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Full Length Research Paper

The ability of T2/B4 primers to detect *Leishmania infantum* among peripheral blood of visceral leishmaniasis patients in Iran

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Leishmaniasis, caused by protozoa of the genus leishmania, is a zoonotic and anthroponotic disease that is endemic through the tropical and subtropical regions. Twelve million people are affected worldwide and 350 million are at risk. Visceral leishmaniasis in Iran is sporadic in almost all part of Iran and the endemic regions have increased in many districts throughout Iran, the Kalybar, Ahar and Meshkin-shahr districts in East Azarbaijan and Ardabil province in the Northwest of the country. Bone marrow aspiration or biopsy followed by demonstration of leishmania parasites by microscopic and/or cultural examination is the most common diagnosis procedure. These methods are invasive and high risk. The polymerase chain reaction (PCR) has been applied as an analytical method to reveal the presence of small numbers of parasites directly in clinical samples. A PCR-based protocol for the detection of *Leishmania infantum* parasites in blood was developed and tested with human samples taken from twenty three new visceral leishmaniasis cases referred from endemic area hospitals of Northwest Iran to Pediatric educational hospital of Tabriz University of Medical Sciences. Four different primer pairs were used which targeted genomic and kinetoplast DNAs. The results showed that the PCR assay's sensitivity was significantly dependent on the PCR primers used.

Key words: Visceral leishmaniasis, kenitoplast DNA-PRC, genomic DNA-PCR.

INTRODUCTION

Leishmaniasis, caused by protozoa of the genus leishmania, is a zoonotic and anthroponotic disease that is endemic through the tropical and subtropical regions (Desjeux, 1996). Twelve million peoples are affected worldwide and 350 million are at risk (Ashford et al., 1992). The incidence of new cases is estimated to be 1.5 to 2 million per year (Desjeux, 2004). Twenty-two species of leishmania have been reported to cause human infections (Kato et al., 2005). The clinical sign of Leishmaniasis is heterogenous and can be roughly classified into three major forms of increasing severity: cutaneous leishmaniasis (CL), mucocutaneous Leishmaniasis (MCL) and visceral leishmaniasis (VL). In the old world, *Leishmania donovani* and *Leishmania infantum* are responsible for visceral Leishmaniasis (Berman, 1997). Visceral leishmaniasis in Iran is sporadic in almost all part of Iran and the endemic regions were increased to as many as districts throughout Iran, as the Kalybar, Ahar and Meshkin-shahr districts in East Azarbaijan and Ardabil province in the Northwest (Mazloumi Gavgani et al., 2002a). VL is a severe systemic disease characterized by destructive infiltration of organs and high rates of fatality in symptomatic patients. Clinical manifestation includes fever, hepatomegaly and/or splenomegaly and pancyto-

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Table 1. Main characteristics of the four PCR methods used in the study.

PCR component	Primer set			
	A	B	C	D
$MgCl_2$(mM)	2	2.5	1.5	1.5
Taq DNA (IU)	1.5	1.5	1.25	1.25
Anneling temp (°C)	65	61	68	58

penia (Nadim, 1978). However, most *L infantum* infections in people are subclinical, and cause the development of a transient humoral response followed by a protective cell mediated immune response, but no clinical symptoms (Mazloumi Gavgani et al., 2002a).

These symptoms are very similar to those seen in other diseases and the laboratory confirmation is needed (mandatory) when the diagnosis is suspected. Bone marrow aspiration or biopsy followed by demonstration of leishmania parasites by microscopic and/or cultural examination is the most common diagnosis procedure (Siddig et al., 1989; Zijlstra et al., 1992). Although microscopic examination of bone marrow aspirate has been reported to be 62 to 93% sensitivity, it is an invasive method (Da silva et al., 2005) and isolation of parasites by culturing is time-consuming, expensive, and difficult (Weigle et al., 1987).

Serological tests show a high degree of cross-reactivity but cannot discriminate between past and current infections; also their sensitivity are low (Kar, 1995). In addition to the conventional microscopic, cultural and serological methods, numerous DNA-based tests have been described particularly the polymerase chain reaction (PCR) (De Bruijn et al., 1992; Piarroux et al., 1993; Rodgers et al., 1990; Uliana et al., 1991; Van Eys et al., 1989). Recently, using PCR for detection of leishmania DNA in peripheral blood has been shown to be sensitive and specific for diagnosis and follow-up of patients with VL (Lachaud et al., 2001; Martin-Sanchez et al., 2004; Lachaud et al., 2002). This study was designed to compare the different primers and their ability to identify parasite in peripheral blood of Iran endemic area patients using PCR.

MATERIALS AND METHODS

This study was performed in Pediatric Educational Hospital and Drug Applied Research Center of Tabriz University of Medical Sciences during 2006-2007. All patients provided written informed consent before participating in the study. Twenty-three VL patients confirmed with DAT and bone marrow aspiration examination tests. Patients were not receiving any pharmaceutical drugs before sampling (New VL cases). 5 ml of each patient blood was collected in sterile single use tube containing 200 µl EDTA 8% solution. Within 4 h mononuclear cells were separated from patients' blood using Ficoll-Paque (Pharmacia Biotech) and hank's buffer. Collected mononuclear cells were used to DNA extraction by standard method using proteinase K, SDS and CTAB. The DNA was precipitated with ethanol and re-suspended in 100 µl sterile distilled water. The DNA extraction was controlled by measurement of the optical density at 260 nm using spectrophotometer. Blood samples from healthy individuals were used as negative control. A negative control tube with 10 µl of sterile, redistilled water as an alternative of DNA were included in each test to detect any contamination. For positive control 10 µl extracted DNA from 10^6/ml promastigotes and for detection of sensitivity of each method, ten healthy individuals blood with different quantities of 72 h-old cultured *L. infantum* MON1 promastigots in RPMI 1640 supple-mented with 10% heat-inactivated fetal calf serum, were used. Four primer pairs were used to distinguish *L.infantum* in extracted DNAs. The target DNA in two pairs was genomic DNA (A and C) whereas in two others the target was kinetoplast DNA (B and D).

The samples were amplified by four different PCR assays, each one with a different set of primer pairs:

A. Forward Primer (5`- CAC CAC GCC GCC TCC TCT CT-3`) and Reverse Primer (5`-CCT CTC TTT TTT CNC TGT GC-3`) (Schönian et al., 1996),

B. Forward Primer (RV1) (5`-GTG GGG GAG GGG CGT TCT-3`) and Reverse Primer (RV2) (5`-ATT TTA CAC CAA CCC CCA GTT-3`) (Lachaud et al., 2002; Reale et al., 1999),

C. Forward Primer (T2) (5`-CGG CTT CGC ACC ATG CGG TG-3`) and Reverse Primer (B4) (5`-ACA TCC CTG CCC ACA TAC GC-3`) (Minodier et al., 1997; Piarroux et al., 1995) and

D. Forward Primer (Fme) (5`-TAT TGG TAT GCG AAA CTT CCG-3`) and Reverse Primer (Rme) (5`-ACA GAA ACT GAT ACT TAT ATA GCG-3`) (Marfurt et al., 2003).

Total reaction volume of each tube was 70 µl as described in Table 1 with different amount of sample DNA. The following conditions were used: initial annealing at 94°C for 5 min, 40 cycles of 94°C for 40 s, variable denaturation temperature according to the primer set (65 °C, 61 °C, 68 °C and 58 °C for A, B, C and D primers, respectively) for 40 s, and 72°C for 50 s followed by a final elongation at 72°C for 7 min. The reaction products were visualized under UV light after electrophoresis of 7.5 µl of reaction solution with 1.5 µl gel loading buffer in 1.5% agarose gel in 1× Tris-boric acid EDTA buffer and staining with 0.5 µg/ml ethidium bromide (Sambrook et al., 1989).

RESULTS

All positive controls with all primers sets (A, B, C and D) gave their specific band (Figure 1). Primer A could not detect the low quantities of parasites (less than 60 parasites).

Primer set A: The size of amplified fragment by this method was about 450-550 bp. From twenty-three patients samples only five samples (21.7%) gave specific band and eighteen samples were negative.

Primer set B: The size of amplified fragment by this method was about 120 bp. From twenty-three samples, six samples (26.1%) gave specific band and seventeen samples were negative. In positive controls artifactual bands were seen.

Primer set C: The size of amplified fragment by this method was about 250 bp. Eight genomic DNA (34.8%)

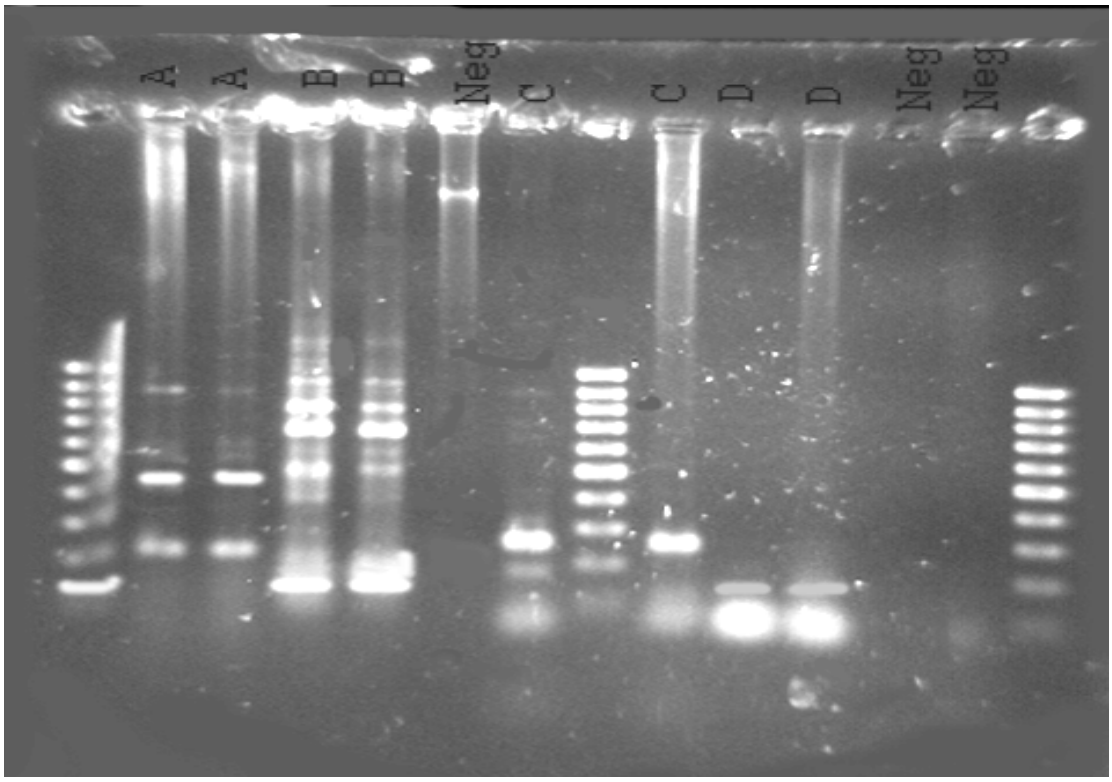

Figure 1. Agarose gel electrophoresis of amplified bands using different primer sets (A, B, C and D; see Materials and Methods) for the detection *Leishmania infantum* among peripheral blood of visceral leishmaniasis patients in Iran

from twenty-three samples gave specific band and fifteen samples were negative.

Primer set D: The size of amplified fragment by this method was about 110 bp. From twenty-three samples, sixteen of them (69.6%) gave specific band and seven samples were negative.

It should be noted that in all four primers bands were not seen in negative controls. Sensitivity of C and D primers for detecting low quantity of parasites in positive controls were more than others. All positive controls (with different concentrations of parasites) had their specific bands with B, C and D primers.

DISCUSSION

The leishmaniases are parasitic diseases that are caused by various species of the protozoon *Leishmania*. These species are endemic in many countries, and infection with these species can cause a wide variety of symptoms, depending on the parasite species (Lachaud et al., 2000).

There are up to half a million new cases of visceral leishmaniasis worldwide every year (Ashford et al., 1992). Visceral leishmaniasis (kala-azar) is a systemic disorder which is fatal if left untreated (Berman, 1997). In Iran, visceral leishmaniasis caused by *L infantum* is zoonotic and domestic dogs act as a principal reservoir host (Mazloumi Gavgani et al., 2000b; Mazloumi Gavgani et al., 2002a), and transmission is by the bite of a phlebotomine sand fly (Mazloumi Gavgani et al., 2007). The parasites can be isolated from either infected organs or lesions and can be cultivated *in vitro*. Several diagnostic tests, such as the indirect immunofluorescence antibody test (IFAT), the enzyme-linked immunosorbent assay (ELISA), microscopic examination of smears, and cultural isolation, are routinely used for diagnosis (Lachaud et al., 2000; Mohebali et al., 2004). The gold standard for VL diagnosis is the detection of parasite in specimens of infected organs. For this, samples that must be obtained by invasive procedures, such as bone marrow (BM), lymph node, or spleen aspirates, are typically needed (Vilaplana et al., 2004). None of the common methods routinely used for the parasitological diagnosis of VL is satisfactory: direct examination shows a poor sensitivity in most centers. *In vitro* cultivation has a good sensitivity but is carried out only in specialized centers, and if the results are negative or if the parasites are insufficient, the results can be obtained only after several weeks; and the specific serology is unreliable for immunocompromised patients and false negative results cannot be excluded. PCR has been shown to be as good as or better than these diagnostic methods, with the advantage that it provides a more rapid result. A number of PCR assays for the diag-

nosis of visceral leishmaniasis due to *L. infantum* (De Bruijn et al., 1992; Piarroux et al., 1993; Rodgers et al., 1990; Uliana et al., 1991) have been developed over the past few years. Blood was used as source of parasite for VL diagnosis in recent studies (Lachaud et al., 2001; Martin-Sanchez et al., 2004; Lachaud et al. 2002, Reale et al., 1999).

In this study, we use PCR as a diagnostic tool for visceral leishmaniasis. Four different kinds of primers using the patients' blood and seeded samples were compared. As the results show the best result was achieved by primer set D which was able to detect more than half of specimens. A and B primer sets did not give acceptable results for molecular detection of leishmania using blood samples as the author declared (Reale et al., 1999). In primer set C the target sequence of PCR amplification is localized at the 5′ end of the previously described repetitive DNA sequence (L42476) of the sequenced clone LA6 from *Pst*I-digested *L. infantum* MCAN/FR/73/LPMA/56 DNA (Piarroux et al., 1993) and showed high sensitivity (97%) and excellent specificity (100%) in other studies (Belli et al., 1998; Delgado et al., 1998; Eisenberg and Jaffe, 1999; Aviles et al., 1999). Primer set D amplified sequences from the gene for the spliced leader RNA (mini-exon). This target is present as tandem repeats (100 to 200 copies) in the genus *Leishmania* and other kinetoplastida, but is absent from the mammalian hosts and the sand fly vectors (Minodier et al., 1997). These primers were used previously by Fernandes et al. (1994) and same results were obtained. Our study showed that this primer set is more reliable and powerful for detection of *L. infantum* from patients in endemic region of North West Iran. We have to mention that using patients' blood to diagnose the disease in comparison with other methods like parasitological method (bone-marrow aspiration) has low risk for patients and the experiment can be repeated several times without any danger to patients.

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