AN EXPERIMENTAL STUDY ON APPLICATION OF PCR IN DETECTION OF KALA-AZAR

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Abstract. A polymerase chain reaction (PCR) technique was developed to detect Kala-azar. Out of 25 patients, 24 were detected, a sensitivity of 96%. The specificity rate, false positive rate, false negative rate, coincidence rate and Youdent's index were 100% (60/60), 0, 4%, 99% and 0.96 respectively. It was shown that the PCR tecnhique can directly detect Kala-azar in samples (1 μl) from peripheral blood.

INTRODUCTION

Polymerase chain reaction (PCR) is a powerful tool to directly detect DNA through amplifying a specific sequence of target DNA, which has been used for diagnosis of several diseases. In order to assess the value of the PCR technique in Kala-azar diagnosis and the possibility to use it for detection in peripheral blood samples, we conducted the following study.

MATERIALS AND METHODS

Leishmania donovani

Leishman – donovan bodies of Shandong strain were cultured by conventional methods (Wang and Wu, 1956), and harvested in 7 days.

Positive serum specimens

Serum specimens from 25 Kala-azar patients were collected by Xinjian Institute of Endemic Diseases. All of these patients were detected as positive by appearance of Leishman-donovan bodies in bone marrow puncture, IFA, ELISA and Dot-ELISA cAg, with an average IFA titer of 1:294 (range 1:64-1:512).

Control serum specimens

A total of 60 serum specimens for control study were selected from 2 patients infected with *Plasmodium vivax*, 2 patients with *Plasmodium falciparum*, 8 patients with *Toxoplasma* (ELISA IgM Ab test confirmed with OD > 2.1 at 1:200 concentration), 10 patients with *Ascaris* infection, 10 patients with liver or lung cancer, and 20 healthy adults.

DNA templates

1.5 ml culture medium of *Leishmania donovani*, and the cell pellets of serum specimens of both controls and Kala-azar patients were suspended in 1 ml normal saline, then centrifuged in a microfuge at 15,000 rpm for 10 minutes, the supernatant solutions were removed and suspended in 50 μl buffer solution (50 mM Tris, pH 8.0, 1mM EDTA, 40 μg/ml protein K), warmed at 55°C for 1 hour, and boiled at 100°C for 5 minutes, centrifuged at 10,000 rpm for 3 minutes, and kept at 4°C until further use.

Polymerase chain reaction

Taq DNA polymerase and dNTP mixture were purchased from Xiehe Medical University, Beijing. Oligonucleotide primers I and II were synthesized with an auto DNA synthesizer (Beckman) by the Institute of Microbiology, Academica Sinica, Beijing. The sequence of the two oligonucleotides were as follows (Rodgers *et al*, 1990):

I:5'-CCCCGTGGGGGAGGGGCGTTCTG-3'

II:5'-AAACTGGGGGTTGGTGTAAAATAGG-3'

The reaction was carried out in an automated DNA thermal cycler, model FR-300 (Fudan Institute of Biomedical Experimental Techniques, Shanghai). PCR reaction condition were: 94°C for 45 seconds (denaturation), 55°C for 1 minute (ex-

tension), and 56°C for 45 seconds (annealing), and after 35-40 cycles, reextended at 72°C for 5 minutes. Ul amplification per well were analysed by gel electrophoresis in 2% agarose, and staining with ethidium bromide (EB), with molecular weight markers, observed under ultraviolet light, photographs were taken by RICOH camera with a red filter.

Data analysis

Sensitivity, specificity, false positive rate, false negative rate, coincidence rate and Youden's index of the PCR technique were calculated as recommended by Lilienfeld (Yin Huyuan, 1994).

RESULTS

Diagnosis of Kala-azar by PCR

Serum specimens from 25 patients of Kala-azar and 60 controls were examined by PCR. 24 out of 25 disease specimens of Kala-azar patients were positive, and all control specimens were negative detected by PCR (Table 1). Data analysis found that sensitivity, specificity, false positive rate, false negative rate, coincidence rate and Youden's index wer 96%, 100%, 0, 4%, 99% and 0.96% respectively.

Table 1

Results of Kala-azar detection by PCR.

		Kala-azar cases	Controls
PCR	+	24	0
	-	1	60

PCR amplifacation products

Results are shown in Figs 1-3. Fig 1 shows representative amplification products from 2 positive samples and 1 of negative samples. Fig 2 shows amplification products at different concentrations of a positive sample. Fig 3 shows amplification products of control serum specimens showing that no band appeared except the marker.

DISCUSSION

Kala-azar is serious parasitic disease of man and animals (Wang and Wu, 1956). It is necessary to establish a technique for early diagnosis of Kala-azar, which will permit treatment of patients quickly and reduce the mortality of the disease. The traditional method for diagnosis of Kala-azar by detection of leishman-donovan bodies in bone marrow puncture cannot give an early diagnosis because of low positive rate (75-80%) and frequent contamination. IFAT and ELISA have limitted application in early diagnosis as a result of high cross-reaction with serum antibodies against other parasites. We have developed Dot-ELISA techniques to detect Kala-azar in the laboratory with 100% sensitivity and 99% specificity by using a 63 kDa protein as detecting antigen (Bao *et al*, 1994). But the technique takes too long to perform and has to be improved further. Although the McAg-AST has higher sensitivity and specificity when used for Kala-azar diagnosis (Hu *et al*, 1989), it is still difficult to apply because of the complexity of the method and many limitation of the test.

The PCR technique is a method for amplification of DNA *in vitro*, and has been widely used for diagnosis of many infections diseases, since it only takes a few hours to amplify a very small amount of genomic DNA or specific fragment of RNA. Smyth *et al* (1992) considered the DNA from only one parasite in the peripheral blood can be detected by PCR. Since Leishman-donovan bodies are develop and reproduce mainly in macrophages of the bone marrow, very few of parasites can be found in the peripheral blood, therefore, PCR takes advantage of higher sensitivity and easy operation in detecting *Leishmania donovani* in peripheral blood samples. Lopez *et al* (1993) reported 95% sensitivity of PCR in detecting 29 Kala-azar patients. 90% sensitivity (57/63) and 100% specificity (0/40) of PCR in detecting peripheral blood of Kala-azar patients were reported by Nuzum *et al* (1995). So it is worth while for to apply PCR in the early diagnosis of Kala-azar.

Our study on PCR on 25 Kala-azar patients and 60 control blood samples showed 96% sensitivity,

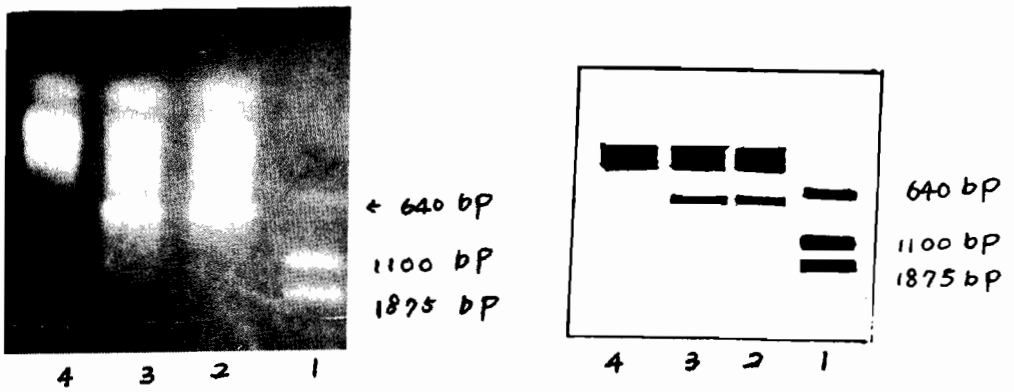

Fig 1–Amplification products of PCR
lane 1: Marker PBR 322
lane 2,3: Serum samples from two patients with Kala-azar
lane 4: Serum samples from control patients with *P. falciparum*.

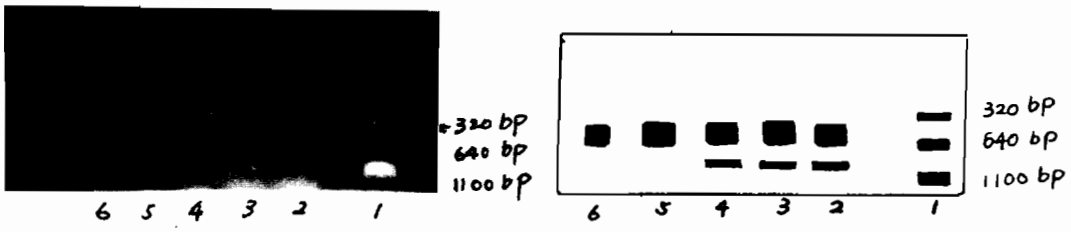

Fig 2–Amplification products of positive samples in different concentrations by PCR
lane 1: Marker PBR 322
lane 2: 100 µl sample
lane 3: 10 µl sample
lane 4: 1 µl sample
lane 5: 0.1 µl sample
lane 6: 0.01 µl sample.

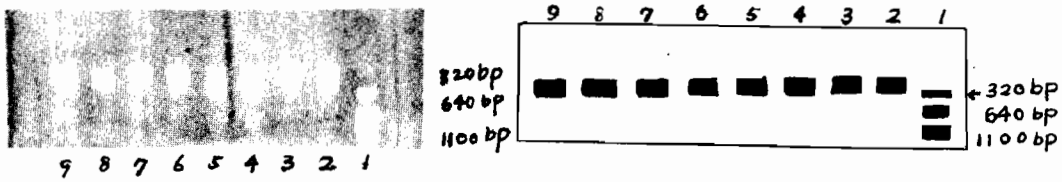

Fig 3–Amplification products of collate serum specimens
lane 1: Marker PBR 322
lane 2: serum sample from patient with *P. vivax*
lane 3: serum sample from patient with *Toxoplasma*
lane 4: serum sample from patient with *Ascaris*
lane 5: serum sample from TB patient
lane 6: serum sample from liver cancer patient
lane 7: serum sample from lung cancer patient
lane 8: serum sample from healthy person

100% specificity, 0% false positive rate, 4% false negative rate and 0.96 Youden's index. It also showed that even 1 μl serum specimen containing parasite DNA could be detected by PCR.

ACKNOWLEDGEMENTS

The authors thank Professor Chu Jinqi, Institute of Parasitic Diseases, Chinese Academy of Preventive Medicine, Shanghai. The study received financial support from WHO/TDR(I.D.No.PDV/CHN/94/01).

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