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RESEARCH ARTICLE

Metagenomics of Mosquito-borne Flaviviruses in Various Geoclimatic Districts of Punjab, Pakistan

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ABSTRACT

Mosquitoes are highly active vectors capable of transmitting various pathogenic and infectious diseases to humans and animals. The present study was designed to identify the major species of mosquitoes and mosquito-borne flaviviruses (Saint Louis Encephalitis, West Nile and Dengue Viruses) prevalent in three districts of Punjab representing the three agro-geoclimatic zones *viz;* Multan, Chakwal and Jhang. The collected mosquitoes were stereoscopically identified to confirm the species and sex of the mosquitoes. Stereoscopic identification confirmed that female mosquitoes were 4334 out of 10675 (40.6%), 2242 out of 7296 (30%) and 2040 out of 6450 (31.6%) from districts Chakwal, Jhang and Multan, respectively. It was concluded that *Culex* species were present in abundance (73.2%) as compared to *Aedes* species (26.7%) in the selected study districts. The results of multiplex RT-PCR depicted that Dengue, West Nile, Japanese encephalitis and Saint Louis encephalitis viruses were prevalent in the mosquitoes of all three selected districts. However, the prevalence of mosquito-borne viruses insignificantly varied (P>0.05) among the three districts. This study has provided a better understanding of the prevailing mosquito species and mosquito-borne viruses in the study districts that can help to devise appropriate control measures.

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INTRODUCTION

Mosquitoes are the hematophagous vectors capable of transmitting pathogens to its vertebrate host through a blood meal. They act as vectors for the transmission of pathogens including viral pathogens causing life-threatening disease such as Dengue (DENV), Yellow fever (YFV), Japanese Encephalitis (JEV), Eastern Equine Encephalitis (EEEV), Western Equine Encephalitis (WEEV), Chikungunya (CHIKV), Zika (ZIKV) etc. Among these, DENV, ZIKV, YFV, and CIKV are transmitted by *Aedes* species while others are contracted by both *Aedes* species and *Culex* species. Mosquito-borne diseases are of primary global health importance as their epidemics are increasing progressively (Franklinos *et al.,* 2019). Approximately 4000 species of mosquitoes have been identified; however, less than 10% are considered as major vectors for the transmission of deadly diseases. About 700 million people are infected with mosquito-borne diseases each year worldwide (Cetin *et al.,* 2010). Mosquito-borne diseases are abundant in tropical and subtropical regions having an adverse impact on economics and commercial yields (Hesson *et al.,* 2019).

Metagenomic analysis of the mosquito-borne viruses can unlock significant aspects of pathogens in animals and humans (Susanta *et al.,* 2014). The genomic study has

also revealed pathogenic evolution and its influence on the mosquito community (Tran *et al.,* 2013). It has facilitated understanding the vector competence of mosquitoes to the transmission of the viral pathogens. The diversity is retained in all the microbial populations, but the relative incidence of the metabolic variation and the difference between metagenomes has anticipated the biogeochemical environment of every surrounding (Gaunt *et al.,* 2005). More than 6.5 million suspected individuals were screened to investigate the presence of malaria during 2018. However, an aggregate of 374,513 confirmed test reports was obtained throughout Pakistan. The predisposing factors for these infections include socioeconomic status, lack of resources, resistance development against insecticides and anti-malarial drugs (Anonymous, 2019).

During the last few years, dengue fever has been reported repetitively in the major cities of Punjab (Lahore, Sialkot, Rawalpindi and Faisalabad) and Sindh (Karachi), Pakistan. Other mosquito-borne diseases like human and animal filariasis (also called elephantiasis), tropical pulmonary eosinophilia and WNV induced disease have also been reported in the human population of Pakistan (Abbas *et al.,* 2014). The favorable humid environment, old-dumped tires, and the presence of natural ponds in and around the cities are among the major factors for the propagation of the vector (Bibi *et al.,* 2015).

In the past, reliable procedures for the diagnosis of mosquito-borne viruses included clinical examination and serological testing. However, these methods are not useful for the detection of specific viral infections due to the presence of similar symptoms caused by different viruses (Papin *et al.,* 2010). In virologic surveillance, detection of the viral genome is an efficient method for disease investigation. The use of multiplex RT-PCR is one of the best molecular methods for the detection of several viral pathogens in a single test (Maher *et al.,* 2008). Hence, the proposed investigation was planned (a) to determine the prevalence of different mosquito species in the selected study districts representing the three agro-geoclimatic zones of Punjab, Pakistan through conventional, and (b) to identify distinct species of mosquito-borne viruses of the family *Flaviviridae* through multiplex RT-PCR.

MATERIALS AND METHODS

Study area: This research work was performed in the Molecular Parasitology Laboratory (MPL), Department of Parasitology, University of Agriculture, Faisalabad (UAF), Pakistan from May 2016 to May 2017. In this research study, three districts of Punjab *viz;* Chakwal (32.9328º N, 72.8630º E), Jhang (31.2781º N, 72.3317º E) and Multan (30.1575º N, 71.5249º E) representative of the three different agro-geoclimatic zones (North, Central and South, respectively) of Punjab province were selected for the sampling of mosquito specimens. In terms of climatic conditions, Chakwal, Jhang and Multan are attributed as hot, hotter and hottest regions, respectively. The minimum and maximum temperatures of the above-mentioned zones range from 4.9-38.9°C, 4.7-39.7°C and 6.3-41°C, respectively (Anonymous, 2017).

Sample collection, preservation & transportation: Adult mosquitoes were collected through convenience sampling method from different localities of the selected study districts. The collection was done by using an EISCO aluminum insect collecting net of 3 cm diameter along with battery-operated aspirator and light baited traps (Florencio *et al.,* 2014). Mosquitoes were collected from domestic areas including livestock farms, roadside ditches, lavatories, and stagnant water pools as shown in Table 1. A total of 153 mosquito pools from Chakwal, 74 from Jhang and 69 from Multan were processed for the detection of *Flavivirus genus.* The collected mosquitoes were submerged spontaneously in RNA *later™* RNA stabilizing reagent (Qiagen) for the immediate preservation of gene expression pattern in tissues.

The ecological information was collected on a predesigned proforma tested through information and formal testing procedures (Thursfield *et al.,* 2018). The data of humidity, rainfall, and temperature of the selected study districts were procured from Regional Pakistan Meteorological Department, Lahore (PMD) (Anonymous, 2017). Samples were transferred to the MPL, UAF and stored at 4ºC until processed.

Taxonomy & sexing of mosquitoes: Prior to RNA extraction, sexing and identification of the mosquitoes were done at species level using a stereoscopic microscope through observation of the morphological characteristics using the standard keys (Becker *et al.,* 2010). Female mosquitoes were pooled randomly into a 1.5 ml centrifuge tube (Eppendorf UK®) according to date, location, and species, contributing 30 mg per pool (Boom *et al.,* 1990).

RNA extraction & cDNA synthesis: The preserved mosquito specimens were processed for RNA extraction according to the standardized protocol of GeneJET RNA Purification Kit (Thermo Scientific, USA). The RNA yield was determined with Thermoscientific ® NanoDrop spectrophotometer 2000 Nanodrop 2000/200c software to ensure the extraction of RNA before cDNA synthesis. These samples were stored at -80°C until further use. During cDNA synthesis, extracted RNA was converted into complementary DNA using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit following manufacturer's instructions. The samples were stored at 2-6°C for short-term storage (Ayers *et al.,* 2006).

Reverse transcriptase PCR & gel electrophoresis: Following sense and anti-sense primers FLAVI-1 (5-AATGTACGCTGATGACACAGCTGGCTGGGACAC-3) and FLAVI-2 (5- TCCAGACCTTCAGCATGTCTTC TGTTGTCATCCA-3) were used for amplification of NS5 coding regions conserved across several species of flaviviruses including YFV, Saint Louis encephalitis virus (SLEV), DENV and JEV (Ayers *et al.,* 2006). Amplification of synthesized cDNA was carried out preparing a 25 µl reaction containing 12.5 µl of Red Dye PCR (2x) Master Mix (GeNeiTM composing of 400 lM of each DNTP, 0.6 units of Taq DNA Polymerase, Tris buffer pH 8.5 and 3 mM of $MgCl_2$), 1 µl FLAVI-1 (sense primer) and FLAVI-2 (antisense primer), 3 µl of cDNA and 7.5 µl nuclease-free water. The PCR thermal cycling was done on Bio-Rad c1000 thermocycler, with an initial incubation at 50ºC for 30 min, followed by incubation at

95°C for 15 min, and 45 cycles consisting of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and elongation at 72°C for 1 min 30 s. Negative controls were set up essentially as described. RT-PCR with these primers resulted in amplicons of different sizes, depending on the virus species. The amplified products were visualized using 1.8% agarose gel. The PCR products for Saint Louis encephalitis virus at 100 bp, WNV at 120 bp, JEV at 143 bp, DENV-1 at 750 bp and DENV-2 at 430 bp were considered positive (Ayers *et al.*, 2006).

Statistical analysis: Prevalence of mosquito-borne viruses, occurring in at least 3% of the total number of samples was included in the analysis. The association of species with ecological parameters were investigated using multiple logistic regression analysis (SPSS 20 for windows). Total processed samples of all the three selected districts have been subjected to statistical analysis. While, P-value indicates the significant ($P<0.05$) and non-significant ($P>0.05$) difference in the prevalence of mosquito-borne viruses among the selected districts and its correlation matrix with the environmental parameters (Thursfield *et al.*, 2018).

RESULTS

Environmental factors significantly impact the mosquito abundance in studied districts of Punjab: The present study has shown that out of a total of 24,421 mosquitoes, the most abundant mosquito species are *Culex* (18,311, 75%) followed by *Aedes* (6110, 25%) in the selected study districts. The relationship of mosquito abundance and climatic conditions vary with changing parameters such as rainfall, temperature and relative humidity. It was observed that highly significant ($P<0.01$) as well as significant ($P<0.05$) correlations were found among mosquito abundance and relative humidity (RH), temperature and rainfall as shown in Fig. 1.

PCR based detection of mosquito-borne flaviviruses in pooled samples: The positive mosquito pools for flaviviruses were determined by analyzing the specific band by PCR in accordance with the expected size of each virus as shown in Fig. 2 & 3. Overall, 296 mosquito pools were processed to investigate the presence of mosquito-borne flaviviruses among the selected districts. A total of 142 (48%) pools were positive for flaviviruses in all the study districts. The prevalence of positive pools was highest in Multan (58%) than Jhang (49%) and Chakwal (43%). However, these results differ non-significantly. The overall prevalence, Odd's Ratio (OR), and Relative Risk (RR) of mosquito-borne flaviviruses among the selected districts *viz*; Chakwal, Multan and Jhang have been shown in Table 2. It was observed that districts Multan and Jhang are at higher risk of mosquito-borne flavivirus infections due to higher OR and RR as compared to district Chakwal.

Prevalence of mosquito-borne flaviviruses in different geo-climatic areas of Punjab: Among the 142 positive pools, a total of 66 pools indicated the presence of mosquito-borne flaviviruses in the respective tehsils of district Chakwal. Saint Louis encephalitis virus was found to be the most abundant (14%) mosquito-borne flaviviruses in Chakwal followed in order by WNV (13%), DENV (9%) and JEV (8%). Similarly, in the district Jhang, 36 positive pools indicated the presence of mosquito-borne flaviviruses with the highest prevalence of Saint Louis encephalitis virus (19%), followed in order by WNV (18%), JEV (8%) and DENV (4%). Moreover, 36 positive pools showed the presence of mosquito-borne flaviviruses in district Multan. WNV was found to be in abundance (28%) followed in order by Saint Louis encephalitis virus (12%), DENV (13%) and JEV (6%).

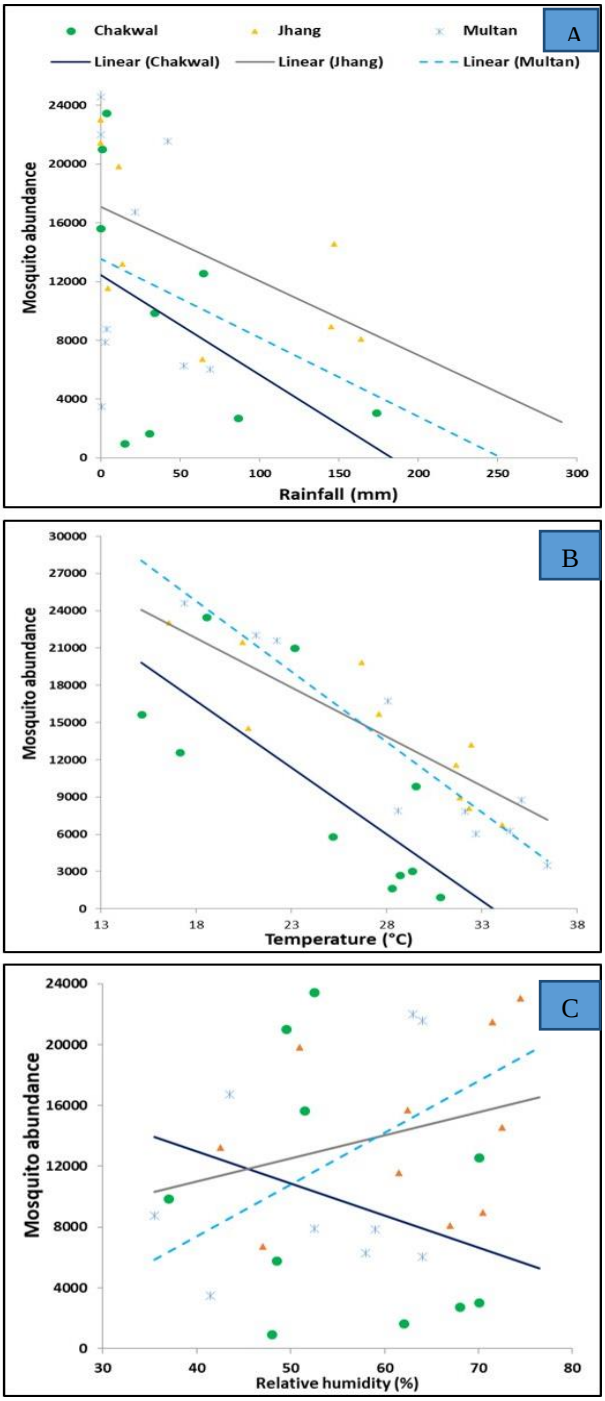

Fig 1: Correlation matrix for mosquito abundance and environmental factors for all selected districts *viz;* Chakwal, Jhang and Multan.
(a) shows correlation between temperature and mosquito abundance.
(b) shows correlation between rainfall and mosquito distribution.
(c) shows correlation between relative humidity and mosquito abundance.

Table 1: Mosquito collection from domestic areas of selected districts

District	Tehsils	Domestic Collection Sites
Chakwal	Choa Saidan Shah, Chakwal, Lawa, Kallar Kahar, Talagang	Choa Saidan Shah, Uthwal, Dhulla, Nurpur, Dhurnal, Kot Sarang, Munrah, Dandashah Bilawal
Jhang	Ahmadpur Sial, Jhang, Shorkot	Samanduana, Sharifabad, Mandi Shah, Bahu lak Bahadur, Bhu Jalal pur, Bhu Khumana Wala
Multan	Shujabad, Multan Saddar, Jalalpur Pirwala, Multan	Aman wala, Sikandarabad, Hamid pur, Billi wala, Manik wala, Jahangir wala, Tehsil Headquarter Hospital

Table 2: Overall Prevalence of Mosquito-borne flaviviruses in the selected districts

District	Total Screened	Positive samples	Prevalence (%)	OR	RR	P-value
Chakwal	153	66	43	*NA*	*NA*	*Ref*
Jhang	74	36	49	1.13	1.29	0.133
Multan	69	40	58	1.35	1.57	0.485
Total	296	142	48.0	-	-	-

OR = Odd's ratio; RR = Relative risk; *NA* = Not Applicable; *Ref* = Reference category.

Table 3: Overall distribution of Mosquito-borne flaviviruses among the selected districts

Sampling districts	Tehsils	Viruses	Pools processed	Positive	Prevalence	OR	RR	P-value
Chakwal	Choa Saidan Shah, Chakwal, Lawa, Kallar Kahar, Talagang	WNV	153	20	13	1.76	1.85	0.167
		SLEV		21	14	1.86	1.85	0.122
		DENV		13	9	1.09	1.14	0.919
		JEV		12	8	*NA*	*NA*	*Ref*
Jhang	Ahmadpur Sial, Jhang, Shorkot	WNV	74	13	18	5.04	4.25	0.013*
		SLEV		14	19	5.52	4.5	0.015*
		DENV		3	4	*NA*	*NA*	*Ref*
		JEV		6	8	2.08	2	0.118
Multan	Shujabad, Multan Saddar, Jalalpur Pirwala, Multan	WNV	69	19	28	6.17	5.4	0.005*
		SLEV		8	12	2.13	2.2	0.303
		DENV		9	13	2.43	2.6	0.199
		JEV		4	6	*NA*	*NA*	*Ref*
Overall	Districts of Chakwal, Jhang and Multan	WNV	296	52	18	2.84	2.4	0.098
		SLEV		43	15	2.27	2	0.145
		DENV		25	9	1.23	1.1	0.372
		JEV		22	7	*NA*	*NA*	*Ref*
	Grand Total		296	142	----	---		----

OR = Odd's ratio; RR = Relative risk; * = Statistically significant difference; *NA* = Not Applicable; *Ref* = Reference category; WNV = West Nile Virus; SLEV = Saint Louis Encephalitis Virus; DENV = Dengue Virus; JEV = Japanese Encephalitis Virus.

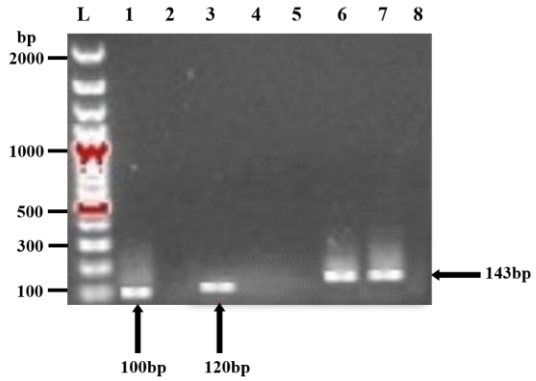

Fig 2: RT-PCR amplicons electrophoresed on 1.8% agarose gel. (L) 100-bp ladder, Lane (1) SLEV 100-bp, Lane (2, 4 & 5) Test Negative, Lane (3) WNV 120-bp, Lane (6 & 7) JEV 143-bp and Lane (8) Negative control.

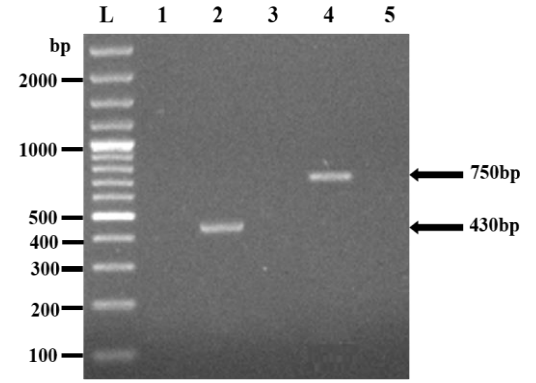

Fig 3: RT-PCR amplicons electrophoresed on 1.8% agarose gel. (L) 100-bp ladder. (1 & 3) Test Negative. Lane (2) DENV-2 430-bp. Lane (4) DENV-1 750-bp and Lane (5) Negative control.

Therefore, out of total 296 pool samples processed, 142 were found test positive showing an overall prevalence of WNV (18%), Saint Louis encephalitis virus (15%), DENV (9%) and JEV (7%) as shown in Table 3. These data describe that mosquito-borne flaviviruses are circulating in different geo-climatic areas of Punjab, Pakistan with variable frequency.

DISCUSSION

The emergence and re-emergence of mosquito-borne pathogenic viruses have been intensified with the passage of time and changing geo-climatic conditions resulting in the global burden of mosquito-borne diseases (Cetin *et al.*, 2010).

The incidence rate of dengue fever has been increased 30 folds over the past 30 years with an estimated 390 million dengue virus infections per year globally (Ebi and Nealon, 2016). After the first case of Dengue virus in Punjab in 1982, hemagglutination assay was used to detect flaviviruses including, West Nile virus, DENV-2 and Japanese encephalitis virus mostly in the residents of Karachi in 1985 (Bhatt *et al.*, 2013). However, the first epidemic of Dengue virus infection was observed in 1994 exhibiting DEN-1 and DEN- 2 serotypes; and the co-circulation of a various number of dengue serotypes ultimately results in the incidence of co-infection (Dhanoa *et al.*, 2016). Additionally, humans and mosquitoes retaining the co-infection provide a niche for viral genome reassortment/combination, resulting in novel strain formation which could increase the disease severity. Such a mechanism has been considered in the development of

Dengue Hemorrhagic Fever (DHS) as well as Dengue Shock Syndrome (DSS) (Ebi and Nealon, *et al.,* 2016). Previous data had shown that Pakistan faced the worst situations in health sectors and economics due to the continuous spreading of mosquito-borne flaviviruses especially Dengue Virus. Pakistan is a developing country with a less intensive disease surveillance system, lacking well- trained entomologists, deprivation of resources, and inadequate formulation of strategic interventions. Inadequate-surveillance system is responsible for the unsatisfactory control of mosquito-borne viruses in Pakistan (Yousaf *et al.,* 2018).

The ecology plays a vital role not only in the dissemination of vector-borne infections but also in the context of developing immediate preventive measures soon after the detection of arthropods as potential vectors of disease (Abbas *et al.,* 2014). The current study has depicted that mosquito abundance/density is correlated with rainfall and temperature in the selected districts of Pakistan. The primary factor which is involved in the dengue epidemic is the supportive climatic conditions particularly the post-monsoon season. During the monsoon season, both humid and hot conditions persist which provides a favorable climate for the growth and survival of *A. aegypti* (Khan *et al.,* 2015). Thus, it has been reported that dengue outbreaks have been observed for the past many years during this post-monsoon season. In the present study, the most abundant mosquito species were *Culex* (75%) followed by *Aedes* (25%). The ecological factors of relative humidity, temperature, and rainfall were significantly correlated with mosquito abundance.

The current study reported that *Culex* is frequently found in rural areas with no proper sanitation system of study districts as already described by Cetin *et al.* (2010). All the above-mentioned components are the major determinants of occurrence and reoccurrence of dengue epidemics in Pakistan (Suleman *et al.,* 2017). During the previous 35 years, *Aedes* mosquitoes have protracted their ecological distribution and hence found abundantly in all the climatic zones of Pakistan resulting in increased dengue infection rate. Moreover, these findings were in accordance with Raza *et al.* (2014) where maximum mosquito abundance was higher during rainfall season. The population of mosquitoes in this study showed a peak in August- September as the temperature ranges from 38–42^{o}C and a reduction in number was noticed from December to March. These findings are in accordance with Alten *et al.* (2000). In this study, the average population of *Culex* was found abundant followed by *Aedes* mosquito in the post-monsoon season which is similar to Akram *et al*. (2009).

A variety of serological testing procedures including rapid diagnostic tests, hemagglutination inhibition assays (HI) and enzyme-linked immunosorbent assays (ELISA) have been practiced for the detection of flaviviruses including dengue virus (Suleman *et al.,* 2017). Among the serological tests, HI test is the simplest one and can distinguish only between primary and secondary dengue infections, ELISA identifying IgG antibodies is more sensitive procedure as compared to HI while monoclonal antibody-based ELISA detecting IgM antibodies is the most efficient detection technique (Parkash and Shueb, 2015). Rapid diagnostic kits are rapid as well as capable to detect anti-dengue antibodies, but these kits provide false-negative results frequently because of cross-reactivity among flaviviruses. But due to cross-reactivity among the flaviviruses a reliable, accurate and specific diagnostic technique must be implemented. Currently, molecular detection has taken a vital place in the diagnosis of infections and detection of the pathogen in vectors. The molecular techniques used for the viral genome detection includes Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Nucleic acid sequence-based amplification (NASBA), Nested PCR and Real-time PCR (Parkash and Shueb, 2015). These molecular techniques have exhibited higher sensitivity and specificity than other conventional methods. Despite all the merits, these techniques have some demerits including laborious, time-consuming, expensive and possible cross-contamination (Suleman *et al.,* 2017).

This study detected mosquito-borne flaviviruses by multiplex RT-PCR. The consensus primers used in this study, have already been used by Ayers *et al.* (2006) from cultured viruses and confirmed the accurate phylogeny of the flaviviruses by Mackenzie *et al.* (2004) and Tang *et al.* (2020). Our data evidenced that 142 (48%) pools were positive for flaviviruses in all the study districts with the highest percent positivity in Multan (58%) than Jhang (49%) and Chakwal (43%). One-step RT-PCR has been considered as a simple and rapid technique for the identification of flaviviruses from the samples including serum of acute-phase febrile patients, the culture of viruses and infected mosquito pools (Tang *et al.,* 2020). Ahamed *et al.* (2017) described that the RT-PCR method is considered to be rapid and differentiates DENV from other members of flaviviruses. Gaunt and Gould (2005) described RT-PCR technique is capable to distinguish 90% of known vector-borne flaviviruses. But this method has not been yet considered as a reliable source for serum samples as RT-PCR of serum samples can only detect virus efficiently before the appearance of neutralizing antibodies (Papin *et al.,* 2010), while the technique implemented in the present study used mosquito for detection of flaviviruses. We found an overall prevalence of mosquito-borne WNV (18%), Saint Louis encephalitis virus (15%), DENV (9%) and JEV (7%) from three districts of Punjab, Pakistan.

The use of rapid and effective molecular techniques could be implemented to counter the emerging and re-emerging flaviviruses outbreaks. Climate change alters the ecological behavior of the mosquitoes; thus, it is important to explore the ecological niche of mosquitoes so that specific measures should be improved for their eradication. The stringent disease surveillance system, disease reporting, advanced diagnostics, trained personnel, and ecological investigations are imperatively required to control the re-emergence of vector-borne diseases in Pakistan.

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Authors contribution: KY, MSS and SA designed the study objectives. UK, AM and HA conducted the fieldwork from specified areas of the study. KY, HA and AM performed the experimental procedures for Mosquito identification, RNA extraction, cDNA synthesis and multiplex RT-PCR. MIA, RA, SA and MS analyzed the results and their interpretation. KY and UK wrote the initial draft of the manuscript and were reviewed by all authors. WA and FA provide technical support. SA, RA, MIA and MSS revised the article and approved the final version.

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