Isolation and Serotyping of Dengue Viruses by Mosquito Inoculation and Cell Culture Technique: An Experience in Bangladesh

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Abstract
Dengue virus isolation and serotyping was carried out in 105 clinically-suspected cases of dengue fever by the mosquito inoculation and cell culture techniques to find a suitable virus isolation technique for a resource-constrained country like Bangladesh and to identify the serotypes responsible for the dengue outbreak in 2000. Virus was identified by direct fluorescent antibody technique (DFAT) using FITC-conjugated anti-flavivirus antibody and serotyping was done by indirect fluorescent antibody technique (IFAT) using serotype-specific monoclonal anti-dengue antibody. Of the 105 cases, 97 (92.4%) were found sero-positive for anti-dengue IgM by ELISA and considered as having dengue infection. Dengue virus was isolated from 44 (45.4%) of the 97 anti-dengue IgM positive cases by Aedes aegypti mosquito inoculation and from 24 (24.7%) cases by C6/36 cell culture. The dengue virus isolation rate was significantly higher (p < 0.01) by the mosquito inoculation than the cell culture inoculation technique. The isolation rate was also higher in patients who presented on the first day of the fever than in those who presented later. Dengue virus was isolated from patients who had a significantly higher mean body temperature (p < 0.01) and the mean anti-dengue IgG level was significantly lower in those from whom virus was isolated (p < 0.01). The serotyping revealed isolation of DEN-3 from 31 (70.5%) cases, which was the predominant serotype, followed by the isolation of DEN-1 from 6 (13.6%), DEN-4 from 4 (9.09%) and DEN-2 from 3 (6.8%) cases out of the total 44 positive cases. This indicates the hyperendemicity of dengue in Dhaka with epidemic potential, which needs careful monitoring by laboratory-based surveillance in order to predict the nature of future outbreaks. This is the first report of dengue virus isolation by mosquito inoculation and C6/36 cell culture in Bangladesh. It was revealed from the study that, for routine surveillance of dengue infection, isolation of virus by mosquito inoculation is a more sensitive, economical, easy and sustainable method. However serotyping by IFAT is more convenient in cell culture.

Keywords: Dengue, dengue haemorrhagic fever, virus isolation, cell culture, fluorescent antibody technique, serotyping, serotypes, Dhaka, Bangladesh.

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Introduction

Dengue is an acute arboviral human disease caused by four serotypes of dengue viruses, which are closely related but antigenically distinct\(^1\). Though classical dengue is a self-limiting febrile illness, its life-threatening severe forms, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) can be fatal unless the associated plasma leakage is treated early. The untreated case-fatality rate may be as high as 40%\(^2,3\). Currently, it is an emerging/re-emerging disease throughout the tropical and subtropical areas and nearly half of the world’s population is at risk\(^4,5,6\). The first reported outbreak of dengue in Bangladesh was the “Dacca fever” recorded in 1964\(^7,8\). After that, the magnitude of infection was generally unknown until the outbreak of dengue in 1999\(^9\). Recently, large outbreaks of DF/DHF have been recorded in Bangladesh\(^10\). During the outbreak in 2000, 5,551 cases and 93 deaths and in 2001, 2,430 cases and 44 deaths were officially recorded\(^10\) and reported\(^11\). These cases were diagnosed clinically, except for a few which were diagnosed serologically. The mosquito inoculation technique\(^12\) and cell culture in C6/36 cell line\(^13\) are widely practised for the isolation of dengue viruses and hence were chosen in the present study as the tool for virus isolation. Serotyping was also carried out to explore the serotypes responsible for the outbreak to compare the different aspects of the methods, including sensitivity and suitability to set up and sustain, in a resource-constraint situation in Bangladesh.

Materials and methods

The study was carried out among 105 clinically-suspected cases of dengue fever during an outbreak which started in June 2000. The patients were selected from the outpatient and inpatient departments of Paediatrics and Medicine and also from patients seeking diagnostic facility in the department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU) during June-December 2000. Inclusion criteria were: patients having fever presented within five days from the onset; body temperature above 100°F at the time of sample collection; fulfilling the WHO case definition of DF and DHF\(^6\), and agreeable to donate two blood samples during the early and convalescence stages, irrespective of age and sex. Exclusion criteria were: febrile cases of more than five days with definite source of infection (e.g. respiratory tract infection, urinary tract infection, meningitis); chronic illnesses like tuberculosis, bronchial asthma, congenital heart diseases, renal failure; and history of bleeding tendency since birth. With all aseptic precautions, 5 ml of venous blood was collected from patients during their first attendance and transferred to a sterile vacutainer. After the separation of serum, it was transferred to labelled cryovials and preserved at −70°C fridges immediately till inoculated into mosquitoes and C6/36 cells for the isolation of viruses and detection of antibody. A second sample of 5 ml venous blood was collected during convalescence 10-14 days after the collection of the first sample and used for the detection of antibody level by ELISA. Blood samples were collected from healthy individuals used for negative control.
Detection of dengue antibody by ELISA

A commercially produced indirect ELISA (Pathozyme Dengue, Omega Diagnostics Limited, Scotland, UK) was used for the detection of anti-dengue IgM and IgG levels from both acute and convalescent sera. The test was performed according to the instruction of the manufacturer and an antibody index (AI) was calculated for both IgM and IgG comparing optical density (OD) of sample and average OD of low positive controls. AI > 1 was considered as positive.

Isolation of dengue viruses by mosquito inoculation technique

Mosquito inoculation technique in *Aedes aegypti* mosquitoes in the department of Virology, BSMMU, was used for virus isolation. One to five days old adult female mosquitoes were collected from fresh laboratory-reared mosquito colony in a cotton-stopper test tube and were immobilized by placing the tube in an ice-filled beaker for 15 minutes. Each mosquito was inoculated intra-thoracically with 0.17 µl of diluted patient’s serum (1:5 dilution with PBS diluents prepared with PBS pH-7.4 with 0.5% gelatin and 5% foetal calf serum). For each patient, 50 mosquitoes were inoculated. After inoculation, mosquitoes were transferred into a labelled plastic drinking glass covered with a piece of mosquito net and incubated at 30 ± 2°C with 75-85% humidity for 14 days and fed with 10% sugar solution. Keeping the plastic glass for 10 minutes in deep freezer killed the survivors. The heads of the freshly killed mosquitoes were dissected along with salivary gland and head squashes were prepared in pre-marked circles on glass slides with the help of 1% silicon-coated cover slip. After air-drying, the mosquito tissue was fixed with chilled acetone at -20°C for 15 minutes and stored at -70°C until the identification of virus isolates and their serotyping.

Cell culture

Cell culture was done in C6/36 cell line with slight modification in the Virology Laboratory, Bangladesh Livestock Research Institute, Savar, Dhaka. *Aedes albopictus* cells (C6/36) adapted to grow at 33°C were used for virus isolation. Cells were maintained on Eagles Minimum Essential Media (MEM) in Earle’s balanced salt solution supplemented with 5% foetal calf serum (FCS), 2% L glutamine, 1% sodium bicarbonate, 2% penicillin, streptomycin, amphotericin B and 1% non-essential amino acid. Twenty-five sq cm screw-capped flat-bottomed flask was seeded with 3 ml of cells at a concentration of 4×10⁵/ml. Fifty µl of patients serum was inoculated into each flask containing confluent mono layer of cells and 200 µl of PBS (pH-7.4) was added. The inoculated flask was incubated at 33°C for 45 minutes with frequent agitation. Five ml of maintenance media (growth media except 1% FCS and 2% sodium bicarbonate) was added and incubation at 33°C maintained for seven days. Regular observation was kept under inverted microscope to detect visible cytopathic effect (CPE). During the incubation period the pH of the medium was maintained at approximately 7 for maximum recovery of virus. At the end of each passage, irrespective of the presence of CPE, slides were prepared from inoculated cells for the
identification of viral antigen by DFAT. Cell suspension was spotted on pre-marked circles of glass slides, air-dried and fixed with acetone at –20°C. After air-drying the fixed slides were preserved at –70°C until the identification of isolated viruses and their serotyping.

**Identification and serotyping of dengue viruses**

The presence of viral antigen of the isolated viruses on glass slides was detected by DFAT using FITC-conjugated anti-flavivirus antibody\(^{(14)}\). Serotyping of the isolated viruses was carried out by IFAT on both mosquito head squashes and spotted slides of cell culture materials using serotype-specific anti-dengue monoclonal antibody and FITC-conjugated goat anti-mouse IgG\(^{(15,18)}\).

**Statistical analysis**

The numerical data obtained from the study were analysed and the significance of difference was estimated by using statistical methods. Data were expressed in frequency, percentage, mean and standard deviation as applicable. Comparison between groups was done by student’s ‘t’ test and ‘Chi square’ test as applicable. All data were analysed by using the computer-based SPSS programme. P value less than 0.05 were considered as significant.

**Results**

Out of the 105 clinically-suspected DF patients, 97 were found positive for dengue IgM antibody by ELISA either in the first sample or in convalescent sera and were considered as recent dengue infections, while eight cases were found negative for antibody. By virus isolation, these were diagnosed as non-dengue febrile illnesses and, therefore not considered for further analysis. In the study, the involvement of all age groups, with adult predominance, was reflected. The mean age of the study patients was 29.2 \(\pm\) 12.9 years and no significant sex difference was found. Out of the 97 dengue patients, dengue virus was isolated from 44 (45.4%) cases by the mosquito inoculation technique and from 24 (24.7%) cases by cell culture (Table 1). The rate of dengue virus isolation was significantly higher by mosquito inoculation than by cell culture inoculation technique (\(p<0.01\)). Dengue virus isolation by mosquito inoculation was positive in 44 cases and negative in 61 cases, whereas among the 44 positive cases by mosquito inoculation, virus was isolated only in 24 (54.5%) cases by cell culture and 20 (45.5%) cases did not yield any virus. All the mosquito negative cases were also negative by cell culture.

**Table 1.** Isolation rate of dengue viruses by mosquito inoculation and cell culture (n=97)

<table>
<thead>
<tr>
<th>Method of virus isolation</th>
<th>Number of cases from which dengue virus was isolated</th>
<th>Isolation rate</th>
<th>(*p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mosquito inoculation</td>
<td>44</td>
<td>45.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Cell culture</td>
<td>24</td>
<td>24.7</td>
<td></td>
</tr>
</tbody>
</table>

\(^{*}\) p value reached from ‘chi square’ analysis
The serotyping revealed that all four dengue serotypes were isolated from the patients, DEN-3 being the predominant (70.5%) serotype during the study period (Figure 1). The rate of isolation of dengue viruses was higher in the serum collected on the first day of fever in both the isolation systems. Virus was isolated from 7 (77.7%) cases by the mosquito inoculation technique and from 4 (44.4%) cases by cell culture, out of the 9 patients whose serum were collected on the first day of fever. The rate of isolation decreased from the second day onwards up to the fifth day by both the techniques. Thus, on the fifth day, of the 26 sera, virus was isolated only from 5 (19.2%) cases by mosquito inoculation and from 2 (7.7%) cases by cell culture (Table 2).

Table 2. Dengue virus isolation rates among dengue cases by mosquito inoculation and cell culture by day of fever

<table>
<thead>
<tr>
<th>Day of fever at which the virus was isolated</th>
<th>Number of cases (n=97)</th>
<th>Positive by mosquito inoculation</th>
<th>Positive by cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st day</td>
<td>9</td>
<td>7 (77.7)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>2nd day</td>
<td>12</td>
<td>6 (50.0)</td>
<td>3 (25.0)</td>
</tr>
<tr>
<td>3rd day</td>
<td>27</td>
<td>14 (51.8)</td>
<td>9 (33.3)</td>
</tr>
<tr>
<td>4th day</td>
<td>23</td>
<td>12 (52.1)</td>
<td>6 (26.1)</td>
</tr>
<tr>
<td>5th day</td>
<td>26</td>
<td>5 (19.2)</td>
<td>2 (7.7)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate rate of virus isolation.

In the present study, it was found (Table 3) that the mean temperature (101.97±1.2°F and 102.0±1.4°F) among the patients from whom virus was isolated was significantly higher than that (101.11±1.4°F and 101.32±1.4°F) of those from whom virus could not be isolated by both the isolation systems (p=0.001 in mosquito inoculation and p=0.041 in cell culture).

Although the mean level of the IgM antibody index was lower in patients where virus was isolated than that of the patients from whom virus was not isolated (Table 4), the difference was not statistically significant (p>0.05), whereas virus isolation in the presence of IgG antibody was lower and the mean AI of IgG was also lower (1.14±1.1 vs 2.35±1.5) in mosquito inoculation and (0.68±0.7 vs 2.17±1.5) in cell culture) in the sera from which virus was isolated than those from whom virus was not isolated by both the isolation techniques was statistically significant (p<0.01).
Table 3. Association between virus isolation and body temperature of dengue patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n</th>
<th>Mean body temperature at °F</th>
<th>SD</th>
<th>Range</th>
<th>*p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolation by mosquito inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>44</td>
<td>101.97</td>
<td>1.2</td>
<td>100.0-104.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>53</td>
<td>101.11</td>
<td>1.4</td>
<td>100.0-104.0</td>
<td></td>
</tr>
<tr>
<td>Virus isolation by cell culture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
<td>102.00</td>
<td>1.4</td>
<td>100.0-104.0</td>
<td>0.041</td>
</tr>
<tr>
<td>Negative</td>
<td>73</td>
<td>101.32</td>
<td>1.4</td>
<td>100.0-104.0</td>
<td></td>
</tr>
</tbody>
</table>

*p value reached from unpaired student 't' test analysis.

Table 4. Relationship between virus isolation and antibody level in acute sera of dengue patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>Mean of antibody index (AI)</th>
<th>SD</th>
<th>Range</th>
<th>*p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM antibody level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mosquito +ve</td>
<td>44</td>
<td>1.07</td>
<td>0.8</td>
<td>0.26-4.68</td>
<td>0.321</td>
</tr>
<tr>
<td>Mosquito -ve</td>
<td>53</td>
<td>1.80</td>
<td>0.9</td>
<td>0.14-4.97</td>
<td></td>
</tr>
<tr>
<td>Cell culture +ve</td>
<td>24</td>
<td>1.07</td>
<td>0.9</td>
<td>0.26-4.68</td>
<td>0.512</td>
</tr>
<tr>
<td>Cell culture -ve</td>
<td>73</td>
<td>1.21</td>
<td>0.9</td>
<td>0.14-4.97</td>
<td></td>
</tr>
<tr>
<td>IgG antibody level</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mosquito +ve</td>
<td>44</td>
<td>1.14</td>
<td>1.1</td>
<td>0.09-2.46</td>
<td>0.001</td>
</tr>
<tr>
<td>Mosquito -ve</td>
<td>53</td>
<td>2.35</td>
<td>1.5</td>
<td>0.11-5.72</td>
<td></td>
</tr>
<tr>
<td>Cell culture +ve</td>
<td>24</td>
<td>0.68</td>
<td>0.7</td>
<td>0.09-2.46</td>
<td>0.001</td>
</tr>
<tr>
<td>Cell culture -ve</td>
<td>73</td>
<td>2.17</td>
<td>1.5</td>
<td>0.11-5.72</td>
<td></td>
</tr>
</tbody>
</table>

*p value reached from unpaired student 't' test analysis.

Discussion

Dengue haemorrhagic fever is hyperendemic in many of the South-East Asian countries, and in some countries, DHF epidemics are occurring at regular intervals\(^5,6\). Though laboratory diagnosis is an essential part of any definitive evaluation of dengue infection\(^6\) and isolation of dengue virus is the confirmatory laboratory test for the diagnosis of dengue infection\(^6\), only a few outbreaks have been adequately studied virologically due to difficulties in isolating dengue viruses. There was no facility for isolating dengue viruses in Bangladesh. Therefore, the study was undertaken to compare two isolation techniques and explore the scope for setting up an easy,
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Economical, sensitive and specific laboratory method for the isolation and serotyping of dengue viruses suitable for Bangladesh. With a view to achieving these objectives, we used the widely-practised mosquito inoculation and C6/36 cell line culture techniques for the isolation of dengue viruses. We used DFAT for detecting dengue virus in mosquito and C6/36 cells and carried out serotyping of the isolates by IFAT to identify the serotypes responsible for the dengue outbreak of 2000.

The superiority of the mosquito inoculation technique over cell culture for the isolation of dengue virus is well-known\(^{12,17,18,19}\) and this has also been reflected in the present study. Dengue virus was isolated from 45.4% of the patients by the mosquito inoculation technique whereas in cell culture, the isolation rate was only 24.7%. Similar findings have been reported from other studies\(^{17,18,19,20}\). The mosquito inoculation technique consistently yielded a higher isolation rate than the cell culture technique from samples collected in the first to the fifth day of fever (Table 2). It was previously reported that the pre-inoculation of human sera in mosquitoes facilitated the isolation and identification of virus in cell culture by enhancing the infective virus titer\(^{21}\). We also had similar findings. An important finding of the present study with regard to the isolation of virus by mosquito inoculation was that three samples collected during the third and fourth day of fever failed to be isolated by cell culture. However, after DFAT, positivity in these serum-inoculated mosquito head squash, the infected mosquito abdomens passed into cell culture resulted in visible CPE within 72 hours and in positive isolation. This finding indicates higher sensitivity of mosquito inoculation.

The low isolation rate in cell culture during the present study was perhaps due to less sensitivity and the toxic effect of serum on the cells. During the present study C6/36 cells frequently showed toxicity of serum even when it was diluted 1:5. The toxicity was found both in negative control serum and test specimens. Frequent susceptibility of C6/36 cells to serum toxicity was also reported from other studies\(^{22,23}\). In a few instances, although there was no visible CPE in the cells during the first inoculation, following the identification of dengue virus antigen in the cells by DFAT, the second passage yielded CPE. A similar absence of visible CPE was also noticed in another study where lower dilution of serum was negative for CPE production\(^{13}\). There have been varied reports on the efficiency of dengue virus isolation by cell culture. Thus, Singh and Paul (1969) reported frequent CPE produced both in C6/36 and Vero cells\(^{21}\) whereas Gubler et al (1984) reported that some viruses grow slowly in C6/36 cells\(^{24}\).

In the present study, it was evident that all four serotypes of dengue viruses were circulating in Dhaka city in 2000, with a predominance of DEN-3. A recent serological study by haemagglutination inhibition test conducted in Chittagong Medical College, situated in the southeastern part of the country demonstrated that except for DEN-1, the other three serotypes were present in Chittagong\(^{24}\). The presence of DEN-3 in Dhaka city was also documented during the first reported outbreak of dengue in Bangladesh in 1964, confirmed by virus isolation and serology\(^{7,8}\). A serological survey was carried out among schoolchildren of Dhaka city in 1982-1983 where among 2,465 samples, 278 (11%) were serologically positive against DEN-1\(^{25}\).
The presence of DEN-1 was also reported in Dhaka during another study in 1980\(^{(26)}\). During the outbreak in 2000, DEN-3, DEN-2 and DEN-4 were detected from the serum of clinically-suspected dengue patients of Dhaka city by the polymerase chain reaction (PCR) analysis\(^{(27)}\). Lack of detection of DEN-1 in this study was perhaps due to fewer number (45) of study subjects.

During the present study, the specific perinuclear fluorescence in the infected neurocytes of mosquito head squash and C6/36 cells were easily identified by DFAT. Similar findings about DFAT were reported in another study\(^{(14)}\). Although background fluorescence created difficulty in IFAT of head squash of mosquito, there was no background fluorescence while carrying out IFAT in C6/36 cells for serotyping. Similar findings were reported from other studies\(^{(13,22)}\).

In the present study, the virus isolation rate was 45.4\% by mosquito inoculation. The isolation rate of dengue viruses by mosquito inoculation method varied widely in different studies carried out elsewhere in the world. It was 32\% in Central Java, Indonesia\(^{(28)}\), during an epidemic of DEN-3 in 1978, while in Jakarta, it was 34\% in 1975-1978\(^{(17)}\). A similar rate (33.3\%) was reported in Pacific Island, Kingdom of Tonga, in 1974 during an epidemic of DEN-2\(^{(29)}\). A lower rate (20\%) and a higher rate (98\%) were also reported in other studies\(^{(30,31)}\). The high rate of virus isolation in the latter study was claimed to be due to the collection of sera at a very early febrile period. The rate of dengue virus isolation employing C6/36 cell culture was 24.7\% in the present study. Report on the efficiency of isolation by mosquito inoculation and C6/63 cell culture is not available. However, a lower rate of dengue virus isolation in C6/36 cell culture has been reported in some studies. An isolation rate of 16.7\% was found in Rio de Janeiro, Brazil\(^{(32)}\), in 1990-91 and 19.2\% was reported from Puerto Rico in 1981\(^{(28)}\). Singh and Paul (1969) had reported a very high isolation rate (88\%) in C6/36 cell culture in India\(^{(21)}\).

The dengue virus isolation rates are influenced by some factors which include the duration of viremia, the number of days after the onset of fever, body temperature and antibody level\(^{(33)}\). It was found during the present study that the virus isolation rate was higher during the early days after the onset of fever (Table 2). It was also found (Table 3) that the mean temperature (101.97±1.2°F) among the 44 patients from whom virus was isolated was significantly higher than that (101.11±1.4°F) of those from whom virus could not be isolated (p<0.01). A similar finding was also reported from Cambodia\(^{(30)}\) where the mean temperature at which the virus was isolated from patients’ sera was 100°F in comparison with 99°F of the patients from whom virus was not isolated (p < 0.01). Vaughn et al (2000) also reported a similar correlation between higher body temperature and viremia\(^{(19)}\). It was also found in the present study that dengue virus isolation was affected by the presence of antibody. Virus was isolated in patients with a lower level of IgM and IgG antibody in the acute stage of the disease (Table 4). Thus, in conclusion, it can be suggested that for the routine surveillance of dengue infection, the isolation of virus by mosquito inoculation is a sensitive, economical and easy method, as, in our experience, maintenance of mosquito colony is easier and less expensive than maintenance of cell culture. However, serotyping by IFAT is more convenient in cell culture.
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