RESEARCH ARTICLE

Determination of frequency of Leishmania tropica in laboratory-confirmed cases of cutaneous leishmaniasis using an in-house conventional PCR test

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Abstract

Objective: To develop a low-cost, in-house conventional polymerase chain reaction method for leishmania tropica for epidemiological surveillance of disease.

Method: The cross-sectional study was conducted at the Department of Microbiology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan, from October 16, 2023, to April 16, 2024, and comprised biopsy samples received for the diagnosis of cutaneous leishmaniasis. Deoxyribonucleic acid was extracted from the samples and was subjected to real-time polymerase chain reaction for the detection of the leishmania genus on a commercially available detection kit. Positive samples were then run by conventional in-house polymerase chain reaction with primers specific to leishmania tropica targetting covering internal transcribe spacer 2 and 18S ribosomal ribonucleic acid region. The in-house conventional polymerase chain reaction was validated by using extracted deoxyribonucleic acid from the promastigote of leishmania tropica as the positive control. The nucleotide sequence was subjected to the basic local alignment search tool, and a phylogenetic tree was constructed on MEGA 5.

Results: Of the 73 suspected leishmaniasis cases, 64(87.7%) were from male subjects. Real-time polymerase chain reaction detected leishmania in 33(45.2%) of the samples, which were then assessed by the in-house polymerase chain reaction specific to leishmania tropica and 28(84.8%) samples were detected positive (p=0.0003). Sequencing of the internal transcribe spacer 5.8S region and subsequent phylogenetic analysis with 1000 bootstraps showed >95% identity with L.tropica. BLAST analysis showed 96–98% sequence identity (query coverage 98–100%, E-value = 0.0).

Conclusions: The development of in-house polymerase chain reaction to test for leishmania tropica was successful in clinical samples, providing a cost-effective alternative to commercially available kits.

Key Words: Leishmania, Leishmania tropica, Commercial PCR, In-house PCR.

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Introduction

Cutaneous leishmaniasis (CL) is caused by parasites of the genus Leishmania, which are flagellated protozoan parasites that are categorised into kinetoplastida order. CL is manifested as single or multiple, occasionally persistent, and frequently ulcerated skin lesions on the exposed areas of the body.[1] Approximately 98 nations in the New and Old World[2] have endemic cases of CL. An estimated 350 million individuals are at risk of developing this condition, and an average of 0.7 million and 1.2 million outbreaks are recorded annually.[2] Leishmania tropica is responsible for anthroponotic cutaneous leishmaniasis (ACL); L. major causes zoonotic cutaneous leishmaniasis (ZCL), which is self-treating, L. aethiopica causes cutaneous leishmaniasis and is only found in limited areas of Africa, and L. infantum primarily causes CL and visceral leishmaniasis (VL). These are the four distinct species that cause widely distributed CL. [3]

In the Middle East and Asia, L. tropica and L. major are primarily associated with CL, whereas CL caused by L. infantum is an occasional disease. However, the regions around the Mediterranean basin, which includes Southern Europe and North Africa, are home to CL caused by L. infantum. Additionally, growing evidence suggests that L. chagasi, which is identical to L. infantum, causes CL in the Americas, albeit it is more frequently associated with VL.[4]

CL has expanded regionally during the past few decades, transcending the regions where it was previously reported. The genetic diversity of the parasite and the existence of several leishmania species with overlapping clinical characteristics occasionally leading to misdiagnosis in endemic areas, highlight the need for the establishment of highly accurate testing methods for the identification of Leishmania species.[5]

In order to limit transmission and initiate early care of the

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infected individuals, an accurate, early and rapid diagnosis of leishmaniasis is very important. Diagnostic testing in laboratories usually relies on molecular methods, like quantitative polymerase chain reaction (qPCR) and conventional PCR, serological assays, and parasitological techniques, like culture and microscopic examination.[6]

For diagnosing leishmania species, PCR amplifications focussing on the ribosomal ribonucleic acid (rRNA) and kinetoplast genes are the most widely employed techniques.[7] The genes encoding the 18S, 5.8S and 28S rRNA are arranged as tandem repeats in most eukaryotic organisms, and these recurring sequences are transcribed sequentially to create a lengthy primary transcript. Several enzymes and small nucleolar RNAs (snoRNAs) extract the internal transcribed spacers (ITSs), and 5′ and 3′ external transcribed spacers (ETSs) from the primary transcript. Only around 12 copies of the rRNA gene repeat are present in each haploid genome in the L. major genome, and they are arranged in tandem arrays on chromosome 27.[8] For L. tropica and L. aethiopica specie, the exact number of copies of rRNA gene clusters are still unknown because of a lack of comprehensive genome sequencing data.

Despite being frequently utilised, microscopic examination has a very low sensitivity, which leads to an underestimation of the infection incidence and a high case count. In underdeveloped countries where access to expensive equipment is limited, availability of qPCR technology cannot be ensured in rural areas. In such settings. leishmania identification can be achieved by the development of in-house PCR techniques that are less expensive, as there is still no gold standard for CL diagnosis.[9,10]

The current study was planned to evaluate the frequency of L. tropica in laboratory-confirmed CL cases using an in-house conventional PCR test.

Materials and Methods

The cross-sectional study was conducted at the Department of Microbiology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, Pakistan, from October 16, 2023, to April 16, 2024. After approval from the institutional ethics review board, the sample size was calculated using the World Health Organisation (WHO) calculator [11] with 5% margin of error, and 95% confidence level. A non-probability consecutive sampling method was used for the sampling process.

Cutaneous biopsy specimen received at AFIP for leishmania PCR were included, while formalin-fixed tissue and biopsy samples having any alternative diagnosis were excluded. Prior to including any participant, written consent was taken.

From the suspected samples, deoxyribonucleic acid (DNA) was extracted by an auto-extraction machine

Table-1A: Polymerase chain reaction (PCR) primer pairs.

Sr. No.	Species	Primer pair	Sequence
1	L. tropica	Forward	5′ACGCACCGCCTATACACAAA 3′
-	-	Reverse	5′ACTACTGCGTTCTTCACCGA 3′

Table-1B: Polymerase chain reaction (PCR) cycling conditions.

Step 1	Initial denaturation	95°C	10 min	1 cycle	Amplicon size
Step 2	Denaturation	95°C	30 sec	30 cycle	
	Annealing	52°C	45 sec		155 bp
	Extension	72°C	30 sec		
Step 3	Final Extension	72°C	10 min	1 cycle	

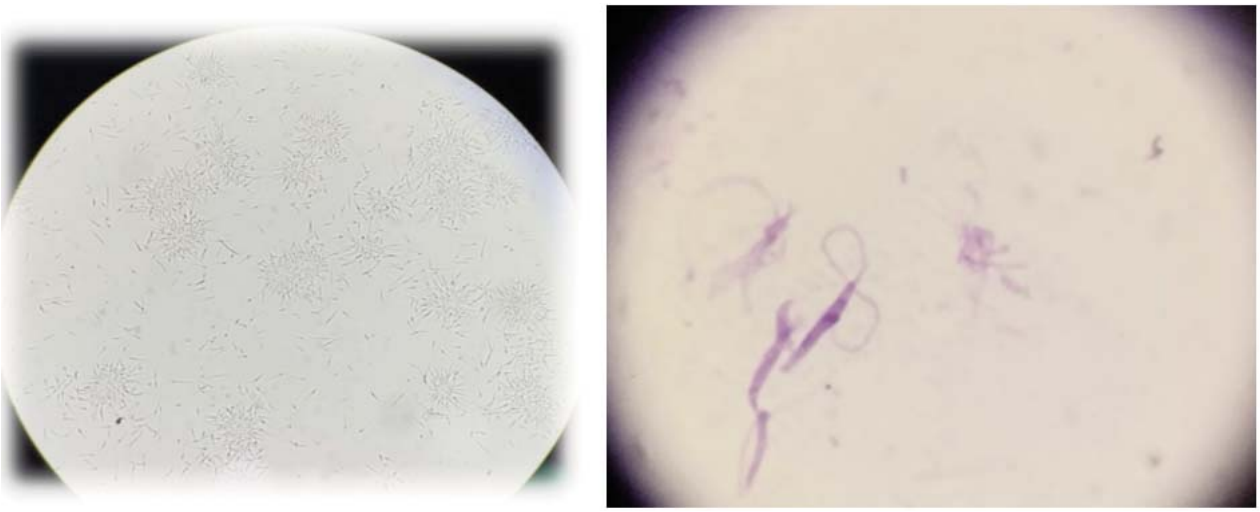

Figure-1: (a) Direct microscopy of leishmania tropica promastigote culture. (b) Giemsa staining of leishmania tropica promastigote culture.

bioPerfectus SSNP-2000B (Jiangsu Bioperfectus Technologies Co., Ltd. (Taizhou City, Jiangsu Province, China). All the samples were subjected to real-time PCR on a commercial kit (VIASURE, CerTest Biotec, Spain) for the detection of the leishmania genus. The positive samples were then run by conventional in-house PCR with L. tropica-specific primers targetting covering internal transcribe spacer 2 (ITS2) and 18S rRNA region (Table 1A-B). The in-house conventional PCR for L. tropica was validated by using the extracted DNA from the promastigote culture of L. tropica as the positive control (Figure 1). Sanger sequencing was performed on patient sample 68, 46 and promastigote PCR product. The nucleotide sequence was subjected to basic local alignment search tool (BLAST) through the National Centre for Biotechnology Information (NCBI)[12], and a phylogenetic tree was constructed on MEGA 5.0 to develop a relationship among the leishmania-positive patient samples. The positive controls were submitted to the Gene Bank[13] to obtain accession numbers.

Results

Of the 73 suspected leishmaniasis cases, 64(87.7%) were from male subjects and 9(12.3%) from female subjects (Table 2).

Table-2: Demographic distribution and polymerase chain reaction (PCR) results in cutaneous leishmaniasis cases.

		n (%)
Gender	Male	64 (87.7%)
	Female	9 (12.3%)
Commercial PCR	Positive	33 (45.2%)
	Negative	40 (54.8%)
In-House PCR	Positive	28 (84.8%)
	Negative	5 (15.2%)

Real-time PCR detected leishmania in 33(45.2%) of the samples (33 out of 73), these 33 genus positive samples were then assessed by the in-house L. tropica-specific PCR which detected 28 out of 33 Real time PCR positive Leishmania samples (84.8%) to be L.tropica. (p=0.0003) (Table 3).

Table-3: Statistical summary of polymerase chain reaction (PCR) results.

Assay	Positive n (%)	Negative n (%)	Total (n)
Real-time PCR (Leishmania spp.	33 (45.2%)	40 (54.8%)	73
In-house PCR (L. tropica)	28 (84.8%)	5 (15.2%)	33

In-house PCR for L. tropica was performed only on real-time PCR positive samples (n = 33). Therefore, no direct comparative statistical test between the two assays was applied

Limit of detection (LOD) of PCR protocol was determined by using 11.5µg/µl, 6.72µg/µl and 0.27µg/µl of DNA from patient sample, and 1.45µg/µl, 0.73µg/µl and 0.145µg/µl DNA from Leishmania tropica promastigoetes culture. In addition, Mycobacterium tuberculosis DNA was used for specificity testing. No PCR product was obtained in Mycobacterium tuberculosis PCR chamber whereas Limit

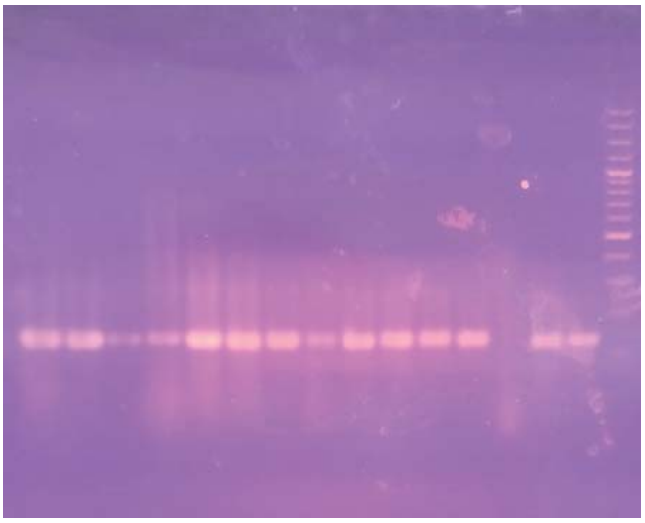

Figure-2: Limit of detection of in-house polymerase chain reaction (PCR) test.
Lane 1-3 11.5µg/µl, 6.72 µg/µl and 0.27 µg/µl of deoxyribonucleic acid (DNA) from patient sample 46.
Lane 4-6 and 1.45µg/µl, 0.73 µg/µl, 0.145 µg/µl of patient sample 70.
Lane 7-12 Patient samples.
Lane 13 Mycobacterium tuberculosis DNA.
L: Ladder.

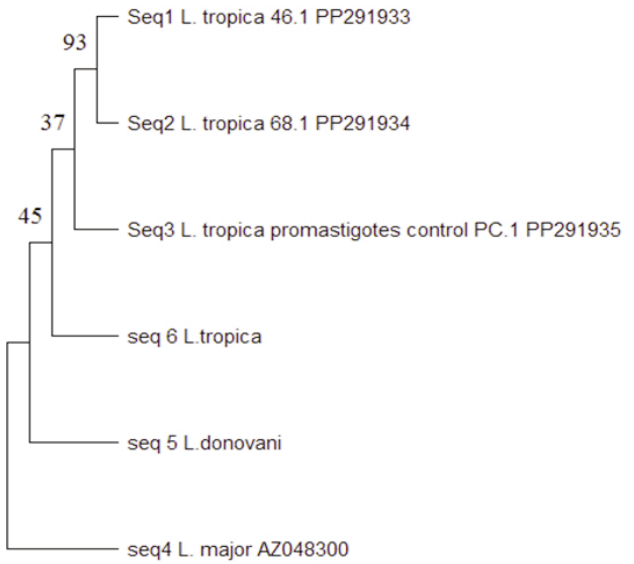

Figure-3: Phylogenetic tree showing relationship among different leishmania species. (93% similarity among patient sample 68 and 46; 45-37% similarity among patient samples and promatigote culture. Leishmania major and donovani served as out group).

Table-4: Accession number of the submitted sequences in Gene Bank.

Sr. No.	Sample No.	Identified species	Percentage similarity	Accession No.
1	Sample 46	Leishmania tropica	98%	PP291933
2	Sample 68	L. tropica	96%	PP291934
3	Positive control	L. tropica	99%	PP291935

Table-5: Comparison of the cost.

Sr.	Cost of real time PCR	Cost of in-house PCR	Comment
1	Need costly equipment real time PCR that cost around 4-5 million PKR	Need conventional PCR device which cost start from 0.5 to 1 million PKR	The in-house device cost almost 5 time lesser
2	96 test kits cost around 0.1 million.	It cost only 0.02 million PKR	Almost 5 time less cost
3	N/A	Need an additional gel apparatus that cost around 0.5 million.	

PCR: Polymerase chain reaction, PKR: Pakistani rupee.

of detection (LOD) was up to 0.145µg/µl (Figure 2).

Sequencing analysis of PCR product of ITS region 5.8S of patient samples and positive controls showed the similarity of the sequenced products (Table 4). The phylogenetic relationship among different leishmania species, including L. tropica, L. major and L. donovani, was also noted (Figure 3).

Discussion

In endemic areas, especially in Asia, CL is becoming a significant health issue due to a rise in incidence and a wider geographic spread. Furthermore, conventional diagnostic techniques take a lot of time and require advanced technology and knowledge.[14] As a result, it is becoming increasingly crucial to standardise and enhance CL diagnostic techniques, particularly in low-resource countries where this disease is most prevalent.[12] Comprehensive CL control and efficient case management necessitate the use of a rapid, precise and economical diagnostic tool, particularly in cases when many leishmania species exist in the same region.[12] Consequently, for epidemiological and case management objectives, species characterisation is essential. ITS region and kinetoplast DNA (kDNA) are two of many genetic markers extensively used for the identification of leishmania parasites in various biological samples.[15,16] Although susceptible methods for detecting specific species of leishmania have been documented, molecular methods that necessitate unique PCR primers for each species are often employed if many leishmania species need to be distinguished in a diagnostic laboratory.[17] However, in the current study, an in-house PCR technique was used, and it was compared with the conventional PCR, showing a significant association between the techniques.

A study by Bousslimi N, et. al.[18] tested rapid, simple and highly sensitive loop-mediated isothermal amplification (LAMP) assays for the DNA-specific detection of distinct leishmania species from the cutaneous lesions. Two LAMP assays were designed to detect and distinguish between L. tropica and L. major species, which targeted the cysteine protease B (cpb) gene. No cross-reactions were found. Similar to the current investigation, both tests were performed on clinical samples of individuals suspected of having CL, and the results were compared with traditional cpb-based, microscopic PCR and kDNA qPCR assays. The LAMP tests demonstrated a specificity of 100%, and sensitivity of 84% to distinguish between the L. tropica and L. major species. However, the LAMP assays demonstrated 100% sensitivity when compared to the results of the diagnostic assessments using latent class analysis (LCA).[19] While the LAMP assay offers rapid amplification under isothermal conditions, the in-house PCR applied in this study relies on widely available thermal cycling platforms, and targets conserved ribosomal regions, facilitating integration into routine diagnostic laboratories. The comparable detection rates observed between the two methodologies highlight that the in-house PCR provides a reliable and cost-effective alternative for L. tropica identification (Table 5), particularly in resource-limited settings where access to LAMP-specific reagents and optimisation expertise may be limited.

Another study[20] developed an enhanced PCR amplifying the rRNA gene's ITS2 sequence, which was aligned from several strains of the Old World leishmania species that cause CL. Using a single set of primers in a single reaction, the universal PCR allowed for the simultaneous identification of L. infantum, L. tropica and L. major. The PCR assay proved capable of effectively amplifying up to as 0.01-0.1pg of leishmania DNA from cultured promastigotes, and it demonstrated 100% specificity (3/3) (95% confidence interval [CI]: 29.24-100%) and 100% sensitivity (22/22) for species determination on specimens from lesion exudates, as well as 100% specificity (11/11) (95% CI: 71.51-100%) and 100%

sensitivity (13/13) (95% CI: 75.29-100%) for species verification on biopsy specimens of CL subjects.[21] However, in the current research, only the frequency of L. tropica was determined from the biopsy specimens of lesion exudates from CL patients by using an in-house PCR technique.

In a study[22] aimed at developing an accurate diagnostic examination of leishmaniasis, a comparative assessment was conducted between one commercially available PCR test (Method B), two in-house PCRs (Methods A and C), and one real-time PCR (Method D) for the identification of leishmania DNA. With the positive and negative controls, each approach under investigation yielded the anticipated outcomes. On the other hand, Method C demonstrated a statistically significant higher positivity rate. The relative sensitivity and specificity of Methods A, B and D were 50.7%, 43%, 40%, and 90.8%, 93.4% and 89.5%, respectively, when compared to Method C. All these studies suggest that there is no standardised approach for the detection of leishmania species. Microscopy technique has low sensitivity which results in underestimation of the disease, while qPCR has high sensitivity. The current study adds to on-going attempts to develop a method for L. tropica diagnosis, and provides an efficient, cost-effective and scientifically reliable method of genus-level detection.

The current study has limitations. Since AFIP is located in an area with moderate prevalence of CL cases, the developed in-house PCR could not be tested on a larger sample size. Besides, due to limited resources, only three of the samples were 18S-sequenced. Multi-centre studies with large-scale sequencing are needed to validate the current findings.

Conclusion

The internally created PCR test could be regularly utilised for identifying suspected cases of CL since it reliably identified the pathogen in already diagnosed cases.

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Conflict of Interest: None.

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AUTHORS' CONTRIBUTIONS:

HUR: Concept, interpretation, drafting, final approval and accountability.

SHN & AZ: Data analysis, revision, drafting, final approval and accountability.

RI, AI & RS: Data analysis, revision, final approval and accountability.