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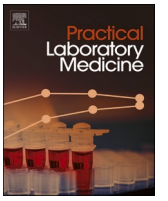

Molecular diagnosis of visceral leishmaniasis from blood samples using different genetic markers: A simple, sensitive and less invasive diagnostic approach

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ABSTRACT

Visceral leishmaniasis (VL), or kala-azar, is a deadly disease with high fatality rates if diagnosis and treatment are delayed. Diagnosis is often delayed due to symptoms that mimic other conditions. Sample isolation and diagnostic procedures are labor-intensive and time-consuming. Rapid immunochromatographic tests cannot differentiate active cases from past infections. In the present study we investigated the utility of peripheral blood samples for molecular diagnosis of VL. Whole genomic DNA from the erythrocyte fraction of blood from VL and cutaneous leishmaniasis (CL) suspected patients was used for PCR using multiple markers (k-DNA, ITS-I, and 18s rRNA). PCR amplification of k-DNA, ITS-I, and 18s rRNA genes yielded positive results in VL symptomatic patients. However, the same PCR approach with peripheral blood samples from CL patients was not significant. Hence, peripheral blood samples can effectively distinguish active VL cases through PCR using multiple markers, offering a less invasive and labor-intensive diagnostic alternative.

1. Background

Leishmaniasis ranges from benign skin lesions to severe visceral infections. The deadliest form, Visceral Leishmaniasis (VL), also called Kala-azar or "Poor man's disease," affects over 300,000 people annually, primarily in Brazil, India, Bangladesh, Sudan, Ethiopia, and South Sudan [1]. Sandflies of the genus *Phlebotomus* transmit the disease in the Old World, and *Lutzomyia* in the New World. In the Indian subcontinent, *P. argentipes* transmits VL. Diagnosis is often delayed due to complex symptoms like hepatosplenomegaly and anemia. Recently, incidence in immunocompromised individuals, such as HIV patients, has been rising. Early diagnosis is crucial for effective treatment [2].

Earlier, leishmaniasis detection relied on in-vitro culture of parasites from clinical isolates [3]. Advanced technologies now include rapid tests and highly sensitive PCR methods, requiring minimal sample amounts [4–7]. Histopathological examination of spleen, liver, and bone marrow aspirates and biopsies has long been used after clinical symptom examination, despite requiring expert collection and potentially causing pain and internal hemorrhage [8–10]. Accuracy depends on parasite load and technician expertise. This study highlights the effectiveness of PCR analysis on peripheral blood samples for VL detection, simplifying the process and

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reducing patient discomfort and pain. The same procedures are applied to other clinical samples, underscoring the method's versatility.

2. Methods

Ethics approval

Ethical clearance for this study was obtained from the Indian Council of Medical Research– Vector Control Research Centre (approval no. IHEC-0619/R/M).

2.1. Sample preparation

3 ml of venous blood from seven VL-suspected (rk39 positive) and PCR-confirmed CL patients were aliquoted into 1.5 ml tubes. Written consent was obtained from all patients. Samples were kept at room temperature for 30 min, then stored at 4 °C until centrifugation. At ICMR- VCRC, Field Station, Kerala, samples were thawed, centrifuged (5000 rpm for 10 min), and 200 µl of the erythrocyte fraction was used for DNA extraction from each sample.

2.2. DNA extraction

The whole genomic DNA extraction was carried out following the manufacturer's protocol for SIGMA Genelute Mammalian Genomic DNA MiniPrep kit (Sigma-Aldrich, USA), from VL suspected and CL confirmed individuals. The final DNA elution was done in 35 µl nuclease free water. The eluted DNA was analyzed for the concentration and purity using nano-drop and was stored at −40 °C until further use.

2.3. PCR diagnosis

Quantitative PCR (qPCR) was used for amplifying and detecting minicircle kinetoplast DNA (k-DNA), and standard PCR was used for amplifying ribosomal markers ITS-I and 18s rRNA. For k-DNA qPCR, the protocol and primers from Castelli et al., 2021 [11] were used, with conditions: UNG step at 50 °C for 150 s, initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 35 s. ITS-I amplification was done with initial denaturation at 95 °C for 5 min, 34 cycles of 95 °C for 30 s, 53 °C for 40 s, and 72 °C for 1 min, and final extension at 72 °C for 7 min [12]. For 18s rRNA, conditions from Sriavastava et al., 2011 [13] were used: initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 50 s, 54 °C for 1 min, 72 °C for 1 min 20 s, and final extension at 72 °C for 7 min. Primer details are in Table 1. DNA from bone marrow aspirate of a confirmed VL patient was used as the positive control, and nuclease-free water as the negative control.

2.4. Gel electrophoresis and confirmation

The amplified PCR products were visualized under UV light on an ethidium bromide-stained 1.5 % agarose gel. A 100 bp DNA ladder served as the marker, confirming the band size of the amplicon.

3. Results

In the present study peripheral blood samples collected from 7 VL suspected patients were processed for molecular diagnosis using different molecular markers. All the DNA samples extracted from the erythrocytes of the VL suspected individuals were positive for k-DNA (qPCR) (Supplementary Table and Fig. 1), ITS-I and 18s rRNA. An amplicon of size 320 bp and 311 bp was amplified for the diagnostic ITS-I and 18s rRNA respectively (Fig. 2). The results were indicative of the confirmation of leishmaniasis infection. On the other-hand, all the CL confirmed patient's blood samples were negative for the above diagnostic PCRs. As it was mainly intended for the diagnosis of VL, no downstream processing of the PCR products were carried out.

Table 1
The details of markers (kDNA-qPCR, ITS1 and 18s rRNA), primer sequence and amplicon size.

Sl. No.	Genetic Marker	Primer	Primer Sequence	Amplicon Size	Reference
1	kDNA	Leish 1	F- 5' GGCGTTCTGCGAAAACCG 3'	-	Castelli et al., 2021
		Leish 2	R- 5' AAAATGGCATTTTCGGGCC 3'		
		Leish Taqman probe	FAM 5' TGGGTGCAGAAATCCCGTTCA 3'		
2	ITS 1	LITSR	F- 5' CTG GAT CAT TTT CCG ATG 3'	320 bp	Schonian et al., 2003
		L5.8S	R- 5' TGA TAC CAC TTA TCG CAC TT 3'		
3	18s rRNA	BHUL18SF	F- 5' CGT AAC GCC TTT TCA ACT CAC 3'	311 bp	Srivastava et al., 2011
		BHUL18SR	R- 5' GCC GAA TAG AAA AGA TAC GTA AG 3'		

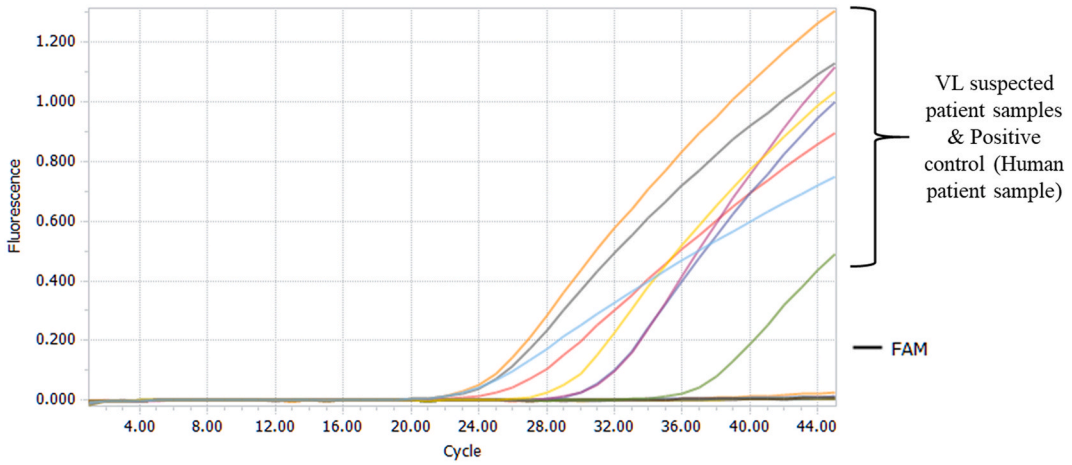

Fig. 1. Amplification curves of detection of k-DNA gene of *Leishmania* using qPCR assay.

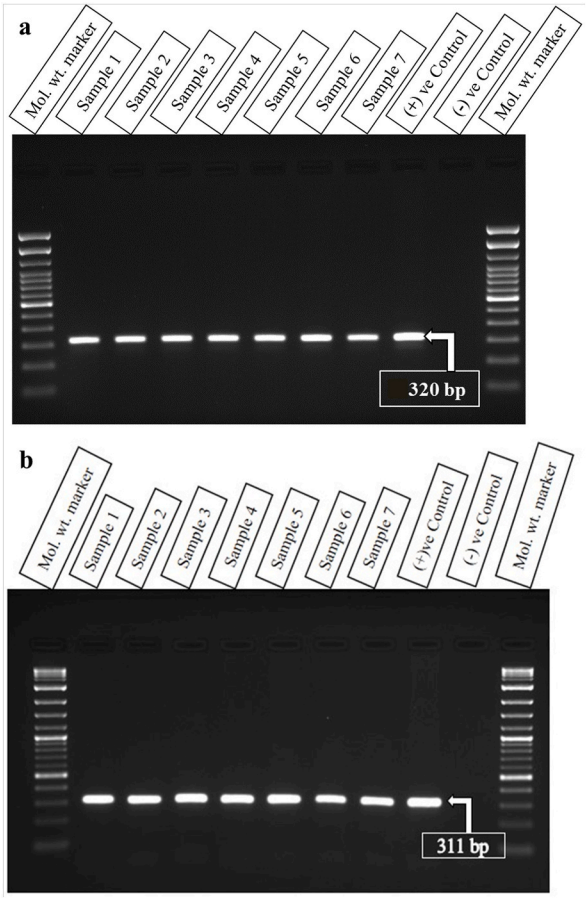

Fig. 2. The gel electrophoresis photograph- (a) ITS-I; (b) 18s rRNA.

4. Discussion and conclusion

The present study aimed to test the sensitivity of diagnostic markers for the molecular diagnosis of kala-azar or VL using the peripheral blood samples. The selected markers were able to detect the presence of *Leishmania* parasite. First amplified gene was the k-DNA that is specific for *Leishmania donovani* complex. It is a highly sensitive PCR able to detect the parasite even in case of low intense

infections. k-DNA amplification denotes the infection of *Leishmania* parasite belonging to *donovani* complex, which includes *L. infantum* and *L. donovani* [14]. The other two markers chosen were also equally relevant diagnostic tool as it is of short fragment size [13,15]. Hence, the infection can be confirmed within a short time. ITS-I PCR yielded an amplicon of length 320 bp. The same marker can be utilized to distinguish the major *Leishmania* species groups through restriction digestion using *Hae III* [15]. The restriction digestion pattern varies with the species and it can be confirmed through electrophoresis. Similar to ITS-I, 18 s rRNA (311 bp) is also a ribosomal marker and is a multicopy gene and the results can be obtained through a short time PCR [13].

Molecular diagnostic methods are always unique and different in comparison to the traditional diagnostic methods, as the results of conventional methods are dependent on the parasitic load and the distribution of parasite in host tissues. The genetic markers selected in the present study, k-DNA, ITS-I and 18s rRNA easily enables the confirmation of leishmaniasis. k-DNA PCR is a primary diagnostic PCR employed in leishmaniasis confirmation [16]. It is primarily preferred over other markers because even in low parasite load the thousands of copies of the mini circles enables high sensitivity to the PCR [6]. This genetic marker is specific to the protists in the group Kinetoplastida, are also utilized in analyzing the infection in other human samples, vector sandflies and the reservoir hosts [4,17,18]. The ITS-I PCR is mainly practiced for characterization of *Leishmania* parasites through RFLP analysis [14,19]. The 18s rRNA and ITS-I are rarely used as primary diagnostic method for leishmaniasis compared to the k-DNA marker [15,20]. And also the sensitivity of the PCR methods may vary with the type of biological sample used.

Our primary objective of the study was to set up an efficient PCR assay using peripheral blood to detect *Leishmania* sp. in VL suspected patients. This sampling method offers advantages such as simplicity, minimal invasiveness, and ease of repetition compared to more invasive methods like bone marrow, liver biopsy, or lymph node sampling. The sensitivity and specificity of all markers were 100%, with negative control tubes remaining consistently negative. Additionally, the diagnosis of CL based on peripheral blood was found to be irrelevant in this context.

CRediT authorship contribution statement

Harish Kumar Shah: Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **K.R. Rajesh:** Methodology. **P.A. Fathima:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **R.S. Aiswarya:** Investigation, Methodology, Writing – original draft, Writing – review & editing. **P.M. Ajithlal:** Investigation, Methodology, Software, Writing – review & editing. **Prasanta Saini:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Ethic statement

Not applicable.

Availability of data and material

The datasets generated and/or analyzed during the current study are available from the corresponding author on request.

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Declaration of competing interest

We hereby declare that the manuscript entitled “Molecular diagnosis of visceral leishmaniasis from blood samples using different genetic markers: a simple, sensitive and less invasive diagnostic approach” by Shah et al. does not have any conflict of interest involved in this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2025.e00448.

Data availability

Data will be made available on request.

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