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Original article

A study on the role of aedes mosquitoes in arboviruses and SARS-CoV-2 infection: A new challenge

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ABSTRACT

Chikungunya, Zika, Dengue Viruses, and now Novel Coronavirus are global health challenges that cause human diseases ranging from febrile illnesses to death. Most of these viruses are mainly vectored by *Aedes* mosquitoes worldwide. Molecular detection of arboviruses was made in female *Aedes* mosquito pools caught from all the seven districts by using a reliable molecular technique, “RT-PCR.” From 216 collections of *Aedes* species, arboviruses were detected in 27, including only Alphavirus genus to determine mosquito abundance and evaluate the potential role of *Aedes aegypti* and *Ae. albopictus* mosquitoes in arboviruses and novel Coronavirus transmission. 5322 mosquitoes were collected using aspirators; 35.31% (n = 2049) were identified as female *Aedes* using morphological keys, pooled into 216 pools, and tested for arboviruses and coronaviruses by using RT-PCR with the help of specific primers. Novel Coronavirus was not detected in this study. Only the *Flavivirus* genus was detected in twenty-seven pools giving an infection rate of 62.96% (n = 17) for DENV2, while DENV3 was 37.03% (n = 10). Furthermore, our results indicated no role of mosquitoes in the spread of Covid-19. Results showed a higher infection rate in urban sites than in rural ones. The detection of arboviruses indicates possible human health risk due to active role of these mosquitoes in spreading of arbovirus in the study area.

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1. Introduction

Old and evolving mosquito-borne pathogens posed a significant challenge to public health authorities and the human population. These pathogens cause significant worldwide disease pressure. Half of the world is at risk of infection by vector-borne pathogens (Anonymous, 2007). The Zika virus, rift valley fever virus (RVFV), yellow fever virus (YFV), dengue virus (DENV), and Chikungunya virus (CHIKV) are the arthropod-borne viruses. They are considered the most common emerging pathogens vectored by *Aedes* mosquitoes in tropical and subtropical countries worldwide (World Health

Organization, 2011). The increase in human activities in forested areas around the globe, especially tropical areas, having hot and humid conditions is an essential factor for the spreading of these arboviruses. (Appawu et al., 2006). A recent increase in the distribution of the urban mosquito vector, mainly *Aedes aegypti*, even in non-endemic regions, is due to a rise in man's traveling (Alan-Barrett, 2010). Infectious diseases caused by arboviral infections transmitted by arthropods are common in several districts of Pakistan; they cause a high morbidity and mortality rate in human beings and animals because they are often misdiagnosed and treated as malaria. (Fig. 1).

Dengue fever is caused by the *Aedes*-borne virus and transmitted by *Ae. aegypti* and, to a lesser degree *Ae. albopictus* in urban and peri-urban areas. Dengue virus has four distinct serotypes, which are DENV1, DENV2, DENV3, and DENV4 (Munoz et al., 2009). In Pakistan, serotypes 2 and 3 are prevalent in different cities in Punjab. Due to unsafe water, packed cities, insufficient sanitation, overprovision of shelters, and squat vaccination exposure, dengue is endemic in Pakistan, and the situation usually attains peaks during the rainy season (Jahan, 2011).

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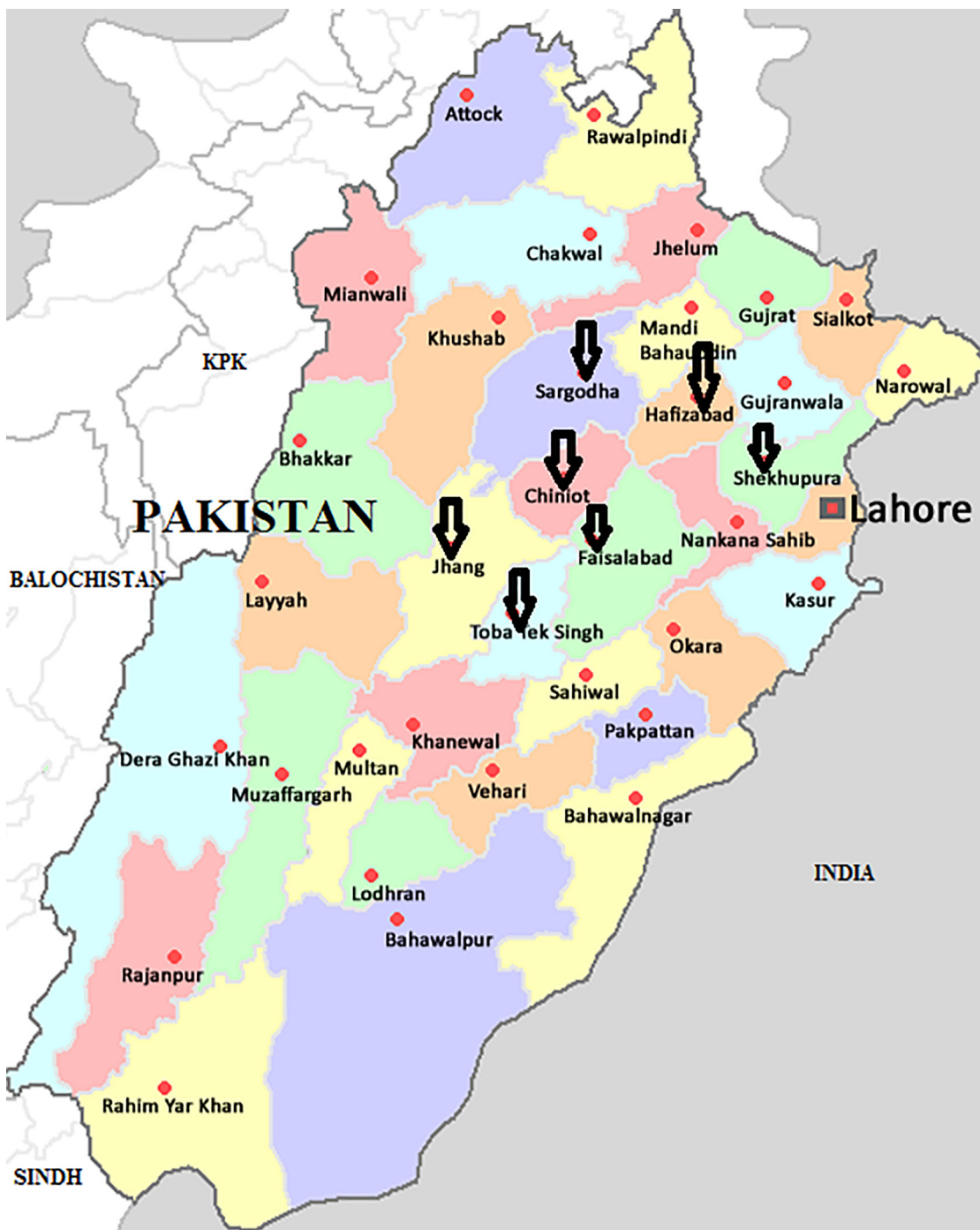


Fig. 1. Map of the Punjab, Pakistan, showing the districts selected for collection of mosquitoes.

Novel Coronavirus belongs to the family Coronaviridae and genera Betacoronavirus cause pneumonia-like symptoms, first appeared in Wuhan, China, in December 2019, and has rapidly increased in pandemic scale. This was named coronavirus disease (Covid-19) on the 11th of February 2020 by WHO (Zhou et al., 2020). The outbreak of this disease has quickly spread worldwide, caused uncountable human deaths, and paralyzed the economy, health, trade, and all other systems of the planet earth in the short period of six months. The genome of SARS-CoV-2 was 85% homologous to bat SARS-like virus and possessed 79% homology with SARS-CoV. In addition to this, pangolins have also been reported to be an intermediate host of SARS-CoV-2 (Lake, 2020).

Aedes mosquitoes play an active role in transmitting the Chikungunya virus (CHIKV) (Borgherini et al., 2007). Chikungunya is now spreading rapidly in Karachi, Pakistan. Chikungunya virus was found circulating in rodents as early as 1983 (Darwish et al., 1983). More than 4000 cases have been confirmed through qualitative RT-PCR by the “National Institutes of Health and Armed Forces Institute of Pathology” in Pakistan (Rauf and Fatima-tuz-Zahra, 2107). Last year, 4868 Chikungunya cases were reported from Sindh, and in Karachi, 73 patients were reported in December 2017.

Arboviruses were detected by using the molecular techniques in the field caught *Aedes* mosquitoes from Al-Madinah Al-

Munawwarah, Saudi Arabia (Ayman et al., 2012), from five sites in Kyela district, Kenya (Bisimwa et al., 2016), from Swat, Khyber Pakhtunkhwa, Pakistan (Khan et al., 2018) by using the molecular technique of real-time RT-PCR.

Pakistan has had multiple arbovirus outbreaks resulting in economic and public health distress. Pakistan, along with other Asian countries, is facing climate change. Winters are getting shorter every year while the summers are becoming longer and harsher. Due to the warmer climate, many arboviral illnesses have occurred in this region, such as dengue, malaria, and Chikungunya. Most Asian countries have bad sanitary conditions that favor the arthropod vectors by providing excellent breeding sites. Rapidly increasing dengue, Chikungunya, and covid-19 cases in Pakistan have created an alarming situation. Due to the lack of efficient measures and fragmentation of natural ecosystems, diseases caused by arboviruses and Coronavirus will become a severe public health problem both in endemic developing countries and many non-endemic countries (Rauf and Fatima-tuz-Zahra, 2107). Thus, detecting viruses in their hosts (mosquitoes) is necessary to determine the viral activities. The objectives of the present study to detect the role of *Aedes* mosquitoes in the transmission of arboviruses and Coronavirus by using RT-PCR, collected from seven districts of central Punjab, Pakistan.

2. Materials and methods

2.1. Sampling of mosquitoes

Adult mosquitoes were collected in the mornings (7–10 am) and evenings (5–7 pm) using a battery-operated aspirator (Florescio et al., 2014) amongst the vegetation and from both indoor and outdoor resting sites. Field samplings were carried out four times a year from May 2018 to May 2020 during four seasons, viz. spring (February to April), early summer (May to July), rainy season (August to October), and Winter (November to January) from the seven districts (Faisalabad, Sargodha, Hafizabad, Shaikhupura, Toba Tek Singh, Jhang and Chiniot) of the province Punjab, Pakistan. Samples were collected separately from urban and rural areas, including city parks, industrial sites, residential areas, from and near water storage containers, old used tyres, grounded vehicles, and trains. Commercial ports (dry ports) and airports were also surveyed to collect the invasive mosquito species (IMS). In addition, sampling of mosquitoes was done from urban and suburban areas, including bushes and shrubs, crops, parks, construction sites, marshes, ponds, forests, and all types of vegetation found in the study area.

2.2. Mosquito analysis

After collection, specimens were brought back to the Zoology Laboratory, Department of Zoology, Government College University, Faisalabad, inside vials closed with muslin cloth after providing a specific field number, date, and time collection, type of habitat, and location. After bringing in the laboratory, adult *Aedes* mosquitoes were sorted out after identification using a microscope (Olympus) and appropriate taxonomic key, according to Becker et al. (2010). Female *Aedes* mosquitoes were pooled (up to 15 mosquitoes per pool) based on collection sites, and season and RNA were extracted on the same day.

2.3. Extraction and analysis of viral RNA

According to the manufacturer's recommendations, a pure Link™ RNA Mini Kit extracted the RNA from pooled mosquito samples.

2.3.1. Lysis and Homogenization:

Sample (2.5 mg mosquito tissues) was put in the RNase free tube and made centrifuge at 2,000 rpm for 5 min at the temperature of 4 °C to form the pellets, then mixed 0.6 ml of lysis buffer with 2-mercapto ethanol to the sample. Vortex was made to continue with high speed up to the lysis of cells. Further homogenization was processed by transferring the lysate to a homogenizer and centrifuging at 12,000 rpm for 2 min.

2.3.2. Binding, washing, and elution

We added 03 ml of 70% ethanol in homogenate and mixed thoroughly by vortexing until the disappearance of a precipitate. After centrifuging, we purged it by removing the supernatant, putting it in the spin cartridge, and then in the same collection tube. Centrifuged the sample at 12,000 rpm for 20 s at room temperature after adding the 700 µl wash buffer I into the spin cartridge and again made purification by discarding the supernatant. After that, centrifuged and dried the membrane of a spin cartridge to which attached RNA. Centrifuged was made again to elute the RNA from the membrane into the recovery tube.

2.3.3. Spectrophotometric analysis

1 µl of RNA was diluted with 39 µl of DEPC treated water. We used a 10ul micro cuvette to take OD at 260 nm and 280 nm to determine the purity and sample concentration. Using the formula that 1 OD at 260 is equal to 40 µg/ml RNA, the A260/A280 ratio is maintained above 1.6.

2.3.4. cDNA synthesis

4 µl of extracted RNA was mixed with 16 µl of a master mix, 2 µl of 10x enzyme mix, and 10 µl nuclease-free water, and the whole volume was brought up to 20 µl. The tube contents were mixed by vortexing for 15 s and then incubated to synthesize cDNA.

2.3.5. Detection of arbovirus by RT-PCR

To detect the arboviruses and Coronavirus in mosquitoes, primers were used from Invitrogen USA. The sequences of these primers were taken from an already published article. Positive samples were further tested to detect the presence of specific viruses by using conserved genes targeting primers, as shown in Tables 1 and 2. The type-specific primers anneal specifically to each of their respective genotype (Lanciotti, 2003; Chan et al., 2020). All the primers are given according to their individual published sequence.

The ordered primers were delivered in the lyophilized form. Each primer was reconstituted to a 1000 µM concentration by adding 100 µl nuclease-free water. In PCR, denaturation of DNA was made by heating at 94 °C for 15 min, then annealing at 57 °C for 60 s, and extension was made at 72 °C for 10 min. Last, 15 µl from PCR solution was analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide at 80 V for one hr. Then the product was observed under ultraviolet light, and different amplified bands were observed and captured with a camera.

3. Results

3.1. Collection of mosquitoes

During this study, 5802 adult mosquitoes were caught from 140 selected sites (10 from urban areas while 10 from rural areas of each district) from May 2018 to May 2020 from seven selected districts, viz. Faisalabad, Sargodha, Shaikhupura, Toba Tek Singh, Hafizabad, Jhang, and Chiniot districts, Punjab, Pakistan. Female *Aedes* were separated from other mosquitoes and pooled into 216

Table 1
DNA sequences of the primers used for detection of arbovirus genera and Coronavirus.

Virus	Target gene or protein	Primer	Sequence (5'→3')	Position	PCR product size (bp)	Reference
Alphavirus	NSP4	VIR2052F	TGG CGC TAT GAT GAA ATC TGG AAT GTT	6971–6997	150	Eshoo et al. (2007)
		VIR2052R	TAC GAT GTT GTC GTC GCC GAT GAA	7086–7109		
Bunyavirus	N Protein	BCS82C	ATC ACT GAG TTG GAG TTT CAT GAT GTC	86–114	251	Bryant et al., 2007
Flavivirus	NS5	BCS332V	GCTGT TCC TGT TGC CAG GAA AAT	309–329		
			FU1	TAC AAC ATG ATG GGA AAG AGA GAG AA	9007–9032	220
		CFD2	GTG TCC CAG CCG GCG GTG TCA TCA GC	9308–9283		
Coronavirus	COVID –19 – S	Spike/Forward Spike/Reverse Probe	CCTACTAAATTAATGATCTCTGCTTACT		30	Chan et al., 2020
			CAAGCTATAACGCAGCCTGTA		21	
			HEX -CGCTCCAGGGCAAACCTGAAAG-IABkFQ		22	
	COVID –19 – N	Nucleocapsid/ Forward Nucleocapsid/reverse Probe	GCGTCTTCGGGAATGTCG		18	Chan et al., 2020
TTGGATCTTTGTCATCCAATTTG				23		
FAM -AACGTGGTTGACCTACACAGST-IABkFQ				22		

Table 2
DNA sequences of the primers used for detection of arbovirus type.

Virus	Primers	Sequence (5'-3')	Region, position	Reference
DENV	D1 F	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	3'UTR, 10520–10541	Lanciotti, 2003
	D2F	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	3'UTR, 10674–10694	
	D2R	CGTCTCAGTGATCCGGGGG	568–586, Size: 482	
	TS-1	CGCCACAAGGCCATGAACAG	232–252, Size: 119	
		TAACATCATCATGAGACAGAGC	400–421Size: 290	
ZIKV 1086	TS-2	CTCTGTGTCTTAAACAAGAGA	506–527 Size: 392	Lanciotti et al., 2017
	TS-3	CCGCTG CCC AAC ACA AG		
	TS-4			

pools, each containing up to 15 mosquitoes on the bases of collection sites and season consisting of 41, 37, 34, 33, 27, 26, and 18 pools from Faisalabad, Sargodha, Shaikhupura, Hafizabad, Toba Tek Singh, Jhang and Chiniot districts respectively.

3.2. Seasons distribution of Aedes mosquitoes

Physical factors (temperature, rainfall, and relative humidity) prevailing in the selected areas had a significant influence on the prevalence of the mosquito population. More rain falls during seasons; summer 18, rainy 18, and rainy 19 resulted in more relative humidity and suitable temperature (30–35 °C), favoring the mosquito population. Hence, the highest mosquito population was observed in these seasons. The Aedes population recorded the highest population when the mean temperature was below 30 °C, as shown in Fig. 2. So, the maximum Aedes population was noted during springs 19 & 20.

3.3. Molecular detection of arboviruses in female Aedes mosquitoes

RNA was extracted from pooled mosquito samples using the Pure Link™ RNA Mini Kit according to the manufacturer's recommendations. Molecular detection was performed in two steps; firstly, screening was made to detect different viruses (Bunyavirus, Alphavirus, Flavivirus, and Coronavirus). From 216 Aedes pools screened, arboviruses were detected in 27 pools belonging to Flavivirus. No sample was found positive for Alphavirus, Bunyaviruses and Coronavirus. The positive mosquito samples (actual pools 4, 9, 12, 15,17, 20, 24, 36, 40, 48, 55, 61, 67, 78, 88, 90, 110, 135, 150,

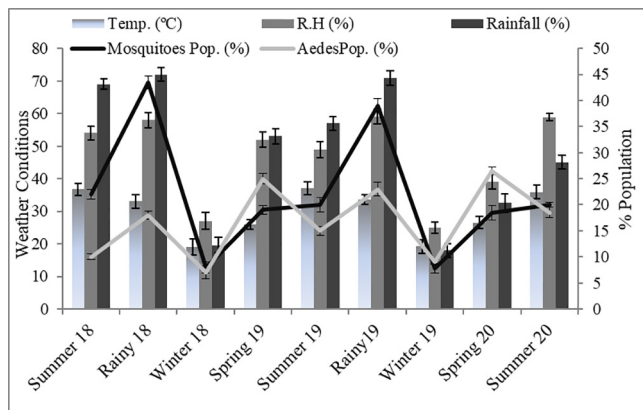


Fig. 2. Relationship of different physical factors with mosquito population as a whole and Aedes mosquitoes.

168, 179, 180, 186, 196, 200, 204, 215) were then further tested by using specific primers (Figs. 3 and 4).

Over all infection rate of DENV2 62.96% (n = 17) while DENV 3 was 37.03% (n = 10). The highest number of DENV2 was detected in mosquitoes sampled from Sargodha 10.81% (n = 5) followed by Faisalabad 09.75% (n = 4), Shaikhupura 08.82% (n = 3), Hafizabad 06.06% (n = 2), Chiniot 05.55% (n = 1) Jhang 03.84% (n = 1), and then Toba Tek Singh 03.70% (n = 1). DENV3 was found to be more positive in Sargodha 10.81% (n = 4) then in Faisalabad 07.31% (n = 3), Toba Tek Singh 03.70% (n = 1), Hafizabad 03.03% (n = 1), Shaikhupura 02.94% (n = 1), Jhang (nil) and Chiniot (nil) as shown in Fig. 4.

Results showed that, higher rate of infection was found in the urban sites than rural ones.

4. Discussion

Mosquitoes play a vital role in spreading arboviral disease throughout the world. To determine high-risk areas where the emergence and circulation of arboviral diseases might occur, the study of arboviral vectors is an initial aspect. The present study investigated *Aedes* mosquito-borne viruses in seven districts (Faisalabad, Sargodha, Shaikhupura, Toba Tek Singh, Hafizabad, Jhang, and Chiniot) of central Punjab, Pakistan. During this study, 5802 adult mosquitoes were collected using battery-operated aspirators from May 2018 to May 2020. Female *Aedes* mosquitoes were separated and selected for this study. The results showed that out of 5802 collected mosquitoes, Female *Aedes* were 35.31% ($n = 2049$), while other than female *Aedes*, mosquitoes were 64.68% ($n = 3753$).

According to our results, female *Aedes* mosquitoes were the most prevalent from February to April (spring season) 2019 and 2020, while the least number in the winter season (November to January) 2018 & 19. This season-wise fluctuation in the population indicated the effect of climate on the reproduction of these mosquitoes. They begin to increase their population size on hot temperatures and humid air availability. Mosquito abundance differed dramatically among the ecologically distinct districts where the mosquitoes were collected due to large livestock, uncontrolled human population, urbanization, and increasing breeding sites in these areas for several species, particularly *Aedes* mosquitoes. These results were similar to the findings of Matthew et al. (2008). These observations are also supported by studies (Rasheed, 2012). Our results are also very close to Akram et al. (2009), who reported the abundance of mosquitoes from March to September. Dhimal et al. (2014) collected vectors (mosquitoes) of malaria, Chikungunya and dengue virus, lymphatic filariasis, and Japanese encephalitis in the same season. Mori and Wada (2007) also reported the most minor population of *Aedes* mosquitoes during the winter (December to January). These results are at par with our findings. Our results also showed that populated areas like Faisalabad and Sargodha provided more suitable habitats for *Aedes* mosquitoes. These results agree with previous surveillance studies conducted in Pakistan, such as in Murree Hills (Qasim et al., 2014), Kasur and Sheikhupura (Oneeb et al., 2016). They

recorded species composition and then analyzed these results with temperature, relative humidity, and rainfall for different species of mosquitoes. They also collected the least mosquitoes in winter (December to January), but they found the maximum population in July due to more rains and breeding sites. These results differ from our findings because we observed the entire people in March to April due to more showers and humidity than in July and August.

Molecular detection of arboviruses was made in female *Aedes* mosquito pools caught from all the seven districts by using a reliable molecular technique, "RT-PCR." From 216 collections of *Aedes* species, arboviruses were detected in 27, including only Alphavirus genus. All samples showed a negative result for Flavivirus and Bunya viruses. The positive mosquito samples were then further tested using specific primers. This result indicates that *Aedes* mosquitoes are involved in arbovirus transmission in these districts. Most infected pools were detected from Faisalabad, Sargodha, and Shaikhupura. These three regions are classified as semi-arid with a high level of human population and industrialization, ideal for reproducing arboviral vectors. Mosquitoes collected in these areas may feed on infected people and become infected with arboviruses. These findings are supported by the study of Anyamba et al. (2001). These results are different from our findings because we found peak dengue infection in March and April.

This study detected dengue virus (DENV2 and DENV3) in the collected mosquitoes. These results are supported by the findings of Jahan (2011), who also observed that serotypes 2 and 3 were prevalent in different cities of Punjab, Pakistan. Khan et al. (2018) conducted field sampling of mosquitoes in Swat, Khyber Pakhtunkhwa, Pakistan, and analyzed field data for epidemiological trends of dengue. They used the reverse transcriptase PCR technique to detect all types of dengue viruses. They found that dengue was at its peak (56%) in September 2013 and 24% in October 2014.

Studies carried out in New California by Dupont et al. (2012) and in Argentina (Domingo et al., 2005) confirmed the involvement of *Aedes aegypti* in Chikungunya and dengue transmission during epidemics. The findings of Cumberland (2009) demonstrated that Filariasis, Japanese encephalitis, Yellow fever, Plague, Dengue fever, Malaria, Zika, and Chikungunya are transmitted among vertebrate animals, including human beings, and create great concern for public health. Indeed, detection of infection of arboviruses in this area indicates that *Aedes* mosquitoes can vector arboviruses, including dengue fever virus. The low positivity obtained for genera-specific could be due to the mosquito preservation method

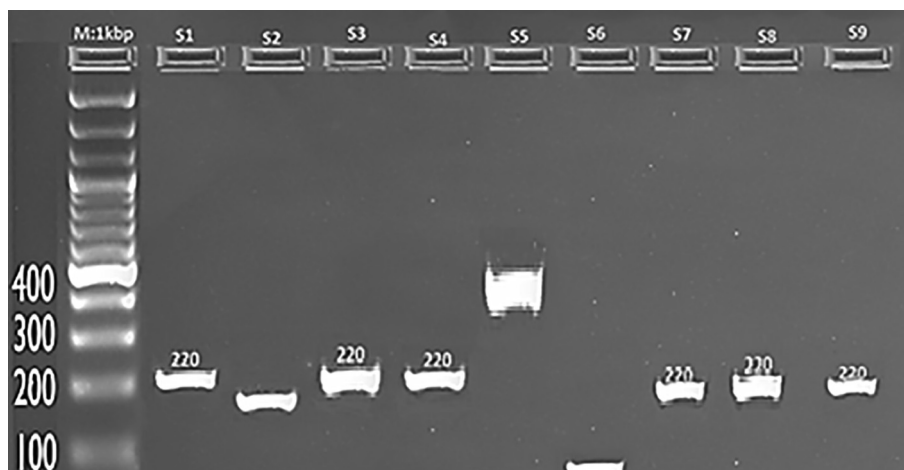


Fig. 3. Visualization of RT-PCR product of the arbovirus and Coronavirus M: Marker(1000 bp), The expected size of amplicon was 220 bp. Samples 1,3,4, 7, 8, and 9 were positive for *Flavivirus*, while samples 2, 5, and 6 were negative for any arbovirus and Coronavirus.

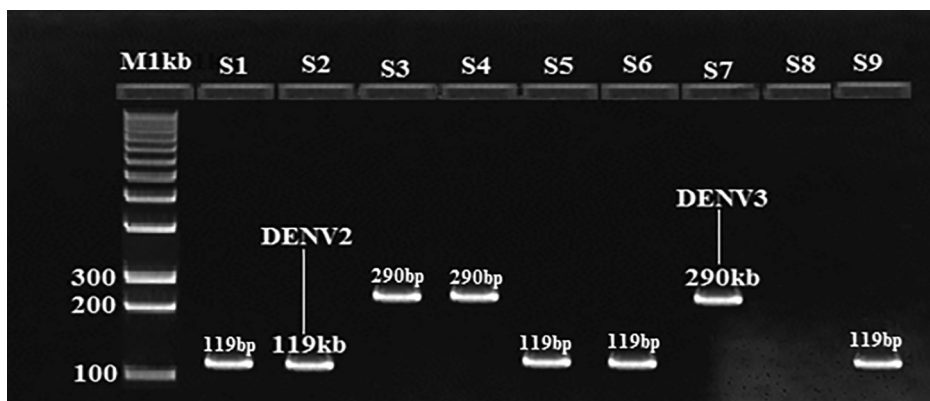


Fig. 4. Visualization of RT-PCR products for Dengue virus. The expected PCR product size was 119 bp for DENV2 and 290 bp for DENV3. M: DNA marker (1000 bp). Samples 1, 2, 5, 6, and 9 were positive for DENV2, while samples 3, 4, and 7 were positive for DENV3.

used and the time between sample collection and laboratory analysis. In this investigation, a new *Aedes* mosquito was used for RNA extraction. The findings of Mbanzulu et al. (2017), Bisimwa et al. (2016), Monika et al. (2016), and Ayman et al. (2012) also supported the results of this study. They also detected arboviruses in the *Aedes* mosquitoes responsible for causing Chikungunya and dengue. Our results indicated no involvement of *Aedes* mosquitoes in the spread of Novel Coronavirus (SARS-CoV-2) involved in COVID-19. This finding is in line with the findings of (Huang et al., 2020; Xia et al., 2020), who also found similar results, but their methodology is different from our studies. They did not use mosquitoes collected from the fields. Instead, they tried to replicate the Coronavirus in mosquitoes under lab conditions. We performed our analysis on field-collected mosquitoes.

5. Conclusions

It has been concluded that there is no involvement of *Aedes* mosquitoes in the spread of Novel Coronavirus (SARS-CoV-2) involved in COVID-19. The low positivity obtained for genera-specific could be due to the mosquito preservation method used and the time between sample collection and laboratory analysis. The detection of arboviruses indicates possible human health risk due to active role of these mosquitoes in spreading of arbovirus in the study area.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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