STUDY THE PREVALENCE OF DENGUE MOSQUITO SPECIES AND VIRUS SEROTYPES IN MULTAN

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ABSTRACT
Dengue, a mosquito instinctive viral septicity is considered as a major unrestricted problem worldwide. Dengue virus is associated with the properties of encased, positive strand RNA viruses. Around 50 million individuals are at danger with 24,000 passing away year after year globally. In Pakistan during the most recent few years dengue has become an evolving vector endured disease. Dengue infection is triggered by DENV1, DENV2, DENV3, DENV4 and DENV5. Present study was carried out to identify the dengue mosquito species and virus serotypes prevailing in Multan city. Fully-grown mosquitoes were sampled with aspirator and larvae with dipper from different nine areas of Multan city. Samples were well-preserved in 70% alcohol and carried to laboratory conditions in Department of Zoology, GC University Faisalabad. Mosquito were recognized and samples were kept at -20°C for PCR. Real time PCR were done to identify the presence of virus in mosquito samples. Analytical results of sample exhibited the existence and nonexistence of dengue pathogen in Multan city. The PCR result shows the presence of dengue virus in Gulgasht colony and jail road. Out 250 collected mosquitoes, 180 (72%) were identified as A. aegypti while 70 (28%) were identified as A. albopictus. These results indicate that A. aegypti was more prevalent in the survey areas of Multan. Significant population of A. aegypti was observed in the months of July (18%), September (18.8%), November (22%), February (22%) and March (19.2%). The results of RT–PCR and gel electrophoresis reveled that only two pools out of nine were positive with dengue viral RNA. The dengue virus serotype was identified as DENV-3 in both negative pools.

Keywords: Dengue, Mosquito species, Prevalence, Virus

INTRODUCTION
There are more than 3,000 species of mosquitoes, but the members of three genera endure the primary concern for the spread of human diseases. Anopheles mosquitoes are the solitary species known to transmit malaria. They also spread filariasis (also called elephantiasis) and encephalitis. There are small insects belonging to the family Culicidae of the order Diptera. This family contains round about 3500 species which have been grouped into 42 genera and 135 subgenera (Rueda, 2008). Culex mosquitoes carry encephalitis, filariasis and the West Nile virus while Aedes mosquitoes carry yellow fever, dengue and encephalitis viruses (Belkin, 1962).

The various serotype of dengue virus are transmitted to humans by infected Aedes mosquito. This mosquito borne dengue viral infection is regarded as a major public health problem throughout the world. Dengue has four distinct serotype including DENV1, DENV2, DENV and DENV5. Dengue virus with serotype DENV-2 and DENV3 are associated with severe disease that is followed by secondary dengue infection (Lanciotti et al., 1992). In Pakistan, serotype-2 and serotype-3 are prevalent in different cities of Punjab including Multan. The geographical distribution of this disease is characteristically associated with the distribution of its principal vector, Aedes aegypti (Jacob, 2000). This mosquito is widely distributed in tropical and sub-tropical areas of the world (Gibbons and Vaughan, 2002). First certified out-break of the dengue detoxification was
The sampling areas of Multan specified for collection of A. aegypti

<table>
<thead>
<tr>
<th>Sampling area</th>
<th>Sampling codes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehari chow</td>
<td>MI</td>
</tr>
<tr>
<td>Shah Rukan-e-Alam town</td>
<td>M2</td>
</tr>
<tr>
<td>Gulgasht colony</td>
<td>M3</td>
</tr>
<tr>
<td>Basti khudabaksh</td>
<td>M4</td>
</tr>
<tr>
<td>Bosan road</td>
<td>M5</td>
</tr>
<tr>
<td>Jail road</td>
<td>M6</td>
</tr>
<tr>
<td>Lodhi colony</td>
<td>M7</td>
</tr>
<tr>
<td>Bhatta colony</td>
<td>M8</td>
</tr>
<tr>
<td>Jalalpurpirwala road</td>
<td>M9</td>
</tr>
</tbody>
</table>

Table 1

The sampling areas of Multan specified for collection of A. aegypti

Entomology in the Zoology department of Govt. College University Faisalabad. RNA extraction was done by trizol isolation protocol (Chomczynski and Mackey, 1995). The tissue's samples were homogenized in 1ml of Trizol reagent per 50 to 100 mg of tissues using a glass Teflon or powder homogenizer. Then samples were incubated for 5 minutes at room temperature and centrifuged to remove cell debris. After that, supernatant was transferred to new tube. The supernatant was added with 0.2 ml of chloroform per 1 ml of Trizol reagent, vortexed vigorously for 15 second and incubated at room temperature for 2-3 minutes. Then sample was again centrifuged at 12,000X g for 15 minutes at 2 to 8 °C. After centrifugation, the mixer was separated into lower red phenol-chloroform phase and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. Then upper aqueous phase was transferred carefully into fresh tube. RNA was precipitated by adding 0.5 ml isopropyl alcohol and incubation was done at 15 to 30 °C for ten minutes. Again centrifugation was performed at the same conditions. A gel like pellet was formed on the side and bottom of test tube. RNA pellet was washed with 75% alcohol. Then overtaxing and centrifugation was performed for 5 minutes. Whole procedure was repeated with ethanol to remove all debris. Re-
dissolving of RNA was done and RNA pellet was dried by air or vacuum for 5-10 minutes. Never dry the RNA pellet by centrifuge under vacuum. RNA sample were partially dissolved in A260/A-280 in ratio 1:6. Then RNA was dissolved in DEPC-treated water by passing solution a few times through a pipette tip. After that, spectrophotometry analysis was done to detect the dengue virus in mosquitoes. Dengue specific primers were synthesized from IDT technology USA. These primers were specific to four genotypes named DENV-1, DENV-2, DENV-3 and DENV-4 (Table 2).

Primers D1 and D2 were fulfilling the criteria and type specific primers (TS1, TS2, TS3 and TS4) were designed to anneal specifically to each of their respecting genomes (Lanciotti et al., 1994) (Table 2).

Target viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by use of reverse transcriptase (RT). Subsequent Taq-polymerase amplification was performed on the resulting cDNA with the upstream dengue virus consensus primer (D1). All relevant aspects of the RT-PCR were initially optimized by use of quantified and purified dengue virus RNA to achieve a maximum level of sensitivity.

Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) using single stranded RNA was used in the presence of a primer to synthesize a complimentary DNA strand. This enzyme was used to synthesize a first strand cDNA up to 7 kb. The tube contents were mixed and then incubated for 2 minutes. The 1 µl of M-MLV RT was added and then mixed up and down. After adding D2 primer, incubation was done for ten minutes. Then mixture was heated at 70 ºC for 15 minutes. Now cDNA could be used as template for amplification in PCR. (M-MLV reverse transcriptase product information sheet, 2015). The amplification process was performed by combining with reverse transcriptase of viral RNA followed by Taq polymerase in a single reaction. This yielded an equal or greater level of DNA product as separate RT reaction and PCRs.

Then reaction was allowed to proceed in a thermo cycler program to incubate it for 1h at 42 ºC and then proceeded with 35 cycles of denaturation at 94 ºC for 30 second. Primer annealing was done at 55 ºC for 1 minute. Then extension was performed at 72 ºC for 2 minutes. Final extension was performed at 72 ºC for 10 min. A second amplification reaction was initiated with 13 µl of diluted material. The reaction mixture was same but the primer D2 was replaced with the dengue virus type-specific primers mixture. That mixture contained TS-1, TS-2, TS-3 and TS-4. This reaction consisted of only 20 cycles. Initial denaturation of reaction was started at 94ºC for 5 minutes. Second step of denaturation was performed again at 94 ºC for 30 seconds. After denaturation, annealing step was performed at 55 ºC for 1 minute. Extension step was done at 72 ºC for 2 minutes. The size of resulting RNA band was characteristic for each dengue virus.

After that, agarose gel electrophoresis was performed. Finally PCR products were run on 1.6% agarose gel electrophoresis at 80 voltages for one hour and gel was observed on GEL Doc apparatus. Different amplified bands were observed and images of results were saved.

RESULTS

Prevalence of dengue mosquito species

From Multan, 250 mosquitoes were collected from nine areas of Multan city. Out of these 180 mosquitoes (72%) were identified as A. aegypti while 70 (28%) were identified as A. albopictus. These results indicate that A. aegypti was more prevalent in the survey areas of Multan (Table 3).

Amplification profile image

Amplification profile image of Aedes aegypti shows that there are total nine pools of collected mosquito which were PCR-assayed using two types of primers already designed and a ladder of 1kb. The result of this gel image shows that two samples out of nine were positive for RNA virus. Sample-3 (M3) and sample-6 (M6) exhibited positive results for DENV-3 serotype. Seven pools showed negative result for dengue (Fig. 3).

Month wise prevalence of dengue mosquitoes

The climate of Multan is hot which become suitable for dengue mosquito growth in September, October and November. The results of survey study reveal that 45 (18% of total collected mosquitoes) individuals of A. aegypti were collected in the month of July. While 19% (47 adults) and 19.2% (48 adults) of total 250 A. aegypti individuals were collected in September and March, respectively. The maximum dengue mosquitoes (55 adults i.e. 22% of total dengue mosquitoes) were collected in the month of November and February (Fig. 3).

Nine pools and one negative control of mosquitoes (250 mosquitoes) were assayed. Every pool comprised of 25 mosquitoes. After applying RT–PCR, the result of gel electrophoresis showed that only two pools out of nine were positive with dengue viral RNA. The dengue virus serotype was identified as DENV-3 in both negative pools (Table 4).

DISCUSSION

Aedes aegypti is one of the major mosquitos transmitting dengue virus in the whole world (Bosio et al., 2005). Millions of people are infected and many people lose their lives due to dengue virus which is spreading day by day (Guzman et al., 2010).

Pakistan is one of the victimized country where dengue breaks out at a large scale. The spread of dengue disease is worse in Multan where 273 patients were brought to hospital and 183 patients showed positive result for dengue serotype-3. Our results also exhibited the presence of serotype DENV-3 in the assayed dengue mosquitoes from Multan. In 1985, a research was conducted to study the prevalence of dengue virus infection in Pakistan. It showed that about 50-60% of the Pakistanis, especially those living in Karachi, were haemagglutination inhibition (HI) antibody positive for West Nile, Japanese encephalitis and DENV-2 Flaviviruses. These cases rapidly increased from July to October in patients ranging from 6 to 20 year age (Chong et al., 1994). The results of present study also demonstrated the prevalence of dengue mosquitoes during July, September, November, February and March with peak incidence in November and February. In 1994, first outbreak of DHF was reported in Pakistan, out of
16 patients, 15 had dengue IgM identified using DEN-2 antigen. It was also observed that in three out of ten patients of dengue virus were infected with DEN-1 and DEN-2 (Paul et al., 1998). In 1995, DEN-2 infection was reported from Baluchistan province. In 1998 from Baluchistan, DENV-1 and DEN-2 were found by using ELISA study (Akrham et al., 1998). These results are not in confirmatory with the results of present study. This variation may be attributed to geographical distribution of the localities surveyed. The current study shows the circulation of DEN-2 and DEN-3. In 2013, dengue outbreak in district swat is the first reported outbreak in literature in Khyber Pakhtunkhwa, in which more than 8000 dengue incidences were recorded from different areas of this region. Among the nine infected areas, the rate of infection was found higher in urban areas as compared to rural and infection were observed in Saidu (35%), Mingora city (27%), Matta (12%), Kanju (09%), Patchapur (04%), Charbagh (07%) and Khwazakhela 06% (Ali et al., 2013). In 2005, outbreak of DHF in Karachi, DEN-3 was reported among the few tested patients. In serum of children in Karachi, DEN-1 and DEN-2 was found using serological studies. DEN-2 and DEN-3 were found to be co-circulated during 2006 outbreak in Karachi (Khan et al., 2006). Similarly in 2013 dengue outbreak in swat, out of 6000 dengue patients (male=4121 and female=1879), the male infection rate was found more as compared to female, the cases confirmation was done for antidengue antibodies in which NS1 positive cases among population were male=3359, female=1171, while IgG, IgM observed were male=762, female=708. The positive blood samples were subjected to RT-PCR method, the serotypes present in this outbreak were found DENV-2 and DENV-3 respectively, which shows similarities to 2005 dengue outbreak during Karachi because of the same serotypes detected in the blood of patients DENV-3 (Jamil et al., 2007). In 2006 outbreak in Pakistan was found to be closely related to DEN-3 in 2004 outbreak in New Delhi (Gupta et al., 2005). In 2008, a dengue outbreak was reported in Lahore infecting large number of citizens of Lahore samples were found to have DEN-4, DEN-2 and DEN-3 infection (Hamayoun et al., 2010). In 2009, it was reported that children living in Karachi had high levels of antidengue IgM antibody. Samples had concurrent infection with serotypes DEN-2 and DEN-3. Studies showed that serotype DEN-2 was dominant in samples of dengue virus infection collected during the period of three years from 2007 to 2009 (The Nation, 2011 unpublished data). In November 2010, it was reported by a private news channel that out of the 5,050 patients, 2,350 patients were from Sindh, 1,885 from Punjab and at 158 patients from Khyber Pakhtunkhwa. The blood samples of the patients had an infection with DEN-2 and DEN-1 (ARY NEWS). These results are partially in confirmatory with the results of present research because results confirm the prevalence of A. aegypti and dengue virus serotype-3 in Multan. The pratial difference in dengue virus serotypes may be due to the geographical variation and difference of dengue mosquito species. Based on the current study, it is concluded that A. aegypti is main source for the spread of dengue virus in Multan. The dengue virus serotype transmitted by dengue vector A. aegypti is serotype-3.

REFERENCES


Table 2

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Genome position</th>
<th>Size in bp of amplified DNA product (primers)</th>
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<tbody>
<tr>
<td>D1</td>
<td>5’-TCAATATGCTGAAACGCGAGAAAACCG-3’</td>
<td>134-161</td>
<td>511</td>
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<tr>
<td>D2</td>
<td>5’-TTGCAACACAGTCAATGTCTTCAAGGTTC-3’</td>
<td>616-644</td>
<td>511</td>
</tr>
<tr>
<td>TS-1</td>
<td>5’-CGTCTCAGTGATCCCGGAGG-3’</td>
<td>568-586</td>
<td>482(D1 &amp; TS1)</td>
</tr>
<tr>
<td>TS-2</td>
<td>5’-CGCCACAAGGGCCATGAACAG-3’</td>
<td>232-252</td>
<td>119 (D1 &amp;TS2)</td>
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<tr>
<td>TS-3</td>
<td>5’-TACATCATCATGAGAGAAGGC-3’</td>
<td>400-421</td>
<td>290(D1 &amp; TS3)</td>
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<tr>
<td>TS-4</td>
<td>5’-CTCTGTGTTTCTTAACACAGAGA-3’</td>
<td>506-527</td>
<td>392(D1 &amp; TS4)</td>
</tr>
</tbody>
</table>

Table 3

Prevalence of dengue mosquito species in surveyed areas of Multan.

<table>
<thead>
<tr>
<th>Total mosquitoes samples</th>
<th>Density of Aedes aegypti (Percentage of total sample)</th>
<th>Density of Aedes albopictus (Percentage of total sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>180(72%)</td>
<td>70(28%)</td>
</tr>
</tbody>
</table>
Fig. 1

Amplification profile image of *Aedes aegypti*.


