Research Article

Prevalence and Molecular Diagnosis of Cutaneous Leishmaniasis in Local population of Dir District, Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

A total of 300 samples were collected from suspected patient to find the prevalence of *Cutaneous Leishmaniasis* as well as Polymerase Chain Reaction (PCR) based identification of respective species in the endemic areas of Dir Lower and Dir Upper (Khyber Pakhtunkhwa Province) Pakistan. These patients including (171) males and (129) females from these areas, irrespective of age, were analyzed using patient's biopsy samples, slide smears and filter paper impressions from lesions. Kinetoplast kDNA-PCR and (Internal Transcribed Spacer 1(ITS1-PCR) were performed using RV1/RV2 and LITS R/L5.8s primers, respectively. Age group of 0-15 years was found to be the most infected with 47.5% of total cases while age group of 45 years and above showed lowest rate (7.0%). Out of 300 patients, 172 (56.17%) were positive by microscopy, 79.5% (238 Patients) by ITS1-PCR-Assay and 280 (93.5%) were confirmed by kDNA-PCR. It was concluded that cutaneous leishmaniasis in prevalent in local population of Dir district. Furthermore, kDNA-PCR is more sensitive and specific method for identification of the parasite, followed by ITS1-PCR as compared to Microscopy and culturing.

Keywords: Clinical Diagnosis, *Leishmania, Cutaneous Leishmaniasis, Mucocutaneous Leishmaniasis, Visceral Leishmaniasis, Leishmania tropica, Leishmania major.*

INTRODUCTION

Leishmaniasis; an inflammatory chronic disease; has causative agent an obligate intracellular parasitic protozoan, haemoflagellate, *Leishmania* which belongs to genus *Leishmania*[1,2]. Parasites of *Leishmania* are found in Asia, Europe and Africa while *Viannia* in tropical and sub-tropical America[3]. Overall, it is an endemic disease of 88 countries[4] in which by 1991 twelve million cases were reported from all over the world with increment of 1.5-2.0 million cases and 100,000 deaths per year[5].

These species can cause *Leishmaniasis* in variety of vertebrates[6] including *Carnivora* (cats & dogs), *Rodentia* (Rats & Gerbils) and *Primates* (humans & monkeys) invertebrates. *Leishmaniasis* in invertebrates such as phleobotomine sand flies and ticks have also been found[7]. Mostly these parasites are transmitted among humans through genus *Phelobotomus* or genus *Lutzomyia* of sand flies [8].

Diagnosis of *Cutaneous Leishmaniasis* in non-endemic area is intriguing but in common practice at endemic area appearance of the lesions seen is used for diagnosing[9] while re-infection or mistreatment can cause hurdles in diagnosis[2] and severity in infection. In later case specialized methods such as histopathological examination and microscopic observation of smears from lesions or cultures[10]. Most advance techniques today used for diagnosing *Leishmania* include observation of smears stained with Giemsa or leishman stains[11] use of monoclonal antibodies, immunohistochemistry, electron-microscopic studies, histopathological studies, immunofluorescence, DNA probes, PCR and ELISA [12].

Being a serious health issue, along with Afghanistan, India[12], Brazil[13], Latin America[14], Iran[15], Iraq[2], Peru, Saudi Arabia and Syria[16] *Cutaneous* as well as *Visceral Leishmaniasis* has been reported in Pakistan in various geographical regions having diverse climate [17]. *Cutaneous Leishmaniasis* is confined mostly to the province of Khyber Pakhtunkhwa [18], Azad Jammu and Kashmir, Balochistan and Punjab [19] while *Visceral Leishmaniasis* is endemic and mostly prevalent in Gilgit Baltistan and Azad jammu and Kasmir [20].

Dir hosts many Afgahn refugee camps particularly in Timargar. Where CL is endemic. L. tropica was previously isolated and characteristed from Afghan regugee camps in Timagara [21]. But there is no data regarding frequency of CL in local population of the said district.

Keeping in view for demand of detailed information, this study was conducted with aim to develop molecular diagnosis using PCR technique and to determine prevalence of *Cutaneous Leishmaniasis* in the endemic areas of Dir Lower and Dir Upper regions of Khyber Pakhtunkhwa province of Pakistan.

MATERIALS AND METHODS

Study Area

Dir Lower

Dir lower is one of the most important regions, both historically and culturally. The district is 1,582 square kilometers in area. It is situated between 34° 22′ and 35° 50′ North and 71 ° 02′ and 72° 30′ East. Apart from the tehsils of Adenzai round Chakdara and Munda in the south-west, Lower Dir is rugged and mountainous. The district is bounded by Swat District to the east, Bajour Agency to the west, Upper Dir to the north, and Malakand District to the south. Timergara is the district headquarters and lies at 2,700 ft (820 m). The population of the Lower Dir district's Councils is 797,852 according to the 1998 census report. The projected male population of Dir lower in 2005 is 514,072 and the female is 523,020.

Dir upper

Upper Dir district is 3,699 square kilometres in area. Almost all of the district lies in the valley of the Panjkora river which rises high in the Hindu Kush mountainous with peaks rising to 16,000 feet (4,900 m) in the north-east and to 10,000 ft (3,000 m), along the watersheds with Swat to the east, Bajour Agency to south west, Chitral to North, Lower Dir to south and Afghanistan to the west. The population of Dir Upper is 575,858.

Sampling

Natives (both male and female) of Dir Upper (Taimer, Baroon and Munjai Regions), and Dir Lower (Warri, Sahib-Abad and Bando Regsions) were surveyed to collect information about number, site and duration of lesions, along with name, locality, age, sex and traveling history. Out of 4130 surveyed persons, 300 suspected *Cutaneous Leishmaniasis* patients including both male and female of various age groups. Samples (Biopsy smears and tissue aspirates) were taken from these patients with prior consent. Those who did not fulfill the required criteria were excluded from study. The samples were labeled and were transported to Microbiology Laboratory, Department of Microbiology at Kohat University of Science and Technology, Kohat and stored at - 20°C prior to PCR.

Isolation of *Leishmania* species

Two types of media i.e. NNN (Novy, Mc-Neal Nicoli) and RPMI (Roswell Park Memorial Institute) 1640 along with 10 % rabbit blood were used for isolation of *Leishmania* species. Slants and Tubes containing normal saline solution were also prepared. 1 ml of normal saline was transferred into each tube after adding antibiotics, Streptomycin and penicillin, to normal saline. Tissue aspirate of skin lesions were inoculated on NNN (Novy Mc-Neal Nicolle) media supplemented with 10% fetal calf serum, 200 µg/ml streptomycin, and 200 U/ml penicillin. The samples were incubated at 26°C for four days. Promastigotes found after examining under microscope were transferred into RPMI1640.

Microscopic Examination

A smear impression from each sample prepared on slide was stained with Geimsa stain and finally examined at 40X and 100X using oil emersion for presence of amastigotes.

DNA Extraction

DNA from each sample was extracted using NUCLEOSPIN® Tissue kit (Macherey Nagel, Germany) according to maunfacture's recommended protocol with little modifications.

Polymerase Chain Reaction

kDNA-PCR and ITS1-PCR were performed using RV1/RV2 (5'-CTGGATCATTTTCCGATG-3') and LITS R/L5.8s (5'-TGATACCACTTATCGCACTT-3') forward primers respectively. PCR conditions were maintained as performed by Andrade et al., 2006[13] with slight modifications. PCR Master Mix (50 µl) included 5 µl isolated DNA, 2.0 mM $MgCl_2$, 200 µM dNTP's, 20 pmol of each primer and 2 U of Taq polymerase (ROCHE BIOTECH) in the PCR buffer. For KDNA primers Rv1/Rv2 PCR conditions were applied as initial denaturation at 95 °C for 5 minutes 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), final annealing temperature of 72°C for 10 minutes and at last hold at 4 °C. While for ITS 1 primers LITSR/L5.8S the samples were initially denatured at 94 °C followed by 35 cycles (denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 and extension at 72 °C for 30 sec) and final extension of 72 °C for 5 minutes and then hold at 4 °C.

Collection of Sand flies

Sand flies were collected using stickp tapes and CDC light traps from indoor and outdoor near the houses of CL patients. The sand flies were preserved in 90 % ethanol till further used for studying morphology and species identification.

RESULTS AND DISCUSSION

Survey Results

Area-wise Prevalence of Cutaneous Leishmaniasis

Samples from Munjai region of Dir Lower area showed highest prevalence rate with 7.31% followed by Sahib Abad (Dir Upper) while the lowest prevalence rate was 2.45% observed in Baroon region (Dir Lower) as shown in Table 1.

Our findings supports the findings of Rehman et al., (2009) [22] and Shoaib et al., (2007) [23] who identified *Cutaneous Leishmaniasis* from various locations of province Khyber Pakhtunkhwa and strengthens the statement that this disease is endemic across the province up to the border of Afghanistan as well as in other provinces of Pakistan.

Gender-wise Prevalence of Cutaneous Leishmaniasis

The prevalence in male was high 56% as compared to females 43% in the two districts. Among Males the highest prevalence 58% were observed in Dir Lower while lowest prevalence 55% were observed in Dir Upper. While the prevalence among females was highest in Dir Upper with a rate of 44%, while lowest Prevalence of 42% were observed district Dir Lower (Table 2).

Similar findings were also shown by Farahmand et al., (2011)[24] who also find out that infection was more prevalent among males (63.8%) than females (36.2%). Bari et al., (2006)[12] showed that most of the patients were males 54 (90%) out of 60 and 10% were females. 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 sandfly) and also sleep without shirt during night while females in this geographical areas cover their body parts completely so prevalence in males was high as compared to females.

Age-wise Prevalence of Cutaneous Leishmaniasis

The people were surveyed on household basis with the age difference of 0-15 years followed by people of age 16 to 30, 31-45 and above 45 years. The highest number of cases, 26% were observed in boys and 21.5% in girls of age group 0-15 years, while the lowest rate of prevalence was in people of age above 45 years. The comparison of age wise prevalence is given in Table 3.

Similar findings were also shown by Ullah et al., (2009) [25] It was highest (43.8%) in 1-15 year group and lowest (7.0%) in 46-60 years group. Kakarsulemankhel in 2004 studied the prevalence of active CL (cutaneous leishmaniasis) in boys (17-22 years) and found 72.93% (1617 out of 2217 boys). However, in the school children (11- 16 years) there were more cases of active CL (45.12 % [2643 out of 5857 children]) and in children of younger age group (5-10 years) active CL was 44.11 % (3210 out of 7236 children). We are also agree with who[26] determined the high prevalence of Leishmaniasis (10.96%) among age group 0-9 years, followed by 6.66% (10-19 years), 5.35% (30-39 years), 5.12% (40-49 years), 3.96% (20-29 years) and lowest prevalence rate 2.94% was observed in age group 50-59 years. Youssefi et al. in 2011[27] studied the greatest rate of leishmaniasis in patients between 21 and 30 years (38.7%) and the least was in those between 51 and 60 years (4.83%). In our study the prevalence in young children was high because young children play outside of their houses so they were exposed to the sand fly bite. The prevalence in young children was also high because of the reason that there immune system is not fully developed.

Lesion-wise Prevalence of Cutaneous Leishmaniasis

All the patients were observed and numbers of lesion were noted. Most of the patients had a single lesion with a rate of 51%, two lesions with the rate of 32.50 % and the percentage of 16.50% were observed in patients with more than two lesions. The overall comparison is given in Table 4.

Talari et al., (2006)[28] reported 117 patients of cutaneous leishmaniasis and determined that Single lesions were seen in 50.9% of patients, double lesions were observed in 24.6% of patients and 29.4% of patients showed multiple (3-15) lesions. Ayaz et al., (2011)[29] carried out study on 339 suspected patients of cutaneous leishmaniasis and found (58.4%) of the cases with the single lesion, two lesions (29.2%) and three or more than 3 lesions (12.38%).

Monthly or Seasonal-wise Prevalence of Cutaneous Leishmaniasis

The samples were collected from endemic areas of Dir Lower and Dir Upper each month from July to December 2011. The highest numbers of prevalence were in September 28%, followed by October with a rate of 21.5%. The lowest prevalence 9% was observed in December.

Durani et al., (2011)[30] found that the disease was prevalent throughout the year in human populations in Northern Pakistan with 375 positive cases in May 2007. Most cases occurred in November (661) and the least number of cases were detected during February 2008 (292). In Southern Pakistan 315 cases were recorded during May and (308) occurred in June 2007. The disease was most prevalent in April 2008 (518 cases) In Western Pakistan 219 positive cases were determined in May while cases were most prevalent in October 2007 (281 cases) and the lowest number of cases were recorded during February 2008. Alavinia et al., (2009)[15] interpreted a data concerning 1453 patients with CL and reported in all months of the year with the highest rate from September to November. The least number of patients were observed in February and March. The seasonal factors play important role in spread of Cl as Sand fly is more active during warmer months of the year as compared to Winter (November to January).

Diagnostic Results

The diagnostic study include microscopy for detection of Amestigote or LD (Leishman and Denoven) bodies, culturing, PCR (Polymerase Chain Reaction), and RFLP (Restriction Fragment Length Polymorphism). The result of each methods and diagnostic tools are given below.

Microscopy Results

Three hundred (300) smears were prepared from samples for microscopic examination for detection of LD bodies or Amestigote form of *leishmanial* parasite. The LD bodies were detected only in 172 (57.5%) of specimens. One hundred twenty eight (42.5%) smears were negative for LD bodies' detection. Both the positive and negative slides smears were further diagnosed and checked for the identification of parasite using PCR. Similar results were also shown by Fazali et al., (2009)[31] who detected Leishmania amastigote in (50%) 24 cases out 48

specimens. Ayaz et al., (2011)[29] microscopically diagnosed only 43.06% (146/339) with the disease. Alsamarai and Aloaidi, (2008)[2] diagnosed 107 patients of suspected cutaneous leishmanaisis and showed 73% of the cases were positive by geimsa stain. Thus microscopy is least sensitive method for detection of CL, although it is 100 % specific.

Culturing of parasites

Among 300 Sample only 90 were inoculated in NNN (Novy McNeal-Nicole) media as well as in RPMI1640 media. Limoncu et al., (1997)[32] successfully cultured leishmania in RPMI 1640 media, NNN media and P-Y (Peptone-Yeast). The reproduction in RPMI 1640 and P-Y (Peptone- Yeast) media appeared very close to each other and reproduction was also observed in NNN media but that was least in numbers. Rowland et al., (1999)[21] cultured leishmania parasite in NNN media. The parasite were successfully cultured in 20 (48%) among 50 specimen, while the remaining were discarded due to fungal or bacterial contamination. Similar results were also determined by Ayub et al., (2003)[33] who cultured Leishmania parasite in Evans Tobie's medium and parasites were successfully cultured in 16 specimen.

Table 1: Area-wise prevalence (%) of *Cutaneous Leishmaniasis*

Areas of sampling		People Surveyed	No of suspected Patients	Prevalence
Dir Lower	Taimer	755	28	3.70
	Baroon	489	12	2.45
	Munjai	410	30	7.31
Dir Upper	Warri	475	23	4.84
	Sahib Abad	336	19	5.65
	Bando	215	08	3.72

Table 2: Gender-wise prevalence of *Cutaneous Leishmaniasis*

Areas	Total Samples	Male	Female	Male Prevalence	Female prevalence
Dir Lower	170	98	72	58 %	42%
Dir Upper	130	73	57	55%	44%
Total	300	171	129	56%	43%

Table 3: Age-wise prevalence of *Cutaneous Leishmaniasis*

Areas	0-15 Years		16-30 Years		31-45 Years		>45 Years	
	Male	Female	Male	Female	Male	Female	Male	Female
Dir Lower	44	36	29	20	18	9	7	3
Dir Upper	34	28	19	16	9	16	11	2
Total	78	64	48	36	27	25	18	5
Prevalence	26%	21.50%	16%	12%	9%	8.50%	5.50%	1.50%

Table 4: Lesion-wise prevalence of *Cutaneous Leishmaniasis*

Areas	One Lesion	Two Lesion	> two Lesion	Total
Dir Lower	85	56	29	170
Dir Upper	68	42	20	130
Total	153	98	49	200
Prevalence	51%	32.50%	16.50%	100%

PCR RESULTS

kDNA and ITS1 PCR

All the 300 samples were analyzed by kDNA PCR using kDNA specific (RV1/RV2) primer. One hundred Sixty (94.28%) specimen among 170 samples collected from Dir Lower, 117 (90%) samples out of 130 collected from Dir Upper were positive using kDNA-PCR assay. A total of 280 (93.5%) among 300 were positive and 16 (5.5%) were negative by kDNA-PCR. Similarly specific primer (LITS R/L5.8s) was used to amplify the ribosomal internal transcribed spacer 1 (ITS1) region. 136 (80%) out of 170 samples collected from Dir Lower, 98 (76%) out of 130 specimens from Dir Upper were positive by using ITS1-PCR. A total of 238 (79.5%) among 300 samples were positive and 61 (20%) were negative, using ITS1-PCR. Bensoussan et al. 2006[34] used three PCR assay for diagnosing the *Leishmania* parasite. Using kDNA PCR

(98%) 77 out of 78 specimens were positive, followed by ITS1-PCR in which (91%) 71 were positive, while only (53.8%) 42/78 were diagnosed by using spliced leader mini-exon PCR. Eroglu et al., (2011)[35] identified causative agent of cutaneous leishmaniasis patients using kDNA and mini-exon PCR. Among 64 suspected cases (68.75%) 44 were positive, while using mini exon PCR only (54.7%) 35 were positive. The present study showed that kDNA PCR was more sensitive than ITS1 PCR as well as microscopy.

Sand fly identification

Using standard morphological key, most of the female sand flies trapped were identified as *Phlebotomus sergenti*, while some of the sand flies were identified as *P. papatasi. P. sergenti* is proven vector of zoonotic CL in many parts of world particularly Afghanistan, *P. paptasi* is the proven vector of Anthroponotic CL[19]. *P. sergenti* was also trapped from Timagara Afghan refugee camp of Dir in 1999 [21] which is in accordance with our present study.

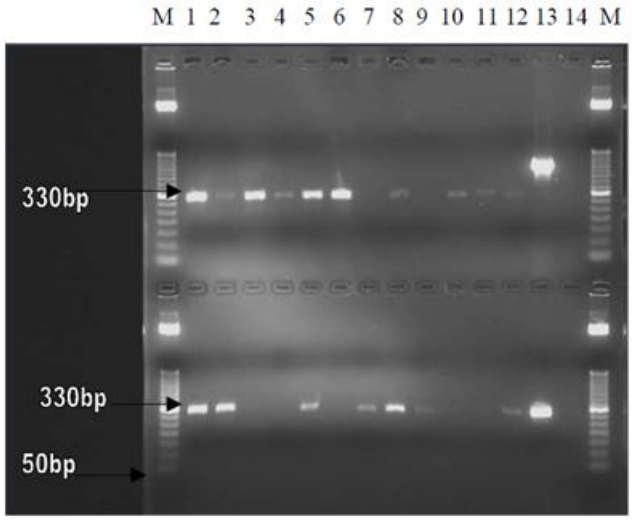

Figure 1: ITS1-PCR analysis of clinical samples isolated from CL patients with band size of 330bp. Upper Gel Lane 1-6,8,10,11 positive samples, Lane 7,9,13 negative samples, lane 12, artefact. Lower gel, lane 1,2,5,7,8,912, positive samples, lane 3,4,6,10,11 negative samples, lane 13 positive control, lane 14 negative control. Ladder 50bp (Fermentas)

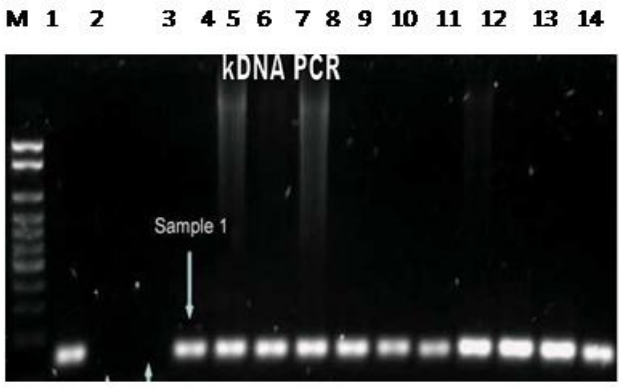

Figure 2: kDNA-PCR analysis of clinical samples isolated from CL patients with band size of 145bp using 50bp DNA ladder (Fermentas). Lane 1 positive control, lane 2 negative control, lane 3 negative sample, lane 3-14 positive samples

CONCLUSION

It can be concluded that *Cutaneous Leishmaniasis* is prevalent in local population of Dir Lower and Dir Upper. For diagnostic study it was concluded that PCR assay may be employed in routine diagnosis of CL particularly in mis diagnosed cases while kDNA-PCR is more sensitive and specific method for detection of the parasite, followed by ITS1-PCR as compared to microscopy and culturing.

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