 1994 and April 1996. The total population (2,300 people) was screened twice yearly to study the epidemiology of VL. People from other villages who presented during these screenings were investigated and were managed along the same lines as the populations of the two villages under investigation.

Clinical examination. Demographic and clinical data were recorded for each inhabitant as reported previously (22). PKDL was diagnosed on clinical grounds and the aspect of the rash (macular, nodular, or maculopapular), its distribution, and history of spread (starting on the face around or on the nose, spreading to the rest of the face, and then spreading to the shoulders, trunk, and upper arms) and its relation to VL (23).

All patients had a recent history of confirmed or suspected VL and had received treatment for VL. Patients with confirmed 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 strongly suggestive of VL plus a positive serological test.

Sixty-three patients with PKDL were examined between April 1994 and April 1996. (i) Lymph node aspirates were taken from 23 patients; (ii) slit skin smears were obtained from 7 patients; (iii) bone marrow aspiration was performed for

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TABLE 1. Comparison of PCR and microscopy for detection of *Leishmania* in lymph node aspirates taken during PKDL

PCR result	No. of samples with the following microscopy result:		
	Positive	Negative	Total
Positive	9	33	42
Negative	0	10	10
Total	9	43	52

4 patients; (iv) bone marrow and lymph node aspirates were obtained from 13 patients; (v) lymph node aspiration was performed and slit skin smears were obtained from 15 patients; and (vi) all three procedures were performed for 1 patient.

Inguinal lymph node aspiration was performed as described by Zijlstra et al. (22) for 52 patients; for 11 of these patients this could be repeated 6 months after they recovered from PKDL. For 23 patients with lesions on the trunk or arms, slit skin smears were made. A tiny amount of material was obtained by scraping the skin at the edge of the lesion with a vaccinostyle or needle. We did not attempt to obtain skin scrapings from facial lesions because this procedure may leave scars, the procedure is painful, and patients object. Smears were stained with Giemsa stain and were examined microscopically. For 18 patients with fever and splenomegaly, bone marrow aspiration was also performed to exclude concurrent VL. For 16 patients both skin smears and lymph node material could be examined at the same time.

For 46 of 63 patients, the LST with *Leishmania infantum* antigen (from the Istituto Superiore de Sanità, Rome, Italy) was done and the result was read after 48 h. For the remaining 17 patients either the LST was already positive at a previous screening or these patients did not show up after 48 h.

Collection of PCR material. A total of 20 to 30 μl of skin, lymph node, or bone marrow aspirate from PKDL patients 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.

To exclude possible cross-reactions, we subjected two *Mycobacterium leprae* PCR-positive skin aspirate specimens from leprosy patients and 125 pg of *M. leprae* DNA to the *Leishmania* PCR. Moreover, five skin aspirate specimens from five microscopically positive PKDL patients and 1 ng of *Leishmania* DNA were tested in the *M. leprae* PCR; this PCR was performed as described by De Wit et al. (3).

DNA isolation. Skin, lymph node, or bone marrow aspirates on filter papers were put between two sheets of clean paper, and samples (punches) were punched out with a paper puncher. After punching of each sample, a clean sheet of paper was punched 10 to 12 times in order to prevent DNA contamination from one sample to the next (13). The same paper puncher was used throughout the investigation, and for every batch of 35 patient samples, 10 negative controls and 3 positive controls were randomly distributed to examine for contamination and inhibition.

DNA was isolated as described previously (10, 13). Two punches (equaling approximately 15 μl of aspirate) were placed in 250 μl 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), and the mixture was incubated overnight at 60°C. 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]).

PCR amplification. PCR amplification was carried out as described previously (13). Briefly, 5 μl 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 $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 μM, 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 789 (5′-GGC CGGTAAAGGCCGAATAG-3′).

The 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.

The amplification reactions were visualized on a 2% agarose gel; 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.

Dilution series. Differences in the *Leishmania* DNA concentrations in lymph node aspirates from VL patients and PKDL patients were detected by making 10-fold dilution series of DNA from the lymph node samples and subjecting these to PCR as described above.

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

TABLE 2. Comparison of PCR and microscopy for detection of *Leishmania* in lymph node aspirates taken 6 months after recovery from PKDL

PCR result	No. of samples with the following microscopy result:		
	Positive	Negative	Total
Positive	0	2	2
Negative	0	9	9
Total	0	11	11

RESULTS

Control experiments. We have tested a total of 104 clinical samples from PKDL patients, 15 positive controls, and 30 negative controls. All negative controls were negative by PCR, whereas all positive controls were positive. Control samples containing *M. leprae* were negative by the *Leishmania* PCR, as were the samples containing *Leishmania* by the *M. leprae* PCR. In conclusion, no contamination, inhibition, or cross-reaction was detected.

Lymph node aspirates taken during PKDL. Lymph node aspirates from 52 PKDL patients were subjected to PCR and microscopy. A comparison of the results is given in Table 1. PCR detected *Leishmania* in lymph node aspirates significantly more often than microscopy did ($P < 0.000001$).

To investigate whether there is a difference in the *Leishmania* DNA concentration in lymph node aspirates from VL patients and PKDL patients, 10-fold dilution series of DNA from lymph node samples from 6 VL patients and 8 PKDL patients were made. We found that, on average, lymph node aspirates from VL patients contained 100 times more *Leishmania* DNA than aspirates from PKDL patients (data not shown).

LST data were available for 46 of the 52 patients whose lymph node aspirates were tested. Twenty-seven of 29 LST-negative PKDL patients were positive by PCR, whereas only 10 of 17 LST-positive PKDL patients had a positive PCR result ($P = 0.0078$).

Lymph node aspirates taken 6 months after PKDL. The data in Table 2 indicate that there was no significant difference in the results between PCR and microscopy with lymph node aspirates taken 6 months after the patients recovered from PKDL ($P > 0.05$).

Slit skin smears taken during PKDL. Slit skin smears from PKDL patients were subjected to PCR and microscopy. A comparison of the results is given in Table 3. PCR detected the presence of *Leishmania* significantly more often than microscopy did ($P = 0.001$).

Bone marrow aspirates taken during PKDL. Bone marrow aspirates from 18 PKDL patients were subjected to PCR and microscopy. One of 18 samples was positive by microscopy and PCR, and one sample was PCR positive and microscopy neg-

TABLE 3. Comparison of PCR and microscopy for detection of *Leishmania* in slit skin smears from PKDL patients

PCR result	No. of samples with the following microscopy result:		
	Positive	Negative	Total
Positive	7	12	19
Negative	0	4	4
Total	7	16	23

ative. The remaining 16 samples were negative by both methods.

Comparison of PCR with lymph node and slit skin smears taken from the same patient. For 16 patients a lymph node aspirate and skin smear material could concurrently be examined by PCR. For 13 of these 16 patients the PCR of a lymph node aspirate and slit skin smear were both positive, whereas for the other 3 patients PCR of both types of samples was negative.

Comparison of PCRs with material from bone marrow, lymph node, and skin taken from PKDL patients. For PKDL patients, PCR detects *Leishmania* significantly more often in slit skin smear and lymph node aspirates than in bone marrow aspirates ($P < 0.000001$). The results in Tables 1 and 3 reveal that there is no significant difference ($P = 1$) between the results of PCR with lymph node aspirates and slit skin smears. When comparing the results of PCRs with slit skin smear and lymph node aspirates taken from the same PKDL patient, we found a 100% agreement (see above).

DISCUSSION

PKDL frequently occurs after the treatment of active VL in India (18) and Sudan (23) but occurs less frequently in Kenya (11). Sporadic cases have been reported from China (9) and the Mediterranean area, where *L. infantum* is the causative agent of VL. Recently, the first case of PKDL in a human immunodeficiency virus-infected person from Spain has been reported (17).

PKDL can easily be confused with a number of skin disorders. Clinical similarities between the skin lesions of PKDL and leprosy may lead to diagnostic difficulties, especially in areas where the diseases are coendemic (4). The microscopic detection of parasites in PKDL lesions is impeded by the limited sensitivity of microscopy (4, 5). Clinical diagnosis may be problematic for patients in whom there is a long interval between active VL and the development of PKDL.

We have evaluated the use of *Leishmania* PCR with different clinical materials for the diagnosis of PKDL. First, we investigated the specificity of the *Leishmania* PCR with *M. leprae* DNA and samples from leprosy patients. No cross-reactivity was found.

PCR and microscopy were both negative for the majority of the bone marrow aspirates taken from PKDL patients. Of the two patients who were PCR positive, one was diagnosed as having a para-kala-azar dermal leishmaniasis case (the simultaneous occurrence of VL and PKDL), and in the other patient the co-occurrence of VL and PKDL was suspected and was confirmed by PCR.

In a previous study, we found all 13 bone marrow aspirate specimens from VL patients to be positive by PCR (13). In contrast, during the present study we found only 2 of 18 bone marrow aspirate specimens from PKDL patients to be positive by PCR. This significant difference ($P < 0.00001$) is probably due to the fact that the parasites are cleared from the bone marrow as cell-mediated immunity develops during or after treatment of VL (24) and apparently are no longer present during PKDL.

It was shown previously that microscopy of lymph node aspirates from PKDL patients is of limited sensitivity (23). Here we report that PCR with these lymph node aspirates is a sensitive method by which parasites can be detected more frequently than by microscopy ($P < 0.000001$).

Indian researchers found that LST-negative PKDL patients were more likely to be positive by microscopy than LST-positive PKDL patients (16). By PCR, we also found that LST-negative persons were positive by PCR (27 of 29) significantly more often than LST-positive individuals (10 of 17) ($P = 0.0078$). For LST-positive patients the cellular immune response is better developed and will kill some of the parasites. Therefore, one may expect that LST-positive patients generally have lower parasite levels than LST-negative patients and therefore more often tend to be negative by PCR.

When studying VL patients, we found 33 of 38 lymph node aspirate specimens to be positive by PCR (13). This is not significantly different from the results obtained with lymph node aspirates from PKDL patients (42 of 52 of whom were positive). On the other hand, comparison of microscopic detection of *Leishmania* in lymph node aspirates from VL patients (31 of 38 of whom were positive) and PKDL patients (9 of 52 of whom were positive) differed significantly ($P < 0.0000001$). The results of the control experiments together with the results of PCR and microscopy for VL patients indicate that the PCR results with lymph node aspirates from PKDL patients are not due to false-positive results. Using 10-fold dilution series of DNA from isolates from VL and PKDL patients, we found that lymph node aspirates from PKDL patients contained relatively low amounts of *Leishmania* DNA compared to the amounts in the lymph node aspirates from VL patients. The low concentration of the parasite may also explain the discrepancy between PCR and microscopy results for lymph node aspirates from PKDL patients.

Comparison of PCR and microscopy results for lymph node aspirates taken 6 months after recovery from PKDL showed that there was no significant difference, and the results of both tests tended to be negative, as may be expected. However, by PCR we still found two lymph node aspirates to be positive. Since PCR in patients who previously had VL is predictive of the future development of PKDL (14), these patients should be monitored closely for a recurrence of PKDL.

In PKDL patients *Leishmania* parasites are scanty and difficult to demonstrate during the stage when the skin lesions are hypopigmented, but their numbers gradually increase with the progress toward the nodular stage of the disease (5). In this study, *Leishmania* was detected in significantly more skin aspirates taken from PKDL patients by PCR than by microscopy. However, we found no significant correlation between the severity or distribution of the disease and the percentage of PCR-positive patients (data not shown). This is probably due to the high number of PCR-positive patients.

There was no significant difference in the PCR results obtained with lymph node aspirates (42 of 52 were positive; sensitivity, 81%) and skin aspirates (19 of 23 were positive; sensitivity, 83%) from PKDL patients before treatment. This was confirmed when we compared PCR with pairs of lymph node and skin aspirates taken from 16 patients.

In conclusion, we have demonstrated that PCR with either lymph node or skin aspirates is more sensitive than microscopy for the diagnosis of PKDL; this may be due to the often low concentration of the parasite. For the diagnosis of PKDL one can use either lymph node or skin material. Since PKDL is often confined to the face, one may prefer to take lymph node material rather than skin material because the aspiration procedure may leave scars.

ACKNOWLEDGMENTS

This work was financially supported by EU grant TS3*-CT93-0245.

We thank Eltahir Fadul, Ahmed Abdel Majid, and Khamis Abdu for collecting samples in the field and Sjoukje Kuijper for performing the *M. leprae* PCR. We gratefully acknowledge the logistical support of the MSF Holland office in Khartoum, Sudan.

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