Research paper

Determinationof the sensitivity and specificity of polymerase chain reaction (PCR) in the diagnosis of visceral leishmaniasis

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Sri Lankan Journal of Infectious Diseases 2014 Vol. 4 (1):22-29

DOI: http://dx.doi.org/10.4038/sljid.v4i1.6036

Key words: Visceral leishmaniasis, PCR, Sandfly.

Abstract

Introduction: Leishmaniasis is a disease caused by a protozoan parasite of the genus *Leishmania,* which is transmitted through bites of infected sandflies. It has been reported that the polymerase chain reaction (PCR) is a more sensitive and specific test for the diagnosis of visceral leishmaniasis than bone marrow examination. This recent study is a renewed effort to validate the role of PCR in the diagnosis of visceral leishmaniasis.

Objective: The objective of this study was to determine the sensitivity and specificity of PCR in the diagnosis of visceral leishmaniasis.

Study design: Cross sectional (validation) study carried out in the Haematology department, Armed Forces Institute of Pathology, Rawalpindi. from 25th March 2011 to 24th March 2012.

Subjects and Methods: A total number of 59 patients with visceral leishmaniasis diagnosed on microscopic bone marrow examination with equal number of negative controls were studied. The subjects were tested for the presence of visceral leishmaniasis by the polymerase chain reaction.

Results: All the 59 patients were found to be positive for visceral leishmaniasis by PCR. None of the negative controls were positive by PCR.

Conclusion: The study validates that PCR is equal to microscopic bone marrow examination in the diagnosis of visceral leishmaniasis.

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Introduction

Leishmaniasis is caused by parasites of the genus *Leishmania*, which are transmitted to humans from human or animal reservoirs by the bites of phlebotomine sandflies.[1] It is transmitted by the bite of infected sandflies, including flies of the genus *Lutzomia* in the New World and *Phlebotomus* in the Old World.[2,3] Although estimated as the ninth largest disease burden among individual infectious diseases, leishmaniasis is largely ignored in discussions of tropical disease priorities.[4] Visceral leishmaniasis is the most severe form in which parasites migrate to vital organs like the liver, spleen and bone marrow.[5] Patients usually present with unexplained fever, generalized weakness, weight loss, nausea and vomiting.[6] On examination, massive splenomegaly is frequently present along with hepatomegaly and lymphadenopathy. The annual incidence of visceral leishmaniasis worldwide is 0.5 million.[7] Of these, 90% cases occur in the Indian subcontinent and Sudan.[8] Other endemic regions are the Mediterranean and Africa. In Pakistan, visceral leishmaniasis is endemic in Azad Kashmir and some areas of Khyber Pakhtoonkhwa.[9] The peripheral blood film usually shows anemia, leucopenia and thrombocytopenia. Bone marrow examination by light microscopy is the gold standard for the diagnosis of visceral leishmaniasis in which *Leishmania donovani* bodies (LD bodies) are seen in the aspirate.[10] Other methods for the diagnosis of visceral leishmaniasis include Giemsa staining of splenic aspirate and lymph node biopsy by which amastigotes are easily identifiable. The sensitivity of the test can be increased by staining the specimen with fluorescent dye-tagged antibodies to the surface receptors of the parasite. Fluorescein isothiocyanate isomer- or rhodamide B isothiocyanate-conjugated antiserum is usually used for this purpose.[11] The polymerase chain reaction (PCR), over the last decade, have been shown to be both highly specific (100%) and sensitive (95%) for the diagnosis of visceral leishmaniasis. The most commonly used targets for primer design have been mini-exon-derived RNA genes, genomic repeats, ribosomal RNA genes and kinetoplastid DNA. The latter is still one of the most appealing targets for primer design due to the highly repetitive copies of minicircle DNA which is present at thousands of copies per cell. The identification of sequence element represented within the kinetoplast DNA (kDNA) of a given species of Leishmania has allowed the design of oligonucleotide primers for species-specific identification of parasites in clinical samples.[12] The use of PCR is therefore helpful in diagnosis of the disease.

Treatment of the disease has been based on pentavalent antimony drugs e.g. Sodium stibogluconate. As Human Immunodeficiency Virus (HIV) co-infected patients do not respond well to antimonials, Amphotericin B is the drug of choice. Other drugs used are paromomycin and miltefosine.[13]

This is the first study conducted in Pakistan to determine sensitivity and specificity of PCR in the diagnosis of visceral Leishmaniasis. Therefore, data presented here would be of value in the diagnosis of the disease.

Methodology

This study was completed in 12 months, from 25th March 2011to24th March 2012 at the Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan. Non-probability consecutive sampling technique was used. A total of 59 patients diagnosed with visceral leishmaniasis were studied. Patients of all ages and both genders referred to the AFIP were included. All patients with inadequate bone marrow sample, taking anti-leishmaniasis drugs and with diseases other than visceral leishmaniasis were excluded from the study.

Patients diagnosed with visceral leishmaniasis were selected from AFIP on designated days by consecutive sampling. An equal number of age and sex matched patients with negative visceral leishmaniasis on bone marrow examination were selected from AFIP as the control group. Informed consent was obtained and a proforma completed for all individuals highlighting the personal details (name, gender, age, and ethnicity), relevant clinical history and physical findings.

Specimen collection

After obtaining informed consent from the guardian (as all of our cases and control patients ranged from 03 months to 08 years of age), bone marrow aspirate (1-2ml) was taken for microscopy and PCR. Ethylenediamine tetra-acetic acid (EDTA) was used as an anticoagulant; at concentrations of 1.5 + 0.5 mg/ml. Bone marrow aspirate (0.5ml-1ml) was taken from the posterior iliac spine in patients above 02 years and from the tibia in patients below 02 years under strict aseptic conditions. Slides were air dried, stained with Leishman stain and examined microscopically for the detection of *Leishmania donovani* amastigotes (LD bodies).

Polymerase Chain Reaction (PCR)

PCR was performed using following techniques

(i) Cell lysis method with Tris – EDTA buffer

Specimen processing and DNA extraction

900µL of Red Blood Cells Lysis solution (Ammonium chloride, EDTA, Sodium Bicarbonate) was added to 300µL bone marrow aspirate, mixed well, left at room temperature for 03-04 minutes and centrifuged at 13000 rpm for 30 seconds. After discarding the supernatant, the pellet was mixed well and left at room temperature for 03-04 minutes. After vortexing for 15 seconds, 300µL White blood Cells lysis solution (Tris, EDTA, Sodium Dodecyl Sulphate) was added and mixed well. 100µL protein precipitating solution (Ammonium acetate) was then added and the mixture vortexed for 20 seconds and centrifuged at 13000 rpm for 01 minute. Isopropanol (300µL) was added, and inverted 50 times until DNA precipitated and a thread was seen. This was washed with 300µL 70% ethanol and centrifuged at 13000 rpm for 01 minute. Hydration solution was added to the pellet and incubated at 56°C for 15 minutes. The resultant DNA was stored at 04˚C.

(ii) Proteinase K Method (*http://www.diva-portal.org/smash/get/diva2:130963/FULLTEXT01.pdf*)
This method was used to extract DNA from bone marrow slides. A few drops of Lysis solution (Tris 50mmol, EDTA 20mmol, SDS 02%) was dropped on the slide and smear scrubbed off into an 1.5 ml eppendorf. 500-700µL lysis solution followed by 25-30 µL Proteinase K (20mg/ml) was added. The mixture was vortexed for a few seconds and then kept at 37°C overnight. 250µL phenol and chloroform were added, vortexed and centrifuged at 11000 rpm for 2 minutes. The precipitate was discarded and procedure repeated. 500µL chloroform was added and after . Vortexing and centrifugation at 11000 rpm for 2 minutes, the supernatant was transferred into another eppendorf and 150 µL ammonium acetate added. The tube was then filled with absolute ethanol, inverted 20-30 times until DNA precipitated and thread was seen and then centrifuged at 11000 rpm for 2 minutes. The supernatant was discarded and 1 ml absolute ethanol was added and centrifuged at 11000 rpm for 2 minutes. After discarding the supernatant, the eppendorf was kept at room temperature for 3-5 minutes. Hydration solution was added and the mixture incubated at 56°C for 15 minutes, vortexed and centrifuged for 15 seconds. The resultant DNA pellet was stored at 4˚C.

b) DNA amplification
PCR mix (dNTPs, MgCl2, KCl, Tris, gelatin, Spermidins 20µL / vial, Taq polymerase 0.5U / vial) and leishmania specific primers (STAT-NAT® Leishmania spp.) 1µL / vial were added to the DNA extract. PCR amplification included a first denaturation step of 2 minutes at 94˚C followed by 40 cycles of 94˚C for 1 minute, 45˚C for 1 minute and 72˚C for 1 minute in a thermal cycler which is used to amplify segments of DNA via PCR. Final extension was carried out at 72˚C for 3 minutes. Negative and positive controls were also applied. 6% PolyAcrylamide Gel Electrophoresis (PAGE) was then carried out. 50µL 10% APS (Ammonium Per Sulphate) and 10µL TEMED (Tetra Methyl Ethylene Diamine) were added in 5ml 6% polyacrylamide solution. This was mixed well and put into electrophoresis apparatus and kept at room temperature for 20 minutes. 3µL of amplified product was mixed with 3µL of bromophenol blue (tracking dye) and loaded into wells. Electrophoresis was carried out at 200V for 20 minutes. The gel was stained with 0.1% AgNO3 for 15 minutes followed by counterstaining with 1.5% NaOH mixed with formalin, and preserved on a filter paper. Visual assessment of the bands of visceral leishmaniasis of the patients was done by comparing with normal and positive controls.

The data was entered into SPSS version 14. The analyzed variables included numerical data like age, presence of hepatomegaly, splenomegaly for which mean and standard deviation were calculated. Percentages were used for qualitative data like gender, results of bone marrow and sensitivity and specificity for PCR.

Results

Demographics
A total of 59 patients diagnosed as having visceral leishmaniasis were enrolled in the study. Collectively, the age of the patients ranged from 03 months to 08 years. Mean age of the patients was 22 months. Out of the 59 patients, 26 were males (44%) and 33 were females (56%). Splenomegaly was present in 58 patients and hepatomegaly in 34 patients.

Polymerase Chain Reaction
PCR was done for of all the cases with bone marrow confirmed VL along with equal numbers of negative controls. All negative controls were negative by PCR as well and all of the patients positive on bone marrow examination were also positive by PCR.

Discussion

Leishmaniasis is a major public health problem which is endemic in many countries in the tropics and subtropics. About 350 million people are considered to be at risk of contracting the disease.[14] Leishmaniasis is considered to be a neglected emerging disease and one of the most important parasitic diseases. The disease occurs in varying presentations, from self limited and even self healing cutaneous form to fatal systemic disease. Visceral leishmaniasis, also known as kala-azar, accounts for an estimated 75000 deaths annually, with more than half of these occuring in Sudan and India.[15] In Pakistan, visceral leishmaniasis is endemic in Azad Kashmir, northern areas and Khyber Pakhtoonkhwa, while cutaneous leishmaniasis is prevalent in Baluchistan and Interior Sind.[16] Visceral leishmaniasis has the most number of fatalities and if left untreated can lead to death.

Clinically, a patient with visceral leishmaniasis presents with unexplained fever with massive splenomegaly and hepatomegaly.[17] Lymphadenopathy may also be present. The blood picture shows pancytopenia. Diagnosis is confirmed by demonstration of LD bodies on bone marrow examination which is the gold standard method for the diagnosis of visceral leishmaniasis. LD bodies can also be found in peripheral blood, splenic aspirate or lymph node aspirate but their sensitivity and specificity is less than bone marrow aspiration. *Leishmania donovani* parasites cause fibrosis of bone marrow, often resulting in a dry tap. Identification of *Leishmania donovani* bodies is therefore difficult due to an inadequate sample having a low number of parasites.

Fortunately, PCR has proved to be a useful method for the diagnosis of visceral leishmaniasis. It has almost 100% sensitivity and specificity.[18] This study was done to validate the PCR in the diagnosis of visceral leishmaniasis considering bone marrow examination as the gold standard. The result of this study has proved that PCR is equally useful to bone marrow examination for the diagnosis of visceral leishmaniasis. The study included 59 patients diagnosed with visceral leishmaniasis by bone marrow examination together with negative controls. All of the patients diagnosed on bone marrow examination were also positive by PCR, while all the negative controls were also negative. Thus, the study showed that PCR was 100% sensitive and specific for the diagnosis of visceral leishmaniasis.

Many studies have been done internationally on this subject. Our result is almost similar to those reported by Maurya R and colleagues (2005). Pretreatment peripheral blood from 101 patients with parasitologically proven (by splenic smear and/or culture) kala-azar, were included in their study of the PCR assay. Their results showed that 100 out 101 patients were positive by PCR while all parasitologically negative controls proven by splenic smear and / or culture (n=150) were also PCR negative.[12] Salotra P and colleagues (2001) also had similar results in a study

conducted in India. PCR was positive in 102 of 107 patients with 96% sensitivity. Peripheral blood was used instead of bone marrow or splenic aspirate as these were invasive procedures.[19]

Antinori *et al.* (2007) showed a 95.7% sensitivity using PCR on bone marrow aspirate samples of 68 patients with visceral leishmaniasis. None of 229 negative controls were positive by PCR showing 100% specificity.[7] Fraga TL *et al*. (2010) used peripheral blood of 40 patients and concluded that PCR of peripheral blood was 95.6% sensitive as compared to PCR of bone marrow aspirates (91.1%) and culture (26.7%).[20] Since our study is the first of this nature in Pakistan, no local studies are available on the subject for purpose of comparison of our results. Therefore, it is suggested that future studies on this subject should be done with sufficient time to allow the study of a larger sample, selected by randomization.

A prospective study on this subject like the one performed by Fraga TL et al. (2010)[20] in which they used peripheral blood as a tool for diagnosis must be undertaken so that painful and invasive procedures like bone marrow could be avoided. Moreover, among the different techniques of PCR, we found real time PCR had the highest benefit for the patient because it was both qualitative and quantitative. It could give the parasite load at the time of diagnosis and could monitor the treatment response allowing modification of treatment regimens when needed. Real time PCR could also help in detecting minimal residual disease.

As Leishmaniasis is still one of the major neglected parasitic diseases, health education by community participation has a vital role for the prevention of the disease. This capability is beneficial both in diagnosis of the disease and for prediction of the disease outcome keeping in mind the financial cost of PCR vs microscopy, since a negative PCR result at the end of treatment is likely to be associated with a favorable outcome. But it needs special funds in countries with resource constrains.

Limitations of the study

- The generalization of the results could have been affected by the use of convenient non probability sampling.
- PCR is an extremely sensitive method and therefore, contamination from non-template PCR present in the laboratory environment (e.g. bacteria, viruses) presents a problem when considering the results.

Conclusion

PCR is an equally sensitive and specific test to bone marrow examination in the diagnosis of visceral leishmaniasis.

Acknowledgement

We are thankful to the Armed Forces Institute of Pathology, Rawalpindi, for providing logistic and financial support required for testing our study subjects.

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