Diagnosis of visceral leishmaniasis by the polymerase chain reaction using blood, bone marrow and lymph node samples from patients from the Sudan

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Summary

We have evaluated the sensitivity of the polymerase chain reaction (PCR) as a diagnostic tool for Leishmania donovani using blood, bone marrow and lymph node samples from Sudanese patients with a confirmed infection. Forty patients were diagnosed by microscopic examination of bone marrow or lymph node samples. The PCR was able to detect parasite DNA in 37 out of 40 blood samples. In bone marrow and lymph node samples, the PCR was able to detect parasite DNA in all 7 and 6 samples, respectively. We suggest that the PCR should be considered as a valuable and sensitive tool for the diagnosis of L. donovani infection. However, if PCR diagnosis is to supplement or even replace microscopic diagnosis in developing countries, a large number of patients with no apparent signs of infection and patients with other diseases have to be tested in order to evaluate its true potential.

keywords visceral leishmaniasis, diagnosis, PCR

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Introduction

Leishmaniasis are a group of diseases caused by protozoan parasites of the genus Leishmania. Depending on the species of parasite and the immune response of the host, the disease spectrum ranges from self-healing cutaneous lesions to a fatal systemic disease (Heyneman 1971). Visceral leishmaniasis or kala-azar, which is caused by L. donovani in the Sudan, is the most serious form of the diseases and is a major health problem in the country. The definitive diagnosis of L. donovani infections is obtained by microscopic demonstration of parasites in stained bone marrow, lymph nodes or spleen aspirates, by culture or animal inoculation of samples from affected organs. However, the sensitivity of microscopy depends on the number of parasites in the samples, which is often low. Culture of Leishmania parasites can be time-consuming and contamination may be a problem, while taking organ biopsies is not easy under field conditions. As a result, much effort has been directed towards the development of new and more sensitive diagnostic techniques.

Among the new and promising diagnostic tools of molecular biology is the polymerase chain reaction (PCR; Saiki et al. 1988). Several primers have been
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developed to amplify specific regions of DNA from *Leishmania* parasites. These include primers recognizing deoxyribonucleic acid (DNA) sequences in the kinetoplast (Rodgers et al. 1990; Smyth et al. 1992), ribonucleic acid (RNA) gene spacers (Van Eys et al. 1992; Hassan et al. 1993) or repetitive nuclear sequences (Piarroux et al. 1993). Recent studies in African patients have shown the promise of the PCR technique in diagnosing *L. donovani* infections (Adhya et al. 1995; Schaefer et al. 1995).

In this study we evaluate the use of PCR in the diagnosis of *L. donovani* infections using blood, bone marrow and lymph node samples from Sudanese patients with parasitologically confirmed infection.

**Materials and methods**

**Patients**

After giving informed consent, 40 patients with visceral leishmaniasis presenting to Soba Hospital, Khartoum, and Gedaref Hospital, Gedaref, Sudan, were included in the study. The clinical history of all patients was obtained and they were clinically examined. The final diagnosis of *L. donovani* infection was made after microscopic demonstration of amastigotes in bone marrow or lymph node aspirates. Patients were treated with sodium stibogluconate (Pentostam) at a dose of 10 mg/kg per day for 4 weeks.

**Collection of patient samples**

Blood samples were collected into heparinized vacuumers. Five hundred μl of whole blood was applied onto nitrocellulose membranes (Millipore, USA), dried at room temperature and stored at 4°C until further use. Bone marrow (app. 50 μl) and lymph node aspirates (app. 20 μl) were also applied onto nitrocellulose membranes, dried and stored as above. In some bone marrow and lymph node aspirates, the number of amastigotes was counted macroscopically according to standard techniques. Blood samples were also taken from Danish control donors. These samples were treated in the same way as the blood from patients.

**Preparation of samples for PCR**

The area of the dried blood, bone marrow, or lymph node sample was cut out from the nitrocellulose membrane and added to proteinase K buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, pH 8.0 and 1% Triton X-100) and proteinase K was added to a final concentration of 100 μg/ml. A volume of 5 times the original sample volume (i.e. blood, bone marrow or lymph node) was used for the digestion performed overnight at 37°C followed by phenol/chloroform extraction and ethanol precipitation (Sambrook et al. 1989). The dried DNA pellet was finally resuspended in dH2O in the same volume as the original sample volume.

**Polymerase chain reaction**

The primers developed by Smyth et al. (1992) were used for the PCR (5’CCA GTT TCC CGC CCC G3’, 5’GGG GTT GGT GTA AAA TAG GCG3’). The PCR reactions (in 20 μl total volume) consisted of the following: 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 20 pmol of each primer, 0.5 μl of DNA and 1 unit of Taq polymerase (Perkin Elmer, USA). Samples were initially incubated at 94°C for 5 minutes before Taq polymerase was added (hot start). The cycling parameters were 94°C for 1 minute, 64°C for 1 minute and 72°C for 2 minutes for 40 cycles on a Perkin-Elmer DNA thermal cycler. The cycling procedure was terminated by an extension at 72°C for 10 minutes. The PCR products were analysed on a 1.5% agarose gel, stained with ethidium bromide and visualized by ultraviolet illumination.

**Results**

In this study we examined the suitability of the PCR technique for the detection of *L. donovani* parasites in blood, bone marrow and lymph node samples from Sudanese patients with a parasitologically confirmed infection. Of the 40 blood samples examined, 37 (92.5% sensitivity) gave an amplification product with the expected size of approximately 800 base pairs (Figure 1). In 3 patients, 19, 26 and 34 (Figure 1, lanes 8, 14 and 20, respectively), we were not able to detect any PCR product. In patient 23 (lane 12) only a very faint amplification product was present.
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In addition to the blood samples, we performed PCR on bone marrow and lymph node samples. Figure 2A–B shows the results from PCR amplification of purified DNA from bone marrow and lymph node samples, respectively. In the bone marrow samples we detected parasite material in all 7 samples (Figure 2A) and in lymph node samples we were able to detect parasite material in all 6 samples (Figure 2B). Of the 5 PCR-negative blood samples we had only a bone marrow and lymph node sample available from patient 19, and this patient tested positive in the PCR (Figure 2A and B, lanes 5 and 3, respectively) for both samples. We did not, however, find any relation between the intensity of the amplified PCR band from blood, bone marrow or lymph node and the number of parasites in biopsies (data not shown).

**Discussion**

In this study we tested the sensitivity of the PCR in the diagnosis of *L. donovani* infections in Sudanese
patients using blood, bone marrow or lymph node samples. We used only samples from patients with a microscopically confirmed infection. In our PCR we were able to detect parasites in a blood volume equivalent to 0.5 µl. When we increased the DNA concentration in the PCR reaction, we often experienced a faint signal or none after agarose gel electrophoresis. This could be due to inhibiting factors in the DNA preparation even after phenol/chloroform extraction. Factors such as heparin and haemoglobin have been suggested as being inhibitory factors for the Taq polymerase (Beutler et al. 1992; Panaccio & Lew 1991). It might be possible, however, to reduce or remove these factors by isolating the buffy coat from the blood samples before purifying the DNA.

Panaccio and Lew (1994) evaluated the influence of several anticoagulants on PCR performance and found an inhibiting effect of heparin salts, but only after several weeks of incubation at room temperature. In our study, however, we immediately processed the blood samples and stored them on nitrocellulose membranes at 4°C before DNA purification and PCR analysis. To ensure that a negative signal in our PCR was not due to a possible inhibition, we also added parasite DNA in an extra PCR reaction for all the patient blood samples and found an amplified band of the appropriate size (data not shown). To ensure that no DNA contamination took place from sample to sample during the DNA purification, we processed the samples as soon as we received them. Because the blood samples were collected over a period of several months, most samples were processed one by one, so no cross-contamination took place. Blood samples from several Danish controls were also processed in parallel and used as negative controls in the PCR. Furthermore, samples from Sudanese patients were tested several times and the samples showed the same result as in the initial testing, indicating that cross-contamination was not taking place.

In the PCR performed on blood, we obtained a sensitivity of 92.5%. A similar level of sensitivity (90%) was reported by Nuzum et al. (1995) using a PCR-ELISA on blood from Indian, Kenyan and Brazilian patients with parasitologically confirmed kala-azar. In a recent study in India, Adhya et al. (1995) reported a sensitivity of 82% using blood from patients with visceral leishmaniasis and a similar level of sensitivity (82%) was reported by Piarroux et al. (1994) in a study on \textit{L. infantum} in France using bone marrow aspirate as a source of DNA. In the same study, microscopic examination of bone marrow aspirate resulted in a sensitivity of only 55%.

One disadvantage of the PCR is that it is indirect, that is, it does not necessarily detect an ongoing infection. We have no detailed knowledge with respect to the half-life of parasite fragments or naked circulating DNA in the blood or tissues. However, Schaefer et al. (1995) were able to detect circulating DNA in blood from a patient 3 years after successful treatment with no apparent relapse.

In this study we isolated the DNA by conventional phenol/chloroform extraction technique. This technique is tedious and time-consuming and it would be advantageous to avoid the use of hazardous chemicals in developing countries, where facilities for handling these chemicals are often limited. Several DNA isolation kits for the purification of DNA that avoid these chemicals are now available commercially. We are testing various protocols for isolation of DNA from blood for use in the PCR in the Sudan.

We found no relation between the intensity of the PCR band and the parasite gradation index for bone marrow or lymph node aspirates. As far as we know, no studies have been undertaken to examine such a possible relation. It could be interesting to see whether patients with a high parasite count in the bone marrow or spleen also have a high number of circulating parasites in the blood.

In conclusion, this study has shown that the PCR technique is a sensitive and promising tool in the diagnosis of \textit{L. donovani} infections from blood from patients with a confirmed infection. Taking a blood sample is faster and less painful than taking a bone marrow or lymph node biopsy for diagnosis. However, if PCR diagnosis is going to supplement or even replace microscopic diagnosis, a large number of patients with no apparent signs of infection and patients with other diseases have to be tested in order to evaluate its true potential. We are investigating the potential of the PCR for detecting subclinical infections in a large number of patients. In addition to its higher sensitivity compared to conventional parasitological diagnosis, PCR has the advantage of characterizing the parasite causing the infection.
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References


