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The Role of Vertical Transmission of Dengue Virus among Field-Captured *Aedes aegypti* and *Aedes albopictus* Mosquitoes in Peshawar, Khyber Pakhtunkhwa, Pakistan

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IA, AI and IK conceived and designed the experiments. JK performed the experiments. JK and IK analyzed the data. JK wrote the paper.

Key words
Dengue, Polymerase chain reaction, Trans-ovarial Transmission, *Aedes aegypti*, *Aedes albopictus*.

ABSTRACT

Despite the fact that dengue is one of the major public health threats in Pakistan causing severe outbreaks since 2005, the molecular epidemiology in mosquito (dengue vectors) is limited. Here, we investigated the vertical transmission of dengue virus (DENV) in the *Aedes* mosquitoes and its (DENV) transmission through other factors mainly tyres trade. DENV was detected in *Aedes aegypti* (57%) and *Aedes albopictus* (43%) adults (n=1050) and larvae (n=550). The samples were collected from four (Shuba Bazaar, Tarnab Farm, Wazir Bagh and Hayat Abad) dengue suspected locations of district Peshawar, Khyber Pakhtunkhwa (KPK), Pakistan. A semi-nested multiplex PCR was used to amplify the cDNA of the four dengue serotypes. Six out of 35 (17.14%) adult mosquito pools were found positive for dengue virus with type-2 (77.77 %) and type-3 (11.11 %) while a single (11.11%) pool of concurrent infection with type 2 & 3 was also detected. The adult *Ae. aegypti* belonging to Shuba Bazaar showed 33% positivity for DENV with minimum infection rate (MIR) 11.1 while that of Wazir Bagh showed 16.67% positivity with MIR 5.5. Similarly, the *Ae. albopictus* of Shuba Bazaar resulted in 22.22% positivity with MIR 7.4. The pooled larvae showed 3 (17.64%) positive out of 17, the pooled *Ae. aegypti* larvae from Shuba Bazaar resulted in 40% positivity (MIR=13.3) while the *Ae. albopictus* showed 33% positivity (MIR=11.1). These results indicated the vertical mode of dengue virus transmission in *Aedes*, highest DENV positivity was observed in mosquitoes (adults and larvae) collected from tyres in Shuba Bazaar. The disease burden in Pakistan is critical requiring large-scale molecular epidemiology and entomological surveillance. Moreover, transportation of mosquitoes via tyre trade necessitates more conservative mosquitoes control strategies in Pakistan especially in the unrestricted transport of tyres from southern part of the country to the northern parts.

INTRODUCTION

Dengue has become an emerging health issue in the world generally and in Pakistan especially. Over 20,000 deaths and 500,000 cases of severe dengue (DHF/DSS) with approximately 50 million to 200 million dengue episodes are reported annually from different parts of the world (Gubler, 2002; Shepard *et al*., 2011).

The people of Pakistan have been suffering from dengue since 1960s. The first dengue case was recorded in Karachi in 1994; whereas, the first dengue outbreak was documented in Karachi 2005 reporting a total of 3940 patients admitted to different hospitals throughout the country (Jamil *et al*., 2007; Khan *et al*., 2008; Idrees and Ashfaq, 2012). From 2006 to 2009, dengue (DENV-2, 3 and 4) caused 7193 morbidities with 120 deaths. Dengue reached a peak of 9000 cases with 51 fatalities reported throughout the country in 2010 (Rasheed *et al*., 2013). The two largest dengue outbreaks were recorded for the first time in the country when Lahore (capital of Punjab Province) faced the burden of 22562 cases with 363 fatalities in 2011, and Swat (district of Khyber Pakhtunkhwa Province) reported 8343 confirmed dengue victims along with 57 deaths. By the middle of October 2014, more than 600 confirmed dengue cases were observed in different parts of the country.

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Dengue virus (DENV) is a flavivirus with an 11 kb single-stranded positive sense RNA, having genetically distinct serotypes (DENV1, DENV2, DENV3 and DENV4). The virus is transmitted by *Aedes* mosquitoes through horizontal as well as vertical transmission. *Aedes aegypti* (primary) and *Aedes albopictus* (secondary) are the key mosquito vectors involved in transmission of the virus. In horizontal transmission, the virus is transmitted through the injection of saliva contaminated with dengue virus after an infected mosquito probes for blood (Halstead, 2008). In vertical transmission, the virus is transmitted through the eggs laid by the *Aedes* mosquitoes (Halstead, 2008; Rezza, 2012).

Vertical transmission of dengue virus in *Aedes* plays an important role in the maintenance of DENV in nature and potentially increases the possibility of dengue outbreaks in endemic and non-endemic areas. The DENV infected eggs can survive for more than a year in dry or cold environments even in the absence of a suitable vertebrate host. This mechanism of vertical transmission allows the virus to survive during inter-epidemics (Rezza, 2012; Rosen *et al*., 1983; Khin and Than, 1983; WHO, 2011). Though dengue vectors have been reported in the region before and after the creation of Pakistan, the incidence of DENV trans-ovarial/vertical transmission in *Aedes* has not been reported previously from Peshawar, KPK. Despite the fact that dengue has been one of the critical health issues in Pakistan insufficient attention has been paid to the molecular epidemiology (Koo *et al*., 2013) of dengue virus in mosquitoes until this current research was undertaken. Eventually, it played a significant role in dengue outbreaks in the region. The present study deals with the indication of trans-ovarial transmission of DENV in wild caught *Aedes* in Khyber Pakhtunkhwa, Pakistan.

MATERIALS AND METHODS

Study area

Peshawar is the capital of Khyber Pakhtunkhwa Province of Pakistan, situated at altitude of 359 meters (1,178 ft) above sea level in between 34.0167° North latitude and 71.5833° East longitude near the Pak-Afghan border. Peshawar features a semi-arid climate with hot summers (May to September) and mild winters (November-March) having the mean maximum over 40 °C (104 °F) and minimum 25 °C (77 °F) temperatures. The monsoon peak is observed in the month of August, and the relative humidity varies from June (46%) to August (76%). Over the last three decades, the density of urban population has increased as a result of internal migration of people (from other parts of KPK and Federally Administered Tribal Areas or FATA) in search of jobs opportunities, better education, and services, and because of immigration of Afghan refugees after 1979 due to Soviet Union attack on Afghanistan. Peshawar has also been a center of trade between Afghanistan, South Asia, Central Asia and the Middle East for centuries. The increasing availability of suitable environments for mosquito breeding, the rising trend in urbanization and human population density, the trade and influx (countrywide and worldwide) of people has significantly increased the recent geographic expansion of dengue in the region.

Collection of larvae and adults

A survey of *Aedes* mosquitoes was conducted in four dengue suspected locations such as Shuba Bazaar (main area with tyre businesses: 34°00’27.77” N, 71° 33’46.0” E), Wazir Bagh (urban area, with parks and swimming pools: 33°59’46.91” N, 71° 34’27.04” E), Tarnab Farm (34° 00’ 59” N, 71° 42’ 23.00” E) and Hayat Abad (33.9861° N, 71.4569° E) (suspected dengue cases recorded during 2011) of district Peshawar. This survey was undertaken from June 2012 to November 2012. Mosquito larvae were collected from tyres (70%), tap water catch basins and house hold water containers (20%) and from multiple natural breeding sites (10%). Swimming pools (in Wazir Bagh Park) are known larval habitats. Adult mosquitoes (n=1050) were captured using a back pack aspirator from the nearby houses at each location, the detail regarding sampling from each collection spot has been shown in Table I. Adult mosquito and larvae (510) collections were made from public as well as private properties. Prior permission for sampling from privately owned premises was taken from their owners. The identification of collected mosquitoes was carried out using the Leopoldo’s key (2004).

Pools formation

A total of 35 adult pools (n=1050) and 17 larval pools (n=510) of *Ae. aegypti* and *Ae. albopictus* were processed, each pool consisted 30 specimens. The 35 adult pools comprised 20 (57%) pools of *Ae. aegypti* and 15 (43%) pools of *Ae. albopictus* (Table I). The 17 larval pools comprised 10 (59%) pools of *Ae. aegypti* and 7 (41%) pools of *Ae. albopictus* (Table I). The number of mosquitoes per pool may be increased or decreased depending upon the sample size. Most often 30 specimens per pool have been processed in literature we therefore, have adopted the same procedure (Halstead, 2008; Rezza, 2012; Rosen et al., 1983; Khin and Than, 1983; WHO, 2011; Savage et al., 1993).

Table I.- Distribution of dengue virus in pools (N = 30/pool) of adults and larvae of *Ae. aegypti* and *Ae. albopictus* from various sites in Peshawar, Khyber Pakhtunkhwa, Pakistan.

Species	Site	Total Individuals	No. of Pools	PCR Positive Pools	Positive Pools (%)	MIR
Adult mosquito						
Ae. aegypti	Hayat abad	90	3	0	0	
	Shuba Bazaar	270	9	3	33	11.11
	Wazir Bagh	180	6	1	16.7	5.5
	Tarnab	60	2	0	0	
	Total	600	20	4	20	
Ae. albopictus	Hayat abad	30	1	0	0	
	Shuba Bazaar	270	9	2	22.2	7.4
	Wazir Bagh	120	4	0	0	
	Tarnab	30	1	0	0	
	Total	450	15	2	13.33	
Both Species	Total	1050	35	6	17.14	
Mosquito larvae						
Ae. aegypti	Hayat abad	30	1	0	0	
	Shuba Bazaar	150	5	2	40	13.3
	Wazir Bagh	90	3	0	0	
	Tarnab	30	1	0	0	
	Total	300	10	2	20.0	
Ae. albopictus	Hayat abad	0	0	0	0	
	Shuba Bazaar	90	3	1	33	11.11
	Wazir Bagh	90	3	0	0	
	Tarnab	30	1	0	0	
	Total	210	7	1	14.29	
Both Species	Total	510	17	3	17.64	

MIR, minimum infection rate per 1000 mosquito larvae.

Table II.- Oligonucleotide sequences used to amplify C-prM gene junction of dengue virus [26].

Primer	Sequence	Genome position	Amplified size
D1F	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	134-161bp	511bp
D2 R	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644bp	511bp
TS1 R	5'-CGTCTCAGTGATCCGGGGG-3'	568-586bp	482bp
TS2 R	5'-CGCCACAAGGGCCATGAACAG-3'	232-252bp	119bp
TS3 R	5'-TAACATCATCATGAGACAGAGC-3'	400-421bp	290bp
TS4 R	5'-CTCTGTTGTCTTAAACAAGAGA-3'	505-527bp	392bp

Minimum infection rate (MIR)

The formula used for calculation of MIR is the following (Savage *et al*., 1993):

$$\text{MIR} = \frac{\text{Number of Positive pools by species}}{\text{total number of that species tested}} \times 1000$$

RT-PCR

A nested RT-PCR (potential tool for rapid and specific detection of dengue virus) developed by Lanciotti and colleagues with minor modifications (Table II) (Lanciotti *et al*., 1992) was used to analyze the pooled individual mosquitoes. This procedure was adopted to

eliminate possible laboratory contamination, and making available additional facts for further studies in future. Samples (ground mosquitoes) (150 µl) were taken, and RNA was extracted with Favorgine RNA extraction kit (CAT# FAVNKOO1-2) according to the instructions of manufacturer. RNA (5 µl) was reverse transcribed, the cDNA (the C-prM junction of the dengue virus genome) was amplified with primers D_1 (Upstream/Forward) and D_2 (Downstream/Reverse) (Table II) (Lanciotti *et al*., 1992) using MMLV-reverse transcriptase (Fermentas, USA) in a single reaction vessel with 50 µl final volume. The thermocycler was programmed to incubate for 45 minutes at 42°C and then 35 cycles at 94°C, 55°C and 72°C. In the second step 10 µl of dengue cDNA from the first reaction was used with four dengue virus (DENV) type specific primers (TS1–TS4, plus D1) (Table II) in order to amplify and identify the four different dengue virus serotypes. Positive controls for DENV-2 and 3 were available from 2011 dengue outbreak in Lahore, Pakistan, where some of the DENV positive samples were preserved at -80°C. Bands of different amplified products were visualized in 2% agarose gels stained with Gel Red (Biotium Inc., USA). depending on the DENV serotype (DENV-2 119 bp and DENV-3 290 bp) after 20 cycles at the same temperatures as the first reaction (Lanciotti *et al*., 1992).

RESULTS

Presence of DENV in adult and larval pooled individual mosquitoes

Six out of 35 adult mosquito pools were found positive for dengue virus (Fig. 1, Table I). The DENV infection rate in the adult mosquitoes was thus estimated to be 17.14%. Furthermore, higher infection of dengue virus was noted in *Ae. aegypti* as compared to *Ae. albopictus*. Similarly, 3 (17.64%) out of 17 larval pools were found positive for DENV, a complete detail of DENV infection rates in mosquito pools has been shown in Table I. This data clearly indicates vertical transmission of DENV-2 and 3 from infected *Ae. aegypti* and *Ae. albopictus* female mosquitoes to their eggs that may have served as an interepidemic reservoir between outbreaks in successive years in Pakistan.

Serotyping of dengue virus

The dengue virus positive pools (n=9) were further processed for serotyping with type specific primers (TS1, TS2, TS3 and TS4) (Table II) in a multiplex PCR (Lanciotti et al., 1992). Among nine, 7 pools showed dengue virus type 2 (77.77 %) and one pool showed serotype 3 (11.11%) where as one pool (11.11%) had concurrent infection of serotype 2 and 3 (Fig. 1, Table I).

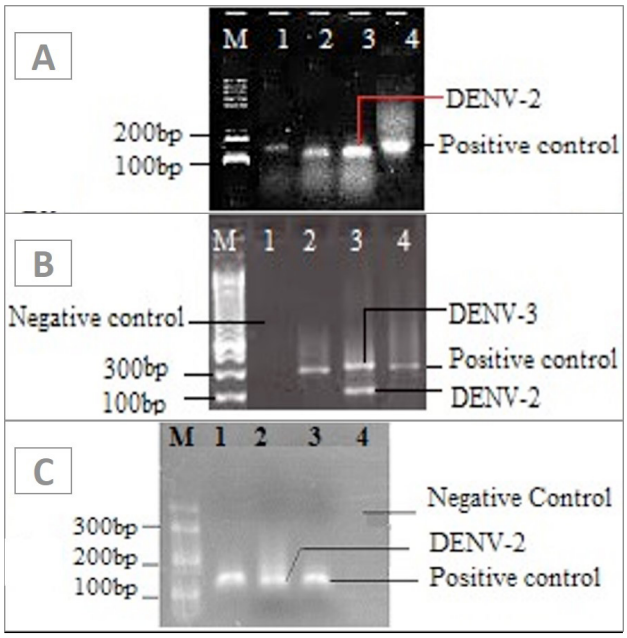

Fig. 1. Agarose gel electrophoresis showing amplified fragments of DENV in different pools of adults and larval mosquito. M molecular weight marker (50 bp ladder) (DENV-2 119 bp and DENV-3 290 bp).

DISCUSSION

All the four dengue virus serotypes (DENV-1 to DENV-4) have been reported from Pakistan. It is believed that dengue virus has been transported from India, where multiple dengue outbreaks have occurred for many decades, to Karachi (Southern part of Pakistan) via tyres with eggs deposited by dengue infected mosquitoes present (Idrees and Ashfaq, 2012; Rasheed *et al*., 2013; Tariq and Zafar, 2000; Dash *et al*., 2011). Dengue was then transmitted in a northerly direction becoming established in Punjab and KPK provinces of Pakistan (Koo *et al*., 2013; Ali *et al*., 2013; Khan *et al*., 2013). This hypothesis is supported by the current study; the mosquitoes were mainly collected (55%) from tyres in Shuba Bazaar, where approximately 300 shops are involved in the tyres trade within and outside the country. The highest viral infection rates were found in these mosquitoes (Table I). Moreover, the serotypes detected (DENV-2 and 3) during 2011-2012 shared genetic similarity with the serotypes found in Karachi in 2005-2009 and in Lahore in 2011 (Koo *et al*., 2013). Therefore, our findings suggest that dengue virus might have been transported to Peshawar, capital of KPK, *via* tyres having DENV infected eggs and larvae though other factors such as dengue infected humans traveling may also be involved in its transmission. Similarity of

serotypes detected in Karachi, Lahore and Peshawar also reveals that the 2012 dengue cases in Peshawar were a continuation of the 2011 outbreak in Lahore (512 km away from Peshawar), and might have been the major cause of epidemics in Swat (172 km from Peshawar) during 2013. This further demonstrates the importance of vertical transmission facilitating the viral circulation. The pooled mosquito (larvae and adults) were processed irrespective of their sex and feeding. Our findings, therefore, suggest that the adult mosquito has either inherited the virus from dengue infected female mosquito via vertical transmission or have taken the blood from dengue infected patient. Moreover, this indicates that vertical transmission of DENV plays a role in viral maintenance in nature and potential transmission to humans.

The role of *Aedes* mosquitoes in the transmission of DENV can be estimated through the minimum infection rates (MIRs), which may serve as a tool for predicting future dengue epidemics in an area (Halstead, 2008). In the present study, we screened 52 pools (35 adults pools and 17 larval pools) consisting of *Ae. aegypti* (57%) and *Ae. albopictus* (43%) (Table I). A total of 9 pools (6 pools of adults and 3 pools of larvae) were found positive for DENV-2 and 3 with MIRs of 11.1, 5.5 and 7.4 for adults of *Ae. aegypti* and *Ae. albopictus* respectively (Fig. 1, Table I). Our minimum infection rates concur with the MIRs reported from other countries (Adams and Boots, 2010; Arunachalam *et al*., 2008; Le Goff *et al*., 2011; Vilela *et al*., 2010). In addition, we observed high minimum infection rates (13.3 and 1.11) in larvae as compared to adults which are incompatible with (Mulyatno *et al*., 2012) who documented higher MIRs (5.7 to 31.9) in adults as compared to larvae (5.5) in *Ae. aegypti*. In the current investigation, the larvae of *Ae. albopictus* demonstrated low MIR (11.11) as compared to *Ae. aegypti* (MIR=13.3). Similar to our results, the others (Lee and Rohani, 2005) also reported lower MIRs (2.35-14.30) in larvae of *Ae. albopictus* than the *Ae. aegypti* (MIR=5.77-40.00). Further, we found high infectivity in *Ae. aegypti* as compared to *Ae. albopictus* (Table I) which is in accordance with (Eva *et al*., 2013). The MIR values in our experiment may, in part, be higher than rates previously documented, because of the greater sensitivity of our virus detection method, RT-PCR, compared with other methods of screening and sample sizes. However, our findings further suggest that entomological and molecular (characterization of DENV in vector mosquitoes) surveillance may be a significant awareness tool for the impending outbreaks in the country. The DENV loaded vector mosquitoes in Peshawar (2012), in fact, became the cause of subsequent devastating outbreaks of dengue in the succeeding year (2013) in the Province.

In the present research we also collected larvae from discarded tyres of automobiles in close proximity to humans. The high value of MIR (13.3 and 11.1 in *Ae. aegypti* and *Ae. albopictus*) and the maximum number (55%) of vector mosquitoes collected from tyres indicates that they are suitable containers for breeding and thus increases the risk of disease burden in an area. This may also indicate that travel and trade are additional contributing factors as explained by other researchers (Ahmad, 2014) regarding the mobility of dengue vectors and dengue transmission all over the world. Moreover, our research was conducted in four different areas of Peshawar namely Hayat Abad, Shuba Bazaar, Tarnab Farm and Wazir Bagh, but Shuba Bazaar is the most congested area of the four places which showed highest dengue infection rates in mosquitoes. This also suggests that the higher MIR in mosquitoes from Shuba Bazaar might be due to more interaction of human population with the dengue infected mosquitoes as well as the improper waste disposal system in the area as noted. Furthermore, the dengue virus was not detected in mosquitoes collected from Tarnab Farm and Hayat Abad. The reason may again be the low population density in Tarnab Farm and thus minimum contact between the local mosquitoes and the people while in Hayat Abad a city of Peshawar, with improved waste management and sanitation that may have reduced the population growth of *Aedes*. On the other hand, dengue virus was detected only in Shuba Bazaar and Wazir Bagh with maximum viral infection rates in *Ae. aegypti* of Shuba Bazaar than that of Wazir Bagh. Shuba Bazaar is the major Bazaar of Peshawar, while Wazir Bagh is the second most congested area of the four sites with no proper waste disposal and cleanness system. These observations indicate that higher population density of humans and improper waste disposal system play an important role in the expansion of dengue in the area. Furthermore, our study suggests the prioritization of surveillance of dengue vectors and their control measures such as insecticidal spraying of adults and eradication of breeding sites to stop/reduce an impending outbreak from spreading.

RT-PCR is an excellent tool in virological surveillance of dengue virus particularly when negative results are observed by other common screening methods such as virus isolation in cell culture (Miagostovich *et al*., 1997). Due to high sensitivity of RT-PCR, it is used in epidemiological studies for large amounts of vector samples (Martins *et al*., 2012) and may also be used in small size of sample even up to less than 20 *Ae. aegypti* mosquitoes with high sensitivity performance (Urdaneta *et al*., 2005; Khan *et al*., 2016). The current study, therefore, suggests incorporating RT-PCR as a routine activity in dengue control programs for better result in Pakistan where monitoring of DENV by

virus isolation and RT-PCR in *Aedes* mosquito is limited.

Another important aspect of our study is to explain the factors that govern vector mosquito contact with humans. The exposure rate of humans to dengue vectors increases the risk of DENV transmission in an area. The Wazir Bagh Park has swimming pools (suitable sites for *Aedes*) and great biodiversity of plants producing favorable breeding habitats with more favorable conditions for the production and maintenance of vector mosquitoes. This Park is visited by an intensive crowed of people daily for sport (especially cricket, football, badminton *etc.*) and other leisure activities and thus provides an excellent contact opportunity for vector mosquitoes to visit humans for blood. The potential of this mosquito (vector) to act as a bridge for the introduction of DENV in peridomestic environments is one of the factors which increase the risk of human infection. In accordance with our results the others (Khan and Khan, 2015; Khan *et al*., 2015, 2016) have also recorded maximum incidences of dengue in highly congested/populated areas of the same Province (KPK). Our study, therefore, suggests about the adoptation of all the self-preventive measures in such conditions to avoid further spreading of the virus; otherwise, this situation could lead to considerable dengue complications in future. Moreover, there is need to strengthen the vector and epidemiological surveillance and operational research on dengue vector(s) densities, bionomics between high and low epidemic-prone areas, rural and urban areas and characteristics of virus. These measures, eventually, will lead to implement community-friendly and sustainable disease management strategies in the country.

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Conflict of interest statement

We declare that we have no conflict of interest.

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