



Sand Fly (Psychodidae: Phlebotominae) Ecology of an Emerging Cutaneous Leishmaniasis Focus in Khyber Pakhtunkhwa with an Updated Species Checklist from Pakistan

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ABSTRACT

Leishmaniasis, an important neglected vector borne disease, endemic in the northern parts of Pakistan. The disease mainly exist in cutaneous form while sporadic cases of visceral leishmaniasis has also been reported from the affected areas. Present study was designed to know the species composition, relative abundance and seasonal variation of leishmaniasis vector in an emerging endemic focus of Nowshera. Sand flies collected by flit method were identified into 4 species of genus *Phlebotomus* and 17 species of genus *Sergentomyia*. *P. sergenti*, the suspected vector of leishmaniasis was also collected in this survey. *Sergentomyia baghdadis* and *S. babu* were the most abundant species and sand fly active season extended from March to November with a unimodal distribution and population reaching its peak during the month of August. A supplementary collection from Charun area of Chitral revealed 12 species of *Phlebotomus* and four species of *Sergentomyia*. Among the total 31 species collected from both areas, six species of *Phlebotomus* (*P. angustus*, *P. comatus*, *P. kabulensis*, *P. rupester*, *P. turanicus* and *P. newsteadi*) collected from Chitral and a single species of *Sergentomyia* (*S. sagodina*) collected from Nowshera are new reports to Pakistan. Current study also added subgenus *Kasaulius* to the *Phlebotomus* of Pakistan. With the addition of these seven species, the checklist of sand flies of Pakistan has increased to 50 species, checklist provided.

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Authors' Contribution

SBR conceived and designed the study and wrote the article. SBR and FZ supervised the research projects. Samiullah and TJ collected the specimens as part of undergraduate research project. SBR, Samiullah and TJ identified the specimens. FZ edited the manuscript.

Key words

Sand fly, Diversity, Ecology, Checklist, *Phlebotomus*, Cutaneous leishmaniasis

INTRODUCTION

Leishmaniasis is a growing vector borne parasitic multi host neglected tropical disease with 350 million people at risk of contracting the disease. Approximately 12 million people get infected yearly with this disease that occur in three clinical forms in human i.e., visceral leishmaniasis (VL), mucocutaneous leishmaniasis (MCL) and cutaneous leishmaniasis (CL) affecting the visceral organs, mucous membrane and skin, respectively. CL is the most common form that causes skin lesions, leaving lifelong scars on exposed parts of the body. An estimated 1 million cases annually occur around the globe, 85% of

which occurs in 10 countries: Algeria, Afghanistan, Brazil, Bolivia, Colombia, Iran, Iraq, Syria, Tunisia and Pakistan (Ruiz-Postigo *et al.*, 2021).

Leishmaniasis is an emerging vector borne disease in Pakistan too. Cases have been reported from all four provinces i.e., Punjab, Sindh, Khyber Pakhtunkhwa (KP), Balochistan and from Gilgit-Baltistan and Kashmir but KP province has the most affected areas of the country. Leishmaniasis is endemic in all seven newly merged districts of KP (previously known as Federally Administered Tribal Area or FATA) with more than 42000 cases reported from district Khyber only during the past one decade. According to WHO cases have been reported from Malakand, Mardan, Nowshera, Bannu, Karak and all merged tribal districts of KP with disease burden mounting to 40,000 cases in 2019 (Ruiz-Postigo *et al.*, 2021).

Sand flies are small hematophagous insects responsible for the transmission of leishmaniasis, bartonella and arboviruses. There are approximately 1000 species of sand flies in which 540 occur in new world and 460 in old world (De Sousa-Paula, 2021). Only 98 of these species act as vectors of human leishmaniasis, 56 of which belong to genus *Lutzomyia* in the new world and

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42 to genus *Phlebotomus* in the old world (Maroli *et al.*, 2013). Species of sand flies such as *Phlebotomus sergenit* and *P. papatasi* are proven vectors of anthroponotic CL and zoonotic CL in the old world respectively, while *P. salehi* and *P. caucasicus* have been incriminated or marked as suspected vectors of leishmaniasis in different parts of the world (Ajaoud *et al.*, 2013; Es-Sette *et al.*, 2014; Azizi *et al.*, 2012). Though importance of sand flies is well established across the world but in Pakistan little work has been done on the systematics of these medically important insects. Few studies have been conducted to investigate the sand fly fauna of leishmaniasis affected areas in Pakistan (Qutubudin, 1951; Nasir, 1958; Barnett and Suyemota, 1961; Ahmad *et al.*, 1960; Lewis, 1967; Burney *et al.* 1981; Burney, 1986; Munir, 1994).

Lewis (1967) reported 27 (10 *Phlebotomus* and 17 *Sergentomyia*) species of sand flies from Pakistan. Later Munir (1994) collected eight species of *Phlebotomus* from Northern areas of Pakistan. Aslamkhan *et al.* (1998) published a check list of sand flies of Pakistan reporting 29 species from Pakistan. Kakarsulemankhel (2003) collected *P. bergeroti*, *P. papatasi* and *Phlebotomus salehi* from Balochistan and then published a checklist of sand flies of Balochistan with six new reports from Balochistan and Pakistan (Kakarsulemankhel, 2004), Ali *et al.* (2016a) worked on the diversity of sand flies of Dir and reported 20 species. Ali *et al.* (2016b) while exploring the sand fly fauna of North Waziristan reported 14 species of sand flies.

Current study was conducted to investigate the sand fly fauna of an emerging leishmaniasis focal point in KP. Knowledge of distribution and abundance of vectors can provide useful information to devise a control strategy to prevent the spread of disease in endemic areas. This study was designed to know the species composition, relative abundance and seasonal variation in two villages of district Nowshera. Additionally, a supplementary collection was carried out from district Chitral, an area located in the Hindukush Mountain range in the north of KP, to gather some preliminary information regarding the sand flies occurring in the area. This paper also provided an updated checklist of sand fly fauna of Pakistan.

MATERIALS AND METHODS

Study area

Nowshera is a district in KP located along the river Kabul. The district consists of 03 tehsils and 47 Union Councils covering an area of 1748 sq km, it lies between latitude 33°42', to 34°09' N and longitude 71°41' to 72° 15' E. The climate of district Nowshera is warm and sub-humid with an average temperature of 30°C. In summer the weather is too hot as it touches 40°C to 45°C, while

in winter the weather is too cold as sometimes it can be as low as 1°C. The area receives an average rainfall of 513 mm/year. The area receives maximum rainfall in the months of February, March, July and August.

Collection methods

A year-round collection of sand flies was performed from December to November in the Manki Shareef and Nizampur areas of district Nowshera (Fig. 1). From each village 15 houses were selected and the collection was performed on monthly basis by flit method. Sand flies were collected from two types of habitats Human dwellings and Animal rooms. The supplementary collection was carried out from Charun area of District Chitral (Fig. 1) by CDC miniature light traps.

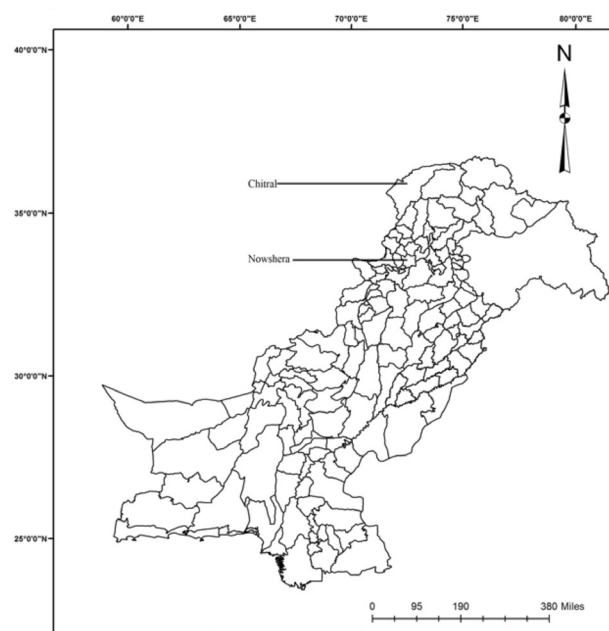


Fig. 1. Map showing the study area in the map of Pakistan.

Flit method

Indoor sand fly collection was carried out by flit method (Lane and Crosskey, 1993). The rooms from where collection was done were either muddy or cemented, but majority of the rooms were cemented. The rooms were of two types based on the basis of usage i.e., animals rooms and human inhabiting rooms. The rooms floors were cleared and white sheets were spread to cover the floor area. Aerosol insecticide spray (Pyrethroids) of various brands (Zending and Kingtox) was performed for 30 sec and then the room was kept airtight and locked for 10-15 min, for the insecticide to kill any sand fly in the room. After 10 min, the room was opened and sheets

were searched for any sandfly killed by the insecticide spray. Using fine entomological forceps the sandflies were carefully transferred to Eppendorf tubes containing 70% ethanol for preservation. The sandflies were kept in the alcohol at room temperature until the identification of the specimens up to species level.

CDC miniature light traps

In Charun area of Chitral battery operated CDC miniature Light Traps (model 512 with air-actuated gate system and LCS-2 photo switch; John W. Hock Company, USA) were hung inside a human dwelling room 30 inches above ground from dusk to dawn to collect sand flies. At dawn the sand flies were collected by fine entomological forceps and preserved in 90% Ethanol.

Mounting and identification sand flies

Sand flies were identified based on internal anatomy which can be only visible if they are mounted permanently on slide. For this purpose, a sand fly was transferred to a glass slide and air-dried to remove any alcohol present in it. The head of the sand fly was detached from the body with the help of fine needle under a stereo microscope (Olympus, SD 30, USA) and positioned, so that dorsal side is visible. The thorax and abdomen were also placed in a position so that all anatomical structures were easily visible. A drop of Puri's media was added to the slide and then covered with cover slip. The cover slip was pressed gently to remove any air bubbles from the media. The slides were left for two weeks to clear the internal anatomy of the specimens. Sand flies were identified under binocular microscope (Nikon E-100) using species identification key described by Lewis (1967) and Artemiev (1978).

Data analysis

Relative abundance and seasonal variation among various species were calculated in Excel, while correlation coefficient of bioclimatic variables with abundance was calculated by using online statistical software (Wessa, 2017).

RESULTS

A total of 5894 specimens of sand flies were collected from Nowshera and Chitral comprising 31 species belonging to two genera *Phlebotomus* and *Sergentomyia*. *Phlebotomus* was represented by six subgenera and 14 species while *Sergentomyia* was represented by five subgenera and 17 species (Table I). Collection from Charun area of Chitral comprised of 16 species classified into two genera and six subgenera. *Phlebotomus* was having 12 species, while *Sergentomyia* was represented

Table I. List of Phlebotomine of Pakistan and the sites of collections Chitral (C) and Nowshera (N).

Names of species	Site of collection	
	N	C
1. Genus <i>Phlebotomus</i> Rondani and Bert, 1840		
A. Subgenus <i>Adlerius</i> Nitzulescu, 1931		
1. <i>P. (Ad.) angustus</i> Artemiev, 1978	-	✓
2. <i>P. (Ad.) comatus</i> Artemiev, 1978	-	✓
3. <i>P. (Ad.) hindustanicus</i> Theodor, 1958		
4. <i>P. (Ad.) kabulensis</i> Artemiev, 1978	-	✓
5. <i>P. (Ad.) longiductus</i> Parrot, 1928	-	✓
6. <i>P. (Ad.) rupester</i> Artemiev, 1978	-	✓
7. <i>P. (Ad.) salangensis</i> Artemiev, 1978	-	
8. <i>P. (Ad.) turanicus</i> Artemiev, 1974	-	✓
B. Subgenus <i>Anaphlebotomus</i>		
9. <i>P. (An.) colabaensis</i> Young and Chalam, 1927		
C. Subgenus <i>Kasaulius</i>		
10. <i>P. (K.) newsteadi</i> (Sinton, 1926)	-	✓
D. Subgenus <i>Euphlebotomus</i> Theodor, 1948		
11. <i>P. (E.) argentipes</i> Annandale and Brunetti, 1908		
E. Subgenus <i>Larroussius</i> Nitzulescu, 1931		
12. <i>P. (L.) kandelakii burneyi</i> Shurenkova, 1929		
13. <i>P. (L.) keshishiani</i> Shurenkova, 1936	-	✓
14. <i>P. (L.) major major</i> Annandale, 1910		
F. Subgenus <i>Paraphlebotomus</i> Theodor, 1948		
15. <i>P. (Para.) alexandri</i> Sinton, 1928	✓	-
16. <i>P. (Para.) andrejevi</i> Shakrizjanova, 1953		
17. <i>P. (Para.) caucasicus</i> Marzinovyskiy, 1917		
18. <i>P. (Para.) kazeruni</i> Theodor and Mesghali, 1964	✓	-
19. <i>P. (Para.) nuri</i> Lewis, 1967		
20. <i>P. (Para.) sergenti</i> Parrot, 1917	✓	-
G. Subgenus <i>Phlebotomus</i> Rondani and Brete, 1940		
21. <i>P. (P.) bergeroti</i> Parrot, 1934		
22. <i>P. (P.) papatasi</i> (Scopoli, 1786)	✓	✓
23. <i>P. (P.) salehi</i> Mesghali, 1965		
H. Subgenus <i>Synphlebotomus</i> Theodor, 1948		
24. <i>P. (Sy.) ansari</i> Lewis, 1958	-	✓
25. <i>P. (Sy.) eleanorae</i> Sinton, 1931	-	✓
2. Genus <i>Sergentomyia</i> Franca and Parrot, 1920		
A. Subgenus <i>Grassomyia</i> Theodor, 1958		
26. <i>S. (G.) dreyfussi turkestanica</i> Theodor and Mesghali, 1964	✓	-
27. <i>S. (G.) indica</i> (Theodor, 1931)	✓	-

Table continued on next page.....

Names of species	Site of collection	
	N	C
B. Subgenus <i>Parratomyia</i> Theodor, 1958		
28. <i>S. (Parr.) africana</i> (Newstead, 1912)		
29. <i>S. (Parr.) babu</i> (Annandale, 1910)	✓	✓
30. <i>S. (Parr.) baghdadis</i> (Alder and Theodor, 1929)	✓	-
31. <i>S. (Parr.) bailyi</i> (Sinton, 1931)	✓	✓
32. <i>S. (Parr.) freetownensis</i> (Sinton, 1930)		
33. <i>S. (Parr.) grekovi</i> (Chodukin, 1929)	✓	✓
34. <i>S. (Parr.) montana</i> (Sinton, 1924,)	✓	✓
35. <i>S. (Parr.) palestinensis</i> (Alder and Theodor, 1927)	✓	-
36. <i>S. (Parr.) sogdiana</i> (Parrot, 1929)	✓	-
37. <i>S. (Parr.) shortii</i> (Adler and Theodor, 1927)		
38. <i>S. (Parr.) sumbarica</i> (Perfiliew, 1933)	✓	-
C. Subgenus <i>Neophlebotomus</i> Franca and Parrot, 1920		
<i>Randanomyia</i> Theodor, 1958		
39. <i>S. (Neo.) hodgsoni hodgsoni</i> (Sinton, 1933)		
40. <i>S. (Neo.) hodgsoni pawlowskyi</i> (Perfiliew, 1933)	✓	-
D. Subgenus <i>Sergentomyia</i> Franca and Parrot, 1920		
41. <i>S. (Ser.) dentata</i> (Sinton, 1933)		
42. <i>S. (Ser.) fallax afghanica</i> Artemiev, 1974	✓	-
43. <i>S. (Ser.) mervynae</i> Pringle, 1953	✓	-
44. <i>S. (Ser.) murgabiensis</i> (Perfiliew, 1939)		
45. <i>S. (Ser.) punjabensis</i> (Sinton, 1927)		
46. <i>S. (Ser.) theodori</i> (Parrot, 1942)	✓	-
E. Subgenus <i>Sintonius</i> Nitzulescu, 1931		
47. <i>S. (Sin.) christophersi</i> (Sinton, 1927)		
48. <i>S. (Sin.) clydei</i> (Sinton, 1928)	✓	-
49. <i>S. (Sin.) hospitii</i> (Sinton, 1929)	✓	-
50. <i>S. (Sin.) tiberiadis pakistanica</i> (Alder, Theodor and Lourie, 1930)	✓	-

by four species. Collection from Nowshera comprised of 5424 sand flies classified into four species in two subgenera of *Phlebotomus* (*P. papatasi*, *P. sergenti*, *P. kazeruni* and *P. alexandri*) and 17 species in 5 subgenera of *Sergentomyia* (*S. babu*, *S. baghdadis*, *S. fallax afghanica*, *S. indica*, *S. bailyi*, *S. mervynae*, *S. hodgsoni pawlowskyi*, *S. tiberiadis pakistanica*, *S. hospitii*, *S. grekovi*, *S. clydei*, *S. montana*, *S. sumbarica*, *S. sogdiana*, *S. palestinensis*, *S. dreyfussi turkestanica* and *S. theodori*) (Table I). Majority of the collection was represented by *Sergentomyia* with *S. baghdadis* being the most dominant species followed by *S. babu*, *S. fallax afghanica* and *S. bailyi* (Table II).

Table II. Relative abundance of sand fly species collected from Nowshera and Chitral.

S. No	Species	Nowshera		Chitral	
		♀, ♂	Relative abundance	♀, ♂	Relative abundance
1	<i>Ph. papatasi</i>	96, 80	3.24	339, 62	85.3
2	<i>Ph. sergenti</i>	74, 136	3.87	12, 20	6.8
3	<i>Ph. kazeruni</i>	2, 2	0.07	-	
4	<i>Ph. alexandri</i>	0, 8	0.15	-	
5	<i>Ph. keshishiani</i>	-		2, 3	1.06
6	<i>Ph. longiductus</i>	-		2, 0	0.43
7	<i>Ph. ansari</i>	-		3, 0	0.64
8	<i>Ph. angustus</i>	-		1, 3	0.85
9	<i>Ph. rupester</i>	-		1, 0	0.2
10	<i>Ph. newsteadi</i>	-		0, 1	0.2
11	<i>Ph. eleanorae</i>	-		2, 0	0.43
12	<i>Ph. kabulensis</i>	-		6, 1	1.49
13	<i>Ph. turanicus</i>	-		2, 1	0.64
14	<i>Ph. comatus</i>	-		1, 0	0.2
15	<i>S. babu</i>	1210, 632	33.96	1, 1	0.43
16	<i>S. baghdadis</i>	1760, 846	48.04	-	
17	<i>S. fallax afghanica</i>	102, 138	4.42	-	
18	<i>S. indica</i>	2, 36	0.7	-	
19	<i>S. bailyi</i>	138, 46	3.39	2, 2	0.85
20	<i>S. mervynae</i>	4, 2	0.11	-	
21	<i>S. hodgsoni pawlowskyi</i>	32, 2	0.63	-	
22	<i>S. tiberiadis pakistanica</i>	0, 4	0.07	-	
23	<i>S. hospitii</i>	6, 2	0.15	-	
24	<i>S. grekovi</i>	24, 4	0.52	1, 0	0.2
25	<i>S. clydei</i>	4, 2	0.11	-	
26	<i>S. montana</i>	4, 6	0.18	1, 0	0.2
27	<i>S. sumbarica</i>	0, 2	0.04	-	
28	<i>S. sogdiana</i>	2, 2	0.07	-	
29	<i>S. palestinensis</i>	2, 6	0.15	-	
30	<i>S. dreyfussi turkestanica</i>	4, 0	0.07	-	
31	<i>S. theodori</i>	2, 0	0.04	-	
	Total	5424		470	

Phlebotomus was represented by only 7.33% of the total collection with *P. sergenti* being the most dominant species of the genus followed by *P. papatasi* (Table II). A supplementary collection from Charun area of Chitral by light traps yielded 470 specimens belonging to 16 species of *Phlebotomus* and *Sergentomyia*. *Phlebotomus* was represented *P. papatasi*, *P. sergenti*, *P. keshishiani*, *P. longiductus*, *P. ansari*, *P. angustus*, *P. rupester*, *P. newsteadi*, *P. eleanorae*, *P. kabulensis*, *P. turanicus* and *P. comatus* while *Sergentomyia* was represented by *S. babu*, *S. bailyi*, *S. grekovi* and *S. montana* (Table I).

The year around collection made from District Nowshera has shown that abundance of sand fly was minimum during the cold months and maximum during the warm rainy season (Monsoon season from July to September). An increase in sand fly density was observed with temperature, precipitation and humidity (Fig. 2). No specimens of sand fly were reported during the month of January and February, however sand flies started appearing during the month of March and April, but abundance was low. Population started building up in May and then a sudden increase was observed from July to September with a peak abundance observed in the month of August, then a sudden decrease was observed in the month of October and only few specimens were reported in November and none in the cold month of December (Fig. 2). Correlating the abundance with climatic variables has shown a positive correlation with temperature, humidity and precipitation, however, this correlation was significant with temperature and precipitation only ($P < 0.05$).

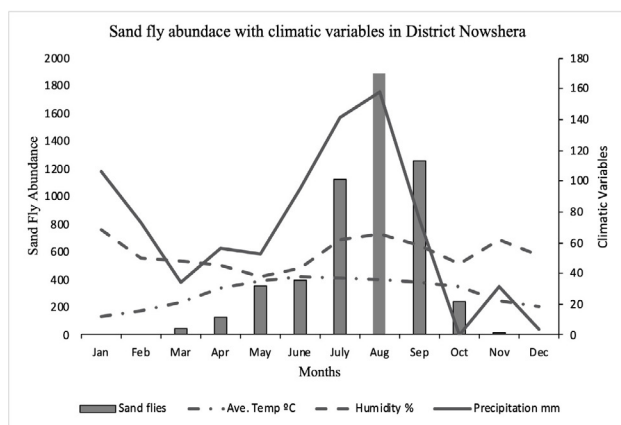


Fig. 2. Sand fly seasonal variation and its relationship with temperature, humidity and precipitation in district Nowshera.

Current study for the first time from Pakistan reported one new subgenus i.e. *Kasaulius* and six new species of *Phlebotomus* i.e. *P. angustus*, *P. rupester*, *P. kabulensis*,

P. turanicus, *P. newsteadi* and *P. comatus* from Chitral and one new species of *Sergentomyia* i.e. *S. sogdiana* from Nowshera. Four of the *Phlebotomus* species i.e., *P. angustus*, *P. rupester*, *P. kabulensis* and *P. turanicus* belonged to subgenus *Adlerius*, while *P. newsteadi* and *P. comatus* were from subgenera *Kasaulius* and *Synphlebotomus*, respectively. *S. sogdiana* belonged to subgenus *Parratomyia* of *Sergentomyia* genus. With the addition of these seven species now the sand fly fauna of Pakistan comprises of 50 species, of which 25 species each belonging to *Phlebotomus* and *Sergentomyia* are listed in Table I which also shows sand flies collected from Nowshera (N) and Chitral (C).

DISCUSSION

There has been regular reports of leishmaniasis from the northern parts of Pakistan since the beginning of this century. The northwestern province of Khyber Pakhtunkhwa has been one of major hotspots of leishmaniasis in Pakistan for last more than one decade. The present study area has been one of the leishmaniasis hotspots in the province. The disease has been endemic in certain parts of the affected areas especially the tribal belt while epidemics or sporadic infections have been reported from areas like in various parts of district Nowshera, Bannu, Lakki Marwat and Karak in KP. Very few studies have been conducted to know the species composition, relative abundance and seasonal variation of sand flies in Pakistan. Some of these studies were conducted on large scale (Lewis, 1967; Munir, 1994; Aslamkhan *et al.*, 1998; Kakarsulemankhel, 2004), while few had been carried out on a local scale in some districts of Pakistan (Kakarsulemankhel, 2003; Ali *et al.*, 2016a, b; Wahid *et al.*, 2020). An epidemic of the diseases had been reported from Manki Shareef and Nizampur areas of district Nowshera. One year sand fly collection from the affected area revealed that the suspected leishmaniasis vectors in Pakistan i.e., *P. papatasi* and *P. sergenti* both were occurring in the study area. The species were also collected from Chitral district. Both these species have been reported by Munir (1994) from hilly areas of northern Pakistan, Azad and Jammu Kashmir and Islamabad, from various parts of KP and Punjab (Aslamkhan *et al.*, 1998), from Balochistan (Kakarsulemankhel, 2003, 2004) and in KP from Dir Upper and Lower (Ali *et al.*, 2016a) and tribal district of North Waziristan (Ali *et al.*, 2016b).

P. sergenti had been incriminated as vector of *L. tropica* in Iran (Oshaghi *et al.*, 2010; Aghaei *et al.*, 2014), Saudi Arabia (Al-Zahrani *et al.*, 1988), Syria (Haddad *et al.*, 2015), Turkey (Limoncu *et al.*, 2020) and Morocco (Mhaidi *et al.*, 2018), while it has been considered as

suspected vector of cutaneous leishmaniasis in Pakistan (Rowland *et al.*, 1999). The present study area of Nowshera faced an epidemic of cutaneous leishmaniasis before this entomological survey and presence of vector species confirms the presence of disease spreading agent in the study area. During the study period both suspected vector i.e., *P. papatasi* and *P. sergenti* were less abundant and was represented by only 3.24% and 3.87%, respectively of the total collection. In contrast to current work, studies conducted in the merged tribal districts of North Waziristan (Ali *et al.*, 2016a) and Bajaur (Wahid *et al.*, 2020) reported high abundance of *P. sergenti* and other *Phlebotomus* species as compared to *Sergentomyia*, but similar observations had been reported from the settled districts of Dir Upper and Lower. High relative abundance of *P. sergenti* had been reported from leishmaniasis endemic areas of Egypt (El-Sawaf *et al.*, 1987) and Morocco (Faraj *et al.*, 2013; Boussaa *et al.*, 2016). In Pakistan presence of high abundance of *P. sergenti* could have been the possible reason that the merged tribal districts are reporting cases of leishmaniasis regularly for last more than one decade, while sporadic cases were reported from the Afghan refugee camp in Dir during late 90's only (Rowland *et al.*, 1999) and a single epidemic in the study area before conduction of current entomological survey.

Our study has reported a unimodal distribution of sand flies in district Nowshera with active season extending from March to November. *S. babu* was collected throughout the active season while *P. papatasi*, *P. sergenti* and *S. baghdadis* were reported from March to October. Seasonal activity of sand flies is influenced by abiotic factors like temperature, rainfall and relative humidity (Maroli *et al.*, 2013). In Pakistan temperature start rising in the month March and favorable temperature generally remains till October and in microhabitats till the start of December. In the hilly areas of Pakistan with cold environmental conditions, shorter active season had been reported for sand flies. In the tribal district of North Waziristan the active season extends from April to September (Ali *et al.*, 2016a), in Dir upper and lower this period extends from May to October (Ali *et al.*, 2016b) and from June to October in the tribal district of Bajaur (Wahid *et al.*, 2020). In Nowshera sand fly population was high during the month of July to September, peaking in the month of August when the temperature is between 35 to 40°C and humidity is high. As these two factors mainly influence the density of sand fly (Gebresilassie *et al.*, 2015), and both these factors remain ideal during the monsoon season from July to September in Pakistan thus explaining the high density and unimodal distribution of sand flies in the study area. Similarly, other studies carried out in Pakistan had reported peak seasonal activity in the months of July

(Ali *et al.*, 2016a; Wahid *et al.*, 2020) and August (Ali *et al.*, 2016b), which coincide with the monsoon season in the country providing ideal conditions for the sand flies to thrive and multiply.

In this study we reported 31 species from two districts of Pakistan i.e., Nowshera and Chitral. Four *Phlebotomus* and 17 *Sergentomyia* species were collected from Nowshera, while 13 *Phlebotomus* and four *Sergentomyia* from Chitral. Majority of these species had already been reported from Pakistan in previous studies (Lewis, 1978; Munir, 1994; Aslamkhan *et al.*, 1998; Ali *et al.*, 2016a, b; Wahid *et al.*, 2020) but six *Phlebotomus* species collected from Chitral and one species of *Sergentomyia* collected from Nowshera are new reports from Pakistan. To date researchers have reported the presence of 37 sand fly species in Pakistan (Zaidi *et al.*, 2017; Wahid *et al.*, 2020), however, literature have shown a different figure. Lewis (1967) reported presence of 10 *Phlebotomus* and 17 *Sergentomyia* species and predicted presence of *P. bergeroti*, *P. eleanorae*, *P. jacusieli*, *P. modfidii*, *P. mongolensis*, *P. newsteadi*, *P. salehi*, *S. sintoni*, and *S. zeylanica* in Pakistan and some of these were then reported in surveys later conducted in various parts of the country. Munir (1994) further added two species i.e., *P. hindustanicus* and *P. salangensis* to this checklist increasing the number of sand fly species to 29 from Pakistan. A large-scale spatial survey further reported three new species from Pakistan, which were *P. eleanorae* and *P. salehi* as predicted by Lewis (1967) and *S. hodgsoni* increasing sand fly species number to 32 (Aslamkhan *et al.*, 1998). A survey in Balochistan reported *P. bergeroti* (Kakarsulemankhel, 2003) and a checklist of sand fly of Balochistan further added six new species raising the number of species to 39 (Kakarsulemankhel, 2004). A study conducted in tribal district of North Waziristan reported *P. caucasicus* and *S. sumbarica* for the first time from Pakistan (Ali *et al.*, 2016a) and *P. ansari* and *P. kazeruni* reported from district Dir were also the first report of these species from Pakistan. To date literature reports 43 species of sand flies comprising of 19 species of *Phlebotomus* and 24 species of *Sergentomyia*. Present study reports six species of *Phlebotomus* from Chitral and one species of *Sergentomyia* from Nowshera for the first time from Pakistan, thus increasing the checklist of sand fly fauna of Pakistan to 50 species.

Sergentomyia is classified into six subgenera (Artemieve, 1978) and except *Parvidens* all occur in Pakistan. *Phlebotomus* is subdivided into 11 subgenera (Lewis, 1982) and eight of them occur in Pakistan, while *Spelaophlebotomus*, *Idiophlebotomus* and *Australophlebotomus* have not been reported from Pakistan, as they are confined to African, East Asian and Australian regions respectively (Lewis, 1982). All four subgenera of

Phlebotomus involved in the transmission of leishmaniasis i.e., *Euphlebotomus*, *Larroussius*, *Paraphlebotomus* and *Phlebotomus* occur in Pakistan, which explains the occurrence of anthroponotic cutaneous leishmaniasis, zoonotic cutaneous leishmaniasis and visceral leishmaniasis in Pakistan (Khan *et al.*, 2021). *Kasaulius* (Lewis, 1982) reported for the first time from Pakistan is represented by a single species *P. newsteadi*. Theodor (1948) provisionally placed this species in subgenus *Euphlebotomus* but Lewis (1978) suggested it to be outside this subgenus and placed it in the newly described subgenus *Kasaulius* (Lewis, 1982). This species had been previously reported from India only. *Adlerius* was previously represented by three species in Pakistan and our study further adds five more species to this subgenus.

Sand flies have cosmopolitan distribution and are distributed worldwide (Andrade and Brazil, 2003). India, the eastern neighbouring country of Pakistan lies in Oriental region, its sand fly fauna consists of 46 species with 11 species belonging to genus *Phlebotomus* and 35 species to genus *Sergentomyia* (Lewis, 1978; Ranganathan and Swaminath, 2015). Both neighbouring countries share 21 species of sand fly. Afghanistan the westerly neighbouring country of Pakistan lies in Palearctic region. 44 species had been reported from Afghanistan, consisting of 22 identified and 2 unidentified species of *Phlebotomus* and 20 species of *Sergentomyia* (Artemieva, 1978). Both countries share 39 sand fly species with *P. caudatus* and *P. zulfagarensis* not been reported from Pakistan, while *P. ansari*, *P. argentipes*, *P. bergeroti*, *P. colabaensis*, *P. eleanorae*, *P. newsteadi*, *S. africana*, *S. bailyi*, *S. fretownensis*, *S. punjabensis* and *S. shortii* not collected from Afghanistan. Literature shows very few studies on the diversity and ecology of sand flies in both these countries and filling these gaps may increase the number of sand fly species occurring in these countries.

Lot of work has been done on the sand fly fauna of Iran and the checklist have 56 species reported from Iran (Kasiri *et al.*, 2000; Zhraei-Ramazani *et al.*, 2013; Kassiri *et al.*, 2011). Both countries share 16 species of *Phlebotomus* and 17 species of *Sergentomyia*. Nine species each of *Phlebotomus* (*P. hindustanicus*, *P. angustus*, *P. rupester*, *P. turanicus*, *P. comatus*, *P. colabaensis*, *P. newsteadi*, *P. argentipes*, *P. nuri*) and 8 species of *Sergentomyia* (*S. babu*, *S. shortii*, *S. montana*, *S. fretownensis*, *S. punjabensis*, *S. fallax afghanica*, *S. murgabiensis*, *S. hospitii*) collected from Pakistan has not been reported from Iran, while 16 species of *Phlebotomus* (*P. blcanicus*, *P. brevis*, *P. halepensis*, *P. mesghali*, *P. nadimi*, *P. ilami*, *P. langeroni*, *P. mascittii*, *P. perfiliewi*, *P. simrnovi*, *P. tobbi*, *P. wenyoni*, *P. jacusieli*, *P. mongolensis*, *P. chinensis*, an unidentified species of subgenus *Paraphlebotomus*) and 7 species of

Sergentomyia (*S. squamipleuris*, *S. iranica*, *S. dolichopus*, *S. antennata*, *S. sintoni*, *S. alderi* and an unidentified species of subgenus *Neophlebotomus*) reported from Iran (Kasiri *et al.*, 2000) have not been observed in Pakistan. All six subgenera of *Phlebotomus* present in Iran are also reported from Pakistan but *Anaphlebotomus* and *Kasaulius* are reported only from Pakistan. Similarly, Iran has all six subgenera of *Sergentomyia* but subgenus *Parvidens* is absent in Pakistan.

In the old world, Iran has reported maximum number of sand flies followed by Pakistan, India and Afghanistan. China has reported 42 (16 *Phlebotomus* and 26 *Sergentomyia*) species (Leng and Zhang, 1993). Kasap *et al.* (2019) gave a checklist of 28 species of sand flies from Turkey, 22 species have been reported from Palestinian territory (Sawalha *et al.*, 2017), 21 from Jordan (Kamhawi *et al.*, 1995), 14 from Egypt (El Sawaf *et al.*, 1987) and 11 species have been reported from Morocco (Boussa *et al.*, 2016). Pakistan shares 2,670 km boundary known as Durand line with Afghanistan and only two species reported from Afghanistan are not present in Pakistan because both these species occur in the west of Afghanistan. Iran also shares boundary with the Balochistan region of Pakistan and 23 species reported from Iran have not been collected from Pakistan. Among these species 5 species i.e., *P. halepensis*, *P. mesghali*, *P. mongolensis*, *S. iranica* and *S. sintoni* has been reported from regions less than 200 kms from Pakistan border. A detailed survey near the Iranian border region of Pakistan may reveal the presence of these species in Pakistan. Similarly, Pakistan also shares a lengthy easterly border with India. Majority of species reported from India have not been collected from Pakistan. Species like *S. eadithae*, *S. siroi*, *S. zeylnica*, *S. kauli* and *S. himalayensis* that occur in the areas near Pak-India border could be expected to occur in Pakistan.

CONCLUSION

In Pakistan few detailed spatial and temporal studies on the diversity of sand flies have been conducted. This study provided a checklist of 50 species of sand flies from Pakistan but detailed surveys of unexplored areas from all over the country may add more species to this list. Further studies will not only enhance the knowledge regarding the diversity of sand flies in Pakistan but will also contribute to the knowledge regarding the vector species that could transmit various form of leishmaniasis in human population.

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IRB approval and ethical statement

This study was reviewed, approved and monitored by the "Ethical Committee" Faculty of Life & Environmental Sciences, University of Peshawar, under applicant certificate number (12/EC/F.LIFE-2020).

Statement of conflict of interest

The authors have declared no conflict of interest.

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