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Molecular Approaches for Enhanced Detection and Identification of *Leishmania* Parasites in Endemic Hot Spots of Dir Lower, Khyber Pukhtunkhwa, Pakistan

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ABSTRACT

Cutaneous leishmaniasis (CL) is a growing health concern in Pakistan, particularly in endemic regions such as District Lower Dir, Khyber Pakhtunkhwa (KPK), where environmental and socio-economic conditions facilitate the spread of Leishmania parasites through infected sandflies. This study, conducted from May to September 2024, employed modern molecular techniques, including polymerase chain reaction (PCR), to detect and identify Leishmania species in the area. Out of 167 samples, 78.4% (131 samples) tested positive for Leishmania, highlighting PCR's higher sensitivity and specificity compared to traditional diagnostic methods like microscopy. Genetic analysis identified *Leishmania tropica* as the most prevalent species, with a 98.85% similarity to previously documented strains. Phylogenetic analysis revealed limited genetic variation within *L. tropica*, suggesting the presence of a genetically uniform parasite population in the region. This research emphasizes the importance of integrating molecular diagnostics into local health surveillance systems for early detection, accurate species identification, and effective disease management. It also offers vital insights into the genetic diversity of *Leishmania tropica*, which can inform targeted control measures to reduce the burden of CL in District Lower Dir and similar endemic areas.

INTRODUCTION

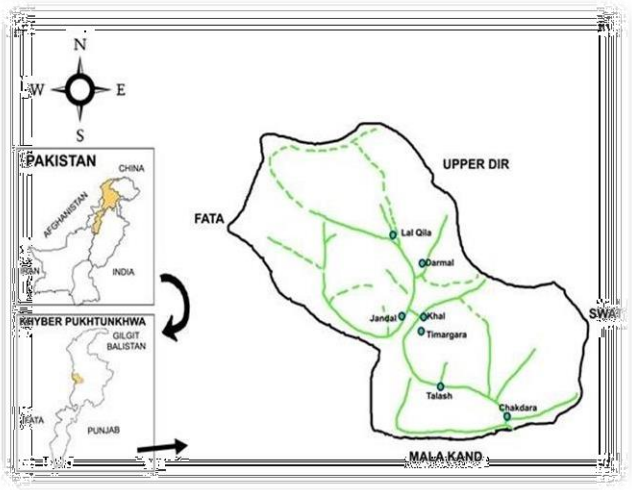
Leishmaniasis, a diverse set of diseases caused by protozoan parasites of the *Leishmania* genus, represents a major global health issue, particularly in tropical and subtropical regions. With over 20 distinct *Leishmania* species implicated in human infections, the disease affects millions of people worldwide and poses significant challenges to both public health and economic development, especially in endemic areas[1]. The *Leishmania* parasites are transmitted primarily by female sandflies, species belonging to the *Phlebotomus* genus in the Old World and the *Lutzomyia* genus in the New World. These sandflies transmit the parasite through their bites, delivering the *Leishmania* promastigotes, which proliferate within the host's macrophages[2]. Leishmaniasis presents in three primary clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). These forms are caused by different *Leishmania* species, each with distinct geographical distributions and clinical manifestations[3]. CL, the most common form, typically results in localized skin ulcers, while MCL can lead to severe mucosal damage, particularly in the nose, mouth, and throat. VL, on the other hand, affects internal organs like the liver and spleen and is the most fatal form of the disease, with high case-fatality rates[4]. Despite its significant impact, leishmaniasis is often considered a neglected tropical disease (NTD), as it primarily affects populations in developing nations, where it can go undiagnosed or misdiagnosed due to lack of resources and awareness[5]. Leishmaniasis is categorized as either anthroponotic (when humans serve as the primary reservoir) or zoonotic (when animals serve as the reservoir), further complicating control efforts[6]. The disease is endemic across 99 countries globally, including regions of Latin America, Africa, Asia, and the Middle East, and it remains

a significant health burden[7]. According to the World Health Organization (WHO), between 20,000 and 30,000 deaths are reported annually due to leishmaniasis, with approximately 12 million people worldwide suffering from the disease[8]. The geographic spread of leishmaniasis is closely linked to environmental conditions, with factors such as climate change, migration patterns, and socio-economic conditions influencing the distribution and prevalence of the disease. For example, the increase in CL cases in Mediterranean regions, such as the Middle East and Central Asia, is linked to internal migration, which can bring populations into endemic areas, thus facilitating the transmission of the parasite[9]. Similarly, in countries like Afghanistan, Pakistan, Syria, and Brazil, CL remains endemic, with outbreaks often occurring in areas with poor sanitary conditions and limited access to healthcare[10]. In Pakistan, CL has become a growing concern, especially in regions such as Khyber Pakhtunkhwa (KPK) and areas impacted by Afghan refugees and military conflicts. Originally identified in the country's northern hills in 1960, CL has since spread across almost every part of Pakistan, with reports of 400,000 cases in 2016, constituting approximately 10% of the global CL burden[11]. CL remains a major health issue in mountainous regions of Pakistan, particularly in the provinces of KPK and Baluchistan, where outbreaks have been reported[12]. Furthermore, both CL and VL are now common in regions such as the Federally Administered Tribal Areas (FATA), exacerbating the overall disease burden. The transmission of CL is primarily facilitated by the bite of female sandflies, which inject the *Leishmania* promastigotes into the host's skin. These sandflies are found in regions across the globe, particularly in tropical and subtropical zones. The sandfly species *Phlebotomus* (Old World) and *Lutzomyia* (New World) are responsible for transmitting various species of *Leishmania*, with each region hosting specific species adapted to local ecological conditions[13]. The *L. major* and *L. tropica* species are found in the Old World, while *L. braziliensis*, *L. mexicana*, and *L. amazonensis* dominate the New World[2]. In these regions, CL is often associated with particular ecological conditions, such as forests in the Americas and dry, exposed regions in the Old World[13]. CL is the most common and widely reported form of leishmaniasis. It is characterized by the development of skin ulcers at the site of the sandfly bite. These ulcers are typically localized but can vary in appearance, ranging from papular lesions to nodules and eventually ulcerating lesions[4]. Although CL is often self-healing in many cases, the scars left behind can cause disfigurement, leading to significant psychological and social consequences for affected individuals[5]. In areas where CL is endemic, the disease significantly impacts the affected communities, both in terms of public health and economic losses due to lost productivity[14]. The distribution of CL is particularly high in countries such as Afghanistan, Iran, Pakistan, Brazil, and Algeria, with these nations accounting for a significant proportion of global CL cases[10]. In Pakistan, as mentioned earlier, the disease is primarily found in the northern regions but has spread across the country due to internal migration and the arrival of refugees from neighboring countries. The disease is also linked to military personnel and peacekeeping forces deployed in endemic areas, such as the Federally Administered Tribal Areas (FATA), where CL transmission has been reported[12]. The species of *Leishmania* responsible for CL vary depending on the geographic region. In the Old World, *L. major*, *L. tropica*, and *L. infantum* are commonly responsible for CL, while in the New World, species such as *L. braziliensis*, *L. mexicana*, and *L. amazonensis* are more prevalent[13]. These species exhibit distinct transmission dynamics, with *L. major* and *L. tropica* generally causing less severe forms of CL, while *L. braziliensis* can lead to more severe forms, such as mucocutaneous leishmaniasis (MCL), which causes severe facial and mucosal lesions[4]. The presence of different *Leishmania* species in endemic regions is influenced by factors such as vector species, environmental conditions, and the presence of suitable reservoirs, which include domestic animals and wild mammals like foxes, rats, and dogs[14]. The life cycle of *Leishmania* is complex, involving both a vector (sandfly) and a mammalian host. Female sandflies transmit *Leishmania* parasites through their bites, during which they inject the promastigote stage of the parasite into the skin of the host. Inside the host's macrophages, the promastigotes transform into amastigotes, which proliferate and cause the lesions associated with CL[2]. The amastigotes are then ingested by sandflies when they take a blood meal from an infected host. Inside the sandfly, the amastigotes transform into promastigotes, completing the cycle[13]. Traditionally, diagnosing CL has relied on clinical presentation, parasitological examination, and serological tests. However, recent advances in molecular techniques, particularly polymerase chain reaction (PCR), have improved the sensitivity and specificity of leishmaniasis diagnosis[2]. PCR-based methods have become a valuable tool in the diagnosis of CL, particularly in detecting low parasite loads that might not be visible through microscopy. PCR is also useful in identifying the specific *Leishmania* species responsible for the infection, which is crucial for understanding transmission dynamics and implementing appropriate treatment strategies[13].

METHODOLOGY

Figure 1

Map of Khyber Pakhtunkhwa (Hussain et al., 2019)



This study, approved by the District Health Officer and

the Bioethical Committee of Quaid-i-Azam University (BEC-FBS-QAU2024-693), was conducted in District Dir Lower, Khyber Pakhtunkhwa (KPK), Pakistan, a region located between latitudes 34°37' to 35°07' north and longitudes 71°31' to 72°14' east. The district spans an area of 1,583 km^2 with a population density of 816.8 persons/km^2 (Almanac, 2021). The study period was from May to September 2024, allowing for a comprehensive collection of samples from endemic areas. This region, marked by its unique environmental and socio-economic factors, presented an opportunity to assess the presence of *Leishmania* parasites in the local population.

For sample collection, lesions were first scrubbed with alcohol swabs or Dettol to remove surface contaminants. A sterile lancet was then used to puncture the lesion, allowing blood and pus to exude. The collected pus was absorbed onto Whatman filter paper with a 2 µm pore size, by pressing the paper against the lesion until sufficient material had been collected. After drying at room temperature, the filter paper was sealed in polyethylene bags containing silica gel beads to preserve the samples. These samples were stored at temperatures between -2°C and -4°C for further molecular analysis.

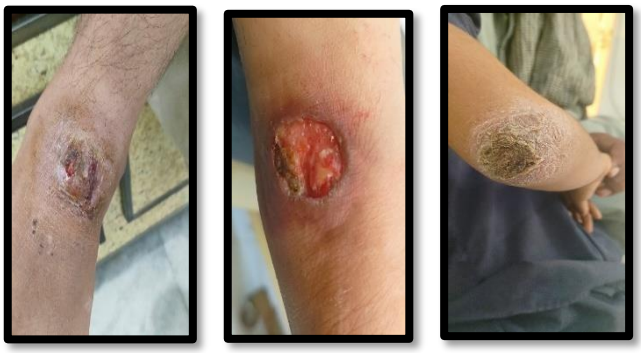
DNA extraction was carried out using a two-day protocol. On Day 1, the filter paper punches were placed in a 1.5 ml Eppendorf tube with 250 µL of lysis buffer, 2.5 µL of Triton X100, and 1.30 µL of proteinase K. This mixture was incubated at 37°C for 24 hours, as described by Shaheen et al. (2020, 2021). On Day 2, the samples were centrifuged at 8050g for 10 minutes, and DNA was extracted using the GeneJET Genomic DNA Purification kit. The procedure involved lysis, ethanol precipitation, washing, and elution steps. The purified DNA was stored at -20°C until further processing.

Gel electrophoresis was performed to assess the quality of the extracted DNA. A 1% agarose gel was prepared with ethidium bromide, and the DNA samples were loaded with loading dye. The gel was run for 1 hour at 90 volts and analyzed under UV light for DNA visualization. For PCR amplification, *Leishmania*-specific primers (LITSR forward: 5'CTGGATCATTTTCCGATG3' and reverse: 5'TGATACCACTTATCGCACTT3') targeting the ITS1 region of ribosomal DNA were used (Ahmad et al., 2013; Bensoussan et al., 2006). The PCR reaction mixture consisted of DNA template, primers, nuclease-free water, and the Dream Green PCR Master Mix. The amplification cycle included denaturation at 94ºC for 3 minutes, followed by 37 cycles of 95ºC for 30 seconds, 44ºC for 30 seconds, and 72ºC for 60 seconds, with a final extension at 72ºC for 5 minutes.

Post-PCR, the amplified products were analyzed using 2% agarose gel electrophoresis, and a 100bp DNA ladder was employed to estimate the size of the amplified bands. The gel was visualized under UV light, and the PCR products were purified using the Solarbio DNA Product Purification Kit to eliminate residual primers and nucleotides. The purified DNA samples were then sent to the Beijing Genomics Institute (BGI) in China for Sanger sequencing. Phylogenetic analysis was performed using MEGA X software to understand the genetic relationships of the *Leishmania* species identified. Descriptive statistics of the collected data were analyzed using SPSS software. This methodology enabled the identification and genetic characterization of *Leishmania* species in the endemic areas of District Dir Lower.

Figure 2

Shows Lesions of Cutaneous Leishmaniasis Patients



RESULTS

Table 1

PCR Test Results for Leishmania Detection in the Study Population

PCR Result	Frequency	Percent (%)
Positive	131	78.4
Negative	36	21.6
Total	167	100

The PCR test revealed that 78.4% (131 out of 167) of the cases were positive for *Leishmania*, while 21.6% (36 out of 167) tested negative. This highlights the high sensitivity of the PCR method in detecting *Leishmania* in the sampled population, reflecting a significant presence of the parasite in the region. The positive results demonstrate the effectiveness of PCR as a diagnostic tool for identifying infections, even when the parasite load is low, which may be missed by traditional diagnostic methods like microscopy.

Table 2

Prevalence and Genetic Similarity of Leishmania Species in District Dir Lower, Khyber Pakhtunkhwa, Pakistan.

Leishmania Species	Prevalence in Study Region	Sequence Similarity (%)	Details
Leishmania tropica	Most prevalent species	98.85%	Confirmed as the predominant species in the study area, showing high genetic similarity to previously documented strains.
Other Leishmania Species	Not identified	-	No other Leishmania species were identified in the study region.

Leishmania tropica was the most prevalent species, accounting for a significant proportion of infections in District Dir Lower, Khyber Pakhtunkhwa, Pakistan.

The 98.85% sequence similarity with previously documented strains of Leishmania tropica confirms its identity and highlights the reliability of molecular

techniques for species-level identification.

Figure 3

Leishmania Species Prevenance

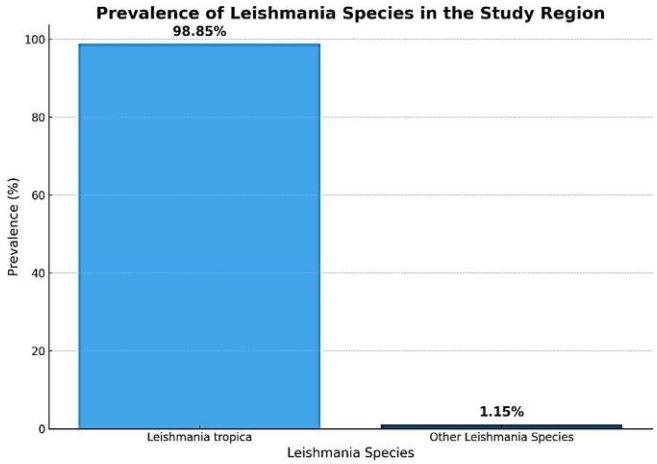
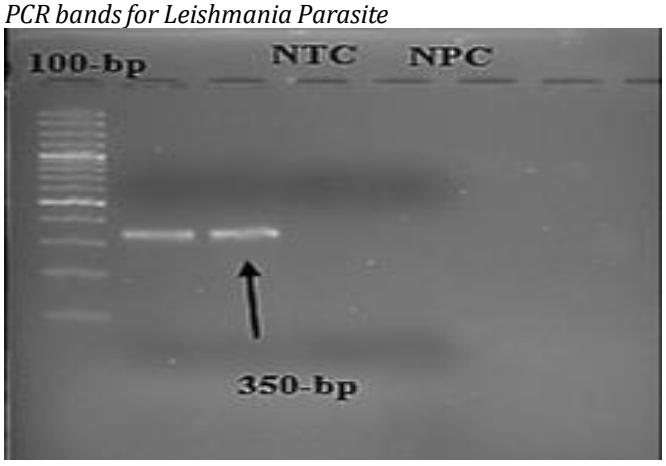


Figure 4

PCR bands for Leishmania Parasite



Sequence and Phylogenetic Analysis

The DNA sequences obtained from Sanger sequencing were initially processed to correct base-calling errors and remove any low-quality regions. This editing procedure was conducted using BioEdit software, a widely recognized and reliable tool for sequence alignment and manipulation in molecular biology. Following the editing process, the sequences were aligned to ensure an accurate and robust comparison across the data set.

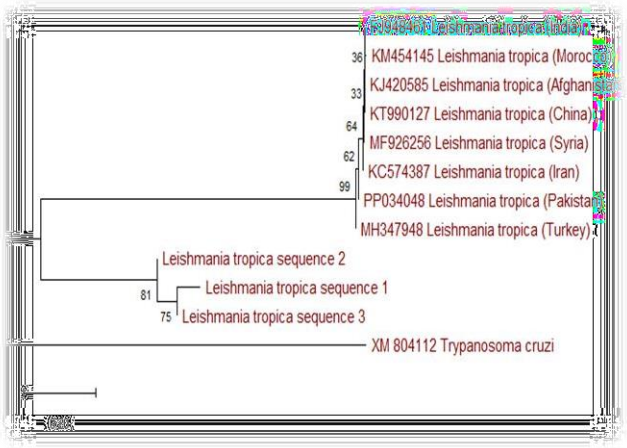
To authenticate the species identity of the sequenced isolates, a BLAST (Basic Local Alignment Search Tool) analysis was conducted by comparing the obtained sequences against the NCBI GenBank database. This comparison revealed a striking 98.85% similarity with previously documented sequences of *Leishmania tropica*, confirming that the isolates from this study were indeed *L. tropica*, thus ensuring species-level identification.

To further investigate the genetic relationships and evolutionary dynamics of the *Leishmania tropica* isolates, a phylogenetic tree was constructed using the Neighbor-Joining (NJ) method, as implemented in MEGA version 10 software. This method allowed for a comprehensive understanding of the genetic relatedness between the isolates. The resulting phylogenetic tree visually represented the evolutionary distances among the isolates, providing insights into their evolutionary relationships.

To root the phylogenetic tree and better interpret the evolutionary divergence, *Trypanosoma cruzi*, a related protozoan species, was included as an outgroup. This approach facilitated a more precise analysis of lineage separation and provided clearer insights into the phylogenetic structure of *L. tropica*. The phylogenetic analysis offered a deeper understanding of the genetic diversity of *Leishmania tropica* in the study region, which could be crucial for understanding the parasite's transmission dynamics and aiding in the development of targeted control measures.

Figure 5

Phylogenetic Systematic Tree/Classification



The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.25073940 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 11 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 623 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

DISCUSSION

The results of this study underscore the significant role of molecular techniques, particularly polymerase chain reaction (PCR), in detecting and identifying *Leishmania* species in endemic regions. The high PCR positivity rate of 78.4% in the 167 samples collected indicates the effectiveness of PCR in accurately identifying *Leishmania* infections, which may otherwise be undetected by conventional diagnostic methods such as microscopy. The use of PCR, specifically targeting the ribosomal ITS1 region of the *Leishmania* genome, has been widely recognized for its high sensitivity and specificity, enabling the detection of low parasite loads that would be missed by standard parasitological methods (Moreira et al., 2018)[14]. This corroborates findings from previous studies where PCR demonstrated higher sensitivity compared to traditional microscopy in diagnosing leishmaniasis (Rasti

et al., 2016)[15].The sequence analysis of the PCR-amplified products revealed a 98.85% similarity to previously documented sequences of *Leishmania tropica*. This result supports the identification of *L. tropica* as the predominant species in the study area, consistent with reports from other endemic regions where *L. tropica* has been implicated as the leading causative agent of cutaneous leishmaniasis (Cosma et al., 2024)[13]. Phylogenetic analysis further strengthened this species-level identification, and the evolutionary tree provided valuable insights into the genetic diversity of *L. tropica* isolates in the study region. These findings align with previous research on the genetic diversity of *Leishmania* species in endemic hotspots (Almeida-Souza et al., 2024)[12].The phylogenetic analysis, conducted using the Neighbor-Joining method, revealed clear clustering of the *L. tropica* isolates, offering a visual representation of their genetic relatedness. The inclusion of *Trypanosoma cruzi* as an outgroup in the analysis facilitated the interpretation of lineage divergence and helped establish evolutionary distances between the isolates. The use of an outgroup is a common strategy in phylogenetic analysis to root the tree and better understand the evolutionary history of the species under investigation (Alvar et al., 2007)[14]. The high genetic similarity among the isolates suggests limited genetic variation within the population of *L. tropica* in the study region, which may have implications for control strategies, as uniformity in genetic traits could influence the effectiveness of treatment and intervention measures.The detection of *Leishmania* species through molecular methods also has significant public health implications. Early and accurate diagnosis is critical for effective treatment and management of cutaneous leishmaniasis, which remains a major health issue in many parts of the world, including Pakistan (Zeb et al., 2021)[18]. As emphasized by WHO (2023)[19], the implementation of advanced molecular diagnostics could improve the early detection and accurate identification of *Leishmania* species, ultimately aiding in timely treatment and reducing the disease burden in endemic regions.Moreover, the findings of this study also highlight the need for continued research into the genetic diversity and transmission dynamics of *Leishmania* species. Understanding the evolution and spread of *L. tropica* in different regions is crucial for developing targeted control measures, especially in areas where the disease is on the rise due to factors such as migration and changes in environmental conditions (de Vries & Schallig, 2022)[20]. Additionally, the insights gained from phylogenetic analysis could contribute to the development of vaccines or more effective treatments tailored to the genetic diversity of *Leishmania* parasites (Steverding, 2017)[17].

CONCLUSION

In conclusion, the molecular methods used in this study, particularly PCR and phylogenetic analysis, have provided valuable insights into the presence and genetic diversity of *Leishmania tropica* in the District Dir Lower, Khyber Pakhtunkhwa, Pakistan. These findings highlight the importance of integrating molecular diagnostics into local health surveillance systems to enhance the early detection and management of leishmaniasis. Furthermore, they emphasize the need for continued research into the genetic characteristics of *Leishmania* species to inform public health strategies and interventions aimed at reducing the burden of this neglected tropical disease.

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