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# Use of PCR on lymph-node samples as test of cure of visceral leishmaniasis

By O. F. OSMAN

*Institute of Endemic Diseases, University of Khartoum, Sudan*

P. A. KAGER

*Department of Infectious Diseases, Tropical Medicine and AIDS,  
Academic Medical Centre (AMC), University of Amsterdam, Amsterdam, The Netherlands*

E. E. ZIJLSTRA, A. M. EL-HASSAN

*Institute of Endemic Diseases, University of Khartoum, Sudan*

AND L. OSKAM\*

*Department of Biomedical Research, Royal Tropical Institute (KIT),  
Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands*

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When the polymerase chain reaction (PCR) was used to test lymph-node aspirates from 35 patients from eastern Sudan, who had had visceral leishmaniasis but were believed cured, leishmanial DNA was detected in samples from 14 of the patients. There were no significant differences between the PCR-positives and -negatives in terms of age, sex, spleen size, malaria status or presence of anti-*Leishmania* antibodies. However, PCR was more often positive in the patients who tested negative by the leishmanin skin test (LST) than in those who gave positive skin tests. Moreover, patients with a positive PCR and a negative LST converted more often to LST positivity than those with a negative PCR and a negative LST. The most important finding was that, during follow-up, eight (57%) of the PCR-positives, but none of the 21 negatives, developed post-kala-azar dermal leishmaniasis (PKDL). In conclusion, PCR-based testing of lymph-node aspirates after treatment may be used as a prognostic marker for the future development of PKDL and may be useful in the follow-up of patients.

Visceral leishmaniasis (VL) is caused by protozoan parasites of the *Leishmania donovani* s.l. complex. During the active disease, parasites may be detected in spleen, bone-marrow or lymph-node aspirates (Zijlstra *et al.*, 1992). Although the leishmanin skin test (LST) is characteristically negative at this stage of the disease (Manson-Bahr, 1961), serological assays, such as direct agglutination tests (DAT), may demonstrate anti-*Leishmania* antibodies in serum samples from >95% of all HIV-negative VL patients (Meredith *et al.*, 1995).

After successful treatment, >80% of ex-

VL patients convert to a positive LST within 2 years (Manson-Bahr, 1961) but seroconversion to a DAT titre considered a negative result may take as long as 7 years (Hailu, 1990). In areas where *L. donovani* s.s. is the causative agent of VL, such as the Indian peninsula and East Africa, a proportion of the ex-VL patients will develop a dermatropic form of leishmaniasis called post-kala-azar dermal leishmaniasis (PKDL) (WHO, 1990).

Although it has been shown previously that PCR can be used to detect low levels of *Leishmania* DNA (Van Eys *et al.*, 1992), the value of PCR in diagnosis of infection with *Leishmania* and follow-up remains to be established. The present investigation, a prospec-

\* Author to whom correspondence should be addressed.  
E-mail: bo@mail.support.nl; fax: +31 20 6971841.

tive study of 35 ex-VL patients from eastern Sudan, was an attempt to discover the factors which may be predictive of the future development of PKDL.

## PATIENTS AND METHODS

### Patients

As part of a long-term study of the epidemiology of VL in eastern Sudan, the population of the village of Um Salala is examined twice yearly: a short history is taken, a physical examination is performed, finger-prick blood is taken for the detection of anti-*Leishmania* antibodies by DAT (Harith *et al.*, 1988), the LST is performed on everybody who was negative in the previous visit and, on indication, aspirates are taken from inguinal lymph nodes or, if needed, bone marrow (Zijlstra *et al.*, 1994, 1995). Some of the blood from those suspected of having malaria is used to make thick films and these are checked for malarial parasites.

### Definition of Study Group

For the purposes of the present study, ex-VL patients were defined as those who had either had parasitologically confirmed VL or who had been diagnosed as suspected cases of VL (without parasitological confirmation), who were positive by the DAT and who had responded to specific treatment.

Between April 1994 and April 1996, the results of clinical examination, DAT and LST were collected for 35 ex-VL patients from whom lymph-node aspirates were collected after treatment and apparent cure. These patients were all followed for 6 months–2 years (i.e. the remainder of the study period). Of the 35, 23 had been treated < 1 year before providing lymph-node aspirates, three had been treated 2 years before and the remaining nine had been treated  $\geq 3$  years prior to sampling. Prior to the aspirations, seven had been diagnosed as having PKDL on clinical grounds and eight had a history of PKDL.

### PCR Samples

A small subsample of each lymph-node aspirate was smeared, fixed, stained with Giemsa

and examined for amastigotes. Another subsample of each aspirate, 20–30  $\mu\text{l}$ /patient, was collected on filter paper (Whatman No. 3) before storage in a plastic bag (one sample/bag) at  $-20^{\circ}\text{C}$ . Blood samples from healthy Dutch volunteers were used both as negative controls and, after addition of  $10^5$  *L. donovani* (MHOM/SD/68/1S) promastigotes/ml, as positive controls.

### DNA Extraction

DNA was isolated as described by Meredith *et al.* (1993). Briefly, a paper punch was used to cut discs from the filter papers on which the aspirates were stored, each paper being held between two sheets of clean paper while punched. After discs were cut for each sample, a clean sheet of paper was punched 10–12 times in order to prevent DNA cross-contamination. Two discs (together equivalent to about 15  $\mu\text{l}$  aspirate) were placed in 250  $\mu\text{l}$  lysis buffer [50 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 1% (v/v) Triton X-100 and 200  $\mu\text{g}$  proteinase K/ml] and incubated overnight at  $60^{\circ}\text{C}$ . The samples were then subjected to phenol/chloroform extraction, ethanol precipitated and redissolved in 50  $\mu\text{l}$  TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 7.5). Positive and negative controls were randomly added to check for contamination and inhibition.

### PCR Amplification

The DNA solution, 5  $\mu\text{l}$ , from each aspirate was added to 45  $\mu\text{l}$  of a PCR mix containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM  $\text{MgCl}_2$ , 250  $\mu\text{M}$  of each dNTP, 500  $\mu\text{M}$  dUTP, 0.5 unit *Taq* polymerase, 0.5 unit uracil nucleotide glycosylase (UNG), 100 pmol primer 174 (5' GGTTCCCTTTCCTGATTTACG 3') and 100 pmol primer 789 (5' GGCCG-GTAAAGGCCGAATAG 3'). Samples were pre-incubated at  $50^{\circ}\text{C}$  for 5 min followed by initial denaturation at  $94^{\circ}\text{C}$  for 10 min and 38 cycles, each consisting of denaturation at  $94^{\circ}\text{C}$  for 75 s, annealing at  $60^{\circ}\text{C}$  for 1 min and extension at  $72^{\circ}\text{C}$  for 2 min.

Amplification reactions were separated on 2% agarose containing 0.1  $\mu\text{g}$  ethidium bro-

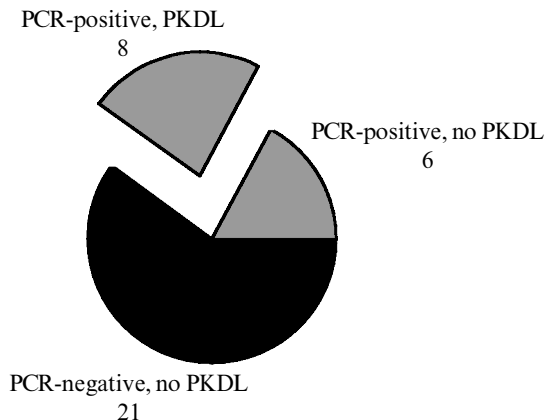


Fig. 1. The results of PCR-based tests for leishmanial DNA in lymph-node samples from 35 ex-visceral-leishmaniasis patients and the development or not of post-kala-azar dermal leishmaniasis (PKDL) during follow-up (6 months–2 years).

mide/ml; a 100-bp DNA ladder (Pharmacia) was used as a marker. Samples were scored 'positive' when a PCR product with a size of 560 bp could be detected.

#### Statistical Analysis

McNemar's  $\chi^2$  test was used throughout.

## RESULTS

#### Microscopy and PCR

Although amastigotes were not seen in the smears of any of the 35 aspirates, leishmanial DNA was detected by PCR in 14 of them (see Fig. 1). Most (eight) of the 14 PCR-positive patients, but none of the 21 PCR-negatives, developed PKDL during follow-up ( $P = 0.0001$ ).

#### LST and DAT

The LST results are presented in Fig. 2 for 33 of the 35 patients (one failed to attend at the time of the inoculations and another did not return for his test result to be read).

Complete DAT data are available for 32 of the 35 patients. Of these 32 patients, 29 (91%) were DAT positive. There was no significant difference between the group of PCR-positive

patients and that of PCR-negative patients as far as DAT positivity is concerned.

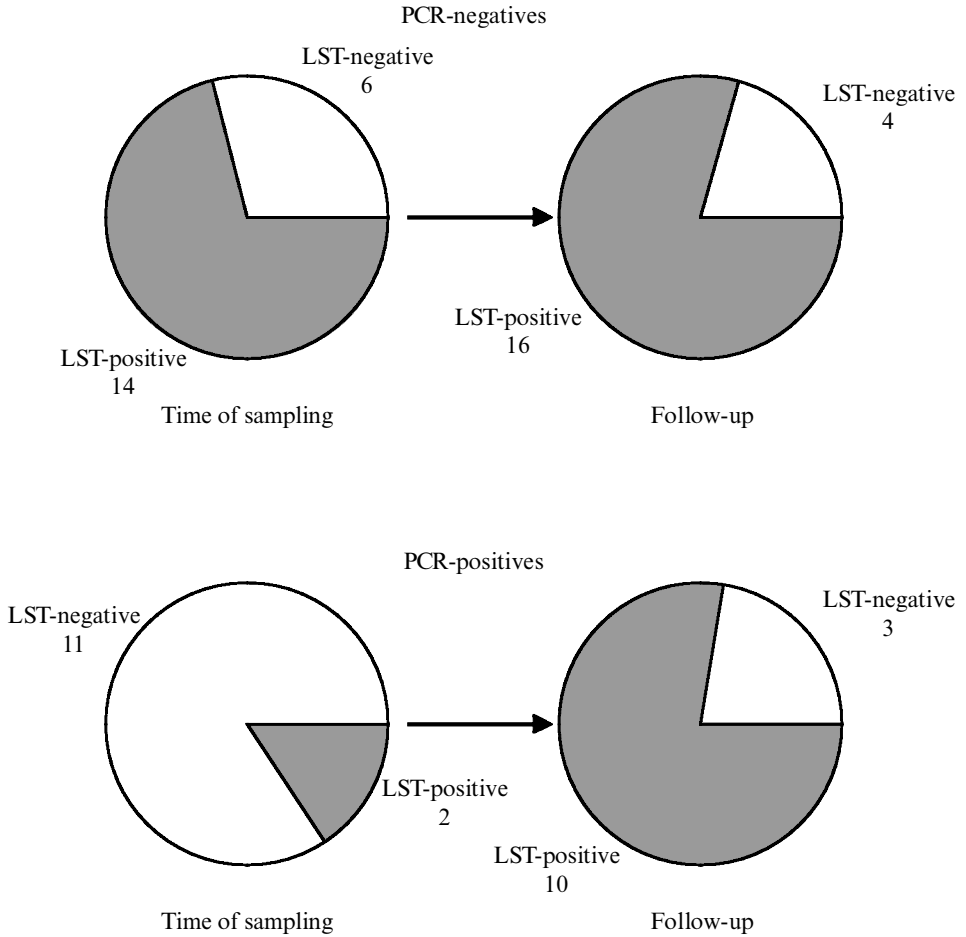
#### Other Parameters

The 35 patients comprised 14 males and 21 females, with a mean age of 7.7 years at the time of sampling. Twenty-five were checked for the presence of malarial parasites in the blood and 14 (56%) of these tested positive. The spleen was palpable in 26 persons (reaching a mean of 3.4 cm below the coastal margin). The PCR-positive and PCR-negative groups were similar in terms of age, malarial infection and splenomegaly.

## DISCUSSION

Although there have been many studies on ex-VL patients, focused, for example, on sero-epidemiology (Manson-Bahr, 1961; Zijlstra *et al.*, 1991), therapeutic efficacy (Van der Poll *et al.*, 1995) or antibody profiles (Ghosh *et al.*, 1995), little is known about the factors which may be used to predict the development of relapses or of PKDL in such patients.

Fifteen of the present 35 patients had had PKDL prior to sample taking: three were PCR-positive and 12 PCR-negative



**Fig. 2.** The results of leishmanin skin tests (LST) of 20 PCR-negative and 13 PCR-positive ex-visceral-leishmaniasis patients at the time of sampling and at the first follow-up visit, 6 months later.

( $P > 0.05$ ). Of the three ex-PKDL patients who tested positive by positive PCR, one was treated for VL 5 years prior to sampling, with PKDL of unknown duration occurring shortly after treatment. At the time of sampling during the present study, this patient's rash had cleared for at least 3 years and the LST gave a positive result. One of the other two ex-PKDL patients who were PCR-positive had had PKDL for 1 month and the other had had PKDL for 18 months. In both, the rash had cleared shortly before sampling and the

LST was negative at the time of the sampling described here; both were found to have converted to a positive LST in the next survey, 6 months later.

None of the 35 patients apparently had a relapse of VL but eight patients (23% of the total group and 40% of those who had not had PKDL before) were found to have developed PKDL during the follow-up period. This incidence of PKDL post-treatment is similar to the value of about 20% reported in India (Thakur and Kumar, 1992) but less than the

incidence observed in eastern Sudan within 1 year of VL (56%; Zijlstra *et al.*, 1995).

Hailu (1990) reported that the DAT remains positive in the majority of VL patients for a long time post-treatment. It was therefore no surprise to find that 91% of the present patients had a positive DAT titre.

Seventeen (52%) of the 33 ex-VL patients available for LST analysis gave a positive result. Six months later, the number of LST positives in this group had risen to 27 (82%), indicating that the development of a cellular immune reaction as measured by the LST lags behind the cure of VL.

Because of the low number of PKDL patients, no significant association between DAT titre or LST result and the development of PKDL could be detected.

By using PCR, very low concentrations of *Leishmania* DNA can be detected and the presence of such DNA can often be demonstrated in lymph-node aspirates (but not in bone-marrow aspirates or peripheral-blood samples) from PKDL patients (unpubl. obs.). Lymph node aspirates were therefore tested in the present study.

Microscopy was much less sensitive than the PCR for detecting *Leishmania* in the samples (0% positive *v.* 40%;  $P < 0.0001$ ). The limited sensitivity of microscopy has been reported previously (El-Hassan *et al.*, 1992; Zijlstra *et al.*, 1995). The results of the PCR indicate that *Leishmania* parasites (or at least leishmanial DNA) are present in more than one third of the ex-VL patients, albeit at low levels.

There were no significant differences between PCR-positives and -negatives with regard to age, sex, spleen size, malaria status or DAT positivity (results not shown). However, at the time of sampling, the results of the LST were more often positive in the PCR-negatives than in -positives (Fig. 2;  $P < 0.001$ ). Perhaps the cellular immune response is better developed in the LST-positives and more effective at eradicating the parasites.

At follow-up, the patients who had had a positive PCR and a negative LST 6 months earlier were found to have converted more

often to LST positivity than those who had been negative by PCR and LST (Fig. 2), indicating an active host-parasite interaction among the PCR-positive patients.

The most important finding of the present study was that, of the 15 PCR-positives, eight (57%) developed PKDL during follow-up and three others had already been cured of PKDL. In contrast, none of the 21 PCR-negatives developed PKDL (Fig. 1;  $P = 0.0001$ ), although 12 of these had already had PKDL and been cured prior to sampling (and therefore possibly no longer at risk of developing this condition). These results indicate that any PCR-positive patient who has never had PKDL may do so in the near future and should be closely followed. The results presented in Fig. 1 strongly indicate the presence of live *Leishmania* parasites in at least 57% of the PCR-positive patients. This strengthens the conclusion from the results of the LST: that there is an active host-parasite interaction in the PCR-positives. Since only PCR-positives appear to be at risk of developing PKDL, the testing of lymph-node aspirates from ex-VL patients by PCR may be a useful screen. It is not known if PCR-positive individuals play a role in transmission, as PKDL patients do (WHO, 1990; Thakur and Kumar, 1992). If they are reservoirs of infection then treatment regimens should probably be modified. This aspect of VL epidemiology requires further study.

In conclusion, LST-negative ex-VL patients are more often found to be positive by PCR than LST-positives. Those who have leishmanial DNA in their lymph nodes, as demonstrated by PCR, are much more likely to develop PKDL than those who appear negative by PCR. Further prospective studies have been started to validate these observations and to assess their implications for treatment and follow-up.

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