Dengue and Dengue Haemorrhagic Fever Outbreak in Pondicherry, South India, during 2003–2004: Emergence of DENV-3


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Abstract

During October 2003 – February 2004, a suspected outbreak of dengue fever and dengue haemorrhagic fever occurred in Pondicherry and the present investigation was carried out to confirm the dengue epidemic through clinical, serological and molecular studies. Analysis of clinical symptoms showed that the younger age group of 1–15 years was the most affected (65%, n=28). Of the 79 serologically-confirmed dengue cases at hospitals and clinics, 64 were positive for IgM antibodies reflecting current (primary) infections. Viral infection was also confirmed by testing IgM-positive samples by intracerebral inoculation of Toxorhynchitis splendens and indirect immunofluorescence using anti-dengue virus antibodies. Nested reverse transcriptase (RT) PCR assay (capsid region) yielded amplicon of the size 290 bp, diagnostic for DENV-3 for 32 samples. Sequence analysis of the amplicon confirmed the serotype and phylogenetic analysis of sequence showed closeness of the Pondicherry isolate to the Peru/Taiwan strains. Three health blocks were found to be the risk areas for the transmission of dengue infection, which also recorded moderate-to-high breeding of Aedes aegypti, and to a lesser extent of Ae. albopictus. High breeding of Ae. aegypti and detection of clustering of cases indicated this vector species playing a major role in the outbreak of dengue in Pondicherry.

Keywords: Dengue outbreak, ELISA, RT-PCR, DENV-3, Aedes aegypti, Pondicherry.

Introduction

Over the last 20 years, classical dengue fever (DF) and its more severe forms, dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS), have emerged as the most important arthropod-borne viral diseases in humans. During this period, dengue has spread throughout the tropical regions worldwide, principally in urban settings. Up to 100 million cases of DF are estimated to occur annually, and roughly 450 000 cases of DHF/DSS are reported, while approximately 2.5 billion people live in areas at risk of dengue virus transmission. The dramatic spread of epidemic dengue fever and the emergence of DHF/DSS occurred after World War II in South-East Asia, where DHF is now one of the leading causes of hospitalization and death. This pattern of epidemic dengue fever and emerging DHF is also being observed in India where it is spreading at an alarming rate, not only in urban settings but also in rural areas where Aedes aegypti has penetrated under the impact of safe drinking water schemes.

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Dengue fever is caused by four distinct serotypes of dengue virus, which are transmitted among humans mainly by *Ae. aegypti*, the urban vector, and *Ae. albopictus* as a maintenance vector in suburban and rural areas. The lack of a vaccine or a cure for this disease make active surveillance systems with appropriate laboratory support essential to provide early warning of dengue fever epidemic and to initiate effective vector control measures. It is crucial to determine the circulating DENV serotype(s) as a previous infection with one of the four serotypes can be an important risk factor for developing DHF/DSS. From the month of October 2003 through February 2004, 92 patients with suspected viral syndrome were reported from different hospitals in Pondicherry. Blood samples collected from these cases were received at the Vector Control Research Centre (VCRC) and screened for anti-dengue antibodies as well as the etiological agent. We conducted this passive survey to identify the circulating DENV serotype that caused the outbreak.

**Materials and methods**

**Study area**

Pondicherry is a coastal town situated in the eastern peninsular India between 11.45° to 12.15° latitude North and 79.35° to 80.00° longitude East. The population of the Pondicherry urban area is about 0.24 million. The clinically suspected dengue cases started reporting from the month of September 2003, which coincided with the rains which usually commence during this month and last for about 4 months in this part of the country. The highest number of cases was reported during the month of November 2003. The mean monthly rainfall and the total rainy days for this period (September till December) were 202.08 mm and 45 days respectively. Although some of the suspected dengue cases originated from the neighbouring Tamil Nadu state, the towns/villages where they resided are very close to or interspersed within the Pondicherry Union Territory and, hence, are geographically interspersed within Pondicherry area.

**Blood samples and virus strains**

A network of passive survey, constituting local government and private hospitals and also private clinics, was established for receiving the blood samples from clinically suspected dengue cases identified as per WHO classification. A total of 92 samples were received from Pondicherry and nearby villages under Pondicherry Union Territory (Figure 1). Blood samples were collected 3–7 days post-onset of fever. The serum obtained from the blood samples was subjected to various investigations or stored at −70 °C till used. Viral stocks of four dengue serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) were received from the National Institute of Virology, Pune, India.

**Screening for anti-dengue antibodies**

Serological testing of blood samples was undertaken using an enzyme-linked immunosorbent assay (ELISA) kit (Immunodagnostica, Germany), as per the procedure prescribed by the manufacturer.

**Clinical history of patients**

A complete clinical history, laboratory and other parameters pertaining to dengue infection diagnosis such as tourniquet test, erythrocyte sedimentation rate (ESR), platelet count, etc., were taken and recorded at hospitals.
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**Detection of virus through mosquito inoculation and immunofluorescence**

The method for mosquito inoculation and detection through an indirect immunofluorescence assay (IFA) was essentially as per the method of Dhanda and Ilkal, except that fourth instar larvae of *Toxorhynchitis splendens* were used instead of *Ae. aegypti* mosquitoes, for intracerebral inoculation of serum samples from dengue-suspected cases.

**Reverse transcription and PCR amplification**

RNA was extracted from serum samples using Tri-Reagent® (MRC Inc., USA). The presence
of dengue viral RNA in the sera samples was checked by a two-step RT-PCR according to the method of Harris et al. [8]. The nucleotide sequences of dengue serotype-specific primers [8] are presented in Table 1. The amplified products were run on 2% agarose gel in 1X TAE buffer and the identity of the serotype of the virus was determined by the size of the amplicon observed. [8] The genetic relatedness of the Pondicherry isolate with other isolates of DENV-3 was deduced through sequencing the capsid gene and phylogenetic analysis. For this the capsid gene was amplified using the procedure stated above, but using primers D1 and TS3. The amplicons (290 bp) were purified using Qiaex II kit (Qiagen, Germany) and sequenced using Big dye terminator in ABA 737 sequencer. The nucleotide sequences obtained were aligned with those of global isolates available in the GenBank and a phylogenetic tree was constructed employing UPGMA and Clustal X software.

**Entomological surveys**

*Aedes* larval surveys were carried out in localities reporting dengue cases and also in adjacent areas (Figure 1) from the month of September 2003 through December 2003, in order to find any correlation of entomological parameters with that of dengue cases. The sample size for the survey was based on the criterion indicated by WHO, [9] and the selected households were thoroughly examined for *Aedes* breeding. The immatures collected from each household were reared individually to adult stage for species identification, and based on emergence data, species-wise house index (HI) and container index (CI) were estimated.

**Results and discussion**

**Serological outcome**

Out of the 92 blood samples collected from suspected dengue cases (as per the WHO criteria), 79 were found positive for anti-dengue antibodies (either for IgM or IgG or both). Forty (50.6%) of them were found positive for only IgM antibodies. These results indicated the occurrence of current (primary) as well as secondary infections in Pondicherry.

**Clinical signs and symptoms**

Of the 79 IgM/IgG-positive cases, 57 (72.15%), 14 (17.72%) and 8 (10.13%) conformed to DF, DHF and DSS respectively as per WHO case definition. An analysis of the clinical picture showed that fever was the most common symptom (98%), followed by hepatomegaly – 46.8% and bleeding – 41.7%. The other clinical parameters were: low platelet count (<100 000/ml) – 29.1%, shock – 25.3%, myalgia – 16%, tourniquet test positive – 6.3%, and convulsion – 1.2% (Table 2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’ to 3’)</th>
<th>Position</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>TCA ATA TGC TGA AAC GCG CGA GAA ACC G</td>
<td>134–161</td>
<td></td>
</tr>
<tr>
<td>TS1</td>
<td>CGT CTC AGT GAT CCG GGG G</td>
<td>568–586</td>
<td>482</td>
</tr>
<tr>
<td>TS2</td>
<td>CGC CAC AAG GGC CAT GAA CAG</td>
<td>232–252</td>
<td>119</td>
</tr>
<tr>
<td>TS3</td>
<td>TAA CAT CAT CAT GAG ACA GAG C</td>
<td>400–421</td>
<td>290</td>
</tr>
<tr>
<td>TS4</td>
<td>TGT TGT CTT AAA CAA GAG AGC TC</td>
<td>506–527</td>
<td>389</td>
</tr>
</tbody>
</table>

**Table 1**: Dengue serotype specific primers used for the detection of dengue virus serotypes
Epidemiological findings

Case distribution

Geographical

Most of the dengue cases came from three areas, viz. Pondicherry urban and its sub-urban areas (Ozhukarai and Ariankuppam), while a few cases were reported from rural areas. Investigations revealed clustering of cases in certain areas. The number of cases ranged from 1 to 3 plus (Figure 1). The possible reasons for the clustering of cases in these areas could be favourable vector breeding sites such as disposable plastic containers (tea cups), discarded grinding stones, coconut shells and air-conditioners.

By age groups

The analysis of the antibody-positive cases with respect to different age groups showed the following: the highest IgM antibody prevalence (66%, n=28) was among children in the age group of 0–15 years, as against other age groups (15–30, 31–45 and >45 years) with the prevalence ranging between 38–60%. The 31–45 years age group showed higher prevalence (92%) of IgG antibodies, while the younger age group exhibited higher percentage of IgM antibodies. The IgM antibody prevalence decreased with increasing age while the IgG antibody prevalence was the reverse (Figure 2).

**Table 2: Signs and symptoms of serologically-confirmed dengue cases**

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Clinical symptoms</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DF and DF with unusual bleeding (n=57)</td>
<td>DHF (n=14)</td>
</tr>
<tr>
<td>1.</td>
<td>Fever</td>
<td>57 (100)</td>
</tr>
<tr>
<td>2.</td>
<td>Myalgia</td>
<td>6 (10.5)</td>
</tr>
<tr>
<td>3.</td>
<td>Haemorrhage</td>
<td>11 (19.2)</td>
</tr>
<tr>
<td>4.</td>
<td>Hepatomegaly</td>
<td>20 (35)</td>
</tr>
<tr>
<td>5.</td>
<td>Shock</td>
<td>12 (21)</td>
</tr>
<tr>
<td>6.</td>
<td>Convulsions</td>
<td>0 (0)</td>
</tr>
<tr>
<td>7.</td>
<td>Elevated ESR (&gt;20%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>8.</td>
<td>Tourniquet +ve</td>
<td>0 (0)</td>
</tr>
<tr>
<td>9.</td>
<td>Platelet count (&lt;100 000/μl)</td>
<td>1 (1.7)</td>
</tr>
</tbody>
</table>

**Figure 2: Prevalence of anti-dengue antibodies (IgG and IgM) among different age groups**

![Figure 2](image-url)
Virus isolation by mosquito inoculation

Out of the 20 sera samples tested, dengue virus was isolated from 10 samples by mosquito inoculation technique, the presence of which was confirmed by indirect immunofluorescence assay (IFA).

Molecular confirmation of dengue isolate

RT-PCR was carried out for all the samples, irrespective of their antibody positivity, using serotype specific primers for capsid region of the dengue virus.\(^8\) Fifteen samples gave an amplicon of the size 290 bp, which is diagnostic of serotype-3 (Figure 3). Out of these 15 samples, 9 were found positive for IgM also. Four samples, which were negative for IgM, were positive by RT-PCR assay. Two of these samples came from a female patient (45 years) and her son (15 years). While the mother manifested clinical features of DHF, she had no anti-dengue IgM antibodies while her son had DF and anti-dengue IgM. Sequencing of the capsid region of DENV-3 isolate from the woman and alignment of the nucleotide sequence obtained with the sequence database (Entrez) showed >95% homology with the DENV-3 sequences, thus confirming the identity of the virus as DENV-3. The phylogenetic tree constructed based on the nucleotide sequences of DENV-3 isolates available in the GenBank and of Pondicherry isolate showed that the latter was genotypically close to the Taiwanese/Peruvian strains.

**Figure 3:** A sample gel showing the identification of serotype of dengue virus isolate from Pondicherry obtained during 2003-2004 outbreak by RT-PCR assay

Lane 1: Negative Control, Lanes 2-18: Patient blood samples, Lane -19: Positive control, M – 100 bp ladder
**Entomological findings**

Entomological surveys were carried out for four months during the period of the outbreak (September through December) when a total of 39 areas in Pondicherry town were surveyed and *Aedes* breeding was recorded in 36 areas. Both *Ae. aegypti* (76%) and *Ae. albopictus* (24%) were recorded from the emergence of immature samples. Among different areas, it was difficult to identify the transmission foci as the vector, *Ae. aegypti*, is a day-biting mosquito and hence the infection may take place at the workplace in the case of adults and in schools in the case of children. However, the analysis of the occurrence of cases in different areas of Pondicherry indicated the existence of at least five high-risk areas for the transmission of dengue. These were: (i) Bharathi Nagar (Karuvadikuppam block); (ii) Pakkumudayanpet (Saram block) and Indira Nagar (Thattanchavadi block), which are located adjacent to each other; and (iii) Muthialpet (Muthialpet block); (iv) Chinnakadai Street (Pondicherry town block); and (v) Thenral Street (Olandai Keerpalayam block). While there was continuous reporting of dengue cases for a period of three months from Karuvadikuppam (September till November) and Thattanchavadi health blocks (October till December), it was only for two months in Saram (October and November) and Ozukarai blocks (November and December). The transmission risk areas are located at a distance of 2–5 km from each other. However, an analysis of the prevalence of anti-dengue IgM antibodies among children below 5 years of age in the area showed that, out of the 10 clinically suspected cases in this age group, 8 were found positive for IgM antibodies. Three of the latter cases were from Indira Nagar and an area adjacent to it (Kamaraj Nagar), indicating that this area could be a transmission focus. Topographically, the clustering of dengue cases could be observed in: (i) coastal areas; (ii) the town proper; and (iii) peri-urban localities of Pondicherry, indicating the occurrence of cases irrespective of the ecological situation in a coastal town such as Pondicherry. The main transmission foci of dengue had moderate-to-high HI and CI values (Figure 1).

In most of the areas, both *Ae. aegypti* and *Ae. albopictus* were found to coexist, with the HI and CI of the former species ranging from 4–26 and 3–58 and the latter ranging from 3–26 and 1–58, respectively, in different areas (Figure 1). In the absence of data of *Aedes* species breeding indoors and outdoors, it is not possible to demarcate the ecological boundaries of *Ae. aegypti* and *Ae. albopictus*. *Ae. aegypti*, with its preference to breed in artificial (man-made) containers and generally encountered indoors, is strongly anthropophilic and accounts for clustering of cases because it bites more than one person due to its nervous nature of feeding. On the other hand, *Ae. albopictus* prefers to breed in natural breeding habitats close to natural vegetation. Gratz,\[10\] who made a critical review of world literature on the vector status of *Ae. albopictus*, sums up that *Ae. albopictus* probably serves as a maintenance vector of dengue in rural areas of dengue endemic countries of South-East Asia and the Pacific islands. Since in the present study the emergence of *Ae. aegypti* (76%) far exceeded that of *Ae. albopictus* (24%), it is likely that *Ae. aegypti* played a critical role in the present outbreak in Pondicherry. Chen et al.\[11\] also observed co-existence of both *Ae. aegypti* and *Ae. albopictus* in coastal areas in Malaysia, the proponderance of *Ae. aegypti*, both indoors and outdoors, was seen as playing a major role in the transmission of DF/DHF.

Thus, the clinical, serological, molecular biological investigations confirmed that the
suspected outbreak was indeed dengue, and that the etiological agent was DENV-3. The involvement of this serotype in dengue outbreaks has also been reported from nearby cities (about 150 km) such as Chennai\textsuperscript{12} and Vellore earlier\textsuperscript{13} and, more recently, in the northern parts of the country.\textsuperscript{14} People from Pondicherry travel frequently to these places and it is likely that they carried the infection. This is the first confirmatory report of an outbreak of dengue in Pondicherry, although a dengue-like fever had been reported during 1964–65.\textsuperscript{15} The emergence of DF as a major public health problem in India with reports of its outbreaks in several parts of the country and all the four serotypes being involved in the epidemics at different places\textsuperscript{16} calls for constant surveillance and for generating public awareness and appropriate action by health authorities.

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**References**


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