

BioScientific Review (BSR)

Volume 6 Issue 1, 2024

ISSN(P): 2663-4198 ISSN(E): 2663-4201

Homepage: <https://journals.umt.edu.pk/index.php/bsr>



Article QR



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Author (s): Asad Ullah¹, Zobia Afsheen¹, Mian Syed Ahmad², Tariq khan³, Aamina Azam⁵, Muhammad Medrar Hussain¹, Muhammad Umair^{2,4}

Affiliation (s): ¹Abasyn University, Peshawar, Pakistan
²Institute of Basic Medical Sciences, Khyber Medical University, Peshawar, Pakistan
³Ahmad Medical Institute, Peshawar, Pakistan
⁴University of Haripur, KP, Pakistan
⁵Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar, Pakistan

DOI: <https://doi.org/10.32350/bsr.61.05>

History: Received: October 12, 2023, Revised: November 20, 2023, Accepted: January 8, 2023, Published: January 30, 2024

Citation: Ullah A, Afsheen Z, Ahmad MS, khan T, Azam A, Hussain MM, Umair M. Molecular investigation of Cutaneous Leishmaniasis (CL) from biopsy samples: a case study of district Mohmand, KPK. *BioSci Rev.* 2024;6(1):109–120. <https://doi.org/10.32350/bsr.61.05>

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Conflict of Interest: Author(s) declared no conflict of interest



UMT

A publication of
The Department of Life Sciences, School of Science
University of Management and Technology, Lahore, Pakistan

Molecular Investigation of Cutaneous Leishmaniasis (CL) from Biopsy Samples: A Case Study of District Mohmand, KPK

Asad Ullah¹, Zobia Afsheen¹, Mian Syed Ahmad², Tariq Khan³, Aamina Azam⁴, Muhammad Medrar Hussain¹ and Muhammad Umair^{2,5*}

¹Department of Health & Biological Sciences, Abasyn University, Peshawar, Pakistan

²Institute of Basic Medical Sciences, Khyber Medical University, Pakistan

³Department of Allied Health Sciences, Ahmad Medical Institute Peshawar, Pakistan

⁴Institute of Pathology and Diagnostic Medicine, Khyber Medical University, Peshawar, Pakistan

⁵Department Medical of Lab Technology, University of Haripur, Pakistan

ABSTRACT

Background: Leishmaniasis is a complex disease with diverse clinical manifestations and remains a significant public health concern, particularly in tropical and subtropical regions around the world. Cutaneous leishmaniasis (CL), being the most prevalent type, causes significant morbidity and social stigmatization. District Mohmand, an arid mountainous region in the KPK province of Pakistan, has an increased burden of CL with no prior molecular study reported on it in this region of Pakistan.

Methods: The current study investigated CL using molecular techniques to detect and evaluate the risk factors associated with this disease in the affected population of District Mohmand. The study was conducted at Tehsil Head Quarter Hospital Mamad Gat, District Mohmand.

Results: Out of 150 samples, 106 were found to be positive and 44 turned out to be negative under microscopy, while 122 were positive and 28 were negative using PCR analysis. Statistical analysis revealed that children between the ages of 1 and 10 years are frequently affected. Similarly, male gender is affected to a greater degree as compared to the female gender. Furthermore, the proportion of facial lesions was 62%, followed by upper limbs (17%), lower limbs (17%), and lesions on multiple sites (4%). These numbers are associated with various factors, such as the presence of domestic animals, lack of bed net usage, migration, and the type of wall and roof.

Conclusion: The study concluded that children are more prone to infection. The PCR method was found to be more reliable for diagnosing CL as compared to microscopy.

Keywords: Cutaneous Leishmaniasis (CL), District Mohmand, ITS1-PCR, microscopy

Highlights:

1. The study reveals the high prevalence of Cutaneous Leishmaniasis (CL) in District Mohmand, Khyber Pakhtunkhwa, employing both traditional microscopy and validated PCR techniques for accurate diagnosis.

*Corresponding Author: : umairkhan.ibms@kmu.edu.pk

2. The study identifies risk variables for CL, including age, gender, location, occupation, length of active lesions, travel history, sleeping habits, and medication use, providing valuable insights into the disease's epidemiology.
3. The study emphasises early detection and comprehensive management of CL, particularly in children, and recommends public health interventions addressing animal interaction, sleeping habits, travel history, and medication use for effective disease control.

1. INTRODUCTION

Leishmaniasis is a complex vector-borne disease caused by different species of the protozoan parasite 'Leishmania' with diverse clinical manifestations [1]. It is a significant public health concern in many regions around the world, particularly in tropical and subtropical areas [2]. It is categorized as Cutaneous leishmaniasis (CL), Mucocutaneous leishmaniasis, and Visceral leishmaniasis [3, 4] with CL being the most prevalent [5]. The infection is confined to the skin, manifesting as skin lesions, ulcers, and nodules and causing significant morbidity and social stigmatization [6].

Leishmaniasis spreads by the species of the genera *Sergentomyia*, *Phlebotomus*, and *Lutzomyia*, with *Phlebotomus* being the most pathogenic [7]. It can spread to humans through the bite of infected sandflies and hence, is categorized as a zoonotic disease (reservoir being wild animals) [8]. However, there is also an anthroponotic form where leishmaniasis is transmitted directly from one individual to another by a sandfly vector [9]. In this case, humans are likely the only reservoir hosts, though occasional cases of animals including dogs being reservoir hosts have been reported [10]. Adult sandflies, both male and female, primarily feed on plant nectar for their nutrition. However, female sandflies also require blood from mammals and birds to lay their eggs [11, 12]. It is through this process of biting an animal host that only female sandflies can transmit the leishmania parasite [9, 13].

The number of leishmaniasis cases has significantly increased in recent decades [14]. The World Health Organization (WHO) has selected leishmaniasis as one of the six diseases for its special program on tropical disease research and training [15]. It ranks as the second highest in terms of both morbidity and mortality among human protozoan diseases after malaria. According to WHO, twelve (12) to thirteen (13) million people are affected by leishmania each year and most of the cases are reported in developing countries including Pakistan, Bangladesh, Nepal, and India. Additionally, an anticipated yearly occurrence of millions of new cases of Cutaneous leishmaniasis and Visceral leishmaniasis is expected, placing approximately 350 million individuals at risk [16].

A molecular study was carried out in District Kohat of the KPK province of Pakistan for the first time to determine the prevalence of CL in local population, which was found to be 5.17%, with active lesions of 3.91% and scar prevalence of 1.26% [17]. Another cross-sectional survey was conducted in an emerging district of North Waziristan Agency, KPK, Pakistan which reported a higher prevalence of CL in the male population [18]. In another study, the epidemiological aspects of CL were investigated during the recent epidemic in District Charsadda in 2019. A total of 150 cases were examined. Statistical analysis revealed the higher frequency of 59.3% in males as compared to 40.7% in females. Moreover, a higher infection incidence of

26.0% was observed in the age group of 5-14 years, followed by 24.0% in the age group 15-25 years, and 20.6% rate in the age group 25-34 years. The infection affected the hand region (34%), leg region (16%), and chest region (14%) [19] of the patients.

Even though the common type of CL is not difficult to detect, the atypical form of the disease might be challenging to diagnose in remote areas. The atypical form of CL is the same as the presence of foreign bodies, leprosy, syphilis, sarcoidosis, deep mycosis granulomas, and tumors, in addition to a few cases of skin cancer, which denote the clinical features of CL [6, 20]. This results in a delayed treatment until the lesion has been confirmed for *Leishmania major* amastigotes through parasitological investigations. The standard approach to diagnose CL involves employing direct smears and other methods during the investigation of prevalent diseases in specific regions [21].

District Mohmand is an arid mountainous region bordering Afghanistan. The CL disease burden is increasing day by day in this district and very little information is available related to CL based its molecular study (*Leishmania Tropica*, *Leishmania Major*) in this region of Pakistan. So, this study was designed to investigate biopsy samples of CL using molecular techniques to detect the causative agents at species level and to evaluate the risk factors associated with this disease in the affected population of District Mohmand.

2. METHODOLOGY

2.1. Ethical Considerations and Informed Consent

This study was approved from the Graduate Committee of Abasyn University,

Peshawar. Before conducting the research, all volunteers were thoroughly informed about the significance of this study. Informed consent, both verbal and written, was obtained from each participant in their native language.

2.2. Risk Factors Evaluation

Detailed information regarding patient demographics was collected using a self-structured questionnaire to evaluate the risk factors of leishmaniasis including gender, age, site, occupation, duration of active lesion, travel history, sleeping habits, and use of medications. The participants of all age groups with visible lesions of CL, regardless of the presence of single or multiple lesions, who were not taking any medications were included in the study [22].

2.3. Sample Collection

Fresh samples of CL were obtained from Tehsil Headquarters Hospital Safi in the merged district of Mohmand, Khyber Pakhtunkhwa. Patient's demographic information were recorded as done by [23]. Sample collection involved 150 patients with CL, specifically targeting the site of skin lesions. Slides were used to collect the samples, which were then carefully dried and stained using leishmania staining techniques. For cases with both mucocutaneous and cutaneous involvement, sufficient material was collected for PCR analysis.

2.4. Giemsa Staining

The suspected leishmanial smears were preserved by immersing them in pure methyl alcohol and allowing the slides to dry naturally. The smears were then treated with diluted Giemsa stain mixed with buffered water (1:20 ratio) for 10 minutes. Afterwards, the stain was carefully removed by rinsing the slides with distilled

water. All the stained slides were dried in the air and prepared for microscopic analysis.

2.5. Microscopy

The smear slides were carefully examined under compound microscope to directly identify the presence of CL amastigotes. Initially, the slides were observed using a 10X magnification lens to scan for any visible indications of the parasites. If no clear identification was made, a drop of cedar wood oil was added to the slide and it was reexamined using a higher magnification of 100X. By focusing on specific regions of the slides, the amastigote forms of CL were detected during the microscopic evaluation. Any positive findings (presence of CL) were documented as positive reports which were then selected for further processing, such as by using molecular detection techniques, to gather more detailed information about the Leishmania parasites present in the samples.

2.6. Molecular Detection of Leishmaniasis

2.6.1. DNA Extraction from Leishmaniasis Species.

The DNA extraction protocol using the Nucleospin II commercial DNA extraction kit (Macherey-Nagel, Germany) was adapted for various clinical samples including tissue smears on slides and old Giemsa-stained slides from CL patients [24]. For tissue samples or biopsy samples or smeared weighing approximately 25 mg, 1.5 ml microcentrifuge tubes were used. To these samples, 180 µl of buffer T1 and 25 µl of proteinase K solution were added. The mixture was vortexed for a few seconds and

then incubated at 56°C in a water bath for 1-3 hours. However, tissue samples of skin from humans were left in the solution overnight to allow maximum digestion by proteinase K. The next day the solution was vortexed and 200 µl of buffer B3 was added. After vigorous vortexing, the samples were incubated at 70°C for 10 minutes.

Subsequently, 210 µl of 96-100% ethanol was added to each sample, followed by thorough vortexing. The solution was then transferred to a Nucleospin tissue column placed in a collection tube and centrifuged at 11,000 x g for 1 minute. The flow-through was discarded and the column was placed back in the collection tube. Then, 500 µl of buffer BW was added to the column and centrifuged again at 11,000 x g for 1 minute. The process was repeated with the addition of 600 µl of buffer B5, followed by centrifugation as before. The flow-through was discarded and the column was centrifuged once more to dry the silica membrane. Finally, 100 µl of prewarmed buffer BE (70°C) was added to the column, incubated for 1 minute at room temperature, and centrifuged at 11,000 g for 1 minute. The eluted DNA was then stored at -20°C until further use.

2.6.2. PCR Analysis of Leishmaniasis.

The procedure began with an initial denaturation step at a temperature of 95°C for 4 minutes. This was followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extension at 72°C for 30 seconds. After the cycling steps, a final extension was performed at 72°C for 5 minutes. PCR was carried out using the master cycler equipment from Eppendorf.

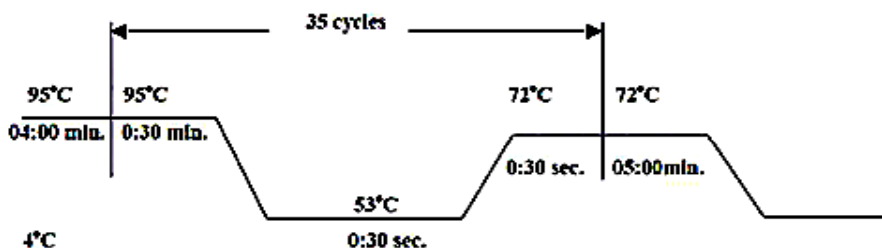


Figure 1. PCR Cyclic Conditions for ITS 1 Primer (LITSR/L5.8S)

Table 1. Primers for Internal Transcribed Spacer

Primer	F/R	Target	Primer Sequences	Length (Bp)	Product size
LITSR	Forward	ITS 1	CTTGGATCATTTCGGATG	19	330bp
L5.8S	Reverse		TGATACCACTTATCGCATT	19	

2.6.3. Gel Documentation. DNA grade agarose from Sigma was used for electrophoresis, along with specific buffers and reagents including 10X TAE (0.4 M Tris-acetate, pH 7.5; 20 mM EDTA), 10X TBE, and ethidium bromide, at a concentration of 10 mg/ml. A 6X DNA loading dye composed of 50% glycerol, 6X TAE, and 1% bromophenol blue was also employed. The DNA ladder used for sizing the DNA fragments included 100 bp and 50 bp ladders obtained from Fermentas (Lithuania) and PR omega (USA), respectively.

To prepare the gel, 2 gm of molecular grade agarose was mixed with 100 ml of 1X TAE or 1X TBE buffer in a clean flask and heated in a microwave for approximately 2-3 minutes until the gel was fully dissolved. After cooling to 40-45°C, 5 µl of ethidium bromide solution was added to the flask and mixed gently. The resulting solution was poured into the gel tank and a comb was carefully positioned to create wells for loading the samples. Once the gel solidified, the combs were removed and the gel was placed in an electrophoresis tank containing 1X TAE or 1X TBE running

buffer.

In the gel, the DNA ladder mix was loaded in the first or last wells and PCR products along with DNA loading dye were loaded in other wells. The gel tank was connected to a power supply and electrophoresis was run for either 60 or 90 minutes at 100 or 120 volts. After electrophoresis, the DNA bands obtained from the Leishmania-specific primer-based PCR were visualized under a UV transilluminator and a clear photograph was captured using a gel documentation system.

2.7. Statistical Analysis

The data collected in the study was evaluated using Statistical Package for Social Sciences (SPSS-22). Chi-square was used for qualitative variables to examine the relationship between different categories/groups. The calculated *p*-value of less than 0.05 was measured as statistically significant which indicated that there is a meaningful association between the study variables.

3. RESULTS

3.1. Microscopic Detection of

Leishmaniasis

A total of 150 samples were collected from both children and adult patients. Both microscopy and PCR were performed for

the identification of CL. Among the 150 tissue lesions samples, 106 (71%) samples were found to be positive. However, 44 (29%) were found negative for CL during microscopy, as shown in the Figure 2.

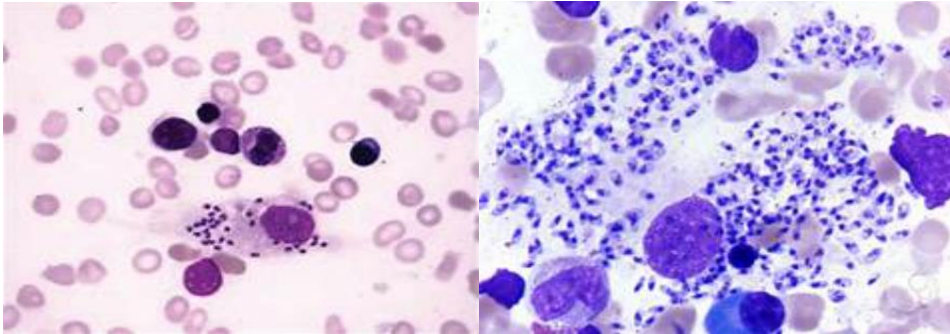


Figure 2. Microscopic Detection of Cutaneous leishmaniasis Amastigotes

3.2. Molecular Investigation of Leishmaniasis

Table 2

After performing PCR for all 150 samples, 122 (81%) samples were found to be positive. However, 28 (19%) samples were found negative for CL, as shown in

Agarose gel electrophoresis results showed DNA fragments from different samples. Band labels are shown in the Figure 3, where “L” represents the ladder.

Table 2. Results of Microscopy Screening and ITS1 PCR

Assay used	Number of Samples		% Of Samples	
	Confirmed positive	Confirmed Negative	Confirmed positive	Confirmed Negative
Microscopy	106	44	71	29
Internal Transcribe Sequences 1, Polymerase Chain Reactions				
ITS1 PCR	122	28	81	19

L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 L

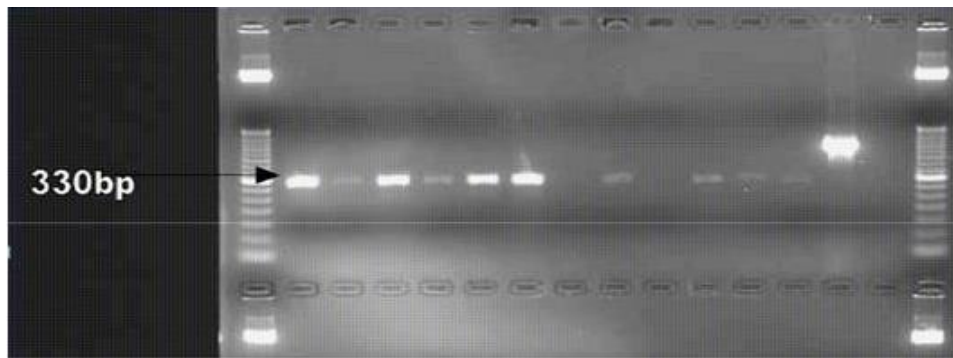


Figure 3. ITS1-PCR Analysis of Clinical Samples

PCR products showed a band size of 330bp. In the gel, positive samples were observed in columns 1 to 6, 8, 10, 11, and 12, while negative samples were seen in lanes 7, 9, and 14. Lane 13 showed an artifact. The 1kb ladder was used as a molecular weight marker.

3.3. Evaluation of Risk Factors of Leishmaniasis

Out of the total 122 positive patients, 55% were male ($n=67$) and 45% were females ($n=55$). Based on the data collected through the questionnaire, it was determined that their age range was 1 to 51 years. The highest frequency of CL was 69% ($n=84$) in the age group of 1-10 years and the lowest frequency was 3% ($n=4$) in the age group of 31-50 years. The prevalence of active CL lesions was analyzed across three different sites. It was found that the face was the most affected part of the body with 62% lesions, followed by 17% lesions each on the upper and lower limbs, and another 4% lesions on multiple sites.

As far the occupation is concerned, 80% ($n=120$) participants were pre-school or school children, 11% ($n=16$) were

farmers, 5% ($n=8$) were laborers, 2% ($n=3$) were unemployed, 1% ($n=1$) was a private employee, while 0.6% ($n=1$) was a government employee. Based on the duration of active lesions, 84% lesions were active for 1-6 months (in a total of 53.17% males and 46.82% females), 3.33% lesions were active for 7-12 months (in a total of 40% males and 60% females), and 10.66% lesion were active for more than 1 year (in a total of 75% males and 25% females).

4. DISCUSSION

The current study included 150 patients visiting Tehsil Headquarters Hospital Safi with skin lesions in the Merged District of Mohmand, Khyber Pakhtunkhwa. Biopsy and glass slide samples of CL were obtained from the study population over a period of 2 months, that is, from October to December, 2022. The current study employed a combination of molecular techniques and traditional methods to investigate disease prevalence, as well as the etiologic agents and factors influencing CL in District Mohmand of Khyber Pakhtunkhwa.

Out of a total of 150 patients, 106 (70.6%) were found to be positive for CL as

a result of microscopy. Whereas, 122 (81.3) were found to be positive through the ITS1-PCR assay. In addition, ITS1-PCR was found to be a more sensitive and specific method as compared to microscopy for the detection of parasites. A similar study was conducted in District Dir where out of 300 patients, 172 (56.17%) showed positive results on microscopy, 238 (79.5%) showed positive results from ITS1-PCR-assay, and 280 (93.5%) showed positive results from kDNA-PCR. Furthermore, kDNA-PCR and ITS1-PCR were found to be more sensitive and specific methods for the detection of parasite, rather than microscopy and culture [25, 26].

Gender wise and age wise prevalence of CL showed that out of 122 positive patients, 84 (68.8%) were in the age group of 1-10 years, 18 (12%) were in the age group of 11-20 years, 7 (5.7%) were in the age group of 21-30 years, 4 (2.6%) were in the age group of 31-40 years, another 4 (2.6%) were in the age group of 41-50 years, and 5 (4.09%) were in the age group of > 51 years. Similarly, the male gender was affected more with a total of 67 patients (55%), as compared to the female gender with 55 (45%) patients. A similar study was conducted previously which also showed the male gender as more affected. It showed that out of the total 278 patients, 142 (54%) were male and 136 (46%) were female. On the other hand, the highest percentage of infection (54%) was found in the age group 1-15 years and the lowest infection rate was found in the age group > 46 years [27]. This similarity with previous studies further supports the results obtained in the current study.

While analyzing the prevalence of active CL lesions across three different sites in this study, it was found that the face is the most affected part of the body. Hence, 62% lesions affected the face, 17% lesions were

found on upper limbs and lower limbs each, and 4% lesions were found on multiple sites. In a similar study previously conducted in Nepal, the majority of lesions were seen on exposed body areas, such as the face, legs, hands, and lips. The ratio was the highest in the facial regions (50%) and the lowest on the hands (17%), legs (20%), and other body parts (13%) [28]. Hence, the results of this study align with the results of previous studies regarding the prevalence of active CL lesions based on different sites.

According to the results obtained using the Chi-square test, it was observed that preschool and school children had a higher CL prevalence of 80%. On the other hand, government employees showed a lower prevalence (0.6%). Similar results were obtained from a study conducted in Ethiopia with the most prevalent cases of CL in pre-school children (60%), followed by laborers (10%), and farmers (7%) [29]. These findings further strengthen the outcomes of this research.

A total of 84% lesions were active for 1-6 months in 53.17% male and 46.82% female patients, 3.33% lesion were active for 7-12 months in 40% male and 60% female patients, and 10.66% lesion were active for more than 1 year in 75% male and 25% female patients. These findings are supported by a study conducted by Kassi et al., where lesions present for a duration of 1-3 months were found to have a prevalence ratio of 55.3%, followed by lesions present for 4-6 months comprising 26.9% of cases [30].

It was found that 87.33% of participants had interaction with animals, 63.33% of participants slept on the floor, 14.66% of participants had a travel history, 13.33% of participants had a migration history, 10.66% of participants used bed

nets, and 23.33% of participants used medications. A previous study conducted by Arif et al. also reported the association of such factors with the patients of CL, hence supporting the fact that they have a role in developing this disease condition [31].

4.1. Conclusion

The occurrence of Cutaneous leishmaniasis (CL) in District Mohmand of Khyber Pakhtunkhwa is substantial and the number of fresh cases is on the rise. So, additional molecular exploratory studies are required to tackle the disease burden. Children are more commonly affected than adults and men are more prone to the infection as compared to women. The combined use of microscopy and PCR in diagnosing CL provides a comprehensive approach to confirm the presence of the *Leishmania* parasite. These diagnostic tools are vital for early detection, proper management, and effective control of the disease, contributing to improved patient outcomes and public health interventions.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

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