Entomological Surveillance and Detection of Dengue Viruses in Vector Mosquitoes as an Early Warning Tool for the Control of Dengue in Pakistan

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ABSTRACT

Dengue viruses spread through the bites of female Aedes mosquitoes to human mostly in urban areas of tropical/sub-tropical countries. Dengue is occurring as epidemic annually in Pakistan since 2006. Recently in 2011 a severe epidemic occurred in Punjab province along with >15000 positive cases and > 400 deaths, especially in the highly populated urban city of Lahore. With neither vaccine nor proper treatment for dengue, prevention of the disease depends upon the surveillance and early diagnosis/detection of dengue virus antigens from mosquito vectors which can serve as an early warning system for forecasting impending outbreaks. In current study 28 entomological surveys were carried out in various localities of Lahore from March-September, 2011 for the collection of Aedes mosquitoes. Two species Aedes aegypti and Aedes albopictus were found common during this period. However, Aedes aegypti were present throughout these months while Aedes albopictus appeared in the months of July-August, 2011. In addition various types of natural and artificial breeding containers were also observed for immature stages of Aedes mosquitoes in all localities, visited during the above mentioned period. The most productive containers were automobiles used tyres for larval production with 94%positivity. Collected mosquitoes were screened for dengue viruses using dengue specific monoclonal antibodies (MAB) as antigen capture Enzyme Linked Immunosorbant Assays (ELISAs). Of the 114 pools of Aedes aegypti females (n=570) screened, 31 pools were found positive for dengue viruses indicating 27.19% infection rate (MIR). However, of the 04 pools of Aedes albopictus females (n=40) screened; only 1 pool was found positive with 25% infection rate (MIR). This is the first report of DENV detection from adult females of Ae. aegypti and Ae. albopictus collected from different localities of Lahore.

Key words: Dengue viruses; Aedes mosquitoes; Pakistan; ELISA; DENV detection

INTRODUCTION

Mosquitoes transmit several diseases such as filariasis caused by Waucheria bancrofti, malaria by Plasmodium (parasites of human), dengue, encephalitis, and yellow fever by Arboviruses. In Pakistan, mosquitoes belonging to 3 genera i.e., Aedes, Culex and Anopheles are in abundance. Lahore district of Punjab has high population densities of these genera (Mukhtar et al., 2003). Mosquitoes belonging to the genus Aedes have been found all over the tropical and subtropical regions of the world (Womack, 1993) and play an important role in the transmission of a number of viral diseases in man which include the West Nile fever, Dengue fever (DF), Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS).

Pakistan is at high risk of infectious diseases, especially, vector borne diseases such as dengue which is one of the most rapidly spreading tropical diseases in South East Asian regions. Many factors are responsible for their spread such as poor supply of drinking water, population growth, climatic changes, improper sanitation, lack of the proper vector control activities and no vaccines (Gubler, 2002). Ae. aegypti and Ae. albopictus are two important species which are involved in transmission of dengue viruses. Dengue is a re-emerging infectious disease caused by four antigenically different viruses (DEN 1-4) belonging to genus Flavivirus, and family Flaviviridae, which contain the ssRNA genome of about 11 kb.

DF affects humans in terms of morbidity and mortality. The disease is now endemic in over 100 countries with 2.5 billion people present in areas of high risk (WHO, 2009). However, South East Asian regions are the most affected areas of the world. Dengue became an epidemic from 2006 to date annually in Pakistan. More recently in 2011, more than 15000 positive cases were reported all over Punjab province along with 417 deaths. Urban city Lahore was on high risk along with many deaths. There is neither proper treatment nor vaccine for the control of DF/DHF throughout the world. Surveillance of cases and mosquito vectors along with early detection of dengue viruses are the only option to control the disease.

Detection of viruses in human sera during an outbreak or in endemic areas will not be of much helpful to prevent any future outbreaks. Therefore,
detection of dengue virus antigen in mosquitoes has provided a reliable tool to find the types of viruses circulating in nature and also help in designing effective vector control strategies. Entomological surveillance of mosquitoes infected with dengue viruses will help to monitor the infection rates within vector population and provides an early warning system to predict future epidemics. Many authors studied recently virus infection rates in vector mosquitoes for developing early warning tool (Tewari et al., 2004; Srissuphanunt et al., 2007). Dengue virus isolation in mosquitoes have been made in wild caught mosquitoes by many authors (Das et al., 2004; Samuel & Tyagi, 2006; Linthicum et al., 2009). There are many techniques available for virus isolation such as cell cultures and suckling mice inoculation but these are laborious, slow, expensive and time consuming techniques. Mosquito inoculation techniques have been reported for detection and replication of Flaviviruses. Although this technique is sensitive for routine virological confirmation of dengue fever, however, it needs large number of infected mosquitoes, special containment facilities, particular technical skill, and time consuming. Insect bioassays such as indirect immunofluorescent assays (IIFA) and Toxo-IFA on individual and pool of mosquitoes, and polymerase chain reaction (PCR) have been used to detect the dengue viral infection in wild caught Aedes mosquitoes (Chow et al., 1998; Romero et al., 1998). All these techniques require special facilities and are not suitable for large scale detection of samples in any epidemic. RT-PCR gives rapid result but is expensive and has danger of contamination. ELISA, enzyme linked immunosorbent assays has been shown to be rapid and sensitive alternative to all of the above mentioned techniques for monitoring arboviruses in wild caught mosquitoes. Therefore, ELISA offers a good potential tool for rapid screening of large number of samples up to the serotype level. Srissuphanunt et al., (2007) detected dengue viruses using indirect ELISA in adult Aedes aegypti and Aedes albopictus. The authors suggested that this method is less laborious than mosquito inoculation and a large number of vectors could be used for screening. Although, it depends on the accumulation of detectable level of antigen in wild caught mosquitoes. ELISA method required standardization with respect to mosquito pool size and interpretation of results based on OD values in each laboratory. The current study was designed with its specific objects as follows:

1. To detect dengue virus infection in adult mosquitoes collected from DF endemic different localities of highly populated urban city Lahore, by direct antigen capture enzyme linked immunosorbent assays (ELISAs).

2. To measure accurately the minimum infection rate (MIR) of Aedes aegypti and Aedes albopictus within the localities.

3. To locate potential, DF risk areas with vector infection and their relation with clinical incidence of dengue fever like cases reported from various localities in Lahore.

**MATERIALS AND METHODS**

**Study Sites**

Lahore is capital of the Punjab and situated along the River Ravi. Lahore has latitude & longitude; 31°33'N and 74°20'E respectively. The total area of Lahore is 1,772 km², and the population of Lahore is 6,318,745 with density 3,566 persons/km². Localities of Lahore which are dengue sensitive areas were selected on the basis of a history of high incidence of Dengue fever mosquitoes (Aedes) and human population along with building structures. The selected areas were: Jallo Park, Mughalpura, Manawan, Railway Station, Wahdat Colony, Chuburji Quarters, Lahore Zoo, Officer’s Colony, Model Town and GCU. In preliminary entomological surveys, different breeding sites of mosquitoes containing egg-larvae-pupae and adults were observed. The presence of adult mosquitoes near water bodies or damp/cool and shady places were found in many of the above selected localities. The most common breeding sites containing rain or fresh water were artificial/natural containers, automobile used tyres, plant pots, desert air coolers, tree holes, open aquarium (indoor), etc.

**Climate**

The average temperature of Lahore, Punjab, Pakistan is 23.9°C (75°F) in 2011. The range of average monthly temperature was 21°C. The warmest average high temperature was 41°C (106°F) in June. The coolest average low temperature was 5°C (41°F) in January & December, 2010. Lahore, receives on average 489 mm (19.3") of precipitation annually or 41 mm (1.6") each month of year 2011. Mean relative humidity for year was recorded as 37.9% and on a monthly basis it ranges from 20% in May to 58% in August. An average range of hours of sunshine in Lahore, Punjab was between 6.8 hours per day in January and 9.9 hours per day in May. Current study was carried out from March-September, 2011. The average temperature, rain fall and humidity of these months are presented in Fig., 1.
Entomological Surveillance

Field Collection of Larvae

On each survey about 50-60% of houses in each of localities were searched both inside and outside for breeding places of Aedes mosquitoes. Different type of containers indoor/outdoor were observed and recorded in each locality. Aedes larvae were collected from each container and brought to the Vector Biology Laboratory, GCU University, Lahore. Each larva identified up to species level. A proportion of larvae were frozen at -20°C while the others were reared up to adults for detecting dengue viruses.

Collection of Adult Mosquitoes from the Field

Mosquitoes resting or flying indoor/outdoor (rooms/front and backyards) of houses were collected during mornings (8-11 am) and evenings (4-6 pm) for 15-20 minutes using CDC back pack aspirator (John W. Hock Company, USA) at weekly intervals from March-September, 2011 from each locality per survey. Four to six researchers spent about 2-3 hours for adult collection per survey.

Adult mosquitoes collected from each locality were brought to Vector Biology laboratory, GCU on ice and identified using morphological keys up to species level. Aedes adult mosquitoes male and female of different species were stored in glass vials (10ml) at -20°C for further detection of dengue viruses, within ten days of collection using antigen capture enzyme linked immunosorbent assays.

Preparation of Adult Aedes Mosquito Homogenates

Collected adult Aedes mosquitoes (alive/frozen) were homogenized using a 1ml glass tissue homogenizers (Iwaki). Head and thorax of each mosquito was removed from the rest of body using sterilized forceps and surgical blades under dissecting microscope (Kruss, Germany) and homogenized mechanically. Pool size was of five-tenth females/ml of 0.01M PBS pH 7.4 / APS pH 7.0 / 20% acetone extracted human serum. Homogenates were centrifuged at 6000 rpm for 5 minutes at 4°C (Beckman couter Allegra 64 Centrifuge) and were stored at -20°C until further processing. Homogenates of laboratory reared Aedes aegypti (colony established in GCU since 2006), and Anopheles stephensi (colony established by NIMRT Lahore) fed on non-infected blood of albino mice and were prepared in the same way as mentioned above, used as negative control for ELISAs.

Antigen Capture Enzyme Linked Immunosorbent Assay (ELISA)

The enzyme linked immunosorbent assay (ELISA) is the most widely used serological tests for antibody or antigen detection. This test involves the linking of the antigen and antibody on a micro liter polyethylene plate in U-shaped 96-well format which is commercially (Titertek) available for performing ELISA. This facilitates use of small volumes of buffers/antibody/antigen and gives ELISA the potential of handling and operating a number of samples rapidly.

Since one of the reactants in the ELISA is attached to solid phase, thus the separation of bound and free reagents is easily made by simple washing procedures. The end result of ELISA is a coloured complex that can be read using specially designed multichannel spectrophotometer or ELISA plate reader (Trinity Biotech, Ireland).

ELISA Procedure

Optimal dilution of dengue specific monoclonal antibody or capture antibody (MAB D3-5C9-1 broadly reactive against all four serotypes, kindly provided by Centre for Disease Control and Prevention, Fort Collins, California) was made in coating buffer (carbonate buffer, pH 9.0 in ratio 1:1000). 100 µl of optimal dilution of MAB was used to coat each well of 96-well ELISA plate and incubated at 37°C for 2 h. The plates were then stored for overnight at 4°C. Plates were flicked in the sink and washed four-five times with 0.01M PBS (pH 7.4) in 0.05% Tween (PBS-TW). To block nonspecific protein binding 100 µl/well blocking buffer (3% BSA in PBS-TW) was added and plate was kept for 2 hours at 37C. Second flicking and washing of plate for four to five times with PBS-TW was followed. Test samples and control samples (mosquito homogenates in PBS) 100µl/well were added in triplicate wells and incubated for 2 hours at 37°C or overnight at 4°C. After washing four-five times with PBS-TW 100 µl of enzyme linked detector antibody MAB 6B6C-1 broadly reactive against Flavivirus (kindly provided by the CDC, Fort Collins, California) diluted in 1:1000 in 0.01 M PBS was added and incubated the plates at 37°C for 2 hours. Following the washing step Ortho phenyl diamine (OPD) 5mg was dissolved in 10ml of 0.1 M (freshly prepared) Citrate Phosphate Buffer. 70 µl/well of ortho phenyl diamine (OPD) was added and kept it for five minutes. Then added 30 µl of 3% hydrogen per oxide (H2O2) to each well and left for 10-15 minutes in darkness. The reaction was stopped by addition of 50 µl of 4M sulphuric acid (H2SO4) in each well and optical density was
recorded using ELISA plate reader (Trinity Biotech, Ireland) at 492 nm wavelength.

Data Analysis

A mosquito pool was considered dengue-positive if its optical density (OD) value was greater than the mean ±3-4 standard deviation (SD) of the negative control (Thenmozhi, et al., 2005; Tewari et al., 2004; Srisuphanunt et al., 2007). Negative control consisted of homogenates of triturated mosquitoes of the same species Aedes/Anopheles laboratory reared with the same number in each pool.

RESULTS

Entomological surveys were conducted in different localities of Lahore from March to September, 2011. In total 28 surveys, 9704 mosquitoes were collected, among them two genera; Aedes and Culex were found. Total 1385 (14.2%) Ae. aegypti, 557 (4.72%) Ae. albopictus, while 7855 (80.9%) genus Culex were found (Table I).

Two species of Aedes (Ae. aegypti and Ae. albopictus) and Culex quinquefasciatus with respect to male and female densities from different localities of Lahore indicated that overall Culex males (4904) and females (2951) were abundant, followed by Ae. aegypti males (634) and females (751) and then Ae. albopictus males (225) and females (239) (Fig., 2).

Monthly distribution pattern of these mosquitoes in the month of March to September 2011 indicated that Ae. aegypti were present throughout these months in high density (176 males and 97 females were found in the month of August 2011). However, Ae. albopictus appeared in the month of July (175 males and 218 females) which afterwards decreased in density in the month of August (50 males and 21 females) onward. Ae aegypti in the last month of collection (September 2011) was in considerable density (84 males and 77 females), indicated high prevalence of this vector (Fig., 2).

Various types of natural and artificial breeding containers were observed for immature stages for Aedes mosquitoes in all localities visited during the above mentioned period. Total ten breeding sites including desert coolers, ice cream cups, plastic cups, used automobile tires, gutter holes, tin containers, tree holes, discarded shoes, flower pots, water dishes/plates (Table II) were found outdoor / indoor. The most productive containers were used automobile tires for larval production indicated 94% positivity.

Inside the houses desert water coolers were found to be the major breeding site (85%) followed by discarded plastic cups (73%), flower pots (60%) and ice-cream cups (52%). Over all Culex larvae were most abundant 5,886 (45.8 %) during this period, followed by Ae. aegypti 4681 (36.43%) and Ae. albopictus 2,280 (17.74%) (Fig., 3).

Over a selected period of seven months (March-September, 2011), about 570 field collected Ae. aegypti female mosquitoes in 114 pools and 40 field collected Ae. albopictus females in 4 pools were screened for dengue viruses using antigen capture enzyme linked immunosorbent assays (ELISA). Among them, 31 pools of females Ae. aegypti and one pool of Ae. albopictus were found to be positive for dengue virus antigen respectively. The minimum infection rate (MIR) was 27.19% for Ae. aegypti and 25% for Ae. albopictus by ELISA (Table III).

DISCUSSION

Dengue virus infections are becoming a serious health problem with explosive outbreaks since 2006 in all over Pakistan particularly in highly populated urban cities like Lahore and Karachi. The number of reported cases are 22,000 from Lahore during 2011 which are highest among all outbreaks occurred in the last few years. Detection of natural mosquito infection with arboviruses forms a key element in any surveillance study and is valuable for incrimination and effective vector control measures. Dengue virus detection in field population of Ae. aegypti and Ae. albopictus serve as predictive model for forecasting impending outbreak (Lee et al., 2005). No previous research was reported regarding detection of dengue viruses from wild caught Aedes mosquitoes in Pakistan. This is the first report of DEN virus detection from adult females of Ae. aegypti and Ae. albopictus collected from different localities of Lahore, Pakistan using dengue virus specific monoclonal antibodies by antigen capture enzyme linked immunosorbent assays (ELISAs).

The two species of Aedes were found in the highly populated urban localities of Lahore. However, Aedes aegypti was most prevalent and widely distributed in all localities. The same was reported by Kumari et al. (2011) in India. Aedes albopictus appeared in rainy wet season i.e., in July-August which indicated that it was more dependent on rainfall as compared to Aedes aegypti, its larval density sharply increased from August-September.
Both species coexisted in many localities of Lahore also reported in China and Malaysia (Jian-feng et al., 2007)

Current study from March-September indicated that female Ae. aegypti were found with dengue viruses in 31 pools from 114 pools while Ae. albopictus indicated only in 1 pool positive out of total 4 pools screened for dengue viruses. These results indicated 27.71% (MIR) pools were positive for Ae. aegypti and 25% (MIR) pools for Ae. albopictus screened for these viruses. In another study conducted by Srisuphanunt et al., (2007) in Thailand, indicated an average of 18.3% (44 of 240) of Ae. aegypti tested individually, were found positive for dengue virus infection by ELISA during the period April-September 2000. The adult females of Ae. aegypti were collected from selected dengue sensitive area in Chonbun District, Bangkok. In current study the positive pools of collected Ae. aegypti mosquitoes were correlated with high risk of DF/DHF reported localities such as Mughalpura, Wahdat Colony, Chuburji, Officer’s Colony, Jallo Park, Manawan and Lahore Zoo during the study period. Clinical data indicated approximately ≥ 50% dengue cases occurred in these areas.

The observed patterns of dengue virus infection in mosquitoes detected by antigen capture ELISA coincides with clinical data and require more intensive investigation using improved diagnostic tools. Dengue virus detection using dengue specific monoclonal antibodies, broadly reactive against all four serotypes of dengue viruses as the capture antibody and detector MAB, broadly reactive against flaviviruses conjugated with horse reddish peroxidase indicated only positive pools which could be further screened by Toxo-IFA system for virus isolation using dengue virus serotypes specific MABs from DENV 1-4 and RT-PCR techniques can be employed for dengue virus amplification.

Acknowledgement

We are thankful to CDC Fort Collins, California USA for providing dengue specific Monoclonal antibodies (MAB D3-5C9-1), detector antibody (MAB CB6-C) and dengue antigens 1-4 used to detect dengue viruses from mosquito homogenates.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Month</th>
<th>Aedes aegypti</th>
<th>Aedes albopictus</th>
<th>Culex quinquefasciatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>March</td>
<td>393</td>
<td>0</td>
<td>1399</td>
</tr>
<tr>
<td>2</td>
<td>April</td>
<td>307</td>
<td>0</td>
<td>2567</td>
</tr>
<tr>
<td>3</td>
<td>July</td>
<td>251</td>
<td>393</td>
<td>3551</td>
</tr>
<tr>
<td>4</td>
<td>August</td>
<td>273</td>
<td>71</td>
<td>118</td>
</tr>
<tr>
<td>5</td>
<td>September</td>
<td>161</td>
<td>0</td>
<td>220</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1385</td>
<td>464</td>
<td>7855</td>
</tr>
<tr>
<td>Percentage</td>
<td>14.2%</td>
<td>4.7%</td>
<td></td>
<td>80.9%</td>
</tr>
</tbody>
</table>

Total mosquitoes = 9704
Fig., 1: Average Temperature, Rain Fall and Relative Humidity of Lahore from March -September, 2011

Fig., 2: Species Composition of Mosquitoes from March to September, 2011
Fig., 3: Number of larvae Collected from March to September, 2011

Table II: Various Breeding Habitats of *Aedes* Mosquitos from March to September, 2011 in Lahore

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Types of natural and artificial breeding containers</th>
<th>Percentage positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Desert coolers</td>
<td>85</td>
</tr>
<tr>
<td>2</td>
<td>Ice cream cups</td>
<td>52</td>
</tr>
<tr>
<td>3</td>
<td>Plastic cups</td>
<td>73</td>
</tr>
<tr>
<td>4</td>
<td>Used automobile tyres</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>Gutter holes</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Tin containers</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Tree holes</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>Discarded shoes</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Flower pots</td>
<td>60</td>
</tr>
<tr>
<td>10</td>
<td>Water dishes/plates</td>
<td>9</td>
</tr>
</tbody>
</table>

Table III: The numbers of field caught *Ae. aegypti* / *Ae. albopictus* positive for DEN viruses by ELISA

<table>
<thead>
<tr>
<th>Species of Mosquito</th>
<th>No. tested</th>
<th>Total No. of Pools</th>
<th>No. of +ve Pools</th>
<th>Percentage +ve Pools</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ae. aegypti</em></td>
<td>470</td>
<td>114^1</td>
<td>31</td>
<td>27.19</td>
</tr>
<tr>
<td></td>
<td>250^2</td>
<td>25</td>
<td>01</td>
<td>04</td>
</tr>
<tr>
<td><em>Ae. albopictus</em></td>
<td>40</td>
<td>04^2</td>
<td>01</td>
<td>25</td>
</tr>
</tbody>
</table>

*1 Each pool contains 05 mosquitoes; *2 Each pool contains 10 mosquitoes; *3 Larvae reared in laboratory indicating vertical transmission
REFERENCES


