Mitt. Österr. Ges.
Tropenmed. Parasitol. 16 (1994)
141 - 146

Armauer Hansen Research Institute (AHRI), Addis Ababa, Ethiopia (Director: Dr. D. Frommel) (1)
All Africa Leprosy Rehabilitation and Training Center (ALERT), Addis Ababa, Ethiopia
(Managing Director: Mr. J. N. Alldred) (2)
Department of Tropical Public Health, Harvard School of Public Health, Boston, MA
(Head of Unit: Dr. Dyann Wirth) (3)
Institute for Clinical Microbiology, Univerity of Erlangen-Nürnberg, Erlangen, F. R. G.
(Director: Prof. Dr. M. Röllinghoff) (4)

Detection of Leishmania aethiopica in paraffin-embedded skin biopsies using the polymerase chain reaction

T. Laskay[1, 4], T. L. Miko[1, 2], H. Teferedegn[1], Y. Negesse[1, 2], M. R. Rodgers[3], W. Solbach[4], M. Röllinghoff[4], D. Frommel[1]

Introduction

Cutaneous leishmaniasis (CL) is a serious public health problem in several areas of the world. Current reports indicate that the prevalence of the disease is increasing in many countries (4). One major focus of CL in the Old World is found in Ethiopia where the aetiological agent is *Leishmania aethiopica* (1, 2, 7). At present diagnosis relies on the detection of the parasite in smears or skin biopsy specimens by histopathological examination and/or by in vitro culture. Isolation in cell culture remains difficult and depends on the presence of sufficient numbers of viable amastigotes. The presence of morphologically intact intracellular amastigotes is necessary also for diagnosing the disease by histopathological examination. In *L. aethiopica*-induced chronic localized cutaneous lesions, however, intact parasites are scarce and therefore difficult to detect by in vitro culture or to visualize in smears or sections.

The polymerase chain reaction (PCR) can amplify minute quantities of nucleic acids, and primers which provide specificity for a defined nucleic acid target can be designed. Recently, *Leishmania*-specific oligonucleotide primers have been described (6, 8, 9, 10, 12). By using a set of primers from the *Leishmania* kinetoplast (kDNA) minicircles, PCR could detect DNA corresponding to 0.01 - 1 *Leishmania* promastigotes (10).

In this study we used PCR to detect *Leishmania* DNA in paraffin-embedded skin biopsies of confirmed cases of cutaneous leishmaniasis. In addition, PCR was used for the detection of *L. aethiopica* in skin biopsy specimens from patients who were clinically suspected of having cutaneous leishmaniasis, but for whom histopathology and in vitro culture and failed to detect the parasites.

Materials and methods

Patients

Patients reporting to the Clinic of Dermatolgy of the All Africa Leprosy and Rehabilitation Training Center (ALERT) in Addis Ababa were studied. Seventeen patients with confirmed cutaneous leishmaniasis (CL) were included in the study. All these patients had clinical signs and symptoms consistent with CL, and Leishman-Donovan (LD) bodies were observed in skin biopsies by histological examination. The number of amastigotes observed microscopically varied between those heavily infected (n = 7) and those in which only occasional amastigotes were seen (n = 5).

As negative controls, skin biopsies from patients with other diseases, i. e. leprosy (n = 12), candidiasis (n = 2), Kaposi's sarcoma (n = 3) and psoriasis (n = 1), all living in areas where CL is not endemic, were used.

A second group of patients (n = 40) was included in the study for which the diagnosis of CL was made clinically. These patients presented skin lesions in accordance with *Leishmania aethiopica* infection by dermatological examination. However, in vitro culture and histopathology did not detect *Leishmania* parasites in the biopsy specimens taken from the lesions of these patients.

Histopathological examination

Punch biopsy samples were taken from skin lesions and fixed with FMA (formaldhyde/mercuric chloride/acetic acid, which consisted of 4% (vol/vol) formaldehyde, 2% (wt/vol) $HgCl_2$ and 3% (vol/vol) acetic acid. The fixed specimens were then embedded in paraffin. Stained sections were examined independently by two senior histopathologists (T. L. M. and Y. G.). Only specimens of those patients whose diagnosis was confirmed by both of the pathologists were included in the study.

DNA extraction and PCR

DNA was extracted from the very same paraffin blocks of skin biopsies which were used for the histological examination. Crude DNA was obtained using the direct boiling method (3, 11). Two 30 µm thick sections were cut from each block (new blades were used for each section to avoid possible cross-contamination with *Leishmania* DNA) and deparaffinized with xylene and ethanol. The dried specimes were then crushed with 50 µl of glass powder in 1.5 ml microcentrifuge tubes using glass rods. 100 µl of TE buffer was added and the tubes were placed in a boiling water bath for 10 min. After spinning the samples at 10,000 g for 5 sec, 20 µl samples of the supernatants were used in the PCR reactions. As positive control 10 pg purified *L. aethiopica* DNA was used.

Leishmania-specific oligonucleotide primers 13A [5'-GTGGGGGAGGGGCGTTCT-3'] and 13B [5'-ATTTTACACCAACCCCCAGTT-3'] (10) were used to amplify a 120 bp long fragment of *Leishmania* kinetoplast DNA (kDNA) minicircles. PCR was run in an Eppendorf (Fremont, CA) microcycler. Each 20 µl sample was amplified for 25 repeated cycles of denaturation at 94° C for 1 min, annealing at 50° C for 1 min, and sequence extension at 72° C for 1 min in the presence of 1.5 U Taq DNA polymerase (New England Biolabs, Beverly, MA) and 200 µM of each of the four deoxynucleotide triphosphates in a total volume of 100 µl. 15 µl of the amplified samples were run in a 1.8% agarose gel (NA Agarose, Pharmacia, Sweden), the gels were stained with ethidium bromide and examined for bands of appropriate size with UV transillumination. For more sensitive and species-specific detection of the amplified DNA, the samples were transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) and hybridized with ^{32}P-α-dATP-labeled total *Leishmania aethiopica* kDNA as described elsewhere (5) and visualized by autoradiography.

Results

Specific and sensitive PCR-amplification of *L. aethiopica* DNA

To determine the sensitivity of the PCR assay serial dilutions of purified *L. aethiopica* DNA were made and amplified. Positive amplification, as determined by the appearance of the 120 bp specific band in the agarose gel, was observed when *L. aethiopica* DNA corresponding to 0.1 promastigote was used as template in the PCR assay (Fig. 1, panel A). The specificity of the amplified band was demonstrated by Southern-blot hybridization using purified *L. aethiopica* kDNA as hybridization probe (Fig. 1, panel B). It is also shown in the autoradiogram, that due to the higher sensitivity of the DNA-hybridization, DNA corresponding to 0.01 *L. aethiopica* promastigote resulted in a positive PCR amplification.

PCR amplification of DNA from patients with cutaneous leishmaniasis

DNA was extracted from paraffin-embedded skin biopsies obtained from 17 patients with histologically proven cutaneous leishmaniasis. Control DNA was extracted from skin biopsy specimens from patients with skin diseases other than leishmaniasis (12 leprosy, 3 Kaposi sarcoma, 2 candidiasis, 1 psoriasis) not living in endemic areas for leishmaniasis. Amplification of DNA from all 17 patients with leishmaniasis showed a positive result (Table 1), four of these are shown in Fig. 2 (lanes 4 - 7). Amplification of the 18 control DNA samples were negative, two of these are shown in lanes 8 - 9 in Fig. 2. These data show that PCR detected *Leishmania* DNA in all histologically proven cases of leishmaniasis and no false positives were found.

Table 1:

PCR-amplification of *L. aethiopica* DNA extracted from paraffin-embebbed skin biopsy specimens.

Patients	Number of PCR-positive specimens
Histologically proven cases of cutaneous leishmaniasis (n = 17)	17
Patients with skin diseases other than leishmaniasis (n = 18)	0

Table 2:

PCR-Amplification of *L. aethiopica* DNA extracted from skin biopsies from patients with clinically diagnosed CL, but for whom the diagnosis could not be confirmed by direct demonstration (histopathology and in vitro culture) of the parasites.

Histological findings	Number of PCR-positive specimens
Group I LD bodies not seen, but histological features are consistent with cutaneous leishmaniasis (n = 22)	7
Group II LD bodies not seen, histological features do not support the diagnosis of cutaneous leishmaniasis (n = 18)	0

PCR-detection of *Leishmania* DNA in histologically not proven cases of cutaneous leishmaniasis

Skin biopsies from patients with clinically diagnosed cases of cutaneous leishmaniasis were analysed by PCR. These patients were diagnosed by a senior dermatologist upon the features of the skin lesion. In vitro culture and histopathological examination of these biopsies, however, did not demonstrate the presence of *Leishmania* in the tissues. Two histopathologists divided the biopsies into two groups. Group I: biopsies histologically consistent with cutaneous leishmaniasis, and Group II: biopsies with histological features not suggesting leishmaniasis. The results are shown in Table 2. In Group II, no positive amplification was observed (representative samples in lanes 10, 14 - 15 in Fig. 2). In Group I, amplification of 7 out of 22 specimens showed a positive result (Tab. 2, and representative samples in lanes 11 - 13, 16 in Fig. 2), which were confirmed by a repeated amplification with a subsequent Southern-hybridization (not shown).

Discussion

In recent years PCR has been widely applied for the detection of infectious agents such as viruses, bacteria and protozoa in clinical specimens. The major advantage of PCR-diagnostics is the extreme high sensitivity. PCR has been demonstrated to be useful for the detection of infectious agents which are difficult to detect by traditional diagnostic methods, such as culture or histology. Both in vitro culture and histopathology requires the presence of a relative high number of viable or morphologically intact microorganisms. In the chronic phase of cutaneous leishmaniasis the number of viable and/or morphologically intact parasites in the skin lesion is very low. In this study we evaluated the PCR for the detection of *L. aethiopica* parasites in the chronic cutaneous lesions.

Detection of *Leishmania* using PCR with various primer sets has been recently reported (6, 8, 10, 12). The kinetoplast DNA minicircle as template has an obvious advantage, the copy number in one single organism being in the range of ten-thousand. Using a common (not species-specific, but *Leishmania*-specific) region of the minicircle DNA RODGERS et al. have reported (10) that DNA corresponding to less than one pramastigote was successfully amplified, and the primers have been successfully used for the detection of *Leishmania* in tissues of mammalian reservoir hosts (13). In the present study we have used the above PCR primers and could confirm the extreme sensitivity. Using hybridization of the PCR products with *L. aethiopica* kDNA we were able to detect DNA corresponding to 0.01 promastigote. This high sensitivity seems to be necessary if crude DNA is used as template. PCR with these primers has previously been used to detect *Leishmania* parasites in a small number of frozen biopsy specimens from suspected cases of cutaneous leishmaniasis in Brazil (10). The routine method of tissue preparation in most pathology laboratories, however, involves fixation and embedding in paraffin. We show here that PCR-amplification of *Leishmania* DNA can also be performed on crude DNA prepared from fixed and paraffin-embedded tissues.

Using crude DNA extracted from paraffin-embedded skin biopsies, we have shown that PCR detected *Leishmania* DNA in all (n = 17) specimens where amastigotes had been found upon histopathological examination. The corresponding negative control tissues were all

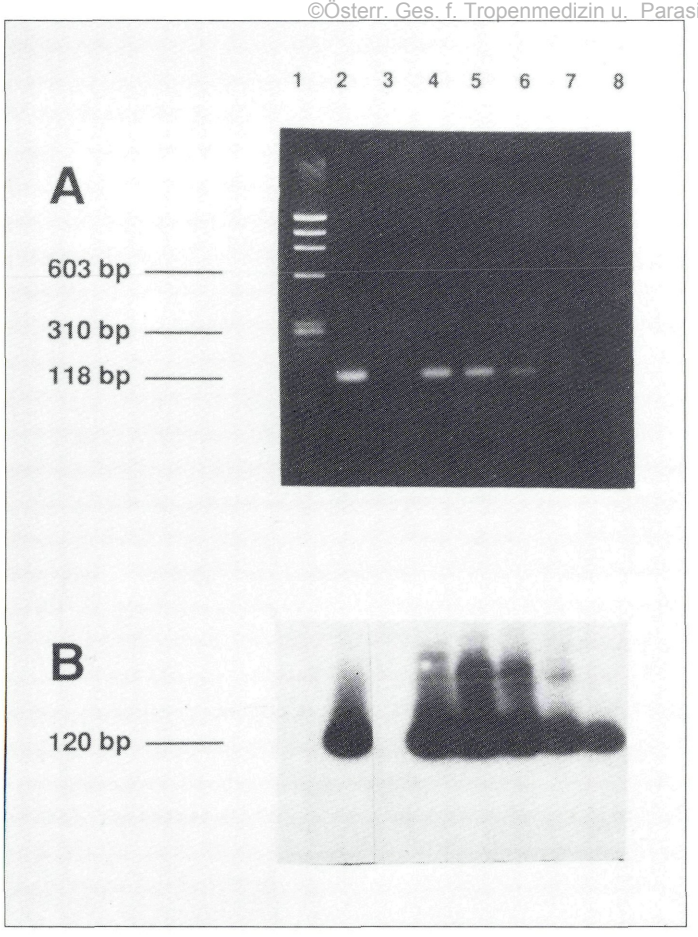

Figure 1:

Sensitivity of PCR-amplication of *L. aethiopica* DNA. MW marker in lane 1. A DNA template of 10 pg *L. aethiopica* DNA as positive control (lane 2) and 100 pg human genomic DNA as negative control (lane 3), and a serial dilution of *L. aethiopica* DNA corresponding to 100, 10, 1, 0.1 and 0.01 promastigotes (lane 4 - 8, respectively) were amplified. PCR products were run in a 1.8% agarose gel and stained with ethidium-bromide (A), and after a Southern transfer hybrizided with radiolabeled *L. aethiopica* kDNA (B).

negative in PCR, no false positives were found. The more interesting question was, however, whether PCR could detect the parasites in skin biopsies from patients diagnosed clinically for having cutaneous leishmaniasis when in vitro culture and/or histopathology could not confirm the diagnosis. PCR detected Leishmania DNA in 7 out of 22 cases for whom, in spite of the absence of amastigotes, the histopathological features of the biopsies were found to be consistent with cutaneous leishmaniasis. All tissues which were histologically not characteristic for leishmaniasis were also PCR negative.

These results indicate that PCR is a sensitive and specific method of potential value in the detection of *Leishmania* parasites in chronic granulomatous lesions, where other diagnostic methods fail to detect the parasites. With assistance of the PCR *Leishmania* DNA can be detected in fixed and embedded tissues. This is a great advantage for most areas where leishmaniasis is endemic. In addition, since samples for PCR can be collected in the field without specific equipment, even without the need for refrigeration, makes PCR an important tool for epidemiological studies.

Summary

In this study the polymerase chain reaction (PCR) was used to detect *Leishmania aethiopica* in paraffin embedded skin biopsy specimens. The *Leishmania*-specific 120 bp long fragment of the kinetoplast DNA (kDNA) minicircles has been amplified from all samples (n = 17) from histologically confirmed cases of cutaneous leishmaniasis (CL), as demonstrated by gel electrophoresis and hybridization with *L. aethiopica* kinetoplast DNA. Control specimens from patients with skin diseases other than leishmaniasis were all PCR-negative. PCR was carried out also on specimens (n = 40) in which *Leishmania* parasites were not detected by histopathology and in vitro culture although the clinical manifestation of the skin lesions was suggestive for CL. Seven of these samples were PCR positive. These data demonstrate that PCR represents a valuable method for the diagnosis of cutaneous leishmaniasis especially in cases where it is difficult to detect the parasites by direct parasitological or histological techniques.

Key words

Leishmaniasis, *Leishmania aethiopica,* polymerase chain reaction.

Zusammenfassung

Nachweis von Leishmania aethiopica in Paraffinschnitten von Hautbiopsien mit Hilfe der Polymerase-Kettenreaktion

In dieser Studie wurden Paraffinschnitte von Hautbiopsien mit Hilfe der Polymerase-Kettenreaktion (PCR) auf eine bestehende Infektion mit *Leishmania aethiopica* untersucht. Von allen 17 Patienten mit histologisch gesicherter kutaner Leishmaniose wurde das *Leishmania-*

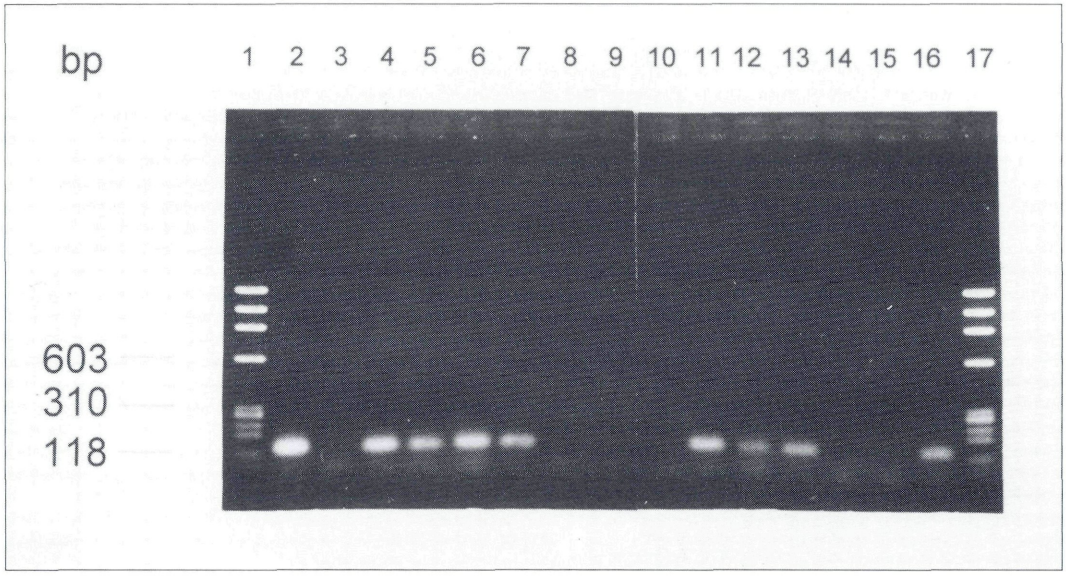

Figure 2:

PCR amplification of *L. aethiopica* DNA extracted from skin biopsy specimens. MW markers in lanes 1 and 17. Ten pg *L. aethiopica* DNA as positive control (lane 2) and 100 pg human genomic DNA as negative control (lane 3), DNA extracted from histologically proven cases of CL (lanes 4 - 7), from negative control biopsies (lanes 8 - 9) and from patients for whom CL was suggestive on clinical grounds but histology and *in vitro* culture were negative (lanes 10 - 16) were amplified. PCR products were run in an 1.8% agarose gel and stained with ethidium bromide.

spezifische 120 bp lange DNA-Fragment amplifiziert. Die Amplifikate wurden mittels Gelelektrophorese und Hybridisierung mit *L. aethiopica* spezifischer Kinetoplasten-DNA verifiziert. Die PCR wurde darüberhinaus an Paraffinschnitten von Patienten (n = 40) mit Verdacht auf kutane Leishmaniose angewendet, bei denen jedoch histopathologisch und kulturell keine sichere Diagnose gestellt werden konnte. Bei sieben dieser Patienten konnte mit Hilfe der PCR Leishmanien-DNA nachgewiesen werden. Diese Daten zeigen, daß die PCR eine sensitve und spezifische Methode in der Diagnostik der kutanen Leishmaniose ist; dies gilt besonders für Fälle, bei denen histopathologische oder kulturelle Methoden keine Aussage liefern.

Schlüsselwörter

Leishmaniose, *Leishmania aethiopica,* Polymerase-Kettenreaktion.

Acknowledgements

The Armauer Hansen Research Institute (AHRI) is supported by the Norwegian and Swedish agencies for International Development (NORAD and SIDA). T. Laskay and W. Solbach received finantial support from the Deutsche Forschungsgemeinschaft (SFB 263).

References

1. ASHFORD, R. W., BRAY, M. A., HITCHISON, M. P., BRAY, A. S. (1973):
The epidemiology of cutaneous leishmaniasis in Ethiopia.
Trans. Roy. Soc. Trop. Med. Hyg. 67, 568-601.

2. BRAY, R. S., ASHFORD, R. W., BRAY, M. A. (1973):
The parasite causing cutaneous leishmaniasis in Ethiopia.
Trans. Roy. Soc. Trop. Med. Hyg. 67, 345-348.

3. CAO, M., XIAO, X., EGBERT, B., DARRAGH, T. M., YEN, T. S. B. (1989):
Rapid detection of cutaneous herpes simplex infection with the polymerase chain reaction.
Journal of Investigative Dermatology 92, 391-392.

4. GRIFFITHS, W. A. D. (1987):
The leishmaniasis in biology and medicine, 617-636.
Academic Press, London.

5. LASKAY, T., KIESSLING, R., DE WIT, T. R., WIRTH, D. F. (1991):
Generation of species-specific DNA probes for Leishmania aethiopica.
Molecular and Biochemical Parasitology 44, 279-286.

6. LOPEZ, M. et al. (1993):
Diagnosis of Leishmania using the polymerase chain reaction: a simplified procedure for field work.
Amer. J. Trop. Med. Hyg. 49, 348-356.

7. MENGISTU, G. et al. (1992):
Cutaneous leishmaniasis in south-western Ethiopia: Ocholo revisited.
Trans. Roy. Soc. Trop. Med. Hyg. 86, 149–153.

8. PIARROUX, R. et al. (1993):
Isolation and characterization of a repetitive DNA sequence from Leishmania infantum: development of a visceral leishmaniasis polymerase chain reaction.
Amer. J. Trop. Med. Hyg. 49, 364–369.

9. PIARROUX, R. et al. (1994):
Comparison of PCR with direct examination of bone marrow aspiration, myeloculture, and serology for diagnosis of visceral leishmaniasis in immunocompromised patients.
J. Clin. Microbiol. 32, 746–749.

10. RODGERS, M. R., POPPER, S. J., WIRTH, D. F. (1990):
Amplification of kinetoplast DNA as a tool in the detection and diagnosis of Leishmania.
Experimental Parasitology 71, 267–275.

11. SHIBATA, D. K., ARNHEIM, N., MARTIN, W. J. (1988):
Detection of human papillomavirus in paraffin-embedded tissue using the polymerase chain reaction.
Journal of Experimental Medicine 167, 225–230.

12. SMYTH, A. J. et al. (1992):
Rapid and sensitive detection of Leishmania kinetoplast DNA from spleen and blood samples of kala-azar patients.
Parasitology 105, 183–192.

13. WILLIAMS, A. O., MUTINGA, J., RODGERS, M. (1991):
Leishmaniasis in a domestic goat in Kenya.
Molecular and Cellular Probes 5, 319–325.

Korrespondenzadresse: Dr. Tamas Laskay
Institut für Klinische Mikrobiologie
Universität Erlangen-Nürnberg

Wasserturmstraße 3
D-91054 Erlangen · Bundesrepublik Deutschland