SYBR green-based detection of *Leishmania infantum* DNA using peripheral blood samples

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Abstract Parasitological methods for the diagnosis of visceral leishmaniasis (VL) require invasive sampling procedures. The aim of this study was to detect *Leishmania infantum* (*L. infantum*) DNA by real time-PCR method in peripheral blood of symptomatic VL patient and compared its performance with nested PCR, an established molecular method with very high diagnostic indices. 47 parasitologically confirmed VL patients diagnosed by direct agglutination test (DAT > 3200), bone marrow aspiration and presented characteristic clinical features (fever, hepatosplenomegaly, and anemia) and 40 controls (non-endemic healthy control-30, *Malaria*-2, *Toxoplasma gondii*-2, *Mycobacterium tuberculosis*-2, *HBV*-1, *HCV*-1, *HSV*-1 and *CMV*-1) were enrolled in this study. SYBR-green based real time-PCR and nested PCR was performed to amplify the Kinetoplast DNA minicircle gene using the DNA extracted from Buffy coat. From among 47 patients, 45 (95.7 %) were positive by both nested-PCR and real time-

PCR. These results indicate that real time-PCR was not only as sensitive as a nested-PCR assay for detection of *Leishmania* kDNA in clinical sample, but also more rapid. The advantage of real time-PCR based methods over nested-PCR is simple to perform, more faster in which nested-PCR requires post-PCR processing and reducing contamination risk.

Keywords Real time-PCR · Nested-PCR · Visceral leishmaniasis · Peripheral blood · Human · Iran

Introduction

Visceral leishmaniasis (VL) is a major public health problem in Iran, with endemic areas including Ardabil and East Azerbaijan in the northwest and the provinces of Fars and Bushehr in the south (Mohebbali et al. 2005, 2011). The disease is frequently fatal if untreated, with almost 500,000 new cases per year and more than 50,000 deaths, so rapid and accurate

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diagnosis is important. Methods of diagnosis that promote early identification of the disease are required to quickly initiate therapy and prevent severe damage. The parasitological techniques performed on bone marrow aspirates that are generally used for detection and identification of VL lack sensitivity (direct examination) or are time consuming (culturing). These techniques require invasive procedures and are difficult to repeat for follow-up of patients. Serological methods such as the Direct Agglutination Test (DAT) have been used for the diagnosis of VL (Mohebbi 2013; Mohebbi et al. 2006). They are effective in detecting active leishmaniasis when large numbers of specific antibodies are present, but has a low sensitivity in immunocompromised patients (HIV-infected) and limited value in evaluating treatment effectiveness, since the antibodies remain detectable for an extended time after clinical cure (Alvar et al. 1997; Desjeux et al. 2003).

Diagnostic methods based on deoxyribonucleic acid (DNA) have made possible more sensitive and rapid detection, characterization of micro-organisms, and noninvasive methods (Noyes et al. 1998; Ghasemian et al. 2011). Over the past two decades, polymerase chain reaction (PCR) has proven a highly sensitive, highly specific alternative to traditional techniques. Several *Leishmania* DNA targets, including the minicircle of kinetoplast DNA (kDNA), internal transcribed spacer 1 (ITS1), and splice leader min-exon have been used for the diagnosis of leishmaniasis in human clinical samples (Castilho et al. 2003; Cupolillo et al. 1995; Marfurt et al. 2003; Victoir et al. 2003). Among the most suitable and sensitive targets for DNA-based diagnosis is the kDNA minicircle (0.8–1 kb in length), since it is present at 10,000 copies per cell and is heterogeneous in sequence and size (Noyes et al. 1998; Marfurt et al. 2003; Brewster et al. 1998).

Development of the SYBR-Green and TaqMan real-time polymerase chain reaction assay has provided sensitive, rapid, accurate, and reproducible diagnosis of VL (Mary et al. 2004; Nicolas et al. 2002; Bretagne et al. 2001; Bossolasco et al. 2003; Vitale et al. 2004). SYBR Green I is an intercalating dye that binds non-specifically to the minor groove of double-stranded DNA. When the dye binds, fluorescence increases over 100-fold, and a specific detection level may be achieved by melting curve analysis (Kubista et al. 2006). Real-time PCR enables elimination of the laborious post-amplification stage (gel electrophoresis preparation) conventionally required for the observation of the amplified product and reduces time required for analysis as well as the risk of contamination.

In the present study, a real-time PCR assay based on SYBR Green I DNA-binding fluorophore dye was used for detection of *L. infantum* DNA in human peripheral blood, and the findings was compared to nested-PCR results. This is the first report of the use of SYBR-green based real-time PCR to detect *L. infantum* DNA in human blood in Iran.

Materials and methods

Patients and controls

Peripheral blood samples were obtained from 47 patients at the Tehran University of Medical Sciences Hospital with confirmed VL. The patients presented characteristic clinical features (fever, hepatosplenomegaly, and anemia) consistent with the parasites in bone marrow aspirate detected by microscopy, Novy-MacNeal-Nicolle (NNN) culture, and positive serology (DAT $\geq$ 3200). Patient age ranged from 7 months to 7 years old with 28 males (60 %) and 19 females (40 %).

As a control group, blood samples from 30 healthy individuals with no history of VL were analyzed. In addition to *Leishmania*, 10 controls from other disease (*Toxoplasma gondii*, *Plasmodium falciparum*, *P. vivax*, *Mycobacterium tuberculosis*, along with viruses *HBV*, *HCV*, *HSV*, and *CMV*) were tested for to determine specificity of the real-time PCR assay. This study was reviewed and approved by the Tehran University of Medical Sciences Ethics Committee and all subjects provided written informed consent.

Microscopy

Bone marrow samples obtained from VL patients were placed on a glass slide, allowed to dry, fixed in methanol, stained with Giemsa in PBS, and examined microscopically at 1,000 $\times$ for *Leishmania* parasites.

Serology

Leishmania antibodies were detected by DAT conducted as described by (Mohebbi et al. 2011, 2006). In brief, DAT antigens were prepared from *L. infantum* promastigotes at the protozoology unit of the School of Public Health at the Tehran University of Medical Sciences. Sera were diluted in 0.9 % saline containing 0.8 % mercaptoethanol. A two-fold dilution series of sera was made, from 1:100 to 1:102,400. Fifty μ l of antigen was added to wells containing 50 μ l of diluted serum and incubated overnight. The cut-off value was defined as the highest dilution at which agglutination was visible as a blue dot compared to negative control wells, which had clear blue dots. Specific *Leishmania* antibodies $\geq$ 1:3,200 were considered positive (Harith et al. 1989).

DNA preparation

Total genomic DNA was extracted from the buffy coat layer obtained from 2 to 5 ml whole peripheral blood

collected in EDTA using the QIAamp DNA Blood mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Following the centrifugation and washing steps, DNA was eluted from the spin column in 100 µl elution buffer and stored at -20°C before use in the PCR-based assay.

The concentration and purity of extracted DNA were assessed by the ratio of the optical density at 260 and 280 nm. Five µl portions of the extracted DNA were used for PCR amplification.

Leishmania nested-PCR-based Assay

The variable segments on minicircles of kinetoplast DNA from *L. infantum* were amplified with two rounds of nested PCR (Noyes et al. 1998). The primers CSB1XR and CSB2XF for the first round, and LiR and 13Z for the second, were used to amplify the variable segments on minicircles of kDNA from *Leishmania* parasites. Each 25 µl, first-round reaction mixture contained 2 µl template DNA, 0.2 mM each dNTPs (Roche, Penzberg, Germany), 1.5 mM MgCl_2 , 1.0 U Taq polymerase, 50 mM Tris-HCl (pH 7.6), 20 pmol of each of CSB1XR and CSB2XF primers. Reactions were cycled in a thermocycler (Corbet Research) under the following conditions: 95°C for 5 min; 35 cycles of 30 s at 95°C , 1 min at 59°C , and 45 min at 72°C ; and a final extension of 5 min at 72°C . The product was diluted 1:9 with ultrapure water, and 1 µl was used as the template for the second round of PCR, which used the same conditions and reaction mixture as the first round, except that LiR and 13Z were used as the primers. Amplicons (PCR products) of the second round were analyzed on 2 % (w/w) agarose gel under UV light. DNA extracted from promastigote cultures of reference strain of *L. infantum* (MHOM/TN/80/IPT1), was run on each gel as positive controls. Negative controls (the products of PCR in which ultrapure water replaced the template DNA) were also run.

An internal control (β -globulin housekeeping gene) was used to ensure that negative results were genuine rather than reflecting PCR inhibition, DNA loading, sample degradation, or absence of human cells.

Real-time PCR assay

A 145 bp fragment of kDNA from *L. infantum* was amplified by SYBR-Green based real time-PCR using specific primers RV1 and RV2. The forward primer RV1 (CTTTTCTGGTCCCTCCGGGTAGG) and reverse primer RV2 (CCACCCGGCCCTATTTTACACCAA) were designed to target conserved DNA regions of the

minicircle of kDNA from *L. infantum*. The amplification was carried out using an ABI StepOne instrument (Applied Biosystems). The PCR reaction was performed in white PCR plates on a volume of 25 µl with 1X Power SYBR Green Master Mix (Applied Biosystems), 5 pmol of each *Leishmania*-specific primer, and 50 ng of the DNA sample. The thermal cycler was settled with two holds pre-incubated at 50°C for 2 min for uracyl-*N*-glycosylase activation and 95°C for 10 min for Taq DNA polymerase activation followed by 40 cycles of amplification of 20 s at 95°C , 20 s at 58°C , and 20 s at 72°C . Fluorescence readings were taken during the extension step (72°C). Following threshold dependent cycling, the melting curve analysis was performed under the following conditions: an initial denaturation step at 95°C for 15 s followed by 1 min at 55°C and continuous heating at $0.1^{\circ}\text{C}/\text{second}$ to 90°C . The melting temperature (T_m , temperature at which a given DNA fragment exists as 50 % each double- and single-stranded) was defined as the peak of the curve. If the highest point was a plateau, the midpoint was identified as the T_m . Calculation of the T_m for each amplicon was done by the equipment software.

Each assay was repeated at least 3 times, and non-template negative control (NTC- H_2O) and positive-control (*L. infantum* reference strain) were included in all amplifications.

Fig. 1 Results of electrophoresis of the products of the nested-PCR-based amplification of DNA extracted from whole blood. The eight lanes contained a molecular-weight 'ladder' (lane 1), test samples identified as *L. infantum* (lanes 2, 3, 4, 5 and 6), the products from reference strains of *L. infantum* (lane 7), and a negative control (lane 8)

Results

Results of PCR

Blood samples from 47 patients with clinical symptoms of VL were examined by nested-PCR and real-time PCR in the Tehran University of Medical Sciences, Faculty of Medicine, Parasitology Department. Of forty-seven patient blood samples chosen for nested-PCR and real-time PCR detection of *Leishmania* DNA, 45 tested positive. The optimal diagnostic sensitivity and specificity of the blood-based PCR assays were 95.7 and 100 %, respectively. *Leishmania infantum* in nested-PCR was detected from the amplification of a 680 bp long sequence (Fig. 1). All of the positive samples in nested PCR were also positive by real time-PCR. We compared the sensitivity and specificity between real time-PCR and nested PCR assessed by the McNemar test. In our study the results indicated no significant difference (P value = 0.29). As all the controls

were negative by both the methods, the McNemar test was not performed for specificity.

Analytical sensitivity and specificity of the PCR assay

The sensitivity of the assay was determined by spiked defined number of cultured promastigotes to the negative *leishmania* blood sample. Tenfold serial dilutions ranging 10,000–1 parasites per ml blood were used to assess the sensitivity of the SYBR-Green based *Leishmania* assay and nested-PCR. The limit of detection tow assay was 1 parasite in a 1 ml blood sample corresponding to 0.025 parasites per reaction (Fig. 2), while non-spiked control blood samples remained negative. A linear standard was obtained with correlation coefficient r^2 of 0.999 (Fig. 3).

There was no signal for the non-*Leishmania* microorganisms tested with this assay, no blood specimen from a healthy donor tested positive (data not shown).

Fig. 2 Analytical sensitivity of DNA amplification assays for *L. infantum*. All assays were performed with DNA extracted from peripheral blood samples experimentally seeded with *L. infantum* promastigote. Lane M, 100 bp DNA ladder marker (Roche Applied Science); lane 1–7, reactions contained 10^5 , 10^4 , 10^3 , 10^2 , 10^1 , 10^0 ,

and 10^{-1} cultured promastigote of *L. infantum* per 1 ml blood, respectively; lane 8, negative control (PCR grade water). **a** Nested-PCR reaction with *L. infantum* DNA. **b** Real-time PCR reaction with *L. infantum* DNA

Fig. 3 Standard curve with various concentrations obtained with SYBR Green 1 based real-time PCR

Typical dissociation curves representing specific T_m generated by the kDNA amplified products of *L. infantum* in VL patients are shown in Fig. 4. The average peak T_m was 80.77 ± 0.2 °C from the corresponding positive control. Any amplification not demonstrating this melting temperature was excluded as a false amplification. The RV1 and RV2 primers, which were specific for kDNA *L. infantum*, resulted in no product in the negative sample (H_2O) and no non-*Leishmania* organisms were detected by this assay (data not shown).

Discussion

Accurate and sensitive procedures for diagnosis of VL are required for the design of an appropriate program for the control of the disease and effective treatment to reduce the morbidity and mortality rate. Currently, the diagnosis of

VL is based on clinical features, parasitological techniques, and detection of anti-*Leishmania* antibodies (Sundar et al. 2002). Visceral leishmaniasis detection using traditional methods is hampered by necessity for invasive procedures, low sensitivity, and the extended time to obtain results. PCR for the detection of *L. infantum* DNA in peripheral blood is a non-invasive alternative for diagnosis offering enhanced sensitivity, and is particularly useful when disease is suspected in patients with negative parasitological results (Reithinger et al. 2007; Fraga et al. 2010).

In the present study, the sensitivity of real-time PCR and nested-PCR was 95.7 %, and the specificity was 100 %. The result of the present study showed comparable efficiency of the real time-PCR assay to the nested PCR, which is being used widely for the diagnosis of leishmaniasis. The existence of one (4.3 %) false-negative could have resulted from factors such as the technique employed for sample extraction, the presence of inhibitors,

Fig. 4 Typical dissociation curve of the amplification products from *L. infantum* showing peak at 80.77 °C and from two negative samples

or the degradation of DNA, particularly kDNA, which is more readily degraded than nuclear DNA (Prina et al. 2007). The kDNA nested-PCR and real-time PCR presented the same analytical sensitivity level (1 parasite/ml) and specificity. The RV primers allow the detection of a single parasite per ml but do not amplify non-*Leishmania* DNA. The assay was considered to have good specificity.

PCR-based assays of kDNA are currently the most sensitive methods for the diagnosis of both visceral and cutaneous leishmaniasis (Bensoussan et al. 2006). This high sensitivity is due to the elevated number of the target kDNA minicircles, approximately 10,000 copies per parasite genome.

The samples tested in the present study were previously found positive by traditional diagnostic methods (microscopy of bone marrow aspiration, culture, and/or serology), before being subjected to testing with this assay. This study demonstrated the value of a real-time PCR assay based on SYBR Green dye for the detection of *L. infantum* as an alternative molecular method for the diagnosis of VL.

The real-time PCR assay was sufficiently sensitive to detect as little as 0.025 parasites per reaction in patient peripheral blood. A slope in the range of -3.6 to -3.1 ,

which correlates to an amplification efficiency of 100 %, indicates that the number of amplified molecules doubles with each cycle of PCR (Kubista et al. 2006). In the present study a slope of -3.02 was obtained, demonstrating high efficiency (Fig. 4).

Real-time PCR is a relatively new advancement for the detection, identification, genetic characterization, and quantification of *Leishmania* parasites (Mary et al. 2004; Wortmann et al. 2005). The main advantages of Real-time PCR over nested-PCR are that (i) it is rapid, with results of the assay after DNA extraction often obtained within 2 h, versus a minimum of 8 h with nested-PCR; (ii) it is far less labor intensive, with one PCR step and no gels to run compared to at least two step PCR and a required gel run for nested-PCR; (iii) there is minimal risk of contamination, as PCR is performed in a closed system during the entire process, and post PCR processing is not required; and (iiii) it is a completely automated assay, and amplicons are identified by their melting temperatures.

This technique has been widely reported for use in the diagnosis of VL, cutaneous leishmaniasis (CL), and a variety of other diseases, including malaria and *Cryptosporidium* infection (Khosravi et al. 2012; Perandin et al. 2004; Soliman and Othman 2009).

A real-time PCR based on SYBR green has been used for identification of the CL agent using Giemsa-stained slides samples from 102 confirmed cases. This technique was found to be a suitable tool for direct diagnosis and characterization of *Leishmania* species (Khademvatan et al. 2011).

Since the symptoms and clinical features of febrile illnesses such as malaria, typhoid, and enteric fever are similar to those of VL, and given that early diagnosis is mandatory in reducing VL mortality and morbidity rates, the use of molecular assays for diagnosis is warranted. Among the available methods, real-time PCR is the most effective, as it is sensitive, simple, and non-invasive. Detection is possible approximately 1 h and 30 min after DNA extraction, it is effective for samples containing a small number of parasites, and it is possible to estimate the parasite load from a clinical sample.

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