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Sensitivity of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood

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PEER REVIEW

Peer reviewer

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Comments

Overall, this is a good study, dealing directly with hospitalized patients. The complication of acute kidney injury associated with visceral leishmaniansis is well detailed.
(Details on Page 14)

ABSTRACT

Objective: To evaluate the effectiveness of PCR and real-time PCR for the diagnosis of human visceral leishmaniasis using peripheral blood samples. **Methods:** DNA extraction was performed using Promega Wizard® Genomic kits. PCR employing RV1/RV2 primers yielded 145-bp amplicons. Real-time PCR was performed with the same primers and SYBR Green ROX Plus mix. These techniques were used to analyze 100 peripheral blood samples from patients with clinical signs of the disease. **Results:** The sensitivity and specificity levels were 91,3%% and 29,6%, respectively, for real-time PCR and 97.78% and 61.82%, respectively, for PCR. **Conclusions:** Real-time PCR proved to be a satisfactory method for the diagnosis of human visceral leishmaniasis.

KEYWORDS

Leishmania, Diagnosis, Visceral leishmaniasis, PCR

1. Introduction

The diagnosis of visceral leishmaniasis (VL) can be made unequivocally by the visualization of Leishmania sp. amastigotes in stained smears of spleen, liver, bone marrow, and lymph node aspirates[1]. The sensitivity of this method has been reported to be 95%-98% for spleen aspirates[2], 76%-91% and 52%-89% for liver and bone marrow aspirates, respectively[3] and 52%%-69% for lymph node aspirates[4]. Parasite culture on axenic media provides sensitivity levels of over 90%[5].

DNA sequencing has been extensively explored in the development of tests based on polymerase chain reaction (PCR), both for the diagnosis of and for the identification of the infectious species[6].

In addition to its high sensitivity and specificity for VL diagnosis, PCR is advantageous in that one of its principal targets in *Leishmania* is the kinetoplast DNA (kDNA), a conserved region occurring in multiple copies-roughly 10 000 per parasite[7] in all *Leishmania* species. The targeting of this high-copy-number DNA further increases the test's sensitivity[8-11].

The RV1 and RV2 primers, developed and adapted, target the *Leishmania infantum* kDNA minicircles, amplifying a 145 bp fragment[12,13]. Early results demonstrated this method's excellent sensitivity for the detection of this species in human macrophages[12], with a detection limit of approximately one parasite for every 106 experimentally

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infected mononuclear cells.

In a subsequent evaluation of two conventional PCR methods[14], found a sensitivity level of 100% for the RV1/RV2 primers when analyzing peripheral blood samples from infected dogs.

The good performance of real-time PCR using primers derived from the RV1/RV2 system was demonstrated for blood and bone marrow samples, allowing the parasite load to be correlated with the clinical status of patients and revealing that parasite quantification is required for the correct interpretation of the PCR results[15].

The RV1 and RV2 primers have been successfully used to identify *Leishmania* species, and to detect *Leishmania* (*Leishmania*) chagasi in dogs and phlebotomines[16-19], as well as in human blood and urine[20,21].

Real-time PCR has proven to be effective both in detecting *Leishmania*[22-23] and in quantifying parasite loads[24].

The implementation of real-time PCR for diagnosing *Leishmania* infection in endemic areas should promote improvements in therapy monitoring and relapse prevention, with extensive benefits for the clinical management of patients and for disease control.

The purpose of this investigation was to evaluate the use of PCR and real-time PCR as tools for diagnosing *Leishmania* infection.

2. Material and methods

2.1. Clinical subjects

The study included 100 subjects with visceral leishmaniasis confirmed by bone marrow aspirates, treated referral hospital in Campo Grande, Mato Grosso do Sul, Brazil. The investigation was approved by the Committee for Ethics in Research in Human Beings of the Universidade Federal de Mato Grosso do Sul.

2.2. Samples

2.2.1. Peripheral blood

Peripheral blood samples were collected in EDTA-coated 4 mL tubes. The material was frozen at -20 ℃ for subsequent extraction.

2.2.2. Marrow bone aspirate smears

Bone marrow aspirates were individually homogenized and used to prepare thin smears (n=100), which were subsequently fixed in methanol and Giemsa-stained. Because VL treatment is typically started following diagnosis based on smears, this method was adopted as the gold standard for the evaluation of the other techniques.

2.2.3. Culture

For the in vitro culturing of the parasite, bone marrow aspirates (n=96; four samples were too small to allow for culture) were seeded in Nicole-Novy-McNeal (NNN) medium, using Schneider's medium at pH 7.2 as the liquid phase, and 20% bovine fetal serum.

2.3. DNA extraction

DNA extraction was performed using a Wizard® Genomic DNA Purification kit (Promega) with 300 μL of peripheral blood. DNA was eluted with sterile demonized water in a final volume of 100 μL.

2.4. Polymerase chain reaction

PCR performed with the RV1/RV2 primers yielded 145 bp amplicons, which were visualized on 2% agarose gels prepared with Tris-acetate-EDTA (TAE) buffer. A total of 25 μL reaction mixture consisted of 1x buffer, 0.20 mol/L dNTPs, 1.5mol/L $MgCl_2$, 0.16 pmol of each primer (RV1/RV2), 4 IU of Taq polymerase (Invitrogen), 19.75 μL of sterile water, and 1 μL of DNA with a concentration of approximately 80 ng/μL - 50 ng/μL. The mixture was cycled in an XP thermal cycler (Bioer) as follows: 5 min at 95 ℃, followed by 35 cycles of 30 seconds at 95 ℃, 1.5 min at 54.5 ℃, and 1.5 min at 72 ℃, with a final extension of 10 min at 72 ℃.

2.5. Agarose gel electrophoresis

Submarine horizontal gel electrophoresis was performed in 2% agarose gels prepared with 1x TAE buffer at pH 8.0. The gels were run at 80 V and 400 mA (0.04-M Tris-acetate, 0.001-M EDTA) and visualized under UV light after ethidium bromide staining (0.5 μg/mL).

2.6. Real-time polymerase chain reaction

A real-time PCR for the detection of L. (L.) chagasi DNA in human blood samples was developed. To achieve high sensitivity, kinetoplast DNA was chosen as the molecular target. The RV1 and RV2 primers were used. Quantification was performed using a standard curve. Amplifications were performed on a Applied Biosystems 7300 model, and each reaction was run using a total of 12.0 μL of PCR mix plus 0.5 μL of DNA (approximately 80-150 ng per reaction). The 12.0 μL PCR mix was composed of 6.25 μL of SYBR Green ROX Plus mix (Taq-Star DNA polymerase, reaction buffer, dNTPs, SYBR Green I, and ROX) code 13-200RTSY, 0.5 μL of each primer (RV1/RV2, corresponding to 10 pmol) (0.4 pmol/reaction), and 4.75 μL of water. Cycling began with 15 min at 95 ℃, followed by 35 cycles of 15 seconds at 95 ℃, 30 seconds at 55 ℃, and 30 seconds at 72 ℃.

In both molecular methods, the reactions were standardized using a DNA control: *Leishmania* (*Leishmania*) chagasi MHOM/BR/74/PP/75, provided by the Laboratory of Leishmaniasis of the Centro de Pesquisas René Rachou, Fiocruz (Belo Horizonte, Brazil). The standard curve was established using L (L.) chagasi DNA extracted from $5x10^6$ parasites from cell culture. Aliquots of serial dilutions (1 μL), ranging from 50 ng to 0.0005 ng of parasite DNA, were introduced into reaction tubes; these dilutions corresponded to 50,000 to 0.5 parasites, respectively.

2.7. Statistical analysis

Statistical analysis was performed with the aid of BioStat software (version 5.0). The level of agreement between the results of the real-time PCR and the other techniques (PCR, smears, and culture) was measured using Cohen's kappa method at a 5% level of significance. The relationship between sensitivity and specificity was analyzed by constructing a receiver operator characteristic (ROC) curve.

3. Results

Of the 46 samples, 42 (91.3%) tested positive by PCR, 42 (91.3%) by real-time PCR, and 46 (100.0%) by bone marrow aspirate smear. Of these 46 samples, 12 (26.0%) tested positive by culturing. Table 1 shows the results for each technique.

Table 1.
Comparison of the results of real-time PCR, conventional PCR (both performed on peripheral blood samples) and parasitological methods (bone marrow aspirate smears and cultures) for patients with clinically suspected visceral leishmaniasis (n=100).

Test	RT-PCR		Agreement (%)	Kappa	*P*
	Negative	Positive			
PCR					
Negative	12	8	69	0.24	0,0119
Positive	23	57			
Bone marrow smear					
Negative	16	38	58	0.20	< 0.0001
Positive	4	42			
Culture*					
Negative	19	64			
Positive	1	12	32	0.05	< 0.0001

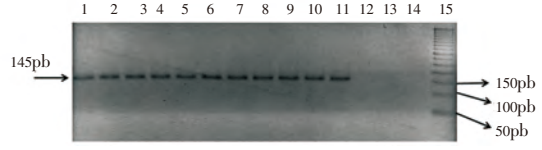

Figure 1. Agarose gel electrophoresis of PCR products generated using primers RV1/RV2. 1: MHOM/BR/2008/MS1139; 2: MHOM/BR/2008/MS1220; 3: MHOM/BR/2008/MS1195; 4: MHOM/BR/2008/MS1252; 5: HOM/BR/2008/MS1251; 6: MHOM/BR/2008/MS1240; 7: MHOM/BR/2008/MS1239; 8: MHOM/BR/2008/MS1186; 9: MHOM/BR/2008/MS1261; 10: MHOM/BR/2008/MS1287; 11: Leishmania (Leishmania) chagasi (MHOM/BR/74/PP/75); 12: MHOM/BR/2008/MS1322; 13: MHOM/BR/2008/MS1324; 14: MHOM/BR/2008/MS1325; 15: 50-bp molecular marker.

3.1. Polymerase chain reaction

Figure 1 depicts the amplifications obtained by PCR using RV1/RV2 primers. The amplicons had an estimated size of 145 bp. Figure 2 shows the amplicon analysis curves obtained for real-time PCR.

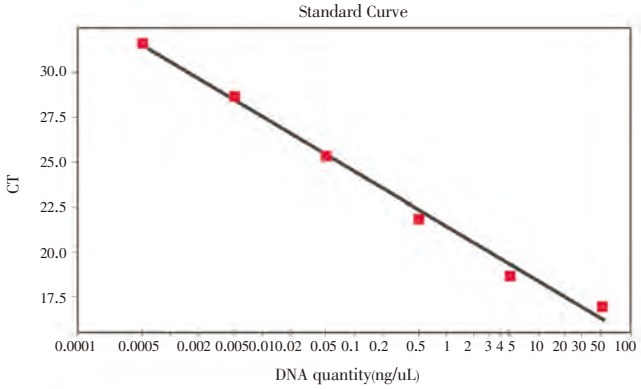

Figure 2. Standard curve constructed using serial dilutions of Leishmania DNA expressed as the DNA quantity per reaction tube from 50 to 0.0005 ng, corresponding from 50 000 to 0.5 parasites, respectively. Each point was tested in duplicate. Slope, – 3.06; PCR efficacy, 112.2%; R2, 0.993.

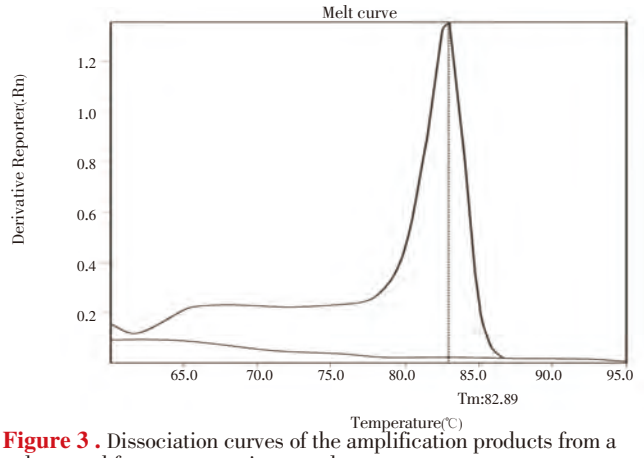

Figure 3 . Dissociation curves of the amplification products from a culture and from one negative sample.

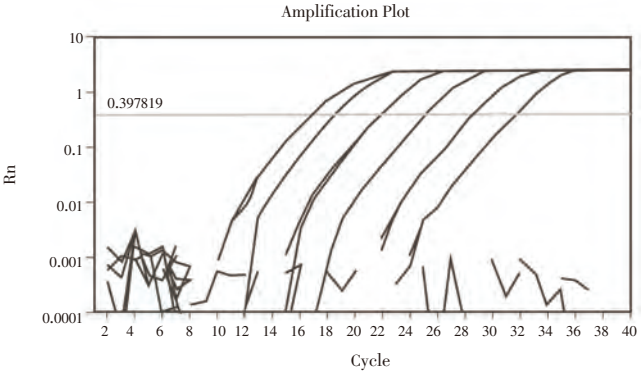

Figure 4 . Amplification curves for different dilutions of parasite DNA and the threshold generated by the StepOne program.

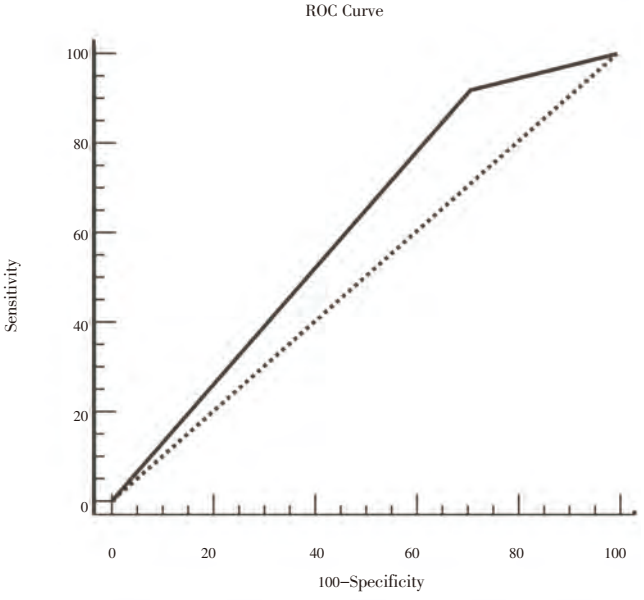

Figure 5. ROC curve for real-time PCR and bone marrow smears.

3.2. Real-time polymerase chain reaction

The minicircle DNA was amplified by real-time PCR, generating a product of approximately 145 bp, which was confirmed by electrophoresis on 2% agarose gels. This observed size corresponded to the expected size.

The sensitivity of the qPCR reaction was tested using serial dilutions of parasite DNA extracted from a known number of parasites. The kinetoplast DNA of L. infantum could be detected at a level corresponding to 0.0005 parasites per

reaction tube, with a dynamic range of 107. The detection limit was 0.0125 parasite/mL of blood, taking into account the amount of biological sample (1 μL of sample DNA) and the elution volume of the extracted DNA (50 μL). To verify the accuracy of the assay, PCR experiments were performed with negative blood spiked with culture promastigotes. Figure 2 presents the standard curve, slope, and efficacy of a typical experiment.

A dissociation curve based on the emission of fluorescence was used to determine the melting temperature (Tm). Figure 3 shows the Tm found for this DNA template.

Figure 4 shows the amplification curves for the different dilutions. Real-time PCR exhibited a higher discriminative power than PCR, in addition to higher sensitivity, as demonstrated by the ROC curve shown in Figure 5. The sensitivity was 91.3%, and the specificity was 29,6%. Table 1 shows the correlation between real-time PCR and the other tests, expressed by the Kappa coefficient.

4. Discussion

In the state of Mato Grosso do Sul, VL remains a public-health issue, as it has now spread to 48 of the 78 countries in the state. There were 1.729 confirmed cases in the period of 2000-2008, 226 of which occurred in 2008 alone[25]. Locally, only parasitological methods (bone marrow smears and culture) are available for diagnosis, at the Laboratory of Parasitology of the Universidade Federal de Mato Grosso do Sul at Campo Grande.

Although parasitological tests are currently the gold standard for the diagnosis of leishmaniasis, the use of peripheral blood provides a less invasive alternative to bone marrow aspirates, exposing patients to lower risks and facilitating sample collection from endemic areas[26].

Culture in specific culture medium, another technique available for parasitological diagnosis, is not routinely used, given the maintenance requirements and necessary growth periods, which can be to 30 days[27].The lower sensitivity of culture-based methods has restricted its use, however, while fostering research into alternatives that offer improved performance[28]. Noted that bone marrow aspirate culture is not the most suitable technique for VL diagnosis, considering the time constraints it places on treatment initiation[29].

PCR for the detection of parasite DNA in peripheral blood is a non-invasive alternative for diagnosis, especially when the disease is suspected in patients with negative parasitological results[26, 30].

In the present study, the sensitivity of standard PCR was 100,0%, in agreement with that reported for this method[26,31] and the specificity was 100%[31-33] when performed with peripheral blood. This method targets kDNA, which increases the test sensitivity, but the test's performance can be reduced by factors such as the technique employed for sample extraction[33], genetic variability[38], and the degradation of DNA, particularly kDNA, which is more readily degraded than nuclear DNA[35].

The low specificity found in this study may be explained by the fact that samples negative by direct observation were positive by qPCR, demonstrating the greater sensitivity of the qPCR method and indicating that its specificity may have been underestimated. These conclusions are supported that fact that a low number of parasites, the quality of the bone marrow aspirate, and the stage of disease progression are factors that can affect the results of direct parasitological examination.

The RV1/RV2 primers provided higher sensitivity (85,0%) and higher rates of positive results than the use of bone marrow aspirate smears for the detection of *Leishmania* amastigotes. With these primers,[36] achieved a 100% level of positive results in peripheral blood samples from infected dogs, a contrasting finding that can be ascribed to differences in the parasitemia levels in dogs and humans[13,14,37,38].

The RV1/RV2 primers allow for the detection of less than one parasite per sample but do not amplify L. amazonensis and L. braziliensis, the causative agents of a number of cases of leishmaniasis in Brazil in general[17] and in Mato Grosso do Sul specifically[16,39,40].

PCR technology has been significantly refined, and real-time PCR now has advantages over traditional PCR, with shorter run times (because electrophoresis is no longer required) and reduced risk of contamination (because amplification can be detected while the tube is still closed) [23]. How does not show the various handling steps required after conventional PCR amplification reduces the risk of contamination of the material and allows, simultaneously, detecting, measuring and comparing the number of parasites in various samples[7,41].

In the present investigation, real-time PCR using the RV1/RV2 primers exhibited higher discriminative power, a feature that corroborates its advantages over the other techniques investigated. Real-time PCR was the only technique able to provide positive diagnoses for two of the patients, corroborating the advantages of applying this technique with RV1/RV2 primers for VL diagnosis, as reported in earlier studies[42,43].

Real-time PCR was sufficiently sensitive to detect as little as 0.001 parasite per reaction using TaqMan probes in human blood samples[15]. This sensitivity of this assay was also higher than that reported in previous studies, owing to the target utilized (kinetoplasts) and the use of TaqMan probes[7,15,24,41,44-46]. A slope of −3.3 corresponds to an efficiency of 100.0%, indicating that the number of amplified molecules doubles with each cycle of PCR[47]. In this study a slope of − 3.06 was obtained, demonstrating high efficiency.

A real-time PCR system with SYBR green was used for the diagnosis of cutaneous leishmaniasis using skin biopsy samples from 100 patients. This technique was found to be more sensitive than microscopy and culture methods, in accordance with the present results[48].

Because the signs and symptoms of other pathologies can mimic those of VL and given that early diagnosis is decisive in reducing the morbidity and mortality rates, the use of molecular methods for diagnosis is warranted. Among the methods available, real-time PCR is particularly advantageous, given its effectiveness for samples containing a small number of parasites and in cases in which differential diagnosis can be crucial in view of the wide range of clinical manifestations.

Conflict of interest statement

We declare that we have no conflict of interest.

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Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT, Brazil, research grant to M.F.C. Matos and student grant to M.S.C. Lima Junior). The authors also wish to express their thanks to the Centro de Pesquisas René Rachou, Fiocruz (Belo Horizonte, Brazil) for donating the DNA controls

Comments

Background

Visceral leishmaniasis, also known as kala azar, is characterized by high fever, substantial weight loss, swelling of spleen, kidney and liver, and anemia. This disease is a concern in countries especially in several African, South-American and developing Asian nations. If not diagnosed early and correctly, the disease could have a fatality rate as high as 100% within two years. This particular study emphasizes molecular diagnosis using real-time PCR in diagnosing visceral leishmaniasis, leading to acute kidney injury in patients admitted to ICUs in Brazil. This study also emphasizes symptoms, co-infections, comorbidities and clinical manifestations, which should help reader of article to understand complications associated with this disease.

Research frontiers

The use of molecular diagnostic technique in diagnosing such a critical disease is a good approach. This will help in early and accurate diagnosing of the disease. The only concern is the affordability of such techniques in public and tertiary care hospitals.

Related reports

A review by, Tavares *et al* 2003, describes the worldwide situation of visceral leishmaniasis with an emphasis on diagnosis, including methods for the detection of antibodies, antigens, parasite DNA and of skin testing. The advantages and problems of each method are discussed and the need for a rapid, sensitive and low-cost diagnostic method for use in field conditions is highlighted. Recent advances in Leishmania genome sequencing, the use of DNA microarrays and protein microarray methodologies and their potential use for leishmaniasis diagnosis are presented. Carlos Alberto P Tavares, Ana Paula Fernandes, and Maria Norma Melo. *Expert Rev Mol Diagn* 2003:3; 657-667. (doi: 10.1586/14737159.3.5.657). Another review by, Paiva-Cavalcanti *et al* 2010 also describes the use of PCR in diagnosis in Leshmaniasis. Paiva-Cavalcanti *et al*. comparison of real-time PCR and conventional PCR for detection of Leishmania (*Leishmania infantum*) infection: a mini-review. *J Venom Anim Toxins incl Trop Dis* 2010; 16: 537 -542.

Innovations & breakthroughs

The use of high-end techniques in diagnosing critical health ailments such as, visceral leshmaniasis is a good approach, especially when reported from a developing nation, Brazil. As we learn from the results of this study, the accuracy of the techniques were on higher side and the other clinical complications, comorbidities associated with disease, which leads to fatality of the patient are well explained and justified. Statistical analysis of data is well done.

Applications

Use of real-time PCR is disease diagnosis, though not new, but definitely a great step towards establishing the early diagnostic method. It will reduce the mortality rates caused by leishmaniansis in developing nations

Peer review

Overall, this is a good study, dealing directly with hospitalized patients. The complication of acute kidney injury associated with visceral leishmaniansis is well detailed. Such studies should help clinician to understand the disease more vividly, and the complications associated with it. It would encourage other clinicians and researchers to do such studies. There is overall novelty in the work. This study has good practical. Authors of this papers should have given more attention towards the redact of the paper. Also the use of diagnostic kits in this research and the results obtained from this study should have been coupled with results obtained with routine diagnostic methods.

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