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Original Article

Molecular Detection of Cutaneous and Mucocutaneous Leishmaniasis in an Endemic Areas of Pakistan

Arshad Ayaz[1], Saddam[2], Nadeem Ullah[3], Abdul Malik[4], Bilal Musa[2], Jamila Bukhari[2], Saira Naz[2], Faheem N Yousaf[2], Wasif Ayaz[5], Sana Tamim[6]

[1] Department of MS Biotechnology, International Islamic University, Islamabad, Pakistan
[2] Department of Health Science, City University of Science and Information Technology, Peshawar, Pakistan
[3] Department of Civil Engineering, City University of Science and Information Technology, Peshawar, Pakistan
[4] Department of Biotechnology, Abdul Wali Khan University Mardan, Mardan, Pakistan
[5] Department of Biochemistry, Quaid e Azam University, Islamabad, Pakistan
[6] Department of Virology and Immunology, National Institute of Health, Islamabad, Pakistan

*Corresponding author: Sana Tamim, sanatamim@gmail.com

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ABSTRACT

Background: Leishmaniasis is a neglected tropical disease with a substantial burden in endemic regions such as Pakistan. Accurate diagnosis and species identification are essential for appropriate case management, epidemiological surveillance, and disease control. Objective: To determine the prevalence and species distribution of cutaneous and mucocutaneous leishmaniasis in endemic regions of Pakistan using SYBR Green real-time polymerase chain reaction (RT-PCR). Methods: This cross-sectional laboratory-based study included 171 clinically suspected patients presenting with cutaneous or mucocutaneous lesions between 2018 and 2019. Clinical specimens were examined by Giemsa-stained microscopy, culture, and SYBR Green RT-PCR targeting the internal transcribed spacer 1 (ITS1) region for species identification. Demographic, clinical, and geographic data were recorded, and descriptive statistics were used to summarize prevalence and distribution patterns. Results: Of the 171 suspected cases, 100 (58.4%) were positive by RT-PCR, compared with 59 (34.5%) by microscopy and 40 (23.3%) by culture. Cutaneous leishmaniasis accounted for 136 cases (79.5%), mucocutaneous leishmaniasis for 31 (18.1%), and mixed presentations for 4 (2.3%). Dry lesions predominated, occurring in 136 patients (79.5%). The highest frequency of cases was observed in the 20-40-year age group (26.9%). Species identification among PCR-positive cases showed Leishmania tropica in 64%, L. major in 22%, L. infantum in 11%, and unclassified species in 3%. The greatest geographic burden was observed in Khyber Pakhtunkhwa. Conclusion: SYBR Green RT-PCR is a sensitive and reliable method for the detection and species-level identification of leishmaniasis. The predominance of L. tropica and the marked concentration of cases in Khyber Pakhtunkhwa emphasize the need for strengthened molecular diagnostics, surveillance, and targeted control strategies in endemic settings. Keywords: Cutaneous leishmaniasis; mucocutaneous leishmaniasis; Leishmania tropica; real-time PCR; Pakistan; molecular diagnosis.

INTRODUCTION

Leishmaniasis is a vector-borne parasitic disease of major global health importance, characterized by a broad clinical spectrum ranging from self-limiting cutaneous lesions to severe mucocutaneous and lifethreatening visceral forms. It is caused by protozoa of the genus Leishmania and transmitted through the bite of infected female phlebotomine sand flies. The disease remains endemic in more than 80 countries, with a substantial burden in tropical and subtropical regions, including South Asia, where environmental, socioeconomic, and geopolitical factors contribute to sustained transmission (1,2).

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Among its clinical manifestations, cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (ML) are the most common forms, often resulting in chronic skin ulcers, permanent scarring, and significant psychosocial stigma (3,4).

Pakistan is recognized as one of the high-burden countries for cutaneous leishmaniasis, with thousands of cases reported annually, particularly in regions bordering Afghanistan and Iran. The disease is highly prevalent in Khyber Pakhtunkhwa and other northern areas, where factors such as population displacement, poor housing conditions, and favorable vector ecology facilitate transmission (5,6). Despite its public health significance, accurate epidemiological data on disease prevalence and species distribution remain limited, partly due to underreporting and reliance on conventional diagnostic methods. The clinical presentation of CL in endemic regions is heterogeneous, with lesions varying in number, morphology, and anatomical distribution, further complicating diagnosis and management (7).

A critical aspect of leishmaniasis epidemiology is the identification of the infecting Leishmania species, as species variation influences disease severity, clinical progression, and therapeutic response. In the Old World, Leishmania tropica, Leishmania major, and Leishmania infantum are the primary species implicated in cutaneous and mucocutaneous forms (8). Traditional diagnostic techniques such as microscopy and parasite culture, although widely used, have inherent limitations, including low sensitivity, inability to differentiate species, and prolonged processing time. These limitations restrict their utility in epidemiological surveillance and targeted disease control strategies (9).

Molecular diagnostic approaches, particularly polymerase chain reaction (PCR)-based methods, have significantly improved the detection and characterization of Leishmania infections. SYBR Green-based real-time PCR assays targeting conserved genomic regions, such as the internal transcribed spacer (ITS) sequences, offer high sensitivity, specificity, and the ability to distinguish between closely related species through melting curve analysis (10). These methods enable rapid and accurate identification of infections even in samples with low parasite loads, making them especially valuable in endemic settings where early and precise diagnosis is essential for effective disease management.

In Pakistan, there remains a paucity of comprehensive studies integrating clinical presentation, prevalence patterns, and molecular species identification using real-time PCR techniques. Existing literature largely relies on microscopy or conventional PCR, with limited data on species-specific distribution and its correlation with demographic and clinical variables. Addressing this gap is essential for improving diagnostic accuracy, guiding treatment strategies, and informing public health interventions. Therefore, the present study aims to determine the prevalence of cutaneous and mucocutaneous leishmaniasis and to identify the predominant Leishmania species in endemic regions of Pakistan using SYBR Green real-time PCR, alongside conventional diagnostic methods. This integrated approach seeks to provide a clearer epidemiological and molecular understanding of the disease in the local context.

MATERIAL AND METHODS

This study was designed as an observational cross-sectional laboratory-based investigation conducted in Pakistan between 2018 and 2019. The study included clinically suspected cases of cutaneous and mucocutaneous leishmaniasis presenting from tropical and subtropical regions, with a particular representation from endemic areas. Participants of all ages and both genders were included. Patients were eligible if they had characteristic skin lesions suggestive of leishmaniasis, including dry or wet ulcers, single or multiple lesions, regardless of prior treatment status. Demographic and clinical information, including age, gender, lesion type, number, anatomical site, duration of disease, and treatment history, was recorded using a structured data collection form.

Specimen collection was performed under aseptic conditions from the active margins of lesions, where parasite load is typically highest (11). After cleaning the lesion area with 70% alcohol, local anesthesia (lidocaine with adrenaline) was administered where required. Multiple needle pricks were made at the

lesion edge, and tissue fluid or blood was collected. Samples were divided for microscopy, culture, and molecular analysis. Thin and thick smears were prepared for staining, a portion was inoculated into culture media, and approximately 200 µL of sample was transferred into sterile microcentrifuge tubes for DNA extraction.

For microscopic examination, air-dried smears were fixed with methanol and stained with Giemsa stain (1:20 dilution). Slides were examined under oil immersion (100× objective) for the presence of intracellular amastigotes within macrophages. Parasite density was graded semi-quantitatively based on standard microscopic fields. Microscopy served as an initial diagnostic screening method (12).

For parasite isolation, samples were inoculated into biphasic culture media consisting of blood agar base supplemented with defibrinated rabbit blood and antibiotics, overlaid with RPMI 1640 medium. Cultures were incubated at 22°C and monitored periodically for up to three weeks for the presence of motile promastigotes. Subculturing was performed to enhance parasite growth where necessary. Culture provided morphological confirmation but was not used for species identification (9,13). Genomic DNA was extracted from culture-positive samples or directly from clinical specimens using a commercially available silica column-based DNA extraction kit (QIAGEN, Germany), following the manufacturer's protocol. Briefly, samples were lysed with proteinase K and lysis buffer, followed by ethanol precipitation, column binding, washing, and elution. Extracted DNA was stored at −70°C until further analysis. DNA quality and presence were confirmed by agarose gel electrophoresis using ethidium bromide staining.

Figure 3. Laboratory workflow for detection and molecular identification of Leishmania. (A) Sample collection using finger-prick technique from lesion margin. (B) Ulcerative cutaneous lesion showing active sampling site. (C) Autoclave used for sterilization of media and laboratory equipment. (D) Biosafety cabinet class II for aseptic handling of samples. (E) Preparation of culture media following sterilization. (F) Addition of defibrinated rabbit blood to prepare enriched media for parasite growth. (G) Real-time PCR system (ABI 7500) used for molecular detection. (H) DNA extraction setup with pipettes, tubes, and reagents. (I) Agarose gel electrophoresis showing extracted genomic DNA.

Molecular detection and species identification were performed using SYBR Green-based real-time polymerase chain reaction (RT-PCR). The internal transcribed spacer 1 (ITS1) region of Leishmania DNA was targeted using genus-specific forward and reverse primers. The reaction mixture consisted of SYBR Green master mix, primers, nuclease-free water, and extracted DNA template in a total reaction volume of 20 µL. Amplification was carried out in an ABI 7500 Real-Time PCR system under standard cycling conditions, including initial denaturation, followed by 40 amplification cycles and a melting

curve analysis stage. Species differentiation was based on characteristic melting temperature (Tm) profiles corresponding to different Leishmania species (10,14,15).

The primary outcome measures included overall prevalence of leishmaniasis, diagnostic positivity rates by microscopy, culture, and RT-PCR, and species distribution identified by molecular methods. Secondary variables included lesion characteristics (type, number, site), demographic distribution (age, gender), and geographic distribution (district and province). Quality control measures were implemented throughout laboratory procedures, including the use of sterile techniques, negative controls in PCR assays to detect contamination, and standardized protocols for sample handling and processing (9,14,15). All procedures were conducted in a biosafety level II environment using appropriate personal protective equipment.

Data were analyzed using descriptive statistical methods. Frequencies and percentages were calculated for categorical variables, including prevalence rates, lesion characteristics, species distribution, and demographic patterns. Results were stratified by age groups, gender, lesion type, and geographic regions where applicable. Given the primarily descriptive nature of the study, no advanced inferential statistical testing was applied. Ethical considerations were observed throughout the study. Informed consent was obtained from all participants or their guardians prior to sample collection, and patient confidentiality was maintained. The study procedures were conducted in accordance with institutional and national ethical guidelines for biomedical research.

RESULTS

A total of 171 clinically suspected patients were included in the study. Among these, 65 (38.0%) were male and 72 (42.1%) were female, while gender data were not recorded for 34 cases (19.9%). The highest proportion of cases was observed in the 20–40-year age group (n = 46, 26.9%), followed by individuals aged >40 years (n = 39, 22.8%). Real-time PCR identified 100 positive cases (58.4%), compared to 59 (34.5%) detected by microscopy and 40 (23.3%) by culture, indicating the highest diagnostic yield for RT-PCR. Regarding lesion characteristics, single lesions were observed in 80 patients (46.8%), two lesions in 28 patients (16.4%), and multiple lesions in 63 patients (36.8%). Overall, dry lesions predominated (n = 136, 79.5%), while wet lesions accounted for 35 cases (20.5%). The most commonly affected anatomical sites included exposed areas such as the hands, feet, and lips.

Clinically, cutaneous leishmaniasis (CL) constituted 136 cases (79.5%), while mucocutaneous leishmaniasis (ML) accounted for 31 cases (18.1%), and mixed presentations were observed in 4 patients (2.3%). Age- and gender-stratified analysis showed a higher frequency of infection among females across most age groups, particularly in individuals aged 20–40 years (n = 26 females vs 20 males) and >40 years (n = 32 females vs 7 males). Among PCR-confirmed cases (n = 100), Leishmania tropica was the predominant species (n = 64, 64%), followed by Leishmania major (n = 22, 22%) and Leishmania infantum (n = 11, 11%), while 3 cases (3%) remained unclassified.

Table 1. Baseline Demographic Characteristics (N = 171)

Variable	Category	n	%
Gender	Male	65	38.0
	Female	72	42.1
	Unrecorded	34	19.9
Age Group (years)	2–5	23	13.5
	6–10	30	17.5
	11–20	33	19.3
	20–40	46	26.9
	>40	39	22.8

Table 2. Diagnostic Yield (N = 171)

Method	Positive (n)	%

RT-PCR	100	58.4
Microscopy	59	34.5
Culture	40	23.3

Table 3. Lesion Characteristics (N = 171)

Lesion Category	n	%
Single lesion	80	46.8
Two lesions	28	16.4
Multiple lesions	63	36.8
Total	171	100

Table 3a. Lesion Type Distribution

Type	n	%
Dry lesions	136	79.5
Wet lesions	35	20.5

Table 4. Clinical Classification (N = 171)

Type	n	%
Cutaneous (CL)	136	79.5
Mucocutaneous (ML)	31	18.1
Mixed	4	2.3

Table 5. Age–Gender Distribution (N = 171)

Age Group	Male	Female	Total
2–5	12	11	23
6–10	10	20	30
11–20	16	17	33
20–40	20	26	46
>40	7	32	39
Total	65	72	171

Table 6. Species Distribution (PCR-positive n = 100)

Species	n	%
Leishmania tropica	64	64

Leishmania major	22	22
Leishmania infantum	11	11
Unconfirmed	3	3
Total	100	100

Table 7. Treatment vs PCR Positivity

Treatment	Total	PCR +	%
Glucantime users	45	7	15.6
Non-users	126	93	73.8

Table 8. Geographic Distribution (N = 171)

Region	n	%
Khyber Pakhtunkhwa	78	45.6
Punjab	42	24.6
Balochistan	29	17.0
Sindh	22	12.9
Total	171	100

Treatment history analysis revealed that among 45 patients who received meglumine antimoniate (Glucantime), only 7 (15.6%) were PCR-positive, whereas among 126 untreated patients, 93 (73.8%) tested positive, indicating a higher detection rate among untreated individuals. Geographically, the highest burden of disease was observed in Khyber Pakhtunkhwa (n = 78, 45.6%), followed by Punjab (n = 42,

24.6%), Balochistan (n = 29, 17.0%), and Sindh (n = 22, 12.9%). Species differentiation based on melting curve analysis demonstrated distinct temperature ranges corresponding to L. infantum (78.0–79.0°C), L. tropica (79.0–79.5°C), and L. major (80.0–80.8°C), confirming reliable molecular identification.

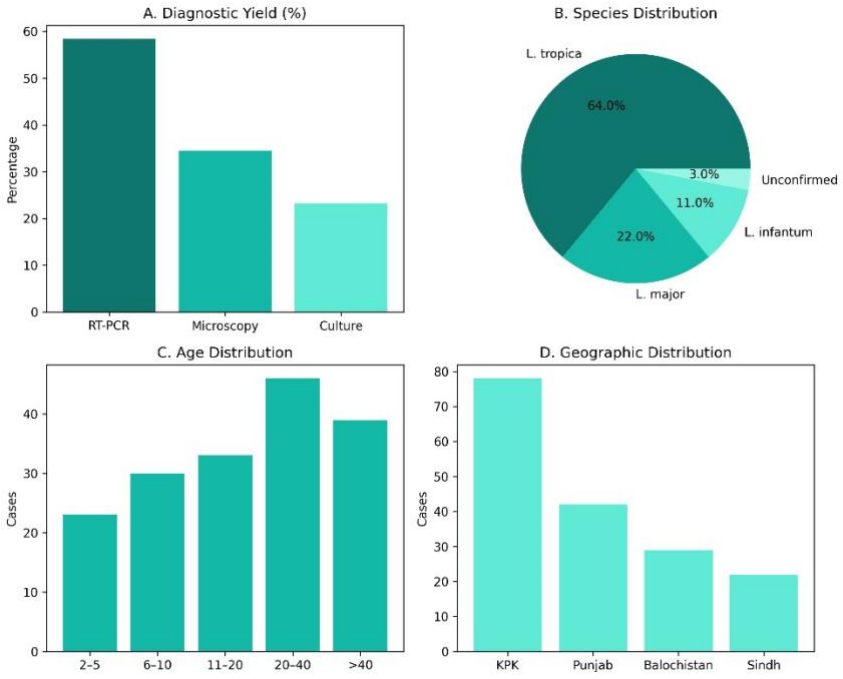

Figure 1 (A) Diagnostic positivity rates across RT-PCR, microscopy, and culture methods, demonstrating the highest detection rate for RT-PCR. (B) Distribution of Leishmania species identified by SYBR Green real-time PCR, with L. tropica

as the predominant species. (C) Age-wise distribution of cases, showing peak frequency in the 20–40-year age group. (D) Geographic distribution of cases across provinces, highlighting the highest burden in Khyber Pakhtunkhwa.

The composite figure illustrates key epidemiological and diagnostic patterns observed in the study. RTPCR demonstrated superior diagnostic sensitivity compared to microscopy and culture, identifying the majority of confirmed cases. Species distribution analysis revealed a clear predominance of Leishmania tropica, followed by L. major and L. infantum, indicating a typical Old World epidemiological pattern. Age distribution showed a concentration of cases in the economically active 20–40-year group, suggesting increased exposure risk. Geographically, the highest disease burden was observed in Khyber Pakhtunkhwa, consistent with known endemicity in regions bordering Afghanistan. Collectively, these findings emphasize the combined clinical, molecular, and geographic dimensions of leishmaniasis transmission in Pakistan.

DISCUSSION

The present study provides an integrated clinical, epidemiological, and molecular characterization of cutaneous and mucocutaneous leishmaniasis in endemic regions of Pakistan. The findings demonstrate a high prevalence of disease among clinically suspected individuals, with real-time PCR identifying over half of the cases, thereby confirming its superior diagnostic sensitivity compared to microscopy and culture. This aligns with previous studies indicating that conventional methods often underestimate disease burden due to low sensitivity, particularly in samples with minimal parasite load, whereas molecular techniques enable detection even in subclinical or early-stage infections (29,46).

The predominance of cutaneous leishmaniasis observed in this study is consistent with regional epidemiological patterns across South Asia, where CL constitutes the majority of reported cases. The high proportion of dry lesions further reflects the classical presentation associated with Old World species, particularly Leishmania tropica. The observed lesion distribution on exposed body parts such as hands, feet, and face highlights the role of vector exposure in disease transmission and supports the hypothesis that occupational and environmental factors significantly influence infection risk (7,24,47).

Species identification revealed Leishmania tropica as the most prevalent organism, followed by L. major and L. infantum. This distribution is consistent with previous reports from Pakistan, Afghanistan, and Iran, where L. tropica is the dominant causative agent of anthroponotic cutaneous leishmaniasis (30,35,41). The presence of L. major suggests a zoonotic transmission component, while detection of L. infantum, although less frequent, indicates the potential for broader ecological diversity. Species-level identification is clinically relevant, as it influences disease progression, risk of mucosal involvement, and therapeutic response, thereby reinforcing the importance of molecular diagnostics in endemic settings (23,25,47).

Demographic analysis demonstrated a higher burden of disease among females and a peak incidence in the 20–40-year age group. The age distribution likely reflects increased mobility and occupational exposure in this population, while the higher proportion of female cases may be attributed to sociocultural and environmental factors, including indoor vector exposure and healthcare-seeking behavior. Similar demographic patterns have been reported in regional studies, although variability exists depending on local transmission dynamics (1,40).

Geographically, the highest concentration of cases was observed in Khyber Pakhtunkhwa, supporting existing evidence that this region represents a major endemic focus of leishmaniasis in Pakistan. Factors such as proximity to Afghanistan, population displacement, inadequate housing, and favorable climatic conditions for sand fly breeding contribute to sustained transmission in this area. Lower prevalence observed in provinces such as Sindh may reflect differences in ecological conditions, vector distribution, or underreporting (22,41).

The comparison of diagnostic modalities clearly demonstrated the superiority of SYBR Green real-time PCR over microscopy and culture. While microscopy remains a rapid and accessible tool, its limited sensitivity and inability to differentiate species restrict its utility. Culture methods, although useful for parasite isolation, are time-consuming and less practical in routine clinical settings. In contrast, realtime

PCR provides rapid, sensitive, and species-specific detection, making it a valuable tool for both clinical diagnosis and epidemiological surveillance. These findings support the growing consensus that molecular methods should be integrated into standard diagnostic algorithms in endemic regions (29,33,46).

Despite its strengths, the study has several limitations. The sampling approach was based on clinically suspected cases, which may introduce selection bias and limit generalizability to the broader population. Incomplete demographic data in some cases may affect the precision of subgroup analyses. Additionally, the study primarily employed descriptive analysis without advanced statistical modeling, limiting the ability to identify independent risk factors. Future studies incorporating larger, population-based samples and analytical epidemiological designs are warranted to further elucidate transmission dynamics.

CONCLUSION

Cutaneous and mucocutaneous leishmaniasis remain significant public health concerns in Pakistan, with a high burden observed among clinically suspected cases. This study demonstrates that SYBR Green real-time PCR provides superior sensitivity for detection and enables reliable species identification compared to conventional microscopy and culture methods. Leishmania tropica was identified as the predominant species, followed by L. major and L. infantum, reflecting the typical epidemiological pattern of Old World leishmaniasis. The concentration of cases in Khyber Pakhtunkhwa and among the 20–40-year age group highlights key demographic and geographic risk patterns. These findings underscore the importance of integrating molecular diagnostics into routine clinical practice and strengthening surveillance strategies to improve early detection, targeted treatment, and effective disease control in endemic regions.

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