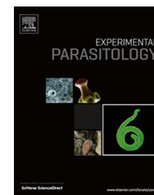
Experimental Parasitology 134 (2013) 206–210

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr



Optimization of single-tube nested PCR for the diagnosis of visceral leishmaniasis

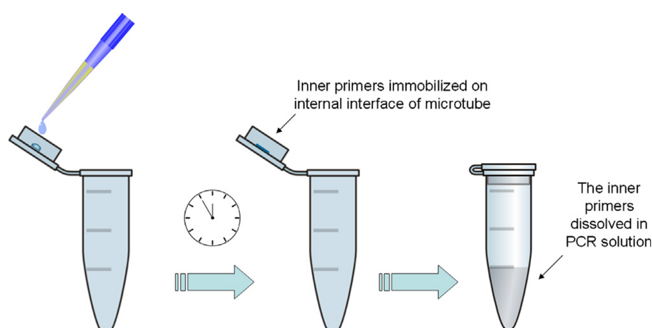
Maria Almerice Lopes da Silva *, Cynthia Regina Pedrosa Soares, Rafael Acioli Medeiros, Zulma Medeiros, Fábio Lopes de Melo

Laboratório de Doenças Transmissíveis, Centro de Pesquisas Aggeu Magalhães – Av. Professor Moraes Rego s/n, Campus da UFPE – Cidade Universitária, Recife, Pernambuco 50670-420, Brazil

HIGHLIGHTS

- We proposed to optimize a single-tube nested PCR (STNPCR) method.
- We compared the performance of STNPCR and nested PCR for detecting *Leishmania chagasi* DNA.
- The detection limit for *L. chagasi* DNA was 1 fg to nested PCR and 10 fg to STNPCR.
- STNPCR is a promising tool for diagnosing visceral leishmaniasis.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 July 2012
Received in revised form 1 March 2013
Accepted 2 March 2013
Available online 16 March 2013

Keywords:
PCR
Diagnosing
Visceral leishmaniasis

ABSTRACT

Conventional nested PCR is a very sensitive and specific method for the diagnosis of visceral leishmaniasis. However, this type of PCR is notorious for contamination problems related to the processing of the product between the first and the second PCR steps. In order to have a PCR method that is just as efficient but without the risk of contamination, we attempted the optimization of a single-tube nested PCR (STNPCR) method. During the first and the second PCR steps, we used the small subunit of ribosomal RNA (ssu rRNA) and the ribosomal internal transcribed spacer (ITS) as targets, respectively. The performances of STNPCR and nested PCR in detecting the DNA of *Leishmania chagasi* were compared. In the case of STNPCR, the inner primers were immobilized on the interior of the tube cap by means of adsorption microtubes and then were solubilized before the second reaction. This procedure eliminated the need to open the microtube, which could have led to false-positive results through cross-contamination. The detection limit for the purified *L. chagasi* DNA was 1 fg by using nested PCR and 10 fg by using STNPCR. We also tested the specificity of the system against other parasites, and observed that *Trypanosoma cruzi* DNA was amplified with a detection limit of up to 1 pg. This study not only presents a promising tool for the diagnosis of visceral leishmaniasis, but also provides a new tool for the diagnosis of Chagas disease, either in mono-infection by *T. cruzi* or in co-infection with *Leishmania* spp.

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1. Introduction

Visceral leishmaniasis (VL) is a systemic protozoan disease caused by species in the *Leishmania donovani* complex that affects thousands of people worldwide (Schönian et al., 2011). Since VL, if untreated, causes high mortality percentages, early diagnosis enables a better prognosis for the patients. Since the methods commonly used for the diagnosis are imperfect, achieving a sensitive, specific, simple, rapid, and low-cost method to date remains a challenge (Arora et al., 2008).

* Corresponding author. Address: Avenida José Américo de Almeida, 151, 301 G, Macaxeira, Recife – PE 52090320, Brazil. Fax: +55 8121012671.
E-mail addresses: almerice_lopes@yahoo.com.br (M.A.L. da Silva), cynthiaregina@msn.com (C.R. Pedrosa Soares), rafacioli@hotmail.com (R.A. Medeiros), medeiros@cpqam.fiocruz.br (Z. Medeiros), fflmelo8@hotmail.com (F.L. de Melo).

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http://dx.doi.org/10.1016/j.exppara.2013.03.003

Many laboratory assays are available for the diagnosis of VL. The gold standard for diagnosis consists of the visualization of the amastigote form of the parasite through examination under a microscope of aspirates obtained from lymph nodes, bone marrow, or the spleen. Although the specificity of microscopy is high, its sensitivity varies depending on the organ examined and the parasite load (Hailu et al., 2005; Silva et al., 2005; Sundar and Rai, 2002). Furthermore, aspiration is an invasive procedure and, thus, often unsuitable (Boelaert et al., 2007).

Immunoassays also have limitations. The indirect fluorescent antibody test (IFAT) has low specificity, presents cross-reactions, and requires trained personnel (Sundar and Rai, 2002). The direct agglutination test has good sensitivity and specificity, and it is characterized by a better performance than the IFAT, but it requires equipment such as microtiter plates and micropipettes, well-trained laboratory technicians and regular quality controls (Chappuis et al., 2007; Gontijo and Melo, 2004). The rK39 immunochromatographic test (ICT) is considered to be excellent method for field application, but it shows cross-reactivity with malaria, typhoid and tuberculosis (Chappuis et al., 2006).

The latex agglutination test (KAtex) has recently been introduced by the Kalon Biological Ltd for the detection of *Leishmania* antigens in the urine of patients affected with active VL (Sarkari et al., 2001). In some studies, this test has shown sensitivity of 68–100% and specificity of approximately 64–100% (Salam et al., 2011; Diro et al., 2007; Sundar et al., 2005; Rijal et al., 2004; El-Safi et al., 2003). KAtex performed better than any other serological test, taking microscopy to be the gold standard. However, a few KAtex-negative results may be found in the cases of parasitologically confirmed VL patients, representing a limitation of this test with regard to the detection of low levels of urinary *Leishmania* antigens (Salam et al., 2011; El-Safi et al., 2003).

Various PCR systems have been developed to identify *Leishmania* spp, and many targets and primer sequences have been described (Aoun et al., 2009; Cruz et al., 2006; Disch et al., 2004; Schallig et al., 2001; Lachaud et al., 2000). Many studies have shown excellent correlations between PCR results and parasitological and serological tests. Thus, PCR is a viable alternative when the tests discussed above are negative or indeterminate (Alam et al., 2009; Antinori et al., 2007; Cruz et al., 2006). Nested PCR involves two sets of primers, used in two successive PCR runs, where the aim of the second run is to amplify the secondary target within the product of the first run (Dupin et al., 2002). However, false positives caused by cross-contamination are a distinctive problem of this technique. In fact, cross-contamination may occur during the opening of the reaction tube for the transfer of the "amplicons" that serve as the template for the second amplification step.

The single-tube nested PCR (STNPCR) method, developed by Abath et al. (2002), involved the immobilization of the inner primers onto the inside of the microtube cap, thereby reducing the risk of contamination during microtube manipulation. STNPCR has already been standardized for detecting *Schistosoma mansoni* (Abath et al., 2002), *Plasmodium falciparum* (Montenegro et al., 2004), *Yersinia pestis* (Souza et al., 2007) and for identifying dengue virus serotypes (Gomes et al., 2007).

In the present study, we developed an alternative to nested PCR tests, in which nested PCR is performed in a single step, thus eliminating the step of opening the tube and therefore the risk of contamination.

2. Materials and methods

2.1. Genomic DNA preparation

Promastigotes of *Leishmania chagasi* (MHOM/BR/1974/PP75) were grown in Schneider's medium and were used to obtain the genomic DNA to assess PCR systems. DNA was extracted from the culture using the GenomicPrep tissue DNA isolation kit (GE Healthcare) in accordance with the manufacturer's instructions. The extracted DNA was quantified by measuring the absorbance at 260 nm. A dilution series of standard DNA (0.05–1 ng) was used to evaluate the limit of detection of the systems. Two µL of isolated DNA were added to a PCR mixture.

2.2. Simple-PCR

Two PCR assays were performed, and each amplified different parts of *Leishmania* ribosomal repeats: (i) part of the ssu rRNA gene by using primers R221 (5′ GGTTCCTTTCCTGATTTACG 3′) and R332 (5′ GGCCGGTAAAGGCCGAATAG 3′) (Van Eys et al., 1992); and (ii) ribosomal internal transcribed spacer 1 (ITS-1), separating the genes coding for ssu rRNA and 5.8S rRNA, by using primers LITSR (5′ CTGGATCATTTTCCGATG 3′) and L5.8S (5′ TGATACCACTTATCGCACTT 3′) (El Tai et al., 2001, 2000). Amplification reactions were performed in volumes of 50 µL. For ssu rRNA PCR, 1.0, 2.0, 3.0, 5.0, and 10.0 pmol of primers were tested. The optimum Mg^{2+} concentration was also evaluated using varying concentrations of $MgCl_2$ (1.5–4.0 mM). The amplification reactions were performed using 2.5 µL of enzyme buffer (100 mM Tris–HCl, pH 8.4, and 250 mM KCl) , 270 mM of dNTP and 2.5 U Taq DNA polymerase (Platinum® - Invitrogen). For ITS-1 PCR, amounts of 25.0 and 50.0 pmol of primers and 1.5, 2.5, and 4.0 mM concentrations of $MgCl_2$ were tested. The amplification reactions were performed using 2.5 µL of enzyme buffer (100 mM Tris–HCl, pH 8.4, and 250 mM KCl), 250 mM of dNTP and 2.5 U Taq DNA polymerase.

The cycling protocols described by Schönian et al. (2003) were adopted: ssu rRNA-PCR consisted of initial heating at 94 °C for 3 min, 38 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 2 min; ITS-1-PCR consisted of initial heating of 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 40 s, annealing at 53 °C for 45 s, and extension at 72 °C for 1 min. Both PCRs ended with a final extension at 72 °C for 6 min. The assays were carried out in triplicate using the same Mastercycler Gradient thermocycler (Eppendorf, Hamburger-Germany). In all the experiments, negative controls were also run.

2.3. Two-step nested-PCR and STNPCR

After optimization of the two simple PCRs, conventional two-step nested PCR was performed on 2 µL of the previous ssu rRNA-PCR products, using the same PCR conditions. For the first reaction, the nested PCR condition was optimized by using 5 and 10 pmol of primers and 2.5 mM Mg^{2+}. In the case of the second reaction, only 50.0 pmol of the inner primers was tested. Several negative controls that contained water instead of DNA were also amplified in each PCR experiment.

To develop the STNPCR method, 10 µL containing 50 pmol of inner primer with traces of bromophenol blue was previously immobilized onto the inside of the microtube cap by incubating the tubes at 37 °C until the solution had dried. The outer primer was added to the reaction mixture. To define the optimal inner:outer primer ratio, 2.0, 3.0, 5.0, and 10.0 pmol of primers were tested in reaction mixture. Negative control samples containing only outer primer, only inner primer, and both primers were also run.

The first round of amplification consisted of initial heating at 94 °C for 3 min, 15 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 2 min. After approximately 1 min of incubation at 95 °C, the tubes were inverted several times to dissolve the inner primer, and returned to the thermocycler for the second round of amplification that consisted of 35 cycles of denaturation at 95 °C for 40 s, annealing at

53 °C for 45 s, extension at 72 °C for 1 min, and a final extension step of 6 min at 72 °C.

2.4. Specificity assay

The specificity test was executed using genomic DNAs from *Leishmania major*, *Leishmania amazonensis*, *Leishmania braziliensis*, *Wuchereria bancrofti*, *S. mansoni*, *Trypanosoma cruzi*, and *Mycobacterium tuberculosis*.

2.5. Electrophoresis

Ten microliters of the PCR products was analyzed on 2.0% agarose gels. Gels were stained with ethidium bromide or blue–green-fluorescent labeling DNA. The DNA bands were visualized with an ultraviolet light transilluminator and photographed.

3. Results

3.1. Simple PCR

The best sensitivity of the assays for which the R221/R332 primers were used (>3 pmol, 2.5 mM Mg^{2+}) was 1 pg of genomic DNA from *L. chagasi*. Thus, we decided to optimize the nested PCR protocol by using 5 and 10 pmol of primers in the presence of 2.5 mM Mg^{2+}. While 2 pmol of primers yielded a sensitivity of 10 pg, no amplification occurred when only 1 pmol of primers was employed. n the case of ITS-1 PCR, in the presence of 2.5 mM Mg^{2+} the sensitivity was 10 fg, irrespective of whether 25.0 or 50.0 pmol of the inner primers were employed.

3.2. Conventional nested PCR and STNPCR

There was no difference in the DNA detection limit from *L. chagasi* when 5 or 10 pmol of the R221/R332 primers was used for the nested PCR of 50 pmol of LITSR/L5.8S. Both nested PCRs achieved a maximum of 1 fg of amplified DNA. Fig. 1 shows the detection limit of the nested PCR with 10 pmol of the outer primer.

When testing the STNPCR, a detection limit of 10 fg was achieved by using both 50:2 and 50:3 pmol ratios between the inner and the outer primers. In the case of the systems corresponding to 50:5 and 50:10 pmol ratios, only 1 pg of *L. chagasi* DNA was amplified.

3.3. Specificity assay

ssu rRNA-PCR did not show any amplification with organisms other than *L. chagasi* and *L. braziliensis*. However, ITS-1-PCR amplified the DNA from *L. major*, *L. amazonensis*, *L. braziliensis* and *T. cruzi*. Products of approximately 300–350 bp were observed for *L. major, L. braziliensis* and *L. amazonensis* and 700 bp for *T. cruzi* (Fig. 2). A dilution series of standard DNA (1–0.05 fg) was used to evaluate the detection limit of the system. ITS-1 PCR amplified up to 1 pg of DNA from *T. cruzi* and up to 100 fg of DNA from both *L. major, L. braziliensis* and *L. amazonensis.*

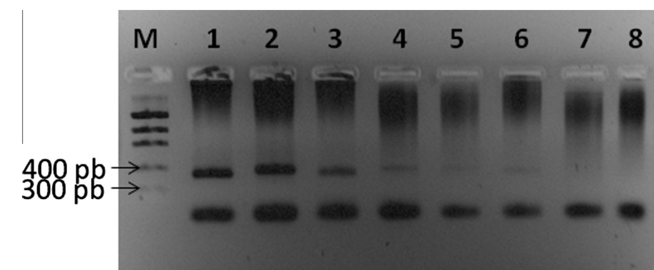

Fig. 1. Nested-PCR (5 pmol/50 μL of external primers, 50 pmol/50 μL of internal primers) analysis of ITS-1 regions (350 pb) amplified from dilution curve of DNA *Leishmania chagasi*: Lanes 1–7: 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 1 fg, 0.1 fg. Lane 8: negative control. A 100 bp ladder was used as molecular size marker (M)″.

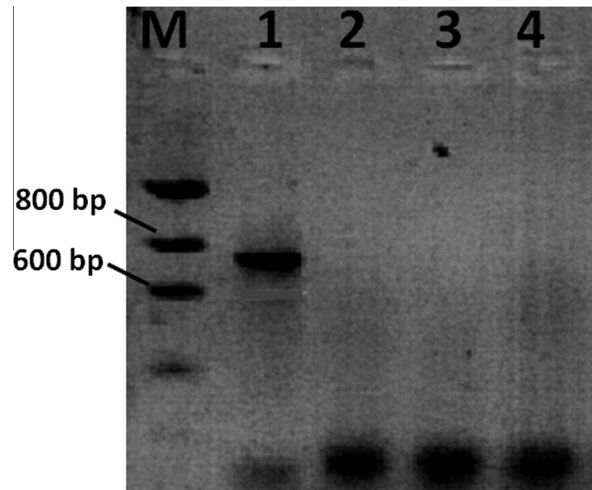

Fig. 2. ITS-1-PCR analysis (50 pmol/50 μL of internal primers): Lane 1: *T. cruzi.* Lane 2: *W. bancrofti.* Lane 3: *S. mansoni.* Lane 4: *M. bacterium.* A high mass DNA ladder was used molecular size marker (M)″.

4. Discussion

Investment in diagnostic procedures has been recognized as necessary for reducing the morbidity and mortality of visceral leishmaniasis, as well as for reducing the risk of its transmission (Andrade et al. 2006). PCR is an attractive alternative to traditional methods for the diagnosis of VL in virtue of its sufficient sensitivity, specificity, and rapidity in meeting the needs of control programs.

The primer pair R221/R332, which was constructed by Van Eys et al. (1992), has been reported to provide the amplification of ssu rRNA. In comparison with direct detection of parasites and serological tests, the use of the various PCR protocols that employed these primers produced good results (Salam et al., 2009; Antinori et al., 2007; Stark et al., 2006; Doncker et al., 2005; Pizzuto et al., 2001). Unlike the previous studies, when we tried to optimize the reaction mixture by using smaller amounts of primers (2–10 pmol), we obtained a sensitivity of only 1 pg of genomic DNA from *L. chagasi*. This modification was designed to optimize the STNPCR method. Preliminary tests with 50 pmol of each primer in the same solution reduced the performance of PCR. This is probably because the two pairs of primers have the same annealing temperature. Additionally, the inner and outer primers would compete for the same targets in the genomic DNA. However, using the conventional nested PCR, a good sensitivity was achieved. There was no difference when using 5 or 10 pmol of the external primer.

Several studies have exploited the amplification of the ITS-1 region for identifying *Leishmania* spp. in bone marrow aspirate or Giemsa-stained smears (Roelfsema et al., 2011; Alam et al., 2009; Amro et al., 2009; Kazemi-Rad et al., 2008; Schönian et al., 2003). The nested PCR is composed of two single PCRs. This setting increases the sensitivity of the assay by diluting potential inhibitors. In addition, this technique improves the specificity by amplifying DNA regions that are included in the product of the first reaction (Schönian et al., 2003). The nested PCR is composed of two single PCRs. This increases the sensitivity of the assay because potential inhibitors are diluted. It also improves the specificity because

amplifies the regions that are internal to the product of the first reaction (Dupin et al., 2002). However, false positives may occur during the opening of the reaction tube for the transfer of the "amplicons" that serve as the template for the second amplification step.

Good results were obtained with the application of PCR in a single tube with inner and outer primers with different annealing temperatures for the diagnosis of pathogenic microorganisms (Minarovičová et al., 2009; Bertolini et al., 2003; Gookin et al., 2002). However, when the two primer pairs are mixed in the same solution, the occurrence of non-specific bands is increased. On the other hand, when the internal primers are physically separated by the use of resins or external components, the enzymatic reaction could be affected (Tao et al., 2004). For these reasons, Abath et al. (2002) developed a new variant of STNPCR in which the sequestration of inner primers consisted of immobilizing them on the internal interface of the reaction cap. This new form of PCR is simple to perform, reproducible, and robust. Thus, this approach was used in our optimization experiments.

In comparing the two optimized nested PCRs, there was no significant difference in detecting the DNA of *L. chagasi* between the new approach proposed in this paper (10 fg) and the conventional approach (1 fg). Since each *Leishmania* spp. cell has 100 fg of DNA (Harris et al., 1998), both PCRs amplified the DNA corresponding to less than one parasite cell. A lower sensitivity is expected for STNPCR because of the depletion of reactants in the mixture. Perhaps the production of pyrophosphates in long cycling protocols also reduces the performance of STNPCR. However, this is not considered to be a severe drawback of STNPCR.

STNPCR is a promising test for the diagnosis of visceral leishmaniasis because in our assay it was very sensitive. The next step is to check its performance in several clinical specimens. This method has already been standardized for detecting *S. mansoni* (Abath et al., 2002), *P. falciparum* (Montenegro et al., 2004), *Y. pestis* (Souza et al., 2007) and for identifying dengue virus serotypes (Gomes et al., 2007).

Frequently, *Leishmania* species co-exist in endemic regions. Even though the ssu rRNA PCR has been shown to be very sensitive, it can identify *Leishmania* parasites only at a generic and/or subgeneric level (Bensoussan et al., 2006; Disch et al., 2006; Schönian et al., 2003; Lachaud et al., 2002; Van Eys et al., 1992). Using ssu rRNA-PCR, only the *L. donovani* and *L. braziliensis* complexes could be identified (Schönian et al., 2003). STNPCR was used to amplify only the DNA from *L. braziliensis* and *L. chagasi*. *L. braziliensis* is the causative agent in the cutaneous form of leishmaniasis, which is quite distinct from the pathology of leishmaniasis caused by *L. chagasi*, a viscerotropic *Leishmania* sp. If a distinction between these species is necessary, then we suggest the use of restriction enzymes and PCR-RFLP, as shown by Schönian et al. (2003). Other *Leishmania* spp. such as *L. major, L. braziliensis and L. amazonensis* could be identified by ITS-1-PCR. Most medically important *Leishmania* species can be distinguished by restriction enzyme analysis of the ITS-1 region that lies between the genes coding for the ssu and the 5.8S rRNAs (Cupolillo et al., 1995; Schönian et al., 2000; Davila and Momen, 2002).

In the specificity assay, amplification of the primer pair LITSR and L5.8S was detected when the genomic DNA from *T. cruzi* was used. Since previous studies reported negative results in this respect, this finding was not expected (Schönian et al., 2003; El Tai et al., 2000). However, using the primer-BLAST tool, it was observed that these primers were able to amplify regions of other species of the family *Trypanosomatidae,* including *T. cruzi*, with products ranging from 300 to 760 bp (Silva et al., 2010).

Our results suggest that STNPCR is a promising tool for the diagnosis of visceral leishmaniasis when a clinical suspicion already exists. This tool now requires standardization and validation with clinical samples. Furthermore, we suggest that this system could be optimized in order to increase its sensitivity for detecting genomic DNA from *T. cruzi*. This would enable the use of this method on clinical samples from endemic regions, such as areas that are co-endemic with leishmaniasis, thereby aiding in differential diagnosis.

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