During 2012, dengue fever (DF) and dengue haemorrhagic fever (DHF) in countries of the World Health Organization (WHO) South-East Asia Region showed an overall increasing trend. A total of 257,204 cases and 1,229 deaths were reported, with a case–fatality rate (CFR) of 0.47. Out of 10 reporting countries, two, namely Bangladesh and Maldives, reported a declining trend. Bhutan and Nepal did not report any dengue deaths, whereas Bangladesh and Maldives reported one death each. The Democratic People's Republic of Korea is a non-endemic country for dengue, owing to climatic factors. During 2012, chikungunya (CHIKV) infection was also reported in three countries of the region, namely India, Indonesia and Thailand. The incidence of CHIKV has shown a declining trend, owing to build-up of herd immunity.

The WHO Western Pacific Region recorded variable dengue activity. Based on reported cases from January to July 2013, the trend in infection showed an increase in Malaysia, New Caledonia, Philippines and Viet Nam, when compared with the same period for 2012. The trend in infection declined in Australia, Cambodia and Singapore. A total of 64,907 cases and 452 deaths, with a CFR of 0.69, was reported. As of July 2013, Solomon Islands in the Pacific sub-Region had reported 6,716 cases with eight deaths, while French Polynesia recorded 219 cases. Infection in the Solomon Islands is with dengue virus type 3 (DENV-3), while in French Polynesia both DENV-1 and DENV-3 are endemic.


We now invite contributions for Volume 38 (2014). The deadline for receipt of contributions is 30 April 2014. While preparing their manuscripts, contributors are requested to please peruse the instructions given at the end of the Bulletin. Contributions should either be sent, accompanied by CD-ROMs, to the Editor, Dengue Bulletin, WHO Regional Office for South-East Asia, Mahatma Gandhi Road, I.P. Estate, Ring Road, New Delhi 110002, India, or by email, as a file attachment, to the Editor at dengue@searo.who.int. Readers who want to obtain copies of the Dengue Bulletin may write to the WHO Regional Offices in New Delhi or Manila or the WHO country representative in their country of residence.

Dr Aditya P. Dash
former Regional Adviser, Vector-Borne and Neglected Tropical Diseases Control (RA-VBN), and
Editor, Dengue Bulletin
World Health Organization
Regional Office for South-East Asia
New Delhi, India.
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1. Abeynayake, Janaki  
   Department of Pathology  
   Stanford University School of Medicine  
   Stanford, CA, USA

2. Aldstadt, Jared  
   Department of Geography  
   University at Buffalo  
   Buffalo, NY, USA

3. Arya, Subhash Chandra  
   Sant Parmanand Hospital  
   Delhi, India

4. Barrera, Roberto  
   Entomology and Ecology Activity  
   Centers for Disease Control and Prevention (CDC), Dengue Branch,  
   San Juan, Puerto Rico

5. Brady, Oliver  
   Spatial Ecology and Epidemiology Group  
   Department of Zoology, University of Oxford  
   Oxford, United Kingdom

6. Carrington, Lauren B  
   Department of Entomology  
   University of California Davis  
   Davis, CA, USA

7. Deen, Jacqueline  
   Global Health Division  
   Menzies School of Health Research  
   Royal Darwin Hospital Campus  
   Casuarina, NT, Australia

8. Despres, Philippe  
   Flavivirus-Host Molecular Interactions and  
   National Reference Centre for Arboviruses  
   WHO Collaborating Centre for Reference and Research on Arboviruses and Viral Haemorrhagic FEVERs  
   Department of Virology  
   Institut Pasteur, Paris, France

9. Duncombe, Jennifer  
   Infectious Disease Epidemiology Unit  
   University of Queensland  
   Brisbane, Australia

10. Esteva, Lourdes  
    Departamento de Matemáticas  
    Facultad de Ciencias  
    Universidad Nacional Autónoma de México  
    México, DF, Mexico

11. Esu, Ekpereonne  
    Department of Public Health  
    Faculty of Allied Medical Sciences  
    College of Medical Sciences  
    University of Calabar  
    Calabar, Nigeria

12. Griffiths, Karolina  
    Liverpool School of Tropical Medicine  
    Liverpool, United Kingdom

13. Hawley, Bill  
    Country Director, CDC Indonesia  
    Hay Simon I  
    Spatial Ecology and Epidemiology Group  
    Department of Zoology, University of Oxford  
    Oxford, United Kingdom

14. Hoffmann, Ary Anthony  
    Departments of Genetics and Zoology  
    Bio21 Institute  
    The University of Melbourne, Parkville  
    Victoria, Australia

15. Hunsperger, Elizabeth  
    CDC, Immunodiagnostic, Development and Research Laboratory,  
    Division of Vector-Borne Diseases  
    Dengue Branch  
    San Juan, Puerto Rico

16. Kay, Brian  
    Queensland Institute of Medical Research  
    Post Office Royal Brisbane Hospital  
    Brisbane, Australia  
    Kurane Ichiro  
    National Institute of Infectious Diseases  
    Tokyo, Japan

17. Lennon, Jeffrey L  
    Liberty University  
    School of Health Sciences  
    Department of Health Professions  
    Lynchburg, VA, USA
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Clinical and laboratory features of dengue fever in the southern lowlands of Nepal

Biswa Neupane, a,b Komal Raj Rijal, a Gyan Bahadur Aryal, a,b Yogendra Shah, b Megha Raj Banjara, a Jeevan Bahadur Sherchand, c Kouichi Morita d and Basu Dev Pandey b#

a Central Department of Microbiology, Tribhuvan University, Kirtipur, Nepal
b Everest International Clinic and Research Center, Kathmandu, Nepal
c Tribhuvan University Teaching Hospital, Kathmandu, Nepal
d Institute of Tropical Medicine, Nagasaki University, Japan

Abstract

Dengue is a mosquito-borne acute febrile illness with an increasing trend and geographical spread in Nepal. This study sought to describe the clinical and laboratory features of dengue fever (DF) cases and compare them with those of non-DF cases among patients presenting at two hospitals in the southern lowlands of Nepal between August and November 2011. A total of 212 samples were collected from patients with suspected dengue infection and analysed for demographic, clinical and laboratory parameters. Myalgia, headache, skin rash, anorexia and thrombocytopenia were the significant features of dengue infection; cases with myalgia, headache and anorexia presented higher sensitivity, whereas cases with skin rash were more specific. Immunoglobulin M (IgM) antibodies were detected in 25 cases by IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA). A combination of simple clinical and laboratory features are potentially able to predict DF among febrile patients, with a high level of accuracy; however, close follow-up of every patient is very important.

Keywords: Clinical symptoms; Dengue fever; Diagnosis; Laboratory features; Nepal.

Introduction

Dengue fever (DF) is a mosquito-borne viral disease caused by one of the four antigenically related but distinct serotypes of dengue virus, DENV-1 to DENV-4, belonging to the genus Flavivirus and family Flaviviridae. It is transmitted principally in tropical and subtropical areas
inhabited by the mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. DENV is the most important flavivirus from the standpoint of human morbidity. Infection by one serotype does not provide lifelong cross-immunity against infection by other serotypes. The infection can be subclinical or it may cause dengue fever (DF) or severe dengue. Early clinical features of dengue infection are variable among patients, and initial symptoms are often non-specific. DF is usually a self-limiting illness with significant morbidity but low mortality if treated properly. However, severe dengue has greater risk of complications, including severe plasma leakage that leads to shock and/or fluid accumulation, with respiratory distress, severe bleeding and severe organ impairment.

DF is a climate-sensitive disease that, in recent years, has become a major public health concern worldwide. Its transmission occurs in tropical and subtropical parts of the world, mainly in urban and suburban areas. In recent years, there has been an increase in receptivity (vector breeding) and vulnerability (virus movement), owing to increased globalization and international travel, resulting in an increase in the frequency of epidemics.

Nepal reported the first case of dengue in 2004. Since then, there has been an upward trend in the incidence of dengue infections. The dengue epidemics that occurred in 2006 and 2010 clearly suggest that DENV has been circulating in Nepal for several years. Dengue surveillance programmes in Nepal are not as effective as they could be and health professionals do not usually consider dengue as a differential diagnosis.

Dengue presents highly complex pathophysiological, economic and ecological problems. Patients need to know the early features that can distinguish DF from other febrile illnesses. Serological and virological diagnosis of dengue requires long, intensive work and advanced laboratory settings, which are often not available in rural areas in Nepal. This study sought to describe the clinical and laboratory features of DF among patients presenting for care at two hospitals in the southern lowlands of Nepal.

**Materials and methods**

**Study area**

This study was carried out in two tertiary care hospitals: Bharatpur Hospital (BH), Chitwan, and Rapti Zonal Hospital (RZH), Dang, during the post-monsoon period from August to November 2011. Both hospitals lie in the southern lowlands of Nepal where dengue has been reported frequently, and serve a mixed population – BH has a capacity of more than 300 beds and RZH has 150 beds.

**Study population**

Patients presenting with a history of fever for >5 days or temperature of >37.8 °C were recruited into the study if they had two or more of the following: myalgia, headache,
Clinical and laboratory features of dengue fever in the southern lowlands of Nepal

arthralgia, skin rash, retro-orbital pain, haemorrhagic manifestation(s) or leukopenia. A case was excluded if routine laboratory testing (whole blood cell count, urine analysis) suggested bacterial infection, any viral infection other than dengue infection, or any other disease. Subjects with previous immunizations for Japanese encephalitis were also excluded from the study. After the patient was assessed and provided treatment, a standard case-report form was completed. About 5 mL of blood from adults and 3 mL of blood from children aged 5 years and younger was collected, and the serum was separated for further serological testing.

**Biological study**

The haematological parameters evaluated were platelet count, total white blood cell (WBC) count and haemoglobin (Hb) levels. These tests were performed with the help of laboratory personnel of the respective hospitals. For the purpose of this study, leukopenia is defined as a WBC count of less than 4000/mm³ and thrombocytopenia as a platelet count of less than 150 000/mm³.

**Serological study**

The serum samples were transferred to Everest International Clinic and Research Centre (EICRC), Kathmandu, maintaining the reverse cold chain for further serological tests by immunoglobulin M (IgM) antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) for DENV (Standard Diagnostics Inc., Korea). The cases testing positive in MAC-ELISA were defined as laboratory-confirmed dengue.

**Statistical analysis**

Data were analysed using SPSS Version 17.0. Values were expressed as mean ± standard deviation (SD). Crude odds ratios (ORs) were calculated. Chi-squared analysis was carried out and the value of significance used for all statistical tests was $P < 0.05$. The tests that were significant in $\chi^2$ were further analysed for sensitivity, specificity and predictive values.

**Results**

During the post-monsoon period from August to November 2011, 2480 febrile patients presented to BH and 1530 to RZH. Of these, 253 (6%) had at least two additional manifestations of dengue fever (see Figure 1). A total of 212 serum samples were collected; dengue infection was confirmed serologically by ELISA in 25 (11.8%) samples, which were classified as DF cases, and the remaining 187 samples were classified as non-dengue fever (non-DF) cases. The number of DF cases for males and females were 11 and 14 respectively. Most of the patients were adults, with a median age of 31 years. The youngest
The highest number of cases was among the age group 15–50 years, which accounted for 14 (56%) of the cases, followed by 7 (28%) in the age group >50 years and 4 (16%) in the age group <15 years (see Table 1). A majority of the cases (36%) were among females in the age group 15–50 years (see Figure 2).

**Figure 1:** Flow diagram of patient sampling for dengue, Nepal, 2011

- 2480 febrile patients presenting to BH and 1530 to RZH, August to November 2011
- Excluded:
  - 2283 (57%) with obvious bacterial infection
  - 567 (14%) with other viral infection
  - 432 (11%) with other infection
  - 346 (9%) with previous Japanese encephalitis vaccination
- 253 (6%) febrile patients with 2 additional manifestations of dengue fever
- 41 (16%) patients with no blood sample taken
- 212 patients included in the analysis
Clinical and laboratory features of dengue fever in the southern lowlands of Nepal

**Figure 2:** Age and sex distribution of DF cases, Nepal, 2011

<table>
<thead>
<tr>
<th>Age and Sex Distribution</th>
<th>Nepal, 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;15 years</td>
<td>2 (2)</td>
</tr>
<tr>
<td>15–50 years</td>
<td>14 (14)</td>
</tr>
<tr>
<td>&gt;50 years</td>
<td>7 (7)</td>
</tr>
</tbody>
</table>

*Male* | *Female* | *Total*
---|---|---
2 | 2 | 4
5 | 9 | 14
4 | 3 | 7

Table 1: Demographic distribution of DF and non-DF cases, Nepal, 2011

<table>
<thead>
<tr>
<th>Demographic characters</th>
<th>DF</th>
<th>Non DF</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BH</td>
<td>RZH</td>
<td>BH</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>10 (40)</td>
<td>1 (4)</td>
<td>46 (26)</td>
</tr>
<tr>
<td>Female</td>
<td>11 (44)</td>
<td>3 (12)</td>
<td>54 (29)</td>
</tr>
<tr>
<td>Age in years, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;15</td>
<td>3 (12)</td>
<td>1 (4)</td>
<td>30 (16)</td>
</tr>
<tr>
<td>15–50</td>
<td>12 (48)</td>
<td>2 (8)</td>
<td>45 (24)</td>
</tr>
<tr>
<td>&gt;50</td>
<td>6 (24)</td>
<td>1 (4)</td>
<td>25 (13)</td>
</tr>
<tr>
<td>Total</td>
<td>21 (84)</td>
<td>4 (16)</td>
<td>100 (53)</td>
</tr>
</tbody>
</table>

BH: Bharatpur Hospital, Chitwan; RZH: Rapti Zonal Hospital.
The patients had symptoms for a median of 4 days (range = 2 to 10 days) prior to hospitalization and their mean temperature at the time of hospitalization was 38.8 ± 1 °C. The most common clinical features among the DF cases were fever (100%), myalgia (84%), anorexia (88%), headache (80%), nausea (60%) and abdominal pain (40%), as shown in Table 2. Myalgia, headache, skin rash and anorexia were found to be significantly associated with DF (P < 0.05). Among the DF cases, 8 (32%) had leukopenia and 14 (56%) had thrombocytopenia, with a mean leukocyte count of 5808 ± 2931/mm³, according to the operational definition set earlier in this paper, while the mean thrombocyte count was 143 800 ± 60 050/mm³. The mean haemoglobin level was 12.5 ± 2.3 g/dL. Moreover, 4 (16%) cases had a haemoglobin level >15.0 g/dL (see Table 2). Thrombocytopenia was found to be significantly related to dengue fever (P < 0.05).

**Table 2:** Clinical and laboratory features of DF and non-DF cases, Nepal, 2011

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>DF, n (%)</th>
<th>Non-DF, n (%)</th>
<th>Odds ratio (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical features</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myalgia</td>
<td>21 (84)</td>
<td>77 (41)</td>
<td>7.5 (2.5 to 22.7)</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Headache</td>
<td>20 (80)</td>
<td>80 (43)</td>
<td>5.4 (1.9 to 14.9)</td>
<td>0.007&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Skin rash</td>
<td>4 (16)</td>
<td>11 (6)</td>
<td>3.0 (0.9 to 10.4)</td>
<td>0.033&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Retro-orbital pain</td>
<td>2 (8)</td>
<td>4 (2)</td>
<td>3.9 (0.7 to 22.9)</td>
<td>0.156</td>
</tr>
<tr>
<td>Anorexia</td>
<td>22 (88)</td>
<td>100 (53)</td>
<td>6.4 (1.8 to 22.0)</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nausea</td>
<td>15 (60)</td>
<td>76 (41)</td>
<td>2.2 (0.9 to 5.1)</td>
<td>0.282</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>10 (40)</td>
<td>53 (28)</td>
<td>1.7 (0.7 to 4.0)</td>
<td>0.259</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>3 (12)</td>
<td>27 (14)</td>
<td>0.8 (0.2 to 2.9)</td>
<td>0.515</td>
</tr>
<tr>
<td>Haemorrhagic manifestation</td>
<td>1 (4)</td>
<td>18 (10)</td>
<td>0.4 (0.1 to 3.1)</td>
<td>0.257</td>
</tr>
<tr>
<td>Laboratory features</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukopenia</td>
<td>8 (32)</td>
<td>45 (24)</td>
<td>0.9 (0.4 to 2.2)</td>
<td>0.790</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>14 (56)</td>
<td>44 (24)</td>
<td>0.3 (0.1 to 0.7)</td>
<td>0.005&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Haemoglobin &gt;15.0 g/dL</td>
<td>4 (16)</td>
<td>25 (13)</td>
<td>1.2 (0.4 to 3.9)</td>
<td>0.719</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>187</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>P value less than 0.05.
The significant features in dengue cases were myalgia, headache, skin rash, anorexia and thrombocytopenia. Using ELISA as a gold standard, the sensitivity, specificity and predictive values of these features in the diagnosis of dengue is shown in Table 3. The sensitivity of myalgia (84%), headache (80%) and anorexia (88%) was high, whereas the specificity of skin rash (94%) was high. The negative predictive values of all the features were significantly higher than their positive predictive values.

**Table 3: Sensitivity, specificity and predictive values of significant features for prediction of dengue cases, Nepal 2011**

<table>
<thead>
<tr>
<th>Features</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Positive (%)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>84</td>
<td>59</td>
<td>21</td>
</tr>
<tr>
<td>Headache</td>
<td>80</td>
<td>57</td>
<td>20</td>
</tr>
<tr>
<td>Skin rash</td>
<td>16</td>
<td>94</td>
<td>27</td>
</tr>
<tr>
<td>Anorexia</td>
<td>88</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>60</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>

**Discussion**

This cross-sectional epidemiological study was conducted in two hospitals of the southern region of Nepal, where dengue has been frequently reported. The authors believe that the study sites represent the scenario of most dengue-endemic regions of less-equipped low-income countries in the post-monsoon period, when optimum conditions for mass breeding and propagation of vector and transmission of the virus are developed. The prospective enrolment of subjects with fever, and selection of cases using established case definitions, helps in the documentation of detailed clinical and laboratory features.

In this study, the clinical manifestations that were most significantly associated with DF as compared with non-DF patients were myalgia, headache, skin rash and anorexia. However, the severity of these clinical symptoms was different in each patient. Many factors may account for the differences seen, such as infection with different serotypes or infection with more than one serotype, either sequentially or concurrently. Differences in host genetics and immune responses may also play a role in the severity of infection. The haematological profile of dengue cases revealed that thrombocytopenia in dengue was statistically significant. The increased destruction of platelets or decreased production of platelets could result in thrombocytopenia. Sensitivity for the prediction of dengue cases was high for myalgia, headache and anorexia, so these features will result in the identification of most individuals who have the disease. Skin rash had higher specificity, so its absence will result in correct
identification of most individuals who are free from the disease. All these features presented better negative than positive predictive values, thus helping the clinician to be more confident that a patient lacking these features does not have the disease.

The frequency of presentation of these features in dengue patients was similar to that in a study in Chitwan, Nepal, during the 2010 dengue outbreak, which reported myalgia (96%), body ache (92%) and nausea (84%) as the most frequent clinical presentations among DF cases.14 Interestingly, in this study, only a single case of severe dengue was noted, according to World Health Organization (WHO) guidelines,5,15 which may be due to the small sample size and short study period. During the early febrile phase, it is often not possible to predict clinically whether a patient with dengue will progress to severe disease.15 Many other infectious diseases present similar signs and symptoms as dengue, in the acute phase of illness. As a result, medical practitioners in hospital settings do not consider a diagnosis of dengue. The introduction of the new WHO South-East Asia Region Comprehensive guidelines for prevention and control of dengue in 201116 should help clinicians to take decisions about where and how intensively the patient should be treated, and enable more consistent reporting in dengue surveillance systems.5 Based on the new guidelines, the probable symptoms of dengue, such as myalgia, headache, skin rash and anorexia, and warning sign like thrombocytopenia, were observed as significant features in this study.

Cases of dengue were more frequent in females than in males and the economically active age group was more susceptible to the infection. These results are not in accordance with previous findings that dengue is a paediatric disease and affects males more frequently than females.17–19 The shift in age may be due to more frequent secondary infections, as dengue has been emerging persistently in Nepal since 2006.

The limitations of the study include the use of a single serum sample for ELISA test instead of paired sera, and the short duration of the study and small sample size. Suspected cases were defined as febrile patients with two additional manifestations of DF. This could have excluded some patients who were likely to have dengue, as dengue patients feel unwell for a longer period.20 Some samples were missed due to failure in blood collection. Furthermore, the samples had to be transported before serological testing and it is possible some positive samples were damaged during transportation. The non-availability of reverse transcriptase polymerase chain reaction (RT-PCR), haemagglutination inhibition (HI) tests and testing for NS1 antigen in the field was a major limitation of the study.

The results of this study are potentially helpful in the ascertainment of dengue cases by clinical and epidemiological criteria, in endemic areas where molecular and serological tests are not easily available. In such areas, all febrile cases should be monitored for the development of severe signs and symptoms. In the study areas, febrile cases revealing thrombocytopenia and evidence of plasma leakage were generally considered to be the criteria for further serological testing of patients for dengue diagnosis. Such practices could lead to underreporting of dengue in endemic areas but overreporting during epidemics. The new 2012 WHO guidelines for dengue diagnosis will be very useful for further studies.
Molecular and virological tests are complex to perform and are less feasible for routine laboratory practices, especially in the resource-limited settings of low- or middle-income countries like Nepal. Therefore, these clinical and laboratory findings are equally helpful for distinguishing dengue from other febrile illnesses at an early stage, which could reduce dengue-associated morbidity and mortality.

**Conclusion**

Diagnosis of dengue in its early stage has been a major problem in low-income countries. The serological, molecular and virological diagnosis is a tedious process in terms of time and technique. Hence, this study has attempted to identify simple clinical and laboratory features of DF that would be useful for distinguishing dengue from other febrile illnesses at an early stage. The use of previous WHO case-classification criteria was rather difficult; however, the introduction of the new South-East Asia guidelines\(^1\) has now simplified the classification and is very useful in resource-poor countries. ELISA has been used to confirm dengue cases. Confirmation by molecular and virological techniques could be more promising in such studies.

**Acknowledgement**

We thank the staff of Everest International Clinic and Research Centre, Kathmandu, for their technical support. We are extremely grateful to the medical superintendents, doctors, nurses, staff and patients of the two hospitals for their kind support during the study. We would also like to thank Mr Shreedhar Subedi and Mr Rabi Pun for their assistance in sample collection.

**References**


Clinical and laboratory features of dengue fever in the southern lowlands of Nepal


A comprehensive review of dengue mortality and its determinants

Zinia T Nujum,# K Vijayakumar and Pamagal Kavithai

Medical College, Thiruvananthapuram, Kerala, India

Abstract
The first objective of the World Health Organization (WHO) global strategy (2012–2020) is to reduce mortality due to dengue by 50%, by 2020. This review attempts a comprehensive study of mortality caused by dengue. The objectives were to find the determinants/predictors of mortality and to discuss the variations in case fatality. A review of articles and published literature was carried out, using a systematic search. Although the general trend of case fatality had been declining in the WHO South-East Asia Region up to 2008, it began to rise in 2009. Fatalities due to dengue occur in all age groups; however, there is evidence of a possible age shift of dengue mortality to older age compared to that seen in previous years. Mortality among pregnant women was higher than among non-pregnant women. Circulation of dengue virus type 2 (DENV-2), annual rainfall, high population density, poverty and “age of endemicity” were independently associated with dengue mortality. Low platelet count, prolongation of prothrombin time, higher blood urea nitrogen, lower bicarbonate, higher activated partial thromboplastin time (APTT), higher serum glutamic-pyruvic transaminase (SGPT), higher serum glutamic oxaloacetic transaminase (SGOT), anaemia and hypoalbuminemia were associated with higher mortality. Non-adherence to WHO dengue-management guidelines and failure to recognize warning signs for severe dengue and shock have been documented as determinants of mortality. Unnecessary transfusions can lead to the risk of pulmonary oedema. Research to provide better biomarkers predicting disease severity is urgently needed. Mortality from dengue can be reduced significantly if severely ill patients access health services in time and receive appropriate clinical care. A surveillance system for dengue mortality needs to be established globally. Evidence-based action and capacity-building is required at all levels of health care.

Keywords: Dengue; Determinants; Endemicity; Mortality; India.

Introduction
Dengue is an avoidable cause of death.1 About 5% of dengue patients develop its severe form, which is more common with second or subsequent infections.2 The minimum estimated incidence of dengue requiring hospitalization is 251/100 000 population.3 In children under

#E-mail: drzinia@gmail.com
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15 years of age, this can be as high as 712/100 000. The case-fatality rate (CFR) and absolute number of dengue deaths are important determinants of the burden of the disease. Primary prevention of dengue through vector-control activities has shown limited success worldwide. Currently, there is no vaccine to prevent dengue, nor is there an antiviral treatment. However, secondary prevention to reduce mortality through improved clinical case management has substantially lowered the mortality rate for severe dengue over the past two decades, from 10% to 20% to <1% in some countries. The first objective of the World health Organization (WHO) Global strategy for dengue prevention and control 2012–2020 is to reduce the mortality due to dengue by 50% by 2020, using deaths in 2010 as the baseline.

Given that dengue mortality is directly determined by the incidence and CFR, the number of deaths is conditioned not only by the factors that facilitate transmission but also by those that influence the severity of the disease and the ease of access to health care. Research to provide better diagnostics and biomarkers predicting disease severity are urgently needed. This review is an attempt to study mortality due to dengue comprehensively, based on experiences from across the world. The objectives were to find the determinants/predictors of mortality, including clinical symptoms and signs and sociodemographic and treatment-related characteristics. The article also aims to discuss the variations in case fatality across studies, places and times.

Materials and methods

A review of articles and published literature was carried out, using a systematic search with the study objectives in mind.

Inclusion criteria

Publications in the English language with a mention of mortality/death due to dengue were included. Studies with observational (case series, cross-sectional, case-control) and record-based/retrospective chart reviews as designs were reviewed (see Table 1). Hospital and community-based studies were available. No age limits were imposed for study participants and no country specifications. Archives (newspapers) and WHO publications were also included. Studies that had any or all of the objectives mentioned in this study stated in their titles/objectives were considered eligible for inclusion. Reports and documents that were available by search, using the keywords, were further scrutinized for eligibility by a search file using keywords in the objectives, namely, dengue mortality determinants/predictors and case fatality.
Table 1: Studies using different methods, and their characteristics

<table>
<thead>
<tr>
<th>Article type</th>
<th>Place</th>
<th>Definition/inclusion</th>
<th>Year</th>
<th>Risk of bias*/strength**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case-control¹</td>
<td>Brazil</td>
<td>Severe dengue</td>
<td>2000–2005</td>
<td>Odds would be underestimated because only severe cases were taken*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Large number of deaths**</td>
</tr>
<tr>
<td>Case series²</td>
<td>Puerto Rico (Latin America)</td>
<td>Deaths suspected to be dengue, with specimen taken for confirmation</td>
<td>2007</td>
<td>Early deaths/patients brought in dead would have been excluded*</td>
</tr>
<tr>
<td>Retrospective chart review⁹</td>
<td>Singapore</td>
<td>Confirmed deaths matching the laboratory test results with mortality data from hospital, cases confirmed by RT-PCR and NS1</td>
<td>2004–2008</td>
<td>Multicentric study**</td>
</tr>
<tr>
<td>Record-based study³</td>
<td>Viet Nam</td>
<td>Hospital admissions from three major hospitals</td>
<td>1996–2009</td>
<td>14-year study, large patient population**</td>
</tr>
<tr>
<td>Ecological study⁹</td>
<td>Latin America and the Caribbean</td>
<td>Suspected and lab-confirmed deaths</td>
<td>1995–2009</td>
<td>Existence of a protocol for studying the aetiology of dengue**</td>
</tr>
<tr>
<td>Retrospective study¹¹</td>
<td>Malaysia</td>
<td>Fatal cases</td>
<td>2006–2007</td>
<td>Small sample size*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Limited documentation of clinical information, especially for patients who were brought in dead and those who died within 24 h*</td>
</tr>
<tr>
<td>Cross-sectional¹²</td>
<td>Pakistan</td>
<td>Clinical diagnosis of dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) above 14 years of age</td>
<td>3-year study published in 2010</td>
<td></td>
</tr>
</tbody>
</table>

NS1: non-structural protein 1; RT-PCR: reverse transcriptase polymerase chain reaction. These are the standard methods for isolation of virus.
Exclusion criteria

Studies with outcomes other than mortality, e.g. outcome of pregnancy in dengue patients, were excluded. Studies on the severity of dengue and its determinants/predictors were avoided, unless there was a specific mention of mortality.

Information sources

Three databases were searched, PubMed, EMBASE and LILAC, using the search engine, Google, to identify studies on mortality due to dengue. While searching in PubMed, research and review articles were searched in all fields. The search terms included “Dengue mortality”, “Dengue” + “mortality”, “Dengue deaths”, “Determinants” + “Dengue deaths” and “Predictors” + “Mortality” + “Dengue”. “Dengue” and “Dengue haemorrhagic fever” (DHF) were cross-referenced with “Mortality”/“Deaths”/“Outcomes”/“Fatal dengue”. The limits used were the title and keywords.

Initial search in PubMed yielded 15 results, of which four were included. Using limits for search, four more studies were reviewed. Another search revealed 20 more results. Eight studies that met the eligibility criteria were included. Cross-references giving important information pertaining to the study objectives were also referred to and cited. In addition, all documents retrieved through Google search using the specific search terms were included. The searches in EMBASE and LILAC were not very productive. Other studies and references were obtained using the Google search.

Results

Case-fatality rate and its trends

The case-fatality rates in the studies reviewed ranged from as low as 0.07% to as high as 4.2% (see Table 2). A few of these case-fatality rates have been derived by calculating percentages using the number of deaths and the population/number of study subjects given in the text. Some of the major outbreaks in the 19th and early 20th centuries that resulted in deaths are listed in Table 3. The CFR prior to the 1950s and the age groups involved are given in Table 4. The trends in case fatality in the WHO South-East Asia Region from 1985–2009 are presented in Figure 1. In a study from Viet Nam, it was seen that the general trend in CFR is declining. However, in children, the CFR had started rising in 2009 compared to 2008. The annual CFRs ranged from 0.10% in 2004 to 0.64% in 1996 (see Figure 2).

The average change in CFR per year was −0.04%, 95% confidence interval (CI) = 0.06% to 0.02%. The annual number of dengue-related deaths in Latin America increased from 242 in the 1980s to 2068 in the 2000s.
Table 2: Case-fatality rates reported in the studies referenced

<table>
<thead>
<tr>
<th>Place, year</th>
<th>Number of cases</th>
<th>Deaths</th>
<th>CFR as % of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerto Rico, 2007(^2)</td>
<td>10 576</td>
<td>40 (11 confirmed)</td>
<td>0.38 (in hospitalized cases 0.6)</td>
</tr>
<tr>
<td>Singapore, 2008(^1)</td>
<td>41 234</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>India, 2012(^1)(^4)</td>
<td>37 000</td>
<td>227</td>
<td>0.6</td>
</tr>
<tr>
<td>Delhi, India, 1996(^13)</td>
<td>8500</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>Pakistan, 2010(^12)</td>
<td>699</td>
<td>19</td>
<td>2.7</td>
</tr>
<tr>
<td>Americas, 1981–1996(^15)</td>
<td>42 246 (DHF)</td>
<td>582</td>
<td>1.38</td>
</tr>
<tr>
<td>Viet Nam, 1996–2009(^3)</td>
<td>132 480</td>
<td>325</td>
<td>0.25 (annual CFR ranged from 0.10 to 0.64)</td>
</tr>
<tr>
<td>Malaysia(^16)</td>
<td>3922</td>
<td>10</td>
<td>0.25</td>
</tr>
<tr>
<td>Malaysia(^17)</td>
<td>48 846</td>
<td>98</td>
<td>0.20</td>
</tr>
<tr>
<td>Philippines, 1997–2008(^18)</td>
<td>34 326</td>
<td>831</td>
<td>2.46</td>
</tr>
</tbody>
</table>

Table 3: Listing of epidemics of dengue/dengue-like illness resulting in mortality in the 19th and early 20th centuries\(^19\)

<table>
<thead>
<tr>
<th>Country (subdivision)</th>
<th>Period</th>
<th>Year</th>
<th>Total number of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia (Queensland)</td>
<td>1895–1926</td>
<td>816</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1895</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1897</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1898</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1905</td>
<td>201</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1906</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1907</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1910</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>
A comprehensive review of dengue mortality and its determinants

<table>
<thead>
<tr>
<th>Country (subdivision)</th>
<th>Period</th>
<th>Year</th>
<th>Total number of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1911</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1916</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>1925–1926</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1926</td>
<td>116</td>
<td></td>
</tr>
<tr>
<td>Australia (Western Australia)</td>
<td>1913–1930</td>
<td>1913</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1914</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1920</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1921</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1923</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1927</td>
<td>29</td>
</tr>
<tr>
<td>Australia (New South Wales)</td>
<td>1926</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>China</td>
<td>1942</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Egypt</td>
<td>1928</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1937</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Greece</td>
<td>1927–1928</td>
<td>1061–1559</td>
<td></td>
</tr>
<tr>
<td>India</td>
<td>1913</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Japan (Okinawa)</td>
<td>1904</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1923–1931</td>
<td>1923</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1924</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1931</td>
<td>468–508</td>
</tr>
<tr>
<td>Japan (Nagasaki/Okinawa)</td>
<td>1943–1944</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Japan (Bonin Islands)</td>
<td>1936</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Lebanon</td>
<td>1945</td>
<td>Unspecified</td>
<td></td>
</tr>
<tr>
<td>Northern Mariana</td>
<td>1927–1929</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Philippines</td>
<td>1943</td>
<td>9</td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Deaths during major outbreaks of dengue before 1950, and age groups affected

<table>
<thead>
<tr>
<th>Country (subdivision)</th>
<th>Year</th>
<th>Number of deaths</th>
<th>CFR/1000</th>
<th>Age groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australia (Queensland)</td>
<td>1897</td>
<td>60</td>
<td>1.0</td>
<td>Children 50%; adults 50%</td>
</tr>
<tr>
<td>Australia (Brisbane)</td>
<td>1905</td>
<td>201</td>
<td>1.0–1.5</td>
<td>&lt;5 years 37.6%; &gt;60 years 35.5%</td>
</tr>
<tr>
<td>Egypt</td>
<td>1937</td>
<td>50</td>
<td>19.3</td>
<td>?</td>
</tr>
<tr>
<td>Greece</td>
<td>1927, 1928</td>
<td>1210, 1061</td>
<td>1.6, 1.6</td>
<td>&lt;16 years 6.62%; &gt;59 years 59.8%</td>
</tr>
<tr>
<td>Japan (Okinawa)</td>
<td>1931</td>
<td>468</td>
<td>4.3</td>
<td>?</td>
</tr>
<tr>
<td>South Africa</td>
<td>1927</td>
<td>60</td>
<td>1.2</td>
<td>?</td>
</tr>
<tr>
<td>Taiwan</td>
<td>1931</td>
<td>26</td>
<td>?</td>
<td>Unknown, but 9 of 11 dead were children</td>
</tr>
<tr>
<td>United States of America</td>
<td>1934</td>
<td>3</td>
<td>1.8</td>
<td>Adults 100%</td>
</tr>
</tbody>
</table>
Determinants of dengue mortality

**Sociodemographic factors related to dengue mortality**

The median age of mortality due to dengue has been reported to be from 25 to 59 years.\(^2,3,16,17,20\) The minimum age of dengue deaths reported in the reviewed literature was 5 months,\(^1\) and the maximum was 86 years.\(^10\) Although the prevalence of dengue shock syndrome (DSS) was highest in children aged 6–10 years, the mortality was highest in younger
children. The mortality decreased with increasing age; 1–5-year-old children were found to have two times higher mortality than 6–10-year-old children and four times higher mortality than 11–15-year-old children. The mortality was higher in children when compared to adult dengue patients (CFR = 0.20% versus 0.11%; \(P = 0.002\)), but among those adults with DSS, the mortality was much higher than in paediatric DSS patients (CFR = 5.5% versus 1.4%; \(P < 0.001\));\(^3\) 45% of deaths occurred in children aged less than 15 years\(^1\) and 48% of deaths occurred in persons aged 15–35 years.\(^17\)

In two studies, females had higher rates of mortality. In one, girls had a higher risk of mortality (odds ratio [OR] = \(-1.57\), 95% confidence interval [CI] = 1.1 to 2.2).\(^3\) In another, adult females constituted 90% of deaths.\(^16\) This may be due to the more robust immune response in females, resulting in females becoming more prone to develop greater inflammatory response or higher susceptibility to capillary permeability.\(^21,22\) This is in spite of the fact that 55% of cases of dengue occurred in males. More deaths among girls, especially those among the paediatric group, were also reported in Viet Nam during 1996–2009, despite the predominance of boys among dengue cases.\(^18,19\) In contrast, another study from Brazil showed that females had lower fatality than males. This may be attributed to the late treatment-seeking behaviour in males in that country.\(^1\) There was also another study in which males constituted 68% of dengue deaths.\(^20\)

Fatalities due to dengue occur in all age groups. However, in many of the early outbreaks of dengue, the occurrence of severe cases and mortality was more notable in children and the elderly. This pattern was consistently observed in the outbreaks, such as the one in Australia in 1926, where children and elderly patients accounted for 22.4% and 41.5% of fatal cases respectively. However, in the 1990s and thereafter, the median age of DHF patients and the proportion of fatalities in certain age groups (>15 years) rose in many countries where the disease is endemic.\(^19\) In general, the incidence of severe dengue and fatality is lower in adults, but in some countries most deaths are seen in adults, which is explained by late recognition, higher incidence of bleeding and delayed blood transfusion.\(^4\) There is a possible age shift of dengue mortality to older age with time, compared to that seen in previous years.\(^20,23\)

The likelihood of mortality due to dengue is higher in the elderly.\(^24\) A study in Brazil found that patients aged 50 years and above had a 2.3 times higher risk of mortality.\(^1\) In older patients constitutional symptoms are less likely and multi-organ involvement and bacteraemia are possible, because a longer period of hospitalization is more common.\(^24,25\) Acute renal failure has a worse prognosis in the elderly with dengue infection.\(^25\) This implies the need for including the elderly in future vaccine trials.

People of white race showed a preponderance of dengue mortality in Cuba,\(^26\) whereas no such preponderance was seen in Singapore.\(^20\) Higher mortality has also been reported among black races (African). This could be due to poor access to health care rather than biological factors.\(^1\) In contrast, the Cuban and Caribbean black population and African populations share a common gene pool that could explain, at least partially, the low incidence of DHF
in those regions. Having had less than four years of schooling was associated with a 1.83 times higher chance of mortality, and living in a rural area had a 2.8 times higher chance of mortality. The higher mortality rate in rural areas may be due to lower socioeconomic status and problems of access to health care.\(^1\)

**Pregnancy as a determinant of dengue mortality**

Pregnant women are 3.4 times more prone to developing severe dengue (OR = 3.38; CI = 2.10 to 5.42). Mortality among pregnant women was higher when compared to non-pregnant women. Dengue during pregnancy can increase maternal mortality. Severe dengue has been associated with maternal deaths, with fatality rates ranging from 2.9% to 22%.\(^{28-30}\) Hospitalization is also twice as high when compared to non-pregnant patients.\(^{25}\) Moreover, the proportion of DHF could still be underestimated, as the identification of plasma leakage syndrome through haemoconcentration or hypoproteinaemia may be compromised from the 7th to the 32nd week of gestation, because of the physiological increase of intravascular volume in this period.\(^{31}\) This underestimation could have resulted in a bias towards higher CFR. Dengue in pregnancy was mostly seen during the third trimester,\(^{32}\) but it is possible that early dengue was missed because of it being less severe. Dengue infection in pregnancy carries the risk of haemorrhage for both the mother and the neonate. In addition, there is a risk of premature birth and fetal death, as well as vertical transmission causing neonatal thrombocytopenia that necessitates platelet transfusions.\(^{33}\) During epidemics in endemic areas, health-care providers should consider dengue in the differential diagnosis of pregnant women with fever, and be aware that the clinical presentation may be atypical and may confound diagnosis. Diagnosis of dengue infection affects management options and obstetricians’ decisions, particularly about the mode of delivery, owing to the potential risk of haemorrhage secondary to thrombocytopenia. Elevated liver enzymes, haemolysis and low platelet counts may be confused with the diagnosis of haemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome, which occurs in women with pre-eclampsia and eclampsia.\(^{32}\)

**Ecological determinants of dengue mortality**

Confluence of factors related to the environment (rainfall), demographics (population density), socioeconomics and biology (circulating serotypes) can contribute to an alarming increase in mortality and the burden of dengue because of their effect on the incidence and case fatality or both. Circulation of dengue virus type 2 (DENV-2) and 4 (DENV-4) serotypes was associated with higher risk of mortality. DENV-2 genomic regions (50- and 30-untranslated regions and E390) give a greater capacity for replication of this serotype, with a high incidence of severe cases and mortality.\(^9\) Studies in Thailand have revealed that the DENV-1/DENV-2 sequence of infection was associated with a 500-fold increased risk of DHF compared with primary infection. For the DENV-3/DENV-2 sequence, the risk was 150-fold, and a DENV-4/ DENV-2 sequence had a 50-fold increased risk of DHF.\(^{34}\) Annual rainfall, high population
density, a low Human Development Index (HDI) and age of endemicity were independently associated with dengue mortality in Latin America. A low HDI as a determinant of mortality has been attributed to the effect of poverty on access to, or the quality of, medical care for dengue.9 However, it is explained that the convergence of other biological factors, such as the coexistence of other neglected diseases,35 increased the risk of complications and death in low- and middle-income countries. Population density was linked more to increasing CFR than to increasing incidence. Therefore, it is hypothesized that where the population density is high, e.g. in urban settings, mortality due to dengue is higher. Viral transmission to humans may also be favoured in urban areas. This may be due to the closer contact between vectors and the vertebrate hosts, more larval habitats, and the existence of appropriate microclimates that promote the survival of mosquito populations. It is also possible that milder cases are underreported in areas of high population density and, therefore, CFR is overestimated.9

Rainfall is associated with a higher incidence of dengue,36–38 as well as dengue deaths and CFR.18 Among the ecological factors, the variable that best explained the variation in dengue mortality was the “age of endemicity”. Age of endemicity means the time since the disease became endemic in a particular region. This usage is obtained from an article from a Latin American country,9 where the annual number of deaths due to dengue increased with increasing age of endemicity and mortality rates tripled every 10 years. The incidence of dengue increases over the years (see Figure 3). As the age of endemicity increases, the likelihood of secondary infections also increases.36,39–43 Furthermore, a longer time from primary to secondary infection increases the risk of mortality.41 Phenomena such as virus–vector–host interactions could eventually lead to changes in the immunity of the population, and to the selection of viral genotypes with higher transmissibility and virulence.43,44

**Figure 3:** Dengue mortality by age of endemicity9
Evidence of secondary dengue infection has been associated with a severe outcome of dengue via antibody-dependent enhancement and T-cell original antigenic sin.\textsuperscript{12,45} Thus, increasing age of endemicity is related to both dengue incidence and case-fatality rate. The incidence is said to rise by 54% and CFR by 99% every 10 years.\textsuperscript{9}

**Time available for intervention and prevention of dengue deaths in hospital**

The time from fever to hospital admission ranged from a mean of 2.9 days to 4.7 days.\textsuperscript{10,11,41} The median time from admission to a positive test result was 2.5 days and the median time from the onset of illness to death was 12 days.\textsuperscript{20} A haematocrit increase of $\geq 20\%$ concurrent with a reduction in platelet count to less than 20 000/μL was associated with the shortest interval to death, which had a median of 3 days.\textsuperscript{46} Dengue patients stayed in hospital for between 7.5 days and 13.7 days\textsuperscript{16,20,41} before death. This time could be considered as the window of opportunity available for the health professional to prevent death.

**Clinical features associated with mortality**

Death in dengue patients is due to either shock or organ failure.\textsuperscript{14,20} The major characteristics of studies referred to in this section on clinical features and the section on investigation-related determinants of mortality are shown in Table 5, which includes the age structure of the different study populations and the place and year of study. Decompensated DSS with evidence of massive plasma leakage, massive bleeding, multiple organ failure and coagulopathy were the primary causes of death. Per rectum bleeding, vaginal bleeding and gastrointestinal bleeding were the commonest sites of severe bleeding.\textsuperscript{16} Prolonged shock is associated with metabolic acidosis and disseminated intravascular coagulation, which may lead to hypoxia/ischaemia and result in both hepatic and cerebral dysfunction. Metabolic acidosis in these patients may be associated with renal failure, in addition to shock. Sixty-six per cent of dengue patients who died had metabolic acidosis on account of prolonged shock.\textsuperscript{2} Metabolic acidosis is found significantly more often in patients with DSS than in those with DHF.\textsuperscript{47}

The presence of encephalitis in dengue patients is significantly associated with mortality. Encephalopathy may be due to acute liver failure from hepatitis.\textsuperscript{48} It may also be caused by the neurotropic effect of the virus\textsuperscript{49} or be a result of secondary bacterial infections.\textsuperscript{14} Coma at the time of being brought to hospital\textsuperscript{14} and impaired consciousness are associated with high mortality and multi-organ involvement.\textsuperscript{49,50} Impaired consciousness was found in 57.1\% of dengue deaths.\textsuperscript{20} The occurrence of seizures is another neurological sign associated with higher mortality.\textsuperscript{2,50} Seizures were documented in 33\% of dengue deaths.\textsuperscript{2}

Hepatic involvement has been incriminated as a significant determinant of dengue mortality in several studies,\textsuperscript{2,20,51,52} since it leads to impairment of liver function and coagulopathy.\textsuperscript{47,53–57} Severe hepatitis was seen in 57.1\% of dengue deaths.\textsuperscript{10} Hence, jaundice
Table 5: Details of the studies used for description of clinical and laboratory findings associated with mortality/severity in dengue

<table>
<thead>
<tr>
<th>Reference number</th>
<th>Clinical/laboratory finding</th>
<th>Children/Adults</th>
<th>Place of study</th>
<th>Year of study/Year of publication**</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>Shock and organ failure, hepatic involvement</td>
<td>Adults</td>
<td>Singapore</td>
<td>2004–2008*</td>
</tr>
<tr>
<td>16</td>
<td>Severe bleeding</td>
<td>Adults</td>
<td>Malaysia</td>
<td>2006–2007*</td>
</tr>
<tr>
<td>2</td>
<td>Metabolic acidosis with hepatic involvement</td>
<td>Children and adults</td>
<td>Puerto Rico</td>
<td>2007*</td>
</tr>
<tr>
<td>12</td>
<td>Metabolic acidosis from hyperventilation</td>
<td>Children</td>
<td>Indonesia</td>
<td>1988*</td>
</tr>
<tr>
<td>14</td>
<td>Hepatitis with encephalopathy</td>
<td>Children</td>
<td>India</td>
<td>2008**</td>
</tr>
<tr>
<td>45</td>
<td>Encephalopathy Mortality rate 22% in children with encephalopathy</td>
<td>Children</td>
<td>Viet Nam</td>
<td>2001**</td>
</tr>
<tr>
<td>46</td>
<td>Encephalopathy</td>
<td>Children</td>
<td>Sri Lanka</td>
<td>2004–2005*</td>
</tr>
<tr>
<td>47</td>
<td>Altered mental status, SGOT&gt;300 and shock</td>
<td>Adults</td>
<td>Pakistan</td>
<td>2010**</td>
</tr>
<tr>
<td>48</td>
<td>Elevated creatine kinase, lactate dehydrogenase and lower albumin and triglycerides</td>
<td>Adults</td>
<td>Colombia</td>
<td>2008**</td>
</tr>
<tr>
<td>49</td>
<td>Hepatic involvement haemophagocytic syndrome</td>
<td>Adult</td>
<td>India</td>
<td>2011**</td>
</tr>
<tr>
<td>50</td>
<td>Encephalitis</td>
<td>5-year-old child</td>
<td>Thailand</td>
<td>1999*</td>
</tr>
<tr>
<td>51</td>
<td>Acute myocarditis</td>
<td>Adults</td>
<td>Taiwan, China</td>
<td>2010**</td>
</tr>
<tr>
<td>52</td>
<td>Gastrointestinal bleeding and bacteraemia markers of fatality Severity marked by hypothermia, leukocytosis and bandemia*</td>
<td>Adults</td>
<td>Taiwan, China</td>
<td>2012**</td>
</tr>
<tr>
<td>53</td>
<td>Platelet count &lt;50 000 /μL and prolonged prothrombin time</td>
<td>Children</td>
<td>Manila, Philippines</td>
<td>1992</td>
</tr>
</tbody>
</table>

SGOT: serum glutamic oxaloacetic transaminase.
*Bandemia refers to the presence of band-form granulocytes in the peripheral blood.
could be a clinical marker of disease severity and mortality. However, jaundice and elevation of liver enzymes occur relatively late in the illness. Hepatosplenomegaly is a clinical feature associated with macrophage activation syndrome, seen in many autoimmune diseases. It may be associated with DENV infection. In contrast to other viral infections, aspartate aminotransferase (AST) levels are much more elevated than alanine aminotransferase (ALT) levels in dengue patients with hepatic involvement. The higher levels of ALT could be due to the release of AST (serum glutamic oxaloacetic transaminase – SGOT) from skeletal muscle and the myocardium, as damage to muscle in dengue infections has been reported.

Both acute and chronic renal failure is associated with mortality in dengue infection. Acute renal impairment occurred in 71.4% of dengue deaths. Severe rhabdomyolysis may occur in patients with dengue fever and this can lead to acute renal failure. However, early and aggressive treatment may prevent acute renal failure and death.

Evidence of secondary bacterial infection has been found to be a determinant of mortality. A high white cell count indicates an associated bacterial infection. Dengue infection usually causes leukopenia. Bacteraemia was documented in 14.3% of patients, at a median duration of 6.5 days from hospital admission and 1.5 days from dengue diagnosis. The common organisms were Pseudomonas and meticillin-resistant Staphylococcus. The onset of bacteraemia after a period following admission points to the possibility of hospital-acquired infection.

Another important determinant of mortality identified in several studies was the presence of comorbidities. Dengue patients with diabetes mellitus, hypertension, chronic anaemia, congestive heart failure, other cardiovascular disease, chronic obstructive pulmonary disease, asthma, renal disease and multiple comorbidities were found to have higher incidence of mortality. The presence of acquired comorbidities like obesity, smoking and alcoholism was also associated with higher mortality.

Haemorrhagic complications, such as epistaxis, gingival bleeding, gastrointestinal bleeding, haematuria and hypermenorrhoea, although rare, are an important cause of death in dengue. Tachycardia at the time of admission was the only predictor of mortality in a Singapore case-control study. Acute respiratory failure is also documented as a clinical determinant of mortality and was present in 66% of dengue deaths. Rarely, cardiac involvement is also seen in patients who have died of dengue. They had cardiogenic shock with evidence of myocarditis, pericarditis, pericardial effusion and global left ventricular hypokinesia. The myocardial injuries could be secondary to the cellular immune response and the production of inflammatory cytokines, or a direct result of DENV infection of the myocardial tissue. These uncommon presentations are often related to poor prognosis and are associated with high mortality in severe dengue.
Investigation findings related to mortality

The platelet count may be a predictor of mortality: the risk of death is six times greater in those with a platelet count $<50,000/\mu L$ than in those with a platelet count $>50,000/\mu L$ in the paediatric population.\textsuperscript{71} According to a study by Feris et al. in an urban hospital, those with a platelet count less than 30,000/\mu L had a three times higher chance of dengue severity and/or mortality.\textsuperscript{72} Prolongation of prothrombin time was associated with higher fatality.\textsuperscript{71} Higher blood urea nitrogen (uraemia), lower bicarbonate (acidosis), higher activated partial thromboplastin time (APTT), higher serum glutamic pyruvate transaminase (SGPT) and SGOT are reported as predictors of mortality.\textsuperscript{51} Haematocrit may not be a sensitive marker of plasma leakage in dengue with severe bleeding. Elevated liver transaminases, creatinine kinase and lactate dehydrogenase, and lower triglycerides and hypoalbuminaemia could be good indicators of vascular leakage or hepatic dysfunction in DHF and could be used as significant markers in identifying cases of severe dengue.\textsuperscript{56} A haemoglobin level of less than 9 mg/dL was associated with a four-times higher risk of mortality and/or severity.\textsuperscript{71}

Management-related determinants of mortality

Successful clinical management of severe dengue may be helpful in preventing dengue deaths.\textsuperscript{2} This requires early recognition and supportive care. For early recognition, there is a need to educate patients and clinicians in identifying dengue and recognizing warning signs for severe dengue, so that anticipatory guidance can be given to minimize delay, and appropriate care can be initiated in a timely manner.\textsuperscript{2} In one study, missed opportunity for the correct diagnosis was found to be responsible for 64% of dengue deaths.\textsuperscript{2} Late hospitalization is cited as a reason for mortality.\textsuperscript{73} The WHO 2009 definition for segregating severe dengue/dengue with warning signs may help in early identification of patients who need close monitoring. Persistent vomiting, hepatomegaly, haematocrit rise and rapid platelet drop, and clinical fluid accumulation, as well as any three or four warning signs were highly specific for DHF or severe dengue. However, none of the warning signs were sensitive.\textsuperscript{74} WHO guidelines (2009) identified 78.6% of cases of dengue, while 36% were identified by the 1997 definition.\textsuperscript{76} However, 100% of severe dengue could be identified. The earliest warning sign was persistent vomiting\textsuperscript{16,20,26} and this was seen in a median time of 1.5 days.\textsuperscript{20} The commonest warning sign at presentation was lethargy, which was seen in 39.3% of cases, and haematocrit change of $\geq 20\%$ with a concurrent platelet count of $<50 \times 10^9/L$ seen in 71.4% of cases was the commonest sign seen within seven days of dengue diagnosis.\textsuperscript{20} Along with persistent vomiting, abdominal pain was also a frequent early warning sign.\textsuperscript{26} However, it is documented that the use of WHO 2009 criteria results in higher workload, lower quality of care, and higher chances of fluid overload and death.\textsuperscript{75} Not adhering to WHO management guidelines,\textsuperscript{4} failure to recognize warning signs for severe dengue and shock, prolonged waiting in the emergency department before admission, and infrequent patient monitoring were the management-related determinants of dengue mortality.\textsuperscript{2}
Automated triage systems detect patients with high body temperature and low systolic blood pressure. It would be useful in dengue-endemic countries for these systems to also identify patients with hypothermia, narrow pulse pressure and age-specific tachycardia in the absence of hyperthermia. This is being suggested because one reason for mortality identified was patients being given a low triage score on account of absence of hyperthermia and hypertension. In countries with a sizeable number of adult patients with severe dengue, it would be important for triage systems to identify patients with chronic hypertension, as low–normal systolic blood pressure may be abnormal for these patients.

Patients need to be monitored closely for warning signs and early signs of shock for 24 h after defervescence, and any signs need to be acted upon promptly.

Appropriate interventions, especially careful intravenous rehydration, and a greater evidence base for interventions, are required to reduce mortality. Fluid overload was found in 33% of dengue deaths and has been identified as a factor related to dengue mortality. Prompt management of shock with intravenous (IV) isotonic crystalloid solutions, followed by colloids to correct refractory shock before the terminal event, is important in prevention of mortality. Patients with prolonged shock and multi-organ failure have poor prognosis and high mortality rate, even with specific treatment. The quality of the evidence for the effect of colloids versus crystalloids for fluid resuscitation in dengue patients for prevention of deaths is low and the direction of effect is uncertain. In one study, one of the 11 patients allocated to 6% hydroxyethylstarch died, compared to three of 16 patients allocated to Ringer’s lactate. A systematic review evaluating the effects of colloids versus crystalloids in critically ill patients has been carried out. It included 74 trials, of which 66 reported mortality data. The review found no evidence that resuscitation with colloids reduced the risk of death compared to resuscitation with crystalloids. There is also a warning from the United States Food and Drug Administration not to use colloids in critically ill patients, especially if there is renal involvement and evidence of coagulopathy.

Administration of platelets/fresh frozen plasma (FFP)/blood transfusion in the event of clinically significant bleeding can prevent death. Reduction in platelet count does not correlate with severity of bleeding. Prophylactic platelet transfusion is not recommended, and does not expedite platelet recovery. Whether reduction in platelet count is associated with bleeding is debated in adults and it is not associated in children. Unnecessary transfusions can lead to a risk of pulmonary oedema. Steroids have no role in reducing mortality or reducing the requirement for blood transfusions. Moreover, they may produce stress ulcers, upper gastrointestinal bleeding, hyperglycaemia and immunosuppression, with increased risk of infection. On the other hand, there is a report of a 24-year-old woman diagnosed with dengue, detected to have haemophagocytic syndrome with haemophagocytic lymphohistiocytosis (HLH), who recovered with administration of corticosteroids. Dengue patients with pleural effusion or ascites may require tapping, but if this is not done with extreme caution, it can result in traumatic bleeding and death. Management of patients with dengue infection with predictors of mortality on presentation warrants admission to a high-dependency unit.
Postmortem findings

Postmortem was done after eight deaths due to dengue in Malaysia. The findings were haemorrhage over the hemispheres (brain) and septum (heart), pleural effusion and lung congestion, blood in the stomach, pericardial and pleural effusion, haemorrhage over the endocardium, fluid in the peritoneal cavity and stomach and enlarged liver and spleen. The histology findings were severe lung congestion with intra-alveolar haemorrhage, spleen congestion, severe fatty infiltration in the heart (suspicious of arrhythmogenic dysplasia) and liver fatty change. Evidence of tubular necrosis of the kidney, inflammation of the heart and pulmonary hypertension were additional postmortem findings in other studies. Tissue from patients who die of probable dengue has to be taken as soon as possible after death and stored at 70 °C or in formalin.

Discussion

The dramatic changes in global demographics since World War II are thought to have facilitated an increase in the absolute size of susceptible host populations, viral serotype/genotype coexistence and increased exposure of subpopulations with previous dengue exposure. Dynamically, changing virus populations have contributed to an increase in dengue incidence and mortality. This review has shown that dengue mortality is more likely in children, the elderly and some particular races. There is a probable shift in the trend towards mortality in higher ages compared to that seen in earlier years. Dengue in pregnancy has to be managed with caution. Among the ecological determinants, increasing age of endemicity and changes in the virulence and types of virus require special emphasis. There is less time available for prevention of mortality, so it is important to identify early predictors of dengue mortality. Involvement of organs, bacteraemia, comorbidities, haemorrhage and some biochemical parameters may be useful predictors for timely and appropriate intervention. The ecological determinants of dengue mortality have not been widely studied, except in Latin America. Studies of these determinants are worth replicating in other regions as well.

Prevention of dengue mortality

Dengue mortality can be reduced by implementing early case detection and appropriate management of severe cases; reorienting health services to identify cases early and manage dengue outbreaks effectively; and training health personnel, along with appropriate referral systems, at primary health-care levels. Investigations are necessary to establish the primary cause of death (including conducting autopsies). It is also necessary for the treatment of plasma leakage with comorbidities, and in pregnancy. Research is needed to find the predictive value of clinical and laboratory criteria in identifying severe cases that may be fatal if not appropriately managed. Activities (triage and management decisions) at the primary- and secondary-care levels, where patients are first seen and evaluated, are critical in determining
the clinical outcome of dengue. A well-managed front-line response not only reduces the number of unnecessary hospital admissions but also saves the lives of severe dengue patients. Early notification of dengue cases seen in primary and secondary care facilities, as well as commonly accepted definitions of outbreak indicators (“triggers”), are crucial for identifying outbreaks and initiating an early response. Systems of reference and counter-reference between different levels of health-care-delivery services need to be established. It is necessary to build capacity and establish quality assurance in both private and public sectors. Evidence-based training material, including dengue courses, need to be established. Integrated surveillance and outbreak response, sustainable vector control, future vaccine implementation and operational and implementational research are all urgently needed to make progress in reducing dengue deaths. Primary health-care settings and the community are ideal sites and targets for this type of preventive management based on health education and active case detection. This involves training of all medical and nursing staff, students and community health workers, as well as reorganizing health care in primary health-care units and hospitals and redistributing available resources during a dengue outbreak.

Opportunities and challenges

For a disease that is complex in its manifestations, management of dengue is relatively simple, inexpensive and highly effective, and can save lives provided correct and timely interventions are instituted. Laboratory tests using NS1 (non-structural protein 1) antigen can provide early diagnosis in febrile patients. Making a diagnosis requires correct timing of specimen collection. Confirmation by laboratory is required because the clinical manifestations are common for many other diseases. First-time (primary) dengue virus infections typically have a stronger and more specific immunoglobulin M (IgM) response; subsequent (secondary) infections show a weaker IgM response but a strong IgG response. These differing patterns of IgM response to infection underscore the need to evaluate the sensitivity and specificity of commercially available tests, especially for diagnosis of secondary dengue virus infections. There are no biomarkers for predicting which admitted patients are likely to develop severe disease. The time between the onset of disease, its diagnosis and death – the window of opportunity to save life – is limited. Other challenges include the wide spectrum of illness, which makes it difficult to set referral criteria for diagnosis. Management protocols for dengue may have to be periodically updated, considering the changing clinical patterns. Also, all levels of health facilities – primary, secondary and tertiary – need to be appropriately equipped in terms of resources to manage dengue cases effectively. Underreporting/failure to designate dengue as an underlying cause of death on death certificates is another major challenge that deters the correct estimation of the magnitude of deaths due to dengue. Various endemic countries, such as Colombia and Brazil, have specific protocols for the etiological study of all fatal cases of dengue, by testing serum samples or samples of other types of tissues. There is a need to develop a protocol for epidemiological investigation of fatal cases of dengue. Dengue death audit, including verbal autopsy at the provider and family level, may be useful to explore the missed opportunities for prevention of mortality.
Limitations of the review

Incomplete retrieval of identified research, reporting bias and the lack of quantitative analysis are major limitations of this review. No cohort studies on dengue mortality/determinants of dengue mortality could be retrieved. The quality of studies has not been measured using any criteria, and hence their results have not been weighed differentially. The CFR reported from several studies has a potential of bias of overreporting/underreporting based on the selection of denominator(s).

Conclusions and recommendations

The case fatality due to dengue may be low, but since the number of incident cases is increasing, the absolute number of deaths may also be increasing. Moreover, there is documentation of exponential marked increase in mortality with increasing age of endemicity. Deaths due to dengue mean lost lives in young children and those in productive years of life. The high mortality in pregnancy needs to be verified by prospective cohort studies, documenting all infections. Mortality from dengue can be significantly reduced if severely ill patients access health services in time, and receive appropriate clinical care. The technical knowledge required to achieve this objective is available; its implementation depends on capacity-building. Simultaneous efforts at prevention of disease outbreaks by integrated vector management and integrated management strategy supported by an effective epidemiological and entomological surveillance can reduce the burden of dengue disease and its mortality. Research is needed into prediction of risk of mortality, and into effective clinical management. Prospective cohort studies may require a large multicentre population base because of the rarity of dengue death; this may require studies at national level. Studies that measure the severity of the disease and its relationship with mortality can be useful to generate evidence of cases and the situations of lives saved despite being severely affected. The review of studies on dengue mortality shows that evidence is either not available or is documented from several countries, including India, which are seriously affected by dengue. A surveillance of dengue mortality globally needs to be put in place to fill these gaps.

This review also reveals that most studies on dengue mortality are confined to clinical settings. There are only limited studies on the virology-related determinants. Studies correlating the vector dynamics and dengue mortality are lacking. A platform bringing together epidemiologists, entomologists and clinical experts to work on the mortality of dengue is required to develop guidelines on the prevention of mortality in dengue. The increasing number of lives lost due to dengue and its expansion, both in incidence and geography, warrant an urgent revamping of the task force and tools to fight the disease, achieve the targets set for 2020, and, ultimately, conquer the disease for ever.
References


A comprehensive review of dengue mortality and its determinants


Identification of dengue infection by different methods in Manaus, Amazonas, Brazil, during 1998–2012

Regina Maria Pinto de Figueiredo,a# Maria Paula Gomes Mourão,a Evaulino F Itapirema,a Adryanne Karolynne Moreno de Matos,a Miriam do Nascimento Melo,a Igor dos Santos Fonseca,a Sergio Pinto,a João Bosco Lima Gimaque,a Felipe G. Naveca,b Michele de Souza Bastosa and Bedsy Dutary Thatcherb

aFundação de Medicina Tropical Doutor Heitor Vieira Dourado, Manaus, AM, Brazil
bInstituto Leônidas e Maria Deane – Fiocruz Amazônia, Brazil

Abstract

The diagnosis of dengue virus (DENV) was performed by different methods in Manaus-Amazonas (Brazil) during the period 1998–2012. Ninety-three serum samples were sequenced, confirming the results of typing by reverse transcriptase polymerase chain reaction (RT-PCR), and virus isolation detected the genotypes of DENV-2/II, DENV-3/III and genotypes I and II of DENV-4. Simultaneous viruses were detected in 15 samples for DENV-3/DENV-4, three for DENV-1/DENV-4, two for DENV-2/DENV-4 and one for DENV-1/DENV-3. All four serotypes of the virus were detected in naturally infected Aedes aegypti.

Keywords: Amazonas; Brazil; Dengue; Genotypes; Serotypes.

Introduction

The arboviruses are a serious public health problem, especially in tropical regions, including Brazil. The Amazon region, in particular, has environmental characteristics and climatic and social conditions that are conducive to the proliferation of certain arboviruses, which are responsible for epidemic outbreaks.¹ Dengue fever (DF), the most prevalent arthropod-borne viral illness in humans, is caused by the dengue virus (DENV).² It is the most widespread arbovirus in the world and is an important cause of morbidity, mortality and epidemics, resulting in high social and economic impact.³ The dengue virus belongs to the genus Flavivirus, family Flaviviridae; the genome is a single-strand of RNA, composed of three genes encoding structural proteins and genes for seven non-structural proteins.⁴ Initial studies using the sequencing E/NS1 region (240 nucleotides) identified five distinct genotypes for DENV-1, DENV-2 and DENV-3. Analysis of the complete sequence of the gene determined four genotypes for the DENV-4 serotype. These genotypes circulated with the other five genotypes.⁵

#E-mail: figueiredormp@yahoo.com.br
In Brazil, the first documented outbreak of dengue occurred in 1981 and 1982 in Boa Vista (Roraima), caused by DENV-1 and DENV-4. In 1990, there was a massive upsurge of cases, as a result of the increased circulation of DENV-1 and introduction of DENV-2 in Rio de Janeiro, where cases of dengue haemorrhagic fever (DHF) were recorded for the first time. Manaus-Amazonas was infested by *Aedes aegypti* in 1996 and the first autochthonous cases of dengue were registered in March 1998, when the Reference Center of Tropical Medicine (Fundação de Medicina Tropical Doutor Heitor Vieira Dourado – FMT-HVD, Manaus, Brazil) implemented a surveillance system for the diagnosis of acute undifferentiated febrile syndromes, with the objective of active and passive surveillance in the Brazilian western Amazonian rainforest, in order to identify and diagnose the etiological agents of acute fever. The first records with laboratory confirmation of dengue occurred in the period 1998–1999. Samples of serum from 81 patients suspected of dengue, from 19 different counties of the state of Amazonas, were sent to FMT-HVD, from the respective health centres in each community. Serum from patients drawn in the municipalities – Autazes, Careiro Coari Iranduba, Manacapuru and Tefe – were seropositive for dengue. In March 1998 in Manaus, a variety of acute febrile diseases with and without haemorrhagic manifestations were diagnosed by detection of specific immunoglobulin M (IgM) antibodies for Mayaro virus (MAYV) and Oropouche virus (OROV). Infections with rubella and parvovirus were also observed.

Coinfections, including distinct dengue serotypes, are probably more common in tropical regions of the world where dengue is hyperendemic, with circulation of all the four serotypes. Risk factors for dengue infections and coinfections include the virulence of the virus and the density of *Aedes aegypti*. In Brazil, one case of coinfection by DENV-1 and DENV-2 was reported in a patient with classic dengue fever from the south-east region, in 2001. Another case of coinfection by DENV-2 and DENV-3 was reported in 2005 in a dengue patient from the north-east region, who recovered without a relapse. During an outbreak of dengue in São José do Rio Preto, in the state of São Paulo, 365 samples were positive for DENV-3, five samples were positive for DENV-2, and eight were positive for St Louis encephalitis flavivirus (SLEV). Among the positive samples, one coinfection with DENV-2 and DENV-3 was detected.

This paper reports four cases of DENV-3/DENV-4 coinfection detected by serological and molecular tests among 674 patients with acute undifferentiated fever, from the tropical medicine reference centre of Manaus City, Brazil, between 2005 and 2010. This study is a review of the laboratory detection of dengue in Manaus since its inception in 1998. Samples were collected from patients treated at a reference centre for tropical medicine in the city of Manaus, Brazil, between 1998 and 2012, and were tested for dengue virus infection by different methodologies.

In 2010, DENV-4 was detected in naturally infected *Aedes aegypti*. Today, all four serotypes of the virus are detected, by the polymerase chain reaction (PCR) method, in naturally infected *Aedes aegypti*. 
Materials and methods

Study area

The Amazon is situated in the northern region of Brazil, bordering in the north with the state of Roraima and Venezuela and Colombia, in the east with the state of Pará, in the south-east with the state of Mato Grosso, in the south with the state of Rondônia and in the south-west with the state of Acre.15 Manaus is a city where eco-tourism is being developed, and the Industrial Pole of Manaus, with over 450 factories of large, medium and small sizes,16 attracts investors from around the world. The city receives many people from these and other states and countries.

Serological diagnosis

Serum samples, collected from patients from the sixth day of illness and stored at –20 °C, were tested for dengue virus infection by detection of anti-dengue immunoglobulin M (IgM)-specific antibodies, using IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA), according to the technique described by Kuno et al.17

Virus isolation

Acute-phase serum samples were used in two tests: one for viral culture by serum inoculation into Aedes albopictus cell line C6/36 and after 10 days of incubation, followed by viral antigen identification with type-specific monoclonal antibodies (kindly provided by the Centers for Disease Control and Prevention), in an indirect immunofluorescence assay.

RNA was extracted directly from serum samples, using the QIAamp Viral RNA Mini-Kit (Qiagen, United States of America [USA]), following the manufacturer’s instructions, and submitted to reverse transcriptase (RT)-PCR, followed by semi-nested multiplex PCR as previously described for DENV detection and typing.18 Reverse transcription was conducted on 5 μL of extracted RNA, with kit AccessQuick™ RT-PCR System (Promega), following the manufacturer’s instructions, and random primers. After incubation, 2 μL of each cDNA was submitted to PCR amplification with D1 and D2 primers, for 35 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, and a final extension of 10 min at 72 °C.

A second round of amplification was performed with 10 μL (diluted 1/100) of the first amplicon; a mixture of type-specific reverse primers (TS1–TS4) and the conserved forward primer D1 was submitted to the same cycling parameters used on the first reaction. Amplicons from the C/PrM region were purified and sequenced in both directions, using the BigDye Terminator Cycle Sequence Kit (Applied Biosystems, USA). The genotypes were detected by a search tool.19

Positive samples of DENV-2 (AM2266; AM2236; AM2238; AM2290) were amplified with the primers that anneal to the envelope region and NS5 of DENV-2 and Flavivirus, respectively.
Identification of dengue infection in Manaus, Amazonas, Brazil during 1998–2012

Results

For the period 1998–1999, virus isolation detected the presence of DENV-1 and DENV-2, the latter being the second serotype associated with dengue epidemic/DHF cases that occurred in 2001. From March 2001 to December 2002, 132 samples were tested; 72% of those tested positive for group B antigen (Flavivirus). Of those that were positive for group B, 21% were identified as serotype DENV-1, 16.8% tested positive for DENV-2, and one sample tested positive for DENV-3, which was an imported case from Salvador–Bahia. Samples collected in 2000 were also analysed. From December 2002 to December 2003, the positivity was 37.11%, 21.65% and 3% for DENV-1, DENV-2 and DENV-3 respectively. From March 2004 to July 2007, dengue cases were confirmed by the detection of IgM antibody; during this period, 215 samples were inoculated into cell culture (C6/36) and isolation was possible in 48.3% of the samples. Of these, 26.6% were DENV-1, 32.6% DENV-2 and 48% DENV-3. It is important to note that in the period from 1998–2004, 90 serum samples showed positivity to group B antigen (Flavivirus) but were negative to dengue, suggesting the occurrence of other flaviviruses in Manaus.

During 2005–2010, in the course of the project, “Evolutionary Molecular Characterization of Dengue Virus” in the state of Amazonas, Brazil, patients were tested for malaria by thick blood analyses, and all patients with negative results were asked to participate in this study. The participants signed an informed consent form that was approved by the FMT-HVD Ethical Committee Board (272/2005). Two blood samples were collected from each patient, one in the acute phase of the disease and the other in the convalescent phase. The RT-PCR technique was standardized and 574 samples from the acute phase were tested. During this period, 44.4% (255/574) of samples were positive for dengue virus by RT-PCR and, of these, 92 were isolated through cell cultures. Of the PCR-positive samples, 93 underwent nucleotide sequencing. During the same period, 110 patients in the convalescent phase returned for collection of a second blood sample, which was tested by MAC-ELISA. All were positive (see Table 1).

Table 1: Samples detected by different tests, Manaus, Amazonas, Brazil, 2005–2010

<table>
<thead>
<tr>
<th>Test</th>
<th>Samples tested, n (%)</th>
<th>DENV-1, n (%)</th>
<th>DENV-2, n (%)</th>
<th>DENV-3, n (%)</th>
<th>DENV-4, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC-ELISA</td>
<td>110 (19.1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RT-PCR</td>
<td>255 (44.4)</td>
<td>7 (2.7)</td>
<td>29 (11.3)</td>
<td>79 (31)</td>
<td>81 (31.7)</td>
</tr>
<tr>
<td>Virus isolation</td>
<td>92 (16)</td>
<td>1 (1)</td>
<td>17 (18.4)</td>
<td>58 (63)</td>
<td>11 (12)</td>
</tr>
<tr>
<td>Nucleotide sequencing</td>
<td>93 (61.1)</td>
<td>2 (2.1)</td>
<td>7 (7.5)</td>
<td>41 (44.1)</td>
<td>30 (32.3)</td>
</tr>
</tbody>
</table>

MAC-ELISA: immunoglobulin antibody-capture enzyme-linked immunosorbent assay; RT-PCR-reverse transcriptase polymerase chain reaction.
Analysis of genotypes of Asian/American (genotype II) sequences for DENV-2, genotype III for DENV-3; and genotype I and II for DENV-4 were identified. The genotype II was detected in samples collected during the outbreak of 2011 (see Table 2); the information on residence and place of origin showed that three positive results for dengue originated from the entire city of Manaus. Table 3 shows the numbers of patients who were in transit or who had been out of the Amazon up to 15 days before the onset of symptoms and were also seropositive for dengue.

**Table 2: Results of genotyping of dengue virus, Manaus, Amazonas, Brazil**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Collection year</th>
<th>Accession numbers</th>
<th>Genotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM110</td>
<td>2002</td>
<td></td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td>AM682</td>
<td>2005</td>
<td>JF923865</td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DENV-3/GIIIa</td>
<td>(DENV-4/GI)</td>
</tr>
<tr>
<td>AM707</td>
<td>2005</td>
<td>JF923879</td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DENV-3/GIIIa</td>
<td>(DENV-4/GI)</td>
</tr>
<tr>
<td>AM726</td>
<td>2005</td>
<td>EU127898</td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DENV-3/GIIIa</td>
<td>(DENV-4/GI)</td>
</tr>
<tr>
<td>AM1041</td>
<td>2006</td>
<td></td>
<td>DENV-4/GI</td>
</tr>
<tr>
<td>AM1444</td>
<td>2006</td>
<td></td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td>AM2176</td>
<td>2006</td>
<td></td>
<td>(DENV-2/GIIa)</td>
</tr>
<tr>
<td>AM2206</td>
<td>2007</td>
<td></td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td>AM2220</td>
<td>2007</td>
<td>JF923873</td>
<td>(DENV-4/GIa)</td>
</tr>
<tr>
<td>AM2236</td>
<td>2008</td>
<td></td>
<td>(DENV-2/GII)</td>
</tr>
<tr>
<td>AM2238</td>
<td>2008</td>
<td></td>
<td>DENV-2(GII)</td>
</tr>
<tr>
<td>AM2258</td>
<td>2008</td>
<td></td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td>AM2266</td>
<td>2009</td>
<td></td>
<td>(DENV-2/GII)</td>
</tr>
<tr>
<td>AMECF</td>
<td>2010</td>
<td></td>
<td>(DENV-3/GIIIa)</td>
</tr>
<tr>
<td>AM28725</td>
<td>2010</td>
<td></td>
<td>(DENV-2/GII)</td>
</tr>
<tr>
<td>AM127941</td>
<td>2011</td>
<td></td>
<td>(DENV-2/GIIa)</td>
</tr>
<tr>
<td>AM130649</td>
<td>2011</td>
<td></td>
<td>(DENV-2/GIIa)</td>
</tr>
<tr>
<td>AM148779</td>
<td>2011</td>
<td></td>
<td>(DENV-4/GIIa)</td>
</tr>
<tr>
<td>AM150201</td>
<td>2011</td>
<td></td>
<td>(DENV-4/GIIa)</td>
</tr>
<tr>
<td>AM161613</td>
<td>2011</td>
<td></td>
<td>(DENV-4/GIIa)</td>
</tr>
</tbody>
</table>

*aThe genotypes presented (GI, GII, GIII) were those with the highest probability, despite the small size of the PCR fragment sequenced; the genotypes were the most likely, although the reliability is not 100%, owing to the small sample size analysed.*
### Table 3: Numbers of patients that were positive for dengue who were in transit or who had been out of the Amazon, Manaus, Brazil

<table>
<thead>
<tr>
<th>Localities</th>
<th>MAC-ELISA</th>
<th>RT-PCR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DENV-2</td>
<td>DENV-3</td>
<td>DENV-4</td>
</tr>
<tr>
<td>Rondônia(^a)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mato Grosso do Sul(^b)</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Venezuela</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Asia</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rio Preto da Eva(^b)</td>
<td>4</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tarumanzinho(^b)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maués(^b)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhamunda(^b)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estrada de Autazes</td>
<td></td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apuí</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR-174(^c)</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Am 010(^c)</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Manicoré(^b)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uricurituba(^b)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carauari(^b)</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presidente Figueiredo(^b)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Careiro do Castanho(^b)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ramal Ipiranga(^b)</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>27</td>
<td>7</td>
<td>15</td>
<td>1</td>
</tr>
</tbody>
</table>

MAC-ELISA: immunoglobulin antibody-capture enzyme-linked immunosorbent assay; RT-PCR-reverse transcriptase polymerase chain reaction.

\(^a\)Brazilian states.

\(^b\)Municipalities of Amazonas.

\(^c\)State highways.
Simultaneous coinfections between viruses were detected in 15 samples for DENV-3/DENV-4, three for DENV-1/DENV-4, two for DENV-2/DENV-4 and one for DENV-1/DENV-3. Two samples with coinfection with viruses DENV-3 and DENV-4 showed severe symptomatology; when the records were analysed, the following laboratory data were recorded: sample AM2112 – platelet count 120 000 /μL, clinical diagnosis: dengue complications. Sample AM750 had disorientation, anuria, petechiae, haemorrhagic shock, sore throat, reducing haematocrit 48.4, and platelets >114 000/μL or mm³.

Simultaneous co-circulation of the four dengue serotypes was observed during the 2011 outbreak in 137 patients with dengue fever: 17 DENV-1; 51 DENV-2; 22 DENV-3; and 31 DENV-4, and coinfections: 6 DENV-3/4; 5 DENV-1/4; 3 DENV-1/2; 2 DENV-2/3. In 2012, from 48 samples tested by RT-PCR, 7 were positive. Of these, 3, 2 and 2 were infected with DENV-1, DENV-2 and DENV-4 respectively.

Discussion

In Brazil, the DHF cases occurred after the introduction of DENV-2 in the state of Rio de Janeiro, and confirmation of DHF by DENV-2 occurred after an epidemic caused by DENV-1, which had occurred four years earlier. This same pattern was seen in Cuba during the epidemic of 1981, with sequential infection by two serotypes (DENV-1 and DENV-2), and persisted for 5 years approximately. Considering the distribution of serotypes of dengue since its introduction in the Amazon in 1998, it can be seen that the dynamics of the epidemic of dengue in Amazonas are similar to those in other regions of Brazil and the Americas. Since the first dengue epidemic in Manaus was observed, there was an interval of at least 3 years before a new serotype was established to cause an epidemic; serotype DENV-1 was the causative agent of that epidemic, but it is likely that this virus was circulating earlier as well, as the positivity of Aedes aegypti had been confirmed in Manaus in 1996. In 2001, cases of DHF were registered in the state of Amazonas, with isolation of the DENV-2 virus for the first time; DENV-2 was also isolated from stored samples, collected in 1999. In 2001, there was a second epidemic of dengue, most likely caused by DENV-2.

In 2002, serotype DENV-3 was first isolated from a patient coming from Bahia; thereafter, autochthonous cases were diagnosed by viral isolation. The emergence of DENV-4 in 2008 did not cause a major epidemic with severe and fatal cases. It is possible that, owing to competition with other serotypes, DENV-4 took some time to become established and the population may have gained immunity from antibodies produced in heterologous infections by heterologous serotypes. In fact, samples obtained in 2005 and 2006, kept at –80 °C in the FMT-HVD collection, and analysed in the second half of 2007, were found to be positive for DENV-4.

Initially, cases of DENV-3/DENV-4 coinfection were detected by serological and molecular tests. The coinfectected patients presented benign clinical manifestations and recovered without sequelae, corroborating the findings in previous reports. Cases of DENV-1/4 and DENV-2/4
were detected subsequently by RT-PCR. Dengue fever in uncomplicated cases of coinfection has also been observed by other authors, supporting the hypothesis that simultaneous infection with more than one dengue virus results in a more severe form of the disease.\textsuperscript{11,27}

The analysis of the sequences obtained showed the presence of genotype Asian/American (genotype II) for DENV-2, and the sequence AM2266 was obtained from the serum of patients with severe dengue, confirming the association of this genotype with the appearance of DHF.\textsuperscript{11} The sequences in this study indicate the presence of genotype III DENV-3, which carries a high potential for distribution and adaptation in various geographical areas of the world. This subtype has been implicated in epidemics of DHF in Asia, Africa and the Americas, and has high potential to cause a pandemic of dengue.\textsuperscript{28} Since its identification in Manaus in 2002, DENV-3 has been associated with cases of classical dengue fever. This situation was also described in Colombia, where a great number of cases occurred after the re-emergence of DENV-3, but it generated mild febrile or primary cases of dengue fever.\textsuperscript{29}

The Asian DENV-4, genotype I, which has been associated with DHF in the Asian continent, was identified in samples collected in 2005 in Manaus; however, it was not associated with serious cases of dengue. In 2011 only, the epidemic frequency of DENV-4 was high in relation to the period after its identification, followed by DENV-1 and DENV-2, whose frequency was very low in the period 2005–2010. This study also identified genotype II DENV-4, which is often associated with cases of dengue fever with mild symptoms. More severe cases of dengue were associated with another serotype. A similar situation has been observed since its introduction in the Americas in 1981.\textsuperscript{30}

There is a very complex interrelationship between the factors involved in the dynamics of the four serotypes of dengue virus that cause serious epidemics, as seen in south-east Asia.\textsuperscript{31} The classic epidemic that was considered benign occurred in 1979 in Cuba, and was caused by DENV-1, but it was soon followed by another in 1981, which was linked to DENV-2 and was surprisingly serious and caused thousands of cases of DHF.\textsuperscript{22} Each specific serotype, when introduced into large cities, becomes involved with the high density of vector present in the urban environment, to cause rapid transmission and explosive epidemics.\textsuperscript{7} The persistence of these infections in human populations is due to the high density of vector and the host stock, renewed through the birth rates.\textsuperscript{32} These conditions were not fully explained in the epidemiology of dengue in Manaus and its trajectory from 1998 to the present.

It can be concluded that Manaus has all dengue serotypes, with genotype II for DENV-2, genotype III for DENV-3 and genotypes I and II for DENV-4, and its epidemiological history is similar to that in other states in Brazil or in other countries in the Americas, as many geographical locations receive travellers from a range of endemic areas. These characteristics explain the entry of new dengue serotypes and genotypes, as well as other arboviruses, in Manaus.
References


Electrochemical identification of unlabelled virus using a capture-and-probe strategy with a nanoporous membrane sensor

Binh Thi Thanh Nguyen, Victor Chih Hao Gan, Yee Sin Leo, Katja Fink, and Chee-Seng Toh

Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, 21 Nanyang Link, Singapore 637371

Communicable Disease Centre, Tan Tock Seng Hospital, Singapore

Singapore Immunology Network, Agency for Science, Technology and Research, Singapore

Abstract

This paper reports a design for the selective identification of virus serotypes based on the rate of diffusive movement of an antibody probe through a nanoporous membrane immobilized with virus particles at the nanochannel walls. This approach utilizes the bioprobe to interrogate the specificity of the virus by means of the strength of the antibody–virus binding interaction. The initial flux of the eluting probe from the nanochannels is readily followed using differential pulse voltammetry. This strategy exploits the large number of nanochannels within the nanoporous membrane, to achieve large signal outputs using a low-cost electrochemical set-up. The method reports an outstanding unambiguous differentiation of dengue serotype 2 from serotype 3 in the human serum sample, for the concentration range 100 to 500 plaque-forming units (pfu)/mL. It achieves identification of a dengue-2-infected clinical serum sample in short total analysis time of 1 h, without any sample pretreatment. This design offers the outstanding promise of a portable, low-cost measuring unit with changeable membranes, each grafted with a different type of antibody to target different viruses and their serotypes.

Keywords: Antibody; Biosensor; Dengue serotypes; Electrochemical detection; Membrane.

Introduction

The diagnosis of diseases using immune-based methods is of scientific and technological significance. Antibodies are useful biomarkers to assess the magnitude and specificity of the immune response, aside from the disease status of patients. A variety of enzymatic amplification methods using labelled antibodies or antigens, along with surface-sensitive

E-mail: cstoh@ntu.edu.sg
Fluidic dengue serotype biosensor

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methods (using unlabelled reagents) such as acoustics, plasmon resonance or impedance, have been reported to detect antibody–antigen interactions. A further strategy to improve specificity is the use of sandwich or multiplicative approaches to magnify the differences between the binding affinities of target and non-target biological molecules for the same antigen or antibody. These methods have proven ease of utility and performance in disease diagnosis globally.

Consequently, immune-based methods that rely on antibody capture under equilibrium conditions are in widespread use, and for many diseases, serological tests remain the standard for disease diagnosis. However, cross-reactivity of antibodies is common, which gives false-positive results and limits the selectivity of these immune-based methods. Conversely, micro- and nano-fluidic methods, when applied to the separation of biological molecules, provide significant advantages over the above immune-based batch analysis methods. These miniaturized flow systems have used successful two-dimensional “lab-on-a-chip” designs, which comprise flow pumps with effective dampers and micro-sized channels grafted with antibodies for protein capture or separation. In addition, these can achieve high sensitivity, fast response and high resolution when coupled to micro- or nano-sized electrodes and nanopore structures.

At present, dengue virus diagnostics are based on direct virus detection using reverse transcriptase polymerase chain reaction (RT-PCR) or NS1 antigen detection, and serological methods based on detection of dengue-specific antibodies. Immunoglobulin G (IgG)-based tests are not recommended, owing to lifelong persistence causing false-positive detection. NS1-antigen-based diagnostics are only sensitive in the early phase of the infection, when virus remains detectable in the blood. A typical laboratory workflow for the diagnosis of dengue is NS1 antigen detection or immunoglobulin M (IgM) enzyme-linked immunoassay (ELISA), followed by serotyping of the infecting dengue using PCR. These combined methods are generally time consuming, each involving multi-step procedures that are costly and require trained personnel. This paper reports a method that demonstrates its potential in differentiating the dengue serotypes of infected patients, concurrently with disease detection. The biosensor’s design comprises electrochemical detection coupled to a continuous diffusion flow of redox-labelled antibody probes through arrays of nanochannels, whose walls are covalently grafted with immune reagents and subsequently immnocapture the virus particles. Because the nanochannel-bound viruses can bind the antibody probe and slow its diffusion movement along the nanochannels, it is possible to differentiate viruses of closely similar serotypes, owing to differences in their binding affinities with the diffusing antibody probe. Previously, detection of unlabelled viruses has been achieved using an antibody-immobilized membrane biosensor with a small organic redox probe. This virus capture-and-probe (CP) method differs from microfluidic protein separation analysis, which resolves multiple components via differentiation in the electrophoretic or convectional flow velocities of analytes.

This immune-membrane virus CP method successfully achieves identification of dengue serotype 2 (DENV-2) from dengue serotypes 3 and 4 viruses (DENV-3 and DENV-4) in infected
patients’ serum samples. Its ability to identify a specific dengue virus serotype within a short total analysis time of approximately 1 h (including the virus-binding equilibration time) is more rapid than the RT-PCR method that is currently being used. Overall, the simple design of the scheme comprises a low-cost potentiostat/galvanostat, no flow-injection instrumentation and a nanochannel membrane separating a two-compartment cell (see Figure 1).

**Figure 1:** a Antibody-grafted nanochannels, followed by incubation in a sample containing unlabelled viruses, followed by elution experiment of ferrocene-labelled anti-DENV-2 antibody; b schematic diagram of the experimental set-up (feed compartment: 100 μL of Fc-IgG, receiver compartment: 500 μL of phosphate-buffered saline pH 7.4, WE: glassy carbon electrode, RE: Ag/AgCl(1 M KCl), CE: platinum wire mesh)
Materials and methods

Materials

Bovine serum albumin (BSA, >98%), ferrocenecarboxylic acid (FcCOOH), phosphate-buffered saline (PBS, 10×), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimidehydrochloride (EDC), 3-aminopropyltrimethoxysilane (APS), dimethyl sulfoxide (DMSO), glutaraldehyde (CH2(CH2CHO)2) and bicinchoninic acid (BCA) protein assay kits were purchased from Sigma Aldrich. Anhydrous sodium carbonate (Na2CO3) was purchased from SCRS) and sodium hydrogen carbonate (NaHCO3) from GCE laboratory chemicals. Sodium bicarbonate buffer (SBB, pH 8.3) was prepared from Na2CO3 and NaHCO3 solution. Propylamine (C3H9N) and N-hydroxysuccinimide (NHS) was purchased from Merck, 85% phosphoric acid (H3PO4) from Lancaster Synthesis; anti-dengue serotype 2 antibody (clone 3H5, isotype IgG, 1.0 mg/mL) from Millipore; hydrochloric acid (HCl, 36.5–38%) from MSR; and Econo-Pac 10DG columns from BioRad. Nanoporous alumina membranes (Anopore) with 60 μm thickness, 13 mm diameter, 100 nm nominal pore size and a porosity of 25–50% were purchased from Whatman. All chemicals and materials were used as received. All solutions were prepared in (1× PBS) with high-purity water from a Millipore milli-Q (Biocel, 18.2 MΩ) water purification system.

Virus cultivation and inactivation

Aedes mosquito cells (C6/36) were infected with dengue serotype 2 viruses (New Guinea C strains) at a multiplicity of infection (MOI) of 0.01 for 1 h at 28 °C. Following this, the medium was removed and fresh growth medium (RPMI, 5% heat-inactivated fetal bovine serum [FBS] and 5 mM 2-mercaptoethanol) was added. Next, the cell culture was incubated at the same temperature for 5 days. After this time, the medium was collected and a plaque assay was carried out to determine the concentration of the dengue virus in terms of plaque-forming units (pfu/mL). The dengue virus sample was subsequently heat inactivated at 56 °C for 30 min.

Preparation of IgG/BSA labelled with FcCOOH

3H5 antibody (1 mg/mL, 900 μL) was concentrated to ~3 mg/mL in 300 μL, using an Eppendorf concentrator 5301 at 4 °C (solution A); 23 mg of FcCOOH was dissolved in 1 mL of DMSO (solution B); 0.14 mmol of EDC and NHS were dissolved in 1 mL of DMSO (solution C). Solution B was added drop-wise into solution C, with stirring. The mixture was subsequently stirred for 2 h at 25 °C. After this time, the mixture was added to solution A and stirred at 4 °C overnight. Free FcCOOH molecules were removed using an Econo-Pac 10DG column. Two bands were observed in the column. The first band contained IgG labelled with FcCOOH (IgG–Fc) and the second band contained the free FcCOOH.
molecules. The first band was eluted out using PBS as eluent, and collected in fractions of 1 mL. A BCA protein assay method was used to identify the IgG–Fc fractions, which were combined and concentrated to ~2 mL, using an Eppendorf concentrator 5301 at 4 °C. The final concentration of IgG–Fc was determined with the BCA protein assay method, using an Agilent Technologies, Carry 100 UV–Visible Spectrophotometer. Cyclic voltammetry of IgG–Fc was performed using an electrochemical station (CHI Instruments, Electrochemical Analyzer model 750D). For the BSA labelling, BSA solution (3 mg/mL, 300 μL) was used to react with solution B, following the same procedure as above.

**Grafting of anti-DENV antibody onto membrane nanochannels**

Grafting of antibody within the nanochannels of the alumina membrane was carried out using the silane–carbodiimide grafting reaction, as follows. The alumina membranes were first immersed in 5% APS in acetone for 1 h. Subsequently, the membranes were washed thoroughly with acetone and dried with nitrogen gas. The membranes were further dried in an oven for 30 min at 45 °C. The membranes were then immersed in glutaraldehyde for 6 h, followed by thorough washing with ultrapure water and drying with nitrogen gas; 30 μL of 100 mg/L of anti-DENV 3H5 antibody solution was drop-casted onto the membrane and kept at high humidity overnight. The membranes were subsequently rinsed with 1 M NaCl to remove any non-specific absorbed antibody, and dried with nitrogen gas; 30 μL of 10⁻⁵ M propylamine was added to each membrane and kept at high humidity for 6 h, to remove excess glutaraldehyde and to improve the hybridization efficiency. After this, the membranes were thoroughly washed using 1 M NaCl followed by ultrapure water, and dried with nitrogen gas.

**Analysis procedure of the immune-membrane capture-and-probe system**

All analyses were carried out at room temperature, in the custom-made acrylic cell where the modified membrane was clamped between the feed and receiver compartments (see Figure 1). At the start of the experiment, the feed compartment contained 100 μL BSA–Fc or IgG–Fc in PBS (pH 7.4) and the receiver compartment contained 500 μL PBS (pH 7.4) solution. A home-made glassy-carbon working electrode (WE), platinum mesh auxiliary electrode (CE) and Ag/AgCl (1 M KCl) reference electrode (RE) from CHI Instruments were placed in the receiver solution. The solution in the receiver compartment was stirred constantly throughout the analysis. The amount of labelled antibody/labelled-BSA (IgG–Fc/BSA–Fc) that crossed the membrane to the receiver compartment was measured using differential pulse voltammetry (DPV) with the CHI Instrument 750D, using the following parameters: potential scan 0.3–1.3 V versus Ag/AgCl (1 M KCl) reference electrode, 10 mV increment potential and a 50 mV amplitude. Repeated DPV scans were applied at intervals of 1 min, to measure the increase in IgG–Fc/BSA–Fc concentration with time in the receiver compartment.
Real sample analysis

Four clinical serum samples derived from patients infected with dengue virus serotype 2 (DENV-2), serotype 3 (DENV-3) and serotype 4 (DENV-4), and serum from an uninfected patient, were used in the clinical sample analysis. These serum samples were collected from patients between 3 and 5 days after the onset of fever, and the virus serotype was validated using RT-PCR assays. A volume of 15 μL of each serum sample was first diluted 2 times with PBS (pH 7.4). The diluted sample was subsequently drop-casted onto the antibody-grafted membrane, followed by incubation for 1 h, to capture the virus. Finally, the probe analysis procedure was carried out. The National Healthcare Group (Singapore) Domain Specific Review Boards had approved the Prospective Adult Dengue Study (Reference No.: 2009/00432), from which clinical samples were derived. Informed consent was obtained from all participants.

Results and discussion

Characterization of the immune-membrane capture-and-probe (CP) set-up

Figure 2 shows the signal response of the electrochemical detector toward ferrocene-labelled BSA as it passes through the same membrane from two different concentrations of feed solutions. As expected, the slopes of two lines are different, owing to the difference of concentrations in the feed solution and, therefore, different drop in concentration.

Figure 2: Signal response of the electrochemical detector, using the DPV technique, toward ferrocene-labelled BSA as it traverses the same nanochannel membrane from two different concentrations of feed solutions prepared in PBS (pH 7.4)
gradient across the membrane, giving rise to a faster rate of protein diffusion for the higher-concentration feed solution. Thus, the signal output can be measured as a form of flux that depends on the rate of diffusion of a probe that traverses the membrane nanochannels.

**Selectivity and specificity of the capture-and-probe system**

The 3H5 antibody, which binds exclusively to a particular region of the envelope domain III (EDIII) protein of DENV-2, was used as the labelled probe to interact specifically or non-specifically with the nanochannel-attached target or non-target virus particles respectively (see Figure 1). Figure 3 shows the DPV signal response towards the labelled probe at the received compartment, using this immune-membrane CP scheme, for a membrane containing anti-DENV-2-grafted nanochannels, equilibrated with DENV-2 virus sample and probed with an anti-DENV-2 antibody as the diffusing probe. Its DPV signal response is distinctly different from that of a membrane without any captured virus (control). To test the specificity of this method, the same experiment was carried out using another membrane grafted with the same anti-DENV-2 antibody but equilibrated with the non-specific DENV-3 virus sample, which is of high structural similarity to DENV-2. Figure 3 clearly shows that the DENV-3-captured membrane gives a different diffusion flux compared to the DENV-2-captured membrane. This can be attributed to the specific interactions of the labelled anti-DENV-2 antibody molecules with the nanochannel-bound DENV-2 virus, which slow the traversing rate of the labelled antibody probe, compared to the nanochannel-bound DENV-3 virus.

**Model**

The following derives a simple relationship between the cross-sectional area of the membrane nanochannels and the permeation rate of the antibody probe traversing the membrane nanochannels, captured with viruses along the nanochannel walls. The initial flux of the antibody probe traversing the nanochannels relies on the concentration gradient of the traversing antibody probes across the nanochannels and the nanochannels’ cross-sectional area, according to Fick’s diffusion law. To explain the effect of the nanochannel cross-sectional area \( A \) (cm\(^2\)) on the overall diffusion rate of the antibody probe, it is more appropriate to describe the permeation rate \( P \) (mol/s) instead of flux \( J \) [mol/(cm\(^2\) s)]:

\[
P = JA
\]

\[
P = -D \frac{(c_f - c_{rec})}{l_m} A
\]

where \( c_f \) and \( c_{rec} \) are the antibody probe concentrations in the feed and receiver solutions, respectively, \( l_m \) is the nanochannel length (60 \( \mu \)m) and \( D \) is the diffusion coefficient of the antibody probe.
Figure 3: a Plot of redox-labelled anti-DENV-2 3H5 antibody concentration at the receiver side (c_{rec}) derived from the electrode signal versus time as the antibody probe transverses the membrane after 1 h incubation with DENV-2 or DENV-3 virus; b plot of the rates of change of antibody concentration in the receiver solution with time (dc_{rec}/dt) for the two different virus samples and cell culture medium as the control. Membranes are prepared using APS covalent attachment with DENV-2 antibody 3H5.
At the feed side of the membrane, where the concentration of the ferrocene-labelled antibody probes equals $c_f$, the antibody probes bind specifically to the nanochannel-bound virus particles. As a result of the specific antibody–virus complexes formed within the nanochannels near the feed side of the membrane, the total cross-sectional area of the membrane nanochannels, $A$, will be less than its initial value $A_0$, thus reducing the initial flux of the antibody probe traversing the membrane. This decrease in the nanochannel cross-sectional area depends on the amount of antibody that is adsorbed onto the nanochannel-bound specific virus, which relies on the fraction of available binding sites ($1 – \theta$) on the virus surface, so that:

$$A = A_0 k(1 – \theta) \quad (2)$$

where $k$ is a proportionality constant and $\theta$ is the fraction of bound sites on the attached virus particle surface.

Assuming simple adsorption behaviour between the antibody and virus surface, the fraction of available binding sites ($1 – \theta$) can be described in terms of antibody–virus binding affinity, $K$, and the concentration of the antibody probe at the feed side of the membrane, $c_f$, following the usual Langmuir adsorption isotherm:

$$1 – \theta = \frac{1}{1 + Kc_f}$$

It is practically useful to express the permeation rate in terms of the rate of change in the receiver concentration:

$$\frac{P}{V_{rec}} = \frac{dc_{rec}}{dt}$$

where $V_{rec}$ is the volume of the receiver solution. Combining these with equations (1) and (2) gives:

$$\frac{dc_{rec}}{dt} = \frac{DA_0 k}{l_m V_{rec}} (c_f – c_{rec}) \frac{1}{1 + Kc_f}$$

(3)

It is assumed herein that, at short times, the receiver concentration remains low at $\sim 0$, so the difference in the feed and receiver concentrations is relatively constant throughout the short analysis time of a few minutes. In the following, the antibody probe concentration in the receiver solution was plotted over time. The probe concentration is calculated from the differential pulse voltammetric currents, which are directly proportional to the concentration of probe solution. From control studies, significant electrode fouling by the protein probe occurs after 10 min, so all experiments were carried out at $\sim 10$ min and less. The experimental rates of change in the receiver concentration were subsequently calculated.
from the gradients of the plot of probe receiver concentration over time and plotted versus time. Figure 3a and b shows the plot of concentration versus time and the rate of change in receiver concentration versus time respectively, using the antibody-grafted membrane to first capture the virus, followed by short-time analysis using labelled antibody probe.

Figure 3b shows that the initial rate of change of receiver concentration for the specific DENV-2 virus is significantly lower than that of non-specific DENV-3 virus and control. This indicates significant binding of the antibody probe to the nanochannel-bound virus that occurs before any antibody probe reaches the sensing electrode, so that the cross-sectional area of the nanochannels, $A$, is significantly reduced in the case of the specific virus, compared to the non-specific virus and control. To derive meaningful information on the antibody–virus binding affinity, the ratio of the initial fluxes (or rate of change of receiver concentration) is calculated from Figure 3b. From inspection of equation (3), the ratio of the initial rates of change of receiver concentration of DENV-3 to DENV-2, i.e.

$$\left(\frac{d c_{\text{rec}}}{d t}\right)_{\text{DENV-3}, \text{time}=0} / \left(\frac{d c_{\text{rec}}}{d t}\right)_{\text{DENV-2}, \text{time}=0} = \frac{1 + K_{\text{DENV-2}} c_f}{1 + K_{\text{DENV-3}} c_f}$$

Since the general specific binding affinity of DENV–IgG is $10^7$ to $10^9$/M, and the feed concentration of antibody probe is in the range of 10 µM,

$$\frac{1 + K_{\text{DENV-2}} c_f}{1 + K_{\text{DENV-3}} c_f}$$

equals close to the antibody-binding affinity ratio for the two viruses, $K_{\text{DENV-2}}/K_{\text{DENV-3}}$. From Figure 3b, a value for $K_{\text{DENV-2}}/K_{\text{DENV-3}}$ of ~1.7 is estimated. This value is somewhat lower than known corresponding ratios of the specific and non-specific binding rate constants of dengue serotypes to 3H5 antibody, which is probably due to non-specific adsorption of antibody probe to the membrane surfaces and nanochannel walls. Importantly, this result also indicates the relative ease of differentiation of the different serotypes over a short few minutes (less than 10 min) of probe analysis time.

The immune-membrane CP set-up is a reusable membrane-based electrochemical system. Unlike all previous redox-label-based immuno sensors, the three main components of the CP biosensor system – the working electrode, redox-labelled antibody and membrane – are distinctly independent, which allows flexible changes to suit the sample. For example, to analyse a dengue-infected sample that could contain dengue serotype 2 or 3 virus, the immune-membrane CP system can readily analyse both targets by switching from a DENV-2 capture membrane to a DENV-3 capture membrane, with the corresponding change in the traversing redox-labelled antibody probe. Since the signal produced depends on the flow
of redox-labelled probes through the membrane, the CP biosensor system is ideally suited for continuous monitoring. Additionally, the sensor’s unique selectivity can be achieved in a relatively short total analysis time of 1 h, which includes the virus-binding equilibration time.

The immune-membrane CP system also offers significant advantages over microfluidic-based protein analysis. First, there is no pump, thus the cost of the biosensor system can be significantly reduced. Second, the detector uses a conventional macro-sized electrode but can provide a highly sensitive response for a small 30 µL volume of 100–500 pfu/mL virus samples. This can be achieved because the parallel multi-arrayed nanochannels provide significant signal output at the electrode, owing to simultaneous elution of the antibody probe from the large number of nanochannels at any one time. Besides lowering the overall cost, this robust design avoids a laborious miniaturization procedure and associated instrumentation, thus suggesting potential utilization in fieldwork. Third, the narrow cross-sectional area of each nanochannel can achieve smaller dimensions compared to microfluidic devices, thus allowing intimate interaction between the diffusing antibody probe and the surface-bound virus, which will greatly improve the separation efficiency.

Clinical sample analysis

In analysis of clinical samples, direct detection of non-labelled target analytes is highly desired because it achieves minimal sample preparation and pretreatment. Using the same grafting method, the 3H5 antibodies were attached onto the walls of the nanochannels of the alumina membrane; the membrane was then incubated in DENV-2 virus solution and subjected to labelled antibody probe elution. This approach achieves virus capture during the equilibration time of less than 60 min, followed by the probe analysis procedure of ~10 min, giving a total analysis time of approximately 1 h. Since the virus is specifically captured using antibody–virus interaction, the grafted antibody approach is potentially applicable to direct detection of unlabelled virus in real sample analysis.

To test the utility of the method in clinical analysis, four serum samples were collected from an uninfected patient and patients infected with DENV-2, DENV-3 and DENV-4 viruses between 3 and 5 days after the onset of fever, and were validated using RT-PCR. Figure 4 shows the plot of concentration of redox probe in the receiver solution and the rate of change of the redox probe concentration in the receiver solution versus time. In the human serum samples, the PanBio ELISA test showed the presence of either IgG or/and IgM in all three serotypes sample (see Table 1). The presence of pre-existing antibodies in the sample can reduce the binding affinity of the virus toward the specific redox-labelled antibody. However, from Figure 4, it is still possible to differentiate DENV-2 from other non-specific viruses, based on the initial rate of change of receiver concentration at time 0 (Figure 4b). Thus, Figure 4 shows that the proof-of-concept can differentiate one virus serotype from two others in clinical serum samples, using the CP biosensor system. The anti-DENV-2 antibody
**Figure 4:** a Plot of redox-labelled anti-DENV-2 3H5 antibody concentration at the receiver side, derived from electrode signal versus time as it transverses membrane incubated with human serum samples infected with DENV-2, -3 and -4. Control is obtained from an uninfected human sample; b plot of the rates of change of antibody concentration in the receiver solution with time for the various human serum samples. Membranes were prepared using APS covalent attachment with DENV-2 antibody 3H5.
**Table 1:** RT-PCR and PanBio ELISA assay results for uninfected and dengue-infected patient serum samples

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Cycle threshold for screening PCR</th>
<th>Genotype</th>
<th>Fever day on enrolment</th>
<th>Infection status</th>
<th>Panbio dengue IgM capture</th>
<th>Panbio dengue IgG indirect</th>
<th>Panbio dengue IgG capture</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-2</td>
<td>24.89</td>
<td>Cosmopolitan, New Clade</td>
<td>5</td>
<td>Secondary</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>IgG capture positive by day 6</td>
</tr>
<tr>
<td>DENV-3</td>
<td>16.21</td>
<td>G1 Malaysia/Indonesia</td>
<td>3</td>
<td>Secondary</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>IgG capture positive by day 5</td>
</tr>
<tr>
<td>DENV-4</td>
<td>26.80</td>
<td>GII</td>
<td>5</td>
<td>Secondary</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
</tbody>
</table>


Probes eluted at the slowest rate of change of the receiver concentration for the DENV-2 sample, which was clearly distinguished from the other samples. Furthermore, the ratio of

\[
\frac{\frac{dc_{rec}}{dt}}{DENV-3 \text{ or } DENV-4, \text{time}=0} = \frac{\frac{dc_{rec}}{dt}}{DENV-2, \text{time}=0}
\]

is in the range of 3.3, which is consistent with the ratio of specific and non-specific binding affinities derived using laboratory-prepared dengue virus samples. This outcome demonstrates the excellent selectivity of the simple and direct analysis procedure without elaborate sample treatment, which should be relevant to larger-scale analysis of clinical samples.

**Conclusion**

The CP biosensor system offers the unique advantage of rapidly differentiating highly similar dengue virus serotypes within a short time. The overall detection scheme relies on diffusive mass transport of the labelled probes across the porous membrane. The sensor is specifically designed for the detection of DENV-2 compared to three other viruses. Importantly, this simple design can potentially be miniaturized further, using microelectrode arrays that are individually addressed at the end of each nanochannel to provide high-throughput analysis.
Acknowledgements

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References


A review on dengue diagnosis and epidemiology by a regional reference laboratory from 1986 to 2011, Rio de Janeiro, Brazil

Flavia Barreto dos Santos, Ana Maria Bispo de Filippis, Eliane Saraiva Machado de Araújo, Monique da Rocha Queiroz Lima, Fernanda de Bruycker Nogueira, Nieli Rodrigues da Costa Faria, Jaqueline Bastos Santos Simões, Simone Alves Sampaio, Priscila Conrado Guerra Nunes, Manoela Heringer da Silva, Dinair Couto Lima, Rita Maria Ribeiro Nogueira

Flavivirus Laboratory, Oswaldo Cruz Institute/FIOCRUZ, Av Brasil 4365, Manguinhos, Rio de Janeiro, RJ, 21045–360, Brazil

Abstract

Dengue fever (DF) activity in Brazil during the past 25 years has been evidenced by a large number of cases in most states. Dengue viruses 1 to 3 (DENV-1, DENV-2 and DENV-3) were introduced in Rio de Janeiro in 1986, 1990 and 2000, respectively. In 2010, DENV-4 re-emerged 28 years after its first isolation. DENV-1 caused an explosive “virgin soil” epidemic in 1986–1987. The introduction of DENV-2 in 1990 caused the first cases of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Introduction of DENV-3 caused severe epidemics in 2002, with the largest number of DF/DHF cases and deaths. In 2007–2008, the country experienced the most severe epidemic in terms of morbidity and mortality and severe cases in children. Phylogeny performed on DENV-2 identified distinct lineages of the Asian–American genotype. In 2009 and 2010, DENV-1 re-emerged and was prevalent in many Brazilian states. Phylogenetic studies also demonstrated distinct lineages of DENV-1. Since 1986, when virus isolation and immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) were first used, laboratory diagnosis has played an important role in disease surveillance and epidemiology. After the introduction of DENV-2 in 1990, the characterization of immune response performed by the haemagglutination inhibition test was replaced by IgG-ELISA. In the 1990s, real-time reverse transcriptase-polymerase chain reaction (rtRT-PCR) and sequencing were used for nucleic acid detection and characterization. rtRT-PCR and immunohistochemistry proved to be essential for the confirmation and study of fatal dengue cases. NS1 capture tests were used for the early diagnosis of DENV infections after 2007. Since the introduction of DENV, a total of 47 346 suspected dengue cases were received by the Laboratory of Flavivirus, IOC/FIOCRUZ, Rio de Janeiro, a regional reference laboratory for dengue diagnosis for the Brazilian Ministry of Health, from March 1986 to December 2011. The authors’ experience has shown that the implementation of new diagnostic techniques over the years has constituted important and reliable tools for dengue surveillance in Brazil.

Keywords: Brazil; Dengue virus; Diagnosis; Epidemiology.

#E-mail: flaviab@ioc.fiocruz.br
Introduction

Currently, an estimated 2.5 billion people living in urban areas in tropical and subtropical countries in South-East Asia, the Pacific and the Americas are at the risk of infection with a dengue virus (DENV). In 2012, the geographical distribution of dengue covered more than 125 countries, and the Americas have hyperendemic dengue with indigenous transmission in almost all countries. In 2012, Brazil reported 565,510 dengue cases, 4,055 severe cases and 284 deaths, or 52% of all dengue cases reported in the Americas. By the second epidemiological week of 2013, the country had already reported 25,879 dengue cases and five deaths.

The state of Rio de Janeiro (RJ) has a land mass of 43,780 km² and 15,989,929 inhabitants, is divided politically and administratively into 92 municipalities in distinct geographical regions, and has experienced dengue epidemics in the last 25 years. It is an important tourist centre, with a major human population passing through its international airport.

Dengue was first recognized in RJ 90 years ago, when Antonio Pedro described a disease clinically resembling dengue fever in the city of Niteroi. Aedes aegypti is considered the main vector for DENV in Brazil, despite the presence of Aedes albopictus. After the eradication of Aedes aegypti in 1950, the country became dengue free. However, the disease re-emerged with re-infestation of the country with Aedes aegypti. Since then, the disease incidence and vector abundance follow seasonal patterns in RJ, with peaks during the summer when both the rainfall and temperatures are high. The Laboratory of Flavivirus at the Oswaldo Cruz Institute, as a regional reference laboratory for dengue diagnosis for the Brazilian Ministry of Health, supports the dengue surveillance programme in the state.

Despite the outbreak caused by dengue virus serotypes DENV-1 and DENV-4 in Boa Vista, state of Roraima, in 1981–1982, and the reintroduction of Aedes aegypti in the 1970s, the minimal circulation of arboviruses in Rio de Janeiro did not encourage research in this field, particularly in light of the state’s public health priorities. However, Dr Schatzmayr, a renowned virologist at the Oswaldo Cruz Institute, envisaged dengue as a potential threat to RJ and attended an International Dengue Course in Venezuela in March 1986, sponsored by the Pan American Health Organization. By April, serology and virus isolation were established at the Laboratory of Flavivirus at the Oswaldo Cruz Institute. In the same month, the first DENV-1 case was isolated during an outbreak of exanthematic disease in the city of Nova Iguaçu, and, since then, RJ has become an important centre of epidemiological studies of dengue, with the introduction of DENV-2 in 1990 and DENV-3 in 2000. DENV-3 was prevalent in the majority of Brazilian states from 2002 to 2006 and, from 2007 to 2008, DENV-3 was displaced by DENV-2. In 2008, Brazil experienced a severe dengue epidemic, with 806,036 reported cases, and RJ alone accounted for 255,818 cases. In 2009, DENV-1 re-emerged in the south-east region and was detected in 50.4% of isolated viruses, displacing
DENV-2 and DENV-3. Despite the introduction of DENV-4 in RJ in 2010 and its subsequent spread to other states of the country, DENV-1 was the most prevalent dengue virus and was responsible for epidemics, with more than 2 million reported cases in Brazil in 2010 and 2011.

The dramatic increase of dengue cases in Brazil has led to the establishment and consolidation of a National Dengue Diagnosis Network in 1989 to monitor DENV transmission and spread. Since then, DENV surveillance has been accepted as one of the most important tools for the prediction of dengue epidemics. Each Brazilian state has a Central Laboratory (LACEN), where samples from suspected dengue cases at health centres and public hospitals are tested. The national network is supported by regional reference laboratories responsible for the five Brazilian regions (North, North-east, Midwest, South and South-east): Evandro Chagas Institute (IEC); the National Reference Laboratory, Adolfo Lutz Institute (IAL); LACEN/Distrito Federal; LACEN/Recife; and the Laboratory of Flavivirus at the Oswaldo Cruz Institute (FIOCRUZ/RJ). The latter maintains a surveillance programme in the state of RJ, to detect the role of the state in the introduction and spread of the disease. This paper reports and analyses the epidemiological and laboratory aspects of data obtained during DENV surveillance performed by a regional reference laboratory in RJ, Brazil, between 1986 and 2011.

**Materials and methods**

**Suspected dengue cases**

The specimens analysed in this study were collected between March 1986 and December 2011. Suspected dengue cases (n = 47,346) were received during a surveillance programme performed by the Laboratory of Flavivirus, IOC/FIOCRUZ, regional reference laboratory for the Brazilian Ministry of Health, located in RJ. Acute serum samples (collected ≤7 days after the onset of the symptoms) were stored at −70 °C and submitted for virus isolation, reverse transcriptase polymerase chain reaction (RT-PCR) and NS1 antigen-capture enzyme-linked immunosorbent assay (ELISA). Convalescent samples (collected >7 days after the onset of symptoms) were stored at −20 °C and used for immunoglobulin M (IgM) antibody-capture (MAC)-ELISA, haemagglutination inhibition (HI) and immunoglobulin G (IgG) antibody-capture ELISA (IgG-ELISA) tests.

**Virus isolation**

Virus isolation was performed by inoculation into C6/36 cells and isolates were identified by indirect fluorescent antibody test, using DENV type-specific monoclonal antibodies.
**Immunoglobulin M antibody-capture ELISA**

MAC-ELISA to detect anti-DENV IgM antibody capture was performed as described previously.\(^{23}\)

**Haemagglutination inhibition test**

The HI test was performed to characterize infections as primary or secondary, as previously described.\(^{24}\)

**Immunoglobulin G antibody detection ELISA (IgG-ELISA)**

IgG-ELISA has been previously described\(^{25}\) and was performed to characterize infections as primary or secondary, in place of the HI test for dengue cases previously confirmed by virus isolation, RT-PCR and/or MAC-ELISA.

**Viral RNA extraction**

Viral RNA was extracted from sera using the QIAamp Viral RNA Mini kit (Qiagen), following the manufacturer’s instructions, and was stored at \(-70\, ^\circ\text{C}\).

**Reverse transcriptase polymerase chain reaction**

RT-PCR for DENV detection and typing was performed as described previously.\(^{26}\)

**NS1 antigen capture ELISA**

The Platelia Dengue NS1 Ag-ELISA kit (Biorad Laboratories, Marnes-La-Coquette, France) was used according to the manufacturer’s instructions.

**Real-time RT-PCR (TaqMan) assay (rtRT-PCR)**

The one-step real-time RT-PCR assay was performed as described previously\(^{27}\) in the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, United States of America), for investigation of fatal dengue cases during the epidemic that occurred in 2002.
Generic reverse transcriptase-nested polymerase chain reaction (generic RT-nested-PCR)

Generic RT-nested-PCR for flavivirus detection was performed as described previously, as an alternative molecular tool to detect and confirm DENV infection during the epidemic that occurred in 2008.

Ethics statement

The samples were drawn from a collection belonging to an ongoing project approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil.

Results and discussion

DENV infection was first confirmed in the state of RJ in April 1986, when DENV-1 was isolated during an outbreak of exanthematic disease in the city of Nova Iguaçú. From 1986 to 1987, DENV-1 was the only DENV type isolated and was detected in 46.8% of cases, with 95% of isolated viruses producing a cytopathic effect in C6/36 cells. Anti-DENV IgM antibody was detected in 926 DENV-1 cases during the early phase of the disease (day 2) and persisted for 3 months after the onset of symptoms. About 59% of cases analysed by virus isolation and/or MAC-ELISA were confirmed, and the infecting DENV type was detected in 80% of isolations performed ≤4 days after the onset of the symptoms. At that time, the evaluation of clinically based dengue surveillance had demonstrated the need for laboratory-based surveillance. A serological survey estimated that, owing to intense DENV transmission from 1986 to 1987, more than one million persons were infected with DENV-1.

In April 1990, 4 years after DENV-1 had first been isolated, DENV-2 was isolated from a 56-year-old patient with dengue in the city of Niterói; however, the first death due to dengue shock syndrome (DSS) occurred in June 1991, after the introduction of DENV-2. The introduction of DENV-2 in 1990 resulted in establishment of the HI test for characterization of immune response, as secondary infections led to hospitalizations and severe cases. In fact, the predominant circulation of DENV-2 from August 1990 to 1991 was associated with more hospitalizations, and dengue haemorrhagic fever (DHF) cases were analysed. A seroepidemiological survey conducted in schoolchildren inhabiting the city of Niterói, RJ, after sequential epidemics caused by DENV-1 and DENV-2, characterized secondary DENV infection in 66% of the children tested by HI test. However, owing to its laborious and time-consuming characteristics, the HI test was replaced with IgG-ELISA in 1995. The protocol described by Miagostovich et al. showed that IgG titres were reliably associated with primary and secondary infections for characterization of the patients’ immune response.
RT-PCR\textsuperscript{36} was also established in 1995 as a rapid and specific molecular approach for detecting and typing DENV, which allowed the identification of DENV-1 and DENV-2 in 41% of previously confirmed dengue cases.\textsuperscript{37}

The implementation of molecular techniques in the 1990s constituted an important tool for DENV diagnosis and characterization of genotypes, with advances in phylogenetic and evolutionary studies. The first complete sequence of a Brazilian DENV-2 was characterized.\textsuperscript{38} The DENV-2 strains isolated from 1990 to 2000 were further analysed and circulation of the Asian/Asian–American genotype was confirmed.\textsuperscript{39}

In December 2000, during a virological surveillance performed in the city of Nova Iguaçu, where DENV-1 was first isolated in 1986,\textsuperscript{13} DENV-3 was isolated from a 40-year-old female presenting with dengue, and primary DENV infection was defined.\textsuperscript{15} DENV-3 was also isolated from naturally infected \textit{Aedes aegypti} collected during an entomological surveillance performed in Nova Iguaçu from July 2000 to June 2001, despite the circulation of DENV-1 and DENV-2 in humans.\textsuperscript{40} Co-circulation of DENV-1, DENV-2 and DENV-3 was later characterized by analysis of 5324 suspected dengue cases received from 2000 to 2001, when 35.3% of the cases were confirmed. DENV-1 was detected by virus isolation and/or RT-PCR in 62.7% of cases, DENV-2 in 24.3% and DENV-3 in 13%. RT-PCR played a definitive role in surveillance, as a rapid diagnostic tool that guided the implementation of control measures as local authorities were notified of confirmed DENV-3 cases. Furthermore, in combination with virus isolation, RT-PCR increased confirmation of cases due to DENV infections. By IgG-ELISA, 59.5% of DENV-3 cases were defined as primary infections. An IgM monotypic response in both primary and secondary infections with DENV-3 was observed in 46% of cases.\textsuperscript{19} By using the different methodologies available in the laboratory (MAC-ELISA, IgG-ELISA, RT-PCR and virus isolation), 53.3% of analysed cases were confirmed. Forty fatal cases were confirmed in the Laboratory of Flavivirus, IOC/FIOCRUZ, and, of these, 20 were positive by at least two methodologies that detected DENV-3 as the only infecting DENV type.\textsuperscript{15,41}

Phylogenetic analysis of DENV-3 isolated in 2001, based on E gene sequencing and complete genome sequencing of a DENV-3 isolated from the liver of a fatal case, characterized the viruses as belonging to genotype III.\textsuperscript{42} This DENV-3 genotype was responsible for a large and severe epidemic in 2002, as the number of DHF/DSS cases (\(n = 1831\)) and deaths (\(n = 91\)) exceeded the total number of DHF/DSS cases (\(n = 1621\)) and deaths (\(n = 76\)) in the entire country from 1986 to 2001.\textsuperscript{43} In the state of RJ, a total of 288 245 cases were reported.\textsuperscript{41} The role of DENV infection in pregnancy was evaluated in women infected by DENV-3 during the 2002 epidemic, and it was shown that the infection increased the risk of premature birth, especially if it occurred in the last trimester of pregnancy.\textsuperscript{44}

In 2003, rtRT-PCR was established in the laboratory as a novel research tool for DENV detection and quantification in suspected dengue cases. The amount of DENV-3 RNA in 42 patients with fatal and non-fatal outcomes during the 2002 epidemic in RJ, and its correlation with primary or secondary infection, was evaluated and a significantly higher virus titre was
found in the samples from fatal cases. Moreover, as more than half of the fatal cases were primary infections, antibody enhancement alone would not have explained the deaths. The rtRT-PCR also yielded the highest rate of positivity in detecting DENV-3 RNA in tissues from those fatal cases. This approach may play an important role for rapid diagnosis of dengue infections, given its accuracy and effectiveness.

DENV-3 was prevalent in RJ and most Brazilian states from 2002 to 2006; however, in 2007, DENV-2 re-emerged and displaced DENV-3. The re-emergence of DENV-2 in 2008 caused the most severe epidemic reported in Brazil and, in RJ, a total of 255,818 cases were reported. Increased disease severity and deaths were observed in children aged 15 years and under. A combination of factors, such as climate, mosquito abundance, susceptible population because of previous low transmission, or viral evolution, could explain the severity of this epidemic. In fact, a phylogenetic analysis of DENV-2 circulating in the 1990s, and after its re-emergence in 2007, identified two distinct lineages within the south-east Asian genotype. Moreover, a study with DENV-2 strains isolated over 20 years confirmed these observations and further characterized these strains.

A recent report by the authors’ study group described the results of active DENV surveillance in RJ during an inter-epidemic period (2004–2005): DENV-3 circulation in 2006, re-emergence of DENV-2 in 2007, and a severe epidemic caused by DENV-2 in 2008. From 2004 to 2006, DENV-2 was not evidenced and its detection rate increased from 10.7% in 2007 to 66.6% in 2008. Confirmation of DENV cases by any of the methodologies used (MAC-ELISA, RT-PCR and virus isolation) ranged from 2.3% in 2004 to 20.7% in 2006, characterizing an inter-epidemic period with case confirmations of 34.4% and 46.8% in 2007 and 2008, respectively. In this study, 69 fatal cases were confirmed and 79.7% were due to DENV-2.

Suspected dengue cases in 2008 were tested by a generic RT-nested PCR, as an alternative molecular tool to detect and confirm DENV infection. By using this approach, 18% of the suspected cases were confirmed. Despite its usefulness in detecting a wide range of Flavivirus, this method was shown to be less sensitive than the conventional RT-PCR that confirmed 42.8% of the cases, maybe because of the use of degenerated primers by the protocol. Another inconvenience is the need for sequencing amplified RNA.

The NS1 antigen-capture ELISA was established in late 2007 as an alternative approach for the early diagnosis of DENV infections. As previously discussed, the most used techniques for dengue serodiagnosis are based on anti-DENV IgM and IgG detection by using MAC-ELISA and IgG-ELISA. However, one of the limitations consists of the variations on the detection rate during the acute phase of the disease. The Brazilian Ministry of Health established this new approach in sentinel clinics throughout the country after the 2008 dengue epidemic, although this occurred before the evaluation of available commercial tests. Evaluation of the sensitivity and specificity of three commercially available dengue NS1 antigen kits was performed and demonstrated its potential use for early laboratory confirmation of acute DENV infection. The highest sensitivity (89.6%) was obtained by the NS1 Ag Strip (Biorad...
Laboratories); however, a lower sensitivity was observed for DENV-3 cases for all three kits.\textsuperscript{53} The usefulness of the NS1 tests was also described as an alternative tool to confirm DENV infection on tissue specimens from fatal cases.\textsuperscript{54}

In 2009, DENV-1 re-emerged in the south-east region of Brazil and was detected in 50.4\% of isolated viruses, thereby displacing DENV-2 and DENV-3. Despite the introduction of DENV-4 in Roraima state in 2010,\textsuperscript{55} and its subsequent spread to other states in the country, DENV-1 remained the most prevalent DENV type and was responsible for epidemics with more than two million cases in Brazil in 2010 and 2011.\textsuperscript{17,18} In the state of RJ, a total of 29 824 dengue cases were reported in 2010, an increase of 274.5\% when compared to 2009. Distinct from what was observed in the whole country, DENV-2 was still prevalent in the state and was detected in fatal-case patients who presented with comorbidities.\textsuperscript{18} For the first time, distinct lineages of DENV-1 were reported in RJ in 2010 and 2011.\textsuperscript{56}

The first DENV-4 cases reported in RJ in 2011 occurred in two young sisters living in Niteroi,\textsuperscript{57} and, at the same time, this DENV type was recovered from an individual \textit{Aedes aegypti} female collected in the field during entomological surveillance. Using molecular techniques, DENV-4 was identified and quantified in a single specimen of vector.\textsuperscript{58} Phylogenetic studies on Brazilian DENV-4 characterized those viruses as belonging to genotype II,\textsuperscript{55,59} and a recent report on DENV-4 isolated in RJ describes the emergence of genotypes I and IIb in the state.\textsuperscript{60}

As reported here, dengue has become a major public health problem in RJ, owing to many factors such as the human-host susceptibility, virus emergence and re-emergence and shifts in circulating DENV types/genotypes, vector abundance, and environmental factors. Since the establishment of dengue activity in RJ and Brazil, laboratory diagnosis of dengue has proven to be imperative in surveillance, by serving as an early warning tool. In this scenario, the implementation of the National Dengue Network in the country, and establishment of the reference laboratories for dengue diagnosis have constituted an important effort aiming to help control the disease.

Overall, during the last 25 years (March 1986 to December 2011), a total of 47 346 suspected dengue cases were received in the Laboratory of Flavivirus, IOC/FIOCRUZ, and 41 614 (87.89\%) were subjected to one or more of the routine diagnosis techniques available (MAC-ELISA, IgG-ELISA, HI, virus isolation, RT-PCR and NS1 ELISA). The yearly distribution of suspected dengue cases diagnosed by the different methodologies from 1986 to 2011 is shown in Table 1 and Figure 1.

A total of 32 374 cases (77.8\% of the total) were tested by MAC-ELISA; 25 037 (60.2\%) were subjected to virus isolation, 181 cases (0.4\%) to HI test and 829 cases (2.0\%) to IgG-ELISA. RT-PCR was performed in 7441 cases (17.9\%) and the NS1 antigen-capture ELISA in 1124 cases (2.7\%). The distribution of diagnosis and confirmation of suspected dengue cases, according to the methodology implemented and used over the 25-year period, is shown in Table 1.
Table 1: Suspected dengue cases confirmed by different methodologies used in routine diagnosis, 1986–2011

<table>
<thead>
<tr>
<th>Year</th>
<th>MAC-ELISA</th>
<th>Virus isolation</th>
<th>HI</th>
<th>IgG-ELISA</th>
<th>RT-PCR</th>
<th>NS1 capture ELISA</th>
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<tr>
<td></td>
<td>Positive/</td>
<td>Positive/</td>
<td>DENV types</td>
<td>Tested</td>
<td>Immune</td>
<td>Tested</td>
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<td>tested</td>
<td></td>
<td></td>
<td>response</td>
<td>response</td>
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<td>1986</td>
<td>252/1558</td>
<td>594/1303</td>
<td>594 DENV-1</td>
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<td></td>
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<td>30/391</td>
<td>22/149</td>
<td>22 DENV-1</td>
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<td></td>
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</tr>
<tr>
<td>1989</td>
<td>57/412</td>
<td>39/177</td>
<td>39 DENV-1</td>
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<td>1990</td>
<td>446/2669</td>
<td>396/1473</td>
<td>234 DENV-1, 162 DENV-2</td>
<td>155</td>
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<td>1991</td>
<td>105/3508</td>
<td>56/841</td>
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<td>1992</td>
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<td>0/79</td>
<td>0</td>
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<tr>
<td>1993</td>
<td>7/119</td>
<td>0/120</td>
<td>0</td>
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<td>10/334</td>
<td>10 DENV-2</td>
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<td>1995</td>
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<td>129/1974</td>
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<td>155</td>
<td>77 P, 64 S</td>
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<td>1996</td>
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<td>85/1118</td>
<td>43 DENV-1, 42 DENV-2</td>
<td>92</td>
<td>50 P, 34 S</td>
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<td>150/756</td>
<td>39/953</td>
<td>33 DENV-1, 6 DENV-2</td>
<td>18</td>
<td>15 P, 3 S</td>
<td></td>
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<tr>
<td>1999</td>
<td>234/1097</td>
<td>18/376</td>
<td>12 DENV-1, 6 DENV-2</td>
<td>26</td>
<td>19 P, 7 S</td>
<td>21/126</td>
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<td>395/1946</td>
<td>76/1224</td>
<td>19 DENV-1, 32 DENV-2, 1 DENV-3</td>
<td>26/178</td>
<td>19 DENV-1, 6 DENV-2, 1 DENV-3</td>
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<tr>
<td>2001</td>
<td>1048/2644</td>
<td>498/2732</td>
<td>330 DENV-1, 116 DENV-2, 52 DENV-3</td>
<td>120/516</td>
<td>49 DENV-1, 32 DENV-2, 39 DENV-3</td>
<td></td>
</tr>
<tr>
<td>Year</td>
<td>MAC-ELISA Positive/tested</td>
<td>MAC-ELISA Positive/tested</td>
<td>Virus isolation DENV types</td>
<td>HI Tested</td>
<td>Immune response</td>
<td>IgG-ELISA Tested</td>
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<td>---------------------------</td>
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<tr>
<td>2002</td>
<td>602/1104</td>
<td>276/1172</td>
<td>7 DENV-1, 4 DENV-2, 265 DENV-3</td>
<td>125</td>
<td>64 P, 45 S</td>
<td>148/353</td>
</tr>
<tr>
<td>2003</td>
<td>244/730</td>
<td>50/481</td>
<td>1 DENV-2, 49 DENV-3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2004</td>
<td>1/143</td>
<td>9/170</td>
<td>1 DENV-1, 8 DENV-3</td>
<td></td>
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<tr>
<td>2005</td>
<td>80/595</td>
<td>28/711</td>
<td>28 DENV-3</td>
<td>6</td>
<td>6 P</td>
<td>80/771</td>
</tr>
<tr>
<td>2007</td>
<td>901/2015</td>
<td>133/2117</td>
<td>10 DENV-2, 123 DENV-3</td>
<td>19</td>
<td>8 P, 10 S</td>
<td>311/1252</td>
</tr>
<tr>
<td>2008</td>
<td>170/321</td>
<td>45/411</td>
<td>33 DENV-2, 12 DENV-3</td>
<td>75</td>
<td>20P, 55 S</td>
<td>179/467</td>
</tr>
<tr>
<td>2009</td>
<td>20/128</td>
<td>12/209</td>
<td>7 DENV-1, 13 DENV-2</td>
<td>5</td>
<td>1 P, 4 S</td>
<td>21/167</td>
</tr>
<tr>
<td>2010</td>
<td>97/253</td>
<td>99/488</td>
<td>31 DENV-1, 67 DENV-2, 1 DENV-3</td>
<td>16</td>
<td>8 P, 8 S</td>
<td>220/596</td>
</tr>
<tr>
<td>Total (%)</td>
<td>9347/32 474 (28.8)</td>
<td>3716/25 983 (14.9)</td>
<td>2,409 DENV-1, 530 DENV-2, 564 DENV-3, 9 DENV-4</td>
<td>181</td>
<td>50 P 132 S</td>
<td>821</td>
</tr>
</tbody>
</table>

DENV: dengue virus; HI: haemagglutination inhibition test; IgG-ELISA: immunoglobulin G antibody-capture enzyme-linked immunosorbent assay; MAC-ELISA: immunoglobulin M antibody-capture enzyme-linked immunosorbent assay; P: primary infection; RT-PCR: reverse transcriptase polymerase chain reaction; S: secondary infection.
In a retrospective analysis, the overall case confirmation, independent of the methodology used, was 33.8%; however, case confirmation during epidemic years was 59.7% and 58% in the DENV-1 epidemic that occurred in 1986 and 1987, respectively; 52.8% during the DENV-3 epidemic in 2002; 52% during the DENV-2 epidemic in 2008; and 47% and 45% during the DENV-1 epidemic in 2010 and 2011, respectively (see Figure 2).

The implementation of RT-PCR in 1997 constituted an important advance in diagnosis, by detecting the infecting DENV type and identifying DENV in cases that were negative by virus isolation. In some years, RT-PCR identified the infecting DENV type in 40% of cases where the virus was isolated. Virus isolation and RT-PCR identified the infecting DENV type in a total of 4990 dengue cases, and characterized epidemics caused by DENV-1 in 1986 and 1998, caused by the co-circulation of DENV-1 and DENV-2 in 1990, and caused by the co-circulation of DENV-1, DENV-2 and DENV-3 in 2001 (see Table 1, Figure 3). An important benefit of the virus isolation is that low-passage viruses are available for molecular characterization and phylogenetic studies.

Despite the limitations and distinct sensitivities and laboriousness that some diagnostic techniques may present, their contribution to disease surveillance is clear. In the authors’ experience, implementation of new techniques may improve diagnosis, increasing viral detection and case confirmation during dengue epidemic and inter-epidemic periods.
**Figure 2:** Suspected dengue cases confirmed by any of the routine diagnosis methods used, 1986–2011

**Figure 3:** Dengue virus serotypes identified by virus isolation and/or RT-PCR by the Laboratory of Flavivirus, IOC/FIOCRUZ, 1986–2011
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References


Dengue investigation by a reference laboratory from 1986 to 2011


Dengue investigation by a reference laboratory from 1986 to 2011


An overview of dengue infections during 2000–2010 in Kolkata, India

Shyamalendu Chatterjee,# Tanuja Khatun, Arindam Sarkar and Debjani Taraphdar

Indian Council of Medical Research (ICMR) Virus Unit, Infectious Disease and Bengal General Hospital (ID & BG) Hospital, 57, Dr SC. Banerjee Road, Beliaghata, Kolkata-700010, West Bengal, India

Abstract

Dengue is one of the major public health threats in Kolkata, India, and it has become endemic in the city. Blood samples from patients with a history of dengue-like illness are routinely referred to the Indian Council of Medical Research (ICMR) Virus Unit, Infectious Disease and Bengal General Hospital, for diagnosis of dengue infection. This paper reports the decade-long findings of the serological and molecular investigations of serum samples collected from febrile cases in Kolkata from 2000 to 2010. A total of 6136 blood samples were collected from different medical colleges and hospitals, from patients with a history of febrile illness of 2–7 days. All samples were tested for the detection of immunoglobulin M (IgM) antibodies, by enzyme-linked immunosorbent assay (ELISA), followed by reverse transcriptase polymerase chain reaction (RT-PCR) test, for the detection of dengue virus (DENV) serotype. Only 1673 (27.2%) samples were found to be ELISA positive throughout this period. The maximum number of dengue cases was detected in the age group ≤10 years, followed by 11–20 years and above, which is inversely proportional to the higher age group. Females (29.8%) were more affected than males (25.4%). Out of 463 IgM-negative acute samples, only 267 (57.6%) had the monotypic infection with different dengue serotypes, and 57 (12.3%) samples had dual infections; only 4 (0.8%) samples had infection with DENV-1, DENV-2 and DENV-3 serotypes. Dengue haemorrhagic fever was found mainly among patients with multiple dengue serotypes in the younger age group.

Keywords: Dengue; Kolkata; Serotype; Virus isolation.

Introduction

The global epidemiology of dengue fever/dengue haemorrhagic fever (DF/DHF) is changing fast.¹ Dengue infection is endemic in many parts of India and epidemics are becoming more frequent. Outbreaks have been reported at regular intervals, from the Indian states of Delhi, Madhya Pradesh, Maharashtra, Punjab, Rajasthan, Tamil Nadu and West Bengal.²³⁹ Dengue virus (DENV), with its four serotypes, is now classified within the Flaviviridae family.¹⁰ Infection
with any one of the four serotypes leads to a mild self-limiting febrile illness, DF, or the more severe forms of the disease, DHF or dengue shock syndrome (DSS),\textsuperscript{11,12} which is common in most countries of south-east Asia, including India. In India, dengue was first isolated in 1946 and DHF was first reported from Calcutta (now Kolkata; West Bengal) in 1963 and again in 1964. Subsequently, DHF cases were reported from Visakhapatnam (Andhra Pradesh) in 1969 and Jallore (Rajasthan) in 1985. Delhi experienced a major DHF epidemic in 1996.\textsuperscript{13}

In the city of Kolkata, sporadic cases of dengue infections are reported every year but no systematic investigation for the detection of dengue infection has yet been established. However, the Indian Council of Medical Research (ICMR) has established a virus laboratory at Infectious Disease and Bengal General (ID&BG) Hospital in Kolkata. This paper presents a comprehensive report on dengue-positive cases from 2000 to 2010, along with the serotype circulating in the city of Kolkata as processed at the ICMR Virus Unit.

**Materials and methods**

**Meteorological data**

The city of Kolkata (22°82’N, 88°80’E) is the capital of West Bengal state and is the seventh largest metropolitan city in area and population and with an area of 1026 km\(^2\) and its urban/metropolitan population is 14 112 536 of which 7 319 682 are males and 6 792 854 are females.\textsuperscript{14} Approximately 7.5% of the total population of India lives in this state. Kolkata has an international airport and a sea port. The main seasons are summer, monsoon, autumn, late autumn and winter. The summer lasts from mid-March to mid-June, with the temperature ranging from 38 °C to 45 °C. The monsoon starts by the middle of June and lasts up to September.

**Patients and clinical samples**

Cases were referred mainly to the Virus Unit of ID&BG Hospital from the outpatient departments of different medical college hospitals, and also from inpatients with a short history of illness. A good number of cases were also referred to the unit by private practitioners. The following criteria were initially considered for selection of DF cases: high fever, headache, retro-orbital pain, nausea/vomiting, malaise/joint pain, generalized skin rash.

In the present study, apart from fever, two or more of these criteria were also considered. The possibilities of bacterial and prokaryotic etiology in the collected samples were excluded through investigation at the respective hospitals. The case histories and investigations of the patients were compiled.

In the case of DHF, the history of illness was revealed by a sudden rise of fever (38.3 °C to 39.4 °C), headache, retro-orbital pain, conjunctival congestion and facial flashing, with
sustained fever for 2–15 days. In addition, some cases had a history of haemorrhagic manifestations, such as petechiae, gum bleeding, haematuria or melaena. In such cases, an intermittent or biphasic course of fever was recorded, where the first phase of fever persisted for 2–7 days and the second bout of fever persisted for 2–3 days. No cases with a history of plasma leakage were observed. In the admitted cases, it was found that fever was accompanied by generalized malaise and lumbosacral pain, and the pulse rate was moderately low (60–70/min). These patients also presented with rashes, which appeared on the second to fifth day of illness, on skin. These were non-pruritic in nature and lasted for 2–7 days. Haematological examination revealed generalized leukopenia and the platelet count was $\leq 100,000/\text{mm}^3$. All the cases were non-diabetic and had no other physiological complications. The liver was either non-palpable or just palpable.

Blood samples were collected by venous puncture, by the health worker and the medical technician. All the samples were transported to the ICMR Virus Unit, maintaining the cold chain. Serum samples were separated and stored at $-80 \, ^\circ \text{C}$ until tested. Informed consent was obtained from all patients before the sample collection. A total of 6136 samples were thus received/collected from the suspected cases during the study period 2000–2010. This study was duly approved by the ethical committee of the National Institute of Cholera and Enteric Diseases, Kolkata, India.

Laboratory diagnosis of recent DENV infections was done mainly by the detection of immunoglobulin M (IgM) antibody by enzyme-linked immunosorbent assay (ELISA). More specific diagnoses were also attempted, either by virus isolation in suitable cell culture, or by detection of the viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR)-based technique. These processes are very specific and were performed within 48 hours of the onset of illness, as the virus disappears after this period.

**Serology**

Serum samples were tested by ELISA for detection of IgM antibody to DENV, using a kit (prepared by the National Institute of Virology, Pune, India) and following the prescribed protocol. Optical density was measured at 492 nm, using an ELISA reader (Titertek Multiskan Plus, Lab systems, Vantaa, Finland, Type-314).

**Tissue culture**

For the isolation of virus, the 436 dengue IgM-negative acute samples were inoculated into the mosquito cell line C6/36, using the tissue culture system. For this purpose, samples from patients with a history of $\leq 1$ day fever, along with any two of the symptoms stated earlier were selected. Out of the 4463 samples collected during 2007–2010, only 463 samples were thus screened; 200 $\mu$L of selected serum were spread over the monolayer of the C6/36 cell line and allowed to adsorb for 120 min in an incubator at 28 $^\circ\text{C}$ with a carbon dioxide
An overview of dengue infections in Kolkata, India

(CO$_2$) concentration of 5%. After adsorption, the excess samples were discarded and 1 mL minimal essential medium (MEM, GIBCO-BRL, United States of America [USA]) supplement with 2% FBS (fetal bovine serum, GIBCO-BRL, USA) and PenStrep (penicillin–streptomycin antibiotic solution, GIBCO-BRL, USA) was added to the 24-well tissue culture plate (Tarsons Pvt. Ltd, Kolkata, India), which was incubated again at 28 °C at 5% CO$_2$. The tissue culture fluid was collected by centrifugation at 1000g for 5 min and the supernatant was kept in aliquots at –80 °C until isolation of RNA followed by RT-PCR. Non-infected C6/36 cell culture was used as negative control. Microscopic observation was carried out regularly for 7 days, to observe the appearance of cytopathic effects. In cases of non-appearance of cytopathic effects, the samples were blindly passaged three times. With the appearance of cytopathic effects, the supernatant was used for identification of the serotype, using the RT-PCR system. Since 2007, with the introduction of the RT-PCR technique in this laboratory, the serotypes of circulating DENV are being identified by this method.

RNA extraction

Attempts were made to isolate the viral RNA from all the tissue culture fluid that produced a cytopathic effect, and directly from the rest of the screened samples, as well as from four different dengue serotype strains, which were used as a positive control. Viral RNA was isolated using a Qiagen viral isolation kit (Qiagen, GmbH, Hilden, Germany), according to the manufacturer’s protocol.

RT-PCR

In this study, primers published by Lanciotti et al. were used. In a single tube, viral RNA was converted to a DNA copy (cDNA) prior to enzymatic DNA amplification by the use of reverse transcriptase (RT) and the DENV downstream consensus primer D2-5’-TTGCACCACAGTCAATGTCTTCAGGTTC-3’ homologous to the genomic RNA of the four serotypes. Subsequent Taq polymerase amplification was performed on the resulting cDNA, with the upstream DENV consensus primer D1-5’-TCAATATGCTGAAACGCGCGAAGAAACCGCGGAGAAACCG-3’. Target RNA was amplified in 25 μL volumes containing the following components: 800 mM deoxynucleotide triphosphates (dNTPs), 8 mM dithiothreitol, 0.24 μM each of the primers D1 and D2, 0.5 U of AMV RT (Promega, Madison, WI, USA), and 0.625 U of Dream Taq DNA polymerase (Fermentas Inc., Waltham, MA, USA). The reactions were allowed to proceed for 1 h at 42 °C and then to proceed at 95 °C for 3 min for initial denaturation, followed by 35 cycles of denaturation (95 °C for 30 s), primer annealing (55 °C for 1 min), and primer extension (72 °C for 2 min), along with final extension (72 °C for 5 min). DENV serotyping was conducted by second-round amplification (nested PCR) initiated with 10 U of diluted material (1:100 in sterile distilled water) from the initial amplification reaction. The total 20 μL of reaction mixture was prepared using 2 μL of diluted first PCR products, 0.8 mM dNTPs, 0.5 U of Dream taq DNA polymerase, and 0.3 μM of primer D1 and 0.3 μM of DENV type-specific primers: TS1 5’-CGTCTCAGTGCAGTTCCGGA-3’, TS2
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5'-CGCCACAAGGGCCATGAACAG-3', TS3 5'- TAACATCATCATGAGACAGGC-3', and TS4 5'-CTCTGTTGTCTTAAACAAGAGA-3'. Dithiothreitol and AMV RT were eliminated. The samples were subjected to initial denaturation (95 °C for 3 min), followed by 20 cycles of denaturation (95 °C for 30 s), primer annealing (55 °C for 1 min), and primer extension (72 °C for 1 min), along with final extension (72 °C for 5 min). The PCR products were analysed by running a 1.5% agarose gel stained with ethidium bromide.

Results

During these study periods, out of 6136 samples, DENV-specific IgM antibody was detected in 1673 (27.2%), and out of 463 ELISA-negative acute samples, RNA was detected in 361 (77.9%) (see Table 1). The overall sex distribution showed that, out of 3522 samples from male individuals, only 893 (25.4%) were positive to dengue IgM antibody, whereas, out of 2614 samples from female individuals with dengue-like illness, only 780 (29.8%) were reactive to dengue IgM antibody by ELISA (see Figure 1). The age distribution of the suspected cases revealed that the maximum percentage positivity to IgM antibody was observed in the age group ≤10 years, followed by the age groups 11–20 years, 21–30 years, 31–40 years and ≥51-years (see Figure 2). Although the highest antibody titre

Table 1: Decade-long dengue IgM and RT-PCR positivity, Kolkata, India

<table>
<thead>
<tr>
<th>Year</th>
<th>ELISA test</th>
<th>RT-PCR test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Number positive</td>
</tr>
<tr>
<td>2000</td>
<td>270</td>
<td>130</td>
</tr>
<tr>
<td>2001</td>
<td>163</td>
<td>82</td>
</tr>
<tr>
<td>2002</td>
<td>177</td>
<td>85</td>
</tr>
<tr>
<td>2003</td>
<td>111</td>
<td>49</td>
</tr>
<tr>
<td>2004</td>
<td>133</td>
<td>51</td>
</tr>
<tr>
<td>2005</td>
<td>2302</td>
<td>954</td>
</tr>
<tr>
<td>2006</td>
<td>438</td>
<td>66</td>
</tr>
<tr>
<td>2007</td>
<td>689</td>
<td>32</td>
</tr>
<tr>
<td>2008</td>
<td>596</td>
<td>68</td>
</tr>
<tr>
<td>2009</td>
<td>461</td>
<td>38</td>
</tr>
<tr>
<td>2010</td>
<td>796</td>
<td>118</td>
</tr>
<tr>
<td>Total</td>
<td>6136</td>
<td>1673</td>
</tr>
</tbody>
</table>

*During 2000–2006, the RT-PCR test was not introduced in the laboratory.
An overview of dengue infections in Kolkata, India

**Figure 1:** Sex distribution of dengue cases, Kolkata, India

**Figure 2:** Age distribution of dengue cases, Kolkata, India
For the isolation of virus in the tissue culture system using the C6/36 cell line, out of the 463 screened acute samples only 66 produced prominent cytopathic effects. Those isolates were identified, by the RT-PCR method, as different dengue serotypes. From 2007 to 2010, all the four serotypes were isolated and identified in the population of Kolkata. The rest of the 397 acute sera, which did not produce any cytopathic effects, were subjected to RT-PCR and only 295 (74.3%) were identified as DENV belonging to different serotypes. This observation also confirmed dual and multiple infections in the dengue cases (see Table 2).

**Table 2: Dengue serotypes identified in Kolkata, India, 2007–2010**

<table>
<thead>
<tr>
<th>Years</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2010</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cases</strong></td>
<td>94</td>
<td>101</td>
<td>112</td>
<td>156</td>
</tr>
<tr>
<td><strong>Culture-positive and RT-PCR-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>12</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>4  DENV-1</td>
<td>3  DENV-1</td>
<td>3  DENV-1</td>
<td>2  DENV-1</td>
<td></td>
</tr>
<tr>
<td>7  DENV-2</td>
<td>5  DENV-2</td>
<td>5  DENV-2</td>
<td>8  DENV-2</td>
<td></td>
</tr>
<tr>
<td>0  DENV-3</td>
<td>3  DENV-3</td>
<td>2  DENV-3</td>
<td>8  DENV-3</td>
<td></td>
</tr>
<tr>
<td>5  DENV-4</td>
<td>6  DENV-4</td>
<td>2  DENV-4</td>
<td>3  DENV-4</td>
<td></td>
</tr>
<tr>
<td><strong>Direct RT-PCR-positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>62</td>
<td>64</td>
<td>62</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>13  DENV-1</td>
<td>16  DENV-1</td>
<td>9  DENV-1</td>
<td>7  DENV-1</td>
<td></td>
</tr>
<tr>
<td>17  DENV-2</td>
<td>9  DENV-2</td>
<td>9  DENV-2</td>
<td>39  DENV-2</td>
<td></td>
</tr>
<tr>
<td>2  DENV-3</td>
<td>9  DENV-3</td>
<td>5  DENV-3</td>
<td>18  DENV-3</td>
<td></td>
</tr>
<tr>
<td>8  DENV-4</td>
<td>20  DENV-4</td>
<td>17  DENV-4</td>
<td>3  DENV-4</td>
<td></td>
</tr>
<tr>
<td>15  DENV-1 + DENV-3</td>
<td>8  DENV-2 + DENV-3</td>
<td>9  DENV-1 + DENV-2</td>
<td>5  DENV-2 + DENV-4</td>
<td></td>
</tr>
<tr>
<td>7  DENV-1 + DENV-4</td>
<td>2  DENV-1 + DENV-4</td>
<td>8  DENV-2 + DENV-3</td>
<td>2  DENV-1 + DENV-2 + DENV-3</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

Dengue infection is a growing health problem, as the incidence of DF and DHF/DSS is increasing. For that reason, monitoring of dengue virus becomes important. Generally, all age groups are affected by DENV infection, with higher incidence in the paediatric age group.\(^20\) In India, DENV-1 and DENV-4 were first reported in 1964,\(^21,22\) and DENV-3 in 1968.\(^23\) DHF was first reported from Kolkata, West Bengal, in 1963 and 1964.\(^24,25\) Although concurrent
An overview of dengue infections in Kolkata, India

Infection with more than one serotype of DENV in the same individual is uncommon, co-circulation of more than one DENV serotype was established in Delhi in 2006.26 The city of Kolkata is a known dengue-endemic zone. The first dengue infection in Kolkata was documented in 1824, almost a century ago, and, after that, epidemics were documented in 1836, 1906 and 1911, affecting almost 40% of the city's population.27 The last large-scale dengue epidemic took place in 2005, and affected the whole state. Although dengue fever was initially known as an urban disease, it is gradually spreading to rural areas.28,29 No active surveillance for the detection of dengue serotypes, in either epidemic or sporadic outbreaks, has yet been established in the state of West Bengal. This decade-long study for the detection of dengue infection reveals the serotypes circulating in Kolkata. During this study period, a total of 6136 blood samples were collected, and initially 1673 (27.2%) cases were reactive to dengue IgM antibody. Females were more affected than males (see Figure 1). As the vector mosquitoes are domestic and peridomestic in nature, females get more exposure than males, as they spend most of their time inside the house. The maximum number of dengue cases was detected in the age group ≤10 years, followed by the age group 11–20 years, owing to the lack of immunity (Figure 2). The inversely proportionate number of cases and dengue positivity in the higher age groups may be explained by the fact that these age groups have acquired substantial immunity, as a result of repeated subclinical infection, or of infection at younger ages. Out of the 463 serum samples from patients with a history of ≤3 days of fever with dengue-like illness, only 66 (14.2%) produced prominent cytopathic effects, and those were identified as DENV by the RT-PCR method. Out of the 463 IgM-negative acute samples, only 267 (57.6%) had the monotypic infection with different dengue serotypes and 57 (12.3%) samples had dual infections, while only 4 (0.8%) samples had infection with DENV-1, DENV-2 and DENV-3 serotypes (see Table 2). Patients with dual or multiple infections had a history of biphasic or triphasic fever, with haemorrhagic manifestation in some cases. This amply reveals DENV infection with multiple serotypes. The epidemic that occurred during 2005 was mainly due to the DENV-3 serotype. In the authors’ observation from 2007 to 2010, DHF was mainly found among patients who were infected with multiple dengue serotypes and was restricted among the young and young adult age groups (0 to 30 years), which may be due to the absence of immunity against all four serotypes.

However, in reality, it was found that a total of 4102 [(4463 – 463) + (463 – 361)] samples from patients with a history of 2–7 days’ febrile illness were truly dengue negative, possibly owing to mishandling of samples, which damaged the IgM antibody/viral titre, or owing to the presence of different etiological agents responsible for dengue-like illness.

This study establishes the co-circulation of all DENV serotypes in the city of Kolkata, which is a hyperendemic zone for dengue. A year-round monitoring of the circulating DENV serotypes among the febrile cases in the city has also established the same observation elsewhere.30 This decade-wide profile of DENV infection in Kolkata has established that, all year round, the young and young adults were the ones who were most affected, indicating that they do not have any protecting antibody. On the other hand, the co-circulation of all DENV serotypes is an indication of impending outbreaks of DHF in Kolkata, which could affect these and other age groups, posing a major health problem for the city.
Acknowledgments

The authors express their sincere gratitude to all the staff members of the ICMR Virus Unit for their continuous help and assistance in carrying out the laboratory investigations and compilation of data. They also gratefully acknowledge the help received from the National Institute of Virology, Pune, India, for providing the specific ELISA kits for the detection of IgM antibody. The enthusiastic help received from the doctors of the medical colleges and hospitals for providing the clinically suspected samples for this study is gratefully acknowledged. The authors are indebted to the Officer-in-Charge, ICMR Virus Unit, Kolkata, for allowing them to carry out the work in the department.

References


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Mapping of dengue vectors and dengue virus activity in Delhi during 2011–2012

Roop Kumari, a RS Sharma, a Kaushal Kumar, a Priya Singh, a
Sampath Krishnan, b AP Dash c and LS Chauhan a

aNational Centre for Diseases Control, 22 Sham Nath Marg, Delhi 110054, India

bOffice of the WHO Representative to India, Nirman Bhavan, Maulana Azad Road, New Delhi 110011, India

cWHO Regional Office for South-East Asia, Indraprastha Estate, Mahatma Gandhi Road, New Delhi 110002, India

Abstract

In India, outbreaks of dengue fever/dengue haemorrhagic fever (DF/DHF) have been recorded in almost all parts of the country, including the National Capital Territory of Delhi, where many outbreaks have, to date, been reported. A severe outbreak was reported from Delhi in 1996 and again in 2006 and 2010. Reports are available for the prevalence of Aedes aegypti in Delhi since 1964. Subsequent studies were carried out from 1996 to 1998 in urban localities of Delhi. The studies revealed that Aedes aegypti was common in urban locations, while Aedes albopictus occurred in either transitional areas/peripheral areas or rural locations. However, it has recently been reported that Aedes albopictus and Aedes vittatus have also adapted to breed in man-made containers in the central urban areas of Delhi, in addition to their natural habitats. Therefore, it is important to know the distribution of infected vector mosquitoes in Delhi, in order to implement control measures to prevent future outbreaks. To detect dengue virus (DENV) activity, Aedes surveys were carried out in different localities and immature Aedes mosquitoes were collected. After rearing them to adult stages, species pools were tested for detection of DENV, using an antigen-capture enzyme-linked immunosorbent assay. The results showed that more than 70% of localities were positive during the monsoon season from July to October. Aedes aegypti was the predominant species (86.89%), followed by Aedes albopictus (11.59%) and Aedes vittatus (1.55%). Out of a total of 476 pools of Aedes mosquitoes (total number of mosquitoes = 3943), 67 pools of Aedes aegypti and 16 pools of Aedes albopictus were found to be infected with DENV. These infected mosquitoes will maintain the infection in the respective localities through vertical transmission. A map has been prepared for the high-risk localities and this is the first such type of report from Delhi in north India. This is important for implementation of appropriate control measures for the prevention of dengue outbreaks in the future.

Keywords: Aedes aegypti; Aedes albopictus; Delhi; Dengue; Dengue virus.

E-mail: dr_roopa@hotmail.com
Introduction

Dengue fever (DF) is a vector-borne disease that has a major impact on public health in many tropical areas worldwide. It is one of the most rapidly growing mosquito-transmitted infections in the world. Dengue has been known in India since 1945 and outbreaks of dengue fever and dengue haemorrhagic fever (DF/DHF) have been reported in almost all parts of the country. The National Capital Territory (NCT) of Delhi is an outbreak-prone area for dengue and many outbreaks have been reported in and around Delhi since 1967. In Delhi, the first major dengue outbreak occurred in 1996, with 10 252 cases and 423 deaths. Subsequently, major outbreaks occurred in 2006 and 2010.

Reports are available about the prevalence of Aedes aegypti in Delhi since 1965. The first Aedes survey conducted in 1965 revealed that Aedes aegypti populations were restricted to the walled (old) city (central part/urbanized areas of Delhi) and the peripheral areas, which presented a semi-urban appearance, recorded only the presence of Aedes albopictus and Aedes vittatus. In the subsequent studies, which were carried out from 1996 to 1998 in newly developed peripheral urban localities of Delhi that had come into existence after the first survey in 1964, Aedes aegypti was the only species that was encountered. Both the studies revealed that Aedes aegypti was common in urban locations, while Aedes albopictus was present in either transitional areas or rural locations. A similar observation was made in Ajmer, Rajasthan, by Kalra et al. However, it has recently been reported that Aedes albopictus and Aedes vittatus are also adapting to breed in man-made containers in the central urban areas of Delhi, in addition to their natural habitats. It is important to know the distribution of vector mosquitoes infected with dengue virus (DENV) in Delhi, in order to implement control measures for the prevention of a potential outbreak. This study was undertaken to detect the presence of DENV in different species of Aedes mosquitoes collected from different localities of Delhi, and to provide evidence of circulating DENV in the natural cycle of vectors.

Materials and methods

Study areas

The NCT of Delhi is the largest metropolis by area and the second-largest metropolis by population in India. It is situated at 77°15'E and 26°15'N. It occupies an area of 1485 km², of which 900 km² is classified as urban. Delhi has a population of 16.7 million (2011 census) http://censusindia.gov.in/2011census/censusinfdashboard/index.html. The city has three major municipal bodies – Municipal Corporation of Delhi (MCD), New Delhi Municipal Council (NDMC) and Delhi Cantonment Board. MCD covers nearly 97% of the total area

1 MCD has since been divided into three corporations – North, East and South.
of the NCT and is divided into 12 zones (Central, City, Civil Lines, Karol Bagh, Narela, Najafgarh, Rohini, Sadar Paharganj, Shahdara North, Shahdara South, South and West). On average, Delhi receives 611 mm of rainfall annually, mainly from July to September. The highest monthly average high temperature is 41 °C in May and the lowest monthly average low temperature is 7 °C in January. The average annual relative humidity is 49.2% and the average monthly relative humidity ranges from 25% in April–May to 73% in August.

Entomological surveys

Entomological surveys for dengue vectors were carried out in different localities of the 12 MCD zones, NDMC and Cantonment areas of Delhi, from February 2011 to February 2012. Repeated surveys were carried out on a monthly basis in 15 fixed sentinel localities, including two control localities where no dengue cases had been reported during the last 3 years. A further 379 localities were surveyed randomly (localities are colonies or urban settlements). Two dairy colonies, namely, Jharoda Dairy and Shahabad Dairy, were selected as sentinel localities, as there was a larger population of cattle there. Therefore, data for these localities were analysed separately. All localities were selected on the basis of confirmed dengue cases reported during the previous 3 years and were also considered different in terms of socioeconomic factors.

In each survey, about 50 houses in each locality were searched, both inside and in peridomestic sites, for breeding of *Aedes*, using single larval techniques. The lawns and grounds of the houses were considered as peridomestic sites. Besides this, the ridge areas, gardens, playgrounds and parks were also surveyed, which were considered as outdoors (OD), in the respective localities. The House index (HI), Container index (CI) and Bretau Index (BI) were calculated. All larvae and pupae collected were reared up to adult stage for species identification.

Dengue virus detection

After the identification of species, the locality, date and sex distribution of different pools were recorded, and pools were tested for the presence of DENV, using an antigen-capture enzyme-linked immunosorbent assay (ELISA). Monoclonal antibody (MAb) D14G2 (1:1000) was used as the capture antibody and MAb-peroxidase conjugate MAb 6B6C-1 as the detector antibody (1:2000). The ELISA plate contained known positive DENV-infected suckling mouse brain homogenate and negative controls (homogenate of uninfected laboratory-reared *Aedes aegypti* pools). Mosquito pools were considered positive for DENV antigen if their mean optical density was greater than or equal to the mean ± 4 standard deviations of the optical density of the normal laboratory mosquito pools.
Statistical analysis

The virus infection rate in mosquitoes was expressed as a minimum infection rate (MIR) per 1000 mosquitoes tested.\textsuperscript{20} Virus infection rates were calculated using SPSS 11.5 software package (SPSS Inc., Chicago, IL, United States of America). The correlation coefficient of two data sets was calculated.

Serological data

Data on the serological diagnosis of dengue in Delhi were collected every month through the reporting agency, namely, MCD, NDMC and respective sentinel localities.

Demographic profile

A house-to-house survey was carried out in selected areas and recorded a complete demographic profile of 50 houses in each selected sentinel site.

Results

Entomological surveillance

The monthly numbers of localities that were positive for \textit{Aedes} breeding are presented in Figure 1, which shows that more than 70\% of localities were found to be positive for \textit{Aedes} breeding during the monsoon season from July to October and the maximum number of localities that were positive was found in September (88.1\%). During the winter season, from December to March, \textit{Aedes} breeding could be detected in fewer localities. After rearing of mosquitoes from every immature stage, adult mosquitoes were identified and it was shown that \textit{Aedes aegypti}, \textit{Aedes albopictus} and \textit{Aedes vittatus} were the prevalent species in Delhi. Analysis of the percentage proportions of different \textit{Aedes} species showed that \textit{Aedes aegypti} was the predominant species (86.89\%), followed by \textit{Aedes albopictus} (11.59\%) and \textit{Aedes vittatus} (1.55\%). Out of the 394 localities surveyed, breeding of \textit{Aedes aegypti} was recorded in 202 localities, while \textit{Aedes albopictus} was breeding in 40 localities and \textit{Aedes vittatus} was found in 13 localities during the study period. Mixed breeding of all three species was also detected in man-made containers during the monsoon and post-monsoon seasons (August–September).
In Delhi, more Aedes breeding was detected in and around houses; 42% of breeding was in peridomestic areas and 21% in indoor areas, whereas outdoor areas contributed only 37% of breeding.

The percentage positivity of Aedes aegypti in different containers in Delhi that were found to be positive for Aedes breeding is shown in Figure 2, which indicates that the mosquito prefers to breed in plastic containers (36%), followed by cisterns, evaporation water coolers and earthen and planted pots, while Aedes albopictus preferred old tyres, bird-feeding earthen pots, planted pots, cement underground tanks (cisterns) and large-sized plastic containers. In the localities with an irregular supply of water, water was stored in plastic drums/containers, cement tanks, buckets and other utensils, which were the most favourable sites for Aedes breeding. Outdoor Aedes breeding was mostly detected during the monsoon and post-monsoon seasons, when rainwater collects in small unused containers.

All containers in Delhi that were positive for Aedes breeding can be classified into three categories:

- **permanent**: permanent sites where breeding was recorded throughout the year were cement tanks at ground level (cisterns); large plastic containers/drums used to store drinking water; overhead tanks; and earthen pots (traditional mud pots to store drinking water, with a capacity of 5–20 L);
- **semi-permanent**: semi-permanent sites where breeding of Aedes mosquitoes occurred at certain periods of time were evaporation water coolers (commonly used for cooling the inside of houses during the hot summer season); potted plants (usually kept indoors or in peridomestic areas in which excess water remained on the soil surface or in the plate below it, made up of either cement or earth); and bird-feeding earthen pots (kept...
Figure 2: Percentage positivity of *Aedes aegypti* in different man-made containers in Delhi during 2011

*Unused tyres, glass, shoes and pits on the cover of manholes.*

outdoors to feed birds with water). Semi-permanent types of breeding sites are also created in construction sites, as water is stored in cement tanks, drums, cisterns and tin containers;

- **temporary**: temporary sites where mosquito breeding was recorded only for a short period of time or in a temporary phase in small water collection, particularly during the monsoon and post-monsoon seasons, were small pits on the covers of manholes; stagnant water on roof surfaces and in unused containers; discarded tyres; discarded unused small plastic/glass/tin containers; etc. This type of breeding mostly occurred outdoors, on roofs or in peridomestic areas around houses, mainly with rainwater collection. As the tendency of the *Aedes* mosquito is to spread its eggs over several sites, plenty of such sites become available during the rainy season. These are the most favourable sites for oviposition. Therefore, breeding increased profusely at these sites.

**Dengue virus detection**

The results of DENV detection from January 2011 to February 2012 (see Table 1) revealed that out of a total of 476 pools of *Aedes* mosquitoes (total number of mosquitoes = 3943), 85 were found to be positive. Out of a total of 371 pools of *Aedes aegypti* that were tested, 67 (18.06%) were found to be positive with DENV, while, out of a total of 86 pools of *Aedes*
Table 1: Dengue virus detection in different species of Aedes mosquitoes from Delhi, January 2011 to February 2012

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Number of pools tested</th>
<th>Number of mosquitoes</th>
<th>Number of pools that were positive</th>
<th>Number of mosquitoes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>F</td>
<td>Total</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>371</td>
<td>1886</td>
<td>1539</td>
<td>3425</td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>86</td>
<td>265</td>
<td>202</td>
<td>467</td>
</tr>
<tr>
<td>Aedes vittatus</td>
<td>19</td>
<td>25</td>
<td>36</td>
<td>61</td>
</tr>
<tr>
<td>Total</td>
<td>476</td>
<td>2176</td>
<td>1777</td>
<td>3953</td>
</tr>
</tbody>
</table>

F: female; M: male.

albopictus, 16 (18.60%) were found to be positive. However, only 19 pools of Aedes vittatus could be made and tested, and DENV antigen was detected in 3 (15.79%), which was the first time from Delhi. However, the role of Aedes vittatus in dengue transmission is not known. Most likely, it acts as a reservoir of DENV.

The average MIR of Aedes mosquitoes in Delhi was found to be 21.76 per 1000 mosquitoes but it was higher for Aedes albopictus (34.26) as compared to Aedes aegypti (19.56). More female Aedes aegypti were infected with DENV, while in Aedes albopictus, the number of infected male mosquitoes was significantly higher. The results showed that male Aedes mosquitoes reared from field-collected larvae were found to be positive for DENV, suggesting vertical transmission of DENV. Infection may transfer to the next generation through vertical transmission and transovarial transmission, so the presence of infected mosquitoes in a locality has epidemiological significance.

Figure 3 shows the distribution of positive pools with DENV in different localities in Delhi. DENV was detected in Aedes aegypti from 52 different localities and in Aedes albopictus from 16 localities. However, DENV was detected in Aedes vittatus mosquitoes for the first time from three localities in Delhi (New Rajender Nagar, Satbarhi and Vasant Kunj). The presence of infected vector mosquitoes in localities may be a risk for the transmission of dengue, with the increase of vector density along with man–mosquito contact.

Table 2 shows the details of localities that are positive for DENV in different species of Aedes mosquitoes, along with the HI, CI and BI of the respective localities. These localities seem to be high-risk areas in relation to dengue transmission, as proliferation of infected dengue vectors has survived there. Despite provision of information to concerned health authorities to control the breeding there, DENV was repeatedly detected in some of the sentinel localities, such as Mehrauli, Sangam Vihar and Sarita Vihar. DENV was detected in all three species of Aedes in Vasant Kunj, where the average HI was 18.00 and BI was 20.00.
The data from sentinel sites show that even with high breeding indices, some localities reported a lower number of dengue cases where MIR was also found to be low. In Jharoda Dairy, North Zone, the average breeding indices were very high (HI = 18.2 and BI = 29) while MIR was 5.7; only one dengue case was reported from that locality. However, in some localities such as Sarita Vihar, HI was comparatively low (9.9) but the MIR recorded was higher (23.8) and 16 dengue-confirmed cases were reported. Data from sentinel localities were analysed and show a significant correlation between MIR with dengue cases and HI, CI and BI. However, there was no significant correlation between HI/CI/BI and dengue cases in sentinel localities.
Table 2: Minimum infection rate (MIR) for different species, larval indices and dengue cases from sentinel localities in Delhi, 2011–2012

<table>
<thead>
<tr>
<th>Locality</th>
<th>Aedes species</th>
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<th>Mosquito tested</th>
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Mapping of dengue vectors and dengue virus activity in Delhi during 2011–2012

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<th>Locality</th>
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<th>Mosquito tested</th>
<th>Number of pools that were positive</th>
<th>MIR</th>
<th>HI</th>
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<td>1.6</td>
<td>0.9</td>
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*aDairy areas; **Control localities.
HI = House Index; CI = Container Index; BI = Bretau Index
Discussion

The results showed that *Aedes aegypti*, *Aedes albopictus* and *Aedes vittatus* were the prevalent species in Delhi, of which *Aedes aegypti* was the most predominant. All three species were found in different parts of Delhi NCT, as there is no limitation to their distribution in the city. In contrast to earlier reports, populations of *Aedes aegypti* were mostly restricted to the central part/urbanized areas of the city. The primary dengue vector, *Aedes aegypti*, is highly domesticated, with a preference for breeding in man-made storage containers. However, *Aedes albopictus* and *Aedes vittatus* prefer natural containers, which are mostly encountered in the peripheral areas of towns. However, these mosquito species have also now invaded peridomestic settings, hitherto the exclusive domain of *Aedes aegypti*. Owing to development activities at the periphery of the city and shortage of water supply, the distribution patterns of different species of *Aedes* mosquitoes have also changed. Recently, Roop K et al. reported that *Aedes albopictus* and *Aedes vittatus* were also adapted to breed in man-made containers in the central urban areas of Delhi, in addition to their natural habitats. Mixed breeding of all three species was also found together in man-made containers in urban localities in Delhi during the monsoon and post-monsoon seasons. During the present study, about one third of *Aedes* breeding was found in outdoor containers; however, Ansari et al. found breeding in both domestic and peridomestic sites. In an earlier survey in September 1965, *Aedes aegypti* breeding was detected only in indoor containers. In comparison to an earlier study in 2008–2009, a higher DENV load in mosquitoes was observed in Delhi during the present study. In this study, pools of about 18% each of *Aedes aegypti* and *Aedes albopictus* were found to be infected with DENV, while in the earlier study, the virus was detected in 10.5% of pools of *Aedes aegypti* and 11.76% of pools of *Aedes albopictus*. 12.5% of pools of *Aedes albopictus* from Tamil Nadu, and 6.1% of pools of *Aedes albopictus* from Kerala were reported to have DENV infection. This During the present study, a higher DENV load in mosquitoes has been detected as compared to earlier study from Delhi. In this study, pools of about 18% each of *Ae. Aegypti* and *Aedes albopictus* were found to be infected with DENV while in earlier studies, DENV was detected in 10.5% pools of *Ae. Aegypti* and 1.76% pools of *Ae. Albopictus*. However, 12.5% pools of *Ae. Albopictus* from Tamil Nadu and 6.1% pools from Kerala were reported to have DENV. Localities with presence of infected vector mosquitoes seem to be high risk areas in view of dengue transmission as infection also maintained by these mosquitoes through vertical and transovarial transmission. The study highlights the need for pre-emptive exclusion of breeding in positive localities to prevent the occurrence of potential outbreaks.
Mapping of dengue vectors and dengue virus activity in Delhi during 2011–2012

Acknowledgments

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References


A comparison of an in-house IgM and IgG assay with a commercial Panbio kit, in a paediatric cohort in Colombo, Sri Lanka

Hasitha A Tissera,a# Ananda Amarasinghe,a Aravinda M de Silva,b Jeroen De Manb and Aruna Dharshan De Silvac

aEpidemiology Unit, Ministry of Health, Sri Lanka
bDepartment of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, North Carolina, United States of America
cGenetech Research Institute, Sri Lanka

Abstract

Dengue is a major public health problem in Sri Lanka. From 2008 to 2010, a prospective study was conducted among children in an urban area of Colombo, to estimate the burden of dengue infection and disease. This article compares the performance of the authors’ in-house assays to Panbio commercial kit assays. It demonstrates that the in-house immunoglobulin G (IgG) assay has a performance that is comparable to the commercial assay, though with the limitation that adequate technical expertise is required. The in-house immunoglobulin M (IgM) assay had an unacceptable sensitivity compared to the commercial assay.

Keywords: Comparison; Dengue; Dengue fever; Dengue IgG and IgM ELISA; PanBio commercial kit assays; Sri Lanka.

Introduction

Dengue has become a major public health problem in many tropical and subtropical countries, with an estimated 390 million infections, 500 000 dengue haemorrhagic fever (DHF) cases and 22 000 deaths annually.1,2 While most infections remain subclinical or present as a mild febrile illness, more severe forms can cause plasma leakage and/or haemorrhage and eventually evolve to shock and death. Therefore, prompt and accurate diagnosis is vital to support proper clinical management.3 A number of rapid diagnostic tests, including immunoglobulin M and G (IgM and IgG) tests, have been developed to assist in the diagnosis of dengue, and their sensitivity and specificity have been tested.4,5

#E-mail: dr_korelege@yahoo.co.uk
Dengue infections are also a major concern in Sri Lanka, where several major epidemics have occurred during the last decade. In 2012, official statistics from the Epidemiology Unit, Ministry of Health, showed more than 40,000 reported cases and 181 deaths from dengue. Between 2008 and 2010, a prospective study among children in an urban area of Colombo was conducted to estimate the burden of dengue infection and disease. This study focused on the performance of “in-house” IgM and IgG enzyme-linked immunosorbent assay (ELISA) tests, which can be used in local settings, by comparing them with commercial Panbio IgM and IgG kits.

**Materials and methods**

The Epidemiology Unit of the Ministry of Health, in collaboration with the Pediatric Dengue Vaccine Initiative (PDVI), Genetech Research Institute, Colombo, and the University of North Carolina School of Medicine, United States of America (USA), established a community-based enhanced surveillance to estimate the burden of dengue infection and disease. The study was based in the Colombo Municipal Council area, which has a high reported annual case-load. An age-stratified sample of 800 children aged ≤12 years was selected in proportion to the population of each census block, to be followed over a minimum of one year. Acute and convalescent blood samples were collected from children who developed a fever (≤7 days of fever), and these were tested for dengue IgG and IgM antibodies. A total of 283 acute blood samples from a randomly selected subset of children with fever from the PDVI cohort was used in this study to compare in-house assays with commercial Panbio kit assays.

Blood was collected and stored on a filter paper disc (Dried Blood Spot – DBS Saver Cards manufactured by ID Biological Systems, Greenville, South Carolina, USA: Ref IDBS1003), or the serum was separated and stored. Each sample was labelled, air dried and transported, in a special container, to the study laboratory at the end of the day. At the laboratory, each spot was stored at –20 °C until testing. Two circles from the blood spot were cut out, using a hole puncher, and added to 250 μL of elution buffer (1 × Tris-buffered saline with 0.2% Tween 20 [TBST], 0.5% Tween 20 and 5% milk), then incubated at 37 °C for 1 h; 100 μL was added to each well for testing.

The in-house dengue IgM-capture ELISA was performed as described in the literature. In brief, 96-well plates were coated with goat anti-human IgM (Sigma, St Louis, MO, USA) in 100 mM carbonate buffer (pH 9.6) by incubating overnight at 4 °C in pH 9.6, 0.1 M carbonate buffer. The plates were washed 3× in TBST, followed by a blocking step with Tris-buffered saline with 0.05% Tween 20 and 3% non-fat dry milk. Eluted serum was loaded in duplicate and incubated (37 °C, 1 h) to capture anti-dengue IgM antibodies from the serum. Unbound antibodies were washed 3× with the above-mentioned buffer. Afterwards
the wells were incubated with a mix of dengue virus 1 to 4 (DENV-1–4) antigens (see below for details; 37 °C, 1 h), followed by 3× washes. Any DENV antigen captured by human IgM was detected by adding mouse anti-flavivirus 4G2 monoclonal antibody (mAb) (prepared in-house) and a secondary human-absorbed alkaline phosphatase (AP)-conjugated goat anti-mouse IgM antibody (Sigma, St Louis, MO, USA). After final incubation with AP substrate, the optical density (OD) was measured at 405 nm.

Dengue IgG-capture ELISA was performed as described.9,10 Plates were coated overnight at 4 °C with mouse anti-flavivirus 4G2 monoclonal antibody in pH 9.6, 0.1 M carbonate buffer. Plates were incubated (37 °C, 1 hour) successively with dengue antigen (see below), eluted serum in duplicate wells and AP-conjugated goat anti-human IgG (Fc portion; Sigma, St Louis, MO, USA) with three washing steps between incubations. OD was measured at 405 nm after final incubation with AP substrate.

The dengue antigen was prepared by growing vero cells inoculated with all four serotypes in separate flasks for ~8–10 days at 37 °C. The supernatant was harvested and frozen down at –80 °C. The supernatant was tested for optimal activity in both IgM and IgG ELISAs and an appropriate dilution was calculated for that batch.

The Panbio IgM and IgG Combo ELISA kit was used according to the manufacturer’s instructions.11

**Statistical analysis**

The sensitivity and specificity were calculated to evaluate the performance of the in-house IgG and IgM ELISA. The results from the Panbio commercial testing kit were used as the standard.

**Results**

The results from the Panbio commercial testing kit were used as the standard to determine the sensitivity and specificity of the in-house ELISA results. Table 1 shows the combined values of the IgG in-house and IgG Panbio ELISA results of 283 samples (the OD cut-off values for Panbio = 8 and in-house = 0.3). This gave a sensitivity and specificity of the in-house ELISA kit of 92.02% and 70.52% respectively. Table 2 shows the values of the IgM in-house and IgM Panbio ELISAs of 281 samples (the OD cut-off values for Panbio = 11 and in-house = 0.2). Accordingly, the sensitivity and specificity of the in-house IgM ELISA kit were 32.10% and 98.00% respectively.
Table 1: Sensitivity and specificity of in-house and Panbio IgG testing, Colombo, Sri Lanka

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<th>PanBio test (gold standard)</th>
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<td>Positive</td>
<td>Negative</td>
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<tr>
<td>In-house test</td>
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<tr>
<td>Negative</td>
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<td>67</td>
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<tr>
<td>Total</td>
<td>188</td>
<td>95</td>
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Sensitivity = 173/(188) = 0.920 = probability of a positive test, given that the gold standard is positive.
Specificity = 67/95 = 0.705 = probability of a negative test, given that the gold standard is negative.
Positive likelihood ratio = 0.920/(1 – 0.705) = 3.119 = the probability of testing positive if the gold standard is positive, divided by the probability of testing positive if the gold standard is negative.
Negative likelihood ratio = (1 – 0.92)/0.705 = 0.113 = the probability of testing negative if the gold standard is positive, divided by the probability of testing negative if the gold standard is negative.

Table 2: Sensitivity and specificity of in-house and Panbio IgM testing, Colombo, Sri Lanka

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<tbody>
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<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
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<td>4</td>
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<tr>
<td>Negative</td>
<td>55</td>
<td>196</td>
</tr>
<tr>
<td>Total</td>
<td>81</td>
<td>200</td>
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</table>

Sensitivity: 26/81 = 0.321.
Specificity: 196/200 = 0.98.
Positive likelihood ratio = 0.321/(1–0.98) = 16.05.
Negative likelihood ratio = (1 – 0.321)/0.98 = 0.693.

Discussion

This study was conducted to evaluate the feasibility of using the in-house IgM and IgG ELISAs as a substitute for the more expensive commercial ELISAs. For the in-house IgG ELISA, the sensitivity (92%) and specificity (70%) were within acceptable ranges and the in-house assay may be used as a substitute for the Panbio commercial kit. The IgG ELISA is not useful for diagnosis in a clinical setting because IgG antibodies only appear late after primary infection.5,12 Moreover, a positive result in the test may be indicative of past exposure, as IgG antibodies persist for decades, if not longer, after infection. Therefore, the IgG ELISA is only useful for surveillance and research purposes, to assess dengue exposure at a population level, or for testing paired serum samples for evidence of increasing antibody levels indicative of current exposure.
The in-house IgM test had a high specificity (98%) but a low sensitivity (32%). The IgM ELISA is an early indicator of an acute infection, which is helpful in clinical management. As such, high sensitivity is important and the in-house assay needs to be improved to achieve a sensitivity that is at least comparable to that of the commercial assays.

The crude cost per test for the in-house ELISA was approximately one third of the cost of the commercial test. However, it is necessary to have facilities for tissue culture (BSL-2 hoods, incubators, microscope and freezer/refrigerator), an ELISA plate reader, experienced technicians and mid-level or senior staff capable of resolving problems that may arise with the assay. This can add substantially to the cost of the assays. Therefore, carrying out the in-house ELISA tests in resource-limited settings will be challenging.

Health-care providers often raise the concern of the high cost of commercial dengue diagnostics. However, while selecting a diagnostic test, its reliability is often given priority over its cost. Nevertheless, low- and middle-income countries face challenges in providing high-quality commercial diagnostics, largely because of their high cost. The authors’ opinion is that the “value” of commercial diagnostics over the in-house testing is greater if they are affordable in routine services.

In summary, this study demonstrates that the performance of the in-house IgG ELISA is an appropriate test for potential use and is comparable to the more costly commercial IgG assays. However, the limitations in technical expertise and the available facilities in local settings will be a major barrier in setting up in-house assays. Further improvements are needed before the in-house IgM assay can be considered to replace the currently available commercial IgM assays.

Acknowledgments

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References


Comparison of an in-house IgG-IgM assay with a commercial PanBio kit in Sri Lanka


Wolbachia-based strategy for dengue control – the way forward
Noor Afizah A and Lee HL#

Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

Abstract
Wolbachia-based vector-control strategies have been proposed as a means to augment the currently existing measures for controlling dengue vector. The successful application of Wolbachia in insect control is critically dependent on the ability of the agent to invade and maintain itself at a high frequency in the natural population, with the goal that the mosquito population will carry the desired genotype. Wolbachia are being introduced into the mosquito vectors of human diseases, including the primary vector for dengue, Aedes aegypti, following the discovery that some strains of Wolbachia can cause pathogen interference as well as shortening the lifespan of mosquitoes, with the hope of causing reduction in viral transmission.

Keywords: Aedes aegypti; Cytoplasmic incompatibility; Dengue; Wolbachia.

Introduction
Dengue, transmitted primarily by Aedes (Stegomyia) aegypti, and, to a much lesser extent, Aedes (Stegomyia) albopictus, is a worldwide public health burden, with an estimated 50–100 million dengue infections worldwide every year. Dengue is endemic in over 100 countries in south-east Asia, Eastern Mediterranean and the Western Pacific. In the absence of effective vaccines and specific treatments, vector control is the only option used to control dengue. Most of the vector-control approaches are directed towards controlling the vector, primarily Aedes aegypti, through the application of chemical adulticides in fogging, and larvacides. However, this conventional method of combating dengue is expensive, unsustainable and normally only used during outbreak occurrence. Moreover, the frequent application of insecticides has created a strong selective pressure and it eventually leads to the development of insecticide-resistant mosquitoes. Other approaches aimed at environmental management of mosquitoes, such as the removal of oviposition sites, the introduction of guppy fish or copepods, as well as application of Bacillus thuringiensis israelensis (Bti), are considered as

#E-mail: leehl@imr.gov.my
practical alternatives. However, these strategies are only temporary measures and not sufficient to reduce the escalating number of dengue cases every year. Thus, this situation has led to a pessimistic thinking that dengue is unstoppable and will remain as a “pandemic threat” across the globe. Therefore, comprehensive and strategic interventions are urgently needed, in order to challenge dengue. In recent years, there has been a resurgence of interest in utilizing the endosymbiont Wolbachia as a biocontrol agent to control mosquito-transmitted diseases, including dengue.

**Wolbachia, the fascinating endosymbiont bacteria**

Wolbachia were first identified in the ovaries of Culex mosquitoes in 1924. Wolbachia species are obligate intracellular rickettsia-like bacteria belonging to the alpha subclass of Proteobacteria and the order of Rickettsiales that live inside the cells of various organs, but most frequently appear in ovaries and testes. Wolbachia infects a wide range of arthropods, including populations of mosquitoes, ticks, flies and nematodes. However, some of the major disease vectors are not naturally infected, including the primary vector of dengue, Aedes aegypti, and all anopheline mosquitoes sampled to date. Morphologically, Wolbachia are characterized as Gram-negative bacteria, coccoid or bacilliform in shape, with size ranging from 0.8 μm to 1.5 μm in length. The bacteria are almost always maternally inherited through host eggs and reach their optimum fitness by altering the reproduction of their host via several mechanisms, including parthenogenesis, feminization, male killing and cytoplasmic incompatibility (CI).

Among all the reproductive modifications caused by Wolbachia, CI has been utilized as one of the tools to fight against mosquitoes. CI is a form of sterility in which, if the same and compatible Wolbachia strain is not present in the egg during embryogenesis, embryonic development will be disrupted. There are several forms of CI caused by Wolbachia: (i) unidirectional CI; (ii) bidirectional CI; and (iii) multiple infections CI (see Figure 1). A Wolbachia-induced CI approach was utilized as early as 1967 by Laven et al. to eliminate an isolated Culex pipiens fatigans population by releasing the incompatible male mosquitoes into the target population. Over a period of time, the sustained repeated release of cytoplasmically incompatible mosquitoes will result in an increasing ratio of incompatible mating and, hence, lead to suppression of the vector population. In a more recent event, another CI-based strategy was demonstrated by releasing Wolbachia-transfected CP male strain (the CP strain was generated by introgressing Wolbachia type originating from Aedes riversi into Aedes polynesiensis) to mate with the wild-type [WT] Aedes polynesiensis. In the field trial, a total of 3800 CP males were released weekly, for a total of 30 weeks. During the period of release of CP strains, a significant reduction in the rate of egg hatching was observed in the trial site relative to the control site. There was no horizontal transfer of Wolbachia from CP strains into WT females, in line with the hypothesized role of males as “dead-end hosts”.
**Figure 1:** Examples of cytoplasmic incompatibility. (i) Uni-directional CI, giving the females a reproductive advantage in which the infected females would be able to successfully mate with the infected or uninfected males. In contrast, for uninfected females, the mating with the infected males will subsequently result in karyogamy failure in diploid species.\(^{15,16}\) (ii) Bidirectional CI involves the individuals that are infected with different, incompatible *Wolbachia* strain, which eventually results in rapid embryonic death. (iii) Multiple infections CI. Male and female individuals can be infected with multiple strains (superinfection). In this situation, incompatible mating occurs between superinfected males and single-infected females.\(^{16}\)

![Diagram of cytoplasmic incompatibility](image)

Source: adapted from Brelsfoard and Dobson, 2009.\(^{14}\)

**Wolbachia** and pathogen interference in *Aedes aegypti*

A successful establishment of artificial *Wolbachia* infection into various insect hosts, including mosquitoes, has permitted broader applications and a deeper understanding of the role of *Wolbachia*. Interestingly, some *Wolbachia* strains are able to cause pathogen interference, including from viruses, in their newly introduced invertebrate hosts, and yet confer no effects
on their original host (see Table 1). For example, the introduction of Wolbachia strain B isolated from Aedes albopictus (wAlbB) into a novel host, Aedes aegypti, via embryonic injections of the eggs, has been shown to induce dengue resistance.\textsuperscript{19} A decrease in the viral proliferation of chikungunya virus (CHIKV) was also observed in wMelPop-infected Aedes aegypti.\textsuperscript{20} wMelPop is one type of Wolbachia strain isolated from laboratory Drosophila melanogaster (fruit fly). The name wMelPop was designated by Min and Benzer (1997) for this particular Wolbachia strain, owing to its popcorn-like appearance when observed under electron microscope.\textsuperscript{26} wMelPop has the ability to overproliferate in the infected tissues and is proven to shorten the lifespan of its original host, Drosophila melanogaster.\textsuperscript{26} Interestingly, a similar life-shortening effect was observed when wMelPop was artificially introduced into Aedes aegypti.\textsuperscript{27} On the other hand, a study conducted by Mousson et al. (2010) showed that Wolbachia helped to modulate the replication of CHIKV in its native host, Aedes albopictus.\textsuperscript{24} The reasons why Wolbachia exhibit an interference with pathogens in the newly introduced host and not in their original host are still unclear.

**Table 1: Summary of Wolbachia effects on pathogen inhibition**

<table>
<thead>
<tr>
<th>Host</th>
<th>Wolbachia type</th>
<th>Pathogen</th>
<th>Wolbachia effect on pathogen inhibition</th>
<th>Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aedes aegypti</td>
<td>wAlbB</td>
<td>DENV</td>
<td>Reduced virus proliferation</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>wMelPop</td>
<td>DENV, CHIKV, <em>Plasmodium gallinaceum</em></td>
<td>Reduced virus proliferation, decrease oocyst accumulation</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>wMel</td>
<td>DENV</td>
<td>Blockage of viral proliferation</td>
<td>21</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>wMel</td>
<td>DCV</td>
<td>Reduced virus proliferation</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>wMel</td>
<td>WNV, CHIKV, LACV</td>
<td>Reduced virus proliferation</td>
<td>23</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>wPip</td>
<td>WNV</td>
<td>Reduced virus proliferation</td>
<td>23</td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>wAlbA and wAlbB</td>
<td>CHIKV</td>
<td>No effect</td>
<td>24</td>
</tr>
<tr>
<td>Anopheles gambiae</td>
<td>wMelPop</td>
<td><em>Plasmodium berghei, Brugia pahangi</em></td>
<td>Decreased oocyst accumulation, reduced filarial worm development</td>
<td>25</td>
</tr>
</tbody>
</table>

CHIKV: chikungunya virus; DCV: Drosophila C virus; DENV: dengue virus; LACV: La Crosse virus; WNV: West Nile virus.
Source: adapted from Brelsfoard and Dobson (2011).\textsuperscript{14}
There are several mechanisms involved in the reduction of parasite burden in Wolbachia-infected mosquitoes. Some evidence has shown that the reduction of parasite burden is due to modification of the host cell membrane, thus inhibiting entry of pathogens from the host. Resource competition between artificially introduced Wolbachia and pathogens inside the mosquitoes may also be another reasonable explanation.20,28 In addition, wMelPop are found to affect host fitness and eventually cut short the lifespan of the infected host. Thus, the pathogen transmission will be reduced as the insect lifespan is shorter than the extrinsic incubation period of viruses and parasites.20,29,30 Apart from that, wMelPop-infected mosquitoes have displayed changes in the blood-feeding behaviour in Aedes aegypti, in which Moreira et al. (2009) have described the phenomenon as “bendy’ proboscis”, which becomes more prominent in older mosquitoes.31 This situation decreases the biting activities of the wMelPop-infected Aedes aegypti, which ultimately limit the vectorial capacity.31 Furthermore, Wolbachia-infected mosquitoes have shown enhanced immune responses towards pathogens. This was demonstrated by Bian et al. (2010), who found that the immune genes such as Defensin, Cercropin, Diptericin, GNBPB1, SPZ1A, Cactus, Rel1 and Rel2 were upregulated in Aedes aegypti in response to infections with Gram-negative bacteria, which might explain the resistance towards dengue virus.19

Wolbachia – from laboratory to open field

On top of Wolbachia-induced CI, which results in embryonic death, the discovery of some Wolbachia strains that can cut short the mosquito lifespan and successfully interfere with pathogen development, including dengue virus, as demonstrated in the laboratory setting, is very exciting. Thus, these are among the key elements that may offer a tool to control vector-borne diseases, including dengue. However, the applicability of a Wolbachia-based control strategy needs to be verified and tested in the open field. The first open field trial utilizing Wolbachia artificially introduced into Aedes aegypti was conducted in Cairns, Australia. For the outset, the goal of the trial is to assess whether the artificially Wolbachia-infected Aedes aegypti would be able to spread into natural Aedes aegypti populations.

There are two types of Wolbachia strains used for this study: wMel and wMelPop. Both strains were proven to reduce the vectorial capacity, with the latter found to halve the lifespan of mosquitoes as compared to uninfected controls in the contained environment.27 This suggests that wMelPop can be utilized to skew the age structure of the mosquito population toward younger individuals, and thereby reduce pathogen transmission.30,32 Nonetheless, this is not a straightforward mechanism, as the effect of wMelPop on longevity appears to be complex and robust. A study conducted by Yeap et al. (2011) suggests that wMelPop invasion is only possible under humid conditions, as the spread of invasion in the population is very unlikely during the dry season, when wMelPop-infected Aedes aegypti suffer significant reduction in fertility and egg hatching.33 Thus, in order to overcome the deleterious effects associated with wMelPop and to ensure successful invasion of wMelPop
into natural populations, a high number of infected mosquitoes needs to be released to secure its persistence across a dry season.\(^{33}\)

The first field release was done in early January 2011 during the wet season in Yorkeys Knob and Gordonvale, Australia, as the trial sites. For these particular locations, both male and female wMel-infected *Aedes aegypti* were released on a weekly basis, for a total of 10 weeks. Constant monitoring was conducted every 2 weeks post-release. It was reported that a total of 100% *Aedes aegypti* were infected with *Wolbachia* in Yorkeys Knob and 90% in Gordonvale 5 weeks after the final release. In January 2012, a second field trial was conducted in Machans Beach (north of Cairns) and Babinda (south of Cairns), with the release of wMelPop-infected *Aedes aegypti*. A promising percentage of wMelPop-infected *Aedes aegypti* was observed in the natural population within 2–3 weeks after the initial release, with 49% and 75% *Wolbachia* infection recorded in Machans Beach and Babinda respectively. However, 1 month after the final release, the numbers of wMelPop-infected *Aedes aegypti* had dropped to less than 50% in Machans Beach, while Babinda recorded 71% positive *Wolbachia* infection.\(^{34}\) This outcome was not surprising, as the *Wolbachia* strain originating from *Drosophila melanogaster* origin was artificially introduced into *Aedes aegypti*, and knowing the limitation associated with utilizing wMelPop, it is possible that *Wolbachia* strains are not able to sustain themselves in the natural environment after a period of time.

Even though the maternally inherited criteria possessed by *Wolbachia* are particularly attractive because they provides a powerful mechanism to invade natural populations,\(^{35}\) the mosquito fitness costs associated with *Wolbachia* infection must not exceed the fitness advantage, as the goal of population replacement is for the entire mosquito population to carry the desired genotype. Given the knowledge of the complexity and deleterious effects associated with wMelPop, this artificially introduced strain must be able to exceed the unstable equilibrium point in order for wMelPop to be able to invade the population rather than losing it from a whole population.\(^{33}\) Nonetheless, a successful invasion of wMelPop in a caged *Aedes aegypti* population has been described by Walker et al. (2011) under a laboratory experimental setting.\(^{21}\) In addition, the results of the field trials in Cairns are highly crucial and can be used as a platform for accumulating key information in developing potential strategies for vector control that utilizes *Wolbachia*-infected mosquitoes. However, assessment of the practicality of utilizing Wolbachia-based vector-control strategies cannot be directly assessed until field trials in dengue-endemic areas have been completed. For this purpose, more field trials will be carried out in other countries, including Brazil, China, Indonesia and Viet Nam, to directly measure the effect of Wolbachia-based strategies on disease impact.

**Future perspective**

The *Wolbachia*-based control strategy serves as a promising platform to be utilized in vector control. The diversity of the *Wolbachia*’s host range makes it the most ubiquitous bacterial genus yet described and therefore compatible to be stably infected even into phylogenetically
distant hosts. Other than Wolbachia-induced CI, which results in embryonic death, the ability of some Wolbachia strains to interfere with pathogen development and to cause reduction in adult mosquito longevity is exciting. However, other questions that may arise prior to envisaging a Wolbachia-based strategy for controlling dengue would be whether artificially introduced Wolbachia would be able to provide complete protection towards all four dengue serotypes and also whether Wolbachia artificially introduced between different insects species can invade and sustain itself in the natural population for a sufficient period of time until the necessary effects have taken place. The proposal to integrate releases of Wolbachia with applications of pesticides to introduce insecticide resistance into the Wolbachia-infected line may offer a solution to facilitate the spatial spread of Wolbachia under field conditions. In addition to balancing the pros and cons of various dengue-control measures, it should be taken into account that there is no such thing as a stand-alone method for combating dengue, and, therefore, a Wolbachia-based control strategy may serve as another feasible candidate to augment the currently existing methods for fighting dengue.

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References


Wolbachia-based control strategy for dengue control – the way forward


Effectiveness of an autocidal trap device for capturing and killing Aedes mosquitoes under field conditions

AH Nurulhusna,a# MS Khadri,a AG Abdullah,a AH Norazlina,a M Khairul-Asuad,a
AK Azim,a AH Hisyamuddin,b ST Sisma and HL Leea

aMedical Entomology Unit, Infectious Diseases Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia
bVector Control Unit, Central Malacca Health District Office, Jalan Bukit Baru, 75150, Malacca, Malaysia
cSchool of Diploma in Applied Parasitology and Entomology, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia

Abstract

A study was conducted in Malaysia to evaluate the effectiveness of an autocidal trap known as the “Institute for Medical Research Autocidal (IMR) Trap (IAT)” in trapping Aedes and other mosquitoes. This device is a modification of the traditional ovitrap used to monitor mosquitoes, especially the Aedes species. The IAT consisted of a black plastic container fitted with a floater, on which a sticky strip was placed. The floater was also covered with a fine wire mesh of copper. Two residential areas, Taman Peringgit Jaya and Taman Kenanga, in the state of Malacca, were selected for the trial. A total of 85 houses were selected and each house was installed with three IATs filled with tap water, two of which were placed indoors and one outdoors. Every 2 weeks the sticky strips were collected and the insects trapped on them were identified, counted and recorded. Three main species of mosquitoes were trapped on these strips: Aedes aegypti, Aedes albopictus and Culex quinquefasciatus. Female Aedes aegypti was the predominant mosquito trapped in the IAT (42.8%), followed by female Culex quinquefasciatus (17.9%) and female Aedes albopictus (5.56%). A mean of two Aedes mosquitoes were trapped/strip/2 weeks/house. On an average, about 21% of the participating houses successfully trapped Aedes species during each visit. Eggs were also laid on the floaters, showing that gravid female mosquitoes were attracted to oviposit in the IATs. The IAT is thus an effective device for trapping and controlling mosquitoes, especially Aedes species.

Keywords: Aedes aegypti; Aedes albopictus; Autocidal trap; Dengue; Trapping device.

Introduction

Aedes aegypti is known for transmitting the dengue virus1–4 and chikungunya virus.5,6 It is a container-breeding insect and prefers to oviposit in stagnant water in discarded containers, such as plastic containers, discarded tyres and septic and water tanks around residential
areas. Occasionally, the larvae can be found in tree cavities around house compounds. The occurrence of dengue fever cases would indicate that the *Aedes* vector population has grown. In the absence of an effective vaccine and specific treatment, the control of dengue and chikungunya depends on suppression of the vector population. However, conventional vector-control measures such as space spraying of chemical insecticides, larviciding, etc., are effective only to a limited extent. Novel vector-control methods, such as use of an autocidal trap (AT), need to be evaluated.

The traditional ovitrap currently used for *Aedes* surveillance was first developed as a surveillance tool for *Aedes aegypti* in the United States of America. The ovitrap was first used by Fay and Perry to provide a sensitive and economical method for detecting the presence of *Aedes aegypti*. In Malaysia, the first ovitrap survey was conducted by Yap, to study the distribution of *Aedes aegypti* and *Aedes albopictus* in small towns and villages of Penang Island. In 1979, Yap and Thiruvengadam conducted a study on the distribution of *Aedes aegypti* and *Aedes albopictus* in different habitats. Chan et al. first reported the successful use of a larval autocidal trap in Singapore to control *Aedes aegypti* in a dengue-endemic urban residential area. Cheng et al. subsequently used a modified ovitrap from which larvae could not escape, and reduced the Breteau Index (BI) by 36% after 1 year. An adulticidal sticky ovitrap was also reportedly successful in trapping adult *Aedes aegypti*.

A new autocidal trap, known as the “IMR Autocidal Trap (IAT)” device was first designed by the Medical Entomology Unit, Institute for Medical Research (IMR), Malaysia. It is a modified version of the traditional ovitrap and is able to trap both gravid female mosquitoes and the larvae. The IAT was subsequently patented by the IMR. The IAT is cost effective, environment friendly, user friendly, durable and safe to use with a minimum amount of maintenance. It consists of five basic components, namely, a black cylindrical plastic container, a floater with a sticky strip, a funnel and a screw cap cover (see Figure 1). The cap of the container has openings to facilitate the entrance of adult mosquitoes. The overflow holes on the container maintain the water at the maximum level. A black-coloured container is used for this purpose, since laboratory and field studies show that both *Aedes aegypti* and *Aedes albopictus* prefer a darker colour for oviposition.

**Figure 1:** Components of the IMR autocidal trap; i: black cylindrical container; ii: floater; iii: sticky strip; iv: screw cap cover; v: funnel
The floater is mounted with copper wire mesh. This fine mesh, 380–420 mesh/cm², is used to trap the larvae hatched from the eggs laid onto the wire mesh. In addition, copper ions released from the wire mesh are toxic to the larvae. Copper does not affect egg hatching but suppresses larval development and induces mortality of early instars of Aedes larvae.\textsuperscript{14}

The other important component of the trap is a sticky plastic strip attached on top of the floater. The sticky surface can trap gravid mosquitoes that are attracted to water in the black container for oviposition. The funnel is used to prevent debris such as dried leaves from getting into the trap and clogging the container, while water can be added through the funnel into the container. The screw cap has four holes to prevent water from accumulating on it and is also fitted with a hanger.

Khalil\textsuperscript{15} first evaluated the IAT under laboratory conditions and reported that it was able to trap both sexes of Aedes mosquitoes, and that up to 64% of adult Aedes mosquitoes were trapped after 96 h. The IAT was also evaluated against another autocidal trap called the “Mosquito Larvae Trap Device”, under laboratory conditions; the IAT showed higher trapping efficiency.\textsuperscript{16} Both studies indicated that the IAT is effective in trapping mosquitoes. However, the trapping efficiency of the IAT under field conditions is yet to be determined. Thus, the main objective of this study is to evaluate the trapping efficiency of the IAT in residential premises.

**Materials and methods**

**Study areas**

The study was conducted in the state of Malacca, which is located in the southern region of the Malay Peninsula near the Straits of Malacca, Malaysia. Two residential areas known as Taman Kenanga (N2°12'3" and E102°13'54") and Taman Peringgit Jaya (N2°13'5" and E102°14'56") were selected. Both residential areas consisted of two different types of terraced houses: single-storey houses with two bedrooms and double-storey houses with three to four bedrooms. Most of the houses have a car porch, a small front garden and a backyard. This site was the first planned residential area established in Malacca in the 1970s. These two localities reported the highest number of dengue cases in Malacca in 2010. Most of the study participants were housewives or self-employed workers, owing to their presence and availability at home during daytime house visits. A total of 85 houses were selected – 19 in Taman Peringgit Jaya and 66 in Taman Kenanga. This study was conducted over a period of 2 months, from 8 July to 3 September 2010.

**Trap deployment**

Three traps per house were installed – two indoors and one outdoors, in Taman Kenanga and Taman Peringgit Jaya, after obtaining consent from the house owners. Each trap was filled with
tap water and placed indoors (bedroom, kitchen, living room) and outdoors (garden, shoe rack, under the tree, gazebo). The autocidal traps and the transparent strips were labelled accordingly, and all information such as the owner’s name, house address and location of the traps was recorded. A total of 170 traps were laid indoors, while 85 traps were deployed outdoors. The first site visit was conducted from 9 to 22 July, the second from 23 July to 5 August, the third from 6 to 19 August and the fourth from 20 August to 3 September 2010.

Data collection and analysis

After 2 weeks, the researchers revisited the participants’ houses and collected the transparent sticky strips. The strips were removed from the autocidal trap floaters and placed in a transparent plastic bag. Insects and mosquito species trapped on the strip were brought to the laboratory for identification. The water level was checked and, if necessary, refilled with tap water to the level of the overflow holes. The traps were also checked for the presence of larvae and eggs. Leaves and other debris were removed and a fresh sticky strip, labelled with the same collection number, was placed into the autocidal trap. The house owner was informed of the presence of trapped Aedes species.

Results and discussion

The highest number of mosquitoes found trapped was on the second visit (241), followed by the fourth visit (211), the third visit (200) and the first visit (175) (see Figure 2).

The largest number of mosquitoes trapped in the IAT were female Aedes aegypti, whose highest number recorded was on the fourth visit (98) and the lowest on the first visit (80). Male Aedes aegypti were also attracted to the trap and the second visit showed the highest number recorded, with 22 males. Aedes albopictus was found trapped throughout the study period, with a total of 46 females and four males. However, during the first visit, no Aedes albopictus were found. The second visit showed the highest number of Aedes albopictus trapped, with a total of 27 females and one male. Beside Aedes species, another mosquito species, Culex quinquefasciatus, was also found trapped. A total of 37 male and 148 female Culex quinquefasciatus were captured. The highest number of Culex quinquefasciatus found trapped was during the fourth visit (67). Some mosquitoes could not be identified because of degradation.

Table 1 shows the mean number of mosquitoes trapped in the IAT during the study period. On an average, female Aedes aegypti were predominantly more attracted to rest/lay their eggs in the IAT, followed by Culex quinquefasciatus and Aedes albopictus. Similarly, for males trapped by the IAT, Aedes aegypti were the highest number, followed by Culex quinquefasciatus and Aedes albopictus. Both sexes of Aedes albopictus were the least attracted to the trap, as compared to others.
Figure 2: Total number of mosquitoes by species collected using the IMR autocidal trap, Malaysia; a: first visit; b: second visit; c: third visit; d: fourth visit

Table 1: Mean number of mosquitoes trapped in the IMR autocidal trap, Malaysia

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Sex (mean ± SD)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Unidentified</td>
</tr>
<tr>
<td>Aedes aegypti</td>
<td>17.50 ± 5.07</td>
<td>88.50 ± 6.76</td>
<td>NA</td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>1.00 ± 0.82</td>
<td>11.50 ± 5.81</td>
<td>NA</td>
</tr>
<tr>
<td>Culex quinquefasciatus</td>
<td>9.25 ± 3.30</td>
<td>37.00 ± 11.61</td>
<td>NA</td>
</tr>
<tr>
<td>Unidentified</td>
<td>7.50 ± 4.29</td>
<td>31.50 ± 17.75</td>
<td>3.00 ± 1.63</td>
</tr>
</tbody>
</table>

NA: not available; SD: standard deviation.

The highest numbers of mosquitoes found trapped on every trip were female Aedes aegypti and Culex quinquefasciatus. Both species are able to rest/lay eggs in the traps. The study also showed that gravid females were attracted to lay their eggs in the traps. Although
female *Aedes albopictus* were also attracted to the trap, their number was much lower, compared to *Aedes aegypti* and *Culex quinquefasciatus*. Most of the traps were placed indoors in the bedroom, living hall and car porch. This is one of the reasons for the lower number of *Aedes albopictus* trapped, compared to *Aedes aegypti* and *Culex quinquefasciatus*, as *Aedes albopictus* is an outdoor-inhabiting species. The percentage of mosquitoes trapped had increased by 16% on the second visit, compared to the first visit. However, on the third visit, the percentage of mosquitoes trapped had decreased by 5%, compared to the second visit.

During the study, a major breeding site of *Aedes aegypti* was found to be a septic tank outside a participating house. An IAT that was set up outside the house under a roof, which was about 7 m from the septic tank, trapped 28 *Aedes aegypti* mosquitoes within 2 weeks (first visit). This finding illustrated that a poor sewage system, with a rusty and broken septic tank, can be a major focus for *Aedes aegypti* breeding.

On an average, *Aedes* mosquitoes were collected from 18 houses out of 85, during each trip. The autocidal trap is also sensitive in detecting the presence of *Aedes*, even though the mosquito population was low. Both gravid mosquitoes and larvae can be collected in the same trap.

Several unidentified mosquito adults were also collected, especially from outdoor traps. These specimens were not identifiable, owing to damage, which was most likely caused by the rain. Heavy rainfall was recorded, especially during the first, third and fourth visits, which resulted in a lower number of adult mosquitoes being collected. This was evidenced by the higher number of mosquitoes trapped during the second visit, which recorded lower rainfall. However, generally, the rain did not affect the trapping because most of the traps were placed inside the house or under the shaded area within the vicinity of the house compound. It is thus recommended that the IAT should be used indoors or in shaded areas.

This short 2-month study showed that the IMR autocidal trap is capable of trapping mosquitoes, especially the *Aedes* species, in residential areas. The IAT managed to trap an average of seven *Aedes* mosquitoes/trap/2 weeks. Since each house used three units of IAT, this was equivalent to 21 *Aedes* mosquitoes/house/2 weeks, which was about 10 *Aedes* mosquitoes/house/week. Therefore, the study to determine the effectiveness of the IAT device should be conducted over a longer period of time, in order to correctly measure the impact of the mosquito population density, especially the *Aedes* species. The IMR autocidal trap can be an effective component of integrated vector management.

**Acknowledgement**

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References


GIS-based surveillance to support dengue control in Thailand, 2009–2011

Anun Chaikoolvatana,a# Pratap Singhasivanon,b Peter Haddawy,c and Wacharapong Saengnilld

aDepartment of Pharmacy Practice, Ubon Ratchathani University, Warinchumrab, Ubon Ratchathani, Thailand

bTropical Medicine Department, Mahidol University, Thailand
cUnited Nations University, Macau, People’s Republic of China
dCollege of Medicine and Public Health, Ubon Ratchathani University, Thailand

Abstract

Dengue fever/dengue haemorrhagic (DF/DHF) fever is the most common vector-borne viral disease of humans worldwide. Recent studies have demonstrated that a geographic information system (GIS) can give promising results in the prediction of changes in the habitats of mosquito vectors as they affect disease transmission. This study aimed to analyse dengue vector indices via a GIS database, in three high-risk provinces in Thailand from 2009 to 2011, as well as to conduct a pilot survey of the attitudes and behaviours of village inhabitants in relation to dengue prevention. Vector indices were investigated in a total of 15 villages from five districts of three provinces, and the prevention behaviours of the village inhabitants in relation to the disease were evaluated through a questionnaire. It was found that dengue vector indices in one of the provinces (Sri-Sa-Ket) were significantly higher than in the other two provinces (P = 0.001, 0.001 and 0.001 respectively). The results showed no statistically significant differences between the dengue indices (House Index, Container Index and Breteau Index) and epidemic periods (P = 0.060, 0.062 and 0.443 respectively). There were no significant differences between the mean scores and either the study periods or provinces (P = 0.672 and 0.358 respectively) regarding dengue perception and prevention behaviours. The overall incidence of the disease increased during 2009–2011 in these high-risk areas. Improvement of surveillance and control strategies needs to be accompanied by improvements in villagers’ incomes, education and awareness.

Keywords: Dengue haemorrhagic fever; Geographic Information System; Perceptions of behaviours; Surveillance; Thailand; Vector indices.

#E-mail: phanunch@hotmail.com
Introduction

Dengue fever/dengue haemorrhagic fever (DF/DHF) is an important public health problem in tropical and subtropical areas. It is the most common vector-borne viral disease of humans, with an estimated 50 million infections occurring annually worldwide, with 500 000 cases and at least 12 000 deaths. No treatment or vaccine is available for dengue, and vector control is the only method to control the spread of disease. The main vector is the mosquito Aedes aegypti. This mosquito is anthropophagic; the females mainly bite humans, and also lay eggs in containers such as jars, cans and used tyres. Vector-control strategies are mainly based on controlling the mosquito population, by elimination of potential breeding sites using 1% temephos sand granules and pyrethroid ULV space fogging. Normally, dengue vector indices, including the House Index (HI), Container Index (CI), and Breteau Index (BI), are used to monitor Aedes aegypti. High incidences of dengue virus transmission are due to various environmental factors, changes in climate, improvements in modes of travel allowing people to be more mobile, and a growing multicultural and multinational population.

The density of mosquito vectors plays a significant role in dengue outbreaks and there are four important epidemiological factors: the agent (Aedes aegypti), the host (sanitation and knowledge and perceptions of DF prevention), the environment (temperature, water resources and remoteness of areas) and economics (poverty).

DF has been detected in Thailand since the 1950s. Dengue in Thailand is high during the monsoon months of June to October. However, since 1976, dengue cases have occurred all the year round. The Ministry of Public Health spends a large part of its annual budget on purchasing chemical insecticides for dengue vector control. However, this effort seems to have failed because the reported number of DF cases remains high. For example, a study in Thailand and Cambodia between 2003 and 2007 revealed that more than 95 000 patients with DF were estimated to have been hospitalized. It also indicated that the incidence of the disease incidence in Thailand was underrecognized by more than 8-fold. A large amount of the national health budget is spent on the treatment of dengue patients. For example, the direct patient cost (treatment cost and the cost of travel, food and lodging) was US$ 66.99 and US$ 61.02 per patient for one episode of dengue, in Bangkok and Suphan Buri respectively.

Therefore, there is an urgent need to review the strategies for the prevention and control of dengue, as well as to conduct research to improve vector-control methodologies. There have been examples of successful vector control in countries such as China, Province of Taiwan, Singapore and Viet Nam, through entomological surveillance and larval source reduction by reducing the availability of Aedes larval habitats. However, in Thailand, dengue surveillance and vector control have not been satisfactory, as the factors contributing to dengue outbreaks still remain; these are: increased storage of water for household consumption and the lack of an exclusive public health programme and staff to concentrate on vector control.
The geographic information system (GIS) is a system that stores and processes data and information. It is able to integrate, analyse and display spatial and temporal data in a geographical context. The system comprises hardware (computer and printer), software (GIS software), digitized base maps, information and a whole set of procedures such as data collection, management and updating. Specific diseases and public health resources can be mapped in relation to their surrounding environment and existing health and social infrastructures. Such information, when mapped together, creates a powerful tool for the monitoring and management of disease. GIS provides a graphical analysis of epidemiological indicators over time, captures the spatial distribution and severity of the disease, identifies trends and patterns, and indicates where there is a need to target extra resources. For dengue surveillance and control, GIS can be used effectively to integrate data temporally and spatially.

Recently, GIS and remotely sensed data were used to evaluate and model the relationship between climatic and environmental factors and the incidence of viral diseases. An earlier study found that remote sensing and geodesy had the potential to revolutionize the discipline of epidemiology and its application in human health. Recent studies have also demonstrated that GIS, satellite imagery, digitized land-use maps and global positioning data were promising in the prediction of changes in the habitats of mosquito vectors as they affect disease transmission.

This study aimed to investigate the implementation of a GIS database via vector indices from 2009 to 2011, in three high-risk areas of Amnart Charoen, Sri-Sa-Ket and Ubon Ratchathani provinces, where there were continual and increasing reported incidences of dengue. Comparisons of dengue vectors focused on the results from three periods – pre-outbreak, outbreak and post-outbreak – of dengue epidemiology. Also, a survey related to the perceptions and prevention behaviours of village inhabitants toward dengue in those high-risk areas was conducted.

**Materials and methods**

**Dengue indices survey**

**Study design**

The study was a descriptive research survey. Data collection was conducted from 2009 to 2011.

**Population**

The study was located in three provinces – Amnart Charoen, Sri-Sa-Ket and Ubon Ratchathani. Five villages in each province were selected because of their high incidence
of dengue cases and high morbidity/mortality rates over the previous three years (2006 to 2008). Fifteen villages were involved in the study and the total population size was 2420 households (see Figure 1).28

GIS development

The GIS database was developed in 2009 using the software ArcMap version 9.3.1. The overall structural functions included installation of primary data, such as dengue vector indices and information on water containers, and secondary data, such as the number of dengue cases per 100 000 population, analysis of data, searches of the target locations, and presentation of the results via figures on the map. The steps of the development were described in a previous article.29

Figure 1: Fifteen high-risk villages for dengue selected for GIS study, Thailand, 2009–2011
**Data collection**

Dengue vector indices, the HI, CI and BI, were collected from indoor and outdoor natural water containers and outdoor artificial water containers at each household of the 15 villages, to determine the presence of *Aedes aegypti* mosquitoes. The locations of each household were mapped by a global positioning system (GPS). In each year of the study, dengue vector indices from the five villages in one province were collected, based on dengue epidemiological periods – pre-outbreak (February to May), outbreak (June to October) and post-outbreak (November to January).

**Analysis of dengue indices**

The morbidity rate was calculated per 100,000 population. Dengue vector indices, HI, CI, and BI, were compared between provinces and within each province during 2009–2011. The indices were assessed according to the following:

1. HI was defined as the percentage of houses found to be positive for larvae:
   - a score above 10 was considered a *high risk of dengue*;
   - a score between 1 and 10 was considered an *average risk of dengue*;
   - a score below 1 was considered a *low risk of dengue*;

2. secondly, CI was defined as the percentage of water-filled containers found to be positive for larvae and the same classification of values as for HI was adopted;

3. finally, BI was defined as the number of larvae-positive containers per 100 houses:
   - a score of above 50 was considered a *high risk of dengue*;
   - a score between 5 and 50 was considered an *average risk of dengue*;
   - A score below 5 was considered a *low risk of dengue*.

The comparisons of the indices were evaluated over the epidemiological periods (pre-outbreak, outbreak, and post-outbreak) and means, standard deviations and percentages were used. Also, the SPSS analytical statistics, including Chi-square, Kruskal–Wallis and grouping variables, were implemented to evaluate the differences in values of the vector indices between the provinces over the 3-year period. The values of the indices for each province were also compared annually. Additionally, buffer zones of dengue mosquitoes were mapped in relation to the households during the pre-outbreak, outbreak, and post-outbreak periods, to observe the spread of dengue mosquitoes around the households in high-risk areas.
Dengue perceptions and prevention behaviours

Nine villages from the three provinces were investigated by questionnaire for dengue perceptions and prevention behaviours. A previously developed questionnaire was tested for both content validity and reliability. Each questionnaire item was scored at a value of either one (1) or zero (0), giving a maximum possible score of 16 points. It was decided that a score between 11 and 16 was considered as a high level of perception, a score between 6 and 10 was considered as an average level of perception, and a score of 0 and 5 was considered as a low level of perception. In Ubon Ratchathani, 96 people were interviewed and completed the questionnaire (pre-test). Seventy people were involved in Sri-Sa-Ket and 52 in Amnart Charoen, making a total of 218 participants. Public health staff provided basic information and self-care for the participants in relation to dengue mosquitoes and DHF prevention. After completion of this exercise, the participants were immediately required to fill out the questionnaire again (post-test). Comparisons of the scores between the three provinces were made. Descriptive statistics of means, standard deviations and percentages were used to analyse the data.

Results

Dengue morbidity rates

Sri-Sa-Ket had the highest dengue morbidity rates over the 3-year period, followed by Amnart Charoen and Ubon Ratchathani provinces. The year 2010 had the highest morbidity rates of dengue compared with other years (see Table 1). All three provinces have been called “relapsed areas”, meaning that dengue cases are still being reported and the nation’s epidemic situation has not been controlled over the last 10 years.

Larval vector survey

Dengue indices – Hi, CI and BI – from five villages of each province were collected during pre-outbreak, outbreak, and post-outbreak periods from 2009 to 2011. Overall, the results revealed that Sri-Sa-Ket had the highest dengue indices compared to the other two provinces. The findings indicated that the high incidence of dengue in Sri-Sa-Ket corresponded with the high morbidity rate shown in Table 1. Comparisons of individual vector indices between provinces over the 3 years showed there were statistically significant differences of HI, CI and BI between provinces ($P < 0.001$, respectively). Similarly, Sri-Sa-Ket had the highest vector indices compared to the other provinces (see Table 2).

The influence of the collection periods on the differences of vector indices was also investigated using the Kruskal–Wallis statistic. The findings indicated that only the HI values of all three provinces over the 3 years were above normal standard values. In 2011,
Table 1: Dengue morbidity rates of three high-risk provinces, Thailand, 2009–2011

<table>
<thead>
<tr>
<th>Province</th>
<th>Morbidity rate per 100 000 population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amnart Charoen</td>
<td>63.36</td>
</tr>
<tr>
<td>Sri-Sa-Ket</td>
<td>97.63</td>
</tr>
<tr>
<td>Ubon Ratchathani</td>
<td>40.72</td>
</tr>
</tbody>
</table>

Table 2: Mean ranks of dengue indices in three provinces in Thailand, 2009–2011

<table>
<thead>
<tr>
<th>Vector index</th>
<th>Province</th>
<th>n</th>
<th>Mean rank</th>
<th>$\chi^2$</th>
<th>df</th>
<th>Asymptomatic significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>House Index (HI)</td>
<td>Amnart Charoen</td>
<td>45</td>
<td>56.50</td>
<td>57.787</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sri-Sa-Ket</td>
<td>45</td>
<td>103.47</td>
<td>23.441</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ubon Ratchathani</td>
<td>45</td>
<td>44.03</td>
<td>44.03</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Container Index (CI)</td>
<td>Amnart Charoen</td>
<td>45</td>
<td>48.32</td>
<td>23.441</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sri-Sa-Ket</td>
<td>45</td>
<td>88.23</td>
<td>88.23</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ubon Ratchathani</td>
<td>45</td>
<td>67.44</td>
<td>67.44</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Breteau Index (BI)</td>
<td>Amnart Charoen</td>
<td>45</td>
<td>49.43</td>
<td>26.476</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sri-Sa-Ket</td>
<td>45</td>
<td>91.12</td>
<td>91.12</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ubon Ratchathani</td>
<td>45</td>
<td>63.44</td>
<td>63.44</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

$df$: degrees of freedom.

vector indices were higher than those in 2009 and 2010. Nevertheless, comparisons of the vector indices of all three provinces showed that HI values were not significantly different for the 3 years ($P = 0.085$). However, CI and BI were significantly different for those years ($P = 0.001$ and 0.003; see Table 3).

The study also investigated the relationship between the epidemiological periods and dengue vector indices for 2009–2011. Generally, the overall dengue vector indices of all three provinces over the three years showed that the HI, CI and BI for the pre-outbreak and post-outbreak periods were higher than the normal standard values (standard values: HI < 10, CI < 10, BI = 5–50; see Table 4). This indicated that all three provinces, especially those 15 villages, had a high risk of dengue vectors, posing the possibility that the number of dengue cases would increase during the epidemic seasons. An evaluation of the relationship between vector indices and the epidemiological periods, by combining all vector indices of the three
### Table 3: Mean rank of vector indices for each year in three provinces in Thailand, 2009–2011

<table>
<thead>
<tr>
<th>Vector index</th>
<th>Year</th>
<th>n</th>
<th>Mean of dengue indices</th>
<th>Mean rank</th>
<th>$\chi^2$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>House Index (HI)</td>
<td>2009</td>
<td>45</td>
<td>21.96</td>
<td>65.32</td>
<td>4.936</td>
<td>0.085</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>45</td>
<td>20.43</td>
<td>60.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>45</td>
<td>26.81</td>
<td>78.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Container Index (CI)</td>
<td>2009</td>
<td>45</td>
<td>8.69</td>
<td>72.90</td>
<td>13.155</td>
<td>0.001*</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>45</td>
<td>4.5</td>
<td>51.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>45</td>
<td>9.33</td>
<td>79.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breteau Index (BI)</td>
<td>2009</td>
<td>45</td>
<td>29.19</td>
<td>65.90</td>
<td>11.844</td>
<td>0.003*</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>45</td>
<td>22.31</td>
<td>54.98</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>45</td>
<td>43.39</td>
<td>83.12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant ($P < 0.05$).

### Table 4: Mean ranks of HI, CI and BI based on epidemiological periods in three provinces in Thailand, 2009–2011

<table>
<thead>
<tr>
<th>Vector index</th>
<th>Epidemiological period</th>
<th>n</th>
<th>Mean rank</th>
<th>$\chi^2$</th>
<th>df</th>
<th>Asymptomatic significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>House Index (HI)</td>
<td>Pre-outbreak</td>
<td>45</td>
<td>78.48</td>
<td>5.618</td>
<td>2</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Outbreak</td>
<td>45</td>
<td>66.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-outbreak</td>
<td>45</td>
<td>59.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Container Index (CI)</td>
<td>Pre-outbreak</td>
<td>45</td>
<td>79.11</td>
<td>5.550</td>
<td>2</td>
<td>0.062</td>
</tr>
<tr>
<td></td>
<td>Outbreak</td>
<td>45</td>
<td>63.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-outbreak</td>
<td>45</td>
<td>61.12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breteau Index (BI)</td>
<td>Pre-outbreak</td>
<td>45</td>
<td>73.24</td>
<td>1.628</td>
<td>2</td>
<td>0.443</td>
</tr>
<tr>
<td></td>
<td>Outbreak</td>
<td>45</td>
<td>68.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-outbreak</td>
<td>45</td>
<td>62.72</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
provinces over the 3 years, revealed no statistically significant differences between these vector indices and the epidemiological periods ($P = 0.060$, 0.062 and 0.443, respectively; see Table 4).

The GIS database had an application to develop buffer zones to see how dengue could spread to neighbouring households. One village, Din-Dum in UbonRatchathani, was chosen for the development of a buffer zone in 2010. This buffer zone graphically showed the areas of households that presented with dengue vectors. Also, it presented the distances that mosquitoes flew from one household to another within the radii of 30 m and 60 m, by researchers’ observations (see Figure 2). Vector indices were mainly found to be high within the 30 m radius of indicated dengue breeding habitats in the risk area, increasing the confidence of the researchers’ predictions of the transmission of dengue in particular risk areas. Figure 3 shows an overview of dengue epidemic within one particular high-risk area (Din-Dum). The spread of dengue vectors in Din-Dum village was mainly in the residential areas. It was revealed that the number of households that had dengue vectors at the pre-outbreak stage was higher than the number at the outbreak and post-outbreak stages. The households with dengue vectors were marked with “red stars” in the pre-outbreak stage. Red-star households decreased during the outbreak period compared to the pre-outbreak stage, implying that the dengue vector-control strategy was effective, owing to the elimination of dengue breeding habitats, resulting in a decrease of dengue vectors. However, the number of red-star households tended to increase during the post-outbreak stage. Overall, it is anticipated that the buffer zone will help health workers to identify dengue risk areas and implement dengue vector-control strategies effectively.

*Figure 2:* The symbols of the buffer zones of mosquito flight distance by 30 m and 60 m, Thailand
Figure 3: An example of the buffer zones during pre-outbreak, outbreak and post-outbreak periods of dengue, Din-Dum village, Thailand
Dengue perceptions and prevention behaviours

Overall, the villagers from the three provinces had high mean post-test scores of dengue perception and prevention behaviours, over the 3 years of study (see Table 5). A comparison of mean scores with the study period (2009 to 2011) found no statistically significant difference of mean scores between those 3 years ($P = 0.672$). Similarly, there were no significant differences of mean scores between the provinces ($P = 0.358$). However, post-test scores were significantly higher than the pre-test scores of three provinces over the 3 years ($P = 0.001$; see Table 6).

Table 5: Mean scores of dengue perception and prevention behaviours in Thailand, 2009–2011

<table>
<thead>
<tr>
<th>Year</th>
<th>Mean (± standard deviation) pre- and post-test score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amnart Chareon (52)</td>
</tr>
<tr>
<td></td>
<td>Pre-test</td>
</tr>
<tr>
<td>Year 1</td>
<td>8.91 ± 3.12</td>
</tr>
<tr>
<td>Year 2</td>
<td>9.87 ± 2.54</td>
</tr>
<tr>
<td>Year 3</td>
<td>9.42 ± 3.21</td>
</tr>
</tbody>
</table>

*total participants of each province.
Mean scores of DHF perception (11–16) = high level; (6–10) = average level; (0–5) = low level.

Table 6: Relationship between mean scores and study periods/provinces/tests for dengue perception and prevention behaviours in Thailand, 2009–2011

<table>
<thead>
<tr>
<th>Item(s)</th>
<th>Comparisons</th>
<th>Mean score</th>
<th>Standard deviation</th>
<th>Pearson correlation</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Year</td>
<td>2009</td>
<td>10.49</td>
<td>2.20</td>
<td>0.107</td>
<td>0.672</td>
</tr>
<tr>
<td></td>
<td>2010</td>
<td>11.04</td>
<td>2.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>11.03</td>
<td>2.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Provinces</td>
<td>Amnart Charoen</td>
<td>11.03</td>
<td>1.84</td>
<td>-0.230</td>
<td>0.358</td>
</tr>
<tr>
<td></td>
<td>Sri-Sa-Ket</td>
<td>9.34</td>
<td>1.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ubon Ratchani</td>
<td>12.18</td>
<td>1.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test</td>
<td>Pre-test</td>
<td>9.21</td>
<td>1.25</td>
<td>0.804</td>
<td>0.0001*</td>
</tr>
<tr>
<td></td>
<td>Post-test</td>
<td>12.49</td>
<td>1.32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant ($P < 0.05$).
Discussion

The high morbidity rates of dengue found in the study could be predicted from the large number of cases that occurred during the epidemic seasons. The influencing factors included dengue vectors, changes of climate (especially temperature and rainfall), insanitary conditions, the impact of globalization, and increased awareness of dengue and its prevention and control. Dengue cases may have come from neighbouring countries with less effective public health systems, such as Cambodia, Laos and Viet Nam, and this may cause an increase in dengue vectors in Thailand’s border provinces.30,31 Climate changes, particularly in temperature and rainfall, play key roles in dengue incidence. A previous report showed that increased temperature and rainfall could increase the survival rates and fecundity of mosquito vectors and decrease the extrinsic incubation period of the virus, causing the spread of more dengue mosquitoes through high-risk and neighbouring areas.32

Normally, all the four dengue serotypes of *Aedes aegypti*, DENV-1, DENV-2, DENV-3 and DENV-4, circulate in Thailand. The serotyping of dengue from patients by the Department of Medical Sciences, Ministry of Public Health, showed changing proportions among the four dengue serotypes, with DENV-1 and DENV-4 being more predominant than the others in the past few years.33 This situation can affect the immunity of the population to specific dengue serotypes, and an abrupt change to a new dengue serotype might then cause an outbreak or more severe cases.

Globalization has resulted in much increased global trade and international travel by varied modes of transport.34 This enhances the potential for the introduction and spread of exotic vectors via local residents and/or international travellers becoming infected while visiting dengue-endemic areas. Other factors, including population growth and inadequate urban infrastructure such as solid waste disposal, also play an important role in dengue epidemics.35 These changes have created conducive, artificial environments for the breeding of the *Aedes* mosquito.36

Sri-Sa-Ket was identified as the area of highest risk of dengue cases and dengue vectors compared to Amnart Charoen and Ubon Ratchathani (see Table 2). The Thai Population Census (2010) indicated Sri-Sa-Ket as the area with the nation’s second-lowest personal income, of approximately 29 174 baht/person/year,37 a factor that could affect the knowledge and awareness of dengue and sanitary standards among the local population. Additionally, Sri-Sa-Ket is a province bordering Cambodia where there is daily trade and movement of people. This trade and mobility, with the growth of towns and neglected mosquito-control efforts, provides an ideal breeding ground for the mosquitoes. As a result, it is anticipated that the spread of dengue will continue and the number of epidemics will increase.

Under normal conditions, there are annual dengue epidemics in Thailand. Government implementation of dengue-prevention and control strategies in high-risk areas should be continued during the epidemics, which should result in a decrease in dengue vectors, and
thus cases, in the subsequent year. However, it is likely that dengue will spread to areas close to the location of a previous epidemic. Therefore, these neighbouring areas should also be monitored and included in the implementation of dengue-prevention and control plans.

Artificial containers such as water tanks and buckets are common sites for the mosquito vector to breed; therefore, it is crucial to implement *Aedes aegypti* habitat control, to minimize dengue transmission. This involves physical and natural control. Physical control involves minimization of dengue vectors by reduction of dengue breeding sites in water containers and water resources, using insecticide chemical products, such as insecticide fogging, 1% temephos sand granules and improvement of sanitation. Natural control of dengue involves the use of alternatives to minimize dengue vectors, such as the use of fish to eat dengue eggs, changes of the acid–base balance in the water to interfere with dengue breeding, and the use of hormones, as well as microorganisms.\(^{38}\) Despite these efforts, the control of the vector may still be far from successful.

Investigation of the effects of epidemic periods on dengue incidence revealed no statistically significant difference between dengue vector indices and epidemic periods. It showed that during pre-outbreak and post-outbreak periods, all dengue vector indices were non-significantly higher than the standard values. Dengue vector indices increased during the pre-outbreak period (February to May) and dropped during the outbreak period (June to October), then gradually increased again in the post-outbreak period (November to January). Hence, strategies to control the dengue vector breeding habitat should be implemented during the pre-outbreak period.

Villagers in high-risk areas should be educated continually regarding control of the dengue breeding habitat, including proper care of water containers inside and outside households, disease prevention by use of mosquito nets and insect repellents, and the use of traditional herbs such as lemon grass, orange skin and lemon. Hospitals need to be alert about inpatients receiving dengue mosquito bites and should prevent this by insecticide fogging around hospital premises.

Generally, it appears that the GIS database system can be used for dengue surveillance and control in the south of north-east Thailand. The GIS technology has the capability to integrate many types of data and to analyse spatial and temporal data to produce new models. The technology is essentially used to determine the health situation of an area, generating and analysing DHF risk areas, prioritizing areas for mitigation and surveillance programmes, monitoring the incidence, and visualizing and analysing or mapping the information in a more interactive manner, for a better understanding. Recent studies have demonstrated the use of GIS imagery and digitized land-use maps, and the use of global positioning data promises improvements in predicting changes in the habitats of mosquito vectors as they affect dengue transmission.\(^ {26,39–41}\)
The information obtained from this research indicates to the Ministry of Public Health that regular evaluation of prevention behaviours within high-risk areas should be focused so that target populations understand and act properly to avoid dengue infection, and campaigns of dengue surveillance and control need to be implemented continuously.

Also, the use of the buffer zone in the study allowed the creation of a housing density map that showed the spread of vectors. Mosquitoes can comfortably fly 30–60 m between breeding sites and house(s) nearby, and the existence of high vector indices raises the expectation of dengue transmission in the surrounding area. Dengue can be found in all the regions and provinces in the country and it is believed that cases emerged from urban settings and spread to rural areas because of people’s movement and transportation of goods. One study showed the disease spreading from the Bangkok metropolitan area to other provinces, at the rate of 148 km per month. Provinces in the central and southern regions experienced more outbreaks in the past 5 years.42

Findings regarding dengue perceptions and prevention behaviours showed that people in high-risk areas generally had high perception and knowledge of dengue and its mosquitoes. However, this knowledge did not translate into action in checking and removing stagnant water from their premises, as the public generally sees the control of dengue as a government responsibility. Public education through the media, pamphlets and posters should be an ongoing process. Also, education programmes tailored for different target groups need to be implemented.

Study limitations

The researchers collaborated with the Office of Disease Prevention and Control Department 743 to develop a database system for dengue survey in the high-risk areas of the region and decided to implement a licensed ArcGIS 9.3.1 for a dengue vector database. All expenditure on this software was subsidized by the Thai Research Fund. The database system is currently a provincial pilot model for dengue control only. Nevertheless, further development of a dengue database system for other areas and populations needs to be implemented through a freeware called “Quantum GIS”, a user-friendly tool for people to access information.

Difficulties in making appointments with householders resulted in fewer participants than hoped completing the survey. As a consequence, the findings may not represent the whole picture of dengue vectors and disease perceptions and prevention behaviours. Village volunteers can be key persons to enhance people’s participation in such projects. Additionally, skills to use a database system are essential and the expense of skill training may have to be considered. The government should support people to undergo GIS training to help overcome the current situation where a number of software systems exist but there are few staff who have the knowledge and skills to use them.
Conclusion

It is impossible to eradicate dengue mosquitoes but control of the disease is possible. Regular control of breeding sites in households, schools and communities, by the participation of local people and authorities, is the cornerstone of dengue prevention and control. This should happen throughout the year but especially during the pre-outbreak period. Early detection, immediate investigation to identify the source, and rapid response by destroying larvae in all breeding sites during the outbreak period, is essential to minimize the number of cases and deaths from dengue.

Additionally, the use of GIS could give health professionals easy access to a large volume of data. Dengue vector indices can also be collected and interpreted for the incidence of dengue transmission. This technology also provides a variety of analytical tools and display techniques for dengue vector indices and the epidemiology of dengue, via maps, graphs, charts, tables and buffer zones. Nationwide use of GIS for dengue and dengue mosquito surveillance and control needs to be encouraged.

Acknowledgements

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References


GIS-based surveillance to support DHF control in Thailand from 2009 to 2011


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Surveillance of dengue in a community cohort in Metropolitan Colombo, Sri Lanka:
part I methods and study population

Hasitha A Tissera,a# Ananda Amarasinghe,a,e,f Aravinda M de Silva,b
Clarence C Tam,c Aruna Dharshan De Silva,d G William Letsone,f
and Harold S Margolisgh

aEpidemiology Unit – Ministry of Health, Sri Lanka
bDepartment of Microbiology and Immunology, University of North Carolina School of Medicine,
Chapel Hill, North Carolina, United States of America (USA)
cDepartment of Epidemiology and Population Health, London School of Hygiene and
Tropical Medicine, London, United Kingdom of Great Britain and Northern Ireland
dGenetech Research Institute, Sri Lanka
ePediatric Dengue Vaccine Initiative, International Vaccine Institute, Seoul, Republic of Korea
fDengue Vaccine Initiative, International Vaccine Institute, Seoul, Republic of Korea
gEl Paso County Public Health, Colorado Springs, Colorado, USA
hCenters for Disease Control and Prevention, Dengue Branch, San Juan, Puerto Rico

Abstract

Dengue is a major cause of childhood fever in Sri Lanka that has caused a number of major
epidemics in the last decade. Despite this, few data exist regarding the epidemiology and burden
of dengue illness in this region. In 2008, a prospective study among children in an urban area of
Colombo, Sri Lanka was started, to estimate the burden of dengue illness and measure the annual
rate of seroconversion for dengue virus. This paper describes the study area, investigation methods
and characteristics of the study population. It is hoped that this landmark study will provide much-
needed information regarding the epidemiology of dengue in the Indian subcontinent and that
the findings will be helpful for conducting future dengue-control activities.

Keywords: Dengue; Disease burden; Epidemiology; Seroconversion; Sri Lanka.

Introduction

Despite historical advances in infectious diseases control, dengue stands out as a disease that
has defied control efforts. Long-term dengue control will require an effective vaccine; several
candidates are in phase II and III testing, while others are likely to enter clinical trials within

#E-mail: dr_korelege@yahoo.co.uk
The design and implementation of dengue vaccination programmes will require detailed information about the disease burden and its distribution, both locally and globally.

Nearly 90% of dengue infections occur in children,\(^3\) in whom mortality during a secondary infection is nearly 15-fold higher than in adults.\(^4\) Despite this, longitudinal studies of paediatric clinical and asymptomatic dengue in the Indian subcontinent are lacking. To date, dengue control in the region has relied on findings from south-east Asia, some of which were generated nearly 40 years ago. Given the dynamic and continuously changing nature of dengue epidemiology, up-to-date information from more locally relevant studies are urgently needed.\(^5\)

In order to address this, a longitudinal study is being conducted to estimate the burden of paediatric dengue illness in Colombo, Sri Lanka. The study objectives include:

- to establish enhanced paediatric fever surveillance;
- to measure the incidence of dengue illness in children;
- to determine the annual rate of dengue seroconversion;
- to describe the dengue strains circulating in Colombo.

This paper describes the study area, the methods used and the characteristics of the study population. It discusses the importance of this study in the local context, and its implications for the understanding of dengue epidemiology in Sri Lanka and dengue control in the south Asian region.

**Materials and methods**

The study commenced in October 2008 and will continue until 2015. It consists of three components: (i) a population census and baseline survey; (ii) a longitudinal study to measure the incidence of clinical dengue; and (iii) baseline dengue prevalence and annual seroconversion surveys.

**Study setting**

The study site is in the Colombo Municipal Council (CMC) area, within the District of Colombo. Colombo District covers an area of 656 km\(^2\) – 1.1% of the total land area in Sri Lanka. The district is in the wet zone of the country, with an average rainfall of 2306 mm/year and an average temperature of 80 °F (27 °C). It had a population of 2,234,146 at the 2001 census, which is approximately 12% of the country’s population. The ethnic composition is 76.4% Sinhalese, 12.4% Tamil, 9.1% Moor and 2.1% other minority groups; 70.4% of the population is Buddhist, 10.9% Muslim, 8.8% Hindu, 7.8% Roman Catholic and 2.0% other Christian denominations.\(^6\)
The CMC, in the western-most part of Colombo District, is Sri Lanka’s main commercial centre (see Figure 1). It covers an area of 37.3 km² – 5% of the district. It had a population of 647,100 residents at the 2001 census. Its population density exceeds 17,000 inhabitants/km²; the country average is 299 inhabitants/km². The CMC provides primary and preventive health-care services to its entire population, free at the point of use. Vaccination coverage among infants for BCG (for tuberculosis), pentavalent 3 (for diphtheria, tetanus, whooping cough, hepatitis B and *Haemophilus influenzae* type b) and measles is 99%. The CMC has a Chief Medical Officer of Health responsible for all public health activities, including childhood immunization and infectious disease prevention and control.

**Figure 1:** Map showing Colombo Municipal Council and its Ward No. 33

The study is being conducted within the Borella North Municipal Ward (No. 33; see Figure 2), which has one of the highest population densities in Colombo and a high dengue case-load. Within the ward are Lady Ridgeway Hospital for Children (LRH), the National Referral Centre of Excellence for Paediatric Care and the main primary and secondary care facility in the ward; the Medical Research Institute (MRI), which acts as the national reference laboratory; and Wanathamulla Municipal Dispensary, which provides primary care to local residents.
Component one: population census and baseline survey

Population census

Since the last national census in 2001, the ward boundaries have changed and new housing units have been built. In September 2008, a census of the area was conducted, to enumerate the paediatric population and determine their health-care-seeking practices in the event of fever.

The ward was divided into census blocks. In a total of 40 blocks (6 blocks were excluded), there were an average of 63.5 housing units (ranging from 8 to 108) per block. Within each block, all housing units were visited by fieldworkers, accompanied by the area’s Public Health Inspector (PHI) or Health Instructor (HI). A housing unit was defined as a group of (related or unrelated) persons who usually reside in a house or other living quarter identified by a municipal assessment number. Some housing units comprised several households, defined as a person or a group of persons who live in the same housing unit and have a common
arrangement for food preparation and consumption. All households in the study area were surveyed. Households that were closed during the first visit were visited twice more on consecutive days and once more during a weekend.

**Baseline survey**

The head of each household was interviewed to obtain the following information about the household: individuals usually living there or staying there at the time of interview; socioeconomic characteristics; and health status and health-care-seeking patterns of children aged under 12 years.

**Informed consent**

Prior to each interview, survey respondents were informed of the purpose of the survey and organizations involved, the voluntary nature of the survey, and the strict confidentiality of any information provided. Verbal informed consent was obtained.

**Data collection and field logistics**

In each block, interviews were conducted by a survey team comprising one male and one female medical officer, and the local PHI/HI. The latter knew the terrain and was known to local residents. Survey teams travelled by private van. Each team was dropped off at a preselected household to begin fieldwork. The principal investigator (PI) was available to assist the teams in the field and visited them several times during the day. To improve rapport, female interviewers administered the questionnaire to female respondents, while male respondents were interviewed by male interviewers. The teams surveyed all households in a block before moving into a new one.

The census information was used to develop a list of households with children eligible for enrolment in the prospective study.

**Training and data quality**

The survey questionnaire was pretested for comprehension and acceptability, in three wards not included in the survey. Questionnaires were administered to five households and modified as necessary. The interviewers were newly qualified medical doctors educated in English and fluent in Sinhalese and/or Tamil. They underwent training sessions covering an overview of dengue, study objectives, informed consent, interview techniques and questionnaire completion. Role-play sessions were conducted with all the trainees alternately acting as interviewers and interviewees. The interviewers also conducted two supervised practice interviews in the community outside the study area. Interviews were conducted in Sinhalese or Tamil, but the questionnaires were completed in English.
Within a week of the original interview, the PI randomly selected five households per block and re-interviewed them on selected questions. The summary sheets of the blocks were checked to ensure all eligible children were included. No discrepancies were found in the information obtained during the first and second interviews.

**Component two: longitudinal fever-surveillance study**

A prospective fever-surveillance study is being conducted among children under 12 years of age living in Ward 33. The study objective is to measure the incidence of clinical dengue.

**Sample size**

The sample size is based around the ability to detect an annual incidence of clinical dengue of 10%, with an absolute precision of 3%. As the survey design involved clustering within households, a design effect of 1.5 was included in the calculation. The estimated sample size was then inflated by 30% to allow for possible loss to follow-up during the study. The final sample size required was 800 children.

**Sampling strategy**

A two-stage sampling design was used, with census blocks as strata and households as primary sampling units. For each age group, the number of children required from each census block was proportional to the population of children in that age group in the block. For each census block, a random list of households was generated. Each household selected was visited until the required number of children were recruited in each block. All eligible children in a single household were invited to participate. This was done to avoid the parental perception that children in a family might receive unequal treatment when seeking medical care. The census indicated that there was an average of 1.5 children per family; it was expected that children from approximately 500 families participating in the study would be recruited.

**Inclusion and exclusion criteria**

To be eligible for enrolment, a child had to be aged less than 12 years, to have been a resident of Ward 33 during the 3 months prior to the census, and to belong to a family that did not intend to move out of the area during the next 12 months.

Children were excluded if they were at increased risk of harm following a blood draw or had haemophilia, leukemia or thrombocytopenic purpura.
Enrolment procedure

Each selected household was visited by a designated team comprising a trained research assistant (RA), a phlebotomist and the area PHI/HI. The RA described the study to the parents or legal guardians, provided an information leaflet and, if necessary, read out consent forms prior to obtaining written informed consent. An enrolment form was completed for every participating child, collecting demographic characteristics, eligibility criteria and anthropometric measurements. All participants were given a personal identification (ID) card, with a unique identifier and contact details for their designated RA. The participants were requested to carry their ID cards when visiting a designated health-care facility during a febrile illness over the next year. Each participating family was provided with a digital oral thermometer and shown how to take an accurate temperature reading.

Fever surveillance procedure

Parents were instructed to inform the RA immediately if their child developed fever. RAs request a temperature reading, record the episode in a fever log and advise patients to visit one of the following:

- a designated room at the Lady Ridgeway Outpatients Department;
- Wanathamulla Central Dispensary;
- one of the two participating private general practitioners (GPs).

These sites were identified in the baseline survey as the health-care providers that local children attended in the event of illness.

Children presenting with fever to any of the study facilities are provided standard care. Attending physicians are trained on up-to-date recommended protocols for outpatient management. Where indicated, patients are referred to Lady Ridgeway Hospital for admission.

One RA is based at every study health-care facility. Following initial assessment and management by the attending physician, the RA interviews the patient using a standard case investigation form. Information recorded includes details of the patient’s medical history, physical examination, laboratory findings, presumptive diagnosis and disposition. A blood sample is collected if the fever is ≤7 days’ duration. Study participants who are unable to, or choose not to, visit a study health-care facility are visited at home by a mobile team who interview the patient and collect a blood sample.

Patients admitted to LRH are followed up daily by an RA, using a standardized form to record information about the patients’ clinical condition during their hospital stay. A similar form is completed upon discharge.
All patients with fever are asked to return to the clinic 2 weeks after fever onset, to provide a convalescent blood sample, collected by finger prick onto filter paper. At this time, a second case investigation form is completed to record details of symptoms experienced during the febrile illness, patient physical examination, final diagnosis and current disposition. Participants are compensated for transport costs and time off from work when attending a study facility (not exceeding US$ 2.50 per visit). Patients who do not return to provide a convalescent sample are followed up at home by the mobile team.

If a participant opts to visit any health-care provider other than the four designated sites, the RA contacts the provider to make arrangements to visit the patient or arrange for a referral to one of the study sites.

Data-collection forms were designed in English and pretested on five eligible children. The forms are administered in a local language but completed in English.

**Surveillance case definitions**

**Fever**

A case of fever is defined as any child within the cohort reported by their parents or guardians as having a temperature of ≥38 °C lasting ≤7 days. Children with a history of fever of ≤7 days’ duration are considered fever cases, even in the absence of a high temperature at the time of the interview. This is because many parents treat their children with antipyretics before seeking medical treatment.

**Dengue**

- **Confirmed dengue fever (DF):** a child with fever in whom laboratory testing provides definitive evidence of dengue infection, either by polymerase chain reaction (PCR) or by serological testing of paired sera.
- **Indeterminate case:** a child with fever in whom laboratory testing is inconclusive, for example, if no convalescent sample is available.
- **Confirmed dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS):** a child with fever that is compatible with the World Health Organization (WHO) clinical diagnosis of DHF/DSS, in whom laboratory testing indicates a definite dengue infection.
- **Probable DHF/DSS:** a case that is compatible with the WHO clinical diagnosis of DHF/DSS but where laboratory testing is indeterminate.
- **Non-dengue case:** a child with fever in whom laboratory testing does not yield evidence of dengue infection.
Increasing the case ascertainment

RAs contact participating families by telephone every 2 weeks to enquire about febrile illness in children. Two full-time field assistants (FAs) are employed to visit all participating households every other week, to identify further febrile episodes. FAs complete a fever log and inform the RAs regarding any fever cases in their designated areas. Additionally, HIs visit each participating household on alternate weeks to enquire about febrile episodes. Thus, each family is visited once weekly, by either an FA or a HI.

Component three: baseline dengue prevalence and seroconversion survey

Collection of baseline and 12-month blood samples

During initial enrolment into the cohort, each child was requested to provide a baseline blood sample collected by finger stick onto a filter paper disc (Dried Blood Spot – DBS Saver Cards manufactured by ID Biological Systems, Greenville, South Carolina, United States of America [USA]: Ref IDBS1003). Each sample was labelled, air dried and transported to the study laboratory, in a special container, at the end of the day. At the laboratory, each spot was stored at –20 °C until testing. After one calendar year, a second sample was collected from each participant, for comparison. Collecting capillary blood on DBS saver cards offers significant advantages in terms of sample collection and storage. Sample collection is simplified, with only a lancet and DBS saver cards needed for the blood draw. Not using needles or drawing venous blood also increases compliance, especially with younger children. Storage space is minimized by saving the blood on thin DBS saver cards and these can be stored long term at –20 °C, compared to –70 °C for blood samples collected in tubes. For resource-limited settings, this can be a major advantage in the numbers of samples that can be collected and stored.

Collection and processing of blood samples

Acute blood specimens are taken by a phlebotomist, either at a participating facility or at home. A local anaesthetic is applied prior to drawing blood from the forearm or wrist veins, into an EDTA anti-coagulated vacutainer tube (BD Vacutainer tubes, BD Diagnostics, New Jersey, USA), using a disposable needle. For children aged less than 1 year, 1–5 years and 6–11 years, a sample of 1 mL, 2.5 mL and 5 mL respectively of whole blood is collected. Each sample is labelled, kept on ice and transported within 4 h to Genetech Research Laboratory, in a vehicle with a reverse cold chain box. Each sample is accompanied by a request form indicating the submitting health-care facility, patient ID number and a short clinical history clearly indicating the duration of fever. A staff scientist checks the sample ID and quality upon receipt. Each specimen is entered into a dedicated logbook and database.
**Laboratory analysis**

Samples are centrifuged at 800 g and 4 °C for 10 min, to separate cells and plasma. The plasma is used for dengue diagnosis and any excess frozen at −80° C. Acute fever samples are tested using an in-house reverse transcriptase-PCR to detect viral RNA and determine the dengue serotype. Acute and convalescent samples are tested using in-house immunoglobulin M (IgM) and immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA). For the IgM assay, an increase of 0.1 optical density (OD) units between acute and convalescent samples is considered indicative of an acute infection; for the IgG assay, a rising OD of >0.2 between acute and convalescent samples, together with a convalescent OD value >0.3, is considered indicative of an acute dengue infection.

If both acute and convalescent samples are available, PCR and IgM/IgG serology are used to make a definitive diagnosis of dengue. At least two of the assays (PCR, IgM seroconversion, IgG seroconversion or increasing titre) must be positive for a definitive dengue seroconversion to be made. If no convalescent sample is available, PCR is used to make definitive diagnosis of dengue infection.

Dengue-positive cases are considered primary infections if the acute sample IgG ELISA has an OD <0.4. If the OD is ≥0.4, the case is considered a secondary infection.

**Virus isolation**

A subset of PCR-positive samples were sent for virus isolation to either MRI, Colombo, or the Duke-NUS Graduate Medical School, Singapore. These isolates are stored at MRI, contributing to a reference collection of Sri Lankan dengue strains for future genetic and vaccine studies. Written informed consent was obtained from participants for the long-term storage and use of blood products.

**Laboratory quality control procedures**

For quality control purposes, a subset of samples tested in Sri Lanka were sent to the University of North Carolina, Chapel Hill, USA, for repeat testing. The goal was to achieve greater than 90% concordance for all serological and PCR assays. If discordant results were obtained for more than 10% of the samples with any particular test, steps were taken to troubleshoot the assay.

**Testing of baseline and annual seroprevalence samples**

Baseline and end-of-year samples were tested using the in-house IgG ELISA. OD values <0.3 were considered flavivirus antibody negative, while OD values ≥0.3 were considered flavivirus antibody positive. A child was considered to have been exposed to dengue virus during the year if a rise in the OD value of 0.1 was observed between baseline and end-of-year samples tested side by side, and the end-of-year sample had an OD value ≥0.3.
Data security, data processing and audit

No names of participating children are recorded in any of the case investigation forms or blood samples sent for testing. Every child is identified by a unique ID, which is used whenever a child visits a study facility for treatment, for sending samples to the laboratory and in all databases storing participant information. The key matching individual children to their IDs is kept in a separate electronic file and stored in a secure location accessible only to the PI. The key is kept for identifying individual children in any emergency (e.g. in the case of adverse events following blood draw), or when a child looses his/her ID card and needs a replacement.

Only the designated study RA handles case investigation forms in the field. Completed forms from all study components are checked at the end of the day for errors and omissions, by the interviewers and the PI. A coding key is prepared and data entered in duplicate into a Microsoft Access database by two trained data-entry operators. Duplicate databases are compared and discrepancies resolved by referring to the original documents.

All computers and databases are password protected and located in a secure room. Databases are accessible only within the study office. Document hard copies are kept in locked cupboards.

The study sponsor conducted two site-monitoring visits to review all study and laboratory procedures, documentation and financial management.

Ethical approval

Local ethical approval for the study was obtained from the Ethical Review Committee of the Faculty of Medicine, University of Colombo. Permission to conduct the study was obtained from the Special Commissioner of the Colombo Municipality and the Chief Medical Officer of Health, CMC. The protocol was also reviewed and approved by the following bodies before the fieldwork was begun:

- The Human Subjects Protection Committee of the Pediatric Dengue Vaccine Initiative, International Vaccine Institute, Korea;
- The Research Committees of the Lady Ridgeway Children’s Hospital and Medical Research Institute, Sri Lanka;
- The Ministry of Health, Sri Lanka;
- The Ethical Review Committee of the University of North Carolina, Chapel Hill, USA;
- The Ethical Review Committee of the London School of Hygiene and Tropical Medicine, United Kingdom of Great Britain and Northern Ireland.
Separate consent is obtained for participation in the cohort, collection of blood samples, and storage and future use of blood and blood products. Participants are free to leave the study at any time. The results of PCR investigations are conveyed to participants within 48 h.

**Characteristics of the study population**

A total of 3126 premises were identified within the study area (see Figure 3). Among these, 2766 were eligible households, of which completed census survey questionnaires were obtained from 2757 (99.7%). Among the households completing the census, 1521 had children under 12 years of age. Children were recruited from 504 of these households, which were selected at random.

**Figure 3:** Recruitment of households into the study, Colombo, Sri Lanka, 2008

The characteristics of households from which children were recruited are shown in Table 1, together with the characteristics of all eligible households. Among the households with recruited children, the vast majority of families owned the house in which they lived and just under two thirds had a monthly income exceeding 15,000 rupees. Over 90% used a tap within the premises as a source of drinking water and 75% used a water seal as a latrine. Most houses had cement or concrete flooring (86.7%) and 61.1% had asbestos roofing. Nearly all households had electricity and owned a radio and a television. Approximately two thirds of households owned a refrigerator. Private transportation was uncommon, with less than one in five households owning a motorcycle and only 3.2% owning a car.
**Table 1**: Characteristics of households in the census and recruited into the study, Colombo, Sri Lanka, 2008

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<th>Characteristics</th>
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<td>1.8</td>
</tr>
<tr>
<td>5001–10 000</td>
<td>55</td>
<td>10.9</td>
</tr>
<tr>
<td>10 001–15 000</td>
<td>105</td>
<td>20.8</td>
</tr>
<tr>
<td>&gt;15 000</td>
<td>322</td>
<td>63.9</td>
</tr>
<tr>
<td>Missing</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Drinking water source</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tap within premises (main line)</td>
<td>457</td>
<td>90.7</td>
</tr>
<tr>
<td>Public taps (main line)</td>
<td>34</td>
<td>6.7</td>
</tr>
<tr>
<td>Protected well within premises</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Protected well outside premises</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Other</td>
<td>7</td>
<td>1.4</td>
</tr>
<tr>
<td>Missing</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Latrine type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water seal</td>
<td>382</td>
<td>75.8</td>
</tr>
<tr>
<td>Pour flush</td>
<td>70</td>
<td>13.9</td>
</tr>
<tr>
<td>Pit</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Other</td>
<td>41</td>
<td>8.1</td>
</tr>
<tr>
<td>Missing</td>
<td>9</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Among the heads of households with recruited children, just under one third had grade 6–10 education, a further 29.8% had General Certificate of Education (GCE) O-level education (up to 16 years of age) and 17.5% had GCE A-level (post-16 level) education (see Table 2). Just over three quarters were married and over half had lived in their residence for 10 or more years. The most common ethnic group was Sinhalese (85.3%).

**Table 2: Characteristics of Recruited Households and All Households**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Recruited households (n = 504)</th>
<th>All households (n = 1421)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td><strong>Floor material</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cement/concrete</td>
<td>437</td>
<td>86.7</td>
</tr>
<tr>
<td>Terrazo/granite/tile</td>
<td>63</td>
<td>12.5</td>
</tr>
<tr>
<td>Sand</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Dung/mud</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Roof material</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asbestos</td>
<td>308</td>
<td>61.1</td>
</tr>
<tr>
<td>Concrete</td>
<td>77</td>
<td>15.3</td>
</tr>
<tr>
<td>Tin sheet</td>
<td>59</td>
<td>11.7</td>
</tr>
<tr>
<td>Tile</td>
<td>58</td>
<td>11.5</td>
</tr>
<tr>
<td>Missing</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Has the following:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electricity</td>
<td>479</td>
<td>95.0</td>
</tr>
<tr>
<td>Radio</td>
<td>443</td>
<td>87.9</td>
</tr>
<tr>
<td>Television</td>
<td>459</td>
<td>91.1</td>
</tr>
<tr>
<td>Refrigerator</td>
<td>335</td>
<td>66.5</td>
</tr>
<tr>
<td>Bicycle</td>
<td>71</td>
<td>14.1</td>
</tr>
<tr>
<td>Motorcycle</td>
<td>95</td>
<td>18.8</td>
</tr>
<tr>
<td>Car</td>
<td>16</td>
<td>3.2</td>
</tr>
<tr>
<td>Van</td>
<td>16</td>
<td>3.2</td>
</tr>
<tr>
<td>Tractor/three-wheeler</td>
<td>106</td>
<td>21.0</td>
</tr>
</tbody>
</table>
Table 2: Characteristics of heads of households among census and recruited households, Colombo, Sri Lanka, 2008

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Recruited households (n = 504)</th>
<th>All households (n = 1421)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td><strong>Educational level</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No schooling</td>
<td>32</td>
<td>6.3</td>
</tr>
<tr>
<td>Grade 1–5</td>
<td>69</td>
<td>13.7</td>
</tr>
<tr>
<td>Grade 6–10</td>
<td>159</td>
<td>31.5</td>
</tr>
<tr>
<td>GCE O-level</td>
<td>150</td>
<td>29.8</td>
</tr>
<tr>
<td>GCE A-level</td>
<td>88</td>
<td>17.5</td>
</tr>
<tr>
<td>Degree or higher</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Don’t know/missing</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Marital status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not married</td>
<td>5</td>
<td>1.0</td>
</tr>
<tr>
<td>Married</td>
<td>393</td>
<td>78.0</td>
</tr>
<tr>
<td>Divorced/separated</td>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>Widowed</td>
<td>96</td>
<td>19.0</td>
</tr>
<tr>
<td>Missing</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinhalese</td>
<td>430</td>
<td>85.3</td>
</tr>
<tr>
<td>Tamil</td>
<td>45</td>
<td>8.9</td>
</tr>
<tr>
<td>Moor</td>
<td>26</td>
<td>5.2</td>
</tr>
<tr>
<td>Other</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Years of residence</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>20</td>
<td>4.0</td>
</tr>
<tr>
<td>1–4</td>
<td>73</td>
<td>14.5</td>
</tr>
<tr>
<td>5–10</td>
<td>48</td>
<td>9.5</td>
</tr>
<tr>
<td>10+</td>
<td>273</td>
<td>54.2</td>
</tr>
<tr>
<td>Since birth</td>
<td>89</td>
<td>17.7</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td>0.2</td>
</tr>
</tbody>
</table>
A total of 802 children aged under 12 years were recruited from these 504 households. In Table 3, the characteristics of recruited children are compared with those of all children identified in the census. Approximately 50% of children recruited were male, and infants made up less than 7% of the study sample. Nearly all children had received a full schedule of immunizations appropriate for their age. One third of children reported having suffered an illness in the past 3 months, and 14% reported having experienced fever. Just under 90% of fevers were reported to have been treated. Treatment for fever was most commonly sought from a government outpatient department (44%) or from a private GP (40%).

No major differences were found between the study sample and the census population of Ward 33, with respect to any of these factors, although the recruited children reported somewhat higher use of private doctors for the treatment of fevers than the census population, and somewhat lower use of government outpatient departments.

**Discussion**

This is the first study of its kind in Sri Lanka and will yield valuable information about the epidemiology of dengue in the Indian subcontinent. It will provide much-needed estimates of the burden of DF in children, which will be important for assessing the potential benefit of future dengue vaccination programmes. Using intensive surveillance, standardized clinical diagnostic criteria, comprehensive laboratory testing and seroprevalence surveys, the study will also give information about the degree of under-ascertainment of clinical and subclinical dengue illness. Such information is crucial for understanding the transmission dynamics of the virus in an urban paediatric population. While childhood dengue continues to be a major problem in Sri Lanka, in recent years, a shift in the age distribution of dengue cases has been observed. The increased burden of dengue among young adults presents new challenges for control. To better understand the epidemiology among older age groups, the cohort is being extended to include adults.

The study will also enable an assessment of the feasibility of PCR and in-house ELISAs as point-of-care diagnostics with rapid turnaround. Current diagnostics, while useful for confirmation, are subject to considerable delay and are of limited use for patient management. A cheap, accurate and rapid test that can provide results to clinicians during the critical acute phase of illness has the potential to greatly enhance medical care and improve treatment outcome. The archiving of samples will be an invaluable resource for future virological and vaccine studies, and isolation and sequencing of strains will shed light on locally circulating strains and their phylogenetic relationship to viruses from other regions of the world.

The high level of participation in this study is a testament to the close relationship between the study team, local health practitioners and the community. Extending this model in Sri Lanka will bring great benefits for public health research and, ultimately, disease prevention and control. In addition, this study is an evidence-generating model for public health interventions, and demonstrates how to set up a community-based cohort...
Table 3: Characteristics of children in the census and recruited into the study, Colombo, Sri Lanka, 2008

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Recruited children ((n = 802))</th>
<th>All households ((n = 2527))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
</tr>
<tr>
<td><strong>Age group, years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1</td>
<td>55</td>
<td>6.9</td>
</tr>
<tr>
<td>1–3</td>
<td>193</td>
<td>24.1</td>
</tr>
<tr>
<td>4–6</td>
<td>196</td>
<td>24.4</td>
</tr>
<tr>
<td>7–9</td>
<td>233</td>
<td>29.1</td>
</tr>
<tr>
<td>10–11</td>
<td>125</td>
<td>15.6</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>408</td>
<td>50.9</td>
</tr>
<tr>
<td>Female</td>
<td>394</td>
<td>49.1</td>
</tr>
<tr>
<td>Missing</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Immunization status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not immunized</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>Partially immunized</td>
<td>9</td>
<td>1.1</td>
</tr>
<tr>
<td>Fully immunized</td>
<td>788</td>
<td>98.3</td>
</tr>
<tr>
<td>Missing</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Illness</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any illness in the past 3 months</td>
<td>265</td>
<td>33.3</td>
</tr>
<tr>
<td>Sought treatment for illness</td>
<td>243</td>
<td>91.7</td>
</tr>
<tr>
<td><strong>Fever</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Had fever in the past 3 months</td>
<td>111</td>
<td>14.0</td>
</tr>
<tr>
<td>Sought treatment for fever</td>
<td>98</td>
<td>88.3</td>
</tr>
<tr>
<td><strong>Sought treatment for fever from:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Government outpatients’ department</td>
<td>43</td>
<td>43.9</td>
</tr>
<tr>
<td>Government inpatient ward</td>
<td>8</td>
<td>8.2</td>
</tr>
<tr>
<td>Private outpatients’ department</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Private inpatient ward</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Private specialist</td>
<td>2</td>
<td>2.0</td>
</tr>
<tr>
<td>Private general practitioner</td>
<td>39</td>
<td>39.8</td>
</tr>
<tr>
<td>Ayurvedic pharmacy</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>Home care</td>
<td>3</td>
<td>3.1</td>
</tr>
</tbody>
</table>
in the setting of a middle-income country in preparation for future vaccines; and how to provide evidence of vaccine needs, including identifying the target populations, cost–benefit assessments and background rates for safety profiles, which are crucial for decision-making prior to vaccine introduction.

**Acknowledgements**

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**Competing interests**

The authors declare that they have no competing interests.

**References**


Modelling and predicting dengue fever incidence in Singapore: an intervention model approach

Yew-Meng Koh*

Department of Statistics, Iowa State University, Ames IA 50011, United States of America

Abstract

Time-series analysis of disease incidence data is a common strategy, owing to the possible dependence that exists between the responses. In this paper, two time-series models for dengue incidence data in Singapore are proposed and compared. The first is a second-order autoregressive model and the second is an intervention model. While both models adequately describe past data and predict future observations, it is observed that the intervention model provides more accurate predictions of future dengue incidence. Furthermore, the intervention model enables predictions of dengue cases in hypothetical future interventions, which may be beneficial in decision-making in regional dengue fever control policy.

Keywords: Dengue fever; Disease prediction; Intervention Model; Singapore; Time series analysis.

Introduction


This paper focuses on the incidence of dengue cases in Singapore for the period 2000 to September 2011. Singapore is a tropical country which, like many other tropical countries, faces the challenge of dengue fever.

The Ministry of Health, Singapore (http://www.moh.gov.sg) releases incidence rates of dengue fever each week. The data used in this paper were obtained by totalling the number of incidences over 4-week periods (i.e. there are 13 4-week periods per year). For the years 2000–2010, there are 13 observations and there are 10 observations for the year 2011 (until September 2011). Thus, there are 153 observations in total. These data are displayed in Table 1 and also in Figure 1.

The following is observed from looking at the dengue fever incidence data (see Figure 1):

- an overall increasing trend, a sharp decline in 2006, then a slow increasing trend again;
- the data display some seasonal behaviour (peak incidence in the middle of each year);

*E-mail: kohym@iastate.edu
there appears to be non-constant variance among observations (see the residual plot in Figure 2 from fitting a second-order regressive [AR(2)] model to the raw, untransformed data). Notice, in particular, the non-constant variance in the year 2005.

The intervention model proposed in this paper improves on the basic AR(2) model and also removes this non-constant variance issue.

**Table 1: Number of new dengue cases in Singapore, 2000 to September 2011**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>First 4-week period</td>
<td>56</td>
<td>72</td>
<td>85</td>
<td>354</td>
<td>297</td>
<td>1071</td>
<td>260</td>
<td>222</td>
<td>617</td>
<td>375</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>Second 4-week period</td>
<td>52</td>
<td>96</td>
<td>77</td>
<td>331</td>
<td>241</td>
<td>1082</td>
<td>240</td>
<td>191</td>
<td>385</td>
<td>485</td>
<td>350</td>
<td>219</td>
</tr>
<tr>
<td>Third 4-week period</td>
<td>40</td>
<td>133</td>
<td>80</td>
<td>341</td>
<td>194</td>
<td>508</td>
<td>197</td>
<td>258</td>
<td>294</td>
<td>320</td>
<td>311</td>
<td>272</td>
</tr>
<tr>
<td>Fourth 4-week period</td>
<td>26</td>
<td>121</td>
<td>139</td>
<td>283</td>
<td>256</td>
<td>507</td>
<td>144</td>
<td>369</td>
<td>360</td>
<td>390</td>
<td>238</td>
<td>291</td>
</tr>
<tr>
<td>Fifth 4-week period</td>
<td>43</td>
<td>152</td>
<td>208</td>
<td>291</td>
<td>436</td>
<td>535</td>
<td>200</td>
<td>607</td>
<td>450</td>
<td>372</td>
<td>238</td>
<td>396</td>
</tr>
<tr>
<td>Sixth 4-week period</td>
<td>39</td>
<td>181</td>
<td>242</td>
<td>739</td>
<td>556</td>
<td>728</td>
<td>315</td>
<td>1145</td>
<td>611</td>
<td>401</td>
<td>326</td>
<td>504</td>
</tr>
<tr>
<td>Seventh 4-week period</td>
<td>57</td>
<td>327</td>
<td>312</td>
<td>568</td>
<td>721</td>
<td>1381</td>
<td>379</td>
<td>1506</td>
<td>564</td>
<td>308</td>
<td>502</td>
<td>745</td>
</tr>
<tr>
<td>Eighth 4-week period</td>
<td>64</td>
<td>323</td>
<td>445</td>
<td>278</td>
<td>1002</td>
<td>1343</td>
<td>318</td>
<td>1210</td>
<td>544</td>
<td>270</td>
<td>499</td>
<td>858</td>
</tr>
<tr>
<td>Ninth 4-week period</td>
<td>61</td>
<td>313</td>
<td>601</td>
<td>290</td>
<td>1182</td>
<td>2081</td>
<td>259</td>
<td>873</td>
<td>584</td>
<td>244</td>
<td>535</td>
<td>512</td>
</tr>
<tr>
<td>Tenth 4-week period</td>
<td>65</td>
<td>241</td>
<td>551</td>
<td>285</td>
<td>1111</td>
<td>2376</td>
<td>214</td>
<td>737</td>
<td>526</td>
<td>241</td>
<td>690</td>
<td>356</td>
</tr>
<tr>
<td>Eleventh 4-week period</td>
<td>47</td>
<td>152</td>
<td>473</td>
<td>313</td>
<td>1083</td>
<td>1270</td>
<td>172</td>
<td>538</td>
<td>643</td>
<td>281</td>
<td>535</td>
<td></td>
</tr>
<tr>
<td>Twelfth 4-week period</td>
<td>52</td>
<td>136</td>
<td>386</td>
<td>344</td>
<td>1053</td>
<td>573</td>
<td>166</td>
<td>512</td>
<td>585</td>
<td>255</td>
<td>384</td>
<td></td>
</tr>
<tr>
<td>Thirteenth 4-week period</td>
<td>61</td>
<td>119</td>
<td>338</td>
<td>316</td>
<td>1160</td>
<td>362</td>
<td>187</td>
<td>469</td>
<td>743</td>
<td>268</td>
<td>347</td>
<td></td>
</tr>
</tbody>
</table>
Previous work on modelling dengue fever incidence and differences between other studies and this paper on dengue fever incidence modelling

Time-series models are often used for modelling disease incidence. This is due to the dependence of observations on one another (i.e. the observations are not independent). As mentioned in the abstract, many authors have attempted to model disease-incidence data using time-series analyses.\textsuperscript{1-4} In references 1, 2 and 4, time-series analysis was used to model dengue fever incidence, while in reference 3, the disease that was modelled was Newcastle disease. In all four references, time-series analysis was used to forecast or predict future incidence rates of the disease.

Often, information from other variables (called covariates) that are thought to influence disease incidence is considered.\textsuperscript{5} One example is climate variables like rainfall levels, temperature and humidity.\textsuperscript{6} As a result, some authors have attempted time-series analyses that incorporate information from these covariates into their time-series models.\textsuperscript{4,7} These kinds
of time-series models are also known as time-series regression models, with the additional covariate variables treated as the regressor variables.7

In this paper, both an ordinary AR(2) time-series model and an intervention model were used for the observations (details of the AR(2) model are given in the Appendix and details of the intervention model are given in the subsection “The intervention model – Model 2”).

The incorporation of the intervention model into the ordinary AR(2) model makes this study different from other studies on modelling dengue fever incidence. This modelling choice was made because of the efforts of the Singaporean government, which implemented a significant anti-dengue campaign in September 2005,8,9 leading to a sharp decrease in dengue fever incidence.

This paper highlights the advantages of using the intervention time-series model,10 as opposed to an ordinary time-series model, which does not take the intervention into account. It also provides a possible extension of the intervention model that can be used to predict incidence rates in the light of possible additional interventions in the future.

The code (using R, the free programming language for fitting many statistical models) for producing all the results and figures in this paper is also included. For more information on the R programming language, see reference 10. This code is commented extensively and can be modified accordingly to suit any other specific situation.

Materials and methods

Time-series analysis is greatly simplified when observations have constant variability. An attempt was made to stabilize the variance by log-transforming the data. Figures 3 and 4 show that the observations now have more homogeneous variability. Henceforth, all analysis was based on the log-transformed dengue incidence values. Throughout this paper, \{Y_t\} represents the time series of log(total new dengue cases between times \(t-1\) and \(t\)). Thus, \(Y_1\) would be log(total new dengue cases in the first 4 weeks of the year 2000), and so on. In this paper, an AR(2) time-series model for the dengue incidence data is proposed (Model 1). Subsequently, the modelling of dengue fever incidence using an intervention model is introduced (Model 2). Then, the performance of the intervention model is compared to that of the ordinary AR(2) time-series model and, finally, the intervention model is extended in a hypothetical situation (Model 3). The method for fitting all three models will be described in detail in the following subsections. The code for fitting all the models is also included in this paper.

For model-comparison purposes, both prediction and variability of prediction will be considered. The closer a prediction is to the actual value and the smaller its variability, the better the prediction. To that end, the last three data points are reserved for model validation and comparison (i.e. \(Y_{151}\), \(Y_{152}\) and \(Y_{153}\) are not used in model fitting).
Modelling the trend and seasonality deterministically, with an ordinary AR(2) model (with no intervention incorporated) – Model 1

Roughly speaking, time-series data $Y_t$ can be decomposed into three components:

- trend;
- seasonality;
- error (or residual).

**Figure 3:** Log (number of new dengue cases in Singapore), 2000 to September 2011

**Figure 4:** Standardized residuals from an AR(2) model fit to log(number of new dengue cases in Singapore), 2000 to September 2011 – Model 1
Trend and seasonality are modelled using a mean function of time $t$, symbolized by $\mu_t$. Examination of the logged data (see Figure 3) highlights the existence of a quadratic trend and some periodic behaviour in the data.$^{11,12}$

First, the trend is modelled deterministically (non-random) by using a quadratic (squared) functional relationship. Then, the seasonal behaviour of the data is also modelled deterministically, using trigonometric functions. The quadratic and trigonometric terms are combined to form the mean function $\mu_t$.

Next, an attempt is made to model the dependence among the residuals $Y_t - \mu_t$ (the random errors that remain in the data after the trend and seasonality have been successfully modelled).

One approach would be to fit an AR(2) structure to the residuals from a linear model fit of the log(dengue) data, which already includes a quadratic time trend and trigonometric terms (owing to the observed periodic structure in the data). It is possible to estimate all parameters simultaneously, using maximum likelihood estimation, a statistical method where values of parameters are taken to be those that maximize the likelihood function.$^{10}$ The following model:

$$Y_t - \mu_t - 1.1506(Y_{t-1} - \mu_{t-1}) + 0.2929(Y_{t-2} - \mu_{t-2}) = W_t$$

with mean function:

$$\mu_t = 4.1184 + 0.0454t - 0.0002t^2 - 0.2968\cos\left(\frac{2}{13}\pi(t - 1)\right) - 0.2116\sin\left(\frac{2}{13}\pi(t - 1)\right)$$

and with $\{W_t\}$ a sequence of identically and independently distributed random variables with zero mean and variance $\sigma^2_W$, was adequate for the incidence data (i.e. the problem of non-constant variance is alleviated; see Figure 4 and compare it with Figure 2), it passes the Ljung–Box diagnostic tests with all $P$ values exceeding the 0.05 threshold (see Figure 5) and there is no more evidence of dependence in the residuals from this model (Figure 6 shows that none of the sample autocorrelation function [ACF] values at non-zero lags are outside the confidence limits [in blue] and none of the sample partial autocorrelation function [PACF] values are outside the confidence limits [in blue]). This indicates that the AR(2) error structure is sufficient to model the dependence in the residuals. Table 2 gives the parameter estimates with their standard errors (all parameters are statistically significant).$^{10}$
Figure 5: P values from Ljung–Box test for Model 1

Figure 6: Sample ACF and PACF plots for Model 1
The intervention model – Model 2

Intervention models are especially useful when a known intervention that is expected to have a significant impact on the values of a time series has occurred or been implemented. A very clear example of this is seen in the Singaporean dengue data set. An intervention was carried out from mid-September to early October 2005, corresponding to time point $T = 76$ in the dengue data set.\(^8,9\) The intervention that took place took the form of media campaigns, checking households for stagnant water (mosquito breeding areas) and widespread fogging to kill mosquitoes.\(^13\) Consistent with this intervention, a very sharp drop in new cases in late 2005 is observed in Figure 1.

**Table 2: Parameter estimates and standard errors for Model 1**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\phi_1$</th>
<th>$\phi_2$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\gamma$</th>
<th>$\eta_1$</th>
<th>$\eta_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimate</td>
<td>1.1506</td>
<td>-0.2929</td>
<td>4.1184</td>
<td>0.0454</td>
<td>-0.0002</td>
<td>-0.2968</td>
<td>-0.2116</td>
</tr>
<tr>
<td>Standard error</td>
<td>0.078</td>
<td>0.0797</td>
<td>0.3665</td>
<td>0.0116</td>
<td>0.0001</td>
<td>0.0827</td>
<td>0.0823</td>
</tr>
</tbody>
</table>

$\sigma^2_w = 0.05494$

**Defining the intervention model**

First, $P^r_t = \begin{cases} 1 & t = T \\ 0 & \text{otherwise} \end{cases}$

is defined as the pulse function at time $T$, and $m_1$ as the effect of the intervention on the original mean function $\mu_t$. 
The time of the intervention is denoted by $T$. Thus, we have:

$$m_t = 0 \text{ for } t \leq T - 1.$$ 

The following steps are utilized in choosing an appropriate intervention model:

- If the intervention impacts the mean function only at $t = T$, then $m_t$ can be modelled by
  $$m_t = \begin{cases} 
  \omega P^T_t & t = T \\
  0 & \text{otherwise}
  \end{cases}$$
  for some constant $\omega$;

- If the intervention effects die out gradually after time $T$, then $m_t$ can be modelled by
  $$m_t = \begin{cases} 
  \delta m_{t-1} + \omega P^T_t & t \geq T \\
  0 & \text{otherwise}
  \end{cases}$$
  for some constants $\delta$ and $\omega$;

- If the intervention only takes effect after a delay of one time unit and the effect dies out gradually, then $m_t$ can be modelled by
  $$m_t = \begin{cases} 
  \delta m_{t-1} + \omega P^T_{t-1} & t \geq T \\
  0 & \text{otherwise}
  \end{cases}$$
  for some constants $\delta$ and $\omega$.

From examining the data, a delay of two time points for the intervention to take effect is observed, and then there is a gradual dying off of the intervention effect. To reflect this observation, $m_t$ is modelled by

$$m_t = \begin{cases} 
  \delta m_{t-1} + \delta_2 m_{t-2} + \omega_0 P^T_{t-1} + \omega_1 P^T_{t-1} + \omega_2 P^T_{t-2} & t \geq T \\
  0 & \text{otherwise}
  \end{cases}$$
  for some constants $\delta_1, \delta_2, \omega_0, \omega_1$ and $\omega_2$.

Another way of stating this is as follows:

$$m_t - \delta_1 m_{t-1} - \delta_2 m_{t-2} = \begin{cases} 
  \omega_0 P^T_{t-1} + \omega_1 P^T_{t-1} + \omega_2 P^T_{t-2} & t \geq T \\
  0 & \text{otherwise}
  \end{cases}$$

A commonly used symbol in time-series analysis is the differencing operator $B$, defined by:

$$B^n f(t) \equiv f(t - n)$$

for any function $f(t)$.
Hence, the effect of the intervention on the mean function $\mu_t$ was modelled as:

$$m_t - \delta_1 B m_t - \delta_2 B^2 m_t = \begin{cases} \omega_0 P_t^\tau + \omega_1 B P_t^\tau + \omega_2 B^2 P_t^\tau & t \geq T \\ 0 & \text{otherwise} \end{cases}$$

which can be rewritten as:

$$m_t = \begin{cases} \left( \frac{\omega_0 + \omega_1 B + \omega_2 B^2}{1 - \delta_1 B - \delta_2 B^2} \right) P_t^\tau & t \geq 76 \\ 0 & \text{otherwise} \end{cases}$$

Here, $T = 76$ (the intervention time). This representation of $m_t$ is used, as it is the syntax by which this model is coded into R, the software that was used in the analysis.

When fitting the intervention model, the quadratic terms and the trigonometric terms from Model 1 are incorporated into the model, together with the AR(2) structure for the residuals. Thus, the complete intervention model fit was Model 2 (once again, the method of maximum likelihood was used to obtain parameter estimates).

$$Y_t - \mu_t - m_t = 1.0852(Y_{t-1} - \mu_{t-1} - m_{t-1}) - 0.2971(Y_{t-2} - \mu_{t-2} - m_{t-2}) + W_t$$

where:

$$\mu_t + 3.5823 = 0.0686t - 0.0003t^2 - 0.2801\cos\left[\frac{2\pi}{13}(t - 1)\right] - 0.1934\sin\left[\frac{2\pi}{13}(t - 1)\right]$$

for all $t \geq 1$ and:

$$m_t = \begin{cases} 0.1606m_{t-1} + 0.813m_{t-2} - 0.6054P_{t-1} + 1.1623P_{t-2} - 0.5997P_{t-2}^2 & t \geq 76 \\ 0 & \text{otherwise} \end{cases}$$

Table 3 gives the parameter estimates with their standard errors (all parameters are statistically significant).
Intervention models for dengue incidence in Singapore

Estimating the effect of a hypothetical second intervention at a later time – Model 3

The intervention model is versatile, in that it can be used to look at the possible effects of other similar (albeit hypothetical) interventions at later times. For this paper, the number of dengue cases if there had been another intervention at time $T = 140$ is estimated. It is assumed that the delay and effect of the second intervention are the same as the first (i.e. the same parameter estimates were used to generate the second post-intervention mean function $M_i$). The model (Model 3) that was fitted to the data is an extension of Model 2.

Model 3 also has an AR(2) structure for the residuals:

$$ Y_i - \mu_i - m_i - M_i = 1.0852(Y_{i-1} - \mu_{i-1} - m_{i-1} - M_{i-1}) - 0.2971(Y_{i-2} - \mu_{i-2} - m_{i-2} - M_{i-2}) $$  \hspace{1cm} (3)

where:

$$ \mu_i + 3.5823 = 0.0686t - 0.0003t^2 - 0.2801\cos\left(\frac{2\pi}{13}(t-1)\right) - 0.1934\sin\left(\frac{2\pi}{13}(t-1)\right) \text{ for all } t \geq 1 $$

$$ m_i = \begin{cases} 0.1606m_{i-1} + 0.813m_{i-2} - 0.6054P_{i-1}^{76} - 1.1623P_{i-2}^{76} - 0.5997P_{i-2}^{76} & t \geq 76 \\ 0 & \text{otherwise} \end{cases} $$

Table 3: Parameter estimates and standard errors for Model 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\beta_0$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\psi_1$</th>
<th>$\psi_2$</th>
<th>$\psi_3$</th>
<th>$\phi_1$</th>
<th>$\phi_2$</th>
<th>$\omega_0$</th>
<th>$\omega_1$</th>
<th>$\omega_2$</th>
<th>$\delta_1$</th>
<th>$\delta_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimate</td>
<td>3.5823</td>
<td>0.0686</td>
<td>-0.0003</td>
<td>-0.2801</td>
<td>-0.1934</td>
<td>1.0852</td>
<td>-0.2971</td>
<td>-0.6054</td>
<td>-1.1623</td>
<td>-0.5997</td>
<td>0.1606</td>
<td>0.813</td>
<td></td>
</tr>
<tr>
<td>Standard error</td>
<td>0.2607</td>
<td>0.0086</td>
<td>0.0001</td>
<td>0.0774</td>
<td>0.0792</td>
<td>0.0793</td>
<td>0.0795</td>
<td>0.2027</td>
<td>0.348</td>
<td>0.2513</td>
<td>0.427</td>
<td>0.4239</td>
<td></td>
</tr>
</tbody>
</table>
Intervention models for dengue incidence in Singapore

Results

The continuity-corrected Akaike information criterion for model comparison

One statistic that could be used to compare different statistical models fit to data is the continuity-corrected Akaike information criterion (the AICc value). The AICc for a given model is defined by

\[ AICc = 2k - 2\ln(L) + \frac{2k(k+1)}{n-k-1}, \]

where \( n \) is the number of data points, \( k \) is the number of parameters in the statistical model and \( L \) is the value of the likelihood of the model, with the maximum likelihood estimates plugged in. It is a measure of model fit that balances goodness with model complexity, i.e. it imposes an increasing penalty on increasingly complex models that would fit the data better. The smaller this value, the better the model is in terms of balancing model fit with complexity.\(^{10}\) In this paper, the AICc values are computed by the software.

Comparing Models 1 and 2

Figure 7 shows the actual data, together with the fitted values from Model 1. It is observed that the fit is satisfactory, with a mean absolute error (MAE) of 0.1865. The AICc value is 9.22. Note:

\[ MAE \equiv \frac{1}{N} \sum_{i=1}^{N} | Y_i - \hat{Y}_i |, \]

where \( \hat{Y}_i \) is the fitted value from the model and \( N \) is the number of observations. The smaller the MAE is, the better the fit of the model to the data.

The fitted values from the intervention model and the actual data are shown in Figure 8. Again, the fit is satisfactory and better directly after the intervention compared to Model 1 (compare Figures 7 and 8). Also, the fitted intervention model passes the Ljung–Box diagnostic tests (see Figure 9) and there is no more evidence of dependence in the residuals from this model (see Figure 10, indicating that the AR(2) error structure is sufficient to model the
Figure 7: Fitted values from Model 1 and actual data

Figure 8: Fitted values from Model 2 and the actual data
Figure 9: $P$ values from Ljung–Box test for Model 2

Figure 10: Sample ACF and Sample PACF plots for Model 2
dependence in the original data). Model 2 has a MAE of 0.1791 and its AICc value is 5.26 (smaller than that of Model 1).

The three reserved log (dengue) values are used to compare Models 1 and 2 (see Figure 11) with regard to the accuracy of their predictions for those values. The intervention model (Model 2) appears to have more accurate predictions, with smaller prediction errors. Prediction error is defined as the expected value of the square of the difference between the prediction and the true value, $E(\hat{Y}_{\text{predicted}} - Y_{\text{actual}})^2$. Model 2 also had a smaller AICc value, indicating better model fit. The next subsection introduces the estimated effect of a second, hypothetical intervention.

**Figure 11**: Predictions and prediction errors from Models 1 and 2 at time points 151, 152 and 153

![Predictions and Prediction error from Models 1 and 2](image)

**Results from Model 3**

Figure 12 shows the actual number of dengue cases and also the estimated number of dengue cases after time $T = 140$ if there had been a similar, second intervention.

Clearly, the intervention model would be a good tool to encourage future interventions, as it can be seen that the estimated number of new cases would be significantly less if another similar intervention had been carried out.
Discussion and conclusion

It is clear that incorporating the knowledge that an intervention took place was beneficial, in that it gave better future predictions with smaller prediction errors. It has also been observed that acknowledging the intervention when modelling the mean structure of the model improved the model fit to the past data (especially in the post-intervention period). Also, the intervention model is useful for estimating the hypothetical effect of future, identical interventions (with the assumptions of similar delayed effect and intensity of intervention). This extension to a hypothetical intervention is especially pertinent in Singapore’s case, owing to the relatively small size of its land area, which makes the aforementioned assumptions more plausible. The estimated positive effects of future interventions may be useful in encouraging the Ministry of Health to include such endeavours in its dengue fever control policies. The methods introduced in this paper (and the code) can be modified to model similar scenarios (for example, when there are multiple interventions that are known to have taken place at different time points).

Appendix: the AR(2) time-series model

Let \( \{Y_t\} \) represent the time series of log(total new dengue cases between times \( t - 1 \) and \( t \)) values.

\[
\mu_t \text{ is a non-random (deterministic) function of time, which models any trend or seasonal behaviour in the time series. } \mu_t \text{ could contain polynomial terms (in our case, quadratic terms) and/or trigonometric terms (sine or cosine functions).}
\]
Let $Z_t = Y_t - \mu_t$ represent the residuals from the data after trend and seasonal behaviour have been modelled by the non-random function $\mu_t$. An AR(2) model models the dependence that exists between these residuals $\{Z_t\}$. Specifically, an AR(2) model assumes that the residuals are related via the relationship $Z_t = \alpha Z_{t-1} + \beta Z_{t-2} + W_t$, where $\{W_t\}$ is a sequence of identically and independently distributed random variables with zero mean and variance $\sigma^2_W$.

The parameters $\alpha$, $\beta$ and $\sigma^2_W$ are unknown and can be estimated via maximum likelihood. Maximum likelihood is the method used in the attached code. See Section 7.3 of reference 10.

**References**


Insecticide-susceptibility status of dengue vectors
Aedes aegypti and Aedes albopictus in India: a review

RK Singh,* S Haq, Gaurav Kumar, PK Mittal and RC Dhiman

National Institute of Malaria Research, Indian Council of Medical Research (ICMR),
Sector-8, Dwarka, New Delhi 110077, India

Abstract
In India, Aedes aegypti is widely distributed and plays a key role in dengue transmission, as a principal vector. In addition, Aedes albopictus, a feral species that breeds in outdoor premises such as tree holes and artificial containers, is surmised as a dengue vector in various areas. This paper reviews studies carried out in India in which insecticides were tested against Aedes aegypti and Aedes albopictus and where insecticide resistance was reported. Public databases were also searched, using relevant keywords. The review of literature on susceptibility status revealed widespread resistance against dichlorodiphenyltrichloroethane (DDT) in adult as well as immature stages of both Aedes aegypti and Aedes albopictus mosquitoes. Adult Aedes aegypti and Aedes albopictus have been found to be susceptible to malathion (organophosphate) and synthetic pyrethroids. There are some reports of tolerance and development of resistance against malathion in the adult as well as larval stages of Aedes mosquito. In general, the aquatic stages of Aedes aegypti are still susceptible to conventional larvicides, namely temephos, Bacillus thuringiensis israelensis (Bti), etc., which are commonly used for larval control in the country. The immature stages of Aedes mosquitoes have shown a tendency to develop induced resistance to temephos under laboratory conditions. In epidemic conditions, spraying with an appropriate insecticide, to which both Aedes aegypti and Aedes albopictus mosquitoes are fully susceptible, may be undertaken for rapid control.

Keywords: Dengue vector; India; Insecticide susceptibility; Review.

Introduction
Mosquitoes belonging to the family Culicidae of the class Insecta are the vectors of various infectious diseases such as malaria, dengue, filariasis, Japanese encephalitis, chikungunya, etc. Dengue is a mosquito-borne arboviral infection found in tropical, subtropical and temperate regions around the world. There are four serotypes of the virus that cause dengue (DENV-1, DENV-2, DENV-3 and DENV-4). The first epidemic of this disease was recorded in 1635 in the French West Indies.1 In South-East Asia, the first confirmed epidemic of dengue haemorrhagic fever (DHF) was recorded in the Philippines in 1953–1954 and in 1958 in Thailand. In India, the first confirmed DHF outbreak was recorded in 1963.2

*E-mail: singhriku@yahoo.co.in
The incidence of dengue has grown dramatically around the world in recent decades. More than 2.5 billion people, i.e. two fifths of the world’s population in tropical and subtropical countries, are now at risk of dengue.³ An estimated 500 000 people with severe dengue require hospitalization each year, a large proportion of whom are children, with a death rate of about 2.5%.³ According to World Health Organization (WHO) estimates, there may be 50–100 million dengue infections worldwide annually. Before 1970, only nine countries had experienced severe dengue epidemics; it is now endemic in more than 100 countries, as dengue cases across the WHO Region of the Americas, South-East Asia Region and Western Pacific Region exceeded 2.3 million in 2010.³ In 2010, 1.6 million cases of dengue were reported in the Americas alone, of which 49 000 cases were severe dengue.⁴ In most of Asian and Latin American countries, DHF has become a leading cause of hospitalization and death among children.⁵ In India, 50 164 dengue cases with 241 deaths, and 15 977 chikungunya cases, were recorded by the National Vector Borne Disease Control Programme (NVBDCP) in 2012.⁴

In India, *Aedes aegypti* and *Aedes albopictus* are the two important vectors of dengue. *Aedes aegypti* is widely distributed and plays a key role in dengue transmission in various states, as a principal vector of dengue fever (DF).⁶ *Aedes aegypti* is a highly domesticated and discordant species and a strongly anthropophilic and nervous feeder that may feed on more than one person. This behaviour greatly increases its epidemic transmission efficiency. *Aedes albopictus* is a feral and concordant species and an aggressive and opportunistic feeder commonly found in peri-domestic areas; it may take the full blood meal in one go from one host, reducing its vectorial capacity. *Aedes aegypti* carries higher vectorial capacity than *Aedes albopictus* in urban epidemic cycles.⁵ Initially, dengue vectors were prevalent in urban areas only. Since cities are growing outwards, these vectors have spread in areas that were, so far, free from dengue.⁷–¹¹

**History of insecticidal spray in India**

In India, during the pre-dichlorodiphenyltrichloroethane (DDT) era (1902–1945), mosquito control was mainly achieved by larval control of malaria vectors, through environmental and engineering methods supplemented by biological and legislative methods.¹² However, with the arrival of DDT, the control strategy changed in 1953, from larval control to adult mosquito control, based on indoor residual spraying of insecticide to control malaria vectors.¹³ In 1958, the control programme was changed to a malaria-eradication programme. This mode of vector control gained much success and became the mainstay in vector-control programmes. By 1964, malaria was almost eradicated, with about one million cases with no recorded death. However, by 1976, malaria re-emerged, with over six million cases with 19 deaths. In 1977, a modified plan of operation, based on annual parasite incidence with macro-level epidemiology, and supported by long-lasting insecticide-treated bed nets and larvivorous fishes, was implemented to control malaria.
In view of the deteriorating situation of malaria in urban areas, an Urban Malaria Scheme (UMS) was started in 1971–1972, in 23 worst-affected towns with over 40 000 population. In urban areas, DDT spraying up to 5 km depth at their periphery was done as barrier spray, irrespective of spleen rates, in addition to anti-larval measures undertaken within the city limits.

Since 1963, after the outbreak of dengue in Kolkata, DF/DHF emerged as a major public health concern in many urban areas. In recent years, several outbreaks of DF/DHF have been reported in cities and the disease is now spreading to most rural areas as well.2,7,8 The dengue vector, Aedes aegypti, which is prevalent in urban areas, predominantly breeds in containers of clean water, stored and used for household purposes. Although there is no specific control strategy for dengue vectors, larvicide with temephos and Bacillus thuringiensis israelensis (Bti) and space spraying/thermal fogging, etc., which is used under UMS, is also now recommended for the control of Aedes mosquitoes. Since the 1980s, temephos, an organophosphate compound, has been used under the public health programme. The recommended application dose of temephos (50% EC) is 1 ppm (1 mg/L in potable water).

Successful implementation of vector-control programmes requires a thorough understanding of vector distribution and its biology and susceptibility to available insecticidal compounds.14 The purpose of the susceptibility test is to determine resistance in a mosquito population, so that alternative control strategies can be implemented in good time, when the insecticide in question is no longer having the desired effect.15 Knowledge of the factors underlying resistance is needed for the implementation of efficient vector-control programmes and resistance-management strategies. This raises the need for country-wide and regular surveys for monitoring the insecticide-susceptibility status of major vectors, detecting resistance genes and assessing their implications for vector-control activities.16 Not much work has been carried out on the susceptibility status of Aedes mosquitoes, but considering the public health importance and complexities in the control of dengue vectors, the available literature on the susceptibility status of Aedes aegypti and Aedes albopictus, with particular reference to India, has been reviewed in this paper.

Materials and methods

The detection and measurement of insecticide resistance in any insect is done by standardized WHO susceptibility tests and cone bioassays,17 biochemical assays (microplate assays), synergist bioassays and molecular characterization. Searches were conducted for studies in which insecticides were tested against Aedes aegypti and Aedes albopictus and insecticide resistance was reported. Public databases were also searched, using relevant keywords. Abstracts were read and full research papers pertaining to insecticide resistance and causes of resistance in dengue vectors under review, with reference to India, were retrieved.
Insecticide-susceptibility status of dengue vectors

Results

Adult insecticide-susceptibility status

*Aedes aegypti*

The insecticide-susceptibility status of the *Aedes aegypti* mosquito is summarized in Table 1. DDT resistance in *Aedes aegypti* mosquitoes was recorded for the first time in 1967 from Jharia, Dhanbad district, now in Jharkhand state. Thereafter, in 1968, DDT resistance was reported in *Aedes aegypti* strains from various parts of India but the species was found to be susceptible to malathion. In 1970, DDT resistance was reported in *Aedes aegypti* strains from Bangalore, Bellary, Delhi, Mettupalayam, Rajahmundry, Varanasi and Vellore, but the species was found to be susceptible to all organophosphorus insecticides except malathion. Furthermore, DDT resistance in *Aedes aegypti* was reported from Maharashtra, Puducherry, Kolkata and Gujarat, but the mosquito populations were susceptible to all major groups of insecticides, including malathion and deltamethrin. Tolerance against dieldrin in *Aedes aegypti* mosquitoes was first reported in 1968 from several areas. In Goa, a high degree of resistance to dieldrin was found in *Aedes aegypti* in 1993, which was susceptible to fenitrothion and malathion. In Shahjahanpur, Uttar Pradesh, biochemical analysis of *Aedes aegypti* mosquito populations revealed that it was resistant to DDT, susceptible to deltamethrin, and exhibited some tolerance to malathion. In Belagola village, Mandya district of Karnataka state, *Aedes aegypti* was found to be resistant to DDT but was susceptible to malathion and deltamethrin. In Delhi, the susceptibility status of *Aedes aegypti* revealed resistance to DDT and dieldrin and tolerance to propoxur and fenitrothion but susceptibility to malathion, deltamethrin, permethrin and lambdacyhalothrin. *Aedes aegypti* collected from different areas of the districts of Bikaner, Jaisalmer and Jodhpur, Alwar, Ajmer and Jaipur in Rajasthan revealed more pronounced resistance to DDT and dieldrin in rural areas, as compared with those collected in urban areas. Biochemical analysis of these mosquito populations showed that resistance to DDT was due to an increase in the kinetics of glutathione S-transferase. In southern India, *Aedes aegypti* was resistant to DDT and dieldrin, but susceptible to propoxur, fenitrothion, malathion, deltamethrin, permethrin and lambdacyhalothrin. *Aedes aegypti* was found to be susceptible to lambdacyhalothrin, cyfluthrin and cypermethrin in Jodhpur city of Rajasthan. In Rajahmundry town of Andhra Pradesh, *Aedes aegypti* was highly resistant to DDT and tolerant to malathion. Recently, *Aedes aegypti* was found to be resistant to DDT but susceptible to malathion, deltamethrin and cyfluthrin in Ranchi city (Jharkhand). Similarly, both the species, *Aedes aegypti* and *Aedes albopictus*, were found to be resistant to DDT but susceptible to malathion, lambdacyhalothrin, deltamethrin, cyfluthrin and permethrin in Koderma (Jharkhand).

A review of literature on the susceptibility status revealed that resistance against DDT is widespread in adult *Aedes* mosquito populations in India. Resistance to dieldrin has also been reported. There are a few reports of tolerance and development of resistance against malathion during adult stages of *Aedes* mosquito, but, in general, the adult *Aedes aegypti* is resistant to DDT but still susceptible to malathion and synthetic pyrethroids such as deltamethrin, permethrin and lambdacyhalothrin (see Figure 1).
Table 1: Insecticide-susceptibility status of adult *Aedes aegypti* in different parts of India

<table>
<thead>
<tr>
<th>State</th>
<th>Organochlorine</th>
<th>Organophosphate</th>
<th>Pyrethroids</th>
<th>Other insecticides</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDT</td>
<td>Dieldrin</td>
<td>Malathion</td>
<td>Fenithion</td>
<td>Fenitrothion</td>
</tr>
<tr>
<td>Andhra Pradesh</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Delhi</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Goa</td>
<td>R</td>
<td>R</td>
<td>VR</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>Gujarat</td>
<td>R</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>S</td>
</tr>
<tr>
<td>Jharkhand</td>
<td>R</td>
<td>VR</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Kerala</td>
<td>R</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>S</td>
</tr>
<tr>
<td>Karnataka</td>
<td>R</td>
<td>VR</td>
<td>—</td>
<td>—</td>
<td>S</td>
</tr>
<tr>
<td>Maharashtra</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Tamil Nadu</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Uttar Pradesh</td>
<td>R</td>
<td>VR</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>West Bengal</td>
<td>R</td>
<td>S</td>
<td>—</td>
<td>—</td>
<td>S</td>
</tr>
</tbody>
</table>

R: resistant (mortality ≤80%); S: susceptible (mortality ≥98%); VR: tolerant (mortality 81–97%). Other insecticides—propoxur, parathion, dursban.
Insecticide-susceptibility status of dengue vectors

Aedes albopictus

Though there are many reports on resistance to insecticides of adult *Aedes aegypti*, there are only a few on resistance in *Aedes albopictus*; these are summarized in Table 2 and Figure 2. Samples of this species collected from different areas in Maharashtra state showed that it was resistant to DDT but was highly susceptible to malathion, deltamethrin, bromophos, propoxur and fenitrothion. Biochemical analysis of these mosquito populations showed that resistance to DDT was due to an increase in the kinetics of glutathione S-transferase. In southern India, *Aedes albopictus* was resistant to DDT and deldrin, but was susceptible to propoxur, fenitrothion, malathion, deltamethrin, permethrin and lambdacyhalothrin. In general, *Aedes albopictus* was found to be resistant to DDT but was susceptible to malathion, lambdacyhalothrin, deltamethrin, cyfluthrin and permethrin in Koderma (Jharkhand).
**Table 2: Insecticide-susceptibility status of insecticides in Aedes albopictus in India**

<table>
<thead>
<tr>
<th>State</th>
<th>Susceptibility status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Organochlorine</td>
<td>Organophosphate</td>
</tr>
<tr>
<td></td>
<td>DDT</td>
<td>Dielradin</td>
</tr>
<tr>
<td>Delhi</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>Kerala</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>Uttar Pradesh</td>
<td>R</td>
<td>—</td>
</tr>
</tbody>
</table>

R: resistant (mortality ≤80%); S: susceptible (mortality ≥98%); VR: tolerant (mortality 81–97%).

**Figure 2: Insecticide-susceptibility status of insecticides in Aedes albopictus in India**
Larval insecticide-susceptibility status

Since there is no separate report on the susceptibility of larval stages of *Aedes aegypti* and *Aedes albopictus*, the insecticide-susceptibility status of larval *Aedes* mosquitoes in general has been summarized in Table 3 and Figure 3. Though most of the insecticides, with the exception of temephos, are not directly used for larvicide against aquatic stages of *Aedes*, they still influence the susceptibility of *Aedes* mosquitoes. The immature stages of *Aedes aegypti* in Vellore were found to be slightly tolerant to DDT, but susceptible to dieldrin.31 Resistance to DDT in aquatic stages of *Aedes aegypti* was reported from Maharashtra, Puducherry, Kolkata and Gujarat but these stages were fully susceptible to other conventional insecticides.23,31,32,36 Similarly, larvae of *Aedes* spp. collected from different areas in Maharashtra state were found to be highly susceptible to malathion, deltamethrin, bromophos, propoxur and fenitrothion.29 In Goa, the immature stages were tolerant to DDT and susceptible to temephos, malathion and fenitrothion.22 In Shahjahanpur, Uttar Pradesh, immature stages of *Aedes aegypti* were resistant to DDT and susceptible to deltamethrin and exhibited some tolerance to malathion.35 In Belagola village, Mandya district of Karnataka state, the immature stages of

*Figure 3: Insecticide-susceptibility status of *Aedes* spp. larvae in India*
<table>
<thead>
<tr>
<th>State</th>
<th>Susceptibility status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Organochlorine</td>
<td>Organophosphate</td>
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<td>DDT</td>
<td>Dieldrin</td>
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<tr>
<td>Andhra Pradesh</td>
<td>—</td>
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</tr>
<tr>
<td>Delhi</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Goa</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>Jharkhand</td>
<td>R</td>
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</tr>
<tr>
<td>Kerala</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Karnataka</td>
<td>VR</td>
<td>—</td>
</tr>
<tr>
<td>Maharashtra</td>
<td>R</td>
<td>—</td>
</tr>
<tr>
<td>Puducherry</td>
<td>—</td>
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<td>Tamil Nadu</td>
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<td>Uttar Pradesh</td>
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</tr>
<tr>
<td>West Bengal</td>
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</tr>
</tbody>
</table>

R: resistant (mortality ≤ 80%); S: susceptible (mortality ≥ 98%); VR: tolerant (mortality 81–97%). *Other insecticides – propoxur, parathion, dursban.
Aedes aegypti were found to be resistant to DDT. In Delhi, the immature stages of Aedes aegypti were found to be susceptible to temephos, fenthion and malathion. In southern India, the immature stages of Aedes spp. were found to be susceptible to temephos and other organophosphorous insecticides, such as malathion and fenitrothion, commonly used under the NVBDCP.

In Jodhpur city of Rajasthan, the aquatic stages of Aedes aegypti were found to be susceptible to lambdacyhalothrin, cyfluthrin and cypermethrin. In Rajahmundry town of Andhra Pradesh, the immature stages of Aedes aegypti were found to be resistant to fenitrothion and fully susceptible to temephos, fenthion and malathion. The immature stages of Aedes aegypti showed variable degrees of resistance to DDT, malathion, carbofuran and bifenthrin in Mandya district, Karnataka. Recently, the immature stages of Aedes aegypti were found to be susceptible to temephos in Ranchi city, Jharkhand, and the immature stages both of Aedes aegypti and Aedes albopictus were found to be susceptible to larvicides such as temephos, fenthion and malathion in Koderma district, Jharkhand. The susceptibility tests on Aedes aegypti larvae collected from different geographical parts of India, namely Delhi, Mumbai, Jodhpur, Chennai and Coimbatore, revealed varying degrees of tolerance to DDT, temephos, fenthion and malathion. In the laboratory, the aquatic stages of Aedes aegypti showed induced resistance to temephos, which showed varying degrees of cross-resistance to fenthion, chlorpyrifos, malathion and DDT. The expression of temephos-induced larval resistance was also observed in adult stages.

The aquatic stages of Aedes aegypti collected from several parts of the country were found to be resistant to DDT, as shown in Figure 2. However, the Aedes mosquito is still susceptible to conventional larvicides, namely temephos, Bti, etc., which are commonly used in the national programme for larval control. The immature stages of Aedes mosquito have shown the tendency to develop induced resistance to temephos under laboratory conditions. The authors’ studies have also shown resistance to temephos in field-collected aquatic stages of Aedes aegypti taken from different localities of Delhi. Although DDT is not used as a larvicide, it is possible that the resistance to DDT in the aquatic stages may be an expression of resistance in adult mosquitoes. Though there is no report of resistance to pyrethroids in India, there are some reports of resistance in Aedes aegypti to pyrethroids from other countries.

Discussion

Resistance to insecticides in mosquitoes is of four types: (i) metabolic resistance; (ii) target-site insensitivity; (iii) resistance to penetration of insecticides; and (iv) behavioural resistance. Of these four mechanisms, metabolic and target-site insensitivity are the major mechanisms. Metabolic resistance is usually associated with increased activity of glutathione S-transferase, oxidases and esterases. Glutathione S-transferase is mainly responsible for resistance to organochlorines. Oxidases mediate resistance to all classes of insecticides, while activity of
esterases is responsible for resistance to organophosphates, carbamates and pyrethroids. Target-site insensitivity results from point mutations in one of the three target sites, namely the genes for the voltage-gated sodium channel, acetylcholinesterase and gamma-aminobutyric acid (GABA). The voltage-gated sodium channel insensitivity mechanism, also termed as kdr (knock-down resistance), mediates resistance to organochlorines and pyrethroids. The acetylcholinesterase-insensitivity mechanism mediates resistance to organophosphates and carbamates, while the GABA-insensitivity mechanism is mainly responsible for resistance to cyclodiene insecticide (organochlorine).42–44

At present, vector-borne disease-control programmes rely heavily on the use of safe and effective insecticides through indoor residual spraying or insecticide-treated nets against adult mosquitoes, and larvicides against aquatic stages. In India, indoor residual spray with DDT, malathion, pyrethroids and insecticide (pyrethroids)-treated bed nets are being used under the malaria-control programme. Temephos has been widely used as a larvicide in India and many other national dengue vector-control programmes, but resistance against temephos has not yet developed in Aedes populations in India. However, resistance to temephos in Aedes aegypti has already been reported from the Americas45–46 and Malaysia.47 This gives an indication that, in the future, resistance to temephos may also develop in dengue vectors in India. Therefore, monitoring of the susceptibility of dengue vectors against insecticides should be done on a regular basis. In addition to normal methods of control (source reduction and larvicidal activities for larval control), pyrethroids have also been used as spray for the control of Aedes aegypti under the beds, tables and other items of furniture in Queensland, Australia.48 In India, Aedes aegypti have also been reported resting on unsprayed surfaces, under the influence of excito repellency of DDT spray, and thus similar control methods have been suggested during epidemic conditions.24 To achieve the desired change in knowledge, attitude, behaviour and practices of the community, information, education and communication campaigns are also very important. Dash et al (2012)49 have pointed out that, in order to meet the growing demand for residential complexes, governments are becoming increasingly dependent on public–private partnership. These complexes have to depend on harvesting rainwater to meet the requirement for potable water. This will not only provide a high potential for the breeding of Aedes aegypti but also for the vector of urban malaria. Therefore, adoption of eco-friendly designs and technologies for varied prototypes becomes essential.

Conclusion

Most of the studies reviewed have revealed that the adult Aedes aegypti and Aedes albopictus mosquitoes are resistant to DDT but, generally, remain susceptible to malathion, temephos, propoxur and fenitrothion. The larval stages of Aedes aegypti and Aedes albopictus have also been found to be resistant to DDT but susceptible to larvicides, namely temephos, fenthion and malathion. Temephos and the bacterial insecticide Bti have very low mammalian toxicity, cause no side-effects and can be used in potable water to kill mosquito larvae. The
Insecticide-susceptibility status of dengue vectors

development of resistance in dengue vectors may adversely affect disease-containment efforts. Thus, synthetic pyrethroids, namely deltamethrin, permethrin and lambdacyhalothrin, should be used safely with precautions. In the absence of a dengue vaccine and effective antiviral drugs, there is an urgent need for strengthening the vector-control options. Under epidemic conditions, indoor residual spraying can be undertaken for rapid control of dengue vectors. A WHO-sponsored project entitled “Eco-bio-social research on dengue in Asia” concluded that vector breeding is complex and the public health response to control these vectors should go beyond larviciding/spraying of insecticides. The study emphasized the need to develop close interaction between different economic sectors and municipal authorities for successful dengue vector control.

Recently, an insect growth regulator (IGR) of proven efficacy (diflubenzuron 25% WP) has been cleared by the Technical Advisory Committee of the Government of India, and should be inducted for anti-larval measures under the NVDBCP. Health-impact assessment studies of development projects, public–private partnerships, rainwater-harvesting provisions, eco-friendly designs and supportive strategies and technologies should be emphasized to check vector proliferation and ecological imbalances. As suggested by WHO, an emergency-action committee and a rapid-action team should be established at national level to tackle the challenge of dengue and to be fully prepared for an epidemic situation, in view of the spread of the disease to rural areas where proper diagnostic and treatment facilities are not yet available.

Acknowledgements

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References

Insecticide-susceptibility status of dengue vectors


[22] Das PK, Rajagopalan PK. Susceptibility of larvae of Culex fatigans (Wiedemann), Anopheles stephensi (Liston) and Aedes aegypti (Linn) to insecticide in Pondicherry. Ind J Med Res. 1979;70:412–416.


Construction and analysis of a questionnaire regarding the dengue campaigns aired on television in Brazil: results and future directions

Rodrigo Drumond Vieira,a# Amanda Amantes,b Silvania Sousa do Nascimentoc and Virgínia Torres Schald

aFluminense Federal University, Faculty of Education, Campus Gragoatá - Rua Prof Mark Valdemar de Freitas Reis, s/n, Block D, Gragoatá, Niterói, RJ - 24210–201, Brazil

bFederal University of Bahia, Faculty of Education, Avenida Reitor Miguel Calmon, s/n, Canela, Salvador, BA - 40110–100, Brazil

cFederal University of Minas Gerais, Faculdade de Educação, Departamento de Métodos e Técnicas de Ensino, Av. Antonio Carlos, 6627, Pampulha, Belo Horizonte, MG - 31270–901, Brazil

dOswaldo Cruz Foundation, Centro de Pesquisas René Rachou, Av. Augusto de Lima – 1715, Barro Preto, Belo Horizonte, MG - 30190–002, Brazil

Abstract

This paper presents the construction and analysis of a large bank of items (573 items) for evaluation of short television films generated to stimulate dengue control and prevention, aired in Brazil during 2010 and 2011. Qualitative and quantitative analysis were conducted by reorganization of attributes, indicators and items, and statistical tests were carried out using SSPS software. From this analysis, a shorter questionnaire was constructed for an analysis of short films about dengue control. This questionnaire was administered to 684 students of different regions in Brazil who watched one short film on dengue prevention in 2012. The results show that the questionnaire was a good instrument to evaluate three attributes (“quality”, “informative capacity” and “level of comprehensiveness”). For these attributes, there was a high level of agreement on endorsement. For the attribute “change of attitudes”, there was no agreement toward endorsement. Also, identification of the change of attitudes had lowest standardized mean, suggesting that this was the least identified attribute in the film analysed. It was concluded that the film was not appropriate to strongly mobilize the participants in relation to the described attributes. Thus, this study suggests the need to reconsider the films about dengue control that are aired, in the Brazilian context.

Keywords: Brazil; Dengue film; Qualitative and quantitative assessment.

#E-mail: rodrigo_vdrumond@yahoo.com.br
Introduction

World populations, including that of Brazil, have dealt with recurrent epidemics of dengue. Dengue is one of the great public health challenges in the world, especially in tropical regions. Prevention and control of dengue is the responsibility of everyone in an affected population.

The efficiency of communication strategies to prevent and control dengue is an important issue. Dengue control demands changes in individuals’ perceptions about life in society, as well as more attention to social responsibility and circulation of information. This entails a collaborative “citizen approach” to develop an understanding that collective well-being is a way to develop personal well-being. However, the binomial “information – change of attitudes” does not feature as a direct aspect of social practices. This led to a consideration of the effectiveness and persuasiveness of the campaigns for dengue control aired in Brazil.

This research is part of a Brazilian national and multicentre project on dengue. This project is currently being conducted in the states of Alagoas, Bahia, Distrito Federal, Mato Grosso do Sul, Minas Gerais, Paraná, Pernambuco and São Paulo. These states are from different regions in Brazil and have had high and low levels (depending on the year and the seasonal variation) of dengue incidence in recent years.

According to recent epidemiological data, there was a total of 204 650 reported cases of dengue in Brazil from 1 January to 16 February 2013. The centre-west (80 976) and south-east region (80 876) had higher incidence, followed by north (18 435), south (12 420) and north-east (11 943).

Among the methods of communication used for dengue control, short television films have featured widely, since they have received large investments from public sources. However, despite the importance of mobilization of these resources, there is little objective knowledge regarding the real impact and effectiveness of these campaigns.

The importance of the persuasive role of information in forming and changing values and attitudes is recognized. Thus, it is important to evaluate the impact and effectiveness of the short films about dengue control. This paper presents a review, validation and analysis of a questionnaire for evaluation of the short TV films aired in Brazil for the campaigns against dengue in 2010, 2011 and 2012.

The multicentre project representatives mobilized 133 respondents for the short films aired in 2010 and 2011; these were professors, PhD students, postgraduate students, undergraduate students from the field of education and health (the majority of the respondents) and professionals of the local health institutions (people with high school and undergraduate degree) of various Brazilian states. The questionnaire was applied in five Brazilian cities: Belo Horizonte, Londrina, Maringá, Matozinhos and Uberaba. In view of the results from its application, a shorter questionnaire was developed and administered to 684 students from different regions in Brazil.
Materials and methods

The construction of attributes and questionnaire validation

The authors proposed a large number of items (the longer questionnaire – 573 items comprising 19 pages) and this was structured as a checklist. The construction of the items represents a synthesis of a wide national and international review of the literature.\(^5\)

The titles of the short films of the 2010–2011 governmental campaigns were: (i) mobilization; (ii) management; (iii) team of combat; and (iv) regional issues. The respondents watched all the films, and qualitative and quantitative analyses were conducted, based on their responses and on the items of the large questionnaire.

From the qualitative analysis, a categorized system was constructed to evaluate the attributes. The attributes are parameters that specify distinct qualities of the instrument, reflecting the respondents’ judgment and, consequently confirming the opinions. In view of these attributes, a series of indicators were outlined and grouped. The questionnaire was analysed based on the classification of the items according to the indicators for each attribute. This first analysis was conducted considering the instrument goals, its items and the respondents’ responses. This process included: (i) construction of the indicators; (ii) checking the respondents’ responses; (iii) re-evaluation of the categories; and (iv) restructuring of the system of categories. This system is presented in Table 1.

The qualitative analysis enabled reorganization of the attributes and indicators and was carried out using a double-check perspective, whereby two researchers constructed and validated the new system of categories. Then, quantitative analysis was developed with the support of SPSS software.

In order to verify the relevance of the instrument in relation to different aspects, the responses of the 133 respondents were used to validate the attributes and indicators.

Bearing in mind the results obtained, the following hypothesis and procedures to restructure the questionnaire were developed:

- questionnaire is too long, so respondents become too tired to respond – need to delineate a smaller number of items/questions;
- presence of random responses – need to redefine the items and reorganize them to improve reception of the questionnaire and make it more understandable;
- non-differentiation among the films regarding there convincing goals – need to restructure the films and the questionnaire itself.
**Table 1:** Attributes and descriptions of the indicators of the television films on dengue control

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Indicators</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Judgment regarding the quality of the film</td>
<td>Video and audio sources</td>
<td>Characteristics that evaluate whether the video and the audio are synchronized with the parameters considered to be good/optimum to hold the viewers’ attention</td>
</tr>
<tr>
<td></td>
<td>Commercial appeal</td>
<td>Characteristics of the film related to its commercial appeal in the sense that the viewers can identify themselves with its content</td>
</tr>
<tr>
<td>Judgment regarding the informative capacity of the film</td>
<td>Communicability</td>
<td>Characteristic related to the film capacity to convey information properly</td>
</tr>
<tr>
<td></td>
<td>Density of information</td>
<td>Characteristic related to the amount and the thoroughness of the content/information conveyed by the film</td>
</tr>
<tr>
<td></td>
<td>Intelligibility</td>
<td>Characteristic that evaluates the explicitness of the film content and whether it is understandable to the viewers</td>
</tr>
<tr>
<td>Judgment regarding the capacity of the film to stimulate change of attitudes</td>
<td>Operational practices against dengue</td>
<td>Characteristic that evaluates whether the film stimulates or models preventive and community actions against dengue</td>
</tr>
<tr>
<td></td>
<td>Content credibility</td>
<td>Characteristic related to the viewers’ confidence in the information conveyed by the film</td>
</tr>
<tr>
<td></td>
<td>Identification with the information from an emotional point of view</td>
<td>Characteristic related to the acceptability to viewers (positive or negative), of the information conveyed by the film</td>
</tr>
<tr>
<td>Level of comprehensiveness of the information conveyed by the film</td>
<td>Sociocultural aspects</td>
<td>Characteristic that evaluates the comprehensiveness of the film content in relation to the viewers’ region, language and social situation</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>Characteristic that verifies the level of comprehensiveness in relation to male and female viewers</td>
</tr>
<tr>
<td></td>
<td>Colour</td>
<td>Characteristic related to the comprehensiveness regarding white, black and mulatto people.</td>
</tr>
</tbody>
</table>
Considering that each film should pursue a definite convincing goal – the results show non-significant differentiation among the persuasive goals of the films analysed. As mentioned earlier, this may reflect the inadequacy of the films or of the questionnaire itself. This pointed to the need for restructuring the questionnaire and a new application. Thus, new analyses were carried out to clarify more about the films and their impact on the students’ responses.

**Construction of the shorter questionnaire**

The larger questionnaire was reduced to 24 dichotomous questions. Each attribute of the previous analysis was related to a set of items derived from the long questionnaire. These items comprise the new questionnaire.

The new instrument (hereinafter called “questionnaire”) presents a heading with information regarding the respondent population, namely sex, age, colour and religion. The analysed data were the responses of 684 students (9–13 years old) from different regions in Brazil (south, south-east, north-east, mid-west). A dichotomous matrix was constructed from the tabulation of responses.

The separation of the 24 questions into four attributes gave an acceptable number of items to evaluate each of these attributes. Since the number of items was different for each grouping, a standardized score was used to evaluate each attribute. In this way, the researchers worked with five score values (four scores for each of the attributes and one total score), varying from 0 to 1, and these represent the endorsement of each attribute, and of the questionnaire in general. Data were analyzed using SPSS software.

One of the short films about dengue that was aired was watched by the respondent population and they answered the questionnaire. The short film was part of the Brazilian campaign against dengue aired in 2012. A transcription is provided next.

[Alarm clock ringing – inside the clock there is the phrase: “It is always time to fight against dengue.”]

Health agent: “In Brazil there are various cities with risk of dengue epidemic, by means of SUS [Sistema Único de Saúde/Unified Health System], thousands of health agents, trained like me, are in the streets for the fight.”

Female citizen: “But it’s pointless if you do not participate.”

Male citizen: “Do your part.”

Health agent: “Every hour is the hour to fight against dengue. Take care of your home, speak with your neighbors and welcome the health agents. [at the same time, on the screen: “THE SUS IS WITH YOU IN THE FIGHT AGAINST DENGUE.”]

Health agent: “Together, we are stronger than dengue.”

Examples of the questions in the questionnaire and their respective attributes are presented below:

(1) Are the sounds of this film well-produced as in advertising?
   ( ) yes ( ) no – “QUALITY”

(2) In this film, do you identify the places where the mosquitoes grow?
   ( ) yes ( ) no – “INFORMATIVE CAPACITY”

(3) Will your daily activities change because of this film?
   ( ) yes ( ) no – “CHANGE OF ATTITUDES”

(4) Do the characters of this film have any resemblance with people you know?
   ( ) yes ( ) no – “LEVEL OF COMPREHENSIVENESS”

Data were analysed to identify which attributes were endorsed more by the respondents and how each attribute was endorsed. In addressing such issues, the film was evaluated in terms of quality, informative capacity, contribution to change of attitudes, and level of comprehensiveness of the information conveyed by the film.

**Results**

Two analyses were conducted, aiming to verify:

(1) the adequacy of the instrument to evaluate the film’s quality, informative capacity, capacity to stimulate change of attitudes, and level of comprehensiveness;

(2) the evaluation of a short film of the Brazilian Campaign against Dengue aired in 2012. This was accomplished by 684 respondents (students) from different regions in Brazil.

**Analysis 1**

The adequacy of the questionnaire to evaluate the attributes in the film was investigated by means of the distribution of the agreement percentage index (API). In this procedure, the percentage occurrence of the total and partial score values of each attribute was identified.

Table 2 shows the percentage corresponding to non-standardized score values for each attribute.

The dispersion around a central tendency is indicative of the level of agreement of the respondents. As seen in Table 2, the participants are in agreement with endorsing the attributes “quality”, “informative capacity” and “level of comprehensiveness”. For these
attributes, a higher percentage is concentrated in three or four score values, while for the attribute “change of attitudes”, the percentage of all values is distributed more equally.

These results are illustrated by the dispersion Figure 1. It can be seen from this graph that there is no agreement among the participants when they evaluate the film’s capacity to stimulate change of attitudes, since there is no peak point (it is almost a homogeneous distribution).
This can be interpreted in two ways:

1. the set of items used to evaluate this attribute is not adequate. The dispersion of the responses indicates that the participants endorse the items differently. This may be a consequence of a weak delimitation of the “change of attitudes” attribute in the questionnaire questions;

2. the attribute is not recognized in the film by the participants. This means the capacity to stimulate change of attitudes is not well perceived by the participants. This is a negative result from the point of view of the campaign.

**Analysis 2**

To verify which attribute is more endorsed, in other words, how the respondents evaluated the film they watched, the means of the standardized scores were analysed.

Table 3 shows the standardized means of the scores for each attribute.

To ensure that these means are statistically different, a paired simple test was carried out. This test was chosen because the sample is not independent (the same sample is evaluated by the data of the four attributes). This method was applied in order to test two response pairs each time. Therefore, this checked whether the means related to the attribute pairs are significantly different. For this analysis, the following hypotheses were formulated:

- H0: $\mu_1 - \mu_2 = 0$ – the difference of the means for each pair ($\mu_1$ and $\mu_2$) is zero, they are equal;
- H1: $\mu_1 - \mu_2 \neq 0$ – the difference of the means for each pair is non-zero; they are statistically different.

**Table 3: Standardized means of scores**

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Mean</th>
<th>$n$</th>
<th>Standard deviation</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity</td>
<td>0.593</td>
<td>684</td>
<td>0.274</td>
<td>0.010</td>
</tr>
<tr>
<td>Informative capacity</td>
<td>0.627</td>
<td>684</td>
<td>0.201</td>
<td>0.007</td>
</tr>
<tr>
<td>Change of attitudes</td>
<td>0.455</td>
<td>684</td>
<td>0.302</td>
<td>0.011</td>
</tr>
<tr>
<td>Level of comprehensiveness</td>
<td>0.490</td>
<td>684</td>
<td>0.210</td>
<td>0.008</td>
</tr>
</tbody>
</table>
### Table 4: Correlations of paired samples

<table>
<thead>
<tr>
<th>Standardized means correlated</th>
<th>n</th>
<th>Correlation</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pair 1  Quantity and informative capacity</td>
<td>684</td>
<td>0.476</td>
<td>0.000</td>
</tr>
<tr>
<td>Pair 2  Quantity and change of attitudes</td>
<td>684</td>
<td>0.602</td>
<td>0.000</td>
</tr>
<tr>
<td>Pair 3  Quantity and level of comprehensiveness</td>
<td>684</td>
<td>0.452</td>
<td>0.000</td>
</tr>
<tr>
<td>Pair 4  Informative capacity and change of attitudes</td>
<td>684</td>
<td>0.640</td>
<td>0.000</td>
</tr>
<tr>
<td>Pair 5  Informative capacity and level of comprehensiveness</td>
<td>684</td>
<td>0.314</td>
<td>0.000</td>
</tr>
<tr>
<td>Pair 6  Change of attitudes and level of comprehensiveness</td>
<td>684</td>
<td>0.466</td>
<td>0.000</td>
</tr>
</tbody>
</table>

### Table 5: Paired samples test

<table>
<thead>
<tr>
<th>Standardized means paired</th>
<th>Paired differences</th>
<th>95% CI of the difference</th>
<th>t</th>
<th>df</th>
<th>Significance (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>SEM</td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Pair 1  Quantity and informative capacity</td>
<td>-0.033</td>
<td>0.251</td>
<td>0.009</td>
<td>-0.052</td>
<td>-0.014</td>
</tr>
<tr>
<td>Pair 2  Quantity and change of attitudes</td>
<td>0.137</td>
<td>0.258</td>
<td>0.009</td>
<td>0.118</td>
<td>0.157</td>
</tr>
<tr>
<td>Pair 3  Quantity and level of comprehensiveness</td>
<td>0.103</td>
<td>0.259</td>
<td>0.009</td>
<td>0.083</td>
<td>0.122</td>
</tr>
<tr>
<td>Pair 4  Informative capacity and change of attitudes</td>
<td>0.171</td>
<td>0.232</td>
<td>0.008</td>
<td>0.154</td>
<td>0.189</td>
</tr>
<tr>
<td>Pair 5  Informative capacity and level of comprehensiveness</td>
<td>0.137</td>
<td>0.241</td>
<td>0.009</td>
<td>0.119</td>
<td>0.155</td>
</tr>
<tr>
<td>Pair 6  Change of attitudes and level of comprehensiveness</td>
<td>-0.034</td>
<td>0.276</td>
<td>0.010</td>
<td>-0.055</td>
<td>-0.013</td>
</tr>
</tbody>
</table>

CI: confidence interval; df: degrees of freedom; SD: standard deviation; SEM: standard error of the mean.
Table 4 shows the correlation between each variable (pair), and Table 5 shows the values obtained in the test and their statistical significance.

For the analysed sample, and with a level of significance of 5%, the null hypothesis is rejected (non-existence of significant difference between each pair of the population’s means). In other words, all the means of the standardized score of each attribute pair are statically different (\( t_1 = -3.520, 683 \text{ df}, P = 0.000 \)); (\( t_2 = 13.946, 683 \text{ df}, P = 0.000 \)); (\( t_3 = 10.420, 683 \text{ df}, P = 0.000 \)); (\( t_4 = 19.291, 683 \text{ df}, P = 0.000 \)); (\( t_5 = 14.866, 683 \text{ df}, P = 0.000 \)) and (\( t_6 = -3.248, 683 \text{ df}, P = 0.001 \)).

For all attributes, a mean for the level of agreement (or endorsement) of around 50% was obtained. The most endorsed attribute was “Informative capacity”, (mean = 0.627, standard deviation [SD] = 0.201), followed by “Quality”, (mean = 0.594, SD = 0.274), and “Level of comprehensiveness” (mean = 0.490, SD = 0.211), and, lastly the “Change of attitudes” (mean = 0.456, SD = 0.303).

This result suggests that the participants’ understanding regarding the attributes was low. They had low recognition that the film they watched had the desirable characteristics to provide a “presentation of high quality”, “sufficient information about dengue”, “stimulus for change of attitudes” and “broad identification with the situation communicated by the film”. This result points to questions that should be considered in future campaigns – for instance, the possibility of attending to all these characteristics in a short film (30 s duration).

Among the endorsed attributes, the “informative capacity” was one that was most recognized by the participants. This means they recognized that the film has important and relevant information about dengue.

The capacity to stimulate “change of attitudes” was the least-endorsed attribute. This result indicates that this attribute is difficult to access, and that the film does not provide a clear message regarding this attribute.

**Discussion**

The analysis presented indicates that the questionnaire is a good instrument to evaluate three attributes (“quality”, “informative capacity” and “level of comprehensiveness”), since the respondents were in full agreement on how to endorse it. For the attribute “change of attitudes”, however, there was no agreement toward endorsement and it was the attribute identified least by participants, in the film analysed. It can be concluded that, for this attribute, the film does not contribute to participants’ change of attitudes.

To support this conclusion, each mean was calculated, and “the change of attitudes” was the attribute with lowest standardized mean. This confirms that this attribute is not well-recognized in the film watched.
The respondents considered the attribute “informative capacity” as the most relevant, although for all these three attributes (“quality”, “informative capacity” and “level of comprehensiveness”), the endorsement was relatively low.

It is concluded that the film is not appropriate to strongly mobilize people in relation to the described attributes. The film watched definitely had a low impact on the participants. Thus, this study suggests the need to reconsider the films about dengue control that are aired, in the Brazilian context.

References


Dengue game debriefing
by health promotion students

Jeffrey L Lennon*

Liberty University, School of Health Sciences, Department of Health Professions, Lynchburg, VA 24502, United States of America

Abstract

This study explored the major themes on the topic of dengue fever by debriefing a group of university health-promotion students after they had played the board game, “Good-Bye to Dengue Game”. Written debriefing was followed by oral debriefing. The themes of game enjoyment, as well as learning about dengue, such as about signs and symptoms and mosquito-related themes, were indicated. The students indicated interest in learning more about dengue. This was the first time that a combination of written and oral debriefing was employed with this dengue game among health-promotion students.

Keywords: Debriefing; Dengue; Dengue game; Health promotion; University students.

Introduction

Various health-educational strategies have been employed to promote the control of dengue fever in communities and schools.1–4 Board games have been used as a specific health-educational strategy to address dengue fever in Venezuela,5 and the Philippines (author’s unpublished observation, University of Alabama at Birmingham, 2001).6,7 An essential part of the educational experience, especially in gaming, is the post-activity debriefing. This allows the game participants to reflect on their play activity.8 Debriefing for games has been accomplished for such topics as dengue,9 and immunity.10

Medical students11 and nursing students have utilized games in their training.12 Health-promotion students may also obtain valuable experience by utilizing games in health-promotion activity, not only by playing games, but especially through post-game debriefing. The health-educational games not only may be useful for health-promotion students to gain knowledge and insight from a game about dengue, but may also be used later in their

*E-mail: jeffchona2@yahoo.com
health-promotion efforts to combat dengue. A key part of this study’s aim is to showcase the debriefing method. It seeks to explore whether the debriefing of the game may produce not only knowledge awareness and positive affective experience, but also present what the learners want, and further need to know about the problem of dengue. An earlier study of a dengue game was performed in the Philippines among primary and secondary students (author’s unpublished observation, University of Alabama at Birmingham, 2001).6,7,9 A further study would assess the possible utility of the dengue game, and its debriefing among a different academic level in a different cultural setting. Consequently, this study seeks to demonstrate the value of a debriefing experience of a dengue game for university-level health-promotion students.

Materials and methods

Design

A descriptive study method was used, through post-experience debriefing of university-level health-promotion students after they had played a dengue-related board game, called the “Good-Bye to Dengue Game”.

The game included a 30-space game board with drawings and content, and also 32 interactive cards. The game was designed to enhance awareness in the areas of dengue control, detection and treatment. It also presented basic information about the mosquito (author’s unpublished observation, University of Alabama at Birmingham, 2001).6 Social cognitive theory was used in the game design and in its previous intervention testing.13

A previous game trial employed an experimentally controlled design that demonstrated significant increases in general dengue-related knowledge, as well as perceived self-efficacy to control dengue through source reduction (author’s unpublished observation, University of Alabama at Birmingham, 2001).6,7 A prior trial among a population of a much lower academic level had already demonstrated that playing the game led to a significant knowledge increase from pre-test to post-test. It was therefore decided that there was no need to conduct another pre-test/post-test study to find out whether playing the game would lead to an increase in knowledge levels. However, it was not known what effect the overall responses from the debriefing of this university student study population would have on positive cognitive and affective responses.

Debriefing

The debriefing included both oral and written components. Twenty health-promotion university students taking a course in infectious disease at a major university in Virginia,
United States of America, volunteered to participate in the study. This was a convenience sample. All students on the course had received a lecture on dengue fever, 2 months prior to the conduct of the study. There were 17 women and 3 men in the study. Health-promotion students in the health-education track of this university conduct practicums and community service activities, not only in-country but also in international settings. These international settings may include dengue-endemic countries.

All students signed an informed consent form. The study was approved by the Institutional Review Board for Research at Liberty University. No individual indicators were placed on any study material or debriefing form. Non-participating students had the opportunity, if they desired, to play the game at a later date.

The written debriefing used an open-ended debriefing form based upon the first seven points of the debriefing used in the immune system game study. This debriefing sequence in the current study was a modification of the debriefing form used in a previous dengue game debriefing study, as well as a modification of the debriefing sequence of Thiagarajan (1991). This type of debriefing method allows participant reflection through the following topics: recollection of the game, feelings or emotions about the game, enjoyment of the game, importance of the game’s topic, new ideas learnt, personal application, game or activity improvements. The debriefing form used in this study is presented in the Appendix.

All participants who had agreed to the study by written consent gathered in a classroom to play the “Good-bye to Dengue Game”, the same game previously tested among primary and secondary schoolchildren in the Philippines. The study involved the only formal game trial population for a comparison. The play of the game and its debriefing had already demonstrated its utility in a dengue-endemic area as a learning tool, as well as a tool to explore affective processes through reflection (author’s unpublished observation, University of Alabama at Birmingham, 2001). The current study sought to explore whether participants from another country and age group could also benefit from playing of the game, and from its debriefing. The study further sought to explore whether health-promotion students could benefit from the play and debriefing of the game.

Prior to playing the game, participants received instructions for 5 min. After this, they played the dengue game continuously in groups of five for 30 min. This was followed by 10 min of written debriefing, utilizing the debriefing form (see Appendix). The written debriefing was followed up by 5 to 10 min of oral debriefing. The debriefing questions were re-read to the students. The researcher asked the students if there were any further questions, or clarifications for any of the debriefing items.
Results

Details of responses

The following lists give the major themes generated from the responses of the participants. The themes of the responses are listed by their respective debriefing question (see Appendix).

(1) Written debriefings: “As a review in your own words, what was the game activity all about?”
- Dengue awareness
- Dengue presentation
- Prevention through removal of stagnant water
- Symptoms and treatment of dengue fever
- Learning about mosquitoes and their life-cycles

Oral debriefing follow-up:
- the game was about dengue: signs/symptoms, treatment, prevention/control, and mosquito lifestyle.

(2) Written debriefings: “How did you feel after the game?”
- Learnt more and felt more knowledgeable
- Felt great
- Felt confident about what I learned about dengue
- Like a winner
- Not good because I lost.

Oral debriefing follow-up:
- most smiled, agreed they felt alright.

(3) Written debriefings: “Did you like the game? Why or why not?”
- 19 out of 20 liked the game
- Why they liked the game:
  - fun
  - learnt new material
  - it was informative
  - it had good information
  - it was challenging
- One person did not like the game because of too many trips to the “hospital” space.
Oral debriefing follow-up:
- most indicated liking the game; no one verbalized displeasure.

(4) Written debriefings: “Did the game cover anything about the game’s topic that is important to you? If so, please explain.”
- Disease prevention
- Need to control stagnant water
- Mosquito breeding sites
- Mosquito stages
- Dengue treatment

Those that answered “no” to debriefing question number 4 already knew the material.

Oral debriefing follow-up:
- overall, the students agreed that the content was important. One student verbalized forgetting about signs, like joint pain as being important.

(5) Written debriefings: “Did you learn some new things about the game’s topic? If so, please explain.”
- Learnt about mosquito breeding sites
- Length of time mosquitoes develop
- Signs/symptoms – such as joint pain
- Where mosquitoes develop – less in dry areas
- Dengue-carrying mosquitoes do not carry malaria as well

Oral debriefing follow-up:
- it was emphasized that not all mosquitoes can transmit dengue.

(6) Written debriefings: “Are there some more things you would like to know about the game’s topic?”
- How to better avoid dengue
- Prevalence of the disease
- More signs/symptoms
- The majority responded no, but that the game was great and informative

Oral debriefing follow-up:
- participants requested more information, especially on medical aspects.
(7) Written debriefings: “Is there anything you can suggest to make the game better?”

- Add colour
- Add more cards
- Fewer “hospital” and “lose turn” spaces
- Have a range of cards, from easy to difficult
- A majority responded no (to changes), as the game was fun or “awesome”

Oral debriefing follow-up:

- participants responded they would have preferred less “going to the hospital: and that adding colour would be an improvement.

Discussion

In their response to Item 1, the health-promotion students were in general agreement with students in the Philippines study, as to the game’s focus on dengue prevention, mosquito aspects and dengue treatment. However, the health-promotion students gave a more specific response related to the mosquito life-cycle. Overall, both the students in the Philippines and the health-promotion students had positive remarks or feelings about the game. The positive effect for the health-promotion students was clearly observed during the oral follow-up time of debriefing for Item 2.

In response to Item 3, both the students in the Philippines study and the health-promotion students indicated that they liked the game because it was fun and also that they learnt from the game. Interestingly, one dissenter in each of the Philippines study and the present study, said that they disliked going to the “hospital” space. However, when observing the students playing the game, the trip to the “hospital” usually brought out the most laughter or excitement among the players, which actually added to the overall spontaneous and “fun” nature of the game.

The oral debriefing follow-up to the written debriefing allowed for clarification and consolidation of information as well as the gaming experience. However, in the follow-up for Item 4, the students discussed what they perceived as important topics. The specific sign of joint pain stood out in the response, in addition to the other responses, which mirrored the written debriefing.

Both the students in the Philippines study and the health-promotion students indicated for Item 5 that they learnt new information about breeding sites of dengue-carrying mosquitoes. Students in both studies also indicated they learnt new material about signs and symptoms. However, the health-promotion students focused on joint pain. The students in the Philippines study listed the signs and symptoms of rashes, fever and bleeding. Also, the students in the Philippines study mentioned learning new topics such as the avoidance of
aspirin and the use of tilapia, a larva-eating fish for larvae control. The health-promotion students also indicated that they learnt about the nature of the dengue-carrying mosquito (*Aedes*), and the fact that it does not carry malaria. The follow-up oral debriefing time also emphasized learning that not all mosquitoes carry dengue.

Item 6 of the debriefing form for the Philippines study asked for things the students would like to do, whereas, for the current study of health-promotion students, this item focused on additional things to know about the game’s topic of dengue. Consequently, the Philippines study students focused specifically on dengue-control measures, such as covering containers, yard clean-up and destroying mosquito breeding sites. On the other hand, the health-promotion students expressed interest in knowing more about signs and symptoms of dengue, and also about dengue prevalence. In the follow-up session, the health-promotion students did not mention dengue control by breeding-site-reduction measures, but expressed interest in learning more about medical aspects of dengue.

The major themes in Item 7 for the students in the Philippines study and the health-promotion students, which asked for suggestions on how to change or improve the game, were nearly a mirror image: add colour to the board, more cards, a range of difficulty in cards, and fewer returns to the “hospital” spaces. The majority of students in both the Philippines study and the current study suggested no change to the game. The follow-up debriefing session of the current study emphasized adding colour, and fewer returns to the “hospital” spaces, despite the fact that, when a student landed on the “hospital” space, it usually resulted in surprise and spontaneous laughter.

This study presented not only the usefulness of playing the dengue game, but also the usefulness of debriefing after playing the game. Multiple themes were identified from the findings of the debriefing. The themes produced from the debriefing could not have been identified from a closed-ended post-test questionnaire format alone. The debriefing provided a means of reflecting about the game’s dengue content. It also generated themes to stimulate further inquiry about dengue by the students, as well as themes to enhance the further design and play of the game. The debriefing of the game also has the potential to provide valuable information for dengue-control programmes or training. Students received first-hand exposure to the health-education tools of dengue game play, as well as debriefing, which they can potentially use in health-promotion activities. Finally, the health-promotion students also experienced that learning about dengue can be fun.

**Appendix: debriefing questionnaire**

1. As a review in your own words, what was the game activity all about?
2. How did you feel after the game?
3. Did you like the game? Why or why not?
Dengue game debriefing by health promotion students

(4) Did the game cover anything about the game’s topic that is important to you? If so, please explain.

(5) Did you learn some new things about the game’s topic? If so, please explain.

(6) Are there some more things you would like to know about the game’s topic?

(7) Is there anything you can suggest to make game better?

References


Dengue vector surveillance in Hong Kong during 2010–2012

KY Cheung and MY Fok*

Food and Environmental Hygiene Department, Hong Kong Special Administrative Region, People’s Republic of China

Introduction

There has been a statutory requirement to notify cases of dengue fever in China, Hong Kong Special Administrative Region (Hong Kong SAR) since 1994.¹ Between 1994 and 2001, the annual number of notifications ranged from 3 to 17 imported cases. In 2002, there were 44 confirmed cases recorded, of which 20 were locally infected. There was another local case recorded in 2003. Over the following 6 years (2004–2009), no local case was reported and the number of imported cases varied between 31 and 58. The results of vector surveillance in Hong Kong have been published up to 2009.² This note updates the surveillance data to 2012. The methodologies continued to be the same as described earlier.²,³ The vector surveillance strategy included some new initiatives for early and effective control of Aedes breeding.

In 2010, four local cases were reported and the number of imported cases also rose abruptly to 79. No local case was reported in 2011 and 2012, while the numbers of imported cases were 30 and 53 respectively (see Table 1).³

Table 1: Number of imported and indigenous dengue fever cases from 2010 to 2012, Hong Kong Special Administrative Region

<table>
<thead>
<tr>
<th>Year</th>
<th>Imported cases</th>
<th>Indigenous cases</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>2010</td>
<td>79</td>
<td>4</td>
<td>83</td>
</tr>
<tr>
<td>2011</td>
<td>30</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>2012</td>
<td>53</td>
<td>0</td>
<td>53</td>
</tr>
</tbody>
</table>

*E-mail: myfok@fehd.gov.hk
Community areas

Since 2011, six areas have been added to the surveillance programme, making the total number of survey areas 44. These additions to the survey areas aimed at expanding the existing programme by including one new area where four local cases were reported in the third quarter of 2010, and five other new areas with a high density of human habitation, such as housing estates and schools. All 44 areas were surveyed every month by six teams of paired staff, to closely monitor the situation of each area and to obtain territory-wide entomological indices collected through ovitraps.

For operational purposes, the ovitrap indices were classified into four different categories:

- level one: indices below 5.0%;
- level two: indices between 5.0% and less than 20.0%;
- level three: indices between 20.0% and less than 40.0%;
- level four: indices at 40% or above.

Different actions were taken based on the levels recorded.

From April 2011, an ovitrap rapid alert system (ORAS) has been introduced to the surveillance programme, to enhance the dissemination of survey results. ORAS involves rapid notification of ovitrap indices reaching/exceeding 20.0% (level 3 and level 4) to targeted government departments, which then alert their relevant subscribers to the results, so that immediate follow-up actions can be taken. This system can promote and better synchronize the mosquito control work carried out by the government and the targeted public groups, at times when dengue vector breeding is becoming extensive.

Port areas

A total of 30 ports, which were categorized into six groups according to their nature, were also surveyed using a methodology similar to that for community surveillance. These six groups were categorized into Hong Kong International Airport; Cross Boundary Check Points on Land (seven ports); Cross Boundary Ferry Terminals (three ports); Container Terminals (nine ports); Public Cargoes Working Area (six ports); and Private Cargoes Working Area (four ports). Twenty ovitraps were used at all ports, except for Shenzhen Bay Control Point and Hong Kong International Airport, where 50 and 766 ovitraps, respectively, were set up.
**Effectiveness of community surveillance**

The Monthly Ovitrap Indices (MOIs) of 2012 followed a similar trend to those in previous years but were generally lower (see Figure 1). The MOIs in the first quarter were maintained at a rather low level of 0.0% to 0.4%. The index then rose sharply in the second quarter, from 4.4% in April to 12.4% in May, which was also the peak MOI of the year. However, the peak index was still lower than the average of previous years (20.3% in June for 2000–2011). The MOI decreased to 11.8% and 5.7% in the following two months. It then slightly bounced back to 7.2% in August, after which the index gradually dropped to 0.3% in December.

**Figure 1: Comparison of the monthly average ovitrap index of 2012, with the average of previous years (from 2000 to 2011), Hong Kong Special Administrative Region**

In 2012, there was one average ovitrap index (AOI) that exceeded 20.0%, in April. The number of AOIs greater than 20.0% increased sharply to five in May and further to six in June. There was no AOI that exceeded 20.0% in July and only one AOI exceeded 20.0% in both August and September. Throughout the year, only one location was recorded with an AOI greater than 30.0%, which happened in May. The AOI of this location dropped slightly in June, but still exceeded 20.0%. Its AOIs were brought down to below 20.0% in the following months. There was also another location where the AOIs exceeded 20.0% for two consecutive months (April, May). Activity of aedine mosquitoes was only detected in a few survey areas in December.

The highest ovitrap index recorded in 2012 was 32.7%. None of the areas surveyed in 2012 had ovitrap indices greater than 20.0% for more than two consecutive months. In
general, areas containing vegetated slopes more often recorded high ovitrap indices (≥20.0%) during summer months, whereas in areas where such a landscape was lacking/minimal, the ovitrap indices recorded throughout the year were usually less than 20.0%.

In addition to the Geographic Information Hub (GIH; a system that allows government departments to access detailed information and results of the surveillances, including locations of positive ovitraps, via intranet), which had been in use for several years, ORAS was also introduced in April 2011. ORAS ensures that anti-mosquito measures are taken promptly when an AOI reaches the alert level of 20% (level three). The targeted groups of subscribers to ORAS include management offices of residential premises, social welfare facilities and schools that fall within the 44 areas of surveillance. Whenever an AOI reaches 20.0%, the subscribers to the system whose premises are situated within the surveillance area concerned are individually notified by the relevant departments, upon the announcement of the AOI. Subscribers are invited to post specially designed alert notices in the common parts of their premises, in order to draw the attention of occupants and staff to take prompt action for mosquito prevention and control. When the AOI drops below 20.0%, usually in the following month, the subscribers concerned will be notified by the relevant departments and they will remove the alert notices in their premises. In 2012, ORAS was activated 14 times in response to the AOIs reaching level three.

**Port surveillance**

In 2012, the Port Monthly Ovitrap Index (PMOI) ranged from 0.0% in January to March, to 0.7% in May and June. The variation in PMOIs showed a trend that was similar to previous years (see Figure 2). The ovitrap indices of all the six port groups were below 20.0%. The highest index of the port groups (7.6%) was recorded twice, in the Cross Boundary Check Points on Land in May and the Public Cargoes Working Areas in June. The ovitrap index of the Cross Boundary Check Points on Land also reached a similar level (7.1%) in June. In 2012, the average PMOI was 0.3%, which was the same as the level in 2011 and lower than that of 2010 (0.5%).

**Discussion and conclusion**

The results of the community and port surveillance indicated that *Aedes albopictus* existed in various areas in Hong Kong SAR and they had a wide distribution, particularly in summer. High ovitrap indices were recorded in some of the areas covered by the surveillance programme, indicating the presence of a considerable number of breeding grounds that needed particular attention. *Aedes aegypti*, the important vector for the transmission of dengue fever and yellow fever, was, however, not detected in all the areas covered by the community and port surveillance programmes.
The dengue vector surveillance programmes served as a tool not only to monitor the local dengue vector distribution but also to provide objective information for the community to take appropriate actions against dengue vectors. Government departments were able to quickly access detailed information and results of the surveillance, including the locations of positive ovitraps, through GIH. Targeted groups of members of the public could quickly receive information of high AOIs via ORAS and take measures to combat the mosquito problem in their premises. With the concerted efforts of the relevant government departments and the public, there were only two survey areas with AOIs exceeding 20.0% for two consecutive months in 2012.

**Figure 2:** Comparison of port monthly area ovitrap index: 2004–2011 and 2012, Hong Kong Special Administrative Region

The AOIs, MOIs and PMOIs were released to the public through press releases and the Internet, to arouse public awareness about mosquito prevention. A detailed and comprehensive advice note on mosquito prevention and control was issued together with the press release. The public was also able to access the information through the Internet. People were advised to pay particular attention to any water accumulation in and near their residences.

As in previous years, the government organized an annual territory-wide anti-mosquito campaign in 2012, to promote community participation and forge a close partnership of government departments and nongovernmental organizations to control the breeding and spread of mosquitoes.
For health education, health talks were organized for schoolchildren, managements of estates and construction sites, as well as local organizations such as area committees at the district level, to disseminate the message of mosquito prevention and control. Training was also organized for pest-control personnel in the government. Operatives of pest-control contractors providing mosquito-control services funded by the government were required to receive proper training on general pest control, as well as on specific control of mosquitoes and dengue fever.

According to the results of the dengue vector surveillance in 2012, *Aedes aegypti* was not detected and the activity of *Aedes albopictus* was, in general, under control. The MOIs were all lower than the averages of the past decade. This indicated that the vector problem had been brought under control in 2012.

Active participation of the government, local organizations and the public at large was the key to the success in controlling the dengue vector. The timely release of the results of dengue vector surveillance to all parties concerned through GIH and ORAS, and the dissemination of the results to the public by press releases and Internet, facilitated prompt remedial actions on vector control. Health education for sustaining the public participation in the prevention and control of the vector continues to be one of the key elements in the mosquito-prevention programme.

**References**


Dengue: pitfalls in diagnosis and management in resource-poor settings

Sher Bahadur Pun#

Clinical Research Unit, Sukraraj Tropical and Infectious Disease Hospital, Teku, Kathmandu, Nepal

Dengue virus (DENV) has spread far and wide and is now endemic in new areas worldwide.¹ It can cause dengue haemorrhagic fever (DHF) and sometimes dengue shock syndrome (DSS), with fatal outcome. Early detection of DENV and prompt response is critical for saving the lives of dengue patients. Available guidelines are the primary source for the diagnosis and management of dengue cases.¹,² It has been recommended that definitive diagnosis can be made on the basis of virus isolation or detection of viral RNA or immunoglobulin M/G (IgM/IgG) in paired sera.

It is, however, impractical for physicians in resource-poor settings to make a definitive diagnosis using currently available guidelines, owing to lack of highly sophisticated laboratory infrastructure. Dengue patients may enter the critical phase 5 or 6 days after illness, with defervescence.² Serological testing is a commonly used laboratory test for dengue in resource-poor settings, but it can be nearly a week from the onset of fever before it is positive, and it is broadly cross-reactive with other flaviviruses.³ This limitation compels physicians to diagnose dengue based merely on clinical manifestations.

After the incubation period, the illness is divided into three phases — febrile, critical and recovery.² Most patients in the febrile phase do not attend health-care centres because they indulge in self-medication without prescription, based on their symptoms; therefore, there is a low chance of early detection of DENV in a patient. In the febrile phase, the patient may present with fever with other symptoms such as headache, retro-orbital pain, myalgia, arthralgia and rashes.² Similar clinical manifestations can also be present in other infectious diseases, which are usually concurrently circulating in the same geographical area. Hence, most physicians will probably miss the febrile phase of dengue fever in patients. Thus, alerting physicians about the possible consequences of severe/complicated form of dengue fever (critical phase) will be missed if clinical symptoms are considered alone.

It has been well documented that the secondary dengue infection is more severe/complicated than primary dengue infection.⁴,⁵ Early identification of secondary infection can drastically reduce mortality among dengue patients, even in resource-poor settings, if health-
care providers are aware of the current geographical distribution of dengue fever. Dengue fever should be made a notifiable disease and should be made part of routine laboratory tests when a patient with fever presents from a suspected dengue area. The conceptual framework shown in Figure 1 can be useful for early detection of secondary dengue infection.

**Figure 1: Seroprevalence study in dengue-suspected areas**

<p>| | |</p>
<table>
<thead>
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<tbody>
<tr>
<td>Seroprevalence study in dengue-suspected areas</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Vector surveillance</td>
<td>Alert/update physicians</td>
</tr>
<tr>
<td>If present, consider dengue in a routine lab investigation</td>
<td>Send serum to reference lab</td>
</tr>
<tr>
<td>Educate and involve local people in dengue control or eradication programmes</td>
<td>If DENV positive, notify to EDCD</td>
</tr>
</tbody>
</table>

EDCD: Epidemiology and Disease Control Division.

Successful management of a complicated case of dengue fever case is another challenge in resource-poor settings. District hospitals are usually not equipped with advanced medical equipment, e.g. intensive care unit and supportive treatment other than fluid-replacement therapy. Bleeding is a serious complication in patients with dengue fever, and has been commonly observed in previous outbreaks.\(^3\)\(^6\) Self-medication with aspirin or non-steroidal anti-inflammatory drugs is a common practice in low- and middle-income countries, which may increase the bleeding tendency in a patient with dengue fever.

Some guidelines have provided good descriptions of the management of the critical phase of dengue fever at a district-level hospital. Unlike other diseases, however, the critical phase of dengue fever is comparatively very short (24–48 hours) and therefore referring patients from a district hospital to a central hospital may not always be feasible in reality, or may even be impossible because of poor road/transport conditions. There is, therefore, an immediate need for well-equipped hospitals and specialists at the district level, where the risk of DENV transmission is the greatest.

Although dengue has, until now, been thought to be a disease of urban areas, it is now evident that it has spread to suburban and even remote areas like the Himalayan foothills,
owing to deforestation, urbanization and climate change.\textsuperscript{3,7} Hence, there is an urgent need for clear guidelines for health-care providers in resource-poor settings on how to make prompt diagnosis of and manage dengue patients.

References


Dengue severity and blood group

James W Young, a Robert V Gibbons, b Alan L Rothman, c Darunee Tannitisupawong, b Anon Srikiatkhachorn, d Richard G Jarman, b Ananda Nisalak, b Suwich Thammapalo e and In-Kyu Yoon b #

a New York University School of Medicine, New York, New York, United States of America (USA)
b Department of Virology, Armed Forces Research Institute of Medical Sciences, 315/6 Rajvithi Road, Bangkok 10400, Thailand
c Institute for Immunology and Informatics, University of Rhode Island, Providence, Rhode Island 02903, USA
d Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA
e Bureau of Vector-Borne Disease, Department of Disease Control, Thailand Ministry of Public Health, Nonthaburi 11000, Thailand

Dengue is an emerging infectious disease that is expanding in incidence and geographical distribution. Since the 1950s, the average number of reported cases by decade has grown exponentially, and geographical extension now includes 120 countries in the Americas, Africa and Asia. 1 Identifying and understanding the risk factors for dengue severity is important for understanding the pathogenesis of the disease. For example, the association of increased dengue severity with secondary dengue virus (DENV) infection, and primary infection in infants with maternal antibody has led to the implication of antibody-dependent enhancement in the pathogenesis of severe dengue disease. However, no single factor can explain which patients progress to more severe disease, and further risk factors and risk profiles are actively being studied. 2

In 2007, Kalayanarooj et al. presented evidence of a relationship between type AB blood and an increased risk of World Health Organization (WHO) grade III dengue haemorrhagic fever (DHF), as compared to either dengue fever (DF) or less severe DHF in patients with secondary infection. 3 The measured and expected numbers of events for grade III DHF were small, but the odds ratio was 10 and the significance of the test was strong ($P < 0.0001$). 3 Older studies failed to find any significant relationship between ABO blood group and severity of dengue disease; 4, 5 however, these earlier studies did not distinguish between primary and secondary infection, did not examine differences between different grades of DHF, and did

#E-mail: Yooni@afrims.org
not have as large a sample. All of these studies enrolled patients that presented to hospitals, and did not include asymptomatic or subclinical infections in their samples.

The present study analysed the association between dengue severity and ABO blood group among primary schoolchildren, in a prospective cohort study conducted in Kamphaeng Phet, Thailand, from 2003 to 2007, which included subjects with clinically inapparent, as well as symptomatic, infections. Inapparent infection was defined as a DENV infection identified by a fourfold rise in haemagglutination inhibition (HAI) antibody against any DENV serotype, between two sequential sera obtained before and after an active surveillance period, without detecting an acute DENV infection during the surveillance period.

Chi-squared tests were used to determine whether there were relationships between ABO blood types and dengue severity. Two-by-two contingency tables were used for each blood group, to compare inapparent infections (266 cases) to symptomatic infections (176 cases), and non-hospitalized infections (404 cases) to hospitalized infections (38 cases). There was no significant relationship between any ABO blood group and these categories of disease severity. Risks between varying grades of DHF and between DHF and non-DHF were not analysed, owing to the low incidence of DHF in the cohort (8 cases total). Table 1 stratifies the measures of dengue severity by ABO blood group.

**Table 1:** Frequency of dengue infections by blood group and disease severity

<table>
<thead>
<tr>
<th>Disease severity</th>
<th>Blood group, n (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O</td>
<td>A</td>
</tr>
<tr>
<td>Inapparent infection</td>
<td>104 a(65.0)</td>
<td>61 (62.9)</td>
</tr>
<tr>
<td>Dengue fever</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-hospitalized</td>
<td>52 a,b (32.5)</td>
<td>27 (27.8)</td>
</tr>
<tr>
<td>Hospitalized</td>
<td>10 a,b (0.6)</td>
<td>8 (8.2)</td>
</tr>
<tr>
<td>Dengue haemorrhagic fever</td>
<td>3 a (1.9%)</td>
<td>1 (1.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>169</td>
<td>97</td>
</tr>
</tbody>
</table>

Note: Percentages given in parentheses are the percentage of each blood group associated with the severity category.

* Blood group O in inapparent infection versus symptomatic infection: \( \chi^2 = 0.210; \ P = 0.646. \)
* Blood group O in hospitalized versus non-hospitalized dengue fever patients: \( \chi^2 = 0.285; \ P = 0.593. \)
* Blood group AB in inapparent infection versus symptomatic infection: \( \chi^2 = 0.101; \ P = 0.751. \)
* Blood group AB in hospitalized versus non-hospitalized dengue fever patients: \( \chi^2 = 0.011; \ P = 0.916. \)

The current findings are not inconsistent with the findings of Kalayanarooj et al., which identified a relationship only between ABO blood group and DHF grade III, i.e. cases with evidence of shock. The present analysis adds to the evidence that blood group may be a risk factor for dengue shock syndrome (DSS) only.
Pathologic mechanism(s) that may explain a link between ABO blood group and DSS have not yet been identified. To the authors’ knowledge, there is no evidence of DENV infection in red blood cells in vivo. A larger number of severe DENV infections with blood group data is needed to explore the relationship between ABO blood group and risk of DSS, which may have both significance in understanding disease pathogenesis and utility as a prognostic factor.

Acknowledgments

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References


Concomitant infection of dengue virus serotypes and malaria in a sickle cell disease patient: a case-study

PV Barde, JK Jatav, PK Bharti, S Godbole and Neeru Singha

Regional Medical Research Centre for Tribals (RMRCT), Indian Council of Medical Research, Nagpur Road, Garah, Jabalpur, Madhya Pradesh, India

Netaji Subash Chandra Bose Medical College, Nagpur Road, Jabalpur, Madhya Pradesh, India

Case-report

Two of the most important vector-borne diseases (VBDs), malaria and dengue, contribute significantly to the overall disease burden globally. The World Health Organization (WHO) estimates that there are between 1.1 and 2.7 million deaths due to malaria worldwide annually, with 300–500 million suspected cases.1 It is estimated that 100 million cases of dengue fever (DF) occur annually, with 500 000 cases requiring hospitalization, resulting in a case-fatality rate as high as 2.5%.2 India is known to be endemic for both these VBDs and the state of Madhya Pradesh (MP) is among the top five malarious states in the country.1 This note reports a case of concurrent infection with dengue virus (DENV) serotypes 1 and 4 and Plasmodium vivax malaria in a patient with sickle cell disease.

A 30-year-old man belonging to the medical fraternity reported to the outpatient department of Netaji Subash Chandra Medical College, Jabalpur, MP (coordinates: 23°10’N 79°56’E), with complaints of recurrent fever (101–103 °F) for more than two days, headache and body-ache, with associated symptoms such as fatigue, nausea and occasional rigors. During clinical examination, he revealed that he had been diagnosed in the past for sickle cell disease (Hb-SS homozygous) using cellulose acetate haemoglobin electrophoresis,3 and had autosplenectomy and right femoral head avascular necrosis. Foreseeing the possible complications, he was admitted to the intensive care unit of the hospital. His blood levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total serum bilirubin (see Table 1) were increased, and a blood smear for malaria parasite on day one was found to be negative; consequently, the sample was sent to the virology laboratory of the Regional Medical Research Centre for Tribals, Jabalpur, MP, for DENV diagnosis. As the sample was drawn in the acute phase of illness, it was subjected to nested reverse transcriptase polymerase chain reaction (nRT-PCR), as described by Lanciotti et al.,4 with a few modifications.5 The results of the tests revealed that the patient was infected with two DENV serotypes, DENV-1 and DENV-4 (see Figure 1). In addition, the PCR products were sequenced using Big Dye

E-mail: neeru.singh@gmail.com
Table 1: Patient parameters, Jabalpur, MP, India

<table>
<thead>
<tr>
<th>No</th>
<th>Parameter</th>
<th>Readings</th>
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<tbody>
<tr>
<td>1</td>
<td>Fever</td>
<td>102 °F</td>
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<tr>
<td>2</td>
<td>Headache</td>
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</tr>
<tr>
<td>3</td>
<td>Body pain</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>Rash</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>Nausea</td>
<td>Yes</td>
</tr>
<tr>
<td>6</td>
<td>Chills/rigors</td>
<td>Mild/recurrent</td>
</tr>
<tr>
<td>7</td>
<td>Pulse (/min)</td>
<td>98</td>
</tr>
<tr>
<td>8</td>
<td>Blood pressure (mmHg)</td>
<td>120/70</td>
</tr>
<tr>
<td>9</td>
<td>Haemoglobin</td>
<td>11.6</td>
</tr>
<tr>
<td>10</td>
<td>Total leukocyte count (/1 μL)</td>
<td>10 500</td>
</tr>
<tr>
<td>11</td>
<td>Hepatomegaly</td>
<td>No</td>
</tr>
<tr>
<td>12</td>
<td>Splenomegaly</td>
<td>Autosplenectomy</td>
</tr>
<tr>
<td>13</td>
<td>Serum albumin (g%)</td>
<td>4.5</td>
</tr>
<tr>
<td>14</td>
<td>Serum ALT (U/L; normal up to 40 U/L)</td>
<td>52</td>
</tr>
<tr>
<td>15</td>
<td>Serum AST (U/L; normal up to 30 U/L)</td>
<td>134</td>
</tr>
<tr>
<td>16</td>
<td>Serum bilirubin (normal &lt;1 mg%)</td>
<td>2.5</td>
</tr>
<tr>
<td>17</td>
<td>Platelet counts (million/1 μL)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Figure 1: Gel picture showing RT-PCR and nested PCR products of the DENV in a patient in Jabalpur, MP, India.
Terminator Cycle Sequencing kit (Applied Biosystems, CA, United States of America) and ABI 3130 XL genetic analyser. The homologies of the sequences were analysed using the Basic Local Alignment Search Tool (BLAST). The DENV-1 sequence was submitted to Gen Bank (Gen Bank accession no. JQ894501). Dengue immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) on this sample gave a negative result. Since fever with rigors continued, the patient was again tested for malaria parasite on day two of admission and the blood smear was found to be positive for Plasmodium vivax. The patient was given antimalarial therapy, according to national programme guidelines, and symptomatic treatment for DENV was initiated along with antibiotic (intravenous ceftriaxone) and antipyretic. The patient was also administered hydroxyurea 500 mg twice a day, and folic acid for management of sickle cell disease. He started showing signs of recovery from day four and was discharged from the hospital on day six. His subsequent follow-up visit to the hospital showed no clinical complications of DENV or malaria.

**Discussion**

Multiple DENV serotype infection, or coinfection with other pathogens, may lead to severe disease. With the presence of both pathogens in this area (DENV and P. vivax) and an abundance of vectors (Aedes aegypti and Anopheles culicifacies/Anopheles fluviatilis), it is quite possible that DENV with malaria can concomitantly infect an individual. The clinical characteristics of dengue and malaria are very similar, and because of overlapping symptoms and possible masking of typical periodic fever of malaria by viral fever, it would be rather difficult to identify such dual infection, making the clinician’s job difficult. In this case, the patient’s blood smear was negative for malaria parasite on day one and his sample was tested for DENV; had he tested positive for malaria parasite, his DENV infection would probably have gone undetected.

This typical case of dual DENV infection along with P. vivax demonstrates co-circulation of multiple serotypes of dengue with malaria in central India.

Despite similar symptoms, the treatments for dengue and malaria are very different, which presents a challenge for treating physicians.

Dual infection with DENV has been reported from other parts of the world as well as India. Simultaneous infections with DENV and other parasites are also reported. However, to the authors’ knowledge, this is the first case-report of concomitant infection of two DENV serotypes (DENV-1 and DENV-4) with P. vivax malaria in a patient with sickle cell disease. The mixed infection of DENV and malaria generally results in severe clinical complications, but this particular patient had comparatively fewer clinical complications, probably because he received prompt and proper treatment. It is known that secondary infection by a heterologous serotype poses a risk for dengue haemorrhagic fever and/or dengue shock syndrome. A few studies have also explained the clinical aspects of dual infections with DENV. It will be worthwhile to study the cytokine, immunological and pathological responses in such
Concomitant infection of dengue and malaria in a sickle cell disease patient

types of cases, which will assist clinicians in patient management in the future. This case-study demonstrates the importance of accurate and timely diagnosis and its significance in treatment.

Acknowledgment

The authors are grateful to the Secretary to Government of India, Department of Health Research, Ministry of Health and Family Welfare, and the Director-General, Indian Council of Medical Research (ICMR), for financial support under ICMR’s Virology Diagnostic Laboratory Network Project, and the Directorate of National Vector Borne Disease Control Programme, New Delhi, for providing kits for diagnosis. The authors also thank Lieutenant General, Dr D Raghunath, for his critical review and suggestions on the manuscript.

References


Guidelines for testing the efficacy of insecticide products used in aircraft*

World Health Organization Departments of Control of Neglected Tropical Diseases and Global Capacity, Alert and Response
(WHO/HTM/NTD/WHOPES/2012.1)

Overview
The purpose of these guidelines is to provide specific, standardized procedures and criteria for testing the efficacy of products designed specifically for killing insects (referred to in this document as “disinsection”) in aircraft; and to assist countries in adopting health-control measures under the International Health Regulations (2005).

The guidelines are intended for use as a companion to other specific WHO technical guidance documents on avoiding the spread of disease vectors through air travel. Their aim is to harmonize the testing procedures used in different laboratories and institutions, in order to generate comparable data for registering and labelling such products by national regulatory authorities. Nevertheless, the requirements for registration of pesticides are determined by the national regulatory authorities.

Methods currently recommended by the World Health Organization for aircraft disinsection
Three methods are currently recommended by the World Health Organization (WHO) for aircraft disinsection: “blocks away”; preflight and “top-of-descent” spraying; and residual treatment. In practice, this involves four techniques.

“Blocks away”
Spraying is carried out by crew members when the passengers are on board, after closure of the cabin door and before the flight takes off. An aerosol containing an insecticide with rapid action is used. The air-conditioning system should be switched off during cabin spraying. The flight deck is sprayed before the pilot boards (when no passengers are on board). The doors of overhead luggage racks should be closed only after spraying has been completed. An aerosol containing 2% d-phenothrin is currently recommended by WHO and should be applied at a rate of 35 g of formulation per 100 m³ (i.e. 0.7 g aerosol index (ai)/100 m³). Cargo holds should also be disinfected.

*http://whqlibdoc.who.int/publications/2012/9789241503235_eng.pdf
Preflight spraying

A preflight aerosol containing an insecticide with rapid action and limited residual action is applied by ground staff to the flight deck; passenger cabin, including toilet areas; open overhead and side-wall lockers; coat lockers; and crew rest areas. The spray is applied before the passengers board the aircraft but not more than 1 h before the doors are closed. A 2% permethrin cis:trans (25:75) formulation is currently recommended for this application, at a target dose of 0.7 g ai/100 m³. This requires application at 35 g of formulation per 100 m³ to various types of aircraft, with a droplet size of 10–15 μm. Preflight spraying is followed by a further in-flight spray, i.e. top-of-descent, as the aircraft starts its descent to the arrival airport.

“Top-of-descent” spraying

Top-of-descent spraying is carried out as the aircraft starts its descent to the arrival airport. An aerosol containing 2% o-phenothrin is currently recommended by WHO for this purpose and is applied with the air recirculation system set at from high to normal flow. The amounts applied are based on a standard spray rate of 1 g/s and 35 g of the formulation per 100 m³ (i.e. 0.7 g ai/100 m³).

Residual treatment

The internal surfaces of the passenger cabin and cargo hold, excluding food preparation areas, are sprayed with a compression sprayer that has a constant flow valve and flat fan nozzle, according to WHO specifications. Permethrin 25:75 (cis:trans) emulsifiable concentrate is currently recommended by WHO, at a target dose of 0.2 g/m² applied at intervals not exceeding 2 months. The emulsion is applied at 10 mL/m², to avoid run-off. Residual sprays are applied by professional pest-control operators and are intended for long-term residual activity on aircraft interior surfaces. In electrically sensitive areas, it may be necessary to use an aerosol instead of a compression sprayer. After treatment is completed, air-conditioning packs should be run for at least 1 h before the crew and passengers embark, to clear the air of the volatile components of the spray. Areas that undergo substantial cleaning between treatments require supplementary “touch-up” spraying.

The pesticide formulations, including spray cans, should comply with national regulations and international standards, as well as with WHO specifications for pesticides. Spray operations should follow international regulations and WHO-recommended procedures, and comply with quarantine requirements in the country of arrival.

Reference

Managing regional public goods for health:
community-based dengue vector control*

*This monograph was prepared on behalf of the Asian Development Bank and the World Health Organization, Manila, Philippines.

Approximately 2.5 billion people are at risk of contracting dengue in the more than 100 tropical and subtropical countries where the *Aedes aegypti* mosquito is found. More than 70% of this population, or 1.8 billion people, live in countries in Asia and the Pacific. Most of these countries have developing or relatively weak economies, and may lack the resources required for addressing the continued emergence of dengue epidemics. Globally, between 50 and 100 million cases of dengue fever occur annually. This includes more than 500,000 cases of severe dengue (previously known as dengue haemorrhagic fever), hundreds of thousands of hospitalizations, and more than 20,000 deaths, mainly among children and young adults. Dengue fever and severe dengue place significant burdens on families, communities, health systems, and economic growth. These burdens are especially acute during epidemics, which result in illness and death, loss of productivity, strains on health-care services, and unplanned government expenditures for implementing large-scale emergency control actions.

Unfortunately, efforts for prevention and control of dengue are proving less than successful in reducing the global spread and negative impacts of the disease. Control programmes tend to mainly comprise emergency responses to epidemics, leaving limited resources and capacity for sustained action. Thus, without sufficient budgetary support and intensified efforts at both the national and community level, maintaining and expanding dengue-control activities will prove difficult. Similarly, innovative and more effective measures for controlling dengue are needed to form a toolkit of vector-control interventions that can be applied across a wide variety of ecological and epidemiological settings. This is particularly true in the face of global climate change, extreme weather events, and associated human responses to these and other environmental changes, all of which could facilitate further spread of the disease.

This report describes a promising, low-cost, year-round vector-control measure that is feasible to implement, is acceptable and safe to the public, and, once established, has minimal recurring costs. Cambodia and Lao People’s Democratic Republic participated in an intervention research project using integrated vector management, to determine whether households would accept the use of guppy fish in their large water storage jars, tanks, and drums, to control mosquito larvae and pupae; and whether development of effective guppy-distribution programmes was feasible. The project also assessed whether household members...
could be motivated through community action and/or school-based programmes to eliminate other, smaller, breeding sites on their property. The project teams used the Communication for Behavioral Impact planning tool of the World Health Organization, in developing the framework for delivering the interventions to selected villages. While both Cambodia and the Lao People’s Democratic Republic incorporated household, community, and school-based approaches, the implementation of each approach was specific to the setting.

The project resulted in a decline in the number of mosquito larvae present in three key water containers (jars, cement tanks and drums). Prior to project implementation, almost 40% of the containers in the Cambodian households had mosquito larvae; by the end of the intervention, less than 3% contained larvae. Similar results were obtained in the Lao People’s Democratic Republic. Further, the project resulted in successful establishment of guppy breeding and distribution systems at the national, provincial, and local levels, in both countries, and generated multisector collaboration between ministries, nonprofit groups, schools, and health centres. In Cambodia, 88% of the water containers contained guppies at the end of the study, while in the Lao People’s Democratic Republic, 76% of the containers had guppies.

The project results indicate that the pilot interventions were effective and successful in mobilizing communities to establish and maintain the guppy fish intervention, and in obtaining high levels of community acceptance of the fish in drinking water containers. Scale-up of the low-cost intervention is recommended in both countries.
Informal expert consultation on yellow fever threat to India and other SEA Region countries: Report of the Consultation, Goa, India, 23–25 March 2011*

*World Health Organization, Regional Office for South East Asia (SEA-CD-235)*

**Recommendations**

The recommendations of the groups were deliberated upon and the following final recommendations of the consultation were made.

**Member States**

1. Member States should review their existing infrastructure for implementation of International Health Regulations (IHR) and strengthen the organizations/agencies responsible for vector surveillance and control at their international airports/seaports, for effective implementation of IHR round the clock.

2. Member States should develop contingency plans for rapid assessment and emergency response in the event of the occurrence of yellow fever, including case verification, containment, control and treatment of cases.

3. Member States should establish at least one national-level yellow fever reference laboratory for diagnosis and quality control of provincial/state-level laboratories.

4. Member States should modify their immigration procedures and forms so that all passengers arriving from yellow-fever-endemic countries are routed to a health desk for confirmation of their vaccination status before an immigration check is done.

*http://apps.searo.who.int/pds_docs/B4810.pdf*
World Health Organization

(1) The World Health Organization (WHO) should encourage Member States to give priority to capacity-building of organizations at airports/seaports, for effective implementation of IHR.

(2) WHO should facilitate the creation of a regional reference laboratory for yellow fever in the South-East Asia Region and capacity-strengthening for production of yellow fever vaccine in the Region.

(3) WHO should promote action aimed at immunization of the entire population of all endemic countries at risk of yellow fever transmission, in order to reduce the chances of its spread to populations living in non-endemic countries.
Instructions for contributors

_Dengue Bulletin_ welcomes all original research papers, short notes, review articles, letters to the Editor and book reviews which have a direct or indirect bearing on dengue fever/dengue haemorrhagic fever prevention and control, including case management. Papers should not contain any political statement or reference.

Manuscripts should be typewritten in English in double space on one side of white A4-size paper, with a margin of at least one inch on either side of the text and should not exceed 15 pages. The title should be as short as possible. The name of the author(s) should appear after the title, followed by the name of the institution and complete address. The e-mail address of the corresponding author should also be included and indicated accordingly.

References to published works should be listed on a separate page at the end of the paper. References to periodicals should include the following elements: name and initials of author(s); title of paper or book in its original language; complete name of the journal, publishing house or institution concerned; and volume and issue number, relevant pages and date of publication, and place of publication (city and country). References should appear in the text in the same numerical order (Arabic numbers in parentheses) as at the end of the article. For example:


Figures and tables (Arabic numerals), with appropriate captions and titles, should be included on separate pages, numbered consecutively, and included at the end of the text with instructions as to where they belong. Abbreviations should be avoided or explained at the first mention. Graphs or figures should be clearly drawn and properly labelled, preferably using MS Excel, and all data clearly identified.
Instructions for contributors

Articles should include a self-explanatory abstract at the beginning of the paper of not more than 300 words explaining the need/gap in knowledge and stating very briefly the area and period of study. The outcome of the research should be complete, concise and focused, conveying the conclusions in totality. Appropriate keywords and a running title should also be provided.

Articles submitted for publication should be accompanied by a statement that they have not already been published, and, if accepted for publication in the Bulletin, will not be submitted for publication elsewhere without the agreement of WHO, and that the right of republication in any form is reserved by the WHO Regional Offices for South-East Asia and the Western Pacific.

One hard copy of the manuscript with original and clear figures/tables and a computer diskette/CD-ROM indicating the name of the software should be submitted to:

The Editor
Dengue Bulletin
WHO Regional Office for South-East Asia
Indraprastha Estate
Mahatma Gandhi Road
New Delhi 110002, India
Telephone: +91–11–23370804
Fax: +91–11–23379507, 23370972
E-mail: dengue@searo.who.int

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The WHO Regional Office for South-East Asia, in collaboration with the Western Pacific Region, jointly publish the annual *Dengue Bulletin*.

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