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THE MOSQUITO MICROBIOME: ANALYSIS FOR ENDEMIC AND EMERGING PATHOGENS OF PUBLIC HEALTH SIGNIFICANCE IN DISTRICT FAISALABAD, PAKISTAN

Iqra Yousaf[1], Shabab Nasir[1,*], Syed Makhdoom Hussain[1], Azhar Rafique[1], Muhammad Umar Ijaz[2], Muhammad Asrar[1], Saif-Ur-Rehman[3], Bilal Ahmad[1], Naveed Ahmad Khan[4] and Mahid Iftikhar[1]

[1]Department of Zoology, Government College University, Faisalabad, Pakistan; [2]Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan;[3]Department of Entomology, Northwest A&F University, Yangling, Shaanxi,712100, P.R. China;[4]Institute of Pure and Applied Biology, Zoology division, Burhanuddin Zakariya University, Multan, Pakistan.
***Corresponding author: flourenceshabab@yahoo.com**

Pakistan being a tropical country, characterized by poor socioeconomic conditions, is more vulnerable to Mosquito-borne diseases. So, this study was proposed to know the prevalence of mosquito-borne diseases in Faisalabad district, Pakistan. For this, mosquito adults and larvae were collected from different habitats with aspirator and dippers respectively from eight towns of Faisalabad district. These samples were carried alive to laboratory and identified with the help of identification keys. *Aedes* female mosquitoes were killed and stored at -20°C for RNA extraction. Slides were prepared from blood of female *Anopheles* mosquitoes for the identification of *plasmodium* under microscope. The genome RNA from female *Aedes* mosquitoes were extracted as per commercially available kits according to manufacturer guidelines and stored at -40 ^{0}C for further use. Using online software, primers and probes for the known human pathogens were designed in consensus with the help of available genome database for different pathogens such as dengue virus. *Plasmodium* was detected from the mosquitoes that were mostly collected from rural areas and this was generally detected from the samples collected from Jaranwala and Samundri town. Dengue virus was detected generally from city areas and serotype 2 and 3 was detected from the *Aedes* mosquitoes. Dengue virus was mostly detected from Lyallpur, Madina and Jinnah towns. Mosquitoes were mostly collected after monsoon season in the months of August-September and in spring (March). The disease pathogens were also detected during these months. Through the surveillance of pathogens, we can focus our attention on the hot spots and apply control measures to control mosquitoes for better management.

Keywords: Faisalabad; Malaria; Mosquitoes; Rural areas; serotypes of dengue fever.

INTRODUCTION

Mosquitoes are vectors of many diseases like dengue, malaria, West Nile virus and zika virus etc (Sritabutra *et al*., 2011). Mosquitoes belong to the family Culicidae and order Diptera. There are more than 100 genera of mosquitoes, but three genera are medically important, i.e., *Anopheles, Culex* and *Aedes* (Samidurai *et al*., 2009).

Anopheles is responsible for spreading of malaria. Malaria is at the top of mosquito borne diseases. According to World Health Organization, about half of the world's population (3.2 billion people) was at risk of malaria during 2015 (WHO, 2016). Normally death rate is more in infants and women, with 3.5 million presumed and confirmed malaria cases annually (Anonymous, 2016). About half of the Pakistani population (more than 140 million) is at risk for malaria with 18 % living in high risk areas (WHO, 2006). Being an agricultural country, Pakistan has a vast network of water channels, rivers and dams for power generation that are good breeding sources for mosquitoes. Out of 23 species of genus *Anopheles*, *Anopheles culicifacies* (Pervez and Shah, 1989) in rural areas and *Anopheles stephensi* in urban areas are considered as main malarial vectors.

Aedes is responsible for spreading of dengue fever, chikungunya, yellow fever and zika virus (Service, 2004). Dengue fever is mostly prevalent in tropical and subtropical regions of the world (Pakistan, India, Bangladesh, Thiland and Brazil etc). According to WHO, more than 50,000 people die with this disease every year. Due to this disease, more than 22,000 casualties were reported per annum mostly in young ones (below 20 years) (Riaz, 2011). Dengue was first reported during 1994 in Pakistan, but huge epidemics occurred in Punjab province during 2010. After that it prevailed all over the country. Dengue is a viral disease that is caused by five different types of flaviviruses, i.e DEN 1, DEN 2, DEN 3, DEN 4 and DEN 5.

Culex is the vector of West Nile virus that is a viral disease. This disease is present in USA and not reported from Pakistan. These diseases are mostly viral so these have neither treatment nor vaccination (Tjahjani, 2008). So, the only solution is to aware the people about these diseases by studying the prevalence in their areas or by management of mosquitoes. Mosquito management is mainly dependent on synthetic insecticides that are more harmful (Gul *et al*., 2019) than their benefits. These insecticides cause not only resistance in insect pests but also cause environmental pollution and health hazards in non target organisms including human beings (Batool *et al*., 2019). Therefore, the study of prevalence of mosquito-borne diseases for mosquito management is best method.

Pakistani Punjab has a vast seasonal and ecological diversity. Faisalabad is an industrial area. It is located in the most populous province, Punjab between 30° 35 minutes to 31° 47 minutes North latitude and 72° 50 minutes to 73° 50 minutes East longitude. This city is more than 100 years old and was named as Lyallpur in 1904 in the honour of Lieutenant-Governor of Punjab, Sir James Broadwood Lyall, for his services during the British rule. Before this, this city was a tehsil of Jhang district. During 70's the present name (Faisalabad) was given after the name of the Late King Shah Faisal of Saudi Arabia. This district consists of 8 towns with an area of 5856 Km^2 and population 6.5 million individuals. Its climate is extreme during both summer and winter; summer temperature may reach up to 50ºC and during winter it lowers below freezing point. Due to industrialization different types of habitats are found here which are responsible for the multiplication of different types of mosquitoes. Due to increase in urbanization and industrialization resulting from fast growing population (Naeem-Ullah & Akram, 2009) along with change in climatic conditions has resulted in recurring epidemics of mosquito borne diseases like dengue fever and malaria. In case of province Punjab, only limited research was done for this purpose (Reisen *et al*., 1981) that needs to be revised as they do not provide in depth studies (Herrel *et al*., 2001). Present studies were therefore carried out to cover the entire canvas about the prevalence of mosquito borne diseases of the Faisalabad, Punjab, Pakistan from different habitats during the winter, summer and rainy seasons.

MATERIALS AND METHODS

***Collection of Mosquitoes*:** Mosquitoes (larvae with standard dipper & adult with aspirator) were collected from different habitats of Faisalabad district. Habitat types like standing waste water around houses, catch basins (tap catch basin, rain catch basin), seepage pools, roadside drains, water channels, rice fields (Suleman *et al*., 1993), park ponds, rock pools (Suleman and Khan, 1993), irrigated fields, wetlands, fish farm (Herrel et al., 2001), tires, tree holes (Akram and Lee, 2004) etc. were explored while capturing the mosquitoes.

Sampling sites: Samples were collected from different towns (Lyallpur, Madina, Jinnah, Iqbal, Chak Jhumra, Jaranwala, Samundri and Tandlianwala Town) of Faisalabad (Fig. 1) from July, 2016 to May, 2017 and at least ten samples were collected from each town.

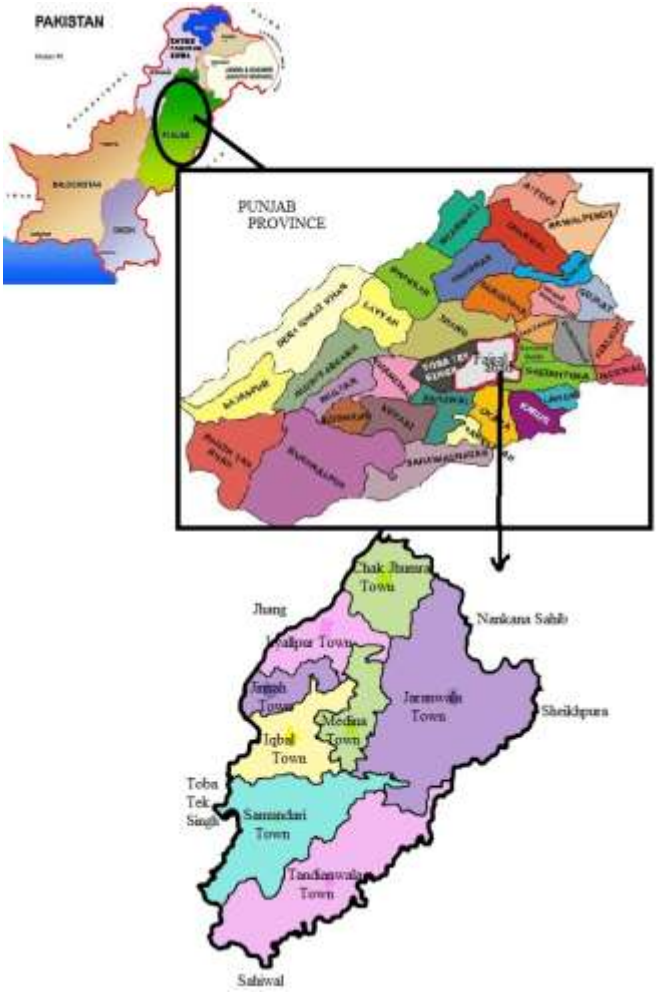

Figure 1. Town wise map of Faisalabad District

***Preservation of Samples*:** The adults (in glass tubes with screen on the top) and immature mosquitoes (in plastic bottles with muslin cloth) were shifted in the lab on the same day of collection for identification with the help of keys (Qasim *et al*., 2014) under microscope. Identified mosquitoes were separated with respect to species and town and then samples were stored in pools of 5 mosquitoes per pool in eppendrof tubes at -20ºC for extraction of RNA (*Aedes* mosquitoes). While adult female *Anopheles* mosquitoes were crushed on the glass slides after killing.

***Plasmodium detection*:** After identification, the female mosquitoes of *Anopheles* were crushed on the slide and blood

Table 1. Designed primer for identification of different types of dengue viruses.

Primer	Sequence	Genome position	Product size
D1	5'-TCA ATA TGC TGA AAC GCG CGA GAA ACCG-3'	134-161	511
D2	5'-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3'	616-644	511
TS1	5'-CGT CTC AGT GAT CCG GGGG-3'	568-586	482(D1 & TS1)
TS2	5'-CGC CAC AAG GGC CAT GAA CAG-3'	232-252	119 (D1 &TS2)
TS3	5'-TAA CAT CAT CAT GAG ACA GAGC-3'	400-421	290(D1 & TS3)
TS4	5'-CTC TGT TGT CTT AAA CAA GAGA-3'	506-527	392(D1 & TS4)

smear was formed at the spot or after some time in the lab for the samples collected from nearby places. Slides were dried, preserved and identified for the presence of *Plasmodium* under stereo microscope.

***RNA extraction*:** The mosquito pools were selected randomly and dengue virus was detected by RT-PCR technique. A total of 110 pools of both species (*Ae. aegypti* and *Ae. albopictus*) were separated and analysed. These pools were homogenized in a sterile homogenizer and RNA was extracted from pooled samples of *Ae. aegypti* and *Ae. albopictus* by using a commercial pure link mini kit (Invitrogen) according to instructions given and then specific type of dengue virus was detected by using the primers given in Table 1 (Lanciotti *et al*., 1995).

For every RT-PCR run, a negative control (without dengue virus) was also used. Finally, 15ul of PCR products were analysed by performing electrophoresis in a 2% agarose gel stained with ethidium bromide and electrophoresed at 80 V for one hour and gel was observed under ultraviolet illuminator and different amplified bands were observed and and captured with camera.

***Data analysis*:** Data were analysed through simple calculations of mean, percentage and graphical presentation by using MS Exel 97-2003. Chi-square test was also applied to calculate the χ^2value and p-values.

RESULTS

The results showed that population of both *Aedes* and *Anopheles* genera was the highest in the month of August and no mosquito larvae or adult was collected during January while all other months showed intermediate results regarding collection (Fig. 5). This figure showed that mosquito population increased from July to September and then population started decreasing and again increased in month of March. *Aedes* larvae were recorded more than adults as shown in the Fig. 5.

Table 2 showed detection of outbreak of dengue virus serotypes in different areas of Faisalabad city. In case of *Ae. aegypti*, 60 pools were tested and 15 (25%) showed positive results, with 10(66.6 %) as dengue type 2 alone and 5 samples as dengue serotype 3 while no pool was found positive for dengue serotype 1 & 4. In case of *Ae. albopictus*, out of 50pools, 8(16%) showed the presence of virus with 5 pools (62.50 %) as type 2, and only 3 pool (37.5%) was found positive for serotype 3 while no pool was found positive for dengue serotype 1 & 4 as shown in Table 2.

For agarose gel, nine samples of both species (*Ae. aegypti*, *Ae. albopictus*) were taken for analysis while NC represents the negative control sample. A ladder of 1kb was loaded in gel. Three samples were found negative for dengue virus while all others were found positive for dengue virus as shown in the Fig. 2. Sample number 2, 4, 5, 7 and 9 were found positive for dengue virus Type 3 while sample number 1 was found dengue virus Type 2 which was detected from larvae of *Aedes* mosquitoes (Fig. 2).

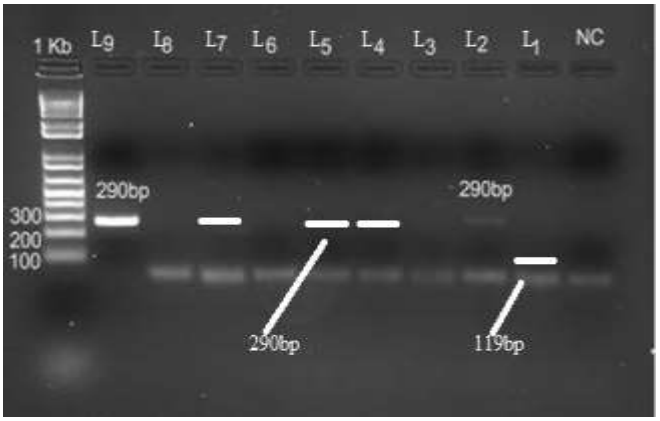

Figure 2. Amplification profile of *Aedes aegypti* and *Aedesalbopictus* populations from Faisalabad with primer D1 and D2. *(NC = Negative control, L1 = Sir Syed Town from Lyallpur town, L2 = Lakkar Mandi from Jinnah town, L3 = Millat town from Lyallpur town,*

Table 2. Detection of positive pools of mosquitoes and specific type of dengue virus through RT-PCR.

Species	Poolsassayed	Total no. of Mosquitoes	Positive pools	DEN1 (%)	DEN2 (%)	DEN3 (%)	DEN4 (%)
Ae. aegypti	60	300	15 (25%)	0	10 (66.6%)	5 (33.3%)	0
Ae. albopictus	50	250	8 (16%)	0	5 (62.5%)	3 (37.5%)	0

Table 3. Mosquito (adults & larvae) presence and detection of pathogens from different Towns of Faisalabad district.

Towns	Mosquito presence				Dengue Virus Presence					*Plasmodium*
	***Aedes*(larva)**	***Aedes*(adult)**	***Anopheles***	**Adult**	**Larva**	**DEN1**	**DEN2**	**DEN3**	**DEN4**	**spp.**
Lyallpur	√√√	√√√	√	√√	√	x	√√	√	x	√
Madina	√√	√√	√	√	X	x	x	√	x	√
Jinnah	√√√	√	√√	√	X	x	√	x	x	√√
Iqbal	√√	√√	√	√√	X	x	√	√	x	√√
Chak Jhumra	√√	√	√√	√	x	x	√	x	x	√√
Jaranwala	√	X	√√√	x	X	x	x	x	x	√√√
Samundari	√	X	√√√	x	X	x	x	x	x	√√√
Tandianwala	√	√	√√	√	X	x	√	x	x	√√
χ^2 value	0.01	1.23	0.02	1.03	1.34	3.93	1.03	0.92	3.23	1.01
p-value	0.04	0.02	0.04	0.03	0.07	1.27	0.03	0.02	1.79	0.04

√= 1-25%, √√= 26-50%, √√√= 51-75%, √√√√= 76-100%

L4 = Lari udda from Medina Town, L5 = Peoples colony from Iqbal Town, L6 = Naimat Colony from Medina town, L7 = Samanabad from Iqbal town, L8 = Nazim abad from Jinnah town, L9 = Chak Jhumra from Chak Jhumra town)

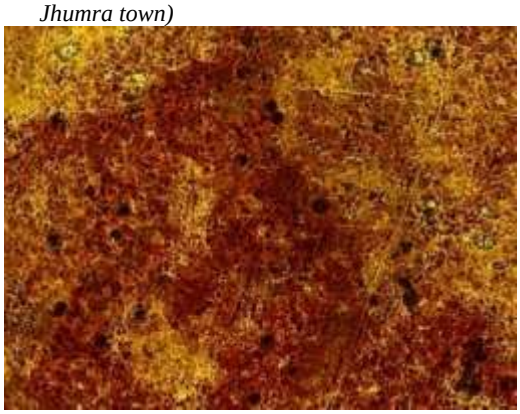

Figure 3.*Anopheles* mosquito samples collected from locality Jaranwala Town showing the presence of *Plasmodium* spp.

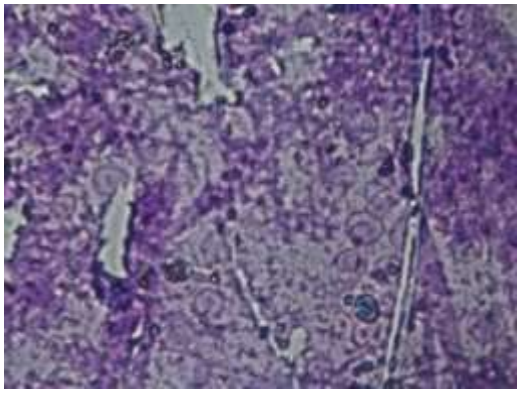

Figure 4.*Anopheles* mosquito samples collected from Samundari *Town* showing the presence of *Plasmodium* spp.

Table 3 showed that Lyallpur town was the most endemic site with respect to presence of *Aedes* mosquitoes (larvae and adults) and Tandianwala town had least number of mosquitoes (*Anopheles* and *Aedes*) while all other sites were statistically different ($p<0.05$) having about 50-75% samples positive for the presence of mosquitoes either larvae or adult of both genera. No adult of *Aedes* species was reported from Jaranwala and Samundari towns.

In case of presence of pathogens, the results showed the presence of vertical transmission of DEN virus from Lyallpur town where both adults and larvae of *Aedes* mosquitoes were found positive for the presence of DEN virus. Mostly towns were found positive with respect to DEN 2 virus followed by DEN 3 and DEN 1 and DEN 4 was not reported from any town. No pool was found positive with respect to dengue virus from Jaranwala and Sumandri towns as shown in Table 3. According to this graph, the rate of infection of dengue virus started during the end of July and was highest in the month of August 2016 followed by the month of September and October due to monsoon season. No disease was recorded in the month of January and February (Fig. 6).

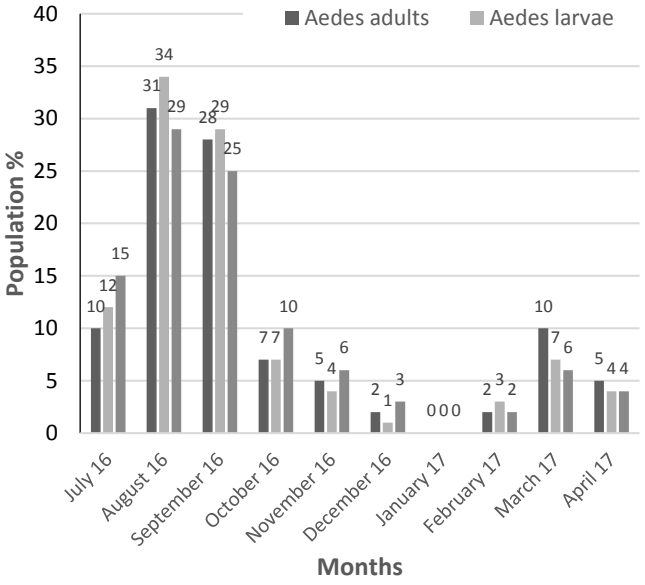

Figure 5. Mosquito collection data from July 16 to April 17.

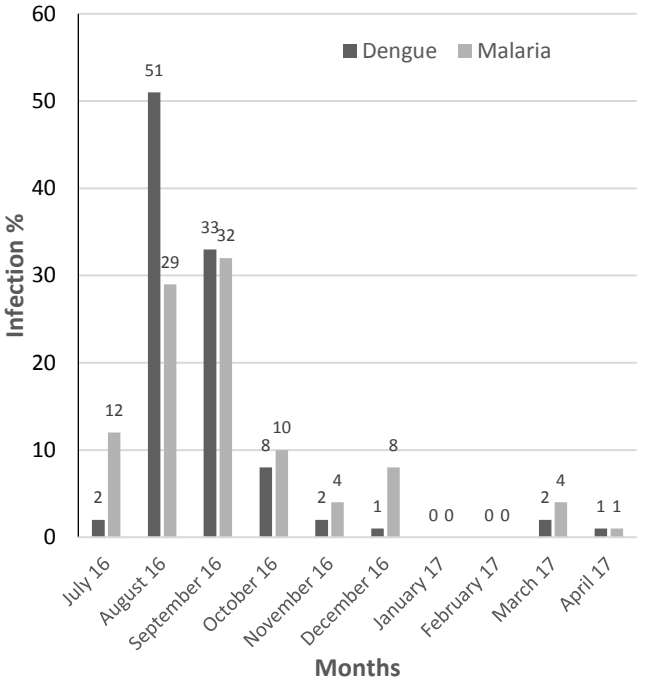

Figure 6. Incidence of diseases during different months of 2016-17.

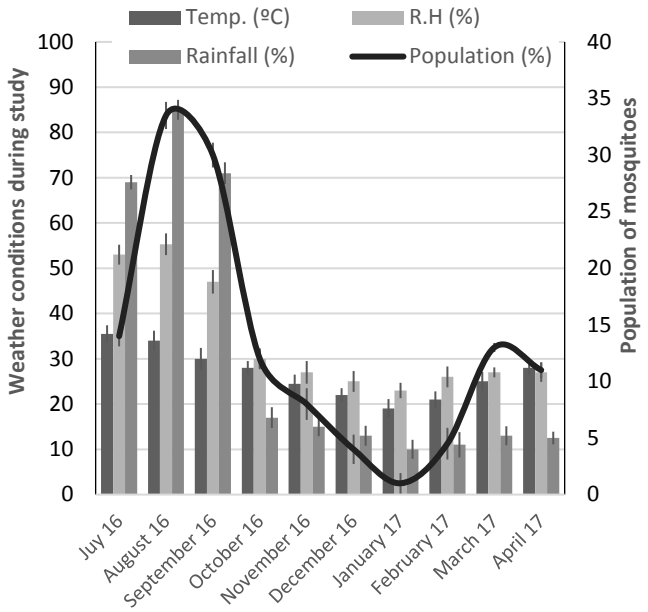

Figure 7. Comparison of meteorological data with mosquito population (%) in district Faisalabad.

All the towns were found positive for the presence of malarial pathogen (*Plasmodium*) as shown in the Fig. 3 and 4. Malarial pathogen was mostly recorded from rural areas than urban.

The comparison of metereological data with mosquito population during different months of the years 2016-17 in different towns of district Faisalabad was shown in Fig. 7. These results showed the above mentioned pattern of mosquito abundance with the highest abundance during rainy season (July-September) followed by decline in the late monsoon season and lower during the winter months (December-January). Data showed that there was again a rise in mosquito population with the start of spring season (February-March) due to changes in mean temperature and average rainfall.

DISCUSSION

During present study, different species of mosquitoes were recorded from rural and urban areas of Faisalabad district. Most of the mosquitoes (larvae and adults) were recorded during August and September and these results are in line with the findings of Ndiaye *et al*. (2006) who also recorded most of the *Aedes* mosquitoes during rainy season. Findings of the present study are also similar with Oneeb *et al*. (2016) who also noted that the month of July yielded maximum population whereas least population was recorded in December due to cool dry weather. Dry period and increase of rainfall consistently influence the mosquito population to increase and further stabilize. However, the decline in mosquito population recorded as the decrease in night

temperature was noticed (Oneeb *et al*., 2016). These results are in agreement with previous surveillance studies conducted in Murree Hills, Pakistan (Qasim *et al*., 2014). They recorded species composition and then analyzed these results with temperature, relative humidity and rainfall to get influential factors involved for the population change of different mosquito species.

Findings of the present work are in line with Akram *et al*. (2009) who reported that abundance of mosquitoes was recorded during March to September in Pakistan whereas least in other months.

During the present study, it was noted that *Ae. aegypti* were collected mostly from residential areas while *Ae. albopictus* from rural areas (fields). These finding were at par with the findings of El-Badry and Al-Ali (2010) and Mukhtar *et al*. (2011). *Anopheles* mosquitoes were mostly observed in rural areas or highly populated areas where there was a problem of water drainage. These results are in agreed with the results of Mukhtar *et al*. (2011). During this study, *Aedes* mosquitoes were analysed through RT-PCR for the presence of dengue virus and was found that both species (*Ae. aegypti* and *Ae. albopictus*) were involved in the transmission of dengue virus. These results are in line with the results of Mukhtar *et al*. (2011) and Khan *et al*. (2016). Moreover, this research showed that *Ae. aegypti* mosquitoes (25%) were involved more than *Ae. albopictus* (16%) in the transmission of dengue virus. These results are in agreement with the results of Mukhtar *et al*. (2011) and Khan *et al*. (2016). Zubaie *et al*. (2016) also found that *Aedes aegypti* is a main vector for the transmission and spread of dengue disease mostly in urban areas of Pakistan. These results were in line with our study. These results are different from the results of Ali *et al*. (2016) who found only DENV-2 from his samples from Lahore city and he also found that *Ae. albopictus* were negative from dengue virus.

Analysis of *Aedes* mosquitoes through RT-PCR revealed that there is an important role of temperature and humidity in high incidence of dengue virus. Results of this study also showed that dengue virus was mostly observed during July to September due to more mosquito population in these months. These results are in agreement with the results of many other researchers (Scott *et al*., 2000; Wu *et al*., 2009; Chen *et al*., 2012; Khan *et al*., 2016).

Results also showed that most of the infected *Anopheles* mosquitoes were found during month of July - August and March. These results are in line with the findings of Oneeb *et al*. (2016) who also noted that the month of July yielded maximum population whereas least population in December.

Conclusion: From this study, it was concluded that mosquitoes and mosquito borne diseases (malaria and dengue) were more prevalent in March-April and August-October than other months of the year. Malaria was mostly recorded from rural areas or from city areas having poor cleanliness conditions.

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