

## PREVALENCE OF CUTANEOUS LEISHMANIASIS IN SURGUL VILLAGE OF DISTRICT KOHAT, KHYBER PAKTUNKHWA

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### ABSTRACT

**Background:** Leishmaniasis is a group of globally widespread parasitic diseases caused by different species of Leishmania, which infect a wide variety of mammals. Leishmania major and Leishmania tropica are two common causes of Cutaneous Leishmaniasis in Pakistan.

**Material and Methods:** The current study was conducted to check the prevalence of Cutaneous Leishmaniasis and to identify the leishmania parasites by using Microscopy and PCR in endemic areas of Surgul village of district Kohat. A total of 60 samples were collected from suspected Cutaneous Leishmaniasis patients with different age groups including both 25 male and 35 female patients.

**Results:** Overall microscopic results showed 70% Prevalence in examined patient in which 76 % of the male patients and 65.71% of female patients were found with Cutaneous Leishmaniasis while these results further confirmed by PCR using RV1/RV2 primers for the extracted DNA. PCR results showed 80% in male while 71.43% in female. In age up to 15 years showed 78.95% followed by 87.50% in male and 72.73% in female microscopically while over all prevalence of CL detected by PCR was 84.21% followed by 87.50% in male and 81.82% in female population.

**Conclusion:** It was concluded from the present work that male were much more infected with CL than female. The present study revealed that PCR is most sensitive than microscopy.

**Keywords :** Cutaneous Leishmaniasis, Leishmania tropica, PCR and parasites.

### 1 INTRODUCTION

Leishmaniasis is a group of globally widespread parasitic diseases caused by different species of Leishmania, which infect a wide variety of mammals. Leishmania major and Leishmania tropica are two common causes of Cutaneous Leishmaniasis (CL) in Pakistan [1]. In Human Population the two main forms of leishmaniasis have been reported from different studies. The infections are found in all ages and most frequently Cutaneous Leishmaniasis infected age group is 10–40 age classes [2]. Cutaneous Leishmaniasis is prevalent in Pakistan and has been reported from all the provinces [3]. It is endemic in Pakistan and is widely spreading day by day [4] where the causative agent is Leishmania tropica, a protozoan parasite Amtul [5].

Biological vectors of Leishmania species in the old world and new world are Sand flies in human and between human and animals [6] in Pakistan, some 29 species of sand flies (Phlebotomus) vector are present [7]. Phlebotomus papatasi was found in Kashmore, Bannu, Dera Ismail Khan, Idak, Tank, Kohat, Lahore Miramshah, Nowshera, Quetta, Rawalpindi [8]. A lot of data and reports of CL concerned to human cases in epidemic situations [9]. There are several methods of laboratory diagnosis of leishmaniasis, including parasitological, immunological and molecular [10]. PCR is becoming a 'gold standard' technique which increased the speed and sensitivity of leishmania diagnosis compared to the microscopy [10].

Different forms of treatments are available including oral, par-

enteral, and topical medications such as pentavalent antimonials, liposomal amphotericin B, miltefosine and paromomycin [11]. Destroying the breeding and resting sites of the vector, control of hyraxes and rodents in the proximity of human dwellings, should also be implemented. Policy should be formulated to control leishmaniasis in the direction of to eliminate stray and feral dogs. Extensive research in epidemiology of leishmaniasis should also be conducted in non endemic areas, too [11].

During last few years, a large number of IDPs (Internally Displaced Persons) have moved to the areas of KPK province like Kohat due to terrorism. Similarly the IDPs being infected with CL are apparently the cause of spreading the disease (leishmaniasis) to native population. Present study was aimed to observe the prevalence of leishmaniasis in endemic region Surgul of district Kohat. The present study was conducted after a preliminary survey in local hospital of Kohat. Most of CL patients that were encountered in Kohat were residents of Surgul village so the present project was aimed to observe the prevalence of leishmaniasis in Surgul village of district Kohat.

## 2 MATERIAL AND METHODS

### 2.1 Study Areas

The present study was conducted in selected Cutaneous Leishmaniasis endemic village Surgul of district Kohat Khyber Pakhtunkhwa during April, 2013 to June, 2013. The village Surgul is located at a distance of 20 km from Kohat University of Science and Technology.

### 2.2 Tissue Samples

During our research duration 60 samples from Surgul village of Kohat were collected for the isolation and identification of leishmaniasis causing pathogen. Active skin ulcers samples of suspected leishmaniasis patients were collected through slide smears. Before taking the sample, 70% ethanols were applied on the infected area of the skin and then lancet was inserted into the periphery of lesion to obtain blood or stinged fluid on the slide. Slides smear were prepared and fixed with methanol. The specimens were transferred and processed in Microbiology department Lab at Kohat University of Science and Technology.

### 2.3 Microscopy

A smear impression from each patient was prepared on slide. The slides were fixed in methanol. It was flooded with Geimsa stain and allowed to stand for 10 to 15 minutes then washed with distilled water. The slide was dried and examined at 40X and 100X using oil emersion.

### 2.4 Molecular Diagnosis

The DNA was extracted using Nucleospin® Blood kit method. First of all a total of 180µl of buffer T1 were added to the slides and then scratch with the help of tip and finally transfer to the eppendorf tube and 20µl of proteinase k (PK) were added to the tube and vortex to mix. The samples were placed at 56 °C in water bath for complete lyses (at least for one hour). The samples were again vortexed.

Two hundred 200µl of Buffer B3 were added and incubated at 70°C for 10 minutes. Then 200µl (96-100%) ethanol were added to the sample and vortex vigoursly. For each sample one Nucleospin® Blood Column was placed into a collection tube. The samples were applied to the column. It was centrifuged for one minute at 11000 rpm. The flow-through was discarded and the column was placed back into the collection tube. Five hundred 500µl Buffer BW were added centrifuged at 11000 rpm for one minutes. Again discarded flow-through and the column were placed back into the collection tube. Now six hundred 600µl of Buffer B5 were added and centrifuged for 1 minute at 11000 rpm again the flow-through were discarded and the column were placed back into the collection tube. The column was centrifuged again to remove the residual ethanol. The Nucleospin® Blood Column were placed into 1.5 ml of micro centrifuge tube and 100µl pre-warmed Buffer BE (70%) were added, incubated at room temperature for one to two minutes and centrifuged at 11000 rpm. The extracted DNA was kept at -20°C till further use.

### 2.5 PCR (Polymerase Chain Reaction)

Two types of primer were used i.e. ITS1 (Internal Transcribe Spacer 1) primer (LITS R/L5.8s) and KDNA (RV1/RV2) for human blood extracted genomic DNA. A PCR was used to amplified the ribosomal internal transcribed spacer 1 (ITS1) region, which separates the genes coding for the smaller sub unit (ssu) rRNA and 5.8S rRNA using the primers LITSR (5'CTGGATCATTTCGGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') which was detected previously [12, 13]. Amplification reaction was performed in volume of 25µl. 5µl of isolated DNA were added to a PCR Master Mix, containing 2.0mM Mgcl<sub>2</sub>, 200µM dNTP's, Twenty (20) Pico mol of each primers and 2ul of Taq polymerase (Roche Biotech) in the PCR buffer.

### 2.6 PCR Conditions

Reaction was amplified in a thermo cycler (Eppendorf, Germany) as follows.

For KDNA primers Rv1/Rv2 an initial denaturation at 95°C for 5 minutes was done followed by 40 cycles (denaturation at 95°C for 30 sec, annealing at 58°C for 30 and extension at 72°C for 30 sec). The samples were then kept at final annealing

temperature of 72°C for 10 minutes and at last hold at 4°C.

### 2.7 Gel Electrophoresis

Amplified products were electrophoresed on a 1.8% agarose gel (Fig 4). DNA ladder of 100-bp (Invitrogen, Corp., California, USA) was used as size marker for comparison of CL DNA to find out the 145bp DNA band for the detection of CL on the methods used previously [12, 13].

### 2.8 Statistical analysis

The data were analyzed and summarize using statistical software Statistix version 9.0 for windows. The results for all variables were presented in percentage (%). Chi Square tests were used to find out positive association among the categorical variables. P<0.005 was considered as significant.

## 3. RESULTS

### 3.1 Gender wise distribution of CL in human beings

The current study was carried out to detect the prevalence of leishmaniasis in human being by microscopy and PCR. A total of 60 samples were collected from the infected population of the study area including 25 male and 35 females. Overall microscopic results showed 70% followed by male 76% and female infected 65.71% with CL while the PCR results showed 80% in male and 71.43% in female population as shown in table 1.

**Table 1. Gender wise prevalence of CL by Microscopy and PCR in human beings**

Gender (n)	Microscopy		PCR		P-value
	Positive	Percent	Positive	Percent	
Male (25)	19	76%	20	80%	P=0.0000<0.05 significant
Female (35)	23	65.71%	25	71.43%	
Overall (60)	42	70%	45	75%	

### 3.2 Age wise distribution of CL by Microscope

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The samples which we were collected from male and female population of the study area were stained with Geimsa Stained and observed under binocular microscope. Overall results showed 70% prevalence including 76% in male and 65.7% in female population. Over all prevalence rate of CL in group I (0 to 15 years) showed 78.95% followed by 87.50% in male and 72.73% in female population while in group II (16 to 25 years) showed overall 70.83% followed by 77.78% in male and 66.67% in female population. Similarly the age group III (26 to 45 years old) showed overall results 66.67% including 66.67% in male and female population. Group IV (Greater than 45 years old) showed 50% overall, male and female population table 2.

**Table 2. Age wise prevalence of CL by Microscopy**

Age groups	♂ n=25 (%)	♀ n=35 (%)	Overall n=60 (%)	P-value
≤15 years	7/8 (87.5)	8/11 (72.73)	15/19 (78.95)	P=0.6107 Non Significant
16-25 years	7/9 (77.78)	10/15 (66.67)	17/24 (70.83)	
26-45 years	4/6 (66.67)	2/3 (66.67)	6/9 (66.67)	
>45 years	1/2 (50)	3/6 (50)	4/8 (50)	
Overall	19/25 (76)	23/35 (65.71)	42/60 (70)	

♂ for Male, ♀ for Female

### 3.3 Age wise distribution of CL by PCR

The present study revealed that PCR is more sensitive and accurate than microscopy. By PCR age wise results showed the highest overall prevalence rate of CL found in age group I (up to 15 years), 84.21% followed by 87.50% in male and 81.82% in female population. In age group II (16 to 25 years) overall results was recorded 70.83% including 77.78% in male and 66.67% in female population. In age group (26 to 45 years old) overall results showed 77.78% possessing 83.33% in male and 66.67% in female population. Similarly the age group IV (Greater than 45 years old), overall prevalence rate showed 62.50% followed by 50% in male and 66.67% in female population as shown in table 3.

**Table 3. Age wise prevalence of leishmaniasis by PCR in human beings**

Age groups	♂ n=25 (%)	♀ n=35 (%)	Overall n=60 (%)	P-value
≤15 years	7/8 (87.5)	9/11 (81.82)	16/19 (84.21)	P=0.7428>0.05 Non Significant
16-25 years	7/9 (77.78)	10/15 (66.67)	17/24 (70.83)	
25-45 years	5/6 (83.33)	2/3 (66.67)	7/9 (77.78)	
>45 years	1/2 (50)	4/6 (66.67)	5/8 (62.50)	
Overall	20/25 (80)	25/35 (71.43)	45/60 (75)	

♂ for Mal, ♀ for Female

### 3.4 Lesion wise Prevalence of CL in male and female population by Microscopy

The samples were collected from 3 different pattern patients of CL. The first group was contained one lesion having overall prevalence 62.07% of CL including 66.67% male and 58.82% female population. Group second having two lesions contained over all 68.75% followed by 71.43% male and 66.67% female population. Group third having more than two lesions showed overall prevalence 86.67% followed by male population 100% and female population 66.67% as shown in fig 1.

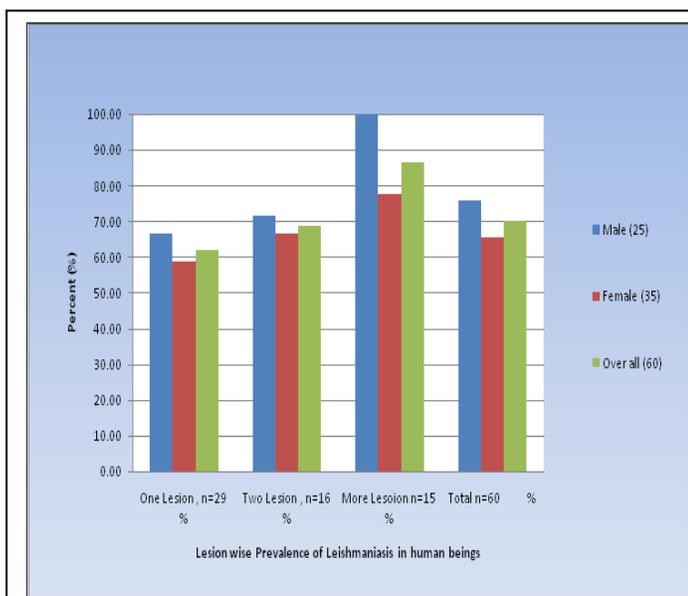


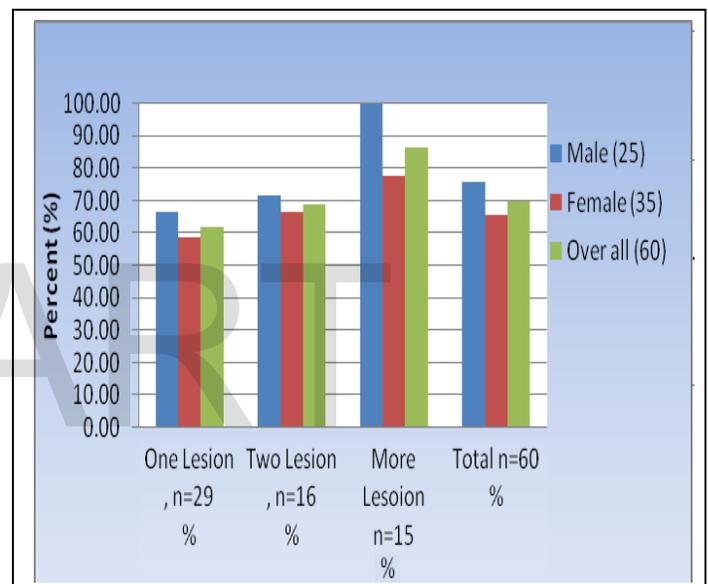
Fig 1. Lesion wise prevalence of Leishmaniasis of human beings by microscope

### 3.5 Lesion wise Prevalence of CL in male and female population by PCR

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The current study conducted by PCR showed slightly more than microscopy. The first group which contained one lesion in out of 29 samples male showed 66.67% and female 58.82% making 62.07% overall prevalence of CL. Group second showed 81.25% over all prevalence in out of 16 samples including 85.71% male and 77.78% female. While the group showed high prevalence rate of CL 93.33%, overall prevalence rate including 100% and 88.89% in out of 15 samples as shown in fig 2.

The entire specimen collected was confirmed and diagnosed by using more specific and sensitive diagnostic tool i.e. Polymerase Chain Reaction (PCR) by using different primers specific for kDNA (Kinetoplast DNA).



## 4. DISCUSSION

Leishmaniasis is one of the highest incident infectious parasitic diseases in the world [14]. The molecular methodology especially PCR is the best way for the detection of CL [5]. Our study was co related to Khan et al., 2013 which is slightly similar to our finding in his finding the microscopy detected CL infection was 60.5% (n= 43/71) in male and 40.4% (n=25/62) in females collectively being 51.2% (n= 68/133), while by kDNA-PCR CL in male was 77.5% (n= 55/71) and in females 72.5% (n= 45/62) collectively was 75.2% (n= 101/133) [10].

The study conducted in Dargai KPK was slightly dissimilar to our study showing more prevalent in local population (61%) than refugees (39%) [15]. It is also showed from the other studies that CL affects both sexes at all ages to varying degrees [16,17] and the disease is found in both genders [18]. The CL was detected about Similar to our findings too which were shown by [19] according to Farahmand et al., showed (63.8%) higher than females (36.2%) and the results obtained by [20]

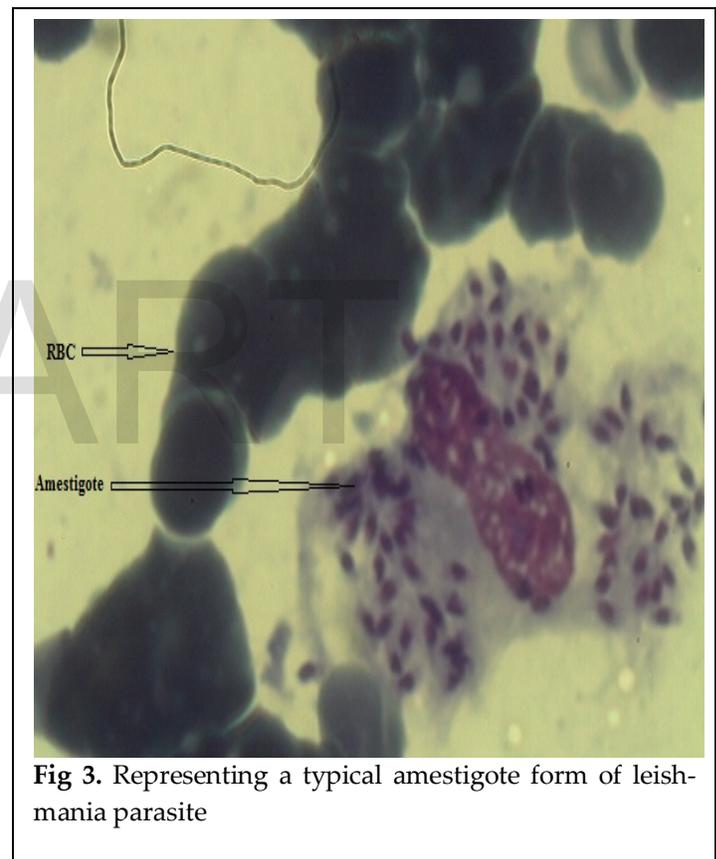
Bari et al., 2006 males 54(90%) out of 60 and 10% were females [21] which was very high in male. The CL infection was more prevalent in men 56.7% - 62.24% than in women 37.75% - 43.3% [22, 23] the infection rate was recorded 58.62 % and 82.8 % respectively by microscopy in the male age group of 21-30 years than females. Similar findings were also shown by Ullah et al., 2009 [14] It was highest (43.8%) in 1 to 15 year group and lowest (7.0%) in 46-60 years group. [18]. Kakarsulemankhel in 2004 studied the prevalence CL in boys (17-22 years) and found 72.93% while in the school going children was (45.12 %) and in younger age group (5-10 years) active CL was 44.11 % [24].

We are also agree with Youssefi et al. in 2011 according to his study the rate of leishmaniasis was very high in patients between 21 and 30 years (38.7%) and the very low in patients between 51 and 60 years (4.83%) [14]. In our study highest results were recorded in children possess up to 15 years age (81.82) by microscopy and (84.21) by PCR which may be by social factor as the children play outside having no proper protection of their bodies near the habitat of sand fly (vector of leishmaniasis). Our findings are quite in comparison to the mentioned studies. The previous work of Khan et al 2013 showed that different body sites of 133/184 suspected individuals of CL (average range, 1-6) lesions were observed. According to his finding most of the patients had a single lesion; some have 2 or 3 lesions, while multiple lesions were found on cheeks and hands [10]. Beside other studies showed that CL lesions on skin ranged between 1-8 per person [25, 17]. The majority 65% (105) patients had a single lesion and 35% (56) patients had 2 lesions being most 78% lesions on face and 26% on upper limb [17] as compared with limbs 30% -37%, Legs 11.5% - 22% and other sites the face was found to be more involved 25% - 41% in CL [21, 23].

We have also found out similar findings related to these studies but variability in the number of lesions was due to the mode and severity of infection caused by *L. tropica* [26]. Talari et al., 2006 studied and showed that single lesions 50.9% double lesions were observed 24.6% and multiple (3-15) lesions showed 29.4% of the patients [27]. Ayaz et al., 2011 result show that (58.4%) patients having single lesion (29.2%) of patients having two lesions and (12.38%) of the patients having three or more than 3 lesions [27]. The factors may be the large size of local population and Malnutrition [14].

demographic village of Surgul District Kohat. It was further concluded that CL is more prevalent in male population than the female population. The high prevalence in male was due to the reason that males are more social and stay more time outside during the evening and night (exposed to bite of sand fly). It was also concluded from the present study that the lower age population of human being especially up to fifteen years old were infected maximum than the other groups.

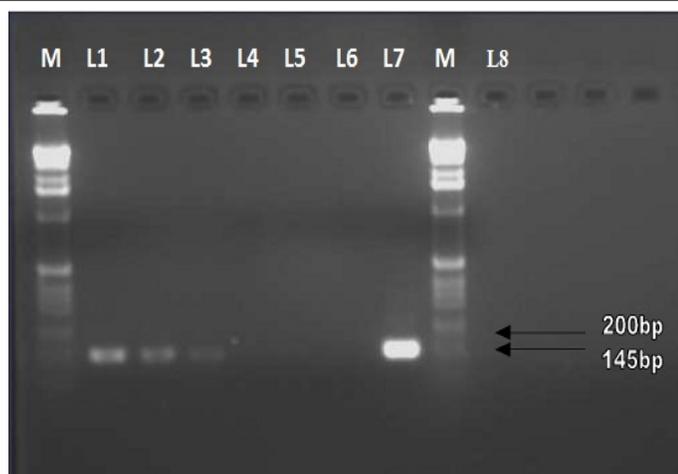
It is recommended that the children should protect to minimize the infection rate of leishmaniasis. It is also recommended that PCR should be used for the detection of leishmaniasis. It was concluded from the current study that PCR is more sensitive than Microscopy.



**Fig 3.** Representing a typical amastigote form of leishmania parasite

## 5 CONCLUSION AND RECOMMENDATION

From our finding it was concluded that CL is prevalent in en-



**Figure 4.** kDNA PCR showing 145bp using (100bp ladder fragment). Lane M represent the ladder, L7 positive control, L8 negative control, L1, L2 and L3 positive samples, L4, L5 and L6 negative samples.

### COMPETING INTERESTS

The authors declare that they have no competing interests.

### AUTHORS' CONTRIBUTION

IK, HA and WA collect epidemiological data write up the manuscript MH, AK, AM, NK, A, SA, MA and MW contributed in literature search and help in manuscript writing. NUA arranged and performed statistically analysis. Final manuscript was readout and approved by all the authors.

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