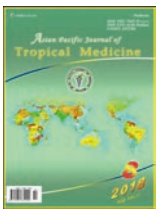

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Molecular detection of *Leishmania* species in human and animals from cutaneous leishmaniasis endemic areas of Waziristan, Khyber Pakhtunkhwa, Pakistan

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ABSTRACT

Objectives: To detect *Leishmania* species in human patients, animal reservoirs and *Phlebotomus* sandflies in Waziristan, Pakistan. **Methods**: Tissue smears and aspirates from 448 cutaneous leishmaniasis (CL) suspected patients were analyzed. To sort out role of the reservoir hosts, skin scrapings, spleen and liver samples from 104 rodents were collected. Furthermore, buffy coat samples were obtained from 60 domestic animals. Sandflies were also trapped. All human, animals and sandfly samples were tested by microscopy, kinetoplastic PCR and internal transcribed spacer 1 (ITS1) PCR followed by restriction fragment length polymorphism for detection of *Leishmania* species. **Results**: An overall prevalence of 3.83% and 5.21% through microscopy and ITS1 PCR respectively was found. However, the statistically non-significant correlation was found between area, gender, and number of lesions. The presence of rodents, sandflies, domestic animals and internally displaced people increased the risk of CL. Using ITS1-PCR-RFLP, *Leishmania* tropica (*L. tropica*) was confirmed in 106 samples while 25 of the isolates were diagnosed as *Leishmania major* (*L. major*). Similarly, 3/104 rodents were positive for *L. major* and 14 pools of DNA samples containing *Phlebotomus sergenti* sandflies were positive for *L. tropica*. None of samples from domestic animals were positive for leishmaniasis. **Conclusions**: In the present study, *L. tropica* and *L. major* are found to be the main causative agents of CL in study area. Movement of internally displaced people from CL endemic areas presents a risk for nearby CL free areas. To the best of our knowledge, we report for the first time *L. major* infection in rodents (*Rattus rattus*) and *L. tropica* in *Phlebotomus sergenti* sandflies trapped in Waziristan, Pakistan.

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1. Introduction

Cutaneous leishmaniasis (CL) appeares as an emerging endemic disease in Afghanistan and Pakistan[1]. It was first reported from Pakistan in 1960 and was limited to northern mountainous region, but now is widely spread in most parts of the country[2] with annually approximately 20 000 CL infected cases have been reported[3,4]. In Khyber Pakhtunkhwa (KP) province of Pakistan, epidemiological and clinical patterns of CL was reported in armed forces deployed in tribal areas for military operation[5].

World Health Organization enlisted this as one of the most serious diseases due to increase number of new cases every year (more than 2 million)[6]. The disease is spread through bloodsucking sand flies (*Phlebotomus*), consisting of 37 species reported from Pakistan[7,8]. The habitat ranges from tropical rainforest to desert and human, dogs, chickens, vertebrates, livestock as well as some mammals serve as hosts[9].

Previously, in some parts of Pakistan, new outbreaks of CL resulted due to frequent movement of internally displaced people (IDPs) to settled urban areas of KP. Positive cases of CL were also reported recently in region of Bannu, Karak, and Kohat because most of IDPs migrated from Waziristan agency settled in these areas (Unpublished data). The recurrent epidemics of leishmaniasis have emerged due to military operations in the tribal areas of KP, Pakistan. As a neglected consequence, a large number of local people migrated from tribal to urban areas. The presence of domestic animals (cows, buffaloes, sheep, goats and pet dogs) in the houses of CL-infected patients or close to these houses have been associated with the prevalence of CL. A positive correlation between prevalence of disease and the presence of domestic animals have been reported in some previous studies[10,11]. Sandflies, animals, and rodents act as carriers for spread of CL from person to person within population.

The purpose of this study was to determine the prevalence of CL in IDPs in Waziristan, molecular detection of *Leishmania* species from human patients, sand flies and reservoir animals by using molecular tools. Moreover, we also performed the identification of the vector species responsible for transmission of CL in this area for the first time.

2. Materials and methods

2.1. Study area and sample collection

The present study was conducted in selected CL endemic villages of North and South Waziristan, Pakistan during 2015-2016. Waziristan covers 15 000 square kilometers with latitude of 33 02' 59" N and longitude: 70 01' 10" in Federally Administered Tribal Areas (FATA). Samples from patients were collected from ulcerating skin lesion scar on clean glass slides with completion of a questionnaire after obtaining informed consent (ethical considerations approved by KUST ethical committee) of the patients including patient's information's regarding name, age, sex, lesions number, site and duration of lesion, present and permanent address, occupation of the patient, type of house, species of domestic animals, physical examination and history of any previous treatment with anti-leishmanial drugs. Furthermore, 104 rodents were captured in steel traps (35 cm × 12 cm × 12 cm) according to methodology standardized for capture of small mammals. A total of 35 traps were set every night in different locations such as corn field and in wild plantations surrounding the houses of CL patients. Rodent species were identified on the basis of anatomical criteria by Department of Zoology, Kohat University. Rodents were euthenised humanely and under aseptic conditions, the spleen and liver were removed and stored at -20 ℃ until further processing. Skin scrapings from suspected skin lesions on ears and body of rodents were obtained. In addition to trap sandflies, reference points were set from houses of CL infected patients. The suspected resting sites and trapping sites of sandflies were within a 1.5 km radius from houses of CL patients. The collection of sandflies was done with sticky papers (castor oil) and CDC light traps (Model 512, John Hock and Co., USA) installed outside and inside of houses in endemic areas. Throughout the study period, 2 traps per site per week were installed in suspected houses of CL patients. The captured sandflies were analyzed soon after their collection. Sandflies to be identified were killed by chilling them in freezer for short time and stored in 70%-80% ethanol until further processing by PCR[12]. Buffy coat was extracted from blood samples obtained from domestic animals sheep (n=8), goat (n=8), cows (n=10), Dogs (n=8), horses (n=8), donkey (n=8), chicken (n=10).

2.2. Microscopy and molecular detection

Giemsa staining was performed on smear impression from CL suspected lesions followed by observation under 100 × oil immersion lens for the presence of amastigotes. The extraction of DNA from human, rodents, sandflies and domestic animal samples was done by using extraction kit Nucleospin® Tissue kit (Macherey Nagel, Germany) as per the manufacturer protocol. In the first round genus specific PCR was performed using kDNA primers RV1/RV2. The positive samples were further used to amplify ITS1 region, which separates the genes coding for the smaller sub unit (ssu) rRNA and 5.8S rRNA using the primers LITSR (5'CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3'). Total reaction volume of 25 μL containing DNA template (5 μL) 2.0 mM $MgCl_2$, 200 μM dNTP's, 20 Pico mol of each primer and 2 μL of Taq polymerase (Roche Biotech) was used. PCR conditions were maintained as: For kDNA PCR, predenaturation was done at 94 ℃ for 3 min followed by 40 cycles (denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s and extension at 72 ℃ for 30 s). The samples were then kept at final annealing temperature of 72 ℃ for 10 min and at last hold at 4 ℃. For ITS1 DNA primers LITSR/L5.8S an initial denaturation at 95 ℃ for 3 min followed by 35 cycles (denaturation at 95 ℃ for 30 s, annealing at 53 ℃ for 30 s and extension at 72 ℃ for 1 min). The samples were then kept at final annealing temperature of 72 ℃ for 5

min and at last hold at 4 ℃. The amplified product was observed on 1.8% agarose gel.

2.3. Restriction fragment length polymorphism (RFLP)

The ITS1-PCR product was digested to carry out the RFLP. Ten μL of amplified product was digested in a 20 μL total mixture containing 10 U of *Hae*Ⅲ (Fermentas) and 2 μL of the appropriate restriction buffer at 37 ℃ water bath overnight. All 20 μL of the digest was used for electrophoresis on 2.5% agarose gel. DNA length standard (100 bp ladder DNA size marker, CinnaGen Co) was used to check the estimated fragment size.

2.4. Identification of sand flies

The sandflies were morphologically identified on the basis of anatomical keys. Detergent solution (1%) was used to wash the sand flies for 5 min and under a stereoscope, the dissection was carried out in 1× phosphate buffer saline. The morphological identification was done by studying anatomical features of heads and genitalia[13].

2.5. Prevalence rate

Prevalence rate of CL was determined by the formula as described[14].

Prevalence rate = No. of patient infected with CL/ total No. of patient examined × 100.

2.6. Statistical analysis

The data was analyzed by using univariate ANOVA using IBM SPSS Statistics 21 and the *P* value <0.05 means significant difference.

3. Results

The CL prevalence rate was assessed based on gender, area and number of lesion using two different methods. Firstly, microscopy was used followed by amplification of kDNA and ribosomal internal transcribed spacer 1 region (Table 1). The overall prevalence was significantly higher in male compared to female through microscopy (2.34% male and 1.48% female) and PCR (3.18% male and 2.02% female) (Table 2). The results demonstrated a high prevalence of CL in children of 0-15 years compared to other age groups (Data not shown). The microscopic as well as molecular analysis revealed that patients with more than two lesion had a high frequency of cases (1.35%) of CL (P<0.05) as compared to other (Figure 1). One of the patient belonging to Razmak of North Waziristan had 8 scar lesions on different parts of the body. Mostly affected persons with active lesions had sought medical intervention within 3-6 months after appearance of the lesions. As CL in Waziristan and KP is thought to be anthroponotic caused by *L. tropica* in which the duration of lesion varies from 3-12 months, while in the case of *L. major*, infectious lesions are thought to be self-healing within 3-6 months, although in the field conditions the story is not always true.

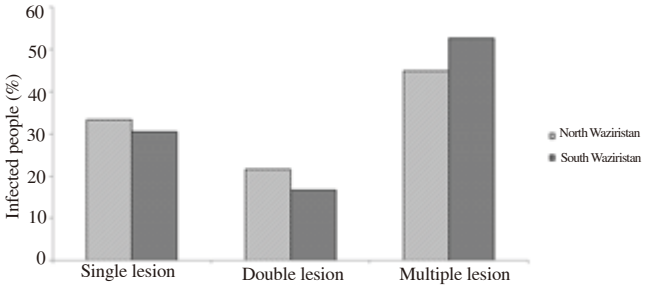

Figure 1. Lesion wise comparison of infected people in South and North Waziristan.

A significant difference (P<0.05) was found in the number of a lesion. A high prevalence (1.27%) was recorded in patients with multiple lesions as compared to single (0.86%) and double lesion (0.53%).

ITS1-PCR-RFLP analysis was performed for identification of different species of *leishmania*. A total of 150 (75 from each region) ITS1 PCR positive random samples were carried out only with the products. ITS-PCR product was incubated and digested by *Hae*Ⅲ enzyme. Three bands approximately 185 bp, 57 bp and 24 bp of *Leishmania tropica* (*L. tropican*) were produced (confirmed through

Table 1

Overall prevalence of CL in Waziristan.

Area of Waziristan	Total population	Suspected patients (*n*)	CL positive samples		CL incidence	
			Microscopy	PCR	Microscopy	PCR
North	3 276	251	129	156	3.93	4.76*
South	1 967	197	72	117	3.66	5.95*
Total	5 243	448	201	273	3.83	5.21*

*Statistical significant (P<0.05).

Table 2

Gender CL prevalence (%) in South and North Waziristan.

Area	Microscopy		PCR	
	Male	Female	Male	Female
North Waziristan	2.25% (74/3 276)	1.67% (55/3 276)	2.74% (90/3 276)	2.01% (66/3 276)
South Waziristan	2.49% (49/1 967)	1.16% (23/1 967)	3.91% (77/1 967)	2.03% (40/1 967)
Total	2.34% (123/5 243)	1.48% (78/5 243)	3.18% (167/5 243)	2.02% (106/5 243)

gene bank). Similarly, for *Leishmania major* (*L. major*) two bands of about 203 bp and 132 bp and three bands for *Leishmania infantum* of about 184 bp, 72 bp, 55 bp size were produced. The ITS1-PCR-RFLP confirmed 76.0% of *L. tropica* and 14.0% of *L. major* specific bands in North Waziristan. However, 65% *L. tropica* and 18% *L. major* specific bands were obtained in samples from South Waziristan (Table 3). Sand flies of the genus *Phlebotomus* and *Sergentomya* were identified both in North and South Waziristan (Table 4). The presence of domestic animals normally influences the populations of the sand fly vectors by providing them with an additional blood source or acting as attractant to make residing people of that house more vulnerable to sand fly bite. Many stray dogs were found in CL endemic villages but none of the dogs was found positive for leishmaniasis. In fact, their increased number in streets can attract sand fly for blood meal thus expanding the risk of CL to the inhabitants. In the present study none of the domestic animal was found PCR positive for leishmaniasis. Previous studies have also shown that domestic animals in CL endemic areas are usually found seropositive but PCR negative. Sand flies and rodents were also collected from different CL endemic villages of the study areas. All the rodents (n=104) were of the same species (*Rattus rattus*) that could be trapped in the present study near houses of CL patients. This is the predominant rodent specie found in study area. PCR analysis showed only 3 of the trapped rodents (*Rattus ratus*) with skin lesions on ears were positive for *L. major*. We trapped different species of sandlfies in present study but the predominant one was *Phlebotomus sergenti* (*P. sergenti*) (n=88) followed by *Phlebotomus papatasi* (71) *sergenti* species (141). So the PCR results showed *L. tropica* in 14 *P. sergenti* sandflies (15% of all *P. sergenti*) and overall infectivity percentage of infected sandflies was 4.6%. Samples collected from domestic animals were negative for leishmaniasis (Table 5).

We reported for the very first-time *L. tropica* infection in 14 female *P. sergenti* and *L. major* infection in this study area.

Table 3

Species identification using RFLP analysis of ITS1-PCR products.

Area	ITS products used in RFLP	Positive samples	*L. tropica*	*L. major*
North Waziristan	75	68	57	11
South Waziristan	75	63	49	14
Total	150	131	106	25

Table 4

Identification of Sandfly in Waziristan.

Genus	Subgenus	Species	Number of sandflies				Total
			North Waziristan	South Waziristan	Male	Female	
Phlebotomus	*Paraphlebotomus*	*sergenti*	40	09	38	11	49
	Phlebotomus	*paptasi*	25	05	23	7	30
Sergentomya	*Sergentomya*	*babu*	25	14	10	29	39
	Sergentomya	*baghdadicus*	43	21	30	34	64
	Sergentomya	*montana*	11	6	12	5	17
	Sergentomya	*sintonius*	22	8	18	12	30
	Grossomya	*indica*	13	12	11	14	25

Table 5

Screening of rodents, sandfly and domestic animals for leishmania parasites.

Species	Type of sample	Total samples	CL positive samples	
			Microscopy	kDNA PCR
Rodents (*Rattus ratus*)	Liver, spleen, skin	104	0	3
Sandfly	Gut	300	0	14
Domestic animals	Buffy coat	60	0	0

The domestic animals were negative for leishmania as diagnosed by microscopy as well as kDNA PCR.

4. Discussion

There is a global change in the epidemiology of leishmaniasis, with the emergence of disease in different parts of the world: a change variously ascribed to population movement and man-made environmental changes. In the present study, we indicated high prevalence in male patients. Similar findings were also reported previously that male has comparatively high prevalence of CL[2,15,16]. Study in Landi Kotal, KP indicated high prevalence in the age group of 4 to 17 years. However, the researchers concluded that use of bed nets during night in the study area might reduce the incidence rate of leishmaniasis[17]. More social activity and mobility of male as they often sleep without shirt expose them to bite of sand-fly. Since most of the people follow Islamic laws in the study region, females always remain covered making them less prone to sand-fly bite. Moreover, Waziristan agency is a big hub for IDPs traveling across the endemic areas of Afghanistan where CL is endemic.

CL as endemic in North, South, and West of Pakistan while East and South-Eastern regions are nonendemic[18]. Moreover, in Northern Pakistan the disease was prevalent in human populations throughout the year[2]. A total of 688 cases of Old World CL from Bajor Agency, Kurram Agency, Khar, Bara, Miran shah, and southern Waziristan and endemic belt along the borders of Afghanistan were reported[19]. Travelling history to Balochistan province also resulted in CL in 256 patients with non-healing chronic ulcers. CL in young children belonging to different areas was also studied in another part of KP[20]. There were 75 (40.5%) cases from Sindh, 20 (10.0%) were from Punjab from known endemic areas, 38 (20.5%) from KP and 52 (28.0%) from Balochistan[21].

It was also reported previously that infection of the *Leishmania*

parasite in children [11- 16 years) showed a high prevalence of 45.12% (2 643 out of 5 857 children)][22]. Moreover, another study reported same outcomes that children are more prone to disease as high prevalence was among 0-9 years age group. (10.96%), followed by10-19 years(6.66%), 30-39 years (5.35%), 40-49 years (5.12%), 20-29 years(3.96%), and lowest prevalence rate 2.94% was found in age group 50-59 years. Furthermore, older people 51-60 years have less prevalence (4.83%)[23]. The high prevalence in children may be due to maximum exposure to sand fly bite as children mostly play outside and their innate immune system is also weak.

The microscopic as well as molecular analysis revealed that patients with more than two lesion had a high frequency of cases (1.35%) of CL (P<0.05) as compared to other. In a previous study a total of 50.9% of patients had single lesions while double lesions were observed in 24.6% of patients and 29.4% of patients showed multiple (3-15) lesions[24]. This difference could be a result from abundance of *P. sergenti* sandflies in the study area and more exposure level due to poverty and social reasons. Limited access to specific antileishmanial drugs, persistence of secondary bacterial infections, development of antimonial resistance and hygienic factors could influence the duration of CL active lesions[22].

In present study we found only 3 rodents out of 104 captured belonging to genus *Rattus rattus* were found positive with *L. major* and none of domestic animals sampled were positive for any *Leishmania* specie. In a recent study from Morocco molecular analysis revealed the presence of *Leishmania* species in 18 specimens: 6 *Rattus rattus* (out of 80 captured; 7.50%), 11 *Mus musculus* (out of 50 captured; 22.00%), and one *Rattus norvegicus* (out of 9 captured; 11.11%)[25]. *L. tropica* induced CL is commonly considered an anthroponotic disease that usually does not involve an animal reservoir[26], but zoonotic transmission through animal reservoirs has been demonstrated in Palestine, Israel and Jordan[27,28]. Some of the animal reservoirs could be possible source of transmission. Similar results have been elucidated by Khan *et al*[8] who reported that these animals are not reservoirs of leishmanial parasite, but they can increase the contiguity of sand fly and humans. Dogs have been found infected with *L. tropica* in Morroco[29], but are not potential reservoirs. A study conducted on animal reservoirs in Addis Ababa, Ethopia, showed rodents *Rattus ratus* were negative for *Leishmania*[30]. These rodents may not be the preferable source for a blood meal. Therefore, detail study of sand fly species, their identification, feeding preferences and habitats in this part of FATA are needed to be investigated in future. The role of animal reservoirs need detail investigation even during the study *L. tropica* was found to be the causative agent in different districts of KP. In previous studies, *P. sergenti* regarded as the susceptible vector of ACL in Afghanistan and Pakistan, but further work is needed to isolate and type *L. tropica* from wild sand fly females[12]. *Phlebotomus paptasi* is also susceptible to carry *L. tropica* and is widely distributed in different parts of Pakistan including KP[31]. In addition, the vector sand flies *Phlebotomus perniciosus* and *P. sergenti* have been found engorged with rodent blood in southern Portugal[32] but also in central Morocco[33] where the sand fly composition is well established[34]. Svobodovà *et al*[35] reported the transmission of *L. tropica* to mice by the bite of *P. sergenti*, a species widespread in our study area[34].

The endemic areas acknowledged during this study present a risk for nearby CL free areas. *L. tropica* and *L. major* are the main causative agents of CL in Waziristan. More molecular epidemiological studies in the endemic areas should be conducted properly with the help of local and provincial government authorities to identify leishmania outbreaks on time. It was concluded that there is a polymorphism in *L. tropica* and a dimorphism in *L. major* therefore the clinical aspect and the microscopic diagnosis are insufficient to identify the species of Leishmania, therefore, the molecular biology highly recommended for the diagnosis and identification of the causative agent. These results suggest the possible involvement of rodent species in *L. major* cycles in study area. The present findings should be taken into consideration when developing control programs to combat this disease in CL endemic areas of Pakistan.

Conflict of interest statement

The authors declare no conflict of interests.

Acknowledgments

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References

[1] Kassi M, KassiM, Afghan AK, Rehman R, Kasi PM. Marring leishmaniasis: The stigmatization and the impact of cutaneous leishmaniasis in Pakistan and Afghanistan.*PLoS Negl Trop Dis* 2008; **2**(10): e259, doi:10.1371/journal.pntd.0000259.g001.

[2] Hussain M, Munir S, Ayaz S, Khattak BU, Khan TA, Muhammad N, et al. First report on molecular characterization of *Leishmania* species from cutaneous leishmaniasis patients in southern Khyber Pakhtunkhwa province of Pakistan. *Asian Pac J Trop Med* 2017; **10**(7): 785-788.

[3] Khan NH, Bari AU, Hashim R, Khan I, Muneer A, Shah A, et al. Cutaneous leishmaniasis in Khyber Pakhtunkhwa Province of Pakistan. Clinical diversity and species-level diagnosis. *Am J Trop Med Hyg* 2016; **95**(5): 1106-1114..

[4] Akram A, Khan HA, Qadir A, Sabir AM. A cross-sectional survey of knowledge, attitude and practices related to cutaneous leishmaniasis and sand flies in Punjab, Pakistan. *PloS One* 2015; **10**(6): e0130929.DOI: 10.1371/journal.pone.0130929.

[5] Hussain M, Munir S, Khan TA, Khan A, Ayaz S, Jamal MA, et al. Epidemiology of cutaneous leishmaniasis outbreak, Waziristan, Pakistan. *Emerg Infect Dis* 2018; **24**(1): 159-161.

[6] WHO. Weekly epidemiological record (WER). Geneva: WHO; 2016; 91(16): 209-216.

[7] Ali N, Ullah A, Wahid S, Khisroon M, Rasheed SB. Distribution, species composition and relative abundances of sandflies in North Waziristan Agency, Pakistan. *Med Vet Entomol* 2015; **30**(1): 89-94.

[8] Khan K, Wahid S, Khan NH, Ali N. Potential resting and breeding sites of sand flies (Diptera: Psychodidae) and their habitat characteristics in Leishmaniasis Foci of Dir districts, Khyber Pakhtunkhwa, Pakistan. *J Med Entomol* 2017; **54**(5): 1390-1396.

[9] Mmc A, Vda A, Mds S, Lds P, Costa DJ, Gil-Santana L, et al. Clinical and immunopathological findings during long term follow-up in *Leishmania infantum* experimentally infected dogs. *Sci Rep* 2017; **7**(1). DOI :https://doi.org/10.1038/s41598-017-15651-8

[10]Bern C, Joshi AB, Jha SN, Das ML, Hightower A, Thakur G, et al. Factors associated with visceral Leishmaniasis in Nepal: Bed-net use is strongly protective. *Am J Trop Med Hyg* 2000; **63**(3): 184-188.

[11]Montesinos-Gelet I, Chabanne JC, Bucheron D. Parler et ecrire pour penser, apprendre et se construire. L'ecrit et l'oral reflexifs. Paris: Presses Universitaires de France. *Revue De Psychoeducation* 2003; **32**(2): 430-431.

[12]Zorrilla V, Santos MBDL, Espada L, Santos RDP, Fernandez R, Urquia A, et al. Distribution and identification of sand flies naturally infected with Leishmania from the Southeastern Peruvian Amazon. *PLoS Negl Trop Dis* 2017; **11**(11): e0006029, DOI:10.1371/journal.pntd.0006029.

[13]Tanure A, Peixoto JC, Duarte R, Pinheiro ADC, Coelho SVB, Barata RA. Identification of sandflies (Diptera: Psychodidae: Phlebotominae) blood meals in an endemic leishmaniasis area in Brazil. *Rev InstMed Trop São Paulo* 2015; **57**(4): 321-324.

[14]Ayaz S, Khan S, Khan SN, Shams S, Saqalain M, Ahmad J, et al. Cutaneous leishmaniasis in Karak, Pakistan: Report of an outbreak and comparison of diagnostic techniques. *Afr J Biotechnol* 2011; **10**(48): 9908-9910.

[15]Bari A, Hasshim R, Mahmood K, Muhammad I, Shahbaz N, Tariq KM. Clinico-epidemiological pattern of cutaneous leishmaniasis in armed forces personnel fighting war against terrorism in Khyber Pakhtunkhwa Province and Fata regions. *J Pakistan Assoc Dermatol* 2011; **21**(1): 10-15.

[16]Farahmand M, Nahrevanian H, Shirazi HA, Naeimi S, Farzanehnejad Z. An overview of a diagnostic and epidemiologic reappraisal of cutaneous leishmaniasis in Iran. *Braz J Infect Dis* 2011; **15**(1): 17-21.

[17]Qureshi NA, Ali A, Rashid U, Ali N. Prevalence of *Leishmania tropica* in school boys of khyber agency, FATA near Pak-Afghan border. *Acta tropica* 2016; **164**: 90-94.

[18]Durrani AZ, Durrani HZ, Kamal N, Mehmood N. Prevalence of cutaneous leishmaniasis in humans and dogs in Pakistan. *Pakistan J Zool* 2011; **43**(2): 263-271.

[19]Rahman S, Abdullah FH, Khan JA. The frequency of old world cutaneous leishmaniasis in skin ulcers in Peshawar. *J Ayub Med Coll Abbottabad* 2009; **21**(3): 72-75.

[20]Shoaib S, Tauheed S, Hafeez A. Cutaneous leishmaniasis: An emerging childhood infection. *J Ayub Med Coll Abbottabad* 2007; **19**(4): 40-41.

[21]Bari AU, Rahman SB. Many faces of cutaneous leishmaniasis. *Indian J Dermatol Venereol Leprol* 2008; **74**(1): 23-27.

[22]Kakarsulemankhel JK. Re-description of species of sandflies of the subgenus *Phlebotomus* (Diptera: Psychodidae) of Balochistan, Pakistan. *Pakistan J Zool* 2004; **36**(2): 143-164.

[23]Youssefi MR, Shojaei J, Jalahi H, Amoli SA, Ghasemi H, Javadian M. Prevalence of cutaneous leishmaniasis during 2010 in Mazandaran Province, Iran. *African J Microbiol Res* 2011; **5**(31): 5790-5792.

[24]Talari S, Shajari G, Talaei R. Clinical study of cutaneous leishmaniasis in a new focus of Iran. *Res J Microbiol* 2006; **1**(4): 366-370.

[25]Echchakery M, Chicharro C, Boussaa S, Nieto J, Carrillo E, Moreno J, et al. Molecular detection of *Leishmania infantum* and *Leishmania tropica* in rodent species from areas of Morocco with endemic cutaneous leishmaniasis. *Parasit Vectors* 2017; **10**(1): 454. DOI 10.1186/s13071-017-2398-8

[26]Ashford RW. The leishmaniases as emerging and reemerging zoonoses. *Int J Parasitol* 2000; **30**: 1269-1281.

[27]Jacobson RL, Eisenberger CL, Svobodova M, Baneth G, Sztern J, Carvalho J, et al. Outbreak of cutaneous leishmaniasis in northern Israel. *J Infect Dis* 2003; **188**: 1065-1073.

[28]Talmi-Frank D, Jaffe CL, Nasereddin A, Warburg A, King R, Svobodova M, et al. *Leishmania tropica* in rock hyraxes (*Procavia capensis*) in a focus of human cutaneous leishmaniasis. *Am J Trop Med Hyg* 2010; **82**: 814-818.

[29]Couez D, Libon E, Wasteels M, Derue G, Gilbeau J. Klebsiella pneumoniae liver abscess with septic endophthalmitis. The role of computed tomography. *J Belge Radiol* 1991; **74**(1): 41-44.

[30]Borraccino A, Lemma P, Iannotti R, Zambon A, Dalmasso P, Lazzeri G, et al. Socio-economic effects on meeting PA guidelines: Comparisons among 32 countries. *Med Sci Sports Exerc* 2009; **41**(4): 749-756.

[31]Killick-Kendrick R, Killick-Kendrick M, Tang Y. Anthroponotic cutaneous leishmaniasis in Kabul, Afghanistan: The low susceptibility of *Phlebotomus papatasi* to *Leishmania tropica*. *Trans R Soc Trop Med Hyg* 1994; **88**(2): 252-253.

[32]Maia C, Dionísio L, Afonso MO, Neto L, Cristóvão JM, Campino L. Leishmania infection and host-blood feeding preferences of phlebotomine sand flies and canine leishmaniasis in an endemic European area, the Algarve Region in Portugal. *Mem Inst Oswaldo Cruz Rio de Janeiro* 2013; **108**(4): 481-487.

[33]Ajaoud M, Es-Sette N, Charrel RN, Laamrani-Idrissi A, Nhammi H, Riyad M. *Phlebotomus sergenti* in a cutaneous leishmaniasis focus in Azilal Province (High Atlas, Morocco): Molecular detection and genotyping of *Leishmania tropica*, and feeding behavior. *PLoS Negl Trop Dis* 2015; **9**(3): e0003687.

[34]Boussaa S, Kahime KM, Samy A, Ben Salem A, Boumezzough A. Species composition of sand flies and bionomics of *Phlebotomus papatasi* and *P. sergenti* (Diptera: Psychodidae) in cutaneous leishmaniasis endemic foci, Morocco. *Parasit Vectors* 2016; **9**: 60.

[35]Svobodovà M, Votypka J, Nicolas L, Volf P. *Leishmania tropica* in the black rat (*Rattus rattus*): Persistence and transmission from asymptomatic host to sand fly vector *Phlebotomus sergenti*. *Microbes Infect* 2003; **5**: 361-364.