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A Simple and Rapid DNA Purification Method for Detection of Leishmania DNA in Peripheral Blood of Patients with Visceral Leishmaniasis.

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Abstract:

Introduction: Recently polymerase chain reaction technique is being used for detection of leishmania DNA in clinical samples of patients suspected with visceral leishmaniasis. Since many of the patients are children and also suffering from anemia and leucopenia, there is a need for the development of a DNA extraction method using small amount of clinical samples. In the present study, a simple and rapid method was introduced for performing the DNA extraction procedure which was capable of removing inhibitors from the whole blood samples and of providing the appropriate DNA for Leishmania PCR amplification.

Materials and Methods: EDTA-peripheral blood specimens were collected from healthy volunteers and frozen at −20°C. The thawed specimens were spiked with a definite number of L. infantum promastigotes. Using in house made lysis buffer and boiling, a DNA extraction method was developed for the purification of Leishmania genome from 500 µl of blood samples. The developed method was then compared with the classical standard method to obtain the highest sensitivity for the diagnosis of visceral leishmaniasis.

Results: The sensitivity of the PCR for the developed method was 10 copies of L. infantum DNA per reaction tube. When clinical samples collected from patients with a definite diagnosis of visceral leishmaniasis were used, no false negative was demonstrated with newly developed method.

Conclusion: Since hazardous materials such as phenol-chloroform are not used during extraction procedure, it seems to be much safer than the standard phenol-chloroform method. However, the use of small amount of sample (i.e. 0.5 µl) and removal of PCR-inhibitors from the whole blood are the major advantages of our developed DNA extraction method.

Key Words: Leishmania, DNA purification, Peripheral blood.

Introduction:

Visceral leishmaniasis (VL), known as kala-azar is endemic in 62 countries, threatening 200 million people. In the severe form of the disease, patients present with fever, weight loss, hepatosplenomegaly and anemia. Untreated cases with symptoms of kala-azar are usually fetal within 2 years. Of crucial importance in the management of this disease is early diagnosis. Definite diagnosis of VL is based on the direct demonstration of the parasite in a splenic aspirate, liver, or bone marrow biopsy. These techniques (especially splenic aspiration and liver biopsy) can be hazardous and require considerable expertise. The parasite may be scanty and therefore difficult to see. Sample preparation is an invasive procedure and thus inconvenient for the patients [(1)]. Although the aspirate can be cultured on specific media, this is not a rapid technique, as the parasite may take between 10 and 21 days to grow.

Non-invasive serological methods such as enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody (IFA), and direct agglutination test (DAT) are rapid and readily adaptable to mass screening, but their suitability for early diagnosis is controversial [(2-5)]. Antibody based detection techniques remain positive for some years after cure, a disadvantage for current diagnosis.

A number of gene amplification techniques such as polymerase chain reaction (PCR) have been developed for the diagnosis of visceral leishmaniasis. Previous studies have made use of PCR as a means detecting leishmania DNA in different sorts of specimens such as bone marrow, spleen aspirate, liver biopsy, lymph nodes and the buffy coat of the blood [(6-12)]. Whole blood has been found to be an appropriate substrate for leishmania PCR, but it contains many inhibitors for DNA polymerase.

Since many kala-azar patients are children who are also suffering from anemia and leucopenia, there was a need for the development of a DNA extraction method using fewer blood samples than required in the mentioned techniques. We managed to introduce a DNA extraction procedure which is simple and rapid because of its ability in removing inhibitors from the whole blood and providing significant amounts of DNA suitable for Leishmania PCR amplification. The developed method was then compared with the classical standard method to obtain the highest sensitivity for the diagnosis of visceral leishmaniasis.

Materials and Methods:

Parasite:

Leishmania infantum was initially isolated from the bone marrow sample of a patient with visceral leishmaniasis. The parasite was maintained at promastigote stage at 25°C in RPMI containing 10% of fetal calf serum. The promastigote containing the medium was centrifuged at 3000 ´g for 10 min and the pellets were washed (for three times) in phosphate buffer saline. After the last centrifugation, the pellets containing promastigots were resuspended in the ste-

rile distilled water and counted by hemocytometer.

Experimental Infections:

The whole EDTA-peripheral blood specimens were collected from healthy volunteers (n=25) and frozen at -20°C until used. The thawed specimens were then spiked with 10-fold dilutions of L. infantum promastigotes. The final concentrations of the parasites tested were corresponding to DNA equivalents of 10^4, 10^3, 10^2, 10 and 1 parasite per PCR tube respectively. The spiked samples were then subjected to different DNA extraction methods, as described below:

DNA Extraction Methods:

Method A: Boiling and Phenol-chloroform: Three hundred microlitres of distilled water was added to an equal volume of treated blood and boiled for 20 minutes. The samples were then subjected to phenol-chloroform extraction. DNA was precipitated with ethanol, and dissolved in 50 of µl Tris-EDTA pH 7.5.

Method B: Proteinase K and Phenol-chloroform: DNA was isolated as previously described [18] briefly, 0.5 µl of the treated blood was incubated overnight at 60°C in an equal volume of lysis buffer which contained 50 mM NaCl, 50 mM tris-HCl (pH 7.4), 10 mM EDTA, 1% ($^{vol}/_{vol}$) Triton X-100 and 200 mg of proteinase K/µl. The procedure was followed as in method A.

Method C: Chelex-100 and Phenol-chloroform: 0.5 µl of 20% ($^{wt}/_{vol}$) chelex-100 was added to 0.5 µl of the treated blood. The samples were then boiled for 10 minutes and the procedure was followed as method A.

Method D: in-house: A total of 500 µl of spiked blood samples was incubated 3 min at room temperature with 1 µl of cold lysis buffer [0.32 M sucrose, 10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1% ($^{vol}/_{vol}$) Triton X-100] and vortexed to make it suspend evenly. The samples were then centrifuged for 5 min, 14000 ´ g at 4°C (MR 1022 Jouan, France). One milliliter of the supernatant was discarded and the above steps were repeated two more times, until no hemoglobin remained. After centrifugation, all the supernatant except a volume of 50 µl was removed. Fifty microliters of the sterile double distilled water were then added to the tube and vortexed. The mixture was incubated at 95°C in a dry heating block (Techne-Dri-Block DB-2D) for 20 min. The samples were then centrifuged at 14000 ´ g for 25 seconds and 10 µl of the supernatant was used as template for the PCR.

Optimization of Leishmania PCR using developed DNA extraction method:

The reaction conditions were thoroughly optimized with blood samples so as to obtain the highest sensitivity and specificity. To do this, standard PCR optimization procedures were performed and different combinations were tested: $MgCl_2$ (0 to 2 mM by increments of 0.5), primers (7.5, 15, 22.5, 30, 37.5 and 45 pmol/tube), and Taq DNA polymerase (1, 1.5, 2 and 2.5 Units/tube). Moreover, annealing tempera-

tures of 50 to 62°C were tested by increments of 1°C.

PCR amplification:

Following several optimization procedures, PCR amplification was performed using the Leishmania specific oligonucleotide primers 13A (5'-GTG GGG GAG GGG CGT TCT-3') and 13B (5'-ATT TTA CAC CAA CCC CCA GTT-3') [13] to amplify a 120 bp fragment of Leishmania kinetoplast DNA (kDNA) minicircles. Ten microliters of the sample were added to 20 µl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 mM of dNTPs, 1 unit of Taq DNA polymerase (AmpliTaq, Roche Diagnostic), and 22.5 pmole of each specific primer. Based on the PCR optimization outcome, magnesium chloride was not added to the PCR mixture. The reactions were performed in an automated thermal cycler (Eppendorf Master Cycle). The samples were denatured for 4 min at 94°C and then subjected to 45 cycles consisted of 1 min of denaturation at 94°C, 1 min of annealing at 59°C, and 1 min of elongation at 72°C and finally 72°C for 5 minutes. Ten µl of each amplified product was analyzed by conventional gel electrophoresis followed by ethidium bromide staining (10 mg/µl). The samples were scored as positive when a PCR product of 120 bp could be detected. Standard precautions were taken to avoid contamination during the PCR. A number of negative controls were run alongside the spiked samples in random order throughout the experiments.

Clinical samples:

The developed DNA extraction method was then applied for the detection of Leishmania DNA in peripheral blood samples of patients with definite diagnosis of visceral leishmaniasis (n=13) as approved by clinical features, bone marrow smears and indirect fluorescent antibody technique (IFA). There was also a patient with the definite diagnosis of visceral leishmaniasis whose sample had been collected after antiparasite treatment.

The blood samples from healthy subjects (n=25) were also included in the experiment as the negative control group.

Results:

Specificity and sensitivity of the PCR assay:

The blood specimens were spiked with serial dilutions of L. infantum promastigotes (10-fold dilution steps from 10,000 to 1 parasite per PCR tube) to establish the detection limit for the PCR assay and determine the ability of DNA extraction method in the removal of PCR inhibitors from blood. Concentrations of 10,000 to 1 parasite/PCR tube were each tested in triplicate. The intensity of the unique amplified 120-bp band was clearly correlated with the number of Leishmania assayed. The sensitivity of each PCR test was assessed with the intensity of the banding pattern in ethidium bromide-stained agarose gels. The sensitivity of the PCR for the methods A, B, and C was 10^3, 10^4, and 10^4 copies of parasite DNA per reaction tube respective-

ly. In fact, only a portion of these reactions was positive, possibly due to the lack of parasite DNA. Using in-house developed DNA extraction method the sensitivity of the PCR improved to 10 copies of Leishmania DNA per reaction tube. The 120 bp band was not detected with the lowest parasite dilution.

The absence of PCR product when DNA was extracted from the blood of the patients infected with Plasmodium vivax, Plasmodium falciparum, Toxoplasma gondii, Brucella and Salmonella indicates that this PCR assay is 100% genus specific.

When 13 clinical samples collected from patients with definite diagnosis of visceral leishmaniasis were used, no false negative was demonstrated with the developed method. PCR was negative on blood samples collected, following antiparasite treatment, from a patient with definite diagnosis of visceral leishmaniasis.

Reproducibility of the DNA extraction method:

To evaluate the reproducibility of the DNA extraction method as well as that of PCR, the whole procedures including DNA extraction, amplification and detection of amplified products were repeated on different days. Negative controls were also included. Similar results were always obtained on different occasions.

Optimization of the Leishmania PCR assay based on the newly DNA extraction method:

The concentration of Mg^{2+} in a PCR mixture is an important yield of the reaction. The lysis buffer, which was used in our study, contained a high amount of Mg^{2+} (i.e. 5 mM). Sample inhibition was observed when standard concentration of Mg^{2+} (i.e. 1.5 mM / tube) was added to the PCR mixture. To overcome this problem, magnesium chloride concentrations of 0 to 2 mM were assessed. Optimum result was achieved when using 0 mM $MgCl_2$.

Discussion:

A large number of nucleic acid extraction methods have been described in literature [14-17]. However, few studies have described an extraction method appropriate for the detection of Leishmania DNA in the whole blood samples. Most likely due to the relatively low levels of parasitemia, as well as to the difficulties usually encountered in DNA extraction from the blood samples.

In our experiments, the highest sensitively was obtained when the in-house method of extraction was employed for the detection of leishmania DNA in the frozen blood samples. The false negative results obtained by methods A, B, and C in higher dilutions of spiked samples could be due to the presence of inhibitors extracted along with the DNA, or to loss of DNA during the procedure. Employing the developed method of DNA extraction, we observed no false-negative results in patients with definite diagnosis of visceral leishmaniasis.

An important function of PCR optimization is to maintain the optimum concentration of Mg^{2+} within the sample reaction tube prior to PCR. Optimum concentration of

Mg^{2+} is essential for the activity of the thermostable enzyme Taq polymerase. Some lysis buffers such as the one used in our study contain a high amount of Mg^{2+} (i.e. 5 mM). The concentration of Mg^{2+} decreased during process of DNA extraction. However, at the end of the procedure a partial amount of this ion remained within the sample tube.

In our study, DNA was extracted from the whole blood by using cell lysate obtained by lysis buffer plus boiling. This is in contrast with other published methods that do not include such steps in their extraction method [11, 18]. This was in line with Nuzum et al [8] studies, in which mononuclear cells isolated from 8 µl of patients' blood was used. Although a high rate of sensitivity was reported (i.e. 90%), a large amount of blood was required, of the anemic children, which is a disadvantage for a DNA extraction method. In our method, only 0.5 µl of patients' whole blood was used.

Osman et al [11]. have used lysis buffer [50 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA; 1% ($^{vol}/_{vol}$) Triton X-100, 200 mg of proteinase K}] and phenol-chloroform for DNA extraction, from EDTA blood samples and from blood samples collected on filter paper. False negative results were found in 16 EDTA blood sample. The possible explanation could be due to the presence of inhibitors not removed by method of extraction, or to loss of DNA during the procedure.

In conclusion, our developed DNA extraction method for detection of Leishmania DNA which is based on the whole blood is simple and short to perform (i.e. maximum 45 min.) as compared to those by others [19,18,11,9,8]. Since hazardous materials such as phenol-chloroform are not used during the extraction procedure, it seems to be much safer than phenol-chloroform method. However, the use of small amount of sample and exclusion of PCR-inhibitors from the whole blood are the major advantages of our developed method.

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