From the Editor's Desk

The endemicity of dengue fever and dengue haemorrhagic fever (DHF) in countries of the South-East Asia (SEA) and the Western Pacific regions continues to rise. During 2007, in the SEA Region, Indonesia, Thailand and Myanmar contributed 62.89%, 25.14% and 6.10% dengue cases respectively, both in morbidity and mortality. When compared with 2006 and previous years, the number of cases in all three countries showed an increasing trend.

Nepal which reported 25 cases for the first time in 2006 reported only three cases in 2007. DENV-2 was found to be circulating in Nepal.

In view of this scenario, WHO developed the biregional Asia-Pacific Strategic Plan (2008–2015) for Prevention and Control of Dengue in 2007. The thrust of this plan will be to strengthen systems in countries to predict and prevent epidemics, improve early recognition and management of cases, support prevention of dengue through integrated vector management and on community participation and research.

The current Volume 31 (2007) of Dengue Bulletin includes contributions from WHO's SEA Region (5); the Western Pacific Region (8); and the American Region (4).

We now invite contributions for Volume 32 (2008). The deadline for receipt of contributions is 30 November 2008. Contributors are requested to please follow the instructions carefully while preparing their manuscripts. Contributions accompanied by CD-ROMs using MS Word for Windows should be sent 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 e-mail as a file attachment to the Editor at dengue@searo.who.int and dengue@whosea.org. Readers desirous of obtaining copies of Dengue Bulletin may contact the WHO Regional Offices in New Delhi, India or Manila, Philippines or the WHO Country Representative in their country of residence.

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The quality and scientific stature of the *Dengue Bulletin* is largely due to the conscientious efforts of the experts and also due to the positive response of contributors to comments and suggestions.
Epidemic situation of dengue fever in Guangdong province, China, 1990-2005

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Abstract

During the period 1990 to 2005, a total of 11 844 cases of dengue fever (DF), with 3 deaths, were reported in Guangdong province. The average attack rate was 1.27/100 000 pop. The disease affected 17 out of 21 cities in the province. DF occurred throughout the year and the epidemic phase extended from July to December. DENV-1, that affected all age groups, appeared to be the predominant circulating virus, although DENV-2, -3 and -4 were also involved. \textit{Aedes albopictus} was the only vector species responsible for the DF outbreaks.

The results of molecular epidemiological studies showed that dengue fever epidemics in Guangdong province were initiated by imported cases from South-east Asia. Control measures comprised of insecticidal fogging and elimination of man-made breeding places of \textit{Ae. albopictus}.

Keywords: Dengue fever; Epidemiological surveillance; Guangdong; China.

Introduction

Dengue fever (DF), an acute febrile viral disease characterized by sudden onset of fever for 2–7 days (sometimes biphasic), intense headache, myalgia, arthralgia, retro-orbital pain, anorexia, nausea, vomiting and rash, is one of the most common and widespread vector-borne arboviral infections in the world. The viruses of dengue fever belong to the family \textit{Flaviridae} and include four serotypes (DENV-1, -2, -3, -4), all of which can classically cause undifferentiated fever, dengue fever and its severe form, dengue haemorrhagic fever (DHF). The global prevalence of dengue has increased dramatically in recent decades. As per WHO estimates, DF/DHF is now prevalent in over 100 countries, posing a threat to more than 2.5 billion people in the tropics and subtropics.\cite{1,2}

In China, in the 1980s, \textit{Aedes aegypti} was reported in Hainan island and Leizhou peninsula, and caused an epidemic affecting one million people. However, \textit{Aedes albopictus}, another common species of mosquito, is known to be the principal vector of DF in Guangdong province since 1990.\cite{3-5} Guangdong
has been a major province in China affected by dengue fever outbreaks in addition to Fujian, Zhejiang and Jiangsu provinces since 1990.\cite{6}

Guangdong province had a population of 85 million (in 2000).\cite{7} It is located in southern China in the subtropical zone, which is suitable for mosquito breeding. The first confirmed outbreak of dengue in the province was reported in 1978.\cite{8-9} Since then, dengue fever has become a major public health concern. The present study focuses on the epidemiological features and molecular characterization of dengue viruses during 1990–2005.

**Materials and methods**

**Data sources**

In China, DF is a notifiable disease. Hence, all health care facilities like hospitals and clinics in the country are required to report clinical and confirmed cases of dengue to the Centers for Disease Control and Prevention (CDC) system: first to the county CDC, which then reports to the provincial CDC. Once these notifications are received, the CDC performs laboratory verification, then conducts further epidemiological investigation, and searches for other dengue cases or clusters.

Based on mandatory notification, we collected and analysed all notified cases of dengue in Guangdong province from 1990 to 2005. However, it is required by law to classify these notifications of dengue into different clinical manifestations {DF, DHF, dengue shock syndrome (DSS)}. Both clinical cases and confirmed cases are reported.\cite{10}

**Case definitions**

A clinical case of dengue is defined as follows:

- Clinical symptoms, such as abrupt onset of fever, severe headache, myalgia, arthralgia, nausea, vomiting, rash, etc.
- When only sporadic cases are found or no local cases have been reported: clinical manifestations of dengue (DF, DHF or DSS) with positive IgG or IgM in a single serum specimen.
- When local cases have been reported: clinical manifestations of dengue (DF, DHF or DSS) with decreased white cell count (<4×10^9/l) and lower thrombocytopenia count (<100×10^9/l).
- The isolation of DENV, by cell culture and virus antigen preparation from culture supernatants of DENV-1, DENV-2, DENV-3 and DENV-4-infected C6/36 cells.\cite{11} The culture supernatants were used as the source of E/M and NS1 antigens for ELISA. The control antigen was prepared by the same procedure from vero cells culture without viral infection.
- Positive test of real-time one-step RT-PCR: The nucleotide sequence of DENV-1 E/NS1 gene segment was isolated. Viral RNA was extracted for use in one-step reverse transcriptase polymerase chain reaction (RT-PCR). Following amplification by RT-PCR, the partial nucleotide fragments of the E/NS1 gene junction were then cloned into the plasmid pblluescript II SK for sequencing. The following primers were used for sequencing:

  Forward primer: 5’–GTCGAGCTCGGATCACAAGGAG–3’

  Reverse primer: 5’–TGATGGTACCGAGACGAGTGGCTGA–3’

  The sequence results were analysed using the DNASTAR software.
• Positive seroconversion or four-fold increase in dengue-specific IgM or IgG antibody from appropriately timed paired serum (with acute-phase sera collected during day 1–7 after the onset of symptoms, and early and late convalescent sera collected during day 8–13 and day 14–30, respectively).
• High-titre dengue-specific IgM and IgG antibody in a single serum specimen where cross-reaction to Japanese encephalitis (JE) had been excluded.

**Vector surveillance**

In Guangdong, surveillance of *Aedes albopictus* was carried out throughout the year, particularly in areas reporting active cases. The investigations included search for *Aedes* breeding places and determining the entomological indices, viz. Breteau index (BI) (number of positive containers for *Aedes* per 100 houses) and Container index (CI) (percentage of containers positive for *Aedes* breeding) until two weeks after the last local case was reported.

**Results**

**Epidemiological information**

During the period of 16 years (1990–2005), a total of 11,844 cases of dengue fever and 3 deaths were reported in Guangdong province (Table). The notification rates ranged from 0.003 to 9.75 per 100,000 population per year. The epidemic peak appeared in 1995. The male-to-female ratio was 1:1.02. All age groups were involved, although most of them (72.07%) were aged 15 to 50 (Figures 1, 2).

An analysis by occupation indicated that out of the 11,470 reported cases, 2,842 were workers, 3,482 farmers, 1,552 students, 749 office clerks, 711 unemployed and household persons and 294 were children. It is noteworthy that medical staff accounted for 0.92% of the cases (106 cases).

**Seasonal distribution**

The majority of the dengue cases (93%) were notified from August to October, although cases were reported throughout the years (primarily

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**Figure 1: Epidemic status of dengue fever in Guangdong, 1990–2005**

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<tbody>
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<td>Cases</td>
<td>374</td>
<td>371</td>
<td>2</td>
<td>359</td>
<td>4</td>
<td>6812</td>
<td>2</td>
<td>632</td>
<td>488</td>
<td>304</td>
<td>401</td>
<td>365</td>
<td>1576</td>
<td>82</td>
<td>49</td>
<td>23</td>
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</table>

Number of cases
**Table: Epidemiological data for dengue fever in Guangdong province, 1990-2005***

<table>
<thead>
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<th>Time</th>
<th>Cases</th>
<th>Incidence (1/100 000)</th>
<th>Outbreak place (number of cases, serotype)</th>
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<tr>
<td>1990</td>
<td>374</td>
<td>0.6</td>
<td>Guangzhou (372, DENV-1, 4)</td>
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<tr>
<td>1991</td>
<td>371</td>
<td>0.57</td>
<td>Guangzhou (258, DENV-1)</td>
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<td>Zhongshan (112, DENV-1)</td>
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<tr>
<td>1992</td>
<td>2</td>
<td>0.003</td>
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<tr>
<td>1993</td>
<td>359</td>
<td>0.53</td>
<td>Foshan (352, DENV-2)</td>
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<tr>
<td>1994</td>
<td>4</td>
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<tr>
<td>1995</td>
<td>6812</td>
<td>9.75</td>
<td>Guangzhou (5337, DENV-1)</td>
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<td>Zhaoqing (983, DENV-1)</td>
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<td></td>
<td>Chaozhou (349, DENV-1)</td>
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<td>1996</td>
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<td>632</td>
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<td>488</td>
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<td>23</td>
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*Only three deaths were reported in 1991 with mortality rate of 0.005.*
imported cases) (Figure 3). Dengue outbreaks occurred from June to November, with peaks during August to October.

**Geographical distribution**

Approximately 63% of cases were reported from Guangzhou (the Capital city), 10% from Chaozhou, 8% from Zhaoqing, and 7% from Foshan. The outbreaks occurred in all these years with the exception of 1992, 1994, 1996 and 2005. Although multiple outbreaks occurred in Guangzhou, Zhongshan, Foshan, Chaozhou and Jieyang, there was no place where DF outbreaks occurred over two consecutive years (Figure 4).

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**Figure 2:** *Age composition of dengue cases in Guangdong province, 1990-2005*

![Figure 2](image)

**Figure 3:** *The seasonal dynamics of DF and Aedes albopictus in Guangzhou province*

![Figure 3](image)
Dengue virus

In Guangdong, all four serotypes, DENV-1–4, caused the dengue outbreaks. With the exception of 1990 and 1993, DENV-1 appeared to be the predominant circulating virus. In addition, DENV-2 and DENV-4 were isolated from patients in Foshan, Zhongshan and Zhuhai city. Many of the index cases of these outbreaks were imported from outside China. For example, index cases were imported from China, Macao Special Administrative Region (SAR), Singapore and Thailand during 2003. All reported cases in 2005 were imported from South-east Asia.

The phylogenic tree of the sequenced 14 strains of DENV-1

The phylogenic tree of the sequenced 14 strains of DENV-1 isolated between 1990 and 2005 has been mapped and it branches into two genotypic groups (Figure 5). The nucleotide sequences showed maximum homology of 99.2% with Indonesian strains, 100% with strains from the Philippines, and 98.8% with strains from Thailand.

Surveillance of vector

*Aedes albopictus* is a highly urbanized species and, in the Guangdong province, it breeds in water stored in man-made containers and natural habitats. The seasonal distribution of *Aedes albopictus* is correlated with a Breteau index which, in turn, is affected by temperature and rain. In Guangdong, the density of *Aedes albopictus* peaked from May to October, and was very low from November to February. [4]
Discussion

We have provided the epidemiological information of dengue spanning over a period of 16 years from 1990 to 2005 in Guangdong province. Our data sources are based on mandatory notification, which have the intrinsic inadequacies such as incomplete reporting, or false positive results due to cross reaction of dengue viruses with Japanese encephalitis vaccine viruses. In addition, dengue notifications in China are not classified by clinical manifestations (DF, DHF or DSS), a majority of them are known to be presented as classical dengue fever. For example, a total of 758 cases of dengue were reported in 1995, of which 12 (1.58%) were DHF. Out of the 978 hospitalized dengue cases in an infectious disease hospital of Guangzhou in 2002, only two were DHF.

A secular trend of increased reporting of dengue outbreak was observed. The developing economy and rapid urbanization within Guangdong have contributed towards increased opportunities for the development and transmission of DF in the province, especially in the Pearl delta region. Risk factors for DF epidemic included: (i) the changing lifestyle of people; for example, more people now grow bonsai and raise fish in tanks near their homes; (ii) more construction sites with poor sanitation conditions; (iii) frequent travel to and from South-east Asia resulting in importation of viruses, especially the Chaozhou and Shantou; (iv) delayed detection and treatment of active disease due to lack of knowledge of DF among primary medical staff; and (v) high densities of vector mosquitoes. The vector control mainly covered adult Aedes mosquitoes control, which remains largely an ineffective intervention.
The evidences from epidemiological investigation and molecular epidemiology supported the hypothesis that the identified dengue cases were imported from other countries into Guangdong. The circulating virus strains in an area were usually different in different years, and different virus serotypes prevailed in different areas in one year. From 1979 to 1999, the circulating DENV-1 strain belonged to two geno-subtypes. It is likely that most of these strains were imported from neighbouring countries such as the Philippines, Indonesia and Thailand, where indigenous dengue epidemics have been confirmed. The results of nucleotide sequencing showed that DENV-1 was closely related to viruses identified in these regions. High mosquito density and favourable natural conditions (such as optimal temperature and rainfall) are responsible for localized outbreaks, once dengue viruses are imported into the province.\textsuperscript{[13]}

For building up an early response to outbreaks, it is necessary to improve diagnostic capacity and strengthen training and reporting awareness of local physicians, particularly in early detection, diagnosis, notification and isolating patients. Secondly, it is important to establish a vector monitoring system to understand population dynamics of Aedes albopictus. Thirdly, comprehensive measures of prevention and control including environmental solid waste disposal and chemical, biological and ecological control should be developed. Lastly, community health education needs to be strengthened to improve awareness of the dengue virus transmission through Aedes albopictus.

From our experience, source reduction and use of non-chemical methods supported by intersectoral and community participation are the most potential methods for control of dengue during interepidemic periods. The Breteau index is the most important indicator to predict impending dengue outbreaks in this province. Maintaining lower BI levels (below 5) is a very effective measure to prevent and control dengue outbreaks. Further studies are required to determine the BI cut-off value for mosquito control to prevent DF outbreaks.

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References


A clinical, epidemiological and virological study of a dengue fever outbreak in Guangzhou, China – 2002-2006

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Abstract

We analysed the clinical and epidemiological characteristics of dengue fever (DF) during the dengue virus (DENV)-1 outbreak in Guangzhou, China. Clinical and epidemiological data of 1342 patients with DF from May 2002 to November 2006 were analyzed retrospectively. The average age was 34.7±13.2 years. The ratio of male to female was 1.05:1. The peak time of the epidemic lasted from August to October. The most common manifestations included fever (100%), headache (85.9%), myalgia (64.5%), bone soreness (46.6%), fatigue (78.2%), skin rash (65.9%) and positive tourniquet test (51.3%). Leukopenia, thrombocytopenia, elevated alanine aminotransferase (ALT), elevated aspartate aminotransferase (AST) and hypopotassemia were found in 66%, 61.3%, 69%, 85.7% and 28.4% of the patients respectively. Only 2(0.15%) patients fulfilled the WHO case definition criteria for dengue haemorrhagic fever (DHF), the others were all diagnosed as classic DF. However, 64(4.8%) patients had severe clinical manifestations (internal haemorrhage, shock, marked thrombocytopenia, myocarditis, sepsis, pneumonia and encephalopathy). Anti-dengue IgM was detected in 90% of patients. Dengue viruses were isolated using the \textit{Aedes albopictus} C6/36 cell line and identified as DENV-1 by RT-PCR. A 346bp fragment from RT-PCR product of every isolate was sequenced to compare with published sequences of other DENV-1 viruses. The nucleotide homology were 97%, 97% and 98% compared with those of DENV-1 strains of dengue fever outbreak in Cambodia, in 1997 and 1999 in China, respectively. In conclusion, the epidemic of dengue fever was caused by DENV-1 infection from 2002 to 2006 in Guangzhou. Patients with severe clinical manifestations are few, but in some of them, the diagnosis of DHF may be missed if the WHO classification is strictly applied, especially in adults.

Keywords: Dengue fever; DENV-1, Severe dengue; Epidemiological and clinical characteristics.

Introduction

Dengue fever (DF) is an acute febrile viral disease frequently presenting with headaches, bone or joint and muscular pains, rash and leukopenia. DF has become one of the most important emerging public health problems in the tropics and sub-tropics. In the past 20 years, there has been a dramatic increase in the number of cases and the severity of illness in countries in South-East Asia, the Caribbean and Latin America.\textsuperscript{1,2} An estimated 50-100 million
people across the globe contract dengue annually, with about 500,000 persons developing the more severe dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) and resulting in about 21,000 deaths.\[3\]

WHO classifies symptomatic dengue virus infection into three categories: undifferentiated fever, classic dengue fever, and DHF.\[3\] According to WHO criteria, DHF is defined by the presence of fever, a haemorrhagic tendency, thrombocytopenia, and some evidence of plasma leakage. DHF is further subdivided, with most severe cases categorized as DSS, when circulatory failure is present. The WHO classification system has been widely applied in research settings and publications. In recent years, several studies reported difficulties with classification, especially in adults with many severe cases being missed: more than two thirds of all adults with severe dengue manifestations were not classified as having DHF.\[4-7\]

The first dengue case in Guangdong was reported in 1978. Infections with all four dengue virus serotypes (DENV-1, 2, 3, and 4) were reported in Guangdong.\[8\] The first dengue outbreak was caused by DENV-3 in 1980. Since then, increasing larger outbreaks have been documented throughout the 1980s and the 1990s, caused by DENV-1, DENV-2, and DENV-4. In this paper, we present a retrospective analysis of 1342 patients to analyse the epidemiological, clinical, laboratory and virological presentations of the dengue infection from 2002 to 2006 in Guangzhou, China.

Materials and methods

Patients and investigative methods

The study was conducted from May 2002 to November 2006 at the Guangzhou No. 8 People’s Hospital. A total of 1360 patients were initially enrolled in this study. Eighteen patients which were imported cases in 2004-2005 were excluded. Thus, 1342 patients (978 in 2002, 54 in 2003, and 310 in 2006) were analysed in Guangzhou where dengue was endemic.

In all patients, a detailed history was taken and clinical examination was done at the time of admission and subsequently during the stay at the hospital. The hospital records for all patients were supported by serological and/or virological confirmation. Serology was performed by indirect ELISA, and/or immunochromatographic test (ICT), and dot immunobinding assay (DIBA) for specific IgM.

Virus isolation

We collected 85 blood samples within five days after onset (36 samples were obtained in 2002, 18 samples in 2003, and 41 samples in 2006). Virus was isolated by *Aedes albopictus* mosquito C6/36 cell micromethod. The patient blood serum was diluted with RPMI 1640 containing 2% FBS to an appropriate concentration and inoculated into the C6/36 cells. The C6/36 cells were incubated at 28 °C, 5% CO\(_2\) for 21 days. Cultures were monitored for cytopathic effect (CPE) daily under the inverted phase contrast microscope. The inoculated cells were passaged once a week. Those with CPE were judged as positive for dengue virus, while those without CPE after passaging three times were declared negative.\[9\]

RT-PCR, DNA purification and sequencing

Viral RNA was extracted from the culture supernatants of infected C6/36 with an improved sodium iodide method. Nucleotides encoding a fragment of the NS2 gene were amplified using RT-PCR. The amplification parameter was as follows: 93° 40s, 55° 45s,
72° 60s, thirty cycles. The products of RT-PCR were then analysed by 1.5% agarose (including ethidium bromide) electrophoresis. The universal primer sequences were P 5’-GACATGGGTATTGGAT-3’, P 5’-TCCATCCATACCCAGCA-3’, which result in an amplification product of 413bp. DENV-1-type specific primer sequences were D1S 5’-GTTGTTCCGCAGACTA-3’, D1C 5’-CATGGTATGTTGGTTT-3’, and the expected size of the amplification product was 346bp. The DENV and DENV-1, 2, 3, 4-type specific primers were provided by Institute of Military Medicine in Joint-Service Department of Guangzhou Military Region. The bands of predicted size were cut and purified using a Golden Bead Product Purification Kit (Songon, China) according to the manufacturers’ instructions. Purified PCR products were cloned on to pMD18-T Vector (TaKaRa, Japan) and delivered to TaKaRa Biotechnology Co. Ltd. for sequencing.[10]

**Sequences analysis**

Sequence homology searches within the GenBank gene database were performed using NCBI BLASTn. In addition, CLUSTALX and TREEVIEW software were used to analyse sequence homology, and constructed its system cladogram. The DENV-1 international and domestic reference strains are as follows: Singapore strain(S275/90), Cambodia strain (Cambodia), West Pacific Ocean strain (WestPac), Thai strain (TH-Sman), Peruvian strain (Peru), Nauru strain (Nauru), Hawaian standard strain (Hawaii), and Guangdong 1995 strain (GD23/95), 1997 strain (GD14/97), 1999 epidemic strain (GD05/99).

**Statistics analyses**

All data were analysed using the SPSS10.0 software package.

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**Result**

### Patients’ characteristics and geographical and time distribution of dengue outbreak

During the study period, we observed a total of 1342 patients (687 male and 655 female, male/female ratio = 1.05:1). The involvement of all age groups, especially an adult predominance, was observed. The mean age of the patients was 34.4±13.1 years and most of them belonged to the 20~29-year age group; less than 15% patients were children (Figure 1).

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**Figure 1: Age groups distribution in 1342 patients with dengue fever**

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**Geographical and time distribution of dengue outbreak**

Dengue fever cases were observed to be distributed in all 12 districts of Guangzhou. There were 872(65%) patients in the predominant outbreak spots where the number of DF cases was above 10.

The peak time of the epidemics was from August to October each year. The number of cases in September reached its maximum, with 587 hospitalized cases, which constituted 43.7% of the total cases.
Clinical and laboratory feature

Of the 1342 DF patients observed from the onset of acute fever, a majority of the cases had the typical signs and symptoms of DF, that included fever (100%), headache (85.9%), myalgia (64.5%), bone soreness (46.6%), fatigue (78.2%), skin rash (65.9%), lymphadenectasis (15.1%), splenohepatomegalia (8.4%), petechia (24.6%), positive tourniquet test (51.3%), internal haemorrhage (3.7%) and pleural effusion (0.22%), as shown in Figure 2.

According to the WHO criteria, the diagnosis of DHF requires the presence of all 4 manifestations, i.e. fever, platelet count less than 100 000 cells/mm³, haemorrhagic tendency, and evidence of capillary leakage (i.e. haematocrit increase for more than 20% from baseline, pleural effusion, ascites, or hypoproteinemia); the additional presence of hypotension or narrow pulse pressure along with clinical signs of shock designates DSS. On the basis of strict WHO criteria, only 2 (0.15%) patients had DHF. However, we found that 64 (4.8%) cases had severe clinical manifestations, including 50 (3.7%) cases who had internal haemorrhage consisting of gastrointestinal tract bleeding (n=34) and/or hypermenorrhea or ecchymosis, as well as rare complications such as myocarditis (n=9), encephalopathy (n=5), shock (n=12), sepsis (n=9), and pneumonia (n=4). Four of the 12 patients with shock did not manifest bleeding signs.

As shown in the Table, most patients had either a low WBC or platelet count during the acute phase of illness. 224 (16.7%) patients had marked thrombocytopenia with a platelet count <50 000 cells/mm, the lowest platelet count was 4500 cells/mm in this case series. Among

Figure 2: Clinical presentation in 1342 patients with dengue fever
those patients tested, over two thirds of them had increased liver enzyme levels, 10.7% and 11.8% of the patients had a 5-fold increase in ALT and AST levels respectively. Hypopotasemia were found in 276 (28.4%) patients.

**Diagnosis of dengue virus infection and identification of DENV-isolated strains and sequence analysis**

In the serological analysis, 1208 (90%) patients were positive for IgM antibody while 467 (34.8%) were positive for anti-dengue IgG.

Forty-four virus isolations (51.7%) were made from the blood samples of 85 cases which were inoculated in C6/36 cells. RT-PCR amplification from the supernatants of the infected C6/36 cells samples was performed using universal primers, which

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**Table: Laboratory parameters in 1342 patients with dengue fever**

<table>
<thead>
<tr>
<th>Item</th>
<th>Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White blood cell count</td>
<td></td>
</tr>
<tr>
<td>&lt;×10^9/L</td>
<td>886 (66.0)</td>
</tr>
<tr>
<td>4–10×10^9/L</td>
<td>376 (28.0)</td>
</tr>
<tr>
<td>&gt;×10^9/L</td>
<td>80 (6.0)</td>
</tr>
<tr>
<td>Blood platelets count</td>
<td></td>
</tr>
<tr>
<td>&lt;100×10^9/L</td>
<td>828 (61.3)</td>
</tr>
<tr>
<td>&lt;50×10^9/L</td>
<td>224 (16.7)</td>
</tr>
<tr>
<td>ALT elevation</td>
<td></td>
</tr>
<tr>
<td>40–200 U/L</td>
<td>782 (89.3)</td>
</tr>
<tr>
<td>&gt;200 U/L</td>
<td>144 (10.7)</td>
</tr>
<tr>
<td>AST elevation</td>
<td></td>
</tr>
<tr>
<td>40–200 U/L</td>
<td>992 (88.2)</td>
</tr>
<tr>
<td>&gt;200 U/L</td>
<td>158 (11.8)</td>
</tr>
<tr>
<td>CK elevation</td>
<td>404 (30.1)</td>
</tr>
<tr>
<td>LDH elevation</td>
<td>611 (45.5)</td>
</tr>
<tr>
<td>Hypopotassiumemia (&lt;3.6 umol/L)</td>
<td>276 (28.4)</td>
</tr>
<tr>
<td>Total bilirubin elevation</td>
<td>72 (5.4)</td>
</tr>
<tr>
<td>BUN elevation (&gt;7.1 umol/L)</td>
<td>15 (1.1)</td>
</tr>
</tbody>
</table>

---

**Figure 3: Agarose gel analysis of the cDNA products from RT-PCR samples isolated from the supernatants of the infected C6/36 cells. The expected size of the amplification product was 346bp with DENV-1 type specific primers.**

Lane 1: Sample 1
Lane 2: DL 2000 marker (GeneRuler)
Lane 3: Sample 2
Lane 4: Sample 3
Lane 5: Sample 4
yielded the 413bp fragment, which was then used for DENV identification and sequencing. All the results were found to be confirmed DENV-1 positive by DENV-1-specific primer and amplified fragment was 346bp located on DENV-1 gene group’s NS2a–NS2b site, see Figure 3. The results confirmed that all dengue viruses belonged to the DENV-1 virus. The nucleotide homology of Guangdong epidemic strain in 2003 were 97%–97% and 98% when compared with that of DENV-1 strain of dengue fever outbreak in Cambodia, in 1997 and 1999 in Guangdong respectively (see Figure 4).

Figure 4: Sequence comparison of four DENV-1 isolated strains from Guangdong and Cambodia

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>GD29/2003</td>
<td>GTTGTTCCGCAGACTAACATCCAGAGAAGTTCTTCTTCAAACATGGGATTGAGTC :56</td>
</tr>
<tr>
<td>GD14/97</td>
<td>.................................................T................................................. :56</td>
</tr>
<tr>
<td>GD05/99</td>
<td>..................................................C................................................. :56</td>
</tr>
<tr>
<td>Cambodia</td>
<td>..................................................a................................................. :56</td>
</tr>
<tr>
<td>GD29/2003</td>
<td>TAGTGCCATCTGGAAGTTACAAATCCTGTGGAGGAGCTGGGGGATGGACTTGCA :112</td>
</tr>
<tr>
<td>GD14/97</td>
<td>................................................................................C............................. :112</td>
</tr>
<tr>
<td>GD05/99</td>
<td>................................................................................C............................. :112</td>
</tr>
<tr>
<td>Cambodia</td>
<td>................................................................................C............................. :112</td>
</tr>
<tr>
<td>GD29/2003</td>
<td>ATGGCATCATGATTTTAAAATTATTGACTGACTTTCAATCACATTAGTTGTGGGC :168</td>
</tr>
<tr>
<td>GD14/97</td>
<td>..................................................................G............C.............. :168</td>
</tr>
<tr>
<td>GD05/99</td>
<td>................................................................................C............................. :168</td>
</tr>
<tr>
<td>Cambodia</td>
<td>................................................................................C............................. :168</td>
</tr>
<tr>
<td>GD29/2003</td>
<td>TACCTTGCTGTCCTTGACATTTATCAAAACAACGTTTTCCTTGCACTATG :224</td>
</tr>
<tr>
<td>GD14/97</td>
<td>.................................................................................................A.......................... :224</td>
</tr>
<tr>
<td>GD05/99</td>
<td>.................................................................................................A.......................... :224</td>
</tr>
<tr>
<td>Cambodia</td>
<td>.................................................................................................A.......................... :224</td>
</tr>
<tr>
<td>GD29/2003</td>
<td>AGACCAAGCTATGGTACTGTAATCTCTCTCCCTATGCTGCTCCAAC :280</td>
</tr>
<tr>
<td>GD14/97</td>
<td>..................................................................................G................................. :280</td>
</tr>
<tr>
<td>GD05/99</td>
<td>..................................................................................G................................. :280</td>
</tr>
<tr>
<td>Cambodia</td>
<td>..................................................................................G................................. :280</td>
</tr>
<tr>
<td>GD29/2003</td>
<td>ACTAACCATG :346</td>
</tr>
<tr>
<td>GD14/97</td>
<td>................................................. :346</td>
</tr>
<tr>
<td>GD05/99</td>
<td>................................................. :346</td>
</tr>
<tr>
<td>Cambodia</td>
<td>................................................. :346</td>
</tr>
</tbody>
</table>
Discussion

From May 2002 to November 2006, Guangzhou experienced a comparatively large-scale dengue fever outbreak. The case specimens collected at different times over four years were analysed by virus isolation, RT-PCR gene identification and sequencing, confirming that this epidemic was caused by DENV-1. The nucleotide homology of the Guangdong epidemic strain in 2003 were 97%, 97%, and 98% compared with those of DENV-1 strain of dengue fever outbreak in Cambodia, in 1997 and 1999 in China, respectively, and these strains belonged to the identical gene type. The initial cases were imported during the epidemic period and subsequently disseminated locally. Although four serotypes had been involved in several episodes of the comparatively large-scale DENV-1 outbreaks since 1995,[7,8] DENV-1 was the most predominant serotype in Guangdong.

DF, as a mosquito-borne infection, would be expected to spread rapidly in populous areas, so the presence of a large proportion of patients in downtown is consistent with the experience elsewhere of dengue infection as an urban phenomenon.[11,12] In our study, 65% of 1342 cases presented in the main outbreak spots in downtown Guangzhou. This DENV-1 epidemic occurred in the rainy seasons from August to October when the abundant rainfall and the temperature favoured high rates of mosquito’s reproduction. Rainfall is considered to be a risk factor for dengue outbreak, especially in urban districts with poor sanitary environment, providing optimal breeding sites for mosquitoes.[13] The main mosquito vector for dengue viruses is Ae. albopictus in Guangdong, which breeds primarily in relatively clean, stagnant domestic water containers during the rainy season.

Our data showed that there was no obvious difference in the patients’ gender distribution. All age groups were affected, but over 80% of them were adults. Children aged 0~10 years comprised only 8.2% of the sample.

In our study, the patients showed most of the typical clinical features of dengue fever, with only 2(0.15%) patients developing DHF according to the WHO criteria. The incidence of DHF was much lower in this study, compared with the reported 2%~6% in populations in the areas where dengue is endemic.[14,15] In an in-vitro experiment, DENV-1 was isolated from samples of DF patients in 2002. DENV-1 is not as virulent as the other types of DENV and DENV-1 infection seems to have mild clinical presentations.[16,17]

In the present study, a few unusual clinical observations and complications such as fulminant hepatitis, myocarditis, encephalopathy, and ophthalmic disease were observed.[18-23] We found that 64(4.8%) of cases had severe clinical manifestations including internal haemorrhage like gastrointestinal tract bleeding and/or hypermenorrhea or ecchymosis, as well as rare complications such as myocarditis, encephalopathy, shock, sepsis, and pneumonia. 16.7% of the patients had marked thrombocytopenia with a platelet count <50 000 cells/mm. More than 10% of the patients had a 5-fold increased liver enzymes. Our result is consistent with other reports.[12,18]

In recent years, the validity of the WHO classification scheme to define severe dengue disease has been found to be inconsistent. Several studies have found that the WHO classification is difficult to apply, with many severe adult cases being missed.[14-4d] Because, according to the WHO criteria, haemorrhagic manifestations without capillary leakage do not constitute DHF, the DSS refers to a condition in which shock is present in addition to all four DHF-defining conditions. The WHO classification is mainly based on studies of children in South-east Asia in the 1960s and
may therefore not be applicable to predominantly adult subjects. Shock and plasma leakage appear to be more common in children, whereas internal haemorrhage is a more frequent manifestation in adults. In addition, the DHF/DSS classification excludes severe dengue disease associated with "unusual manifestations". Therefore, a new definition for severe dengue is urgently needed. A large multicentre study should be performed to establish a more robust dengue classification scheme for use by clinicians, epidemiologists and scientists involved in dengue pathogenesis research.

In brief, the DF epidemic in Guangzhou from 2002 to 2006 was caused by DENV-1. Most cases had the typical clinical manifestation of dengue fever and were diagnosed as typical DF. But in some patients, the diagnosis of DHF may be missed if the WHO classification is strictly applied, especially in adults. A new definition for severe dengue may be needed.

Acknowledgements

We are grateful to the CDC of Guangzhou and the CDC of Guangdong province for their continuing epidemiological survey and virological laboratory support. This work was partially supported by grants from the Foundation of Guangzhou Municipal Science and Technology Bureau. We thank Alfredo Garzino-Demo for critically reading this manuscript.

References


Circulation of dengue serotypes in five provinces of northern Thailand during 2002-2006

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Abstract

Dengue haemorrhagic fever is an epidemic infectious diseases caused by dengue virus. It is a major disease prevalent in all provinces of Thailand. This study was to determine the circulating dengue serotypes by reverse transcription polymerase chain reaction (RT-PCR). A total of 1116 seropositive acute samples were analysed from DF/DHF patients in five provinces of northern Thailand (Chiangmai, Lampang, Lamphun, Mae Hong Son and Phrae) during the period January 2002 to December 2006. Five hundred and fifty-nine samples were found positive, of which 47.2%, 30.6%, 18.4% and 3.8% were affected with DENV-2, DENV-1, DENV-4 and DENV-3 respectively. From 2002 to 2005, the predominant dengue serotype was DENV-2, whereas DENV-1 was predominant in 2006. There was an apparent increase in the percentage of DENV-4 from 2005 to 2006. Our results indicated that all four dengue serotypes were circulating in this region and the annual change of predominant serotypes was the cause of the severity of the disease.

Keywords: Dengue haemorrhagic fever; Dengue serotype; Northern Thailand.

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Introduction

Dengue is a mosquito-borne viral infection caused by four distinct dengue virus serotypes DENV-1–4. It is the most prevalent arbovirus in tropical and subtropical regions of Africa, the Americas, the Eastern Mediterranean, the Western Pacific and South-East Asia.[1,2] In Thailand, the first dengue outbreak occurred in Bangkok in 1958, initially in a pattern with a 2-year cycle, and subsequently in irregular cycles, as the disease spread throughout the country. The largest outbreak was reported in 1987, with an incidence rate of 325 cases/100 000 population. In recent years, increasingly larger dengue outbreaks have occurred. There were 99 410, 127 189 and 114 800 cases of dengue reported to the Bureau of Epidemiology in 1997, 1998 and 2002 respectively.[3] In 1999, the Ministry of Public Health initiated a dengue control programme to reduce the incidence rate to less than 50 cases/100 000 population.[4] In northern Thailand, many provinces are located approximately 250–300 metres above the sea level, with some urban areas and large rural areas. There were 13 915, 11 092, 6147, 6992 and 6914 dengue cases reported during the five-year period 2002–2006, with incidence rates of 119.4, 91.5, 51.3, 58.9 and 58.1 cases/
Circulation of dengue serotypes in Thailand

100,000 population respectively,\(^5\text{–}^8\) with 120 deaths.

Gubler et al.\(^9\) and Lam et al.\(^10\) reported that virological surveillance, which involves monitoring of dengue virus infection in humans, has been used as an early warning system to predict outbreaks. Such surveillance, based on the isolation and identification of dengue viruses infecting the human population, provides an important means of early detection of any change in the prevalence of dengue virus serotypes. Each dengue serotype has characteristics that affect the nature of dengue epidemic and disease severity. Nisalak et al.\(^11\) reported that the predominant dengue serotype in the outbreaks in Bangkok during 1997–1998 was DENV-3. Anantapreecha et al.\(^12\) detected the predominant serotypes DENV-1 and DENV-2 in six provinces across Thailand during 2001–2002. However, dengue serotypes in five provinces of northern Thailand have not yet been well elucidated, thus the present study, which is aimed at clarifying the pattern of circulating dengue serotypes in this region with a view to better understand the epidemiological complexities of the epidemics of dengue infection.

Materials and methods

Patients

A total of 1116 seropositive acute samples with positive anti-dengue IgM antibody were analysed for dengue serotype by RT-PCR at the Regional Medical Sciences Centre, Chiangmai (RMSc_CM), Thailand. These samples were collected from DF/DHF patients in five northern provinces that included Chiangmai, Lamphun, Lampang, Mae Hong Son and Phrae during 2002–2006 (Figure 1).

Figure 1: Map of five provinces of northern Thailand

RNA extraction

Viral RNA was isolated by using QIAamp viral RNA Mini Kit (QUIAGEN, Cat. no. 52904). Briefly, the serum (100 μl) was added and mixed with 400 μl of AVL/RNA carrier solution (lysis buffer). After incubation at room temperature for 10 min, 400 μl of absolute ethanol was added to the solution. All the solutions were then transferred to a spin column and were spun at 8000 rpm for 1 min. The RNA was then washed by adding 500 μl of AW1 (washing buffer 1) and spun at 8000 rpm for 1 min and following the same procedure with AW2 (washing buffer 2). Finally, the RNA was eluted by adding 60 μl of elution buffer and spun at 8000 rpm for 1 min. The eluted RNA was kept in –70 °C until use.

RT-PCR

RT-PCR method was performed as previously described by Yenchitsomanus et al.\(^13\) Briefly,
5 μl of RNA solution was mixed with reagents of one step RT-PCR kit (QIAGEN, Cat. no. 210212) and specific oligonucleotide primers of dengue-envelope (E) gene; DUL and DUR. The cDNA synthesis was performed at 50 °C for 30 min in a thermal cycler (MJ research PTC-100, USA) followed by 40 cycles of PCR step including 94 °C for 5 min, 94 °C for 1 min, 45 °C for 1 min and 72 °C (5 min for the last cycle). 1 μl of the primary PCR product was used as the template for the second PCR with four serotype-specific primer pairs; D1L, D1R, D2L, D2R, D3L, D3R, D4L and D4R (Table 1). The PCR step was the same as above with the annealing temperature set at 62 °C. Negative and positive dengue controls were used. The secondary PCR products were analysed in 2% agarose gel electrophoresis and then visualized by ethidium bromide staining.

### Results and discussion

The number of seropositive acute cases and dengue serotypes in the five provinces during 2002-2006 are shown in Table 2. Five hundred and fifty-nine dengue viral cases were detected with an average positivity rate of 50.0% by RT-PCR. All the four dengue serotypes were detected during this study. The total numbers of positive dengue cases were analysed. DENV-2 was the predominant serotype (47.2% cases), followed by DENV-1 (30.6% cases), DENV-4 (18.4% cases) and DENV-3 (3.8% cases).

The circulation of dengue serotypes in northern Thailand by year during 2002-2006 is shown in Figure 2. From 2002 to 2005, the predominant serotype was DENV-2 as 46.0%, 59.7%, 70.3% and 44.8% of cases were of this serotype, respectively, followed by DENV-1 (42.0%, 32.3% and 25.0% cases) from 2002 to 2004 respectively and DENV-4 (26.6% cases) in 2005. In 2006, the predominant serotype had changed to DENV-1 (42.8% cases), followed by DENV-4 (33.1% cases). DENV-3 was found to be the least circulating serotype during 2003 to 2006 and not found at all in 2002.

The distribution of the predominant dengue serotypes by provinces were analysed when more than five positive dengue virus samples were detected. In Chiangmai province, the predominant serotypes were DENV-1 (48.7% and 56.9% cases) in 2002 and 2006, and DENV-2 (54.8%, 71.4% and 52.0% cases) in 2003, 2004 and 2005 respectively. In Lampang province, the predominant serotypes were DENV-2 (100.0%, 62.5%, 79.3% and 40.0% cases) from 2002 to 2005 respectively and DENV-1 (72.0% cases) in 2006. In Lamphun province, the predominant serotypes were DENV-2 (66.7% and 50.0% cases) in 2005 and 2006. In Mae Hong Son province, the predominant serotypes were DENV-2 (75.1% cases) in 2005 and DENV-1 (80.0% cases) in 2006. In Phrae province, the predominant serotype was DENV-2 (75.1% cases) in 2005 and DENV-1 (80.0% cases) in 2006. The comparison of the predominant dengue serotypes among the five provinces by year was analysed. In 2002, DENV-1 was predominant in one province (Chiangmai) and DENV-2 was predominant in two provinces (Lamphun and Lampang). In 2003, DENV-2 was

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### Table 1: Primer sequences of dengue virus and serotyping by RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUL</td>
<td>GCTGTGTCACCCAGAGTGGCCAT</td>
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<tr>
<td>DUR</td>
<td>TGGCTGGTCACAGACAATGGTT</td>
</tr>
<tr>
<td>D1L</td>
<td>CGGCTTCAATCCCAAGAG</td>
</tr>
<tr>
<td>D1R</td>
<td>CCTTAGTTCAAGCTTTCAC</td>
</tr>
<tr>
<td>D2L</td>
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</tr>
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<td>D2R</td>
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</tr>
<tr>
<td>D3L</td>
<td>CAAATGCTTGAAATACCTTG</td>
</tr>
<tr>
<td>D3R</td>
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<td>D4R</td>
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---
Circulation of dengue serotypes in Thailand

In 2004, DENV-2 was predominant in three provinces (Chiangmai, Lampang and Lamphun). In 2005, DENV-2 was predominant in four provinces (Chiangmai, Lampang, Mae Hong Son and Phrae) and DENV-4 was predominant in one province (Lamphun). In 2006, DENV-1 was predominant in three provinces (Chiangmai, Lampang and Mae Hong Son), DENV-2 was predominant in one province (Phrae) and DENV-4 was predominant in one province (Lamphun). This result has shown that the spread of predominant DENV-2 was increasing from two provinces to four provinces during 2002–2005; after that it rapidly decreased in all provinces. In 2006, DENV-1 was predominant in three provinces. This finding provides an important starting point of change to predominance from DENV-2 to DENV-1 in the northern region and was a

<table>
<thead>
<tr>
<th>Year</th>
<th>Province</th>
<th>Seropositive acute sample</th>
<th>Positive dengue by RT-PCR</th>
<th>DENV-1 (%)</th>
<th>DENV-2 (%)</th>
<th>DENV-3 (%)</th>
<th>DENV-4 (%)</th>
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<tr>
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<td>Phrae</td>
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<tr>
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<td>2 (100)</td>
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<tr>
<td></td>
<td>Phrae</td>
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<tr>
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<td>Total</td>
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<td>37 (59.7)</td>
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<td>1 (100)</td>
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<tr>
<td></td>
<td>Phrae</td>
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<td>0 (0)</td>
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<td>0 (0)</td>
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<td>104 (70.3)</td>
<td>2 (1.4)</td>
<td>5 (3.3)</td>
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<td>4 (26.6)</td>
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<td>Mae Hong Son</td>
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<td>Phrae</td>
<td>29</td>
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<td>4 (22.2)</td>
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<tr>
<td></td>
<td>Total</td>
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<td>31 (20.2)</td>
<td>69 (44.8)</td>
<td>13 (8.4)</td>
<td>41 (26.6)</td>
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<tr>
<td>2006</td>
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<td>2 (3.9)</td>
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<td>31 (21.3)</td>
<td>4 (2.8)</td>
<td>48 (33.1)</td>
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</tbody>
</table>

Total **1,116** (**559** (50.0%)) **171** (**30.6**) **264** (**47.2%) **21** (**3.8%) **103** (**18.4%)
predictive indicator of the continuous pattern of DENV-1 predominance in the next year.

From the 1116 seropositive acute samples collected from confirmed DF/DHF patients with positive anti-dengue IgM antibody by MAC-ELISA for this study, only 559 (50.0%) cases could be found positive for dengue virus. It is possible that some patients visited the hospital in the late period of viremia, as it has been reported that the samples from patients which were collected on fever day 1 were 50% positive for dengue virus. Moreover, other factors could be influenced by the outcome of laboratory determination, such as sample collection, handling and storage from community hospital to the Regional Medical Sciences Centre, Chiangmai.

Conclusion

Our study found that all the four dengue serotypes were circulating continuously in five provinces of northern Thailand, with one serotype emerging as the cause of a periodic dengue infection. These results were similar to the ones in the central and north-east regions. The pattern of fluctuation in the predominant dengue serotype in our study during 2002–2004 is DENV-2 as per the annual report of dengue serotype in Thailand. While DENV-2 was still prominent in 2005 and changed to DENV-1 in 2006 as per our study, but DENV-1 was initially the main serotype reported during 2005–2006. It is possible that since the northern region is mostly rural and mountainous area, the spread of the new serotypes was delayed. It is
concordant that DENV-4 was found increasing during 2005–2006, which was first detected in August 2005 (data not shown), whereas a reported case with DENV-4 was initially detected in Bangkok in April 2005. Our study found that DENV-3 was the least reported serotype. Nisalak et al. reported that DENV-3 was the least predominant dengue serotype in Bangkok in 1997–1998.\[11\]

Our study has shown the pattern of dengue virus serotypes in five provinces of northern Thailand from year to year and provided some insight into the dengue epidemic situation in this region. This information should be beneficial in the long-term dengue surveillance, and future work can focus on using this pattern of dengue serotype circulation to develop a predictive model of DF/DHF in Thailand.

**Acknowledgements**

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**References**


Clinical diagnostic delays and epidemiology of dengue fever during the 2002 outbreak in Colima, Mexico

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\textsuperscript{e}Department of Mathematics and Statistics, Arizona State University, P.O. Box 871804, Tempe, AZ 85287-1804, USA
\textsuperscript{f}School of Medicine, Universidad de Colima, Colima, Col., Mexico

Abstract

Dengue fever is a re-emergent and challenging public health problem in the world. Here, we assess retrospectively the epidemiological and clinical characteristics of the 2002 dengue epidemic in the state of Colima, Mexico. This study is carried out by analysing a database containing demographic, epidemiological and clinical information. Of the 4040 clinical dengue cases diagnosed in the hospitals of the Mexican Institute of Public Health in the state of Colima, 548 cases were confirmed by laboratory tests, and 495 cases presented at least one haemorrhagic manifestation. Of the total clinically diagnosed cases, the most common symptoms observed were: fever (99.6%), headache (92.4%), myalgia (89.4%) and arthralgia (88.6%). The most common haemorrhagic manifestations were: petechiae (7.1%), gingivitis (3.4%) and epistaxis (3.6%). The median time between the onset of illness and visit to the health care clinic (diagnostic delay) was 1 day (interquartile range [IQR]: 0-3). For cases presenting haemorrhagic manifestations, the diagnostic delay was higher (median: 2 days, IQR: 0-4) than for non-haemorrhagic cases (median: 1 day, IQR: 0-3). The proportion of males presenting haemorrhagic manifestations was higher than females (Fisher Exact test; p<0.01). Moreover, the age group 0-5 years presented a lower proportion of cases with haemorrhagic manifestations compared with the age group of 6 years and older (p=0.0281). No significant differences were found between the diagnostic delays in the case of males and females.

Keywords: Dengue fever; Clinical diagnostic delay; Haemorrhagic; Colima; Mexico.

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Clinical diagnostic delays and dengue fever in Colima, Mexico (2002)

Introduction

Annually there are approximately 100 million cases of dengue worldwide. It is endemic in Africa, the Americas, the Eastern Mediterranean, South-East Asia and the Western Pacific regions. The challenges seem daunting. For example, in Singapore, dengue is a major public health problem despite the fact that a set of extraordinary control efforts have been put into place over the past few years. The etiological agent is a Flavivirus with four different serotypes (DENV-1–4). The primary vectors of dengue are mosquitoes of the species Aedes aegypti and Aedes albopictus. Humans are infected when bitten by feeding infectious females. Those who recover may become permanently immune to the serotype involved and partially immune to the other serotypes. Susceptible vectors acquire the infection when feeding on infectious humans. Female mosquitoes are responsible for the transmission of the virus since males are non-blood suckers and feed primarily on plants and flowers.

Cases of dengue are classified as asymptomatic, clinically non-specific flu-like symptoms, dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF and DSS are the severe forms of the disease. Dengue attack rates vary from 40% to 50% but may be as high as 80% to 90%.

Mexico was declared dengue-free when the principal vector, Ae. aegypti, was eliminated in 1963. However, Ae. aegypti reappeared two years later and the disease returned. All the four dengue serotypes are now circulating in Mexico since 1980. DHF has become a public health problem in the country since 1994. In 2002, over 30 Latin American countries reported a total of over a million cases of classical dengue as well as more than 17 000 cases of DHF. We report the results of a retrospective study on the epidemiological and clinical characteristics of the 2002 (serotype DENV-2) dengue epidemic in the state of Colima, Mexico.

Materials and methods

The epidemiological and clinical characteristics of dengue fever cases recorded during the 2002 epidemic (January through December) in the state of Colima have been studied. The state of Colima is located on the Pacific coast, has a tropical climate, and has a population of 488 028 (Figure 1). The data used include cases diagnosed at the hospitals of the Mexican Institute of Public Health (IMSS). The IMSS are a collection of state hospitals that provide primary health services to 60% of the population in the state. The remaining population receives health care services from the hospitals of the Mexican Health Ministry and the private sector.

Figure 1: Map of the state of Colima, Mexico, with divisions by municipalities Armería (1), Colima (2), Comala (3), Coquimatlán (4), Cuauhtémoc (5), Ixtlahuacán (6), Manzanillo (7), Minatitlán (8), Tecomán (9), Villa de Alvarez (10)
Clinical diagnostic delays and dengue fever in Colima, Mexico (2002)

Patient record data are stratified by municipality where the dengue cases were diagnosed (Figure 1); the week of symptom onset and diagnosis; the IgM antibody test result; patient’s age and gender; and diagnostic delay (as presented later in the paper). Furthermore, non-haemorrhagic recorded symptoms included: fever, headache, myalgia, arthralgia, retro-orbital pain, exanthema, diarrhoea, vomit, nausea, pruritus, chills, photophobia, abdominal pain, conjunctivitis, nasal decongestion, cough, hepatomegaly and splenomegaly. Haemorrhagic recorded symptoms included: petechiae, ecchymosis, ascites, pleural effusion, gingivitis, epistaxis, haematemesis and melena. We classified a patient as “delayed” when clinical diagnosis was made two days after the onset of symptoms.

The World Health Organization (WHO) case definition of probable dengue cases requires the presence of fever or chills and at least two symptoms from: myalgia, arthralgia, retro-orbital pain, headache, rash, or some haemorrhagic manifestation (e.g. petechiae, haematuria, haematemesis, melena). The laboratory testing was only carried out in a small subset of the clinically diagnosed dengue cases through anti-dengue IgM antibody tests (ELISA).

We classified cases of DHF following as closely as possible all the four requirements for the WHO definition of DHF (fever, haemorrhage, thrombocytopenia, and signs of plasma leakage). Difficulties arose because basic measurements, such as platelet counts, were conducted in only 1227 (30%) of the cases and signs of plasma leakage were assessed only clinically (pleural effusion and/or ascites). The difficulties in characterizing DHF cases using the WHO system have led some investigators to use modified classification schemes. Here, we used the number of haemorrhagic symptoms as a measure of disease severity and classify dengue patients as haemorrhagic whenever one haemorrhagic symptom was reported. Dengue cases that did not exhibit haemorrhagic manifestations were classified as non-haemorrhagic.

We characterize variations using the mean and standard deviation (SD). Proportions are compared using Fisher’s exact test of independence, which uses a hypergeometric sampling distribution for cell frequencies. Population distributions are compared using the non-parametric Wilcoxon test. Results are deemed significant when the p value is less than 0.05. Some records are not complete. For example, the date of symptom onset was only recorded in 2242 patient records. Hence, the number of records (denoted by N) used is included in the analyses.

Results

The 2002 dengue epidemic in Colima, Mexico, began in January, peaked in September and died out in December (Figure 2). Four thousand and forty cases were clinically diagnosed, including 495 cases with haemorrhagic manifestations. A total of 555 clinical dengue cases (14%) were subjected to ELISA test and 548 cases were positive for anti-dengue IgM antibodies. Thrombocytopenia was detected in 528 cases but platelet counts were only recorded for 1227 cases (30%). Both thrombocytopenia and haemorrhagic symptoms were present in 203 cases (17%, N=1227), but only 8 cases could be classified as DHF under the WHO classification. The distribution of dengue cases by municipality is given in Table 1. The mean age of a dengue case was 24.61 ± 16.30 (SD) years (Figure 3A). The attack rate was about 9.5 dengue cases per 10000 persons. This rate was reported among two age groups of 5-14 and 25-34 years. The male/female ratio was about 1:1 with 2045 (50.6%) males and 1993 (49.4%) females. We found no significant difference between the age distribution of non-haemorrhagic and haemorrhagic cases (Wilcoxon test, p=0.5018, N=4007).
Figure 2: The weekly number of clinical haemorrhagic and non-haemorrhagic dengue cases during the course of the 2002 dengue epidemic in Colima, Mexico

Table 1: Number of dengue cases reported by municipality and classified as haemorrhagic and non-haemorrhagic

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Population size</th>
<th>Total cases</th>
<th>Total cases per 1000 people</th>
<th>Haemorrhagic dengue</th>
<th>Non-haemorrhagic dengue</th>
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</thead>
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<td>Armería</td>
<td>28 015</td>
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<td>2.78</td>
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<tr>
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<td>891</td>
<td>13.44</td>
<td>128</td>
<td>763</td>
</tr>
<tr>
<td>Total</td>
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<td>4040</td>
<td>8.28</td>
<td>495</td>
<td>3545</td>
</tr>
</tbody>
</table>
Figure 3: **Age (A) and effective diagnostic delay (B) distributions of dengue cases presenting haemorrhagic and non-haemorrhagic manifestations during the 2002 dengue epidemic in Colima, Mexico**

The most common symptoms in non-haemorrhagic dengue patients were fever (99.6%), headache (92.4%), myalgia (89.4%) and arthralgia (88.6%), while the least common symptoms were hepatomegaly (3.3%) and splenomegaly (2.3%). For haemorrhagic dengue cases, the median number of haemorrhagic manifestations was 1 (interquartile range [IQR]: 1-2) with a range from 1 to 7. The most common haemorrhagic manifestations were petechiae (7.1%), gingivitis (3.4%) and epistaxis (3.6%), while the least were ascites (0.3%) and pleural effusion (0.1%). The relative frequency of symptom appearance is displayed in Table 2.

The median diagnostic delay was 1 day (IQR: 0-3) with a range of 0 to 22 days (N=2242). Significant differences between the diagnostic delay distributions of non-haemorrhagic and haemorrhagic cases were obtained (Figure 3B, Wilcoxon test, p=0.0004, N=2242). In fact, the proportion of cases that experienced haemorrhagic manifestations and a diagnostic delay greater than two days was significantly higher than those with a diagnostic delay less than or equal to two days (17.4 vs 9.9%, p<0.0001, N= 2283; Fisher’s exact test). The median diagnostic delay for haemorrhagic cases was 2 days (IQR: 0-4; range: 0-14) that is higher than for non-haemorrhagic cases, for which the median
Table 2: Frequency of clinical symptoms presented in dengue cases during the 2002 outbreak in Colima, Mexico

(Because some data were not completely recorded, we provide both the numerator (n) and denominator (N) used to compute the frequencies)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Positive, % (n/N)</th>
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<tbody>
<tr>
<td><strong>Non-haemorrhagic manifestations</strong></td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>99.6 (4009/4025)</td>
</tr>
<tr>
<td>Headache</td>
<td>92.4 (3707/4013)</td>
</tr>
<tr>
<td>Myalgia (muscle pain)</td>
<td>89.4 (3586/4010)</td>
</tr>
<tr>
<td>Arthralgia (joint pain)</td>
<td>88.6 (3550/4006)</td>
</tr>
<tr>
<td>Retro orbital pain</td>
<td>73.3 (2936/4004)</td>
</tr>
<tr>
<td>Chills</td>
<td>58.1 (2334/4018)</td>
</tr>
<tr>
<td>Nausea</td>
<td>52.5 (2107/4015)</td>
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<tr>
<td>Vomit</td>
<td>36.7 (1476/4023)</td>
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<tr>
<td>Photophobia</td>
<td>33.2 (1331/4006)</td>
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<td>Abdominal Pain</td>
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<tr>
<td>Exanthema (skin rash)</td>
<td>30.3 (1217/4019)</td>
</tr>
<tr>
<td>Conjunctivitis (pink eye)</td>
<td>26.1 (1046/4010)</td>
</tr>
<tr>
<td>Pruritus (itching)</td>
<td>25.6 (1027/4015)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>18.3 (736/4019)</td>
</tr>
<tr>
<td>Cough</td>
<td>17.5 (702/4013)</td>
</tr>
<tr>
<td>Nasal decongestion</td>
<td>17.3 (694/4003)</td>
</tr>
<tr>
<td>Hepatomegaly (liver enlargement)</td>
<td>3.3 (128/3938)</td>
</tr>
<tr>
<td>Splenomegaly (spleen enlargement)</td>
<td>2.3 (90/3940)</td>
</tr>
<tr>
<td><strong>Haemorrhagic manifestations</strong></td>
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<tr>
<td>Petechiae (small purplish spots)</td>
<td>7.1 (268/3761)</td>
</tr>
<tr>
<td>Epistaxis (nosebleed)</td>
<td>3.6 (136/3748)</td>
</tr>
<tr>
<td>Gingivitis (bleeding gums)</td>
<td>3.4 (126/3747)</td>
</tr>
<tr>
<td>Haematemesis (vomiting blood)</td>
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<tr>
<td>Ecchymosis (bruising)</td>
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<td>Haematoma</td>
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<td>Ascites</td>
<td>0.3 (10/3741)</td>
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<tr>
<td>Pleural effusion</td>
<td>0.1 (5/3733)</td>
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</table>

diagnostic delay was 1 day (IQR: 0-3; range: 0-22). The proportion of cases with a diagnostic delay less than or equal to 2 days was significantly higher for children (<15 years) than for adults (>15 years) (73% vs 67%, p=0.001, N=2283, Fisher’s exact test). No significant association between short diagnostic delay (less than or equal to 2 days) and gender (p=0.36, Fisher’s exact test, N=2281) was found. The median diagnostic delay turned out to be 3 days (IQR: 0-4) (N=114) for cases with both haemorrhagic manifestations and thrombocytopenia.

A significant association between the presence of haemorrhagic manifestations and age was found. In fact, the 0-5 years age group supported a lower proportion of cases with haemorrhagic manifestations than the age group of 6 years and older (8.7 vs 12.7%, p=0.0281, Fisher’s exact test, N=4007). A significant association between the presence of haemorrhagic manifestations and gender was also identified. In fact, the proportion of males (13.6%) with haemorrhagic dengue was higher than for females (10.8%) (p=0.0072; N=4038; Fisher’s exact test).

Discussion

A retrospective study on the clinical and epidemiological characteristics of dengue was carried out during the 2002 epidemic in Colima. The levels of association significance were assessed between disease severity and epidemiological, clinical and demographic variables. We did not find a correlation between dengue disease and gender. The 1:1 female/male ratio is in agreement with other dengue studies conducted in Nicaragua,[15] Thailand[16] and Taiwan.[17] Males in our study were statistically more likely to experience haemorrhagic manifestations, a finding that agrees with a study conducted in Nicaragua.[15] We found a significantly higher median
diagnostic delay for cases presenting haemorrhagic manifestations than for non-haemorrhagic cases.

Not surprisingly, this finding indicates an important correlation between the disease’s clinical evolution and diagnostic delay. Diagnostic delays may complicate the clinical state of the patient while facilitating the transmission of dengue in the population. In other words, although interventions have shown to be incapable of reverting a dengue epidemic,[18] reductions in the diagnostic delays can reduce the final epidemic size. Mathematical models of dengue transmission have shown that educational campaigns aiming at shortening the diagnostic delays can lead to significant reductions in the final epidemic size.[19] Control strategies that benefit from a prompt identification of dengue cases include the use of nets and screens, the application of insecticides to clothing and the application of mosquito repellents. Moreover, strategies aiming at the elimination or reduction in the number of breeding sites through the use of larvicidal control including ovitraps[20] and malathion spraying for adult control can also reduce the dengue burden.

A fever alone is usually not enough to motivate patients to seek medical care. It is only the presence of severe symptoms (e.g., haemorrhagic manifestations) that motivate to seek medical care. This observation may explain the significant association that we found between diagnostic delays and the presence of haemorrhagic manifestations. Specifically, we found that the proportion of cases with a diagnostic delay of less than or equal to 2 days was significantly higher for children (< = 15 years) than for adults (> 15 years). This is in agreement with the findings by Ahorlu et al.[21] who studied the socio-cultural determinants of treatment delay for childhood malaria in southern Ghana. Ahorlu et al.[21] found that families with similar economic situations who have sick children will be more likely to seek treatment. Gender exhibited no correlation with the length of the diagnostic delays. The most common explanations for diagnostic delays in malaria studies in sub-Saharan Africa include poverty and the inability to pay for treatment.[22] This perspective could not be assessed here as most of the patients comprised only the insured individuals by IMSS. In summary, a combination of cultural factors, the inability to distinguish dengue illnesses from traditional fevers at the early stages of the disease, and the differentiated treatment are the main factors behind differences in diagnostic delays.

The impact of dengue diagnostic delay distributions and their association with clinical, demographic and epidemiological factors have not received much attention. This contrasts, for example, with epidemiological studies dealing with other infectious diseases such as pulmonary tuberculosis.[23] However, there are some relevant studies. For example, an estimate of the median diagnostic delay of 5 days has been reported for the 1996 dengue epidemic in north-eastern Brazil,[24] that is, a significantly longer median diagnostic delay than our estimate of 1 day. Guzman et al.[25] found that the average time from fever onset to hospitalization associated with the 1997 dengue outbreak in Cuba was about 2.9 days. On the other hand, a median diagnostic delay of 2 days was reported for malaria-stricken travellers returning to Sweden during 1994 to 2001.[26]

We found a lower proportion of haemorrhagic cases in the age group 0-5 years than those in the age group of 6 years and older. Secondary dengue infections have been associated with the presence of haemorrhagic manifestations, a phenomenon explained by the theory of antibody-dependent enhancement.[5]

Seasonal effects are also critical. The peak of the epidemic in Colima occurred in mid-September, which correlates well with the
Clinical diagnostic delays and dengue fever in Colima, Mexico (2002)

A peak in the rainfall. A similar pattern has been reported for other dengue outbreaks {Nicaragua (1998), El Salvador (2000) and Bangladesh (2000)}.

In contrast to dengue hyperendemic areas where dengue haemorrhagic fever is primarily a disease of children under 15 years, we found a lower proportion of haemorrhagic cases in the age group 0-5 years compared to the age group of 6 years and older.

The number of dengue infections per municipality was determined by the location of the patient’s address albeit the actual dengue infection may have occurred at work because dengue mosquitoes are daytime feeders.

This study was limited by the lack of complete medical records. Difficulties were encountered in rigorously following the WHO classification scheme for DHF, because the signs of plasma leakage were assessed only clinically (pleural effusion and/or ascites). The number of DHF cases may in fact be as high as 203, that is, the number of cases for which the other three requirements of the WHO classification scheme for DHF (fever, haemorrhage and thrombocytopenia) were satisfied. On the other hand, dengue cases with severe haemorrhage, not accompanied by increased vascular permeability, have been reported from several regions of the world including Indonesia, China, India, Philippines, Thailand, South Pacific and Latin America. To improve the tracking of dengue, workers at IMSS hospitals would need to collect as complete patient information as possible. Efforts to follow the WHO classification guidelines as closely as possible would be quite helpful. Dengue symptoms can be confused in the epidemic and non-epidemic situations with other exanthematous and non-exanthematous viral diseases such as measles, rubella, enteroviruses and influenza. We recognize that there are limited resources for laboratory testing. In the 2002 dengue epidemic in Colima, only 14% of the clinical dengue cases were tested in the laboratory for anti-dengue IgM. The low proportion of clinical dengue cases tested serologically make it difficult to validate some of our findings.

Our findings indicate that an educational campaign in the community with the objective of informing the population about early symptoms of dengue infection could lead to not only reductions in diagnostic delays but also to reduce the final size of a dengue epidemic.

Acknowledgement

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References

Clinical diagnostic delays and dengue fever in Colima, Mexico (2002)


Exposure to the risk of dengue virus infection in an urban setting: ecological versus individual heterogeneity

Maria Glória Teixeira, Maurício Lima Barreto, Maria da Conceição N. Costa, Leila Denise Alves Ferreira, Vanessa Morato, Pedro Fernando Vasconcelos and Sandy Cairncross

Abstract

The dynamics of dengue virus circulation in the intra-urban spaces of large cities and the risk factors for the occurrence of such infections are still not well known. Although it has been established that poverty is one of the determinants of the majority of infectious and parasitic diseases, in the case of dengue this is still a matter of some controversy. This study had the objective of describing the distribution of dengue seroprevalence and seroincidence in different intra-urban spaces within a large and complex city in north-eastern Brazil. The study investigated whether there is any relationship between the intensity of virus circulation and the population’s living conditions or between group immunity and Aedes aegypti infestation rates. The variability in the risk of such infections was also examined. A prospective study was conducted by means of serological investigations among a sample of people living in 30 different spaces (“sentinel areas”) in the city of Salvador, which was selected according to extreme differences in living conditions. High rates of seroprevalence (67.7%) and seroincidence (70.6%) were found for the circulating serotypes (DENV-1 and DENV-2). Similar to what has been occurring in south-east Asia, the seroincidence was high (55%) even when the group immunity had already been partially established (42%) and the Ae. aegypti infestation rates were relatively low (<3%). Contrary to the ecological analysis, at the individual level, substantial heterogeneity in dengue exposure was observed. This paper discusses this apparent contradiction, highlighting its implications for the effectiveness of vector control strategies.

Keywords: Dengue; Prospective study; Herd immunity; Seroprevalence; Seroincidence; Spatial distribution; Aedes aegypti; Epidemiology.

Introduction

The dynamics and determinants of dengue virus circulation in urban areas, particularly in large cities, are not well-established. Epidemiological studies of dengue infections have been neglected, even though they are important for developing dengue prevention and control strategies, as has recently been recognized in the dengue research agenda of WHO.
Exposure to the risk of dengue virus infection in an urban setting

The re-emergence of this disease in several continents and its potential virulence is of great concern to public health worldwide. Moreover, the available control strategies are based on vector control, and these have not always been effectively implemented to prevent transmission of the disease.\(^\text{[3,4]}\) Unlike yellow fever, restrictions on human movement through quarantine are not applicable for the control of dengue for lack of vaccines.

In the large urban centres that are infested with *Aedes aegypti*, large numbers of susceptible individuals and high population density\(^\text{[3]}\) probably facilitate more extensive transmission of dengue virus as mosquito vectors, because of multiple probing/feeding behaviours are likely to infect multiple individuals in different households. However, the risk of exposure to dengue virus infection in relation to the range of social and economic conditions in big cities remains unclear. These risk factors are related both to low income areas and areas with more favourable conditions.\(^\text{[5,6]}\)

In Salvador, a large city in north-eastern Brazil, the dengue virus was first detected in January 1995, and, by the end of 1999, there had been two recorded epidemics. The mean annual incidence over that period reached 691 reported cases per 100 000 inhabitants.\(^\text{[7]}\) We carried out a serological study of the prevalence and incidence of dengue virus infection in this city,\(^\text{[8]}\) at a time when only DENV-1 and DENV-2 were circulating.\(^\text{[7]}\) The present study attempts to examine the relationship between the spatial distribution of dengue virus circulation and the population’s living conditions, group immunity and *Ae. aegypti* infestation rates, and also to find out any variability in the risk of such infections.

Materials and methods

This prospective study on dengue seroprevalence and seroincidence was carried out in Salvador, Bahia state, Brazil. This city had more than 2.3 million inhabitants in 1998 and presented marked differences between specific areas with regard to socioeconomic conditions and environmental sanitation. The present study considered 30 spatial units of analysis that were named “sentinel areas”. These were selected by stratified sampling using data obtained from the Brazilian Institute for Geography and Statistics\(^\text{[9]}\) regarding sanitation system coverage and income levels, which were taken to be estimators of living conditions, using the following strata:

1. High: more than 80% of the homes were connected to the sanitation system and more than 50% of the families had an income of more than five minimum salaries (80 US dollars at the time of the study) (6 areas).
2. Medium: 50% to 80% of the homes were connected to the sanitation system and more than 50% of the families had an income of between one and four minimum salaries (19 areas).
3. Low: less than 50% of the homes were connected to the sanitation system and more than 50% of the families had an income of less than one minimum salary (5 areas).

This selection strategy has been described in more detail elsewhere.\(^\text{[10]}\)

To determine the number of individuals for the serological surveys, a seroprevalence of 50% was assumed because this was the mean observed in previous surveys in Brazilian state capitals.\(^\text{[6,11]}\) With this, and assuming precision of less than or equal to 3% and a confidence level of 5%, the sample size was estimated to be 1503 individuals. After adding 30% to compensate for possible losses, the result was 2149 individuals. Using the database of a demographic census carried out in 30
sentinel areas in 1997, when 68 749 individuals were counted, a random draw for the participants was made (without replacements). These individuals were then grouped in areas by taking the home address into consideration.[12]

Two surveys were carried out. The first, in 1998, is referred to here as “the seroprevalence survey”. The second, one year later in 1999, is termed “the seroincidence survey.”

After the approval of the study protocol by the Ethics Committee for Scientific Research of the Gonçalo Moniz Research Centre (Oswaldo Cruz Foundation, Bahia), a structured questionnaire was formulated during May through July 1998. The data sought in the questionnaire included: name, address, sex, age, educational level and history of vaccination against yellow fever. Just after the interview, these individuals were provided with clarifications regarding the nature of the study and they were asked to sign an informed-consent form. Following this, the first blood sample was collected. Three individuals previously vaccinated against yellow fever were excluded in order to avoid false positive serological test results due to cross-reactions. A second blood sample was collected a year later, from the individuals who were negative during the first serological survey, or had positive reactions to only one serotype of the dengue virus.

Blood samples were collected in 10 ml sterilized vacuum tubes, and the serum was separated by centrifugation and stored at −20 °C. These samples were sent in thermal boxes containing ice to the arbovirus laboratory of the Evandro Chagas Institute. There, the haemagglutination inhibition (HI) test,[13] as modified by Shope,[14] was carried out using antigens for the four serotypes of the dengue virus and four other flaviviruses (YF, Rocio, Ilhéus and St. Louis encephalitis), although these do not circulate in Salvador. There is a controversy regarding the interpretation of the serological response to flaviviruses, which differs between the first (primary) infection and any subsequent (secondary) infection with another flavivirus or serotype (flaviviruses show an increasingly strong response on subsequent infection and serological cross-reactions are frequently observed).

Thus, for the interpretation of serological response, the WHO[15] criteria was followed, i.e. HI titres of 1:20 or higher, exclusively for a specific dengue serotype, or titres four times higher for one serotype than for another (DENV-1 or DENV-2), were considered positive and specific for that serotype (primary response). Titres indicative of secondary response also followed the WHO criteria.[15] These were also confirmed by IgG enzyme-linked immunosorbent assay[16] and were considered positive to both serotypes, meaning that infection with both DENV-1 and DENV-2 had occurred.

For each sentinel area, the seroprevalence and seroincidence rates of dengue infection were estimated both unadjusted (crude) and with standardization by age using the indirect method (Rothman),[17] and the total composition of the study sample as the reference population. Since the interval between the two surveys was one year, the seroincidence was expressed as an annual rate per cent. The prevalence ratio (PR) and relative risk (RR) of dengue virus infection with 95% confidence intervals (CI) were estimated by taking as the reference standard the sentinel areas with the lowest seroprevalence (area 427) and seroincidence (area 7), respectively, among the areas in the highest socio-sanitary stratum. For the three strata according to living conditions, the respective seroprevalence and infection incidence were calculated after Rothman,[17] and the chi-squared test for trend was applied.

From the information collected using the questionnaire, the frequency indicators were
estimated for each area by taking into consideration the proportions of individuals according to sex, age greater than or equal to 15 years, schooling (assuming that individuals were at risk if they were 15 or more years old and had not completed elementary schooling) and mean family income less than or equal to two minimum monthly salaries. The mean population density was obtained from the 1996 census. Calculations of Pearson’s correlation coefficient were used to investigate the existence of associations between the variables of interest.

In April 1999, health workers trained and supervised by the research team visited and inspected all the houses in the 30 sentinel areas. The existence of foci of Ae. aegypti was checked and records were made of these visits. The unit of analysis for these data was the sentinel area. Every building with one or more breeding sites containing the larvae of this mosquito was considered to be positive, and the Premises Index (PI) was estimated as the percentage of positive buildings. The infection incidence was calculated for different PI bands (less than or equal to 3%; 3.1% to 5%; 5.1% to 10%; and over 10%) using covariance analysis, with adjustments for age and mean seroprevalence (herd immunity indicator). The preventable fraction was estimated by considering individuals who lived in areas with PI less than or equal to 3% to be “non-exposed”.

The data were entered using Epi-Info 6.0 and analysed using SAS and STATA.

Results

Among the 1515 individuals who took part in the seroprevalence survey, 58% were female and 71% were aged 15 years or older, mainly in the age groups 15 to 29 (33%) and 30 to 49 (29%). The majority (68%) had had schooling for eight years or less. Around 25% reported that their family income was less than two minimum salaries and 50% earned from two to less than five minimum salaries. There were 595 individuals in the seroincidence survey, out of 860 who were eligible according to the criteria established, which represented a loss of 31%. The great majority of these losses were due to changes of address, and it was not appropriate to locate these losses since this was an ecological study of sentinel areas. Nonetheless, the social and demographic structure of the sample remained similar to what was found in the first survey. Among the sentinel areas, the population density varied widely, from a maximum of 49 980 to a minimum of 1834 inhabitants per km².

The mean seroprevalence was 69% (ranging from 16% to 98% among the sentinel areas), and this distribution was little changed after standardization for age. The prevalence ratio (PR) indicated a risk of positive findings that ranged from 0.36 in sentinel area 1011 to 2.20 in area 1054, which were both in the medium stratum of living conditions (Table 1).

As shown in Table 2, the stratum of lowest living conditions had the highest mean seroprevalence (74.0%), and this trend was statistically significant ($\chi^2 = 8.386; p = 0.004$). It can be seen from Table 3 that the seroprevalence presented a positive correlation ($r = 0.4914; p = 0.006$) with population density and a weak negative correlation ($r = -0.2778; p = 0.137$) with the proportion of individuals aged 15 years or over who had had less than eight years of schooling. No statistically significant association was found with mean income ($r = 0.0571; p = 0.764$).

The mean seroincidence was 71.0% per year. Three areas in which the sample size was less than four were not taken into consideration; in the others, the seroincidence ranged from 50% (area 323) to 90% (area 678) (Table 1). In the areas in which the seroprevalence was
Table 1: Seroprevalence and seroincidence (crude and standardized) for dengue, study population, population density and premises index (PI) according to sentinel area and strata of living conditions in Salvador – Bahia, Brazil, 1998-1999

<table>
<thead>
<tr>
<th>Sentinel area</th>
<th>Stratum</th>
<th>Population density/km²</th>
<th>Seroprevalence*</th>
<th>Seroincidence**</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Sampled No.</td>
<td>Crude (%)</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>H</td>
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<td>M</td>
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<tr>
<td>208</td>
<td>M</td>
<td>26 558</td>
<td>77</td>
<td>74.0</td>
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</table>

*: 1998; **: 1999; H: High living conditions; M: Middle living conditions; L: Low living conditions
lower, the incidence of infection indicated by a change in serological status was high, except in area 571. There was only one area (575) in which no individuals seroconverted in the second survey, although the number of initially negative individuals was only two and this area had the lowest PI (0.27%) (Table 1). The relative risk of seroincidence among the areas in the second survey ranged from 0.64 to 1.52, excluding area 575, in which there were no new cases. In addition, there was a statistically significant negative correlation between the incidence of infection and the proportion of individuals aged 15 years or over, who had not completed elementary schooling. For mean income and population density, a negative correlation was also found, but without statistical significance (Table 4).

Differing from the seroprevalence survey, the highest unadjusted or standardized seroincidence rates were in the stratum of highest living conditions (Table 2), but the chi-squared test for trend did not show statistical significance (\( \chi^2 = 1.332; p = 0.248 \)).

A comparison between the incidence adjusted for age and the mean seroprevalence in the sentinel areas (herd-immunity indicator), when grouped according to the PI ranges considered (Figure), revealed that the lowest seroincidence (55%) was in the group with PI less than or equal to 3% and the highest (77%) was in the group from 3% to 5%. The differences were statistically significant at the 5% level only between the first and second PI groups (\( p < 0.01 \)) and between the first and fourth groups (\( p = 0.02 \)).

### Table 2: Seroprevalence (%) and seroincidence (%) of dengue, prevalence ratio (PR), relative risk (RR) and confidence interval (CI: 95%) according to living condition strata in 30 sentinel areas of Salvador – Bahia, Brazil, 1998-1999

<table>
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<tr>
<td></td>
<td></td>
<td>Standardized (%)</td>
<td>PR</td>
<td>CI: 95%</td>
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<tr>
<td></td>
<td>Standardized (%)</td>
<td>PR</td>
<td>CI: 95%</td>
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<tr>
<td></td>
<td>Standardized (%)</td>
<td>PR</td>
<td>CI: 95%</td>
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<tr>
<td>High</td>
<td>64.8</td>
<td>68.8</td>
<td>1.0</td>
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<tr>
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<td>68.7</td>
<td>69.2</td>
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<tr>
<td>Low</td>
<td>74.0</td>
<td>78.4</td>
<td>1.19</td>
<td>1.07; 1.33</td>
</tr>
</tbody>
</table>

Test for trend: *\( \chi^2 = 8.386, p= 0.004 \); **\( \chi^2 = 1.332, p= 0.2484 \)

### Table 3: Seroprevalence and seroincidence for two serotypes of dengue virus in Salvador, Brazil, 1998-1999

<table>
<thead>
<tr>
<th>Serology</th>
<th>No. examined</th>
<th>1 serotype</th>
<th>2 serotypes</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Seroprevalence</td>
<td>1515</td>
<td>386</td>
<td>25.5</td>
</tr>
<tr>
<td>Seroicidence prior immune status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>331</td>
<td>77</td>
<td>23.3</td>
</tr>
<tr>
<td>Positive for one serotype</td>
<td>264</td>
<td>219</td>
<td>83.0</td>
</tr>
<tr>
<td>Total</td>
<td>595</td>
<td>296</td>
<td>49.8</td>
</tr>
</tbody>
</table>
Table 4: Spearman correlation coefficient \((r)\) for association between crude and standardized seroprevalence and seroincidence for dengue virus and selected variables for residents of 30 sentinel areas in Salvador – Bahia, Brazil, 1998-1999

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Education (proportion (\geq 15) years old with less than middle school)</td>
<td>-0.26</td>
<td>0.17</td>
<td>-0.28</td>
<td>0.14</td>
<td>-0.50</td>
<td>0.01</td>
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<tr>
<td>Average income</td>
<td>-0.04</td>
<td>0.85</td>
<td>-0.06</td>
<td>0.76</td>
<td>-0.09</td>
<td>0.63</td>
</tr>
<tr>
<td>Population density (inhab/km(^2))</td>
<td>-0.49</td>
<td>0.01</td>
<td>-0.49</td>
<td>0.01</td>
<td>-0.17</td>
<td>0.36</td>
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<tr>
<td>Premises Index (PI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.21</td>
</tr>
</tbody>
</table>

In the individual analysis, it was observed that, among the individuals who in the seroprevalence survey had been negative, 38% presented a risk of being infected by the two serotypes within a period of approximately one year. Among those who had been positive for one of the serotypes in the first examination, 83% had a second infection within this period (Table 4).
Discussion

The high seroprevalence level for dengue virus infection found in this study (69%), and the subsequent high incidence level (71%), were surprising. This was particularly so given the short period of time (around four years) that had elapsed since the virus was introduced into Salvador. These findings demonstrate the force of the transmission of this virus in Salvador, given that surveys carried out in other Brazilian state capitals after similar periods of time of virus circulation revealed lower mean seroprevalence (41% to 44%). One plausible explanation for the rates that were so high in Salvador may relate to the almost complete lack of vector control measures at the outset of virus transmission in this city. Such measures were only had waned. On the other hand, in other cities such measures had already been implemented routinely even before the introduction of the dengue virus.

It is worth emphasizing that the results from the present investigation have made it possible to estimate that, between 1995 and 1999, around two million individuals in Salvador were infected by the dengue virus. This means that the population was at a higher risk for haemorrhagic dengue, given that DENV-3 started circulating in 2002.

The association found in 1998 between dengue seroprevalence and precarious living conditions (Table 2) disappeared in the seroincidence for the subsequent year. In other words, the risk of being infected by the dengue virus became practically the same between the different economic strata of the population as viral transmission became established in Salvador. This distribution, which was relatively homogeneous between the strata, has also been observed in Fortaleza and São Luís do Maranhão. The risk in Fortaleza was even found to be slightly higher in the districts with better socioeconomic indices. These facts strongly suggest that dengue in Brazil is a disease that affects all social classes.

Despite the statistically significant associations found between dengue seroprevalence and population density and also between seroincidence and degree of PI and schooling, it can be seen that the risk of infection was high in almost all the areas, including in the areas with good living conditions. It is likely that this dynamics is at least partially due to the fact that in Salvador high population density and PI are found both in areas with precarious living conditions and in those where economically more favoured populations live, even if some people may have been infected away from home, for instance at school or at work.

This possible similarity of exposure to the risk of being infected by the dengue virus, in different intra-urban environments, differentiates this agent from those of the great majority of infectious and parasitic diseases. In particular, it differentiates dengue from the microorganisms whose transmission routes are connected with the environment and which predominantly affect poor populations.

In contrast to the uniformity of exposure suggested by the ecological comparison described above, our results indicate substantial heterogeneity in exposure to dengue at the individual level. Since there is no evidence of variation in the susceptibility of naive individuals to dengue infection, our finding of a higher incidence of seropositivity (83%) among individuals who were initially seropositive to one serotype, is strongly indicative of heterogeneity in the degree of exposure among the individuals of the population of this city. The lack of variation in exposure between neighbourhoods, as described above, suggests that the main differences are between individual households or people.

These results suggest puzzling and apparently contradictory conclusions regarding
Exposure to the risk of dengue virus infection in an urban setting

the degree to which exposure to dengue infection varies across the population, unrelated to the observed *Ae. aegypti* density in each area. The lack of association between mosquito density and dengue seroincidence is particularly remarkable in view of the way in which differentials existing between individuals can be expected to be amplified in ecological comparisons. It is hard to see how people’s movement about the city could explain this phenomenon; by bringing each individual into contact with various local environments, one would expect mobility to render their exposure more homogeneous rather than the contrary.

The larval survey was carried out by the research team in 100% of the households, which is grounds for confidence in this indicator, but the use of only one measure of the vector population constitutes one of the limitations of this study. Perhaps an option to refine our results might have been to count viable pupae, or adult females, per person. However, this was not possible, for reasons of cost and operational complexity. For these same reasons, most vector control programmes use only larval indicators in their routine monitoring. Though the larval densities vary widely during the course of a year, the geographical patterns remain consistent through time. So, our results have disturbing implications for the effectiveness of vector control strategies.

An approach that takes into account the lifestyles that potentially favour greater exposure to the risk of being infected by the dengue virus ought to form one of the lines of research for elucidating this question. This is because the differences may be related both to the public and the private domain, since the environment of the home and its surroundings have a decisive influence on the transmission of dengue virus.

Another important finding from this study relates to the high incidence of dengue virus infection even when the infestation indices were relatively low and group immunity had already been partially established (42%), which also expresses the transmission strength of this agent. It was found that seroincidence did not vary with PI when this was greater than 3%. This finding has strong implications for *Ae. aegypti* control programmes, since it suggests that it is necessary to intensify vector control and bring *Aedes* densities under 3%, before an observable impact can be registered. This finding is in agreement with the observation in Singapore that from the early 1990s the incidence of dengue rose considerably, even when the PI was less than 2%. The lack of a safe and effective vaccine against the virus increases the need for improved strategies and technologies for vector control, and for epidemiological studies to identify changes in infection patterns, such as the locations of transmission foci, the age group of peak incidence, and falling herd immunity. The last of these was one of the most important factors in the re-establishment in recent years of intense dengue virus circulation in Singapore.

It can be understood, therefore, that the results from this investigation should be considered at the time of defining dengue control policies and improving vector control measures. On the one hand, observation that the dengue virus in our environment does not respect social spaces strengthens the principle that vector control measures must always be universally applied in each territory. On the other hand, the identification of specific risk factors in the domestic domain may indicate a need for other evidence-based interventions which can help to eliminate the disease from cities such as Salvador.

**Acknowledgements**

The authors acknowledge the financial and technical support of the National Centre of Epidemiology, Ministry of Health, Brazil, especially its Director, Dr Jarbas Barbosa da Silva Junior.
References

Exposure to the risk of dengue virus infection in an urban setting


Detection of transovarial dengue virus from field-caught *Aedes aegypti* and *Ae. albopictus* larvae using C6/36 cell culture and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques

*A. Rohani*, I. Zamree, H.L. Lee, I. Mustafakamal, M.J. Norjaiza and D. Kamilan

Abstract

Larvae of *Aedes aegypti* and *Aedes albopictus* were collected from a wide variety of artificial containers. Most samples were collected from used tyres and water-holding containers located in residential urban or rural areas. The identified mosquito larvae were pooled according to the species, date and locality and stored at –70 °C. A total of 378 pools of *Ae. aegypti* and 553 pools of *Ae. albopictus* were collected. Virus isolation was carried out using cell culture (C6/36 clone) of *Ae. albopictus* and virus detection by reverse-transcriptase polymerase chain reaction (RT-PCR). Transovarial transmission of dengue virus was demonstrated in both *Ae. aegypti* and *Ae. albopictus* in nature. Infected larvae were recovered from 16 localities (10 in Terengganu; 5 in Kuala Lumpur and 1 in Pahang). The study showed that both the cell culture and RT-PCR techniques can be used to detect dengue virus from mosquito larvae.

**Keywords:** Dengue; *Aedes aegypti*; *Aedes albopictus*; Transovarial transmission; Malaysia.

Introduction

Dengue is an acute arboviral human disease caused by four serotypes of dengue viruses, which are closely related but antigenically distinct. Dengue viruses are transmitted to humans through the bite of infected mosquitoes. For many years, members of the subgenus *Stegomyia*, especially *Aedes aegypti* (Linn.) and *Aedes albopictus* (Skuse.) have been recognized as the primary vectors of dengue.[1,2,3] *Ae. aegypti* breeds in clean water collected in and around human settlements. *Ae. albopictus* can be found not only around and near human habitation, like *Ae. aegypti*, but also in forests and plantations.

The emergence of dengue has been linked to economic and ecological changes that caused dramatic expansion of the urbanized vector of dengue viruses. Industrialization and rapid human population growth led to...
Detection of transovarial dengue virus from field-caught larvae of Ae. aegypti and Ae. albopictus

uncontrolled urbanization in Malaysia. In the absence of proper water supply (for example in the squatter (slum) areas), residents have to store water for domestic use, creating the ideal ecological niche for Ae. aegypti and Ae. albopictus. These mosquitoes prefer to feed on humans and to lay eggs in artificial containers. The increase in air travel also contributed to the expansion of dengue, providing the means for viremic people to move very rapidly from one place to another.[4]

The prevention and control of dengue outbreaks depends upon the surveillance of cases and mosquito vectors. Vector surveillance allows timely implementation of emergency mosquito control measures to limit an impending outbreak from spreading.

In Malaysia, a comprehensive mosquito control programme administered by the Vector-Borne Disease Control Programme, Ministry of Health, incorporates source reduction, public health education, community participation and law enforcement against mosquito breeding. The Aedes control strategy has focused mainly on surveillance for the elimination of Aedes larval breeding habitats and emergency control of adults using insecticidal fogging during outbreaks. Although this strategy has successfully reduced the Aedes mosquito population to a relatively low level, as indicated by the overall House index (HI), it has not prevented the emergence of progressively larger outbreaks in recent years.[5]

Despite extensive research on vaccine development, there are at present no known methods of controlling dengue except by limiting the mosquito vectors. Virological surveillance, which involves the monitoring of dengue virus infection in humans, has been used as an early warning system to predict outbreaks.[6,7] Such surveillance is based on isolation of dengue virus from human serum by cell culture or mosquito inoculation and type-specific identification by immunofluorescence or multiplex RT-PCR. This approach is less effective since the virus is already circulating in the human population. A more effective approach is to detect the virus in mosquitoes before it is introduced into the human population. This way, preventive vector control measures can be undertaken immediately to offset an outbreak. Surveillance of mosquitoes infected with dengue viruses provides an early warning sign for risk of transmission in an area and the specific predominant circulating serotype in the vector population.[8] Control programmes can be prioritized and focused more effectively in specific localities.

The objective of this study was to detect dengue virus from field mosquitoes using C6/36 cell culture and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques.

Materials and methods

Larval collections

Third and fourth instar Aedes aegypti and Aedes albopictus larvae were collected from the field in major towns in Malaysia, i.e. Terengganu, Pahang and Kuala Lumpur. The larvae were collected during dengue outbreaks for each state in 2005. Mosquito larvae collected in the survey were identified using standard taxonomic keys. Identified mosquito larvae were segregated according to species, site and date. Mosquito larvae were stored in pools of 10 larvae per pool in cryogenic vials at –70 °C for future virus isolation studies. The mosquito pools were selected randomly and dengue virus detection was carried out by RT-PCR and C6/36 cell culture technique. A total of 931 pools of Aedes mosquitoes were separated equally and assayed by C6/36 cell culture and RT-PCR.
Detection of transovarial dengue virus from field-caught larvae of Ae. aegypti and Ae. albopictus

Detection of dengue virus using reverse transcriptase-polymerase chain reaction (RT-PCR)

Extraction of RNA

The larval pools were homogenized in a sterile homogenizer and RNA was extracted using High Pure Nucleic Acid Kit (Roche, Germany). The procedures of RNA extraction were as follows: 200 μl sample were added subsequently into 1.5 ml eppendorf tubes containing 50 μl proteinase K. This was briefly vortexed and incubated at 27 °C for 10 minutes. Subsequently, 100 μl of isopropanol was added and vortexed briefly. The solution was then pipetted into the high pure filter tubes, placed into collection tubes and were then centrifuged at 14 000 rpm for 1 minute. The flow-through was discarded and filter tubes were placed into new collection tubes. Five hundred microlitres of inhibitor-remover wash buffer were added into the upper reservoir and centrifuged at 14 000 rpm for 1 minute. The flow-through was discarded and the filter tubes were placed into new collection tubes. A volume of 450 μl wash buffer was added to the upper reservoir and centrifuged at 14 000 rpm for 1 minute. The flow-through was discarded and again the filter tubes were placed into new collection tubes. The wash step was repeated and again the filter tubes were placed into new collection tubes. The wash step was repeated and continued with a short spin for 15 seconds. The collection tube was discarded and the filter tubes were inserted into clean 1.5 ml eppendorf tubes. RNA was eluted by adding 35 μl elution buffer into the middle spot of the filter tubes and centrifuged at 14 000 rpm for 2 minutes. The extracted RNA was kept at −70 °C until used.

Detection of viral RNA using consensus primers

RNA isolated from each pool was subjected to the RT-PCR assay using Lanciotti’s dengue consensus primers (TCAATATGCTGAAACGCCAGAAACCG and TTGCACCAACAGTCAATGCTTCAGGTC). The primers correspond to genome positions 131 to 161 and 616 to 644 of dengue virus respectively. Master mix was prepared using Titan One Tube RT-PCR Kit. Each reaction required 9.75 μl of double distilled water, 2 μl of dNTP mix, 1.25 μl of DTT, 0.5 μl RNase inhibitor, 5.0 μl of RT-PCR buffer, 0.5 μl of enzyme mix, 0.5 μl of dengue universal sense primer and 0.5 μl of dengue universal anti-sense primer to reach 20 μl total volume of master mix.

RNA products were prepared by heating the tubes at 65 °C for 5 minutes by block heater. Five microlitres of each RNA product were added to the master mix and then centrifuged at 8000 rpm. The RT step was carried out at 51 °C for one 30 minutes to produce cDNA which was then amplified by the following PCR steps: 92 °C for 3 minutes as initial denaturation, 92 °C for 30 seconds as denaturation steps, 51 °C for 45 seconds as annealing step and 72 °C for 1 minutes as extension step. The cycle was repeated 41 times before final extension at 72 °C for 5 minutes. For every RT-PCR run, a positive control (a confirmed dengue isolate) and negative control (C6/36 cell culture without dengue virus) were included.

The PCR products were analysed by performing electrophoresis in a 2.0% Nusieve PCR gel (FC Bio, USA) stained with ethidium bromide and run at about 100 volts. The gel was viewed under ultraviolet illuminator (Ultra Lum Ins., California, USA) and the image of the resulting band was captured with a Polaroid camera.

Dengue virus detection using Ae. albopictus clone C6/36 cells

The method employed was modified from Maneekarn. The C6/36 cells were grown in...
minimum essential medium (MEM) growth media supplemented with 10% fetal calf serum (FCS) for 2 days until cell monolayers were formed in culture tubes. Pooled mosquitoes were homogenized on ice in 1.5 ml MEM medium with 5% fetal calf serum. 100 ul each of the homogenate were passed through 0.2 um filters and inoculated into respective culture tubes. The culture tubes were then vortexed and left to incubate for 2 hours at ambient temperature for adsorption. Maintenance medium containing 2% FCS was added and the culture tubes were incubated at 28 °C for 7 days.

Smear preparation

The culture tubes were vortexed and centrifuged after 7 days’ incubation period. Cells from the sediments were transferred onto the Teflon-coated, 12 well slides where each slide would have a maximum of 7 test smears, 4 positive control and a negative control. The smears were left to dry at 28 °C for 4 hours in a class II biohazard cabinet with the air blower on. The smears were then fixed with cold acetone for 20 minutes. The cultures were stored at –20 °C for confirmation by a second or third passage in cell culture if only the initial results were positive.

Peroxidase-antiperoxidase (PAP) staining

PAP staining procedures were performed as previously described by Igarashi. The cells that were fixed with cold acetone reacted with dengue anti-serum at 1:1000 at room temperature for 40 minutes. The antibody is universally reactive to all 4 dengue serotypes. The cells were rinsed in PBS and reacted with rabbit anti-mouse IgG at 1:1000 for 40 minutes. The cells were rinsed in PBS again and then reacted with goat-anti rabbit IgG at 1:1000 for 40 minutes. The cells were then rinsed in PBS and exposed to peroxidase-rabbit anti-peroxidase complex at 1:1000 for 40 minutes followed by washing and peroxidase reaction, using 0.2 mg/ml of 3,3 diaminobenzidine (DAB) and 0.2% H2O2 as the substrate. The cells were observed under a normal light compound microscope. The positive samples were reconfirmed by a second passage in cell culture.

Minimum Infection Rate (MIR)

The MIR was used to compare virus infection rates in Ae. aegypti and Ae. albopictus larvae at the sampling area. The MIR was calculated as (number of positive pools by species ÷ total number of that species tested) X 1000.

Virus Infection Rate (VIR)

The VIR was calculated as (number of mosquitoes by species infected with dengue virus ÷ total number of that species tested) X 100.

Results and discussion

In urban areas of Malaysia, artificial containers are the major larval habitats of Aedes mosquitoes in and near human habitation. The type and location, presence of shade and water conditions usually associated with water receptacles are known to affect breeding of Aedes mosquitoes.

The dominant mosquito larvae collected in the present survey were Ae. albopictus, whereas only very low number of containers were positive for Ae. aegypti larvae. Ae. aegypti tend to be more selective in nature compared to Ae. albopictus. Ae. aegypti larvae preferred heavily shaded areas. This preferential choice of shaded breeding area was due to the stability of water temperature in the artificial containers and therefore more conducive to breeding.
A total of more than 500 containers from 422 collection sites were examined and yielded a total of 9310 *Aedes* larvae comprising 5530 of *Ae. albopictus* and 3780 of *Ae. aegypti*. *Ae. albopictus* was more abundant than *Aedes aegypti*. The preferred sites for *Ae. albopictus* included artificial receptacles: tyres – 23.9%; plastic containers – 23.5%; tins – 16.5%; and earthen jars – 7.8% (Table 1). All these containers were found not only around houses and shops in urban areas but also in rubber plantations, coconut plantations and in the vicinity of houses in suburban areas. Overall, 179 artificial containers were positive for *Ae. aegypti* breeding and the preferred sites included artificial receptacles: tyres – 26.3%; plastic containers – 21.2%; tins – 16.2%; and paint cans – 9.5% (Table 1).

A total of 378 *Ae. aegypti* pools were selected randomly for virus detection. Of these, 33 pools were positive for dengue virus by cell culture and 19 pools were positive by RT-PCR. A total of 553 *Ae. albopictus* pools were assayed and 17 pools were positive by cell culture technique and 6 pools were positive by RT-PCR.

<table>
<thead>
<tr>
<th>No.</th>
<th>Type of containers</th>
<th>No. of breeding sites</th>
<th>No. of pools</th>
<th>% containers containing larvae</th>
<th>No. of breeding sites</th>
<th>No. of pools</th>
<th>% containers containing larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Tyres</td>
<td>47</td>
<td>87</td>
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<td>58</td>
<td>99</td>
<td>23.9</td>
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<td>Plastic containers</td>
<td>38</td>
<td>95</td>
<td>21.2</td>
<td>57</td>
<td>151</td>
<td>23.5</td>
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<tr>
<td>3.</td>
<td>Tins</td>
<td>29</td>
<td>54</td>
<td>16.2</td>
<td>40</td>
<td>105</td>
<td>16.5</td>
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<td>4.</td>
<td>Paint cans</td>
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<td>29</td>
<td>9.5</td>
<td>9</td>
<td>22</td>
<td>3.7</td>
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<tr>
<td>5.</td>
<td>Glass</td>
<td>14</td>
<td>19</td>
<td>7.8</td>
<td>13</td>
<td>32</td>
<td>5.3</td>
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<td>6.</td>
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<td>21</td>
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<td>Earthen jars</td>
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<td>19</td>
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<td>7.8</td>
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<tr>
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<td>Others</td>
<td>5</td>
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<td>2.8</td>
<td>7</td>
<td>19</td>
<td>2.9</td>
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<tr>
<td>9.</td>
<td>Flower pots</td>
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<td>–</td>
<td>–</td>
<td>4</td>
<td>17</td>
<td>1.6</td>
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<tr>
<td>10.</td>
<td>Polystyrenes</td>
<td>3</td>
<td>7</td>
<td>1.7</td>
<td>5</td>
<td>11</td>
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</tr>
<tr>
<td>11.</td>
<td>Cement</td>
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<td>11</td>
<td>1.7</td>
<td>2</td>
<td>7</td>
<td>0.8</td>
</tr>
<tr>
<td>12.</td>
<td>Coconut shells</td>
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<td>5</td>
<td>1.7</td>
<td>12</td>
<td>20</td>
<td>4.9</td>
</tr>
<tr>
<td>13.</td>
<td>Metals</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>4</td>
<td>9</td>
<td>1.6</td>
</tr>
<tr>
<td>14.</td>
<td>Water tanks</td>
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<td>–</td>
<td>–</td>
<td>3</td>
<td>5</td>
<td>1.2</td>
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<tr>
<td>15.</td>
<td>Banana leaves</td>
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<td>1</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>16.</td>
<td>Washing machines</td>
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<td>2</td>
<td>0.6</td>
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<td>–</td>
<td>–</td>
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<td>Drums</td>
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<td>1</td>
<td>1</td>
<td>0.4</td>
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<td>18.</td>
<td>Umbrellas</td>
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<td>–</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>19.</td>
<td>Zink</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>20.</td>
<td>Frying pans</td>
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<td>1</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>21.</td>
<td>Flower vases</td>
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<td>2</td>
<td>0.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>179</td>
<td>378</td>
<td></td>
<td>243</td>
<td>553</td>
<td></td>
</tr>
</tbody>
</table>

*10 larvae per pool*
The positive pools were collected from tyres, plastic containers, abandoned washing machine, glass and wheel barrow. We detected dengue virus in larvae by RT-PCR and cell culture technique because it was easier to collect larvae from field compared with adult mosquitoes. Infection rates of *Ae. aegypti* and *Ae. albopictus* in the survey are expressed as MIR and compared. Our study showed that MIR for *Ae. aegypti* was higher compared to *Ae. albopictus* despite higher number of *Ae. albopictus* samples. More positive pools came from tyres and plastic containers. Table 2 and 3 summarizes the results of the virus isolation. The figure shows agarose gel electrophoresis of RT-PCR of dengue virus. The correct size of the DNA product (480 bp) was obtained for each of the positive pools after amplification with universal dengue primers. The serotyping showed that the viruses belonged to DENV-1 and DENV-3.

Our findings suggest the occurrence of transovarial transmission of dengue virus by *Ae. aegypti* and *Ae. albopictus* in nature. Infected larvae were recovered from 16 localities (10 in Terengganu; 5 in Kuala Lumpur and 1 in Pahang). The virus infection rates (VIR) were higher in *Ae. aegypti* (13.7%) compared to *Ae. albopictus* (4.2%). Field isolations of dengue virus from *Ae. albopictus* larvae were reported in China\[16\] and Brazil.\[17\] Chung\[14\] and Rohani\[3\] reported that field-caught male mosquitoes of both *Ae. aegypti* and *Ae. albopictus* are capable of being infected with dengue virus in the natural environment. If the mechanism of infection in these infected maternal parents and the sex ratio for infection in the offspring was equal, it is likely that a similar proportion of females in the field were also infected via the same mode of transovarial transmission, without the need for the presence

### Table 2: Detection of dengue virus by RT-PCR

<table>
<thead>
<tr>
<th>Species/area</th>
<th>Species</th>
<th>No. of mosquito pools assayed per area</th>
<th>No of dengue virus detected (pool)</th>
<th>MIR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Terengganu</strong></td>
<td></td>
<td></td>
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MIR – Minimum infection Rate per 1000 mosquito larvae
* 10 larvae per pool
Detection of transovarial dengue virus from field-caught larvae of *Ae. aegypti* and *Ae. albopictus*

<table>
<thead>
<tr>
<th>Species/area</th>
<th>Species</th>
<th>No. of mosquito pools assayed per area</th>
<th>No. of dengue virus detected (pool)</th>
<th>Min. infection rate</th>
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</table>

MIR – Minimum infection rate per 1000 mosquito larvae
*10 larvae per pool

Table 3: Detection of dengue virus by C6/36 cell culture

In Malaysia, *Ae. aegypti* is the main vector involved in dengue virus transmission. *Ae. albopictus* plays an important but secondary role.[19] However, in an urban and suburban environment, they can share a breeding site.[20] *Ae. aegypti* is a very efficient vector for dengue viruses, highly receptive to oral infection, well adapted to urban environment (e.g. laying eggs only in artificial containers), and feeding exclusively on humans. On the other hand, *Ae. albopictus*, which is not highly orally receptive...
Detection of transovarial dengue virus from field-caught larvae of Ae. aegypti and Ae. albopictus

Figure: Detection of dengue virus in pools of Aedes mosquitoes electrophoresis of RT-PCR products on 3% agarose gels. Lane M – marker; lanes 1 - positive control of laboratory infected Ae. albopictus; lane 2 - negative control of uninfected Ae. albopictus; Lane 3, 4 - RT-PCR products from field Ae. albopictus pools positive for dengue virus; Lane 5 - negative control of uninfected Ae. aegypti; Lane 6, 7 - RT-PCR products from field Ae. aegypti pools positive for dengue virus; Lane 8 - positive control of laboratory infected Ae. aegypti; Lane 9 - negative control from cell culture and lane 10 - positive control from dengue virus culture.

to dengue virus, is present mainly in rural areas and does not feed exclusively on humans. Ae. albopictus could bridge a putative sylvatic and an urban cycle of dengue since it colonizes both rural and suburban breeding sites.\textsuperscript{21,22}

This study showed that both cell culture and RT-PCR techniques can be used to detect dengue virus from mosquito larvae. The results showed that the rate of dengue virus detection was significantly higher by cell culture technique (28.0%) than RT-PCR technique (9.5%). However, no conclusive observation can be made because these field-collected larvae were separated randomly for the study. Our study observed heavy cytopathic effects in dengue-infected C6/36 cell culture as observed by Singh and Paul.\textsuperscript{23} Gubler\textsuperscript{24} reported that some viruses grow slowly in C6/36 cells.

The mosquito inoculation technique and cell culture in C6/36 cell line are widely practised for the isolation of dengue virus from human serum. The availability of a number of tissue culture systems for the propagation of dengue virus has greatly facilitated studies on this agent. However, the low efficiency of infected cells and the slow development of virus within them remain major obstacles. Dengue-infected cells need 7–10 days to grow and, therefore, virus detection methods using mosquito cell line are time-consuming and relatively labour-intensive. Another
disadvantage to this method of flavivirus detection in field-caught mosquitoes has been the requirement to keep captured mosquitoes alive or frozen fresh to detect viral infection.

RT-PCR for the detection of flaviviruses in human clinical samples has been used for nearly a decade now.[25] The accuracy and speed of the RT-PCR assay makes it an appealing test for the diagnosis of dengue and epidemiologic surveillance. The present study indicates that PCR could detect dengue virus in mosquitoes using larvae collected from the field. Similar findings were also reported by Chao et al. (2007).[26] These techniques constitute practical molecular diagnostic and epidemiological tools for the virologic surveillance of dengue virus-infected Aedes mosquitoes to serve as an early warning system for dengue outbreaks.

In typical infections many types of mosquito tissues are eventually infected and the virus can persist in these tissues throughout the life of the vector. It is generally assumed that once a mosquito becomes infected with dengue it retains that infection for life.[27] Therefore, the collection of wild-caught mosquitoes would appear to be a reasonable approach to virus detection compared with attempts at virus recovery in human populations, which typically have very short periods (4-5 days) of detectable viremia.

Active surveillance for dengue virus infected mosquitoes can be an effective way to predict the risk of dengue infection in a given area. A need exists for better techniques to conduct timely, accurate and meaningful vector surveys and virus detection for estimating transmission risk among susceptible population in endemic areas. PCR could be considered for processing large numbers of samples in operational programmes, mapping areas with different levels of endemicity and stratification of areas, provided the technique is further refined to have higher specificity and sensitivity. A better understanding of dengue virus-vector relationships in the field and their association with human disease can help establish reliable vector population target levels for initiating measures to control and reduce virus transmission.

Acknowledgements

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References


Detection of transovarial dengue virus from field-caught larvae of Ae. aegypti and Ae. albopictus


A new approach to classify risk in dengue infection using bioelectrical impedance analysis

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bFaculty of Electrical Engineering, Universiti of Teknologi MARA, Malaysia
cDepartment of Cardiology, Hospital Pantai Putri Ipoh, Perak, Malaysia
dHealthmedic Research/Universiti Kebangsaan, Malaysia

Abstract

The study on risk classification was conducted on 183 hospitalized dengue patients (4 DF, 179 DHF). The severity of the risk criteria was determined based on three blood investigations, namely, platelet (PLT) count (less than or equal to 30 000 cells per mm³), haematocrit (HCT) (increase by more than or equal to 20%), and either aspartate aminotransferase (AST) level (raised by 5-fold the normal upper limit) or alanine aminotransferase (ALT) level (raised by 5-fold the normal upper limit). The patients were divided into three groups based on their risk factors and the corresponding bioelectrical impedance analysis (BIA) (i.e. bioelectrical tissue conductivity (BETC) parameters), namely, resistance (R), phase angle (α), body capacitance (BC) and capacitive reactance (Xc) were obtained and quantified. Using logistic regression analysis, Xc was found to be the best predictor in predicting the risk and severity in dengue patients. Subsequent two-way analysis of variance (ANOVA) found that there was a statistically significant relationship between the group categories and the change in Xc.

Hence, it was shown that BIA, as reflected by the Xc value, can effectively segregate between the lower-risk (female mean Xc = 60.70Ω, range 58.09Ω–63.43Ω; male mean Xc = 62.17Ω, range 59.92Ω–64.46Ω) and the higher-risk dengue patients (female mean Xc = 43.99Ω range 42.05Ω–52.09Ω; male mean Xc = 49.25Ω range 49.25Ω–52.09Ω), with the control data ((female mean Xc = 69.41Ω, range 67.09Ω–71.74Ω), and (male mean Xc = 64.19Ω, range 61.37Ω–67.15Ω)).

Keywords: Dengue infections; Bioelectrical impedance; Classify; Risk; Reactance.

Introduction

Dengue fever was first reported in Malaya (the former name for Malaysia) after an epidemic in Penang in 1902.1,2 Since then the disease has become endemic in the country with cases reported throughout the year. In 1998, there were 27 373 dengue cases with 58 deaths reported, as compared to 19 544 cases with 50 deaths in 1997. This has shown an increase
of 7829 cases or 40.1% in one year.\[^3\] However, the dengue haemorrhagic fever (DHF) case-fatality rate (CFR) increased to 1.7%.\[^4\] In 2001, there was a sharp increase in dengue cases in the country according to the vector-borne disease unit of the Ministry of Health, Malaysia.\[^5\] In 2003, the serologically confirmed dengue cases totaled 14,170, which number was slightly lower than the previous year (15,493). The CFR dropped to 4.5% as compared to 5.05% in 2002. The most deaths were seen in the 5–9-year-old age group, with adults mainly in the 20–35 years age groups. The dengue cases have seen a rise of 16% every year since 2003.\[^6\] Fatalities related to dengue cases reached record levels in 2004, when 102 fatalities were reported.\[^6\] In January 2005, the number of infections in parts of Malaysia rose significantly, with about 250 suspected dengue cases reported each week in Kuala Lumpur, compared to an average of 100 cases weekly in December 2003. An increase of 34.97%, representing 1416 cases, was recorded during the second week (Jan 9-15) of the year compared to the previous week (1049 cases).\[^7\] Forty-four dengue patients died in the first four months of 2007 out of 16,214 cases reported, compared to 21 deaths and 10,244 cases in the same period last year.\[^6\]

Due to the constantly high fatality rate, an accurate determination of the prognosis of the disease plays a very important role in the patient management, so that the high risk patients can be treated more intensively in their early phase to improve survival. A non-invasive prognostic system for dengue infection is yet to be developed and deployed in Malaysia. Due to the endemic dengue phenomenon in the country, there is a real and obvious need for such a prognostic system to be made available as soon as possible.

This paper describes a novel non-invasive approach to monitor and classify the daily risk in dengue patients using the single-frequency bioelectrical impedance technique. The research findings illustrate that any change in the value of reactance ($X_C$) will indicate the changes in electrical conductivity of the body and this can be used to monitor and classify the risk severity in dengue patients.

### Principles of bioelectrical impedance in humans

Impedance ($Z$) is a measurement of the ability of a medium to conduct current. All conductive materials have impedance, including living tissues. The impedance of various organisms is known as bioelectrical impedance, which may vary seasonally, diurnally, and when stimulated with an assortment of stimuli. An example of the said stimulus includes physiological changes that occur when a body is affected by fever caused by bacterial and viral infections.

Bioelectrical impedance analysis (BIA) is the assessment of changes in electrical tissue conductivity that indicate altered body composition. The impedance of the body is made up of two components. The resistance ($R$) is the index of conductivity determined by energy-dissipating characteristics of the body. The reactance ($X_C$) is the index of conductivity determined by the energy-storing characteristics of the body.

In the healthy living body, the cell membrane consists of a layer of non-conductive lipid material sandwiched between two layers of conductive protein molecules. The structure of cell membranes makes them capacitive reactive elements which behave as capacitors when exposed to an alternating current (Figures 1a & 1b).\[^8\] Although the total body water and extracellular water offer resistance to electrical current, only cell membranes offer capacitive reactance. Since fat tissue cells are not surrounded by cell membranes, reactance is
not affected by the quantity of body fat. Typical total body BIA measurements display the vectors of resistance and reactance, which are intrinsically based on a series network of resistors and capacitors (Figure 2).

Biologically, the cell membrane functions as a selectively permeable barrier separating the intracellular and extracellular fluid compartments. It protects the interior of the cell while allowing passage of some materials to which it is permeable. The cell membrane maintains a fluid osmotic pressure and ion concentration gradient between the intracellular and extracellular compartments. This gradient creates an electrical potential difference across the membrane which is essential to cell survival. Damage to the cell membrane, and its functions, is as lethal to the cell as direct damage to the nucleus itself.
Resistance is indirectly related to the extracellular mass and reactance ($X_C$) is indirectly related to the intracellular mass. Low $X_C$, which correspond to the inability of cells to store energy and indicates of cell breakdown in selective permeability of cell membranes. On the contrary, high $X_C$, indicates large quantities of intact cell membranes and body cell mass.\textsuperscript{9,10,11}

**Serological study**

Blood samples were taken from patients with a clinical diagnosis of DF and DHF during admission and discharge to be used in the serological tests. Sera were stored at $-20^\circ\text{C}$ for serological tests. Total white cell count (TWBC), platelet counts (PLT) and haematocrit (HCT) or packed cell volume (PCV) determination were done using an automated STKS counter (Street Scientific, Holliston). This unit was calibrated everyday with a standardized specimen to ensure that the accuracy and precision lay within the acceptable range.

All the dengue patients’ serum samples were tested in the HUKM for serological evidence of acute dengue virus infection using IgM capture enzyme-linked immunosorbent assay (ELISA)\textsuperscript{12,13,14,15} or PanBio (2001)\textsuperscript{16} dengue duo IgM and IgG rapid strip test. If the findings of ELISA were inconclusive, additional haemagglutinin-inhibition (HI) assays were performed at the WHO collaborating Centre for Arbovirus Reference and Research (dengue and dengue haemorrhagic fever) in Department of Microbiology, University of Malaya Medical Centre (UMMC), Kuala Lumpur.

**Bioelectrical impedance measurement**

All patients were required to abstain from eating and drinking for four hours and from alcohol and physical exercise for 12 hours prior to the BIA measurement. These protocols were implemented to ensure accurate body composition results. Informed consent was obtained for each patient and anthropometrics measurements (height and weight) were taken at admission. The clinical data comprised of date of onset of fever, daily surveillance of symptoms, signs, physical examination, blood results from laboratory tests and the bioelectrical impedance measurements. The
clinical data, weight and bioelectrical impedance measurement were taken daily, on admission, and upon discharge. This in vivo technique involved the application of a small average constant current of less than 1 mA at a single frequency of 50 kHz through the human body, and measuring the body’s bioelectrical tissue conductivity (BETC) parameters, namely, $R$, phase angle ($\alpha$), body capacitance (BC), and capacitive reactance ($X_C$) via four surface electrodes.

Two electrodes were placed on the patient’s right hand, one at the base of the knuckles and another slightly above the wrist joint. Another two electrodes were placed on the right foot, one near the base of the toes and the other slightly above the ankle joint. A constant current was applied to the base of the knuckles and base of the toes, and the signal was picked up by the other two sensor electrodes (slightly above the ankle and wrist joint) as shown in Figure 3.

**Study protocol and risk classification**

The clinical diagnosis and severity of dengue haemorrhagic fever grade I to IV were based on WHO recommendations. Since the patients were admitted at different stages of their illness, the daily progress of the patients was dated with reference to the day the fever subsided (‘Fever day +0’). Hence, ‘Fever day +0’ is defined as the day the fever subsided, i.e. when the body temperature fell below 37.5 °C. Days prior to the fever day +0 were designated as ‘Fever day –1’ (one day before the fever subsided), etc. Days after the fever subsided are designated as ‘Fever day +1’ (one day after the fever subsided) and so on.

In this study, the severity of dengue risk criteria was determined based on the earlier research findings and confirmed haematological profile (PLT, HCT, aspartate transaminase).
aminotransferase (AST), alanine aminotransferase (ALT)) analysis finding in this research. An exhaustive literature review conducted by Bandyopadhyay et al. reveals the difficulties of using the WHO case classification for DHF. It found that most clinicians reported difficulties in meeting all the four criteria: (i) fever; (ii) haemorrhagic tendencies; (iii) thrombocytopenia (100 000 cells per mm$^3$); and (iv) HCT >20% set by WHO. Therefore, most clinicians used the modified classification in their study. This study also faced the same problem and the criteria of the risk were not based on WHO classification solely; therefore, some modification was made. Thus, the severity of dengue risk criteria was determined based on the following blood investigations: (i) platelet (PLT) count was less than or equal to 30 000 cells per mm$^3$; (ii) haematocrit (HCT) increase by more than or equal to 20%; and (iii) aspartate aminotransferase (AST) rose by 5-fold the normal upper limit for AST and alanine aminotransferase (ALT) rose by 5-fold the normal upper limit for ALT. The patients were divided into three groups based on their risk factors: (i) Group 1 (lower risk group) accounted for DHF patients who did not experience any of the defined risk criteria, or experienced only one of the three risk criteria; (ii) Group 2 (higher risk group) accounted for DHF patients who experienced two or more risk criteria; and (iii) Group 3 (control data) was the control group for healthy female and male subjects. Once the dengue patients were classified according to their groups, the corresponding BETC parameters were subsequently obtained and quantified.

**Statistical analysis**

Data were analysed using the SPSS statistical package version 10.01 for Windows 1998. Kolmogorov-Smirnov test and simple linear regression were used for examining the normality assumption and for tests of significance. If the variable was not normally distributed, natural logarithm was used to transform the variables. Using logistic regression analysis, $X_c$ was found to be the best predictor in predicting the risk factors in dengue patients. Subsequent two-way analysis of variance (ANOVA) found that there was a statistically significant relationship between the group categories and the change in $X_c$. The mean of HCT and $X_c$ in the Groups 1 and 2 with fluid infusion and without were compared to find any significant changes.

**Results and discussions**

**Clinical dengue patients**

The 142 healthy controls (91 females and 51 males) had their BIA measured. The clinical data and BIA measurements were made on 97 male and 86 female DF and DHF patients during their hospitalization. The ages of the patients varied between 12 and 83 years (mean of 30.8 years). The length of hospital stay ranged from 2 to 18 days (mean of 4.7 days). Three female and one male patients were clinically diagnosed as DF, 83 as DHF I (39 females and 44 males), 92 as DHF II (40 females and 52 males) and 4 female patients as dengue shock syndrome (DSS). No DSS or fatal case was reported in the male DHF patients.

The detailed descriptive analysis of haematological profile (97 males and 86 female dengue patients) for PLT, HCT, AST and ALT are tabulated in Table 1. For example, the minimum platelet count recorded on the day of fever defervescence (‘Fever day 0’) was 8000 cell/mm$^3$, while the maximum was 136 000 cell/mm$^3$, with a mean of 37 400 ± 25 cell/mm$^3$ for the male patients. The females’ minimum and maximum documented platelet counts were 7000 cell/mm$^3$ and 162 000 cell/mm$^3$ respectively with a mean of 48 000 ± 35
Risk measurement of dengue infection using bioelectrical impedance analysis

Table 1: Haematological profile for PLT, HCT, AST, and ALT in dengue patients

<table>
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<tr>
<th>Fever days</th>
<th>PLT (1000 cells/mm³)</th>
<th>HCT (ratio)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
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<td>Range</td>
<td>Mean±SD</td>
<td>Range</td>
<td>Mean±SD</td>
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<tr>
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<td>(8–136)</td>
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<td>(32.5–58.6)</td>
<td>44.7±6</td>
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<tr>
<td>+1</td>
<td>(7–151)</td>
<td>48.4±32</td>
<td>(24.4–65.7)</td>
<td>43±6</td>
</tr>
<tr>
<td>+2</td>
<td>(10–212)</td>
<td>67.1±4</td>
<td>(28.3–52.2)</td>
<td>41.9±4</td>
</tr>
<tr>
<td>+3</td>
<td>(12–259)</td>
<td>90.1±4</td>
<td>(29.2–51)</td>
<td>41.6±4</td>
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<tr>
<td>+4</td>
<td>(22–195)</td>
<td>99.8±5</td>
<td>(29.7–49.9)</td>
<td>41.5±5</td>
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Average mean ALT 4.4-fold than normal upper limit for ALT
Average mean AST 5.3-fold than the upper limit for AST

<table>
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<tr>
<th>Fever days</th>
<th>PLT (1000 cells/mm³)</th>
<th>HCT (ratio)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
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<td>Range</td>
<td>Mean±SD</td>
</tr>
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<td>(12–289)</td>
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<td>(13–259)</td>
<td>88.3±53</td>
<td>(26.7–45.7)</td>
<td>36±4</td>
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<tr>
<td>+4</td>
<td>(14–258)</td>
<td>113.4±67</td>
<td>(24.2–40.5)</td>
<td>34.6±4</td>
</tr>
</tbody>
</table>

Average mean ALT 8.7-fold than normal upper limit for ALT
Average mean AST 28.7-fold than the upper limit for AST

The reference value for normal range of PLT, HCT, ALT and AST are based on Berhman and Kliegman[26]

cell/mm³. The mean platelet counts observed for both male and female dengue patients was low on the day of fever defervescence (‘Fever day 0’) and remained low for the next 2 to 4 days (‘Fever days 1 to 4’). In this study, many DHF patients with platelet count (mean ± SD) of lesser than 100 000 cell/mm³ were observed to be not ill (Table 1). However, it was observed that the criteria of platelet count <30 000 cell/mm³ identified the more severe cases.

The distribution of serum transaminase is also shown in Table 1. The mean levels of ALT and AST on ‘Fever day 0’ for male subjects were 177.9±138 U/l (range 12–580 U/l) and 291.1±241 U/l (range 79–696 U/l) respectively. On
the other hand, the female mean levels of ALT and AST on ‘Fever day 0’ were 267.7 ± 458 U/I (range 13–2270 U/I) and 755.5 ± 586 U/I (range 341–1170 U/I) respectively. The results indicate that both male and female dengue patients had liver involvement (hepatocyte injury), ranging from mild to moderate degrees, as indicated by the elevation in the transaminase levels (>5-fold greater than the normal upper limit of AST and ALT).

It was also observed that the AST level was higher than that of ALT level in female dengue patients, while in the male patients, it only occurred on fever days 0 and +1. The female mean levels of AST and ALT were higher (the average for fever days 0 to +4 was >9-fold greater than the normal upper limit of AST and ALT) than the value for male subjects for all fever days (the average for fever days 0 to 4 was <5-fold greater than the normal upper limit for the AST and ALT), as illustrated in Table 1. These findings are in agreement with those of Nguyen et al. and Kuo et al., who documented that the elevation in the transaminase levels were <5-fold and >10-fold greater than the normal upper limit for AST and ALT respectively. Thus, the criteria of 5-fold rise above the normal upper limit for AST and ALT were chosen.

The mean HCT levels for both female and male dengue patients were not much elevated with subsequent fever days. For example, on ‘Fever day 0’ the female and male HCT values were 38.9 ± 7% and 44.7 ± 6% respectively. The overall descriptive analysis of haematological profile (Table 1) showed that constant abnormalities occur in PLT, AST and ALT, while HCT only has a moderate elevation.

In the group’s risk categorization, 91 (49.7%) dengue patients were in the lower risk group, while 92 (50.3%) were in the higher risk group. The detailed dengue diagnosis breakdown is shown in Table 2. The higher risk group consists of 92 DHF patients, which was made up of 32 (17.5%) DHF I, 56 (30.6%) DHF II, and 4 (2.2%) DHF IV/DSS patients. The detailed descriptive analysis of haematological profile for PLT, HCT, AST and ALT in both lower and higher-risk patient groups in the duration of ‘Fever day 0’ to ‘Fever day +4’ are tabulated in Table 3.

It can be observed that both groups produced abnormal PLT, HCT, ALT and AST levels. Patients in the higher-risk group (Group 2) produced the lowest PLT values with an average of 7000 cells/mm³ for both genders. The distribution of serum transaminases (AST and ALT) indicate that the females in Group 2 had their ALT levels raised by 76-fold (2353/31) the normal upper limit (<31), whereas the AST levels were raised by 167-fold (5179/31) the normal upper limit (<31). On the other hand, the ALT and AST values for males in Group 2 were only raised by 14.5-fold and 18.8-fold the normal upper limits of ALT and AST, respectively (Table 3). These results show that patients in the higher-risk group (Group 2) had a high degree of liver involvement. In addition, the HCT values for female (55.9) and male (65.7) subjects in Group 2 had exceeded the upper normal values (37–47) of HCT at 47 and 54 respectively (see Table 3).

### Table 2: Dengue diagnosis breakdown according to risk group

<table>
<thead>
<tr>
<th>Groups</th>
<th>DF</th>
<th>DHF I</th>
<th>DHF II</th>
<th>DHF IV/DSS</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower risk (Group 1)</td>
<td>4</td>
<td>51</td>
<td>36</td>
<td>Nil</td>
<td>91</td>
</tr>
<tr>
<td>Higher risk (Group 2)</td>
<td>Nil</td>
<td>32</td>
<td>56</td>
<td>4</td>
<td>92</td>
</tr>
<tr>
<td>Dengue diagnosis cases</td>
<td>4</td>
<td>83</td>
<td>92</td>
<td>4</td>
<td>183</td>
</tr>
</tbody>
</table>

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**Table 4**: Shows the value of HCT and $X_C$ for the dengue patients in Groups 1 and 2, without or with intravenous fluid. At ‘Fever days 0-4’, the mean values of HCT and $X_C$ without fluid infusion (HCT = 39.05, $X_C = 61.43$ Ω) or with fluid infusion (HCT = 37.56, $X_C = 62.89$ Ω) in Group 1, do not differ much. For Group 2, at ‘Fever days 0-4’ the mean values of HCT and $X_C$ without fluid infusion (HCT = 37.75, $X_C = 51.31$ Ω) or with fluid infusion (HCT = 40.13, $X_C = 47.92$ Ω) in Group 2 also do not differ much. However, when these mean values of HCT and $X_C$ were compared between Groups 1 and 2, it was observed that the value of $X_C$ was always lower in the higher risk group (with or without fluid infusion) than the lower risk group.

The interesting results indicate that the fluid infusion does not alter the cell membrane (average reactance value) for ‘Fever days 0-4’ Group 1 (lower risk) patient without fluid infusion (61.43 Ω) and with fluid infusion (62.89 Ω), while Group 2 (higher risk) patient without fluid infusion (51.3 Ω) and with fluid infusion (47.92 Ω). However, the value of $X_C$ is different between the groups (Group 1, $X_C = 62.89$ Ω; Group 2, $X_C = 47.92$ Ω for fluid infusion) or (without fluid infusion Group 1, $X_C = 61.43$ Ω; Group 2, $X_C = 51.3$ Ω). Reactance can be the indicator for the dengue severity by the low value of reactance in the higher-risk group as compared to lower-risk group.

### BIA result analysis

Table 4 shows the value of HCT and $X_C$ for the dengue patients in Groups 1 and 2, without or with intravenous fluid. At ‘Fever days 0-4’, the mean values of HCT and $X_C$ without fluid infusion (HCT = 39.05, $X_C = 61.43$ Ω) or with fluid infusion (HCT = 37.56, $X_C = 62.89$ Ω) in Group 1, do not differ much. For Group 2, at ‘Fever days 0-4’ the mean values of HCT and $X_C$ without fluid infusion (HCT = 37.75, $X_C = 51.31$ Ω) or with fluid infusion (HCT = 40.13, $X_C = 47.92$ Ω) in Group 2 also do not differ much. However, when these mean values of HCT and $X_C$ were compared between Groups 1 and 2, it was observed that the value of $X_C$ was always lower in the higher risk group (with or without fluid infusion) than the lower risk group.

In lower-risk (Group 1) dengue patients with the fluid infusion, the HCT showed the decreased value, while the $X_C$ was high. On the other hand, for the higher-risk (Group 2) dengue patients with fluid infusion, the HCT showed an increased value, while the $X_C$ was low. At this moment, we do not have data on normal individual of the degree of $X_C$ in relation to the HCT, whether affected by the fluid infusion or not; however, in the lower-risk Group 1 the HCT tends to drop with fluid infusion with $X_C$ ($X_C = 62.89$ Ω) being higher, approaching the normal healthy individuals. Thus, we extrapolate further that in Group 1 the intracellular cell membrane is not much affected. On the other hand, in the higher-risk Group 2, with fluid infusion, the HCT continues to increase and this is reflected further by the $X_C$ ($X_C = 47.92$ Ω) that is relatively low. This shows that the intracellular cell membrane is low and is affected by the severity in dengue, and low $X_C$ is indicative of cell breakdown in selective permeability of the cell membrane. Thus, $X_C$ is the indicator for the degree of severity in dengue patients.
Theoretically, reactance in BIA is a measure of the volume of cell membrane capacitance and an indirect measure of the intracellular volume or body cell mass. Any changes in the cell membrane can be measured by reactance indirectly. In a normal healthy subject, if there is a fluid infusion, it will go to the intravascular space, and if there is an excessive fluid it will go to the bladder and output as urine. It should not leak out in the extravascular space. However, in dengue patients, the fluid will leak into the extravascular space via the fine capillary, and, in this case, the volume of the cell membrane and the skin are the major organs involved. This degree of fluid leakage can be indirectly measured by the reactance value which is well-recognized as one of the importance of increased vascular permeability in the pathophysiology of DSS. In the case of severe dengue, prompt and vigorous volume replacement therapy is required with extreme care to avoid fluid overload. If more fluid is infused, there will be leakage; however, it will prevent the patient from hypovolemic shock.\(^{[27,28]}\)

The degree of reactance value according to the WHO classification and the research classification for days 0–4 are illustrated in Figures 4 {a(i), b(i), c(i),d(i), and e(i), respectively}. The graphs in Figures 4 a(i) to e(i), which is classified by WHO, do not show any significant difference of reactance value in the degree of DHF I till IV. However, our proposed classification shown in Figures 4 a(ii) to e(ii) is more precise sub-

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**Table 4: Summary of reactance value with dengue patients with or without fluid intravenous**

<table>
<thead>
<tr>
<th>Fever days</th>
<th>Groups</th>
<th>Fluid infusion No=0, Yes=1</th>
<th>Reactance (Xc), Ω Mean±SD</th>
<th>HCT (ratio) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0</td>
<td>61.99 ± 12</td>
<td>39.92 ± 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>63.00 ± 14</td>
<td>38.84 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>53.31 ± 10</td>
<td>40.41 ± 6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>48.60 ± 12</td>
<td>41.28 ± 10</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>61.77 ± 10</td>
<td>39.04 ± 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>62.22 ± 12</td>
<td>39.41 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>49.58 ± 8</td>
<td>39.67 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>46.69 ± 10</td>
<td>40.20 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>62.50 ± 8</td>
<td>40.40 ± 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>57.92 ± 9</td>
<td>36.23 ± 6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>50.41 ± 11</td>
<td>37.84 ± 7</td>
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<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>48.55 ± 12</td>
<td>39.50 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0</td>
<td>62.20 ± 12</td>
<td>38.77 ± 4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>65.87 ± 11</td>
<td>37.01 ± 4</td>
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<tr>
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<td>0</td>
<td>51.16 ± 12</td>
<td>36.98 ± 6</td>
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<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>48.18 ± 12</td>
<td>39.86 ± 4</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0</td>
<td>58.67 ± 14</td>
<td>37.14 ± 4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>65.42 ± 16</td>
<td>36.34 ± 5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>52.07 ± 16</td>
<td>36.91 ± 5</td>
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<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>47.58 ± 14</td>
<td>39.84 ± 4</td>
</tr>
<tr>
<td>0-4</td>
<td>1</td>
<td>0</td>
<td>61.43 ± 11</td>
<td>39.05 ± 5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>62.89 ± 12</td>
<td>37.56 ± 5</td>
</tr>
<tr>
<td>0-4</td>
<td>2</td>
<td>0</td>
<td>51.31 ± 11</td>
<td>37.75 ± 6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>47.92 ± 12</td>
<td>40.13 ± 7</td>
</tr>
</tbody>
</table>
classification in the groups of DHF I till DHF IV. For example, in Figure 4 a(ii), dengue patients within DHF I and DHF II themselves can be sub-classified to their risk categorization (lower risk or higher risk). From Figures 4 a(ii) to e(ii), dengue patients in DHF I and DHF II can be sub-classified to their risk category {lower-risk (Group 1) and higher-risk (Group 2)} and there is a significant difference (p≤0.05) in the reactance between Groups 1 and 2 in DHF I and II for ‘Fever days 0 to 3’ {See Figures 4 a(ii) to d(ii)}. For ‘Fever day 4’ there is only significant difference (p≤0.05) in reactance between lower risk (Group 1) and higher risk (Group 2) in DHF I {See Figure 4e(ii)}. From Figures 4 a(i) to e(ii), it can be observed that there were many dengue patients from the DHF I who were at equal risk
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Figure 4 c(i): The reactance classified according to WHO classification at ‘Fever day 2’

Figure 4 c(ii): The reactance classified according to research proposed classification at ‘Fever day 2’

Figure 4 d(i): The reactance classified according to WHO classification at ‘Fever day 3’

Figure 4 d(ii): The reactance classified according to research proposed classification at ‘Fever day 3’

(indicated by the low reactance) as in the degree of DHF II patients. Surprisingly, there were many dengue patients from DHF II that were not at as much risk (indicated by high reactance) that they can be classified in the degree of DHF I. Thus, low reactance is the indicator for dengue severity regardless of the status of the degree of DHF in patients of DHF I and II.

Table 5 summarizes the mean reactance classification results in the duration of ‘Fever day 0’ to ‘Fever day +4’ for both the lower- (Group 1) and higher-risk (Group 2) groups. The results indicate that reactance can strongly differ between the higher- and the lower-risk groups. The females in the higher-risk group (Group 2) produced 44.0 Ω on average compared to 60.7 Ω produced by the females in the lower-risk
In addition, the males in Group 2 also produced a lower reactance value of 50.6 Ω compared to 62.2 Ω produced by those in Group 1 (see Table 5).

The trend for the overall mean reactance values for both genders in comparison with the lower- (Group 1) and higher-risk groups (Group 2) are shown in Figures 5 (a) and 5 (b). For female dengue patients, it can be observed that the mean reactance value decreases gradually with ‘fever days’. For example, ‘Fever day 0’ recorded a mean reactance value of 47.51 Ω and this decreases to 42.14 Ω on ‘Fever day 1’ (Figure 5(a)). On the other hand, the lower-risk group shows an increasing trend beginning on ‘Fever day +1’ at 59.14 Ω and ending at 59.68 Ω on ‘Fever day +4’.

Male patients, however, showed an increasing mean reactance value with fever days for both risk groups (Figure 5 (b)). The mean reactance value increases from 60.95 Ω (‘Fever day 0’) to 66.49 Ω (‘Fever day +4’) for patients in Group 1. Group 2, on the other hand, recorded an increase from 50.10 Ω (‘Fever day 0’) to 52.93 Ω (‘Fever day +4’).

It can be noted that although both groups show an increasing trend of improvement, the higher-risk group always experienced a lower value of reactance compared with the lower-risk group. Therefore, it may be concluded that male patients showed a positive response to clinical management and generally recovered faster than female patients. Female patients, thus, are at a higher risk than the males, especially those categorized in the higher-risk group (Group 2). The finding that the three fatal cases were all females supports this conclusion.

The mean reactance values with fever days for the DSS cases (3 fatal, 1 survived) are shown in Figures 6 (a) and 6 (b). The trend of the three fatal DSS cases (case 1, case 2, and case 3) in female patients is similar with the trend of the higher-risk group (Group 2), shown earlier in Figure 6 (a). Case 1 was a 12-year-old girl diagnosed with DSS with no past medical history, and presented with bleeding (i.e. vomiting and coughing blood, nose bleeding and tarry blood stool on admission day). She showed a decreasing trend of mean reactance value beginning on ‘Fever day -4’ (reactance = 58.9 Ω) and ending at ‘Fever day +2’ (reactance = 25.5 Ω), when she died due to multiple organ failure.
Case 2 was a 21-year-old female DSS patient without past medical history, who was admitted on ‘Fever day 0’ and manifested with bleeding (blood in the urine) and recorded a reactance value of 46.4 Ω. She obviously did not respond to the treatment and died on ‘Fever day +2’ with a reactance value of 22.5 Ω. Case 3 was a 45-year-old female DSS patient with a history of diabetes. She was admitted on ‘Fever day 0’ (\(X_C = 55.3\) Ω), and her reactance value gradually decreased until ‘Fever day +2’ (\(X_C = 24.9\) Ω). The value subsequently showed a slight increase on ‘Fever day +3’ (\(X_C = 29.2\) Ω) and ‘Fever day +4’ (\(X_C = 31.4\) Ω), before she died on ‘Fever day +5’ (\(X_C = 27.7\) Ω) due to multiple organ failure.

The sole DSS survivor was a 32-year-old female with no past medical history and bleeding manifestation. She was admitted at ‘Fever day –1’ (reactance 40.6 Ω) and developed hypotension on ‘Fever day 0’ (reactance 36.3 Ω). Her reactance gradually decreased to a minimum of 20.7 Ω on ‘Fever day 3’ before showing a slight increase on ‘Fever day +4’ to 25.1 Ω. This value gradually increased to 59.9 Ω at ‘Fever day 9’ and reached a maximum of 67.2 Ω on ‘Fever day +13’. The mean reactance value subsequently stabilized and decreased to 55.4 Ω on her discharge (‘Fever day +16’). She survived the

---

**Table 5: Summary of mean reactance value for lower-risk and higher-risk females and males**

<table>
<thead>
<tr>
<th>Fever days</th>
<th>Groups</th>
<th>Gender</th>
<th>Mean ± Standard Error (Ω)</th>
<th>95% Confidence Interval Minimum-Maximum (Ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>1</td>
<td>Female</td>
<td>60.70 ± 1.02</td>
<td>58.09 – 63.43</td>
</tr>
<tr>
<td>0-4</td>
<td>2</td>
<td>Female</td>
<td>43.99 ± 1.02</td>
<td>42.05 – 45.97</td>
</tr>
<tr>
<td>Control data</td>
<td>3</td>
<td>Female</td>
<td>69.40 ± 1.02</td>
<td>66.15 – 72.75</td>
</tr>
<tr>
<td>0-4</td>
<td>1</td>
<td>Male</td>
<td>62.17 ± 1.02</td>
<td>59.92 – 64.46</td>
</tr>
<tr>
<td>0-4</td>
<td>2</td>
<td>Male</td>
<td>50.65 ± 1.01</td>
<td>49.25 – 52.09</td>
</tr>
<tr>
<td>Control data</td>
<td>3</td>
<td>Male</td>
<td>67.15 ± 1.03</td>
<td>63.11 – 71.52</td>
</tr>
</tbody>
</table>

**Figure 5 (a): Mean reactance value for female patients in lower- and higher-risk groups**

**Figure 5 (b): Mean reactance value for male patients in lower- and higher-risk groups**
episode primarily due to the absence of bleeding and organ complications. Details of the reactance values for DSS female patients (3 fatal, 1 survived) are presented in Table 6.

**Conclusion**

A new approach to monitor and classify dengue patients using bioelectrical impedance analysis (BIA) has been described. The findings show that reactance ($X_c$) was found to be a potentially useful tool in classifying the risk in dengue patients.

**Acknowledgements**

This work was financially supported in part by Sultan Iskandar Johor Foundation Malaysian and University of Malaya. Our highest appreciation to Dean of Faculty of Faculty of Medicine, Universiti Kebangsaan Malaysia, the management and staff of Hospital Universiti Kebangsaan Malaysia (HUKM) for hosting and supporting this research and to all the dengue patients involved in this study. The HUKM Ethics Committee’s approval code is D-004-2002. We would also like to thank Dr Sharifah Ismail for the clinical data collection, Prof. Lucy Lum Chai See for clinical advise and Prof. Dato’ Lam Sai Kit from the University of Malaya for the serological support.

**Table 6:** Reactance values for fatal (Cases 1 to 3) and survived (Case 4) DSS female patients

<table>
<thead>
<tr>
<th>Fever days</th>
<th>Case 1 reactance ($\Omega$)</th>
<th>Case 2 reactance ($\Omega$)</th>
<th>Case 3 reactance ($\Omega$)</th>
<th>Case 4 reactance ($\Omega$)</th>
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<tbody>
<tr>
<td>-4</td>
<td>58.9</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>-3</td>
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References


Utilization of a geographical information system for surveillance of Aedes aegypti and dengue haemorrhagic fever in north-eastern Thailand

Anun Chaikoolvatana, Pratap Singhasivanon and Peter Haddawy

Abstract

The study aims to develop a geographical information system (GIS) for surveillance of Aedes aegypti and dengue haemorrhagic fever (DHF) in north-eastern Thailand. There are three steps in the development of the GIS – collecting primary and secondary data, analysing the data and searching the target location, and presenting the results via figures on a map. Two sub-districts in each of the five districts in Ubon Ratchathani province with high incidences of DHF cases in the last three years were investigated. Primary (e.g. House Index (HI), Container Index (CI) and Breteau Index (BI)) and secondary data (e.g. number of DHF cases/100 000 population) were collected. The time period was divided into two phases, a low disease incidence phase (February–March 2007) and a high disease incidence phase (June–July 2007). The GIS was developed via ArcView programme 3.2a. The primary data of Ae. aegypti indices including HI, CI and BI indicated a rise in the rainy season period compared with the dry weather period, and the secondary data showed a similar rise and fall in the number of DHF cases in the rainy and dry weather periods respectively. GIS technology can help in planning, implementation and evaluation of the dengue control measures.

Keywords: Geographical information system (GIS); Ae. aegypti indices; Geographical positioning system (GPS); Dengue haemorrhagic fever (DHF).

Introduction

Dengue haemorrhagic fever (DHF) caused by the mosquito species Aedes aegypti is one of the world’s major health problems. In Thailand, the reported cases of DHF vary from 20 000 cases to more than 100 000 cases annually. Recently, the incidence of DHF has increased in many provinces in the north-eastern part of the country, including Ubon Ratchathani, Si Sa Ket, Yasothon and Amnat Charoen. Ubon Ratchathani is ranked as the province with the fourth highest incidence of DHF in Thailand with 172.20 cases per 100 000 population (Figure 1).

A geographical information system (GIS) is an initiative to support dengue control in Thailand. It is an automated computer-based
system with the ability to capture, retrieve, manage, display and analyse large quantities of spatial and temporal data in a geographical context. Roads, residential buildings and other relevant data can be obtained and mapped to form the base map layer using the software Arcview GIS®. Other layers, such as patient populations, mosquito breeding, dengue case incidences, addresses, sensitive areas and containers, can also be mapped into the GIS. Among the publications on the use of GIS in DHF surveillance is included one concerning the development of a database system of mosquito breeding habitats. This led to the development of a system called “Mosquito Information Retrieval System (MIRS)”.[13] Another study conducted at the Notre Dame University developed a computer programme called “MODABUND” that aimed to provide basic information about mosquitoes such as breeding habitats, life cycles, types, epidemiology, diseases transmitted by mosquitoes and means of surveillance and control.[12] However, there have been limitations that include lack of professionals, restricted budget, inadequate knowledge of GIS applications and a lack of updated data. As a result, significant benefits of the use of a GIS in dengue surveillance and control have not been as successful as anticipated.[10,11,18,19] Because of these limitations, researchers are trying to develop a GIS programme to help public health officers gather data and information on DHF to assist in the control of this health problem.

This study is an attempt to develop a GIS for surveillance of *Ae. aegypti* and DHF in north-eastern Thailand.

**Materials and methods**

**Study area**

Ubon Ratchathani is located in north-eastern Thailand, about 629 kilometres from Bangkok (Figure 1). The terrain is generally flat with the river Moon flowing through the central area. The total area of the province is 15,517 sq. km. with highlands and mountains in the east of the province. The total population is about 1,600,000. There are three distinct seasons, dry (March-May), rainy (June-September) and cold (October-February). Average temperatures are from 25 °C to 35 °C depending on the season. The average rainfall is 1300–1800 mm per year.

**Implementing an ArcView programme 3.2a**

The GIS requires a language command programme called “Avenue” with the Dialog Design function of ArcView software 3.2a®. Users follow the menu and extract the required data. The overall structural functions include:
Figure 2: Development programme of a geo-database for surveillance of Ae. aegypti and dengue haemorrhagic fever in Ubon Ratchathani province

(a)

(b)
Use of GIS for DHF surveillance in Thailand

- Installation of primary and secondary data
- Analysis of data and a search of the target location
- Presentation of the results via figures on a map

In this study, epidemiological, digital, satellite and Global Positioning System (GPS) data were incorporated into GIS databases to better understand the spatial distribution of DHF cases (see Figures 2(a) and 2(b)).

**Process**

**Process of GIS development**

The project started in February 2007 and concluded in July 2007. The species of dengue mosquito studied was *Ae. aegypti*. Ubon Ratchathani province in the lower north-eastern part of Thailand was selected as the study site due to its high incidence of DHF cases. The study focused on two sub-districts in each of the five districts in the province with the highest incidences of DHF cases in the last three years. Within each sub-district, one village was selected as part of the study, making a total of 10 villages.

Two categories of data were collected:

**Primary data included:**
- dengue vector indices
- water storage containers

To quantify the relative density of mosquitoes, House Index (HI), Container Index (CI), and Breteau Index (BI) were taken as indicators of the density. For CI, all natural and artificial indoor and outdoor containers, such as water jars, tyres, cement tanks and ant guard jars, etc. in every house, were inspected to determine the presence or absence of *Ae. aegypti*. The primary data were collected by the visual larval survey. The positions of the houses in the ten villages were mapped using a GPS tool. Also, data including village names, house addresses, demographic primary data, breeding sites and containers were mapped and imported into a GIS software for further construction (ArcView 3.2a®).

**Secondary data included:**
- number of reported DHF cases/100 000 population

Secondary data on patient cases were reported by a primary care unit (PCU), local hospital or central hospital and collected by the staff of the Office of Disease Prevention and Control Department 7 (DPC), Ubon Ratchathani, using WHO case definition. Both primary and secondary data were collected by the same process. Data collections were divided into low disease incidence (February–March 2007; dry weather) and high disease incidence (June–July 2007; rainy season).

**Method of data analysis**

**Analysis of the primary data**

Dengue vector indexes were calculated into three variables:
- House Index (HI): Percent houses positive for containers with *Ae. aegypti* larvae or pupae
- Container Index (CI): Percent of water-holding containers positive for *Ae. aegypti* larvae or pupae
- Breteau Index: Number of containers positive for *Ae. aegypti* larvae or pupae per 100 houses.

**Analysis of secondary data**

The total number of DHF patients was presented as the number of reported cases of DHF/100 000 population of the province.
Results

Primary data

Dengue vector indices (HI, CI and BI) for each village during low incidence and high incidence periods are presented in Figures 3(a) and 3(b).

It may be seen from Figure 3(a) that during the low disease incidence period, HI ranged from 15.04 in village Pakhuaiwangnong to 50.88 in Nacharoen village while during the high disease incidence period it ranged from 25.16 in village Ang Hin Tai to 56.36 in Nongpaung village. CI and BI also followed similar trends as indicated in Figures 3(a) and (b).

Secondary data

The total number of reported DHF cases per 100,000 population in Ubon Ratchathani province are given in Figure 4. Comparisons were

Figure 3: *Ae. aegypti indices (HI, CI, and BI) for 10 villages*

3(a): *Low disease incidence period (February–March 2007)*

3(b): *High disease incidence period (June–July 2007)*
made between the low disease incidence period and high disease incidence period (Figure 4).

**Discussion**

The results show that the number of DHF cases increased during the high disease incidence period compared to the low disease incidence period, suggesting a positive correlation between the peak rainfall period in June-July and the high density of *Ae. aegypti* mosquitoes and high incidence of DHF cases. This finding is similar to that identified in other South-East Asian countries. Since the spread of the disease is facilitated by the movement of viraemic people, source-reduction methods should get priority in vulnerable areas (hotels, hospitals and other places of congregations). Generally, such practices undertaken in the low disease incidence period lead to a reduction in the vector receptivity (breeding potential) at the beginning of the wet season and this will have an impact on the number of DHF cases.

In Ubon Ratchathani, *Ae. aegypti* population density remains high in most areas and the incidence of DHF normally tends to increase every other year. This indicates that when an outbreak occurs, it spreads rapidly to nearby areas. The clustering of DHF cases in some big centres such as Muang, Detudon and Warin chamrap has also been attributed to the characteristics of *Ae. aegypti* because of its multiple feeding probe to complete one blood meal. In other south-east Asian countries, it has been reported that most infections occur among children who spend most of their time indoors and are bitten by the usually day-biting *Ae. aegypti*. The high incidence of DHF continues to occur due to lack of standard

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**Figure 4:** Total number of reported dengue haemorrhagic fever cases in Ubon Ratchathani province during low and high disease incidence periods

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mosquito control policies, poor sanitation and poor preventive measures.

The introduction and implementation of an effective remote-sensing tool such as a geographical information system (GIS) into the routine activities of the Disease Prevention and Control Programme staff needs to be considered. This tool can address some limitations regarding *Ae. aegypti* and DHF surveillance like updating, collecting, editing, locating and analysing dengue indices and DHF case data. By this process, rapid and effective prevention and control strategies can be developed prior to the onset of the disease that would eventually reduce both the morbidity and mortality rates of dengue infection.

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References

Use of GIS for DHF surveillance in Thailand


Transmission thresholds and pupal/demographic surveys in Yogyakarta, Indonesia for developing a dengue control strategy based on targeting epidemiologically significant types of water-holding containers

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\textbf{Abstract}

All water-holding containers (ca. 3000) associated with approximately 320 residences in Yogyakarta, Indonesia, were examined for the presence of \textit{Aedes aegypti} (L.), \textit{Aedes albopictus} Skuse, and \textit{Culex quinquefasciatus} Say pupae in four replicate surveys conducted during two dry seasons (1996 and 1998) and two wet seasons (1997 and 1999). Less than 6\% of these receptacles had pupae. \textit{Ae. aegypti} pupae collected were ten times more than \textit{Ae. albopictus} (ca. 1600 vs. 160 respectively); \textit{Cx. quinquefasciatus} was rarely encountered. From a dengue perspective, the epidemiological significance of a particular type of container is a function of the number of \textit{Ae. aegypti} pupae per person – calculated simply as the ratio of total number of \textit{Ae. aegypti} pupae recovered in that type and the number of residents, ca. 2800. Overall, there was an average of 0.57 \textit{Ae. aegypti} pupae per person. Assuming an overall herd immunity of 33\% and an average temperature of 29 °C, we estimate the transmission threshold in Yogyakarta to be approximately 0.43 \textit{Ae. aegypti} pupae per person. By eliminating mosquito production in two common household containers – wells and used tyres the number of \textit{Ae. aegypti} pupae would be reduced from 0.57 to 0.29 per person, below our estimate of the transmission threshold. An assessment of the effectiveness of this strategy is currently being conducted in a multi-year study in Yogyakarta using an insect growth regulator (IGR) for mosquito control.

\textbf{Keywords}: Dengue; Epidemiology; Prevention and control; Risk assessment; Targeted source reduction and control; Sustainable; Community-based; Transmission threshold.

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Introduction

Dengue in Indonesia

Dengue and dengue haemorrhagic fever (DF/DHF) was first observed in Indonesia in 1968 in Surabaya and Jakarta, the two largest metropolitan cities on the island of Java.\textsuperscript{[1]} While this initial epidemic involved less than 60 cases, the case-fatality rate exceeded 40%. Since then the incidence of DF/DHF has increased dramatically in Indonesia and has spread geographically to all regions of the country. The DHF incidence fluctuates monthly and typically reaches its peak in December and January every year, except in large cities such as Jakarta, Bandung and Surabaya, where the highest incidence is reported in April and May. Currently, dengue is the eighth leading cause of hospitalization among Indonesian children.\textsuperscript{[2]}

Similar trends of progressively larger epidemics interspersed with quieter, inter-epidemic years in the neighbouring countries of Cambodia, Myanmar, Laos, Thailand and Viet Nam reflect waxing and waning human population “herd” immunity, urbanization, the movement of people, and the influence of weather anomalies associated with El Niño/Southern Oscillation (ENSO) events. In the face of the most significant ENSO event of the century, 1998 witnessed the largest epidemic on record in Indonesia with 72,133 reported cases and 1,414 deaths; the case-fatality rate (CFR) in this epidemic was 2.0%, reflecting several decades of improved clinical management.\textsuperscript{[2]}

Past dengue control efforts

The World Health Organization’s Regional Office for South-East Asia (WHO/SEARO) conducted an external review of the dengue/dengue haemorrhagic fever prevention and control programme of Indonesia in June 2000.\textsuperscript{[2]} The following brief history of the Indonesian dengue prevention and control programme reflects this report.

The Indonesian Ministry of Health has considerably modified its strategies to control dengue over the past three decades. Initially, adult control using perifocal space spraying of insecticides with portable and vehicle-mounted thermal fogging and ultra low volume (ULV) machines was the government’s recommended method and response for most areas. The protocol specified treatment within a 100-metre radius of reported DHF cases. In the 1980s, the strategy changed to include the addition of extensive larviciding using temephos (1% sand granules). The policy was to treat all breeding sites in dengue-endemic urban areas a single time each year, timed ideally to precede the onset of the transmission season. The Ministry of Health subsequently modified this strategy to target only those urban areas reporting DHF for three consecutive years, wherein re-treatments were scheduled with a frequency of three months. This selective larviciding programme was implemented between 1986 and 1991. Beginning in 1992 and continuing until the present, the national strategic emphasis has been larval control involving community efforts, health education and intersectoral coordination. Currently, national efforts have focused on organizing working groups at the village level under the general guidance of local health centre personnel. This programme, called Bulan Gerakan (or 3M), emphasizes intensive health education using mass media, women’s groups and schoolchildren, community-based breeding source reduction, and door-to-door house inspections to monitor for larvae, and to clean containers and apply temephos as necessary. An important member of the 3M programme is the Family Welfare Education Women’s Movement (Pendidikan Kesejahteraan
Keluarga or PKK). The role of the PKK is education of the house-owner about larval inspections, methods to store water safely, and the elimination or cleaning of breeding containers. An additional role of the PKK involves community-based group education and monitoring programme results. Finally, authorities developed health education programmes for elementary schoolchildren and for use with the mass media.

Unfortunately, with only a few notable exceptions, these efforts have not been successful in controlling dengue. However, a pilot project in the city of Purwokerto, aided with funding from Rotary International has shown promise. The project organized a strategy of community partnership based on dasa wisma (ten houses) in several villages in the area. The results were encouraging to the extent that Rotary and others funded an extension to this effort that targeted 11 major urban areas in Indonesia. Patterned after the Purwokerto project, it included educational programmes for the public and medical personnel. The extended project had the endorsement and support of the Indonesian government, WHO, the US Public Health Service, and importantly, mayors’ offices. While funding was available and key public officials remained prominently associated with the project publically, e.g., the mayor’s wife, the effort did reduce dengue cases (Sustriayu Nalim, personal communication).

Proposed targeted source reduction and control efforts

The goal of the present work is to build on the foundation of the 3M and PKK programmes by reducing the number of types of breeding containers that must be controlled or eliminated to only a select few that are responsible for most of the adult vector production. We believe it is possible to estimate the degree of reduction required and to identify the types of containers that are particularly important by some recent developments outlined below. This strategy targets only the most epidemiologically important types of breeding containers. We measure the epidemiological importance of each type of container in the environment using the statistic the total standing crop of Ae. aegypti (L.) pupae per hectare or per person associated with each particular type.

Results from prior research

Transmission models

Recently, there has been a movement in the epidemiological community to recognize the pervasive influence of the environment and climate on various vector-borne diseases. The efforts of Martens et al. and Patz et al., for example, have documented substantial ties of disease activity to environmental features and climate trends for dengue, schistosomiasis and malaria. The work of Bouma et al. establishing statistical relationships between weather anomalies associated with El Niño and malaria in Colombia is especially encouraging in the context of developing early warning/mitigation systems for weather-driven infectious diseases.

Recognizing these ties, mathematical epidemiologists and public health specialists are beginning to construct disease models incorporating environment and climate parameters. A number of researchers have recently been involved in these types of studies on the dengue system and have developed simulation models and estimates of transmission thresholds. These results have had a degree of success and are being evaluated by the public health community. The algorithms of the dengue models take into account key factors known to influence dengue epidemiology; the result is a software tool used by researchers and public health practitioners that is orientated toward site-specific simulation.
Validation studies compared model output with field and laboratory observations at sites in Asia and the Americas.\textsuperscript{8} Funding from WHO and the US National Institutes of Health (NIH) has led to ongoing evaluations of transmission thresholds derived from the models in Viet Nam and Peru respectively. The hope is that the published thresholds\textsuperscript{11} can be used in tropical locations to predict disease vulnerability, assess control measures and provide guidance in targeting the especially important classes of breeding containers. The recent development of the pupal/demographic survey, coupled with estimates of transmission thresholds, make the results of simulation studies with the dengue models available to operational control programmes in the developing world.\textsuperscript{10,11,12} The models are currently being re-written and extended through funding from the Tahija Family Foundation (Jakarta) and the Innovative Vector Control Consortium (IVCC) funded by the Bill and Melinda Gates Foundation (the citation for IVCC/dengue is: Hemingway J, Beaty BJ, Rowland M, Scott TW, Sharp BL. The Innovative Vector Control Consortium: Improved Control of mosquito-borne diseases. Trends in Parasitology 2006 (22): 308-312.)

**Transmission thresholds and the pupal/demographic survey**

The expense and ineffectiveness of drift-based insecticide aerosols to either prevent or control dengue epidemics has led to suppression strategies based on eliminating larval breeding sites.\textsuperscript{13} With the notable, albeit short-lived, exceptions of Cuba and Singapore, these source-reduction efforts have met with little documented success. Public health workers attribute failure to two factors: inadequate participation of the communities, and a strategy that entailed destruction or treatment of virtually every breeding container in the environment. The transmission thresholds for dengue based on the standing crop of *Ae. aegypti* pupae per person\textsuperscript{11} were developed for use in the assessment of risk of transmission and to provide targets for the actual degree of suppression by type of breeding container required to prevent or eliminate transmission in source-reduction programmes. When coupled with field observations from pupal/demographic surveys (as reported herein for Yogyakarta), it is possible for the first time for control specialists to know how important the various types of containers in the environment are in terms of contributing to the transmission threshold.\textsuperscript{10,11} This strategy of concentrating on only the types of containers most responsible for the majority of adult vector production and hence transmission, e.g. outdoor drums and tyres vs typically low-producing indoor vases and domestic containers, was recently evaluated in a 9-country study in the Americas and SE Asia with WHO/TDR funding (citation is: Focks DA, Alexander N. Multicountry study of *Aedes aegypti* pupal productivity survey methodology. Findings and recommendations. 2006. TDR/IRM/Den/06.1)). The WHO’s Tropical Diseases Research (TDR) programme has commissioned a review article on the current state of the science for entomological surveying for dengue risk assessment and control.\textsuperscript{14} Central in this document are the concepts of the pupal/demographic survey, transmission thresholds and targeted source reduction and control of especially productive containers. There is a growing recognition that adherence to the current strategy of attempting to eliminate or control all containers, irrespective of productivity or time of the year, is doomed to continued failure.\textsuperscript{10}

**Dengue early warning system (EWS)**

In the last several years, there has been a call by the directors of national anti-dengue programmes in Indonesia, Thailand and Viet Nam for the operational need of an early warning system (EWS) that would provide sufficient lead time (1 to 3 months) to permit mobilization of
control operations. In response to this call, preliminary EWSs for Yogyakarta and Bangkok were developed; they are based on logistic regression analysis.\[15\] The predictor variables are sea surface temperature (SST) anomalies over the tropical Pacific and monthly cases of dengue in each city. The predicted variable is the probability of an epidemic year forecast 1 to 3 months before the peak transmission season. The Java EWS was sufficiently accurate to be operational. The Yogyakarta EWS gave perfect 1- and 2-month forecasts; the 3-month forecast incorrectly classified one year in the 14-year period of record.

**Biological control agents**

A very promising recent measure is the application of biological agents like *Mesocyclops*, *Micronecta* and larvivorous fish. In Viet Nam, *Mesocyclops*, a tiny copepod crustacean, have been found to be good predators of *Ae. aegypti* larvae. Since February 1993, *Mesocyclops* have been released into water containers of 400 houses of a hamlet in My Van district, Hai Hung province. In March 1994, this measure was supplemented by a campaign to eliminate discarded containers (which are too small for *Mesocyclops* to survive), and education about how to maintain adequate populations of *Mesocyclops* in domestic water containers. After 17 months, *Ae. aegypti* had been completely eliminated and this result has been sustained until today. Since July 1995, mobilization of the community for peridomestic hygiene and use of *Mesocyclops* to control *Aedes* larvae was implemented for 1600 houses in one commune in Thuong Tin district, Ha Tay province. Working together with the network of health collaborators, primary-school pupils, local authorities and health staff, and using the system of local communication, health education campaigns were organized in order to improve people’s knowledge and to mobilize every member of the community. Almost all big water containers in the commune received *Mesocyclops* or larvivorous fish; it is now very difficult to find discarded containers, and mosquito indices have been reduced almost to zero. From 1996, this model has been extended to three provinces, Nam Ha, Hai Hung and Hai Phong in northern Viet Nam.\[16\]

**Goals of the present work**

The purpose of this report is to provide an analysis of four annual pupal/demographic surveys conducted in Yogyakarta between 1996-1999 highlighting the epidemiological significance of the twenty-some types of *Ae. aegypti*-breeding containers in the environment. From this analysis, and using the estimates of transmission thresholds for dengue, we propose to develop a targeted source-reduction/control strategy for Yogyakarta that will require substantially less effort than the traditional community-based efforts without targeting where the goal is to control or eliminate all containers irrespective of their contribution to the adult population of *Ae. aegypti*.

**Methods**

**Study site**

Yogyakarta, a city of over 520 000 people, is the provincial capital of Yogyakarta, located in central Java. The province is divided into administrative districts called *kabupatens* with each district divided into progressively smaller units beginning with sub-districts called *kecamatans*, and these, in turn, divided into *kelurahans*, and further divided into *rukun warga* (RW), and finally into *rukun tetangga* (RT), the smallest administrative unit composed of approximately 50-80 families each. The study site was located in *kecamatan* Gondokusuman, within Yogyakarta city. The actual study area covered approximately 6.34 ha, from which
323 houses were selected and subsequently sampled over four time periods. Disease and demographic data were derived from a recent study conducted in the same area.\textsuperscript{17} An average of five houses were chosen from each RW where a dengue case had occurred and included 64 RWs distributed among five kelurahans (Kota Baru = 4 RWs; Terban = 11 RWs; Baciro = 21 RWs; Klatren = 16 RWs, and Demangan = 12 RWs). The location of each sampled house was provided coordinates in 1999 using a hand-held geographical positioning device (Magellan GPS 300\textsuperscript{tm}, San Dimas, CA). Demography data were taken from each house, including number of permanent residents, house size and land area. Climatological data (daily rainfall and maximum/minimum ambient temperatures) were obtained from the local Meteorology and Geophysics Agency (Station Bulaksumar, University of Gadjah Mada).

The pupal/demographic survey

Each premise was sampled consecutively for immature stages of container-breeding mosquitoes during four different time periods between 1996 and 1999, with two sample surveys during wet seasons and two during the dry periods of the year.\textsuperscript{*} A team of three or four people would visit each house and carefully inspect the inside and around the outside perimeter all natural and artificial containers for preimaginal stages of mosquitoes. The number and type of containers present at each house were recorded as well as the number of permanent residents. The presence or absence of mosquito larvae in each container was recorded without regard to the number present. Collections concentrated on quantifying pupal abundance by type of container. With the aid of a flashlight, hand-held fine-meshed netting devices and pipettes were used to remove all pupae from the container with captured specimens placed in white trays for easy observation. While in the field, all pupae would be first immobilized using hot water supplied from a thermos and immediately placed in labelled plastic bags containing 70\% ethyl alcohol and sealed. Bag labels included house number, container type, location (indoor or outdoor) and number of pupae collected. Additionally, all information, including lot and house size, was hand-recorded in a field logbook and later transcribed into a computerized database. The only water-holding containers not surveyed in this study were residential wells, which are known to harbour \textit{Ae. aegypti} and \textit{Culex quinquefasciatus} Say larvae.\textsuperscript{18}

Specimen identification

Preserved pupae were returned to the laboratory for identification. Using an illustrated key developed for this purpose, pupae were examined using a stereomicroscope and easily identified for species and sex.\textsuperscript{19} For the purposes of this study, pupae were identified as either \textit{Ae. aegypti}, \textit{Aedes albopictus} Skuse, or \textit{Cx. quinquefasciatus}, with all other pupae identified only to genera.

Results

With very few exceptions, the four pupal/demographic surveys conducted in the kecamatan Gondokusuman returned to the same ca. 316 houses each year. The residents associated with these houses numbered approximately 2800 (Table 1). Each exhaustive survey collected pupae from the ca. 3000 water-filled containers associated with the study houses. Approximately 5.5\% of the containers were positive for one or more pupae of \textit{Ae. aegypti}.  

Targeted source reduction/control strategy for dengue control

Table 1: Summary statistics for pupal/demographic surveys conducted in Yogyakarta, Indonesia, between 1996 and 1999

<table>
<thead>
<tr>
<th>Survey date</th>
<th>Season (Rainfall, avg. temp.)</th>
<th>Number of</th>
<th>Water-filled containers</th>
<th>Total pupae recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Residents</td>
<td>Inhabitants</td>
<td>Total</td>
</tr>
<tr>
<td>8-22 May 96</td>
<td>Dry (116, 27.9)</td>
<td>323</td>
<td>2865</td>
<td>2345</td>
</tr>
<tr>
<td>23 Jan-7 Feb 97</td>
<td>Wet (841, 27.0)</td>
<td>319</td>
<td>2859</td>
<td>3354</td>
</tr>
<tr>
<td>15-30 Sep 98</td>
<td>Dry (51, 28.1)</td>
<td>313</td>
<td>2757</td>
<td>3118</td>
</tr>
<tr>
<td>30 Mar-19 Apr 99</td>
<td>Wet (747, 27.3)</td>
<td>310</td>
<td>2680</td>
<td>3096</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1265</td>
<td>11161</td>
<td>11913</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td>316</td>
<td>2790</td>
<td>2978</td>
</tr>
</tbody>
</table>

*Total precipitation (mm) two months prior to survey and average temperature during survey (°C)

Figure 1: Plot of 30- and 60-day rainfall accumulations before surveys and the average of daily average temperature (°C) in Yogyakarta, Indonesia [Approximate dates of surveys are indicated by vertical, downward-pointing arrows. Total precipitation during the two months prior to each survey and the average temperature during survey are presented in Table 1]

Ae. aegypti, Ae. albopictus or Cx. quinquefasciatus. Approximately ten times more Ae. aegypti pupae were recovered than Ae. albopictus (ca. 1600 vs 160); Cx. quinquefasciatus was rarely encountered in the sites being examined. The average temperatures during the dry- and wet-seasons surveys were not substantially different, averaging 28.0 °C and 27.2 °C respectively (Figure 1). However, the accumulated rainfall for the 60 days prior to each dry-season survey was only about 10% of the wet-season accumulations (ca. 84 vs. 794 mm).
Targeted source reduction/control strategy for dengue control

Types and numbers of water-filled containers as a function of season and location

A total of 71 different types of water-filled containers were observed during the four surveys; many of these types, however, were only seen once or at most only a few times during the surveys. The 3, 5 and 11 most common types of containers accounted for >60%, >75% and 90% of all water-filled containers (Table 2). Some types were

Table 2: Most frequently encountered types of containers during surveys conducted during the dry and wet seasons in Yogyakarta, Indonesia

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Type</th>
<th>Dry</th>
<th>Wet</th>
<th>Mean</th>
<th>Proportion of mean</th>
<th>Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bird watering dish</td>
<td>670</td>
<td>681</td>
<td>675</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>2.</td>
<td>Bucket</td>
<td>549</td>
<td>619</td>
<td>584</td>
<td>0.20</td>
<td>0.42</td>
</tr>
<tr>
<td>3.</td>
<td>Storage in water closet a</td>
<td>538</td>
<td>549</td>
<td>543</td>
<td>0.18</td>
<td>0.61</td>
</tr>
<tr>
<td>4.</td>
<td>Water container (large) b</td>
<td>251</td>
<td>220</td>
<td>235</td>
<td>0.08</td>
<td>0.68</td>
</tr>
<tr>
<td>5.</td>
<td>Plastic water container</td>
<td>196</td>
<td>232</td>
<td>214</td>
<td>0.07</td>
<td>0.76</td>
</tr>
<tr>
<td>6.</td>
<td>Water container (large)</td>
<td>112</td>
<td>152</td>
<td>132</td>
<td>0.04</td>
<td>0.80</td>
</tr>
<tr>
<td>7.</td>
<td>Clay water container</td>
<td>91</td>
<td>90</td>
<td>91</td>
<td>0.03</td>
<td>0.83</td>
</tr>
<tr>
<td>8.</td>
<td>Refrigerator water pan</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>0.02</td>
<td>0.85</td>
</tr>
<tr>
<td>9.</td>
<td>Padasan</td>
<td>41</td>
<td>56</td>
<td>48</td>
<td>0.02</td>
<td>0.87</td>
</tr>
<tr>
<td>10.</td>
<td>Flower pot</td>
<td>19</td>
<td>78</td>
<td>48</td>
<td>0.02</td>
<td>0.88</td>
</tr>
<tr>
<td>11.</td>
<td>Flower vase</td>
<td>45</td>
<td>41</td>
<td>43</td>
<td>0.01</td>
<td>0.90</td>
</tr>
<tr>
<td>12.</td>
<td>Bottle</td>
<td>34</td>
<td>44</td>
<td>39</td>
<td>0.01</td>
<td>0.91</td>
</tr>
<tr>
<td>13.</td>
<td>Plant axil</td>
<td>2</td>
<td>77</td>
<td>39</td>
<td>0.01</td>
<td>0.92</td>
</tr>
<tr>
<td>14.</td>
<td>Tin can</td>
<td>17</td>
<td>53</td>
<td>35</td>
<td>0.01</td>
<td>0.93</td>
</tr>
<tr>
<td>15.</td>
<td>Tyre</td>
<td>10</td>
<td>47</td>
<td>28</td>
<td>0.01</td>
<td>0.94</td>
</tr>
<tr>
<td>16.</td>
<td>Drinking glass</td>
<td>4</td>
<td>51</td>
<td>27</td>
<td>0.01</td>
<td>0.95</td>
</tr>
<tr>
<td>17.</td>
<td>Pool, pond, tank</td>
<td>22</td>
<td>22</td>
<td>22</td>
<td>0.01</td>
<td>0.96</td>
</tr>
<tr>
<td>18.</td>
<td>Bowl</td>
<td>3</td>
<td>30</td>
<td>17</td>
<td>0.01</td>
<td>0.97</td>
</tr>
<tr>
<td>19.</td>
<td>Drum</td>
<td>11</td>
<td>16</td>
<td>14</td>
<td>0.00</td>
<td>0.97</td>
</tr>
<tr>
<td>20.</td>
<td>Fish pond</td>
<td>16</td>
<td>4</td>
<td>10</td>
<td>0.00</td>
<td>0.97</td>
</tr>
<tr>
<td>21.</td>
<td>Pan</td>
<td>7</td>
<td>8</td>
<td>8</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td>22.</td>
<td>Cover or lid</td>
<td>4</td>
<td>11</td>
<td>7</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td>23.</td>
<td>Plate, dish</td>
<td>2</td>
<td>12</td>
<td>7</td>
<td>0.00</td>
<td>0.98</td>
</tr>
<tr>
<td>24.</td>
<td>Clay water pot (small)</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>0.00</td>
<td>0.98</td>
</tr>
</tbody>
</table>

a Indonesian: Bak mandi
b Indonesian: Bak air
exclusively found either indoors or outdoors and others could be found in both locations. By location, 34 container types were observed indoors and 66 types outdoors. Rainfall accumulations preceding the surveys influenced the number of types of containers in the environment (Table 2). During the two dry-season surveys, a total of 26 and 40 different types of containers were observed indoors and outdoors respectively; the corresponding numbers for the two wet-season surveys were 29 and 60 different types respectively. However, somewhat surprisingly, the average number of water-filled containers was largely independent of the season of the surveys (Table 1); the average number of water-filled containers in the environment was 2732 and 2770 for the dry- and wet-season surveys respectively.

Numerically, the most common types of containers observed were (in descending frequency) bird watering dishes, buckets (ember), water storage container in water closet (bak mandi), large water tanks (bak air), plastic water containers (tempayan) and large water storage containers. These particular containers accounted for ca. 80% of all water-filled containers. Thirty-five of the 71 types observed were never found positive for the pupae of any species in any of the surveys. Table 2 provides a list of the 24 most common container types in descending order of abundance. Of the nine most common types, accounting for 87% of all containers, there were no significant changes in abundance as a function of season, suggesting that the most common types of containers are not rain-filled but filled manually. Those container types listed in Table 2 that are more commonly found in the wet season are also those that are located primarily outside of the residence, e.g. flowerpots, plant axils, tin cans and tyres.

Figure 2: Frequency of being positive for larvae in the four surveys conducted between 1996 and 1999 in the 18 most common types of water containers
Prevalence of larvae by season and container type

The average proportion of containers with larvae in the dry and wet seasons was 0.128 and 0.173 respectively. The prevalence of larvae in the 18 most common types of containers is presented in Figure 2. Recent evaluations of the utility of the traditional Stegomyia indices (the House, Container and Breteau indices, and various related derivations) concluded that: (i) they are of only limited operational value for measuring the entomological impact of larval control interventions; (ii) that they are not proxies for adult vector abundance; and (iii) are not useful in the development of targeted control strategies.\[14] Neither are they useful for assessing transmission risk because they do not take into consideration the epidemiologically important variables, including adult vector and human abundance, temperature and herd immunity in the human population. For these reasons, we will confine our analysis to the pupal data.

Table 3: Summary of numbers of Ae. aegypti and Ae. albopictus pupae collected and containers positive for the same by location (indoors or outdoors) based on survey year and season

<table>
<thead>
<tr>
<th>Year</th>
<th>Data</th>
<th>Indoor</th>
<th>Outdoor</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1996 (Dry)</td>
<td>Ae. aegypti pupae collected</td>
<td>1006</td>
<td>388</td>
<td>1394</td>
</tr>
<tr>
<td>1997 (Wet)</td>
<td></td>
<td>987</td>
<td>1149</td>
<td>2136</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td></td>
<td>858</td>
<td>371</td>
<td>1229</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td></td>
<td>935</td>
<td>653</td>
<td>1588</td>
</tr>
<tr>
<td>Averages</td>
<td></td>
<td>947</td>
<td>640</td>
<td>1587</td>
</tr>
<tr>
<td>1996 (Dry)</td>
<td>Ae. albopictus pupae collected</td>
<td>0</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>1997 (Wet)</td>
<td></td>
<td>39</td>
<td>356</td>
<td>395</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td></td>
<td>0</td>
<td>33</td>
<td>33</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td></td>
<td>2</td>
<td>165</td>
<td>167</td>
</tr>
<tr>
<td>Averages</td>
<td></td>
<td>10</td>
<td>149</td>
<td>160</td>
</tr>
<tr>
<td>1996 (Dry)</td>
<td>Containers with Ae. aegypti pupae</td>
<td>89</td>
<td>46</td>
<td>135</td>
</tr>
<tr>
<td>1997 (Wet)</td>
<td></td>
<td>82</td>
<td>120</td>
<td>202</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td></td>
<td>71</td>
<td>43</td>
<td>114</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td></td>
<td>85</td>
<td>58</td>
<td>143</td>
</tr>
<tr>
<td>Averages</td>
<td></td>
<td>82</td>
<td>67</td>
<td>149</td>
</tr>
<tr>
<td>1996 (Dry)</td>
<td>Containers with Ae. albopictus pupae</td>
<td>0</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>1997 (Wet)</td>
<td></td>
<td>3</td>
<td>61</td>
<td>64</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td></td>
<td>1</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>Averages</td>
<td></td>
<td>1</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>1996 (Dry)</td>
<td>Number of wet containers</td>
<td>1026</td>
<td>1319</td>
<td>2345</td>
</tr>
<tr>
<td>1997 (Wet)</td>
<td></td>
<td>1221</td>
<td>2133</td>
<td>3354</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td></td>
<td>1322</td>
<td>1796</td>
<td>3118</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td></td>
<td>1244</td>
<td>1852</td>
<td>3096</td>
</tr>
<tr>
<td>Averages</td>
<td></td>
<td>1203</td>
<td>1775</td>
<td>2978</td>
</tr>
</tbody>
</table>
Table 4: Average total numbers of pupae collected as a function of season and location

<table>
<thead>
<tr>
<th>Season</th>
<th>Species of mosquito</th>
<th>Inside</th>
<th>Outside</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry</td>
<td><em>Ae. aegypti</em></td>
<td>932</td>
<td>380</td>
<td>1312</td>
</tr>
<tr>
<td></td>
<td><em>Ae. albopictus</em></td>
<td>0</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td><em>Cx. quinquefasciatus</em></td>
<td>0</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Wet</td>
<td><em>Ae. aegypti</em></td>
<td>961</td>
<td>901</td>
<td>1862</td>
</tr>
<tr>
<td></td>
<td><em>Ae. albopictus</em></td>
<td>20</td>
<td>260</td>
<td>281</td>
</tr>
<tr>
<td></td>
<td><em>Cx. quinquefasciatus</em></td>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

Figure 3: Total number of *Ae. aegypti* pupae recovered during the four surveys in Yogyakarta by type of container and location (indoors or outdoors) [Containers are distributed across the horizontal axis in descending order of total standing crop in that type. The container types shown are the 18 most productive classes of containers]

Standing crop of pupae as a function of the number of containers, container type and season

The average proportion of containers with pupae in the dry and wet seasons was 0.047 and 0.061 respectively. Of the pupae collected in all surveys, independent of season, *Ae. aegypti* accounted for 89.8%, *Ae. albopictus* 9.0% and *Cx. quinquefasciatus* 1.2% of the total (Table 1). A summary of pupal collection as a function of survey, container location, species of mosquito
and season (Table 3) indicates that *Ae. aegypti* can be found both in indoor and outdoor containers, and that the number of pupae coming from outdoor containers increases during the rainy season; the standing crop of *Ae. aegypti* indoors is remarkably constant and independent of season. Outdoor breeding accounts for virtually all additional production during the rainy season. In contrast, *Ae. albopictus* pupae are essentially found only outdoors and in rain-filled containers. It is therefore not surprising that the average standing crop of *Ae. aegypti* is somewhat less a function of rainfall (dry season average—1312 vs wet season—1862, an increase of ca. 42%) than *Ae. albopictus* (dry season average—38 vs 281 in wet season, an increase of 640%). Table 4 provides an average of total pupal collections for the two seasons by mosquito and location. While the numbers collected are low and preclude confident statements, *Cx. quinquefasciatus* immatures in these surveys were only found outdoors and their abundance seems to be independent of rainfall. *Aedes albopictus* immature abundance is a strong function of rainfall and they are only found outdoors.

The total numbers of *Ae. aegypti* pupae recovered in the four surveys are combined to provide the best estimate of production by container type (Figure 3). The total numbers of *Ae. aegypti* pupae in each of the types of containers highlights an important point: the epidemiological importance of a class of containers is not simply a function of the abundance of the containers, but rather the product of the containers’ abundance and average standing crop of pupae (Table 5). The water storage containers located in water closets (bak mandi) account for 22% of all containers but 50% of all *Ae. aegypti* pupae. The classes “large water container” (bak air) and “tyre” account for 6% and 1% of all containers, yet they are responsible for 13% and 6% of all pupae respectively. The large water tank (12% of all containers) contributes essentially nothing to *Ae. aegypti* production.

<table>
<thead>
<tr>
<th>Container type</th>
<th>Proportions of total: Containers</th>
<th>Ae. aegypti pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage in WC</td>
<td>0.217</td>
<td>0.496</td>
</tr>
<tr>
<td>Water container (lg)</td>
<td>0.063</td>
<td>0.135</td>
</tr>
<tr>
<td>Tyre</td>
<td>0.012</td>
<td>0.055</td>
</tr>
<tr>
<td>Clay water container</td>
<td>0.041</td>
<td>0.042</td>
</tr>
<tr>
<td>Bird watering dish</td>
<td>0.119</td>
<td>0.041</td>
</tr>
<tr>
<td>Bucket</td>
<td>0.150</td>
<td>0.036</td>
</tr>
<tr>
<td>Flower pot</td>
<td>0.016</td>
<td>0.036</td>
</tr>
<tr>
<td>Trash can</td>
<td>0.000</td>
<td>0.035</td>
</tr>
<tr>
<td>Tin can</td>
<td>0.016</td>
<td>0.026</td>
</tr>
<tr>
<td>Container</td>
<td>0.081</td>
<td>0.016</td>
</tr>
<tr>
<td>Flower vase</td>
<td>0.015</td>
<td>0.014</td>
</tr>
<tr>
<td>Fish pond</td>
<td>0.003</td>
<td>0.014</td>
</tr>
<tr>
<td>Refrigerator water pan</td>
<td>0.030</td>
<td>0.013</td>
</tr>
<tr>
<td>Pool, pond, tank</td>
<td>0.007</td>
<td>0.011</td>
</tr>
<tr>
<td>Bowl</td>
<td>0.008</td>
<td>0.005</td>
</tr>
<tr>
<td>Drum</td>
<td>0.007</td>
<td>0.005</td>
</tr>
<tr>
<td>Water tank (lg)</td>
<td>0.123</td>
<td>0.004</td>
</tr>
</tbody>
</table>

* Indonesian: Bak mandi
  b * Indonesian: Bak air

The total number of *Ae. albopictus* pupae recovered in each survey was correlated with the number of water-holding containers present (0.84). The likely explanation is that *Ae. albopictus* largely breeds in outdoor containers, which are substantially filled by rainwater; there is seasonality in abundance as a function of the number of containers (Table 4). In contrast, the total number of *Ae. aegypti* pupae recovered in each survey was independent of the number of water-holding containers present (correlation: −0.10). This is a bit unexpected in so far as *Ae. aegypti* production in outdoor containers increases substantially in the rainy season (cf. Tables 3 and 4). The total numbers of *Ae. aegypti* and *Ae. albopictus* pupae caught in each survey were only slightly correlated (0.32).
positive houses were more than three times more likely to remain positive over time than a negative premises that was to become positive over the course of a year.[21,22] These persistently productive premises were given the name key premises. Their notion was that, in government suppression programmes with limited resources, it might improve effectiveness to focus on former key premises for subsequent visits rather than use a systematic approach of visiting every house. It should be noted that the effectiveness of such a strategy would be dependent not only on the existence of a clumped distribution of productivity at the house level, but also on the persistence of productive houses between years.

With this in mind, we looked into the nature of the distribution of pupae per household. The results presented in Figure 4 clearly indicate a non-linear distribution associated with the existence of key premises. The second question – the persistence or stability of such households between years – is addressed in Table 6. Here, the number of Ae. aegypti pupae recovered at each premises in each of our sequential surveys is summed; and the premises are then sorted in descending order by this sum. Table 6 presents the 28 most productive households, our key premises. While they account for only ca. 8% of all homes surveyed, they accounted for 51% of all Ae. aegypti pupae recovered. The important conclusion to be drawn from this, however, is that unusually high (or low) counts of pupae in a particular survey are not highly correlated with subsequent or previous counts; this is further corroborated by the low correlation of Ae. aegypti pupae per house (Table 7) – average correlations for surveys separated by 1, 2 and 3 years were 0.11, 0.00 and 0.13 respectively. This lack of inter-year correlation is also observed with the number of Ae. albopictus pupae per house – average correlations for surveys separated by 1, 2 and 3 years were 0.08, 0.14 and 0.00 respectively (Table 8).

**Figure 4: Frequency histogram of the numbers of Ae. aegypti pupae per house [Note that the frequency of the 0 to 5-interval class (0.80) is off scale]**

<table>
<thead>
<tr>
<th>Frequency</th>
<th>0.06</th>
<th>0.05</th>
<th>0.04</th>
<th>0.03</th>
<th>0.02</th>
<th>0.01</th>
<th>0.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40-60</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60-80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80-100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100-120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120-140</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>140-160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>160-180</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180-200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Year-to-year variability in production at the household level**

**Especially productive households – key premises**

In an effort to facilitate the location of positive premises and containers in Queensland, Australia, Tun-Lin et al. used various forms of statistical analyses to develop the Premise Condition Index (PCI). In essence, they were looking for proxies or surrogates to detect the presence of high-level outliers among containers and premises.[20] They found that the condition of the house, the degree of shade and tidiness of the yard, both observable without entering the house or yard, were strongly correlated with both the proportion of positive premises and the numbers of infested containers. If only premises with the highest PCI scores were surveyed, they found that the probability of finding a positive home or container was increased approximately fourfold. These particular houses represented <10% of all sites, yet they accounted for 35% of all positive containers. An important observation was that
Table 6: Listing of the households responsible for the highest production of Ae. aegypti pupae during all four surveys

[By way of explanation, the first row of data are the results for house TE179. A total of 247 Ae. aegypti pupae were recovered at this household during the surveys conducted between 1996 and 1999; this total represents 0.039 of all production. The table indicates that about 8% of the houses were responsible for ca. 51% of all production observed. The table indicates that unusually high counts of pupae in a particular survey are not highly correlated with subsequent or previous counts.]

<table>
<thead>
<tr>
<th>House identification</th>
<th>Survey</th>
<th>Total Ae. aegypti pupae</th>
<th>Proportion of production</th>
<th>Cumulative proportion of production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1996 (Dry)</td>
<td>1997 (Wet)</td>
<td>1998 (Dry)</td>
<td>1999 (Wet)</td>
</tr>
<tr>
<td>TE179</td>
<td>56</td>
<td>174</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>KL113</td>
<td>0</td>
<td>192</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BA306</td>
<td>0</td>
<td>0</td>
<td>18</td>
<td>167</td>
</tr>
<tr>
<td>TE158</td>
<td>0</td>
<td>27</td>
<td>157</td>
<td>0</td>
</tr>
<tr>
<td>BA322</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>163</td>
</tr>
<tr>
<td>BA283</td>
<td>36</td>
<td>0</td>
<td>0</td>
<td>127</td>
</tr>
<tr>
<td>DE086</td>
<td>17</td>
<td>89</td>
<td>39</td>
<td>10</td>
</tr>
<tr>
<td>BA287</td>
<td>31</td>
<td>0</td>
<td>0</td>
<td>114</td>
</tr>
<tr>
<td>DE034</td>
<td>0</td>
<td>12</td>
<td>90</td>
<td>35</td>
</tr>
<tr>
<td>KL111</td>
<td>6</td>
<td>125</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>KL123</td>
<td>65</td>
<td>16</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>TE219</td>
<td>1</td>
<td>123</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KL103</td>
<td>0</td>
<td>0</td>
<td>23</td>
<td>82</td>
</tr>
<tr>
<td>DE038</td>
<td>0</td>
<td>72</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>KL104</td>
<td>0</td>
<td>47</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td>KL128</td>
<td>24</td>
<td>45</td>
<td>18</td>
<td>1</td>
</tr>
<tr>
<td>BA259</td>
<td>0</td>
<td>0</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>BA286</td>
<td>1</td>
<td>12</td>
<td>21</td>
<td>50</td>
</tr>
<tr>
<td>BA297</td>
<td>52</td>
<td>0</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>DE077</td>
<td>57</td>
<td>14</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>TE180</td>
<td>0</td>
<td>32</td>
<td>35</td>
<td>8</td>
</tr>
<tr>
<td>BA299</td>
<td>71</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DE041</td>
<td>68</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>KL112</td>
<td>0</td>
<td>72</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>KL093</td>
<td>58</td>
<td>2</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>BA256</td>
<td>71</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>KL119</td>
<td>10</td>
<td>8</td>
<td>0</td>
<td>53</td>
</tr>
<tr>
<td>DE046</td>
<td>29</td>
<td>3</td>
<td>25</td>
<td>11</td>
</tr>
</tbody>
</table>
Targeted source reduction/control strategy for dengue control

Not surprisingly, the number of residents per household is rather consistent. Correlations of the number of household residents observed during the four surveys declined from an average of 0.79 for surveys conducted within one year of each other to an average of 0.69 for surveys separated by two years; the correlation between the two surveys conducted three years apart, i.e. those of 1996 and 1999, was 0.64 (Table 9).

Table 7: Correlations between the total number of Ae. aegypti pupae per house over time
[Average correlations for surveys separated by 1, 2 and 3 years are 0.11, 0.00 and 0.13 respectively]

<table>
<thead>
<tr>
<th>Survey</th>
<th>1996 (Dry)</th>
<th>1997 (Wet)</th>
<th>1998 (Dry)</th>
<th>1999 (Wet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997 (Wet)</td>
<td>0.11</td>
<td>1.00</td>
<td>0.12</td>
<td>-0.01</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td>0.01</td>
<td>0.12</td>
<td>1.00</td>
<td>0.10</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td>0.13</td>
<td>-0.01</td>
<td>0.10</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 8: Correlations between the total number of Ae. albopictus pupae per house over time
[Average correlations for surveys separated by 1, 2, and 3 years are 0.08, 0.14 and 0.00 respectively]

<table>
<thead>
<tr>
<th>Survey</th>
<th>1996 (Dry)</th>
<th>1997 (Wet)</th>
<th>1998 (Dry)</th>
<th>1999 (Wet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997 (Wet)</td>
<td>0.08</td>
<td>1.00</td>
<td>0.17</td>
<td>-0.02</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td>0.30</td>
<td>0.17</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td>0.00</td>
<td>-0.02</td>
<td>0.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Number of water-holding containers per house

There is significantly more correlation between the numbers of natural and artificial water-holding containers per house between seasons than the number of pupae per household (Table 10). Average correlations for surveys separated by 1, 2 and 3 years are 0.59, 0.57 and 0.49 respectively.

Table 9: Correlations between the number of people per house over time
[Average correlations for surveys separated by 1, 2, and 3 years are 0.79, 0.69, and 0.64, respectively]

<table>
<thead>
<tr>
<th>Survey</th>
<th>1996 (Dry)</th>
<th>1997 (Wet)</th>
<th>1998 (Dry)</th>
<th>1999 (Wet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997 (Wet)</td>
<td>0.80</td>
<td>1.00</td>
<td>0.71</td>
<td>0.68</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td>0.69</td>
<td>0.71</td>
<td>1.00</td>
<td>0.86</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td>0.64</td>
<td>0.68</td>
<td>0.87</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Number of people per household

Table 10: Correlations between the total number of artificial and natural water-holding containers per house over time
[Average correlations for surveys separated by 1, 2, and 3 years are 0.59, 0.57, and 0.49, respectively]

<table>
<thead>
<tr>
<th>Survey</th>
<th>1996 (Dry)</th>
<th>1997 (Wet)</th>
<th>1998 (Dry)</th>
<th>1999 (Wet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997 (Wet)</td>
<td>0.60</td>
<td>1.00</td>
<td>0.55</td>
<td>0.64</td>
</tr>
<tr>
<td>1998 (Dry)</td>
<td>0.49</td>
<td>0.55</td>
<td>1.00</td>
<td>0.62</td>
</tr>
<tr>
<td>1999 (Wet)</td>
<td>0.49</td>
<td>0.64</td>
<td>0.62</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Targeted source-reduction strategy for Yogyakarta

In conclusion, while there are definitely key premises in Yogyakarta (Figure 4), the lack of correlation between years (Table 6) in productivity probably makes any attempt to develop proxies (co-variates) for the rapid identification of especially productive households unlikely to succeed. The source of variability in *Ae. aegypti* production at the household level between years is not obviously related to rainfall, nor changes in the numbers of residents or wet containers. However, we cannot discount the possibility that the lack of correlation is due to variability in pupation on a daily basis. Because the key premise concept has potential for control, further study using the total pupation over perhaps a week is warranted.

Temperature to use in estimating the transmission threshold

For temperature, we could use the average mean temperature for the first five months of the year, 27.5 °C when ca. 60% of all cases period. Given that most transmission occurs during the wet season (Figure 6) and that there are more pupae per person then (0.47 vs 0.67), we will use the wet season survey results.

Wet or dry season estimates of pupae per person

Figure 5 includes the annual number of cumulative DHF cases in Yogyakarta province for the period 1985–2001 and Figure 6 includes the cumulative monthly DHF cases for the same period. Given that most transmission occurs during the wet season (Figure 6) and that there are more pupae per person then (0.47 vs 0.67), we will use the wet season survey results.


are observed. Yet, epidemic transmission is often associated with anomalously high temperatures, almost 29 °C, for the Yogyakarta time series (data not shown). Therefore, to be conservative, we will use a value of 29 °C.

**The value of seroprevalence to be used in estimating the transmission threshold**

This one is a bit more difficult and we will have to make a crude estimate. Graham et al. conducted a serosurvey in the late 1990s in Yogyakarta and estimated that the annual seroconversion rate among children aged 4 to 9 years was, for serotypes 1–4, 0.048, 0.077, 0.042 and 0.034 respectively. In the endemic situation, the rate of seroconversion declines with age as those susceptible to infection become increasingly rare. We estimate the age-specific antibody prevalence rate up through age 80 by multiplying the 4–9-year-olds’ rates for each year by one less the prevalence in the previous year. Now, to estimate the average overall prevalences for each of the dengue strains, we need a population estimate of the human age distribution in Yogyakarta. For this we used the WHO demographic data for Indonesia. Taking the average of the product of the age-specific prevalence and age proportions gives us the overall population seroprevalence for each serotype – 0.54, 0.66, 0.51 and 0.45 for serotypes 1–4 respectively (average: 0.54). The transmission thresholds for 0.33 and 0.67 from the table of transmission thresholds given in Focks et al. are 0.43 and 0.96 respectively. Again, to be conservative, we will use the value associated with a seroprevalence of 0.33, i.e., a strategy to bring the area-wide abundance of Ae. aegypti to somewhat below 0.43 Ae. aegypti pupae per person.

**Identification of the targeted classes of containers**

In Table 11 are listed the most productive classes of containers observed in the wet season surveys. We see that if we controlled the wells and a single type of container, the Storage in WC (Bak mandi), we would achieve a standing crop of Ae. aegypti of 0.37 pupae per person. With the elimination of just these two types of containers, we are already below our transmission threshold. Again, to be conservative, if we add to our list of types of containers to be controlled – Wells, Water container (lg) (Bak air) – a fourth type, Tyre, we can anticipate being at levels of 0.29 and 0.23 pupae per person respectively, if all of the targeted types are completely controlled.

**Discussion**

The three targeted types of containers account for 45% of all production, they also account for 65% of all containers. Is this much of a strategy, given the number of containers we are trying to control? We think so for the following reasons: wells, bak mandis and bak airs are in known locations within the house and are typically masonry in construction. This means that they are easy to find, identify and control with several methods currently available in Indonesia, e.g. IGRs (Altocid and Sumilarv) and temephos (Abate). Tyres are similarly easy to locate and identify.

**Research topics**

**Most containers are negative for larvae and pupae**

Why is this so? Is it because they are used domestically and frequently used, cleaned or emptied? Is it because of natural biological
Targeted source reduction/control strategy for dengue control

control, or perhaps because they do not receive oviposition? Could an understanding of this phenomenon aid in controlling productive containers?

Some containers are especially productive and account for the majority of breeding

Studies on the mechanisms promoting productivity are needed. A related issue is the time course of production – are productive containers only episodically so, with intervals of low or no production? Alternatively, are productive containers more or less continuously so? Studies on the mechanisms leading to certain classes of containers being especially productive are also needed. Time series of daily pupation in undisturbed containers in the field would be useful.

**Table 11:** Types of containers most responsible for the observed standing crop of Ae. aegypti pupae per person observed during the wet season surveys; averages for the two surveys are shown

[Number refers to the average number of containers observed by type; the average total number of water-holding containers in the wet season surveys was 3724. Proportion of production is the average proportion of all Ae. aegypti observed in the wet season surveys. Accumulation refers to a summing of the column to the left in a downward direction; the Type “Storage in WC” accounted for 0.456 of all production, that type and the next most productive type, “Water container (lg)” account for 0.571 of all production, etc. Pupae per person refers to the actual contribution of that type of container. Balance if removed is the number of Ae. aegypti pupae per person that would remain in the environment if that Type were controlled and the ones above it. The average number of pupae per person in the environment was 0.672]

<table>
<thead>
<tr>
<th>Type</th>
<th>Number</th>
<th>Proportion of production</th>
<th>Accumulation</th>
<th>Pupae per person</th>
<th>Balance if removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage in WC(^a)</td>
<td>1697</td>
<td>0.456</td>
<td>0.456</td>
<td>0.31</td>
<td>0.37</td>
</tr>
<tr>
<td>Water container (lg)(^b)</td>
<td>429</td>
<td>0.115</td>
<td>0.571</td>
<td>0.08</td>
<td>0.29</td>
</tr>
<tr>
<td>Tyre</td>
<td>311</td>
<td>0.084</td>
<td>0.654</td>
<td>0.06</td>
<td>0.23</td>
</tr>
<tr>
<td>Flower pot</td>
<td>223</td>
<td>0.060</td>
<td>0.714</td>
<td>0.04</td>
<td>0.19</td>
</tr>
<tr>
<td>Trash can</td>
<td>223</td>
<td>0.060</td>
<td>0.774</td>
<td>0.04</td>
<td>0.15</td>
</tr>
<tr>
<td>Bucket</td>
<td>174</td>
<td>0.047</td>
<td>0.821</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Bird watering dish</td>
<td>144</td>
<td>0.039</td>
<td>0.860</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td>Tin can</td>
<td>130</td>
<td>0.035</td>
<td>0.894</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Fish pond</td>
<td>86</td>
<td>0.023</td>
<td>0.918</td>
<td>0.02</td>
<td>0.06</td>
</tr>
<tr>
<td>Clay water container</td>
<td>65</td>
<td>0.017</td>
<td>0.935</td>
<td>0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>Container</td>
<td>59</td>
<td>0.016</td>
<td>0.951</td>
<td>0.01</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\) Indonesian: Bak mandi
\(^b\) Indonesian: Bak air
Seasonality in transmission and the utility of an early warning system

A recent National Research Council (USA) publication investigating the feasibility of developing practical and sustainable early warning systems (EWSs) for infectious diseases concluded that EWSs would provide significant utility where mitigation methods were available. Their primary value lies in the ability temporally to focus scarce resources for control in those periods when epidemics were likely. Cases of dengue and DHF occur in virtually every province and during every month of the year in Indonesia. Does the seasonality of dengue transmission in Indonesia suggest that there would be merit in gaining the ability to time suppression activities to precede peak transmission periods based on an EWS? Initial attempts to develop an EWS for dengue in Yogyakarta have been promising and will be pursued.

Given that the first five months of the year account for an average of 60% of all cases and the remaining months report about ca. 5% each, it seems likely that control would typically be continuous. Perhaps the utility of validated EWSs for Indonesia would be to forecast epidemic years as an aid to the national programme in securing adequate funding for anticipated epidemic years.

Acknowledgement

This work was partially supported by a number of institutions including the Jean and Julius Tahija Family Foundation, the Office of Global Programmes, National Oceanographic and Atmospheric Administration (NOAA), Gadjah Mada University, and the Navy Medical Research Center, Silver Spring, MD. We thank all of the staff who dedicated long hours in the field to carefully collecting mosquito specimens and the data used in this study. We are especially thankful to Lely Sianturi, Saptoro Rusmiarto, Yoyo R. Gionar, Dwiko Susapto, and the health staff of Gondokusuman for their diligence and hard work during the years of investigation. We are also grateful to Iqbal Elyazar for data set preparation.

References


Targeted source reduction/control strategy for dengue control


Elevated levels of soluble tumour necrosis factor receptor 1, thrombomodulin and soluble endothelial cell adhesion molecules in patients with dengue haemorrhagic fever

Beti Ernawati Dewi, Tomohiko Takasaki, T. Mirawati Sudiro, R.H. Nelwan and Ichiro Kurane

Abstract

The pathological mechanism of dengue haemorrhagic fever (DHF) is still poorly understood. Previous studies have suggested that immune responses contribute to an increase in capillary permeability. We examined the levels of cytokines and activated endothelial substances in serum samples collected from DHF patients in Indonesia. We measured the levels of soluble TNF-R1, MCP-1, GM-CSF, IL-17, TNF-α, soluble thrombomodulin, soluble E-selectin, soluble ICAM-1 and soluble VCAM-1. The levels of activated endothelial substances such as sE-selectin, sICAM-1 and sVCAM-1 were higher in DHF patients than in healthy controls. High levels of soluble activated endothelial substances suggest that endothelial cells are highly activated. When compared with patients with other febrile illness (OFI) or healthy controls, the levels of sTNF-R1 were higher in DHF patients. A similar trend was observed in the level of thrombomodulin. MCP-1, GM-CSF and IL-17 were not detected in serum samples from any patients and healthy controls. TNF-α was detected in 5 (9%) of 53 patients. The results suggest that endothelial cells are highly activated in DHF patients and TNF-α is one of the factors which contributes to the activation.

Keywords: Tumor necrosis factor receptor-1; Thrombomodulin; Vascular cell adhesion molecule-1; Intercellular adhesion molecule-1.

Introduction

Dengue virus infection continues to increase in tropical and sub-tropical countries. Dengue virus causes a spectrum of clinical illnesses ranging from a mild disease as dengue fever (DF) to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). It is estimated that up to 100 million individuals are infected with dengue virus, and 500 000 cases of DHF...
Elevated level of cytokines on DHF patients

and 25,000 deaths occur annually. The pathological mechanisms of DHF are still poorly understood. The role of host immune responses have been examined in a number of studies. These studies have suggested that different types of cytokines and abnormalities in coagulation may cause an increase in the capillary permeability, the hallmark of DHF.

The absence of massive structural damage, a short-lived nature of the plasma leakage syndrome and a remarkably rapid recovery of children with DSS all suggest that altered permeability is induced by soluble mediators. It has been reported that serum levels of tumour necrosis factor alpha (TNF-α) are elevated in patients with dengue haemorrhagic fever. The role of TNF-α in the increased permeability of vascular endothelial cells has been postulated.

TNF-α starts to affect cell function by binding to the specific, high-affinity receptors on the surface; TNF-R1 (p55 TNFR) and TNF-R2 (p75 TNFR). Soluble forms of TNF-R1 and TNF-R2 are present in the serum and appear to play a role as modulators of the biological function of TNF-α, modifying the function and availability of TNF-α. Measuring sTNFRs in serum offers some advantages compared with direct qualification of TNF-α, because TNF-α is rapidly cleared from circulation, and some assays are unable to detect TNF-α bound to soluble TNFRs. Soluble TNFRs are very stable and their serum concentrations correlate well with those of TNF-α.

Most of the biological effects induced by TNF-α have been shown to be mediated by TNF-R1. TNF-alpha induces expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin under the control of TNF-R1. TNF-R1 also plays a key role in TNF-alpha-dependent responses that include proliferation, differentiation and apoptosis. Wachtel et al. (2001) showed that down regulation of tight junction components such as occludin by TNF is mediated via TNF-R1.

In the present study, we examined the level of sTNF-R1 in serum samples from DHF patients. In addition, we also measured the levels of thrombomodulin and activation molecules released from endothelial cells in the serum samples.

Materials and methods

Study population

Blood specimens were collected from 53 patients admitted to Cipto Mangunkusumo Hospital, Jakarta, Indonesia, between May and August 2002. After informed consent was obtained, blood specimens were obtained from adult patients with suspected dengue infection. The patients were diagnosed as dengue virus infection by clinical findings and laboratory confirmation – IgM-capture ELISA (PanBio, Brisbane, Australia) and IgG-capture ELISA (PanBio, Brisbane, Australia). The patients were clinically classified as DHF according to the criteria of the World Health Organization; grade I: fever associated with non-specific constitutional symptom and positive torniquet test as the only haemorrhagic manifestation; grade II: the same symptom with grade I with the spontaneous haemorrhagic manifestation; grade III: circulatory failure manifested by rapid, weak pulse with narrowing of the pulse pressure (<20 mm of Hg) or hypotension; grade IV: profound shock with undetectable blood pressure and pulse.

Fifty-three patients are included in this study – 25 females and 28 males, with an average age of 26.75±10.77 years. Eighteen patients were classified as DHF grade II, and 24 patients as DHF grade I; 11 patients as non-dengue infection (other febrile illness, OFI).
Plasma samples were also obtained from 20 healthy donors with a mean age of 37.29±9.67 years as controls.

**Serum sample**

Peripheral blood (5–10 ml) was drawn into sterile tubes containing EDTA. Blood samples were collected everyday from the day of admission until the days when body temperature and thrombocyte count became normal. Blood specimens were centrifuged at 800 rpm for 10 minutes and the plasma was separated and then stored at –80 °C until use. The plasma samples were transported to the NIID, Tokyo, Japan, in dry ice and stored at –80 °C until use.

**Measurement of level of cytokines, chemokines and activated endothelial substance by standard ELISA**

Levels of tumour necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin 17 (IL-17), granulocyte macrophage colony stimulating factor (GM-CSF), soluble E-selectin, soluble vascular cell adhesion molecule-1 (sVCAM-1) and ICAM-1 were assessed with ELISA kits, according to the manual of the manufacturer (BioSource International Inc., USA). Levels of soluble tumour necrosis factor receptor 1 (s TNF-R1) were measured using a human sTNF-R1 immunoassay (R&D Systems, Inc., USA). Levels of thrombomodulin were measured with soluble CD141 ELISA kits (Diaclone Research, France).

**Statistical analysis**

Differences were statistically analysed by Mann-Whitney U-test. The difference was considered to be significant, when p value was less than 0.05.

**Results**

**Level of TNF-R1**

Levels of soluble TNF-R1 in DHF patients, OFI patients during disease days 1–15 and healthy controls are shown in Figure 1. The highest mean levels were 230.51±67.07 pg/ml on day 4 after onset of fever in patients with grade II and 245.02±47.72 pg/ml on day 10 after onset of fever in patients with grade I. The highest mean level was 184±77.64 pg/ml in patients with OFI, and the mean level in healthy controls was 59.37±15.12 pg/ml. The levels of soluble TNF-R1 in patients with grades I and II were significantly higher those in healthy controls, and those in patients with OFI on days 7, 8 and 9 after onset of fever.

**Level of thrombomodulin, sE-selectin, sVCAM-1 and sICAM-1**

The levels of thrombomodulin, soluble E-selectin, soluble VCAM-1 and soluble ICAM-1 were assessed as markers for activation of
Elevated level of cytokines on DHF patients

Endothelial cells. The levels of thrombomodulin are shown in Figure 2. The highest mean titres were 35.42±34.44 ng/ml on day 10 after onset of fever and 18.26±12.94 ng/ml on day 7 after onset of fever in patients with grade II and grade I respectively. The highest mean level in patients with OFI was 14.5±10.24 ng/ml. The levels of thrombomodulin in DHF grade II and I were significantly higher than those in healthy controls. When DHF grade II and grade I were compared with OFI, the levels were higher in DHF grade II and I on days 6 and 7 (p<0.05).

Figure 2: Variation of level of thrombomodulin in the plasma of DHF patients, other febrile illness (OFI) patients and healthy controls on days after onset of fever
[The symbol represents the thrombomodulin value obtained from individual patients]

The levels of soluble E-selectin are shown in Figure 3. The highest mean level was 119.82±75.18 ng/ml on day 4 after onset of fever in DHF grade II and 114.2±86.67 ng/ml on day 10 after onset of fever in DHF grade I. In OFI patients the highest mean level was 65.45±21.20 ng/ml at 7 days after onset of fever. In healthy controls the level was 43.11±10.02 ng/ml. The level of E-selectin was higher in DHF grade II and grade I than in healthy controls (p<0.05).

Figure 3: Variation of level of soluble E-selectin in the plasma of DHF patients, other febrile illness (OFI) patients and healthy controls on days after onset of fever
[The symbol represents the soluble E-selectin value obtained from individual patients]

The levels of soluble VCAM-1 in patients and healthy controls are shown in Figure 4. The highest mean level found in DHF grade II was 291.23±149.05 ng/ml on day 4 after onset of fever, and that in DHF grade I was

Figure 4: Variation of level of soluble VCAM-1 in the plasma of DHF patients, other febrile illness (OFI) patients and healthy controls on days after onset of fever
[The symbol represents the soluble VCAM-1 value obtained from individual patients]
Elevated level of cytokines on DHF patients

The highest level of cytokines and activated endothelial substances were detected on the same days after onset of fever. For example, in DHF grade II patients, the highest mean levels of sTNF-R1, sE-selectin, sVCAM-1, and sICAM-1 were detected on day 4 after onset of fever. In DHF grade I, the highest levels of sTNF-R1, sE-selectin, sVCAM-1, and sICAM-1 were detected on day 10 after onset of fever.

Figure 5: Variation of level of soluble ICAM-1 in the plasma of DHF patients, other febrile illness (OFI) patients and healthy controls on days after onset of fever
[The symbol represents the soluble ICAM-1 value obtained from individual patients]

Level of other cytokines

The plasma samples were also examined for the levels of TNF-α, MCP-1, GM-CSF and IL-17. TNF-α was detected in 5(9%) of 53 patients with DHF, but were not detected in any of the healthy controls. MCP-1, GM-CSF and IL-17 were not detected in plasma samples from any of the DHF patients or healthy controls.

Discussion

It has been hypothesized that DHF is caused by soluble mediator induced in immune responses. Recent studies have demonstrated the elevation of multiple mediators in DHF patients, including IL-6, tumor necrosis factor alpha (TNF-α), IL-10, IFN-gamma (IFN-γ) and IL-2. In the present study we did not find high levels of TNF-α; however, levels of TNF-R1 were higher in DHF patients than in healthy controls or patients with OFI. Direct qualification of TNF-α is often difficult because TNF-α is rapidly cleared from the circulation and some assays cannot detect TNF-α bound to soluble TNFRs.

It has been known that TNF-α has many effects on the endothelial cell barrier functions. TNF-α induces the release of cytokines and chemokines, the production of enzymes, such as cyclooxygenases-2 and nitric oxide synthesis. TNF-α also increases the
Elevated level of cytokines on DHF patients

expression of adhesion molecules on endothelial cells. Ferrero et al. 2001 showed that TNF induced alteration of endothelial permeability in vitro and in vivo via TNF-R1. High levels of TNF-R1 in DHF patients suggest that TNF-α may play important roles in the development of DHF, because the level of sTNFRs in serum correlate well with the level of TNF-α and because most of the biological effects of TNF-α have been attributed to TNF-R1.

Endothelial cells line the inner surface of blood vessels, and play an important role in vascular functions as a barrier between blood and interstitial compartments. It is known that hormones, cytokines and neurotransmitters regulate endothelial cell functions. We found that levels of activated endothelial substances – sICAM-1, sVCAM-1 and sE-selectin – were elevated in DHF patients. Elevation of these substances in serum indicates that endothelial cells are activated during dengue virus infection.

In addition, we measured the levels of an endothelial cell injure marker, thrombomodulin. We found that mean levels of thrombomodulin were elevated in DHF patients compared to those in healthy controls and in patients with OFI. Thrombomodulin is an endothelial cell surface glycoprotein and possesses an anticoagulant property. This protein was immobilized onto polytetrafluoroethylene surfaces to create biomaterials with enhanced haemocompatibility. Thrombomodulin as a thrombin-binding protein serves to initiate the protein C anticoagulant pathway. High levels of thrombomodulin in the microcirculation suggest that thrombin might accumulate and ultimately lead to microvascular occlusion. It is well-known that thrombin increases the permeability of endothelial cells. We feel that high levels of thrombomodulin in DHF patients reflect the functional changes of vascular endothelial cells in patients with DHF.

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References


Elevated level of cytokines on DHF patients


Elevated level of cytokines on DHF patients


Increased dengue virus-infected endothelial cell apoptosis caused by antibodies against nonstructural protein 1

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Abstract

Vascular dysfunction is a hallmark of severe dengue haemorrhagic fever (DHF) in dengue virus (DENV) infection. Both viral infection and immunopathogenesis are involved in the vasculopathy of DHF. We previously showed that antibodies against DENV nonstructural protein 1 (NS1) may cross-react with endothelial cells. A further study demonstrated the pathogenic role of anti-NS1 antibodies on endothelial dysfunction by inducing cell death and inflammation. In this study, we investigated the effect of anti-NS1 antibodies on DENV-infected human endothelial cells. We found increased binding activity of anti-NS1 antibodies to endothelial cells after DENV infection. Either DENV infection or anti-NS1 treatment caused endothelial cell apoptosis. Importantly, co-treatment with anti-NS1 antibodies increased DENV-induced endothelial cell apoptosis. The generation of nitric oxide (NO) could be detected in anti-NS1-stimulated, but not DENV-infected, endothelial cells. Furthermore, anti-NS1-induced endothelial cell apoptosis was NO-dependent, whereas DENV infection-induced apoptosis was NO-independent. These results suggest an additive effect but distinct mechanisms between anti-NS1 antibodies and DENV in endothelial cell damage. These findings indicate that both viral infection and cross-reactive antibodies may be involved in dengue pathogenesis by causing endothelial dysfunction.

Keywords: Dengue virus (DENV); Autoantibody; NS1; Endothelial cells; Apoptosis.

Introduction

Dengue virus (DENV) causes human diseases like mild dengue fever (DF) and severe dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) in most tropical and subtropical areas of the world. Sequential infection with different DENV serotypes may influence the severity of the disease. Antibody-dependent enhancement (ADE) of DENV infection is responsible for DHF in the secondary infection due to the presence of cross-reactive and non-neutralizing antibodies from prior infection. In addition to ADE, viral variation and immunopathogenesis are involved in the progression of DHF. We previously found that anti-DENV nonstructural protein 1 (NS1) antibodies cross-reacted with endothelial cells and induced these cells to undergo apoptosis. Autoimmunity-mediated endothelial cell damage may, therefore, also

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contribute to the pathogenesis of dengue disease.

Vasculopathy, coagulopathy and thrombocytopenia are the hallmarks of severe dengue haemorrhagic syndrome. Previous studies showed the manifestation of vasculopathy characterized by endothelial dysfunction caused by direct viral cytotoxicity as well as immune-mediated inflammatory responses and cellular damage. In addition, we demonstrated that anti-NS1 antibodies present in dengue patient sera caused endothelial cell apoptosis. Furthermore, NS1 protein can be expressed in a glycosyl-phosphatidylinositol (GPI)-linked form on the surface of DENV-infected cells. In the present study, we investigated the apoptotic effect of DENV infection in human endothelial cells in the presence of anti-NS1 antibodies.

Materials and methods

Cell cultures

Human umbilical cord vein endothelial cells (HUVECs) were cultured in modified M-199 medium as previously described. Human microvascular endothelial cell line-1 (HMEC-1) was passed in culture plates containing endothelial cell growth medium M200 (Cascade Biologics) composed of LSGS (2% fetal bovine serum, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, and antibiotics. C6/36 cells were cultured in DMEM medium containing 10% fetal bovine serum and antibiotics. Fetal bovine serum was pre-incubated at 56 °C for 30 minutes to inactivate complement before cell culture. For experiments, 1000 U/ml trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) were used to detach cells.

Viruses

DENV-2 (PL046, Taiwan-isolated) was maintained in C6/36 cells. Briefly, a monolayer of C6/36 cells was incubated with DENV-2 at a multiplicity of infection (MOI) of 0.01 and incubated at 26 °C in 5% CO₂ for 5 days. The cultured medium was harvested and cell debris was removed by centrifugation at 900 × g for 10 minutes. After further centrifugation at 16 000 × g for 10 minutes, the virus supernatant was collected and stored at -70 °C until use. The virus titre was determined using a plaque assay.

Generating anti-NS1 antibodies

Recombinant DENV-2 NS1 was expressed and purified as previously described. To generate antibodies against DENV NS1, we immunized BALB/c mice intraperitoneally, once with 25 μg of recombinant NS1 protein emulsified in complete Freund’s adjuvant and then four times with 25 μg of recombinant NS1 protein emulsified in incomplete Freund’s adjuvant. Sera were obtained 3 or 4 days after the last immunization. The immunoglobulin G (IgG) fractions from hyperimmunized mouse sera and normal mouse sera used for the control were purified using a protein G-Sepharose affinity chromatography column (Amersham Pharmacia, Uppsala, Sweden).

Detecting binding activity

Endothelial cells (5 × 10⁵) were washed with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde in PBS at room temperature for 10 minutes, then washed again with PBS. Mouse anti-NS1 IgG or control IgG were then incubated with cells at 4 °C for 1 hour. After the cells had been washed three times with
PBS, they were incubated with 1 μl of 1 mg/ml fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 4 °C for 1 hour and then washed again with PBS. The binding activity of mouse anti-NS1 or control IgG to endothelial cells was analysed using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA) with excitation set at 488 nm.

**Detecting cell death**

Cells were fixed with 70% ethanol in PBS for a terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) reaction using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions, and then analysed using flow cytometry.

**Detecting caspase-3 activity**

Caspase-3 activation was determined using the ApoAlert caspase-3 colorimetric assay kit (Clontech) according to the manufacturer’s instructions. Relative optical density (OD) measurements were performed using a microplate reader (Molecular Devices).

**Detecting nitric oxide production**

The production of nitric oxide (NO) was assessed as the accumulation of nitrite (NO₂⁻) in the medium using a colorimetric reaction with the Griess reagent. Briefly, the culture supernatants were collected and mixed with an equal (1:1) volume of Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H₃PO₄). The absorbance was measured at 540 nm using a microplate reader. NaNO₂ was dissolved in double-distilled H₂O and used for the standard control (from 1 to 50 μM).

**Statistical analysis**

Using the Student’s unpaired t-tests in SigmaPlot version 8.0 for Windows (Cytel Software Corporation, Cambridge, MA), P-values were determined for all data from three independent experiments. Statistical significance was set at \( P < 0.05 \).

**Results**

**Binding activity of anti-NS1 to DENV-infected human endothelial cells**

Previous studies in our laboratory showed the presence of anti-endothelial cell autoantibodies in dengue patient sera and that the levels of anti-endothelial cell autoantibodies in DHF/DSS patient sera were higher than those in DF patient sera.\(^{[11,12]}\) Further studies demonstrated that anti-NS1 antibodies accounted for the cross-reactivity and the induction of endothelial cell death. After DENV infection, the expression of NS1 protein was detected in patient plasma and on cell surface.\(^{[15-19]}\) In this experiment, HUVECs were infected with DENV-2 at MOIs of 1 and 10 for 12, 24, and 48 hours, and the binding ability of anti-NS1 antibodies was analysed using flow cytometry. We found greater anti-NS1 time-dependent endothelial cell binding activity in DENV-infected cells than in mock-infected cells (Figure 1).

**Effect of anti-NS1 in DENV-induced endothelial cell apoptosis**

Our previous studies\(^{[11-14]}\) showed that anti-NS1 antibodies induced endothelial cell apoptosis. Another study\(^{[15]}\) showed that DENV infection caused endothelial cell cytotoxicity. In this experiment, we further investigated the effect
Anti-NS1 enhances DENV-induced endothelial cell apoptosis

First, HUVECs were infected with DENV-2 at an MOI of 1 for 12, 24, and 48 hours, or treated with 5 or 25 μg of anti-NS1 for 24 hours. Cell apoptosis was measured using a TUNEL reaction and then flow cytometric analysis. Consistent with previous studies, both DENV infection and anti-NS1 antibodies induced apoptosis (data not shown). We then assessed the effect of DENV-induced endothelial cell apoptosis in cells co-treated with anti-NS1 antibodies. We found that anti-NS1 treatment time-dependency increased DENV-induced endothelial cell apoptosis (Figure 2).

Figure 1: Binding activity of anti-NS1 to DENV-infected endothelial cells

[HUVECs were infected with DENV-2 (MOI 1 and 10) for 12, 24, and 48 hours, then incubated with 5 μg of mouse anti-NS1 IgG or control IgG and then FITC-conjugated anti-mouse IgG. The binding ability was analysed using flow cytometry. A set of representative histograms (A) and the percentages of HUVECs reactive with anti-NS1 IgG are shown as mean ± SD of triplicate cultures (B). The data related to their histograms are labeled (a-h). #: statistically significant compared to the group treated with anti-NS1 alone.

* and #: P < 0.05; **: P < 0.01; *** and ###: P < 0.001]

Figure 2: Additive effect of DENV and anti-NS1 antibodies on endothelial cell apoptosis

[HUVECs were treated with 5 μg of mouse anti-NS1 IgG or control IgG for 12 and 24 hours with or without DENV-2 (MOI 1) pre-infection for 24 hours. They were then analysed using a TUNEL reaction and flow cytometric analysis. A set of representative histograms (A) and the percentages of apoptotic cells are shown as mean ± SD of triplicate cultures (B). The data related to their histograms are labelled (a-h).

*: P < 0.05; **: P < 0.01; ***: P < 0.001]
Anti-NS1 enhances DENV-induced endothelial cell apoptosis

Effect of nitric oxide in anti-NS1- and DENV-induced endothelial cell apoptosis

Our previous studies showed that anti-NS1 caused endothelial cell apoptosis via a nitric oxide (NO)-mediated pathway. In the present study, the effect of DENV infection on NO production was investigated. First, anti-NS1 but not DENV infection caused NO production in endothelial cell HMEC-1 (Figure 3). Furthermore, there was no effect of DENV infection on anti-NS1-induced NO production. Using NO synthase inhibitor L-NAME, we next examined the effects of NO on anti-NS1- and DENV-induced endothelial cell apoptosis by determining the activation of caspase-3. The results demonstrated only anti-NS1- but not DENV-induced endothelial cell apoptosis via a NO-regulated signalling (Figure 4).

Discussion

In the present study, we demonstrated that anti-NS1 antibodies increased DENV-infected endothelial cell damage. A previous study had showed that DENV caused complement activation and apoptosis in cultured endothelial cells. A recent study further demonstrated that anti-NS1 antibodies increased complement activation. Here we showed an additive effect between anti-NS1 antibodies and DENV in endothelial cells. DENV infection increased the binding activity of anti-NS1 antibodies to endothelial cells. There are at least two possible explanations for this. One is the NS1 expression on the surface of DENV-infected cells as previously described, and the other, which still needs to be confirmed, is the increased expression of self-antigens caused by DENV infection. These two possibilities are not mutually exclusive. Our preliminary study (unpublished data) using proteomic analysis

Figure 3: Anti-NS1 but not DENV infection induces NO production in endothelial cells

Figure 4: Anti-NS1- but not DENV infection-induced caspase-3 activation is NO-mediated
Anti-NS1 enhances DENV-induced endothelial cell apoptosis

identified several candidate autoantigens present on the surface of endothelial cells. We are currently investigating whether DENV infection may upregulate the expression of these candidate autoantigens.

Although anti-NS1 binding activity was increased after DENV infection, further mechanistic studies indicated that the causes of endothelial cell apoptosis by anti-NS1 antibodies and DENV infection were distinct. Anti-NS1 treatment without DENV infection caused cell apoptosis through a NO-regulated pathway. DENV infection-induced endothelial cell apoptosis was, however, not mediated by NO. There are at least two possibilities for the distinct mechanisms of apoptotic induction between anti-NS1 and DENV infection. One is that DENV infection may induce apoptosis via a NO-independent manner as evidenced in Figure 4. The other, which remains to be investigated, is that GPI-linked NS1 protein[17] on the surface of DENV-infected cells does not involve a NO-mediated pathway by anti-NS1 stimulation. Because anti-NS1 but not DENV causes a NO-mediated apoptotic pathway, it is likely that DENV-increased autoantigen expression is not involved in the additive effect of endothelial cell apoptosis after anti-NS1 binding. This yet needs to be confirmed.

In this study, the induction of endothelial cell cytotoxicity was demonstrated. We previously showed that anti-NS1 antibodies caused inflammatory activation that was mediated by the production of cytokines, chemokines and adhesion molecules.[20,21] A previous study[15] also showed that DENV infection led to chemokine production in endothelial cells. Whether the combination of DENV and anti-NS1 antibodies may increase the inflammatory response requires further investigation.

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References


Anti-NS1 enhances DENV-induced endothelial cell apoptosis


Role of nitric oxide in the pathogenesis of dengue

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Abstract

The free radical nitric oxide (NO) has emerged in recent years as a fundamental signalling molecule for the maintenance of homeostasis, as well as a potent cytotoxic effector involved in the pathogenesis of a wide range of human diseases. The presence of NO during dengue infection as well as its experimental antiviral and apoptotic effects have been documented. In this regard, increased serum NO levels in dengue fever and basal levels in the haemorrhagic form of dengue have been reported. Clinical and experimental data suggest that NO could act as a beneficial factor during dengue infection by its antiviral and apoptotic effects; however, more intense investigations are required.

Keywords: Dengue virus; Nitric oxide; Dengue haemorrhagic fever, Pathogenesis.

Introduction

Dengue fever (DF) is the most important arboviral disease affecting public health in the developing world. The disease is endemic in many countries and causes epidemics frequently. These epidemics involve an estimated 100 million cases annually worldwide, causing great health, social and economic burden.\textsuperscript{[1,2]} Dengue virus (DENV), a RNA virus belonging to genus, Flavivirus, family Flaviviridae, has four serotypes (DENV-1 to 4). Clinically, the disease may be asymptomatic or may range from a mild febrile illness, DF, to the severe, life-threatening form, dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS), which can be caused indistinctly by any serotype.\textsuperscript{[3-5]}

The discovery that mammalian cells have the ability to synthesize the free radical nitric oxide (NO) has stimulated an extraordinary impetus for scientific research in all fields of biology and medicine. NO is produced by three isoforms of nitric oxide synthase (NOS), the inducible form (iNOS), and the two constitutive isoforms endothelial NOS (eNOS) and neuronal NOS (nNOS). Since its early description as an endothelial-derived relaxing factor, NO has emerged as a fundamental signalling device regulating virtually every critical cellular function, as well as a potent mediator of cellular damage in a wide range of conditions. Recent evidence indicates that most of the cytotoxicity attributed to NO is rather due to peroxynitrite, produced from the diffusion-controlled reaction between NO and another...
free radical, the superoxide anion. Peroxynitrite interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms. These reactions trigger cellular responses ranging from subtle modulations of cell signalling to overwhelming oxidative injury, committing cells to necrosis or apoptosis. In vivo, peroxynitrite generation represents a crucial pathogenic mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer, neurodegenerative disorders and viral infections.\[6-9\]

Previous reports have suggested a possible role of NO in DF and DHF/DSS pathogenesis,\[10-17\] however, very little information is available with regard to its role during the human dengue infection. Therefore, evidence from in vivo and in vitro conditions suggesting the possible role of NO in dengue infection is presented in this review.

**Nitric oxide in dengue infection**

NO is ubiquitous physiological-free radical that is responsible for many pathological disorders.\[6-9\] Its presence in human dengue infection was reported for the first time by Valero and collaborators.\[10\]

In this regard, increased serum NO levels in DF and normal levels in DHF patients were reported. There was no relationship between the viral serotypes and the increased values of NO. Interaction of dengue virus with primary human platelet cultures did not induce further regulation of NO production.\[10\] These data suggest that increased levels of NO in DF patients could have a protector effect during the disease, and, on the contrary, decreased levels could be the cause of the deleterious effects observed in DHF. In addition, it seems to be that virus-platelets interaction is not involved in the increased production of NO, suggesting other sources for this molecule. In this regard, monocyte/macrophages are the most important targets of DENV,\[18\] and are capable of producing high amounts of NO; thus, Mo/MΦ-DENV interaction may induce increased amount of NO. In accordance with that suggestion, increased expression of inducible nitric oxide synthase in monocytes from DF patients has been reported,\[13\] which translates into sustained NO production,\[13,15,16\] suggesting the possible source of NO found in patients with DF.

Since endothelium is an important NO producer, the direct or indirect interaction of DENV with the endothelial cells could induce an increased amount of serum NO in these patients.\[6-8,16,17\] However, increased levels of NO in patients with dengue seem to be controversial. Other investigators\[19\] have reported levels of serum NO significantly lower than those of normal controls in Asiatic children with DF and DHF, suggesting that the endothelial damage renders the endothelium incapable of producing NO. These data are different from the increased amount of serum NO found in patients with DF reported by others.\[10\] This different response to the virus infection could be related to the widespread prevalence of human dengue resistance genes in the Americas’ populations compared to Asian populations. The people of countries in South-East Asia have high rates of severe dengue disease compared to consistently low case-fatality rates in American countries reporting DHF.\[20\] Virtually, nothing is known about dengue resistance gene(s) in the pathogenesis of dengue. Are the manifestations of DF suppressed by this gene(s), or does the gene(s) selectively dampen dengue vasculopathy?\[20\] The expression of this gene(s) could involve different cell targets during the disease leading
Role of nitric oxide in the pathogenesis of dengue

to differential production of NO. Future research on dengue infections should emphasize population-based designs to shed light on the heterogeneity of dengue in populations living in the Western and Eastern hemispheres.

Anti-viral effect of nitric oxide

The beneficial effect of NO in DF could be related to an antiviral effect of the molecule. Previous studies have shown that NO could have antiviral effect on dengue-infected cells. In this regard, decreased expression of dengue virus antigens in NO-producing mononuclear cells has been reported. In addition, treatment of those cultures with an iNOS inhibitor (N\(^\text{-}\)-methyl-L-Arginine) induced increased viral antigens detection.\(^{[13]}\) The effect of NO exogenous donor (S-nitroso-N-acethylermicillinamide or SNAP) on DENV replication in neuroblastoma cell cultures has also been reported. DENV-2-infected cells treated with SNAP showed suppression of viral RNA synthesis and, consequently, decreased viral proteins and viral progeny production.\(^{[12]}\) This effect was also observed in DENV-1-infected C6/36 cell cultures treated with sodium nitroprussiate, a NO donor.\(^{[7]}\) Takhampunyak et al.\(^{[14]}\) showed the inhibitory effect of NO on DENV-infected LLC-MK2 cell cultures using SNAP. This report found a diminished cellular accumulation of viral RNA during viral RNA synthesis. This inhibitory effect of NO seems to be related to the inhibition of DENV-2 RNA-dependent RNA polymerase (RdRp) domain of NS5, with further suppression of viral RNA synthesis, viral structural proteins, and decreased number of infectious particles in the culture medium.

Recently, Ubol et al.\(^{[21]}\) reported DENV-2 clinical isolates derived from DF and DHF cases, which are NO-sensitive and NO-resistant, respectively, to endogenous NO produced by THP-1 cells (human monocytic leukemia cell line). These findings were also related with amino acids changes in the RdRp and Methyltransferase (Mtase) domains of NS5. Additionally, when compared the gene expression on DENV-2 infected THP-1 cells, NO-resistant but no NO-sensitive isolates, induced the immune-related genes IL-6, IL-7, IL-8, RANTES, and MCP-3, all of which are potent mediators of tissue damage as well as coagulopathy. These findings need to be carefully studied to determine if this effect is particularly restricted to DENV-2 or to its Asian genotype, or if NO-resistant strains occurs in the nature for the other serotypes.

All the previous data suggest a possible antiviral mechanism of NO in vivo, and suggest a beneficial role of increased serum levels of NO reported in DF.\(^{[10]}\) Lower levels of NO found in patients with DHF could be related to the described DENV action on target cells. In this regard, Chareonsrisuthigul et al.\(^{[11]}\) showed a different antiviral response in experiments using DENV-infected THP-1 cell cultures untreated and treated with an anti-DENV-enhancing antibodies (a model of infection via ADE). These experiments showed suppression of NO production in ADE cultures due to disruption of transcription of the IRF-1 (an iNOS gene transcription factor) and blocking of the activation of STAT-1. These findings could be related to the pathogenesis of DHF/DSS and could suggest a mechanism for basal serum NO levels in DHF patients.\(^{[10]}\)

This antiviral effect of NO is not restricted to the dengue virus. Several studies have reported a NO antiviral effect on an extensive list of viruses including SARS-coronavirus, hantavirus, Ross River virus, vesicular stomatitis virus, Crimean-Congo haemorrhagic fever virus, and members of the genus Flavivirus, including Japanese encephalitis.\(^{[22-29]}\)
Nitric oxide and apoptosis

The dengue virus may be an apoptosis inducer by direct or indirect mechanisms. Infection of Mo/MΦ or endothelial cells with DENV results in apoptosis, however, the mechanisms of apoptosis induction remain unclear. The free radical NO has emerged in recent years as a fundamental signalling molecule for the maintenance of homeostasis, as well as a potent cytotoxic effector. Under normal conditions, NO produced in low concentration acts as a messenger and cytoprotective (antioxidant) factor; however, when the circumstances allow the formation of substantial amounts of NO, this molecule could induce both oxidative and nitrosative stresses, which form the basis of the apoptosis generally attributed to NO.

Previous investigations have shown the presence of apoptosis in patients both with dengue and in experimental dengue infection. However, there is little information about the role of NO in apoptosis during dengue infection. In this regard, antibodies against dengue virus nonstructural protein 1 (NS1), which has cross-reactivity with endothelial cells (AECA), induce apoptosis via a NO-mediated mechanism in experimental dengue virus infection. Endothelial cells undergo apoptosis via the mitochondria-dependent pathway that is regulated by NO production, suggesting that NO-regulated endothelial cell injury may play a role in the disruption of vessel endothelium and contribute to the pathogenesis of vasculopathy. Other investigators have shown increased apoptosis in dengue virus-replicating Kupffer cells associated with increased expression of inducible NO synthase and production of NO. Apoptosis could avoid the release of viral particles and, together with the phagocytosis and digestion of apoptotic cells, represent mechanisms to prevent viral progeny. However, damage of endothelial cells and monocytes/macrophages could induce severe forms of dengue infection. Further investigation is required to determine the modulation of NO in order to provide the therapeutic strategies for dengue infection by preventing the AECA-mediated endothelial cell apoptosis and to determine the in vivo mechanism of NO-mediated apoptosis induced by DENV during the course of human dengue infection.

Conclusion and future perspective

NO, on the basis of its physiological chemistry, provides a conceptual framework, which helps to distinguish between the beneficial and toxic consequences of NO, and to envision potential therapeutic strategies for the future. The high levels of NO may be beneficial during dengue infection by its antiviral and apoptotic effects; however it could also induce severe forms of the disease by damage to endothelial cells. Further investigations are required to determine the source of NO during human dengue infection, its role in the pathogenesis of DF and DHF/DSS, its role in dengue infection according to the prevalence of human dengue resistance genes, and the presence of NO-resistant strains in all the DENV serotypes.

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Comparative oviposition preferences of *Aedes* (Stegomyia) *aegypti* (L.) to water from storm water drains and seasoned tap water

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### Abstract

The comparative oviposition preferences of *Aedes aegypti* to water from storm-water drains* and seasoned tap water were evaluated in the laboratory. The sample was collected from concrete storm-water drains with stagnant clear water in a dengue-endemic site, Taman Samudera, Selangor. *Ae. aegypti* adults were given a blood meal and released into the cage. Gravid females were given a choice between drain water and seasoned tap water for egg deposition. In a no-choice test, there was no significant difference in the numbers of eggs, larvae, pupae and adults colonized from the drain water and seasoned tap water (p>0.05), indicating that *Ae. aegypti* oviposit their eggs on a substrate which is readily available. In a choice test, the number of eggs laid by *Ae. aegypti* in drain water (1630.67 ± 204.26) was significantly more than that in seasoned tap water (221.33 ± 53.18) (p<0.05). The number of eggs was 6-fold higher in the drain water compared to seasoned tap water. The oviposition activity index was 0.71, indicating that the drain water was more attractive compared to seasoned tap water as an oviposition substrate. The pH and biochemical oxygen demand (BOD) values of both drain water and seasoned tap water were not significantly different (p>0.05), indicating that water from the drain did not contain high organic content. Significant water conductivity (p<0.05) and the presence of bacteria could have contributed to the site selection for egg laying by *Ae. aegypti*. The drain water successfully supported the colonization of the immatures, with the emergence of 824.33 ± 13.96 adult mosquitoes. The ratio of male and female mosquitoes was 1:1. This study concluded that the concrete drainage system with clear stagnant water provides a suitable medium for the colonization of dengue vector *Ae. aegypti*.

### Keywords:

Oviposition; *Aedes aegypti*; Drain water, Seasoned tap water; Malaysia.

### Introduction

During the 19\(^{th}\) century, dengue was considered a sporadic disease, causing epidemics at long intervals. However, dramatic changes in this pattern have occurred and currently, dengue ranks as the most important mosquito-borne viral disease in the world. In the past 50 years, its incidence has increased 30-fold, with large outbreaks occurring in five of the six World Health Organization (WHO) regions. At present, dengue is endemic in 112 countries in the world.\(^{11}\)

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*Water from storm-water drains will be mentioned as ‘drain water’ throughout the text.
Dengue has remained endemic in Malaysia since the first case was documented in 1901–1902.\[^2\] The disease was made notifiable in 1973.\[^3\]

*Aedes aegypti* is a domestic mosquito while *Aedes albopictus* is semi-domestic in urban areas. *Ae. aegypti* breeds exclusively in artificial containers such as earthen jars and plastic containers which contain relatively clean water in and around houses.\[^4\] The typical habitats of *Ae. albopictus* are artificial containers, tree holes and bamboo stumps near human dwellings.\[^5\]

The ability of gravid mosquito females to distinguish among potential oviposition sites that will or will not support the growth, development and survival of their offspring is critical for the maintenance of the mosquito population. Once the blood meal large enough to initiate ovarian development has been obtained and the temperature-dependent egg maturation period has been completed, mosquitoes must locate suitable oviposition sites.\[^6\]

In Malaysia, rapid urbanization has resulted in the creation of suitable habitats for *Ae. aegypti*. The present study was conducted to examine the suitability of drain water for the breeding of *Ae. aegypti* mosquitoes.

**Materials and Methods**

**Mosquito colony**

*Ae. aegypti* mosquitoes** (F 967) used in this study came from the colony maintained in the insectarium of the Medical Entomology Unit, Institute for Medical Research, Kuala Lumpur, Malaysia. The colony, established 30 years ago, produced test mosquitoes that are pathogen-free and homogenous.

**Collection of drain water from study site**

The drain water was collected from Taman Samudera (N03°13.987’, E101°41.918’), Selangor state, Malaysia, which is a dengue-endemic area, located 16 km from Kuala Lumpur city centre. Taman Samudera covers 30 ha of land which consists of approximately 400 double storey terrace brick houses.

The ovitrap surveillance conducted in a previous study by Chen et al.\[^7\] indicated that *Ae. aegypti* was the principal mosquito, both indoors and outdoors, as reflected by ovitrap collections.

Taman Samudera is an urban residential site, generally clean. This site had minimum artificial larval habitats such as containers and tyres. The major larval habitat appeared to be the concrete drain with clear stagnant water. The drain water was collected from five different drains in Taman Samudera for this study. All samples of the drain water were pooled together for colonization.

**Preparation of seasoned tap water**

Seasoned tap water (de-chlorinated water) was prepared by collecting the tap water directly from pipe into a plastic container and seasoned for several days.

**Measurement of water parameters**

The pH, conductivity and biochemical oxygen demand (BOD) of the drain and seasoned tap waters were determined. The bacterial fauna of the waters were determined by plating the water sample on Nutrient Agar. Each bacteria colony was isolated and identified at the Bacteriology Unit, Institute for Medical Research.
Comparative oviposition preferences of Aedes (Stegomyia) aegypti (L.) to water from storm water drains and seasoned tap water

Laboratory evaluation of oviposition preference

Preparation of ovitrap

Ovitrap as described by Lee\textsuperscript{[8]} was used in this study. Each ovitrap consisted of a 300 ml plastic container with straight, slightly tapered sides. A filter paper in cone shape was placed in the ovitrap to serve as a resting and oviposition site. Each ovitrap was filled with drain water or seasoned tap water to a level of 5.5 cm.

No-choice test

The ovitrap described as above was filled with drain water and placed into a cage (30 cm X 30 cm X 30 cm). A total of 30 laboratory-bred 5-days-old Ae. aegypti were given a blood meal by feeding on a guinea pig. The gravid females were released into the cage and left for a week. For all tests, 10% sugar solution was provided. At the end of the week, the ovitrap was removed. The filter paper was dried and the eggs were counted. The filter paper was then placed into a tray holding drain water for the eggs to hatch. The larvae, pupae and adult emergence were counted and recorded. Three replicates were conducted. The same procedure was repeated by using seasoned tap water.

Choice test

Two ovitraps each holding drain water and seasoned tap water were placed in a cage (30 cm X 30 cm X 30 cm). The experimental procedure was then carried out according to the no-choice test.

Data analysis

The relative attractiveness of test sample was expressed as an oviposition activity index (OAI) calculated according to Kramer and Mulla,\textsuperscript{[9]} where

\[ \text{Oviposition Activity Index (OAI)} = \frac{\text{NT}-\text{NS}}{\text{NT}+\text{NS}} \]

[NT denotes the number of eggs laid on test water (drain water) and NS denotes the number of eggs laid in control water (seasoned tap water). Index values range from +1 to -1. Positive values indicate that more eggs were deposited in the test water than in the control water, and that the test water was attractive. On the other hand, if more eggs were deposited in the control water than in the test water, negative index values will be obtained indicating that the test water acted as repellent]

Statistical analysis

\( T \)-test analysis (SPSS v.10) was used to determine the significant difference of the physical measures and the number of eggs, larvae, pupae and adult obtained between drain water and seasoned tap water. All levels of statistical significance were determined at \( p<0.05 \). The hatchability and emergence rate of Ae. aegypti in drain water and seasoned tap water were also calculated.

Results and discussion

The oviposition preference of Ae. aegypti to drain water and seasoned tap water from the no-choice test (Table 1) shows that there was no significant difference in the numbers of eggs, larvae, pupae and adult colonized from drain water and seasoned tap water (\( p>0.05 \)), indicating that Ae. aegypti oviposit their eggs on a substrate which is readily available. However, in the choice test (Table 2), the number of eggs laid by Ae. aegypti in drain water (1630.67 ± 204.26) was significantly more than in seasoned tap water (221.33 ± 53.18) (\( p<0.05 \)). The number of eggs was 6-fold higher in drain water compared to seasoned tap water. Furthermore, the oviposition activity index (OAI) was 0.71, indicating that drain water was more attractive compared to seasoned tap water as an oviposition substrate.
Comparative oviposition preferences of Aedes (Stegomyia) aegypti (L.) to water from storm water drains and seasoned tap water

The hatchability and emergence rates of Ae. aegypti in drain water and seasoned tap water (Figure) indicated that there was no complete hatchability and emergence in all the preferred water. The majority of eggs placed in drain water and seasoned tap water did hatch, with 69.0–82.0% hatchability in drain water and 63.0–74.0% in seasoned tap water. In both choice and no-choice studies, drain water and seasoned tap water successfully supported the colonization of Ae. aegypti immatures. In the choice test, a total of 824.33 ± 13.96 adult mosquitoes emerged from the population that was colonized in the drain water; and a total of 125.67 ± 44.95 adult mosquitoes emerged from the colony in the seasoned water (Tables 1 and 2).

**Figure:** Hatchability rate and emergence rate of Ae. aegypti in drain water and seasoned tap water

![Graph showing hatchability and emergence rates](image)

**Table 1:** Mean numbers of eggs, larvae, pupae and adult obtained from 30 gravid female Ae. aegypti in no-choice test

<table>
<thead>
<tr>
<th>Type of water</th>
<th>Mean number ± SE</th>
<th>Adult</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Egg</td>
<td>Larvae</td>
<td>Pupae</td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drain water</td>
<td>1630.67±204.26</td>
<td>1120.00±181.79</td>
<td>990.00±113.01</td>
<td>495.00±60.36</td>
<td>447.67±40.38</td>
<td>942.67±99.30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seasoned tap water</td>
<td>1262.00±116.69</td>
<td>928.67±114.04</td>
<td>846.67±129.34</td>
<td>413.00±70.87</td>
<td>396.33±79.00</td>
<td>809.33±145.17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-test</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td>p&gt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p<0.05 = Significant; p>0.05 = Not significant; SE = Standard error

**Table 2:** Mean numbers of egg, larvae, pupae and adult obtained from 30 gravid female Ae. aegypti in choice test

| Type of water     | Mean number ± SE | Adult         | OAI     |         |         |         |         |         |         |         |
|-------------------|------------------|---------------|---------|---------|---------|---------|---------|---------|---------|
|                   | Egg              | Larvae        | Pupae   | Male    | Female  | Total   |         |         |         |         |
| Drain water       | 1319.67±70.14    | 1080.00±76.05 | 877.67±8.29| 404.00±24.27| 420.33±29.55| 824.33±13.96| 0.71    |         |         |         |
| Seasoned tap water| 221.33±53.18     | 139.67±46.19  | 130.67±46.73| 65.33±24.90| 60.33±20.37| 125.67±44.95|         |         |         |         |
| t-test            | p<0.05**         | p<0.05**      | p<0.05**| p<0.05**| p<0.05**| p<0.05**|         |         |         |         |

* = p<0.05 (Significant); ** = p<0.01 (Highly significant); SE = Standard error
Comparative oviposition preferences of Aedes (Stegomyia) aegypti (L.) to water from storm water drains and seasoned tap water

There was no significant difference in the ratio of males and females colonized from both types of water (p>0.05). The ratio of males and females obtained from the drain water and seasoned tap water was 1:1 (Tables 1 and 2).

The physical measurement of drain water and seasoned tap water is as shown in Table 3. The pH and BOD values of both waters were not significantly different (p>0.05), indicating that water from the drain did not contain high organic content, i.e. the water was clean and clear.

Conductivity is a measure of the flow of electrical current, made possible in the water by ions in solution. The conductivity value obtained for drain water was 0.33 ± 0.06 and was significantly higher than seasoned tap water (0.08 ± 0.00) by four-fold (p<0.05), indicating that conductivity may play a role in mosquito oviposition preference.

Paradise and Dunson[10] reported that high concentration of Na⁺ in the solution may serve as a nutrient for the growth of microbial populations, thus increased microbial production, in part, through the food chain, leading to the support of larger numbers of mosquito larvae. Paradise and Dunson[10] also reported that in addition to direct uptake via anal papillae, mosquitoes may acquire Na⁺ through filter-feeding on microbes and may grow faster under condition of high concentration of Na⁺ due to decreased allocation of energy toward acquiring Na⁺ or increased production of food sources.

<table>
<thead>
<tr>
<th>Type of water</th>
<th>pH: Mean ± SE</th>
<th>Conductivity (ms/cm): Mean ± SE</th>
<th>BOD (mg/l): Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drain water</td>
<td>7.69 ± 0.09</td>
<td>0.33 ± 0.06</td>
<td>3.19 ± 0.43</td>
</tr>
<tr>
<td>Seasoned tap water</td>
<td>7.87 ± 0.03</td>
<td>0.08 ± 0.00</td>
<td>3.60 ± 0.10</td>
</tr>
</tbody>
</table>

p<0.05 = Significant; p>0.05 = Not significant; SE = Standard error

Table 3: Physical measure for drain water and seasoned tap water

<table>
<thead>
<tr>
<th>Water</th>
<th>Gram negative</th>
<th>Gram positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drain 1</td>
<td>Xanthomonas maltophilia</td>
<td>Nil</td>
</tr>
<tr>
<td>Drain 2</td>
<td>Flavimonas oryzihabitans</td>
<td>Nil</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas gladiali</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aeromonas hydrophila</td>
<td></td>
</tr>
<tr>
<td>Drain 3</td>
<td>Pseudomonas alcaligenes</td>
<td>Nil</td>
</tr>
<tr>
<td>Drain 4</td>
<td>Flavobacterium spp</td>
<td>Staphylococcus epidermidis</td>
</tr>
<tr>
<td>Drain 5</td>
<td>Alcaligenes like grp-1</td>
<td>Corynebacterium renal group</td>
</tr>
<tr>
<td>Seasoned tap</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Table 4: Bacteria isolated from drain water and seasoned tap water
Microbacterial examination from drain water and seasoned tap water is presented in Table 4. A total of 9 bacteria were found in 5 different drain water samples collected from Taman Samudera. In the seasoned tap water, no bacteria were found growing on the Nutrient Agar.

Since bacteria are important as nutrients for *Ae. aegypti*, it is reasonable that microbes and microbial metabolites influence site selection for egg laying. Certain species of bacteria have been shown to influence oviposition behaviour. Trexler et al. found that water containing *Psychrobacter immobilis* (from larval-rearing water), *Sphingobacterium multivorum* (from soil-contaminated cotton towels) and an undetermined *Bacillus* species (from oak leaf infusion) elicited significantly higher oviposition than control water without bacteria. Benzon and Apperson reported that two dominant species of bacteria, *Acinetobacter calcoaceticus* and *Enterobacter cloacae*, were identified in holding water, which contributed to the oviposition response of gravid *Ae. aegypti*. Hazard et al. also reported isolating bacterial species from an infusion of alfalfa hay that produced chemical stimulants of oviposition in *Ae. aegypti* and *Culex quinquefasciatus*.

Ikeshoji et al. isolated *Pseudomonas aeruginosa* and found that it produced an oviposition stimulant or attractant for *Ae. aegypti* and *Cx. pipens molestus* Forskal in vitro from decanoic acid. Two bacteria of the family Pseudomonadaceae were isolated in this study, i.e. *Pseudomonas gladiili* and *Pseudomonas alcaligenes*. In studies by Ikeshoji et al., it was shown that *Ae. aegypti* preferred water which had this family of bacteria.

Trexler et al. also reported that a combination of the bacterial species would elicit a stronger oviposition response, increasing the percentage of eggs that are deposited in the test containers.

Larvae of many mosquito species eat bacteria or depend on them to condition organic matter into suitable food. Our study indicates that at least 9 species of bacteria were isolated from the drain water; however, the species that commonly attract *Ae. aegypti* to oviposit their eggs have not been studied. The survival rates of offspring might be increased if an ovipositing female has the ability to discriminate among habitats based on bacterial quantity and species composition. Thus, further studies are required to explain this phenomenon.

Vythilingam et al. also reported that *Ae. aegypti* in the field is able to lay eggs in outdoor drain water. Hence, dengue vector control measures should now target the concrete drain system as well, being a potential source of *Aedes* breeding. In this study, we did attempt to detect the presence of *Aedes* breeding in the drains under field conditions but it was not possible because most portion of the drainage system was covered with concrete slabs. In Singapore, preventive dengue control measures include larviciding of drains in the residential sites, as *Ae. aegypti* and *Ae. albopictus* have been consistently found breeding in the drains (Rama Chandramogan, SWRO–NEA, *per. comm.*).

**Acknowledgments**

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Comparative oviposition preferences of Aedes (Stegomyia) aegypti (L.) to water from storm water drains and seasoned tap water

References


Evaluation of a grass infusion-baited autocidal ovitrap
for the monitoring of Aedes aegypti (L.)

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Abstract
Autocidal ovitraps used for the monitoring of Aedes (Stegomyia) aegypti (L.) (Diptera: Culicidae) in Singapore are routinely stocked with cow-grass (Axonopus compressus) infusion. The optimum concentration of this infusion was determined to be a 1:4 dilution of a stock infusion prepared by fermenting dried cow-grass in tap water at a rate of 10g/l. Aedes aegypti females confined in an empty room and offered an ovitrap baited with cow-grass in competition with a range of common oviposition sites (plastic bottle, paper cup, plastic pail, flower pot plate, Coca-Cola can, and vase) laid significantly more eggs in the ovitrap than in the next most competitive container. The number of eggs laid in the ovitrap did not change with the competitiveness of the other sites offered, nor with the number of other sites, but it did change – albeit non-linearly – with the number of mosquitoes released. Implications for the interpretation of quantitative ovitrap data for the monitoring of mosquito populations are discussed.

Keywords: Aedes aegypti; Autocidal ovitrap; Mosquito surveillance; Hay infusion; Oviposition site.

Introduction
Aedes aegypti (L.) is a container-inhabiting mosquito that is commonly found in urban areas. It is the primary vector of the dengue virus which infects hundreds of thousands of people every year, causing dengue fever (DF) and the potentially fatal, dengue haemorrhagic fever (DHF). No effective drug or vaccine against dengue has been developed despite several decades of research. Hence, past and current dengue control efforts are focused on reducing the population of Ae. aegypti.

Many control methods have been developed for Ae. aegypti, including adulticiding and larviciding with insecticides and/or breeding-source reduction. These interventions are adequately supported by health education and legislation. The commonly employed methods for the surveillance of Ae. aegypti include the inspection of premises for larvae and pupae and the use of ovitraps. Most

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Operational surveillance systems depend on such surveys to gather their house (premise) (the percentage of houses infested with larvae or pupae), Breteau (the number of positive containers per 100 houses) and larval-density indices. However, Focks and Chadee found no correlation between these traditional indices and the measured absolute densities of Ae. aegypti and stated that the Stegomyia indices used as epidemiological indicators of dengue transmission should be viewed with caution. They also concluded that pupal survey could provide an indication or estimation of the vector density. Direct sampling of the adult population requires very labour-intensive manual catching because the diurnal and essentially domestic adult Ae. aegypti do not respond well to light traps, even if the traps are supplemented with dry ice. A more detailed review of Stegomyia sampling methods was being carried out by Focks (2003), and it is not necessary for this paper to re-visit it.

Ovitrap had been used to provide useful data for Aedes control operation and had been reported to be more sensitive than the traditional Stegomyia indices in detecting low mosquito populations. Ovitrap are devices designed to attract female mosquitoes to lay eggs which can then be counted and identified. The original ovitrap, developed in the United States for the surveillance of Ae. aegypti, is a dark-coloured, water-filled container supplemented with a wooden paddle or velour paper strip as oviposition substrate. This design was first used in Singapore in 1969 as a supplementary measure for the control of Ae. aegypti in the Paya Lebar International Airport, Singapore. Subsequently, the design was modified by adding a floating plastic ring with fine wire mesh to prevent the escape of emerged adults. Chan et al. demonstrated that the resulting autocidal ovitrap was superior to common domestic containers in attracting Ae. aegypti to oviposit.

Ovitrap are now commonly used as a surveillance or monitoring tool in the field. Lee and Rawlins et al. found that Ae. aegypti surveillance using ovitraps is more sensitive than visual inspection for larvae. In the case of Ae. albopictus, studies by Ang et al. and Yap et al. indicated that the ovitraps they used attracted more ovipositioning females of that species than natural and other artificial containers.

In situations where the vector populations have been reduced to such low levels that larval searches are largely unproductive, ovitraps may be a useful indicator of the Aedes population. However, a distinction has to be made between using ovitraps to detect the presence of a species in a given area, and using quantitative ovitrap data as an indicator of the size of a mosquito population. The latter may only be feasible under certain conditions, since ovitrap catches are likely to be affected by factors other than the number of female mosquitoes, such as availability of alternative breeding sites, predation on the eggs, the physical surroundings of the ovitrap, etc. In the Philippines, Schultz found that ovitrap catches did not reflect population changes. Studies conducted in Singapore showed that simultaneous adulticiding and source reduction resulted in no change in ovitrap catches (Giger, unpublished data). This may suggest that any effect of a reduction in the mosquito population was cancelled out by the effect of a reduction in the number of alternative breeding sites. Alternatively, the house index in Singapore is as low as less than 1% and this may also have an effect on ovitrap catches.

The ability of gravid mosquito females to distinguish among potential oviposition sites that will or will not support the growth, development and survival of their progeny is critical because a wrong choice may result in the death of many larvae and affect the
Autocidal ovitrap for monitoring Ae. aegypti


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propagation of the species. Gravid females of many species show a high degree of preference in selecting specific oviposition sites.[14,15] Aedes aegypti uses both physical and chemical cues in its oviposition site selection.[16] These cues include the colour of the container, the texture of the oviposition substrate, and the water quality. Over the years, various aspects of the ovitrap have been improved, such as the structure of the oviposition substrate and the ovitrap as a whole, the water used, etc. Different types of organic materials – leaves, grass, sod, etc. – have been fermented to create infusions attractive to gravid Ae. aegypti. [17,18,19,20] Recent reports have shown that hay infusions were more attractive to ovipositioning Ae. aegypti females than clean water.[10,18]

However, Chadee et al.[19] indicated that there were no significant preferences by gravid Ae. aegypti mosquitoes to lay eggs in tap water or various concentrations of hay infusion. They suggested that this could be due to the variability in oviposition response in mosquitoes from different areas or variability in the type of hay used and differences in the methodology of preparation. Organic infusions are easy to make but different mosquito species will need different optimal infusions. The mass of the organic material and the fermentation time will determine the properties of the infusion.

In Singapore, ovitraps are routinely baited with cow-grass infusion that has been shown in the laboratory to be more attractive to female Aedes mosquitoes than clean water (Lam-Phua, unpublished data). Cow-grass (Axonopus compressus) is growing throughout the country in great abundance. It is cut on a monthly basis and usually disposed of as waste, making it easily available for this alternative use.

The study presented here consisted of three experiments, aiming to determine the concentration of cow-grass infusion that works best in the field (“Optimum concentration of infusion”); evaluate the competitiveness of the autocidal ovitrap baited with cow-grass infusion against various artificial containers commonly associated with Ae. aegypti breeding (“Ovitrap competitiveness”); and ascertain the effects of mosquito population size and availability of alternative breeding sites on the ovitrap catch (“Effects on ovitrap catch”).

Materials and methods

Mosquito colony origin and maintenance

Aedes aegypti larvae were collected in Singapore between January and August 2002 and reared to adults. Their offspring were reared in plastic trays (25 cm × 31 cm × 6 cm) with aged tap water, limiting the number of larvae to 800 per tray to avoid overcrowding. The colonies were maintained at 28 ± 1 °C, 79 ± 3% RH, and a photoperiod of approximately 9:15 hours (L:D). First instar larvae were initially fed with instant yeast (Bake King, Singapore). Subsequent instars were fed with dog chow (Glen Forrest Stockfeeders, Western Australia). The adults were kept in Plexiglas cages (30 cm × 30 cm × 30 cm) fitted with cotton cloth on two sides and were continuously provided with a 10% sucrose solution. The F1 and F2 generations were used in the experiments. The females were blood-fed on guinea pigs 3–5 days after emergence and used for the experiments two days later. This timing ensured that the oviposition peak four days after the blood meal[21] fell within the one-week duration of each replicate.
Preparation of infusion

Fresh cow-grass was collected by a contractor, cleaned of foreign matter, dried under the sun and packed in zip-loc bags until further use. Following a modification of the method of Reiter et al.,[18] stock infusions were prepared by fermenting dried cow-grass in tap water at a rate of 10 g/l. Grass and tap water were contained in a 50-litre plastic trough and were fermented for one week at a temperature of 28 ± 1 °C. The infusion was then strained through a screen to remove the cow-grass, and kept in plastic canisters at 28 ± 1 °C for not more than 8 weeks.

Study site

All experiments were carried out in 16 empty rooms (3.5 m × 3.0 m, 2.5 m high; concrete walls, floor and ceiling) in the same block of flats. All windows and openings were sealed with fine netting to prevent the mosquitoes from escaping while providing sufficient ventilation within the rooms. Throughout the study, the relative humidity and temperature inside the rooms was 78 ± 11% RH and 30 ± 1 °C.

Experiment 1: Optimum concentration of infusion

The stock cow-grass infusion was diluted with week-old aged tap water to obtain a range of concentrations. For each of the 48 replicates, four autocidal ovitraps[8] (height 155 mm, internal diameter 103 mm) with two hardboard oviposition paddles each (82 mm × 32 mm × 4 mm) and a floating plastic ring with fine wire mesh to prevent the escape of emerged adults were used (Figure 1). Each ovitrap was filled with 700 ml of cow-grass infusion at four different concentrations (0%, 25%, 50% and 100%) and randomly assigned to the four corners of a room. Ten gravid females were then released into the room and left undisturbed for 7 days. At the end of that period, larvae found in the ovitraps were counted and discarded, while the oviposition paddles were packed in individual plastic bags and brought back to the laboratory for examination. Eggs laid on the paddles were counted under a stereomicroscope, excluding empty egg shells. The total number of larvae and unhatched eggs were tabulated for each ovitrap. Data analysis was carried out on the total number of the eggs laid during the 7-day period, i.e. the number of larvae plus eggs laid on the paddles. The differences between data points were tested for significance using ANOVA (SPSS Ver 15.0).

Figure 1: Autocidal ovitrap. The two wooden paddles allow mosquitoes to lay eggs and the float plastic ring with fine wire mesh helps to prevent the escape of emerged adult mosquitoes
Experiment 2: Ovitrap competitiveness

Autocidal ovitraps baited with the optimum infusion determined in experiment 1 were tested against six different artificial containers that are commonly found in households and are known to attract oviposition by *Ae. aegypti* in the field. These containers were: plastic bottle (height 204 mm, base diameter 60 mm, transparent); paper cup (height 115 mm, opening diameter 80 mm, white with green pattern); plastic pail (3 litres, blue); flower pot plate (plastic, diameter 130 mm) under a potted money plant (*Scindapsus aureus*); aluminium Coca-Cola drink can (height 80 mm, diameter 58 mm, red and silver); vase (height 200 mm, opening diameter 65 mm, pink) with two lucky bamboo plants (*Dracaena* sp.). All the artificial containers were filled with week-old aged tap water to simulate field conditions.

For each of the 48 replicates, three different artificial containers and one ovitrap were randomly assigned to the four corners of a vacant room. The selection of containers for each replicate was random, with the condition that each container was included 24 times. Ten gravid females were then released into the room for 7 days, after which any larvae in the containers and ovitraps were counted, and the containers and oviposition paddles were brought back to the laboratory and thoroughly examined for eggs. The total number of larvae and unhatched eggs were tabulated for each container and ovitrap. Data analysis was carried out as in Experiment 1.

Experiment 3: Effects on ovitrap catch

In this experiment, one ovitrap was placed in a vacant room together with either 1, 3 or 9 pails as alternative breeding sites, and in each of these situations either 1, 3 or 9 gravid mosquitoes were released and left undisturbed for 7 days. Thus, there were nine different designs, each of which was replicated four times. Data collection and analysis were carried out as in Experiments 1 and 2.

Results

Experiment 1: Optimum concentration of infusion

In order to determine the optimum concentration of cow-grass infusion, two rounds of replicates were conducted. In the first round, the four concentrations offered were 0% (i.e. aged tap water only), 25%, 50% and 100% of the stock infusion. In this round, significantly more eggs were laid in the ovitraps with 25% infusion than in ovitraps containing any other concentration (p<0.01; Figure 2). There was no significant difference in the amount of eggs laid in the ovitraps with 0%, 50% and 100% infusion. In the second round of replicates, the range of concentrations was narrowed down to 0–45%. Here the numbers of eggs laid in the ovitraps with 0%, 50% and 100% infusion. In the second round of replicates, the range of concentrations was narrowed down to 0–45%. Here the numbers of eggs laid in 15%, 30% and 45%, respectively, were not significantly different, but all three of these concentrations received significantly more eggs.

Figure 2: Mean number of eggs laid in ovitraps containing different concentrations of cow-grass infusion

(Error bars: standard error)
than 0% (p<0.01). Hence, concentrations between 15% and 45% can be regarded as optimum concentrations. For practical reasons (ease of dilution of stock infusion by vector control officers in the field) the concentration of 20% stock infusion was selected for the standard infusion to be used in the remaining experiments and subsequent application in the field.

**Experiment 2: Ovitrap competitiveness**

The second experiment aimed to determine how well the autocidal ovitrap with cow-grass infusion competes with other types of potential breeding sites commonly found in the field. Pitted against three different containers at a time, the ovitrap attracted an average of 77.1% of the eggs laid in each replicate (Figure 3), which is significantly more than any other container (p<0.01). Among the remaining containers, the pail attracted significantly more eggs than any one of the others (p<0.01). There were no significant differences in the egg count among the other containers.

To determine the effect of the presence or absence of the most competitive alternative breeding site (the pail) on the absolute egg count in the ovitrap and the total egg count in each replicate, the replicates with a pail present were compared with the replicates without a pail. The presence of a pail did not have any significant effect on the absolute number of eggs laid in the ovitrap (mean absolute egg count ± standard error; with pail 538.5 ± 37.4; without pail 506.8 ± 32.3; p>0.05), but the average total egg count in each replicate was significantly higher with the pail present (with pail 764.1 ± 32.9; without pail 595.5 ± 29.4; p<0.01).

**Experiment 3: Effects on ovitraps catch**

The third experiment was designed to determine the respective effects of the mosquito population size and the number of alternative breeding sites on the number of eggs collected from ovitraps. The most competitive container in experiment 2 (the pail) was used as an alternative breeding site.
The number of alternative breeding sites had no significant effect on the number of eggs laid in the ovitrap, but the number of mosquitoes did (Figure 4, significantly different values are marked with a, b: p<0.01; c, d, e: p<0.05). However, the ovitrap catch did not increase linearly with the number of mosquitoes released into the room; with nine mosquitoes, the number of eggs in the ovitrap was, on average, less than twice that with three mosquitoes.

Discussion

As a product of fermentation, grass infusion may vary greatly in its attractive properties, depending on the quality and relative amounts of grass and water used, duration of fermentation, ambient temperature during fermentation, subsequent dilution and possibly other factors. However, if grass infusion is to be used in ovitraps for the purpose of quantitative monitoring of mosquito populations, its properties should ideally be constant and reproducible in order to allow comparisons over time and between ovitraps. In an attempt to standardize our cow-grass infusion as much as possible, we used dried grass and controlled the ratio of grass to water, ambient temperature and duration of fermentation to prepare a stock infusion. Experiment 1, carried out to determine the optimum dilution of the stock infusion, demonstrated that ovipositing females preferred infusions prepared from 15–45% stock infusion. Taking pragmatic considerations into account, the optimum concentration for the subsequent experiments as well as the application in operational monitoring was thus defined as a 1:4 dilution (to obtain a concentration of 20%) of the stock infusion.

The competitiveness of the autocidal ovitrap against a range of other containers had previously been tested for Ae. albopictus with all containers in close proximity. In Experiment 2 we attempted to evaluate that competitiveness for Ae. aegypti and in a setting more representative of the situation in the field, where ovitraps are not placed immediately next to competing breeding sites. However, due to operational constraints, our experiments had to be conducted within the confines of single rooms, which is still very different from the field situation, where the closest water-bearing container may be far away from the ovitrap and may be in a different room of a building or otherwise physically separated. Furthermore, the mosquitoes in our experiments were confined for one week in the vicinity of the same four breeding sites, which is unlikely to be the case in the field. Nevertheless, both experiments 2 and 3 demonstrate that, in a setting which provides the mosquitoes with ample opportunity to investigate all available oviposition sites, the ovitrap was much more competitive than any of the other containers tested. This high acceptability of the ovitrap with grass infusion to ovipositing females – even in the presence of other potential breeding sites – means that the ovitrap is well suited for the application as a monitoring tool in the field.

Even a very competitive ovitrap might be expected to receive fewer eggs in the presence of alternative oviposition sites. However, the results of experiment 2 reveal that the presence of a pail (the most competitive alternative container) did not decrease the absolute number of eggs laid in the ovitrap but, rather, increased the total number of eggs laid. Similarly, in experiment 3, the number of alternative breeding sites had no bearing on the ovitrap catch. These findings may be explained by the fact that all available oviposition sites were offered within one room and the mosquitoes were confined to that room. Ae. aegypti females are known to
Autocidal ovitrap for monitoring Ae. aegypti
distribute their eggs among a number of oviposition sites ("skip oviposition").[22,23] In the field, mosquitoes would encounter different oviposition sites sequentially, and the quality of an alternative breeding site encountered before an ovitrap may well affect the mosquito’s subsequent exploration and, thus, its chances of finding the ovitrap in the first place. On the other hand, after laying a clutch of eggs in an ovitrap in the field, the mosquito may well move out of the vicinity of the ovitrap and not return, a behaviour which would have been prevented in our experiment, leading to repeated oviposition in the ovitrap. These speculative arguments require further investigation, but they illustrate that the results of the experiments reported here do not rule out that the catches of ovitraps set in the field are affected by the number of alternative oviposition sites in the area.

In Experiment 3, the number of eggs laid in the ovitrap increased with the number of the mosquitoes present. This increase, however, was non-linear, with fewer eggs laid per mosquito when more mosquitoes were present. While the total number of eggs laid in each replicate increased more or less linearly with the number of mosquitoes, the ovitrap collected a decreasing share of these eggs. In other words, each mosquito, on average, laid fewer eggs in the ovitrap (the most attractive container) when there were more mosquitoes competing for the same oviposition sites. This finding, as well as the result of experiment 2 where more eggs were laid, in total, with two attractive containers available (ovitrap and pail) than with only one (ovitrap), suggests that there is a limit to the number of eggs an ovitrap can accommodate. The nature of such a restriction to oviposition remains unclear, but this finding agrees well with studies that demonstrated that female Ae. aegypti lay fewer eggs in containers with higher existing egg density.[24,25]

This study has established that the autocidal ovitrap baited with a standardized cow-grass infusion is highly competitive against oviposition sites commonly encountered in the field. Consequently, this ovitrap is well suited for Ae. surveillance in Singapore as far as the choice of container and infusion is concerned. The present knowledge is insufficient, however, to conclude whether quantitative data collected with these ovitraps would be able to provide a measure of the mosquito population in the field. To determine that, more research is required into the competitiveness of the ovitrap under true field conditions, as well as the nature of the apparent limit to the capacity of the ovitrap.

Acknowledgment
We would like to thank Mr Khoo Seow Poh, Director-General for Public Health, for his permission to publish this paper. Our greatest thank go to our lab attendant, Mr Semawi Bin Syed, for all the hard work done by him.

References


Development sites of Aedes aegypti and Ae. albopictus in Nakhon Si Thammarat, Thailand

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Abstract

This study investigated how the seasons affect the development sites of Aedes larvae in three topographical areas: mangrove, rice paddy fields and mountainous areas. We examined how the number of Aedes larvae varied in different types of water containers. Water containers were categorized into the following groups: indoor/outdoor containers, artificial/natural containers, earthen/plastic containers, containers with/without lids and dark/light-coloured containers. Samples were collected from 300 households in both the wet and dry seasons from three topographical areas in Nakhon Si Thammarat province with 100 households per topographical area. The results showed that in the wet season, there were higher numbers of Ae. aegypti and Ae. albopictus larvae than in the dry season. Moreover, the number of Ae. albopictus larvae was higher in mountainous areas than in mangrove and rice paddy areas, in both the wet and dry seasons. The number of positive containers was higher in outdoor containers than in indoor containers, higher in artificial containers than natural containers, higher in earthen containers than plastic containers, higher in containers without lids than containers with lids, and higher in the dark-coloured containers than light-coloured containers in the three topographical areas in both wet and dry seasons. The number of positive earthen containers was higher in mangrove areas than in rice paddy and mountainous areas. The number of positive plastic containers was higher in rice paddy areas than mountainous and mangrove areas.

Keywords: Aedes larvae; Season; Topography; Larval development site.

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Introduction

Dengue fever is caused by dengue viruses of the family Flaviviridae, transmitted principally by Aedes aegypti, and, possibly Ae. albopictus, in the tropical and subtropical regions of the world. Two clinical features, namely, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) may lead to death. No effective vaccine or chemotherapy is currently available for the prevention or treatment of dengue fever; therefore, prevention and control of the disease depend on vector surveillance and control measures. Transmission cycles of the dengue virus depend on the interrelationship between the virus and its mosquito vector, which is influenced by environmental conditions. Adult female Aedes mosquitoes acquire the dengue virus by biting infected humans during the viremic phase, which usually lasts for 4–5 days, although it may last up to 12 days. The virus is transmitted...
Development sites of Ae. aegypti and Ae. albopictus in Thailand

to other persons via bites from infected mosquitoes. The mosquitoes that adversely affect people in southern Thailand are primarily Ae. aegypti L. and Ae. albopictus Skuse. An epidemic of DHF occurred in the Samui Islands in 1966 and 1967 where Ae. aegypti and Ae. albopictus were abundant, and were responsible for the transmission of the dengue virus. Ae. aegypti breeds in a wide assortment of domestic containers, whereas Ae. albopictus is more likely to be found in natural containers, such as bamboo stumps and coconut shells, or in artificial containers outside the houses such as tyres, opened cans and plastic bottles. In southern Thailand, Ae. aegypti and Ae. albopictus have been found in forested habitats as well as in a variety of other habitats in rural and suburban areas.

Since most Thai households store water for cooking and bathing in a variety of jars and cisterns, Ae. aegypti is a more important threat for DHF. Ae. albopictus is encountered in the peripheral areas of towns where it replaces Ae. aegypti populations.

This study investigated the number of mosquito larvae (i.e. Ae. aegypti and Ae. albopictus) in different types of containers (i.e. indoor/outdoor containers, artificial/natural containers, earthen/plastic containers, containers with/without lids and dark/light coloured containers) in the wet and dry seasons in three topographical areas in Nakhon Si Thammarat, Thailand.

Materials and methods
Data collection

The mosquito larval survey was conducted in Nakhon Si Thammarat province (8° 32' 16.5" Nakhon Si Thammarat

Figure 1: (a) Map of Thailand; (b) Map of three topographical areas of study

(a) (b)

Mangrove, Rice paddy and Mountainous areas
Development sites of Ae. aegypti and Ae. albopictus in Thailand

N latitude, 99° 56' 50.7" E longitude) in two seasons: (i) dry season (March-April 2006) and (ii) wet season (October-November 2006). We conducted the mosquito larval survey in three topographical areas: mangrove, rice paddy and mountainous areas (Figure 1). People in these three topographical areas differed in terms of their main occupations. In the mangrove areas, the main occupations of the people are fishing and aquaculture, while in the rice paddy and mountainous areas, the main occupations are mostly agriculture and farm-related.

Samples were collected in households in all sub-districts in the nine districts comprising the study area using the stratified simple random sampling technique. Nine districts were divided into three categories: mangrove, rice paddy and mountainous areas. The mangrove areas included Khanom, Sichon and Pakpanang districts; the rice paddy areas included Praprom, Chalermprakiet and Huasai districts; and the mountainous areas included Thungyai, Bangkhan and Thungsong districts (Figures 1a, 1b). One hundred sampling units were assigned to each topographical area. The topographical areas were assigned as strata, one collected household identified as a sample unit. A structured questionnaire was constructed to obtain the information by personal interview. There were a total of 300 households sampled in the dry season. The same 300 households were re-sampled in the wet season in order to compare the difference in the number of Aedes larvae in various water containers between the two seasons.

Entomological studies

We collected all mosquito larvae from both indoor and outdoor containers using fine-meshed fishnets. The outdoor larval surveys were conducted within 15 m of the houses. Very small water containers were emptied out through the fishnet. Larger water containers were sampled by dipping the net in the water, starting at the top of the container and continuing to the bottom in a swirling motion that sampled all edges of the container. All live mosquito larvae were collected in plastic bags, taken to the 11.2 Vector-Borne Disease Control Centre laboratory, Nakhon Si Thammarat. All mosquito larvae were preserved and identified up to the species level using Rattanarithikul and Panthusiri’s keys. In this study, the first and second instars and pupae were discarded because immature mosquitoes at these stages could not be identified.

There were a total of 29 container categories in this study. Water containers were categorized into the following groups: indoor/outdoor containers, artificial/natural containers, earthen/plastic containers, containers with/without lids and dark/light-coloured containers. Indoor containers were all containers inside the house that held some water such as earthen jars, cement tanks, plastic containers, flower vases, ant guards, ornamental pots and refrigerators with plates. Outdoor containers were all containers within 15 m of the house, such as earthen jars, cement tanks, plastic containers, ornamental pots and natural containers. Artificial containers included earthen jars, flower vases, used cans, discarded tyres, metal boxes, preserved areca jars and foam containers. The preserved areca jars were earthen jars used to preserve areca nuts. Natural containers were composed of areca/coconut husks, banana trees, coconut shells, tree holes and bamboo clumps. Earthen containers were composed of small/large earthen jars, cement tanks, plastic containers, plastic bottles and plastic buckets. Lids were made of cement, plastic, wood and metal. The dark-coloured containers were containers that were black, brown, dark grey, deep green, deep blue and red. On the other hand, the light coloured containers were containers that were white, light pink, light blue and yellow.
Aedes larval index: Container Index (CI) was worked out as per standard WHO guidelines. The CI was defined as the percentage of water-filled containers positive for Aedes breeding.

**Statistical analysis**

The number of *Ae. aegypti* and *Ae. albopictus* larvae in different types of water containers, both indoor and outdoor, were analysed using independent sample *t*-tests. Chi-square tests were used to test the differences between the following factors: (i) indoor/outdoor containers; (ii) artificial/natural containers; (iii) earthen/plastic containers; (iv) containers with lids and without lids; and (v) dark/light-coloured containers in all three topographical areas, two seasons and their interactional terms. All significant tests were two-tailed.

**Results**

*Aedes* larvae and container types

*Aedes* larvae were found in 15 out of 29 types of water containers in all three topographical areas (Figures 2a-f). There were no *Aedes* larvae found in two types of indoor containers in mountainous areas (Figures 2a-f). *Ae. aegypti* larvae were found in 11 out of 29 types of water containers (Figures 2a-f). From 11 out of 29 containers, *Ae. aegypti* larvae were found in two types of indoor containers (i.e. cement tanks and ant guards) and nine types of outdoor containers (i.e. small jars, large jars, plastic tanks, used cans, tyres, metal boxes, plant pots, animal pans and preserved areca jars) (Figures 2a-f). *Ae. albopictus* larvae were found in 10 out of 29 types of water containers. All 10 out of the 29 water container types where *Ae. albopictus* was found were outdoor containers (i.e. small jars, large jars, cement tanks, plastic tanks, used cans, tyres, metal boxes, animal pans, preserved areca jars and coconut shells (Figures 2a-f). The number of *Ae. albopictus* larvae in metal boxes was higher than *Ae. aegypti* larvae in both mangrove and mountainous areas in the dry season (mangrove area: $t_{98} = –2.372$, $P<0.05$; mountainous area: $t_{117} = –2.919$, $P<0.01$, Figures 2b, f).

The number of *Ae. aegypti* larvae was higher in the wet season than in the dry season, this did not differ among the three topographical areas, and there was some interaction between topographical areas and seasons (Table 1). The number of *Ae. albopictus* larvae were higher in the wet season than in the dry season, higher in mountainous areas than in mangrove and rice paddy areas in both

<table>
<thead>
<tr>
<th>Season</th>
<th>Topography</th>
<th>Statistical test</th>
<th>$\chi^2$</th>
<th>df</th>
<th><em>P</em></th>
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<td>Rice paddy area</td>
<td>Mountainous area</td>
<td>$\chi^2$ topology</td>
<td>$\chi^2$ season</td>
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<td>156</td>
<td>228</td>
<td>36</td>
<td>135</td>
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<tr>
<td>Wet</td>
<td>Dry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of <em>Ae. albopictus</em> larvae</td>
<td>526</td>
<td>452</td>
<td>817</td>
<td>280</td>
<td>183</td>
</tr>
</tbody>
</table>

*P<0.001
Development sites of Ae. aegypti and Ae. albopictus in Thailand

Figure 2. *Ae. aegypti* (■) and *Ae. albopictus* (□) larval occurrence in three topographical areas: (a)-(b) mangrove areas, (c)-(d) rice paddy areas, and (e)-(f) mountainous areas in the wet and dry season.

Indoor containers: CTI = cement tanks, AG = ant guards. Outdoor containers: SJO = small jars, LJO = large jars, CTO = cement tanks, PTO = plastic tanks, UC = used cans, T = tyres, MB = metal boxes, PP = plant pots, AP = animal pans, PAJ = preserved areca jars, PC = plastic containers, AH = areca husks, CS = coconut shells.

(a) Mangrove area in wet season

(b) Mangrove area in dry season

(c) Rice paddy area in wet season

(d) Rice paddy area in dry season

(e) Mountainous area in wet season

(f) Mountainous area in dry season
the wet and dry seasons, and there was some interaction between topographical areas and seasons (Table 1).

Positive indoor and outdoor containers

There were more outdoor containers that were positive than indoor containers, more positive containers in the dry season than in the wet season, no interaction between indoor/outdoor containers and season, no differences in indoor/outdoor containers among the three topographical areas, and no interaction between indoor/outdoor containers and topographical areas (Table 2).

Positive artificial and natural containers

There were more artificial containers that were positive than natural containers (Table 3). There were some differences between the number of positive artificial and natural containers in the wet and dry seasons (Table 3). There was some interaction between artificial/natural containers.

Table 2: Container Index (CI) and the number of positive indoor and outdoor containers in three topographical areas in the wet and dry seasons

<table>
<thead>
<tr>
<th>Factors</th>
<th>Container Index</th>
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<td>Indoor Outdoor</td>
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<tr>
<td>Season</td>
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</tr>
<tr>
<td>Wet</td>
<td>0.19 3.27</td>
<td>2 117</td>
<td>$\chi_1^2=247.531^{**}$</td>
</tr>
<tr>
<td>Dry</td>
<td>0.43 4.08</td>
<td>4 148</td>
<td>$\chi_1^2=4.018^*$</td>
</tr>
<tr>
<td>Topography</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mangrove</td>
<td>0.34 3.23</td>
<td>2 103</td>
<td>$\chi_1^2=253.520^{**}$</td>
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<tr>
<td>Rice paddy</td>
<td>0.49 3.41</td>
<td>4 79</td>
<td>$\chi_2^2=2.801$</td>
</tr>
<tr>
<td>Mountainous</td>
<td>0 4.75</td>
<td>0 89</td>
<td>$\chi_2^2=4.761$</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.001

Table 3: Container Index (CI) and the number of positive artificial and natural containers in three topographical areas in the wet and dry seasons

<table>
<thead>
<tr>
<th>Factors</th>
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<td></td>
<td>Artificial Natural</td>
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<tr>
<td>Season</td>
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<tr>
<td>Wet</td>
<td>3.03 1.16</td>
<td>106 13</td>
<td>$\chi_1^2=210.779^{***}$</td>
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<tr>
<td>Dry</td>
<td>4.51 0.24</td>
<td>149 3</td>
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<td>4.57 0.38</td>
<td>97 6</td>
<td>$\chi_1^2=210.779^{***}$</td>
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<tr>
<td>Rice paddy</td>
<td>2.90 0.77</td>
<td>76 3</td>
<td>$\chi_2^2=3.218$</td>
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<tr>
<td>Mountainous</td>
<td>4.00 1.81</td>
<td>82 7</td>
<td>$\chi_2^2=1.248$</td>
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</table>

*P<0.05, **P<0.01, ***P<0.001
Development sites of Ae. aegypti and Ae. albopictus in Thailand

There were no differences in artificial/natural containers among the three topographical areas and no interaction between artificial/natural containers and topographical areas (Table 3).

**Positive earthen and plastic containers**

There were more positive containers among earthen containers than plastic containers and no differences in earthen/plastic containers between the wet and dry seasons, (Table 4).

**Positive containers with and without lids**

There were more positive containers among containers without lids than containers with lids, more positive containers with lids and without lids in the dry season than the wet season. No

<table>
<thead>
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<td>0.71</td>
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*P<0.01, **P<0.001

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<tr>
<td>Season</td>
<td>Wet</td>
<td>1.31</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>Dry</td>
<td>3.53</td>
<td>3.25</td>
</tr>
<tr>
<td>Topography</td>
<td>Mangrove</td>
<td>2.65</td>
<td>2.81</td>
</tr>
<tr>
<td></td>
<td>Rice paddy</td>
<td>2.28</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>Mountainous</td>
<td>2.12</td>
<td>4.18</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.001

Table 4: Container Index (CI) and the number of positive earthen and plastic containers in three topographical areas in the wet and dry seasons

Table 5: Container Index (CI) and the number of positive containers with and without lid in three topographical areas in the wet and dry seasons
Development sites of Ae. aegypti and Ae. albopictus in Thailand

There were more positive containers among dark-coloured containers than light-coloured containers. There were no differences in dark/light-coloured containers among three topographical areas (Table 6).

Discussion

Aedes larvae and container types

Our results showed that Ae. albopictus larvae were found only in outdoor containers but Ae. aegypti were found in both indoor and outdoor containers. This indicates that Ae. aegypti and Ae. albopictus have different preferred development sites that slightly overlap. Many studies have shown that Ae. albopictus prefers to lay eggs in outdoor natural containers and Ae. aegypti tends to lay eggs in artificial indoor water containers that are regularly filled with water even during the dry season. With increasing housing development, good tap water systems, and effective solid-waste management that do not necessitate water-catching natural containers and solid-waste to accumulate on the premises, the breeding potential for Ae. albopictus may decline in the future. In highly urbanized areas in Thailand such as Bangkok, Ae. aegypti has replaced Ae. albopictus.

This study clearly demonstrates that the number of Aedes larvae was higher in the wet season than in the dry season. Many studies have reported the same findings in many countries such as Thailand, Fiji and the U.S. Long rainy seasons, with peculiar water-use patterns of the residents, create favourable conditions leading to a high number of Aedes larvae in the rainy season. People living in Nakhon Si Thammarat prefer rain- and well-water to piped water for drinking and cooking purposes, and, for this reason, rain- and well-water are always stored in water containers in and around the house.

Previous studies have shown that the number of Ae. aegypti and Ae. albopictus varies depending on topography. This could be due

Table 6: Container Index (CI) and the number of positive dark- and light-coloured containers in three topographical areas in the wet and dry seasons

<table>
<thead>
<tr>
<th>Factors</th>
<th>Container Index</th>
<th>No. positive container</th>
<th>Statistical test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dark Light</td>
<td>Dark Light</td>
<td></td>
</tr>
<tr>
<td>Season</td>
<td>Wet Dry</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.59 2.51</td>
<td>95 24</td>
<td>$\chi^2_1$ =146.129*** $\chi^2_2$ =4.018* $\chi^2_1$ =8.728**</td>
</tr>
<tr>
<td>Topography</td>
<td>Mangrove Rice paddy Mountainous</td>
<td>2.91 1.98 2.58 0.99</td>
<td>92 11 62 17 81 8</td>
</tr>
</tbody>
</table>

*P<0.05, **P<0.01, ***P<0.001
to the different needs for water-storage containers in topographic types. There was no tap water available in mangrove areas; people in this area have to store water for drinking and washing in large water containers, especially in the dry season. Our results showed that there were more *Aedes* larvae in the mangrove areas than in other areas in the dry season. However, our results showed that there were higher numbers of *Aedes* larvae in mountainous areas than in other areas in the wet season. This could be because there was a higher amount of rainfall in mountainous areas during the wet season. Waste products such as cans, bottles, tyres, etc., might collect rain-water creating many suitable larval development sites for *Aedes* larvae in the area. In addition, there were many rubber plantations around the human dwellings in the mountainous areas. It is a common practice to suspend tapping temporarily in rubber plantations leading to accumulation of rainwater in sap-collecting containers and small shallow ponds between rows of rubber trees. Therefore, these would provide an ideal developmental site for *Aedes* mosquitoes, especially *Ae. albopictus*. Sumodan's study showed that *Ae. albopictus* laid eggs in sap-collecting containers and their eggs could fully develop into mature adults during that period.

**Positive indoor and outdoor containers**

Our results support previous findings in Khonkean, Thailand, and Pattani, Thailand, that the number of positive containers was higher in outdoor than indoor containers. However, Kittayapong and Strickman found that containers placed inside houses had more larvae than those placed outdoors and under eaves or in the bathroom. Most indoor water-storage containers are cleaned regularly one to three times per week. This kind of routine cleaning practice helps to eliminate *Aedes* larvae in the indoor containers, especially *Ae. aegypti* larvae.

**Positive earthen and plastic containers**

Our results showed that the number of positive containers was higher among earthen containers than among plastic containers. This result supports Luemoh et al.'s study that there were a lower number of *Aedes* larvae in plastic water containers, both with lids and without lids than clay or cement containers. This could be for two possible reasons. First, people in Nakhon Si Thammarat prefer to store their water in earthen containers than plastic containers. This may lead to mosquitoes that prefer to oviposit eggs in earthen water containers over time. Second, previous studies show that female mosquitoes prefer to oviposit in darker coloured and less transparent containers over lighter coloured and more opaque containers. Earthen containers usually are dark brown colour and completely dark, while plastic containers are more often light coloured and transmit light. Third, temperatures in earthen containers tend to vary less than in plastic containers. This might promote full mosquito larval development more than in highly variable temperatures.

**Positive containers with and without lids**

Our results support previous findings that the number of positive containers was higher in containers without lids than containers with lids. This could be for two possible reasons. First, lids could prevent gravid mosquito females from ovipositing in water storage containers. From a behavioural study, Ae. *aegypti* females actively sought the narrow gaps...
between the lid and the lip of the water containers to reach oviposition sites, although the number of eggs deposited was less in containers with lids. Second, lids may prevent leaf litter, insects and organic materials falling into the water containers. These water containers with lids will have fewer nutrients available. High nutrient water is a signal of good food in terms of quality and quantity known to attract ovipositioning females. Therefore, water containers with lids might attract less ovipositioning females due to less nutrients being available for mosquito larvae required during larval development.

Positive dark- and light-coloured containers

A significant amount of research has been done on colour cues for oviposition behaviour in mosquitoes. For example, Toxorhynchites r. septentrioinalis, Tx. r. rutilus, Tx. moctezuma and Tx. Amboinensis prefer to oviposit in black rather than white containers. In addition, Yonoviak's study investigated how the colour of water containers affects mosquito diversity and abundance and found that black- and red-coloured artificial treeholes attracted higher mosquito species diversity and abundance than green-coloured artificial containers. Our results support previous findings that the number of Aedes larvae was higher in dark-coloured containers. Therefore, in order to minimize the number of Aedes larvae in water storage containers, people might use light-coloured water storage containers in their houses.

Acknowledgements

This work was supported in part by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Programme (Grant No. PHD/0201/2548), the Institute for the Promotion of Teaching Science and Technology, and CXKURUE, the Institute of Research and Development, Walailak University. We thank John A. Endler and David Harding for their comments on the previous versions of this manuscript. We also thank the 11.2 Vector-Borne Disease Control Centre, Nakhon Si Thammarat, for mosquito larval identification and students from Yothinbamrung School for field surveys in this study.

References


Behavioural responses of deltamethrin- and permethrin-resistant strains of *Aedes aegypti* when exposed to permethrin in an excito-repellency test system

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Abstract

This study compared the behavioural avoidance responses of the permethrin-resistant and deltamethrin-resistant strains of *Aedes aegypti*, a primary vector of dengue haemorrhagic fever (DHF) in Thailand. The background of biochemical-based resistance mechanism assay of these two strains revealed a significant increase of esterase activity and monoxygenase levels when compared with a laboratory-susceptible strain. Glutathione-S-transferase activity was found to increase only in the permethrin-resistant strain. The DNA sequence of knockdown resistance (*kdr*) mutation in the voltage-gated sodium channel (IIS6 region) was determined but the leucine to phenylalanine amino acid substitution, which is commonly associated with resistance to pyrethroids in many insect species, was not found in either strain. The behavioural escape response of both contact irritancy and non-contact repellency when exposed to permethrin at standard field dose (0.25 g/m²) was observed by using an excito-repellency test chamber. The results showed that in contact trials, the permethrin-resistant strain showed a lower irritancy response when compared with the deltamethrin-resistant strain. This was probably due to the higher levels of resistance to this insecticide for the permethrin resistance strain. For the repellency test by non-contact trials, the response was not significantly different between the two strains. This may be because the repellency effect was much weaker than that of the irritancy effect. This study indicated that the behavioural response of mosquitoes differs according to different pyrethroid compounds and to the physiological resistance mechanism of the mosquitoes. However, further work is necessary to understand how these responses are mediated.

Keywords: Behavioural response; *Aedes aegypti*; Insecticide resistance; Excito-repellency test chamber.

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Introduction

Insecticide resistance in *Aedes aegypti*, a primary vector of dengue haemorrhagic fever (DHF), has been reported in many parts of the world. In Thailand, fenitrothion, deltamethrin and permethrin are the primary insecticides used in thermal fogging and ultra-low volume sprays for the control of adult *Aedes aegypti* mosquitoes during DHF outbreaks. The widespread use of insecticides has led to selective insecticide resistance in vector mosquitoes reducing the efficacy of control measures.

Insecticide resistance can be grouped within four categories:

1. **Behavioural resistance** where the insect behaviour is modified to avoid contact with the insecticide;
2. **Penetration resistance** where the composition of the insect’s exoskeleton becomes modified in a way that inhibits insecticide penetration;
3. **Target-site insensitivity** where the chemical site of action for the insecticide is modified, limiting the affinity of the insecticide for the target site and reducing its ability to disrupt the function of the target site; and
4. **Metabolic resistance** where detoxification enzymes rapidly break down the insecticide so it is no longer toxic to mosquitoes.

Although several reports of insecticide resistance in *Ae. aegypti* have been published, most of them describe biochemical resistance. The impact of these compounds on *Ae. aegypti* in terms of behavioural resistance has not been studied.

In this paper, permethrin and deltamethrin-resistant strains were examined for target-site insensitivity and for behavioural resistance.

Materials and methods

Test populations

A susceptible colony of *Ae. aegypti* from the Department of Medical Sciences, Ministry of Public Health, Thailand, was used as a laboratory-susceptible strain.

Two selected strains of *Ae. aegypti*, i.e. one deltamethrin-selected strain which showed significant elevation of esterase and monoxygenase activity as compared with laboratory-susceptible strains, and the other the permethrin-resistant strain which had a significant increase in glutathione-S-transferase activity when compared with the deltamethrin-resistant strain and the susceptible strain. The details to obtain these two resistant strains were described elsewhere.

PCR and sequencing of partial genomic DNA of sodium channel gene

Adult female mosquitoes, from permethrin- and deltamethrin-selected strains were used for the detection of point mutation in the segment 6 of domain II (IIS6) of sodium channel gene. The mosquitoes were homogenized individually and total genomic DNA was extracted by phenol chloroform method. The DNA pellet was diluted in TE buffer before using as PCR template. PCR was performed by using AegF, (5’ AAC TTA CTC ATT TCC ATG G3’) and Dg2, (5’ GC (T/G/A) AT (C/T) TT (A/G) TT(G/A/T/C) GT (G/A) TC (G/A) TT (G/A) TC 3’) primers. Nested PCR was carried out using primers Dg1, (5’ TGG AT (T/C/A) G (A/C) (A/G) (T/A) (C/G) (A/C/T) ATG TGG GA (T/C) TG 3’) and Dip2, (5’TGG GAC AAA AGC AA (G/A) GCT AAG 3’) primers. The PCR products were subjected to DNA sequencing (Bioservice Unit, Thailand).

Behavioural resistance test

Tests were carried out to compare the behavioural responses of the two strains of *Ae. aegypti* exposed to 0.25 g/m² permethrin by
Behavioural responses of deltamethrin- and permethrin-resistant strains of Aedes aegypti

using excito-repellency test chambers (Figures 1 and 2) developed by Chareonviriyaphap et al. Generally, behavioural responses, or insecticide avoidance, can be categorized as contact irritancy and non-contact repellency. Irritancy results from physical contact with chemical-treated surfaces whereas repellency is a response from a distinct distance without physical contact with insecticides.

The test system consists of two treated test chambers and two paired control chambers. Prior to the exposure, mosquitoes were starved for at least 24 hours. Twenty-five female mosquitoes were carefully transferred into each of the 4 test chambers (Figure 3). Mosquitoes were allowed a 3-min resting period to permit adjustment to the chamber conditions. Observations for behavioural responses were taken at 1 min intervals for 30 mins. After each test was completed, the number of dead or knockdown specimens was recorded separately for each exposure chamber, paired control chamber and external holding cage, which was the receiving box connected to the exit portal for collecting escaped mosquitoes. Escaped specimens and those remaining inside the chambers, for each treatment, were held

Figure 1: Excito-repellency test chamber 1

Figure 2: Excito-repellency test chamber 2
Behavioural responses of deltamethrin- and permethrin-resistant strains of Aedes aegypti

Results

PCR and sequencing of the partial genomic DNA of sodium channel gene

To investigate whether alteration of the sodium channel gene was involved in resistance mechanism, a 400 bp of genomic DNA sequence comprising the S6 trans-membrane segment of domain II (IIS6) in the sodium channel gene was PCR-amplified and sequenced. The result revealed that the leucine (L, CTC) to phenylalanine (F, TTC) substitution was not found from the mosquitoes sequenced, although all mosquitoes showed the highest resistance against insecticide from each strain.

Behavioural test

Even though this was the preliminary study, as the sample size was not sufficient for statistical evaluation, it can be seen that in contact trials the deltamethrin-resistant strain showed higher contact escape response (27.3% within 30 mins) compared with the permethrin-selected strain. The difference in escape response was not observed in non-contact trials. The percentage of the mosquitoes remaining in the exposure chambers under contact and non-contact conditions are shown in Figures 4 and 5 respectively.

Figure 3: Excito-repellency test chamber 3
**Figure 4:** Escape pattern of permethrin- and deltamethrin-resistant populations in contact trial with 0.25 g/m² permethrin

**Figure 5:** Escape pattern of permethrin- and deltamethrin-resistant populations in non-contact trial with 0.25 g/m² permethrin
The percentage of mortality of the escaped mosquitoes was a little lower than those remaining in the test chamber for the two strains, both in contact and non-contact trials (Table).

**Table**: Escape response and percent mortalities of permethrin- and deltamethrin-resistant populations when exposed to 0.25 g/m² in contact and non-contact trials

<table>
<thead>
<tr>
<th>Test condition</th>
<th>Strain</th>
<th>Treated chamber</th>
<th>Control chamber</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. tested</td>
<td>% escaped</td>
<td>No. tested</td>
<td>% escaped</td>
</tr>
<tr>
<td></td>
<td>Treatment chamber</td>
<td>Control chamber</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Escaped</td>
<td>Did not escape</td>
<td>Escaped</td>
<td>Did not escape</td>
</tr>
<tr>
<td>Contact</td>
<td>Permethrin-resistant</td>
<td>99</td>
<td>11.1</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Deltamethrin-resistant</td>
<td>99</td>
<td>27.3</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Lab-susceptible</td>
<td>96</td>
<td>25.0</td>
<td>93</td>
</tr>
<tr>
<td>Non-contact</td>
<td>Deltamethrin-resistant</td>
<td>99</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Lab-susceptible</td>
<td>95</td>
<td>35.8</td>
<td>95</td>
</tr>
</tbody>
</table>

From this study the lower irritancy response in the permethrin-selected strain was probably due to its higher levels of resistance to this insecticide, which may have influenced the escape response because of increased duration of exposure time with this insecticide. Similar results were observed with *Anopheles gambiae* by Chandre et al. These authors demonstrated that resistant mosquitoes could tolerate higher-dose permethrin and stayed longer than susceptibles.

For a repellency test by non-contact trials, the response was not significantly different between the two strains. This may be due to repellency effect which was much weaker than that of the irritancy effect.

Even though the permethrin-resistant strain of *Ae. aegypti*, which has increased metabolic detoxification enzymes involved in its resistance, does not change the target-site insensitivity but it showed a behavioural resistance to permethrin. This study provides important information because the biochemical resistance in mosquito vectors can mediate their behavioural response to that insecticide when applied as a space spray or on surface-treated areas for adult control.

**Discussion**

**PCR and sequencing of the partial genomic DNA of sodium channel gene**

The results suggested that other mechanisms, such as the increase of detoxification enzymes, may be involved in the resistance, or another site of point mutation which is not commonly associated with resistance to pyrethroids in many insect species may be involved in *Ae. aegypti* resistance. This requires further studies.

**Behavioural test**

In our experiment, the study was designed to know the contact and non-contact responses of the permethrin-resistant strain and the deltamethrin-resistant strain of mosquitoes to permethrin. So, no bait or similar attractant was used to avoid any confusion in the results.
References


Perceived self-efficacy to plan and execute an environmental action plan for dengue control among Filipino University students

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Abstract

This study sought to evaluate the change in the perceived self-efficacy in students exposed to a training module with field exercise to conduct an environmental action plan for dengue control. Eighteen education students at a Filipino university participated in the study. A pretest-post-test design was employed. Evaluation was conducted through a nine-point self-efficacy scale. Field exercises were conducted on the grounds of the university campus. The results demonstrated statistically significant increases in perceived self-efficacy scores.

Keywords: Dengue control; Environmental action plan; Self-efficacy; Students.

Introduction

Dengue fever/dengue haemorrhagic fever (DF/DHF) is at present the leading mosquito-borne viral disease worldwide.[1] Dengue especially affects school-age children and youth.[2] Schools, especially in the tropical and subtropical dengue-endemic areas, are frequently the breeding sites for Aedes aegypti, the carrier of the dengue virus.[3,4] Teachers and students therefore potentially provide key links between the schools, the students and the community in dengue control.[5] Teachers have the potential not only to be trainers to their students in dengue control but also can serve as role models on their school campus grounds. In addition, teachers can serve as facilitators for community action, especially as their students are able to carry out dengue control activities, such as environmental action plans for their own homes and neighbourhoods.

Mosquito larval control through source reduction of mosquito breeding sites remains a primary emphasis in dengue control.[6] Various international projects lay stress on source reduction for dengue control at the community level and at schools.[7]

A number of studies have described the mobilization of schoolchildren through involvement of teachers. In the English-speaking Caribbean, teachers trained children as community-change agents through an environmental health model.[8] In Colombia, eleventh graders, after 20 hours of training, conducted community-wide dengue control

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communication. One creative approach was the use of calendars in classrooms by teachers. In Malaysia, schoolchildren distributed weekly worksheets to households. In Puerto Rico, fourth graders had a dengue educational booklet integrated into class work. Their teachers received specific training as a part of the programme. In Honduras, primary-school children, after receiving training by teachers, conducted household mosquito control surveys. Also in the study, parents and teachers had an increased awareness about mosquito control. However, no study has been done to show that teachers, or students who had been trained, had undergone field exercises to prepare them in dengue control, let alone examine the participants’ confidence levels through their experience.

The health behaviour theory provides a framework to conduct health education and health promotion programmes. Self-efficacy, a construct of Social Cognitive Theory, is a powerful predictor of behaviour. One’s efficacy beliefs are central to this construct. According to Bandura, “Perceived self-efficacy refers to beliefs in one’s capabilities to organize and execute the courses of action required to produce given attainments.” Self-efficacy significantly increased in two dengue-related studies, one in Thailand, and the other in the Philippines. In Thailand, self-efficacy to carry out source-reduction measures to reduce mosquito breeding sites increased through a community-based programme. Self-efficacy to perform dengue prevention and control measures significantly increased as a result of the play of a board game among Filipino elementary and high-school students.

No study has yet demonstrated the use of an environmental action plan for teachers or future teachers to increase self-efficacy of mosquito larval prevention and control measures to control dengue. Therefore, this study sought to examine whether university education students (future teachers) may increase their perceived self-efficacy to conduct dengue-related mosquito control measures through the use of environmental action plan training and experiential field activity to control mosquito breeding sites in a university setting. In this study, perceived self-efficacy is operationalized to be defined as the beliefs one has in one’s capability to execute an environmental action plan as later described in this study. This refers to the self-reported self-efficacy without validation through behavioural indicators.

Materials and methods

Participants

Eighteen students from the Foundation University College of Education course entitled, “Personal and Community Hygiene” in Dumaguete city, Philippines, a dengue-endemic city participated in the study in 2005. The student group included 14 females and four males and had no previous exposure to the environmental action plan. Participation in the evaluation of the study activities was voluntary. All the students participated till the completion of the exercise. The majority of the students were primary-school trainees while the remainder were secondary-school trainees. The breakdown of the number of first-, second- and third-year students was seven, nine and two respectively. There were no fourth-year students, as all of them had previous exposure to the environmental action plan. The average age of the students was approximately 20 years.
Perceived self-efficacy to plan and execute environmental plan for dengue control

Materials

A nine-item self-efficacy questionnaire was distributed to the participants. This questionnaire covered such topics as confidence in the following: regular removal of disposable containers, cover reusable containers, change water in flower vases, removal of used tyres, turn over open coconut shells, participation in environmental clean-up, as well as identification of mosquito breeding sites. It was previously distributed to elementary and high school student trainees[17] as well as student-teachers.[18] This questionnaire was pre-tested with student-teachers at a seminar. Similarly, an environmental action-plan format was also utilized by the students. This format was also previously conducted by education students[19] as well as discussed in training for student-teachers.[18]

Procedures

Prior to attending a teaching session on the use of an environmental action plan for dengue-related mosquito control, students were given the self-efficacy questionnaire. The teaching session opened with a learner-centred, problem-posing self-discovery, action-oriented module addressing mosquito larval control, and thus the need of source reduction. The training included visual survey and area mapping of potential mosquito breeding sites, followed by instruction on creation of environmental action plans for the control of potential mosquito breeding sites. The action plan steps included: identification of mosquito breeding sites, number of mosquito breeding sites, action to be taken, how frequently the control activity should be undertaken, and by whom. The following day the students were assigned in groups to conduct visual surveys, mapping and creation of environmental action plans in and around designated buildings and areas around the campus. Each designated campus area had its own environmental action plan. However, there was a core set of environmental problems common to all environmental action plans in this study. This included such topics as the identification of disposable water containers and covering of open containers. Three days later the students received a post-test of the self-efficacy questionnaire.

Data analysis

For reliability, a Cronbach’s alpha was calculated on the questions of the pretest questionnaire by SPSS 15.0 for Windows. The possible differences between the pretest and post-test self-efficacy scores were analysed by paired t-test. All t-tests were two tailed. Statistical significance was set at the .05 level. The analyses were calculated on MS-Excel.

Results

The Cronbach’s alpha for reliability for the self-efficacy questionnaire was .92. This score indicated that the study’s results were reliable. Behavioural capability (knowledge) was assessed through the successful completion of all steps of the environmental action plan by all groups. This included identification of the core environmental problem sets in all environmental action plans.

The results of the two-tailed paired t-test with a critical t of 2.111 revealed a significant difference between mean self-efficacy scores, \( t(17) = 2.517; p < 0.0222 \). The sample mean self-efficacy score for the post-test was significantly higher than the pretest (for pretest, mean = 5.574, variance = 2.296; for post-test, mean = 6.439, variance = .419). The increased difference in the mean scores was .865. This resulted in a 15.52% increase of mean scores, from pretest to post-test.
Perceived self-efficacy to plan and execute environmental plan for dengue control

Discussion

This study’s results demonstrated a significant increase in the perceived self-efficacy mean scores of dengue-related environmental control measures from the pretest to the post-test. This suggested that the training accompanied with field exercise to conduct the environmental action plan for the control of mosquito breeding sites was effective as a means to increase perceived self-efficacy to carry out environmental control activities through the use of environmental action plans.

This study had its limitations in its small size, the absence of a control group, as well as the absence of environmental clean-up behavioural observations. However, since higher-level education students at this university had undergone training in the environmental action plan for dengue control, the group of students in this study remained a valuable sample of education students previously unexposed to the environmental action plan for dengue control.

Why the concern about perceived self-efficacy or efficacy beliefs related to dengue control? Typically, sustained self-efficacy, and, additionally, sustained positive environmental behavioural change, ought to be the concern. However, before one can get to these important issues it is desirable to get a programme’s start on a firm footing. According to Bandura, “Efficacy beliefs influence goals and aspirations. The stronger the perceived self-efficacy, the higher the goals people set for themselves and the firmer their commitment to them. Self-efficacy beliefs shape the outcomes people expect their efforts to produce.”[14] That is why Bandura stresses the importance of self-efficacy as an indicator of behavioural intent. A new or special activity may heighten enthusiasm or spark interest, but not necessarily elevate self-efficacy. Some activities may demonstrate initial observable behavioural changes. However, without a high level of perceived self-efficacy, the sustainability of such activities may falter. Consequently, increasing perceived self-efficacy may be important before investing time, energy and activities in a dengue control programme.

This study demonstrated that university education students were able to increase their perceived self-efficacy to perform dengue control activities through a single training and simple environmental action plan activity. Education students eventually become student-teachers, and, in time, teachers. Increasing one’s perceived self-efficacy to perform specific dengue-related mosquito control activities during the university course may aid future teachers to potentially increase their skills to carry out dengue control activities. This will put future teachers in a better position to become school and community leaders for the control of dengue.

Acknowledgements

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References


Dengue fever (DF) has been increasingly reported among children in Mumbai. The common clinical and laboratory features seen in dengue-infected children are: fever, thrombocytopenia, elevated serum transaminases, elevated partial thromboplastin time (PTT), hypotension, vomiting, haemoconcentration, leukopenia and hepatomegaly.[1] Though dengue virus (DENV) is a member of the Flaviviridae group of viruses which include a number of neutropic viruses,[2] immune-mediated central nervous system’s (CNS) involvement following dengue infection has been rarely reported. Reports of only four children of Guillain Barre Syndrome (GBS) due to DENV have been published.[3-6] We report a 2-year-old boy who presented with GBS due to dengue infection.

Case report

A 2-year-old boy born of non-consanguineous marriage presented in the monsoons with weakness of lower limbs since day 1. The weakness was progressive and now had even involved the upper limb. He had fever with diarrhoea four days back. There was no history of any immunization or injections taken in the recent past. He had been immunized till age.

On examination, he was conscious and oriented. He had hypotonia with reduced power in all four limbs (lower limb involved more as compared to the upper limb). He had diaphragmatic weakness though respiration was not laboured. Superficial and deep tendon reflexes were absent though gag reflex was present. There was no cranial nerve involvement or sensory involvement. Other systemic examination was normal. Thus, he was diagnosed as a case of ascending progressive motor polyneuropathy, most likely Guillain Barre Syndrome. His haemogram revealed thrombocytopenia [Platelet = 1 04,000/cumm] and normal haemoglobin and WBC count. His serum electrolytes liver function tests and renal function tests were normal. HIV ELISA and HBsAg were negative. Nerve conduction velocity showed absent ‘F’ waves in lower limbs suggestive of axonal radiculopathy. Dengue IgM by Panbio Kit was positive. [1.33 AI (Positive = > 0.9 AI)]. The child was given intravenous immunoglobulin (IVIg) (2 gm/kg) following which his weakness improved and he had regained full power in upper limb and power of grade 3 in lower limbs after 15 days of IVIg. His thrombocytopenia resolved within seven days of presentation. Thus, this child had dengue polyneuropathy.
Discussion

Dengue is a common arboviral infection affecting patients in South-East Asia, including India, and South America. CNS involvement is known with this viral infection though its actual frequency is unknown.\(^2\)\(^,\)\(^7\) The common CNS manifestations are altered sensorium and seizures\(^2\)\(^,\)\(^7\) and thought to be due to prolonged dengue haemorrhagic fever (DHF) with fluid extravasation, cerebral oedema, hyponatraemia, liver failure, renal failure and direct neurotropic effect of dengue virus.\(^2\)\(^,\)\(^8\) Immune-mediated nervous system involvement has been described in the form of GBS and post-infectious-disseminated encephalitis rarely.\(^4\) GBS is a post-infectious polyradiculopathy known to occur post-gastrointestinal infection with \textit{Campylobacter jejuni} and other infective agents, like \textit{Mycoplasma}, human cytomegalovirus (CMV), Ebstein-Barr virus and herpes virus. It rarely occurs due to demyelination post-dengue infection. The nerve injury in GBS is mediated by immunological mechanisms. DHF and dengue shock syndrome (DSS) occur due to immunopathological mechanisms whereby secondary exposure to dengue viruses lead to a more severe disease. Immune enhancement is thought to play a major role in the pathogenesis of enhanced dengue infection, leading to cross-reacting antibodies and demyelination.\(^2\)\(^,\)\(^7\) GBS, in the form of polyradiculopathy of a primarily demyelinating nature with an associated axonal component,\(^5\)\(^,\)\(^4\) has been found as was seen in our patient who had absent “F” waves in lower limbs suggestive of axonal radiculopathy.

DHF and DSS are characterized by thrombocytopenia. We found that 92.3% of patients infected with DF have thrombocytopenia.\(^9\) Similarly, our patient had fever prior to the episode of GBS and thrombocytopenia at the time of presentation in the monsoon season (a time when dengue tends to peak in Mumbai), which made us suspect dengue-associated GBS which was confirmed by positive dengue IgM test.

Previous reports have found complete recovery of dengue-associated GBS with intravenous immunoglobulin (IVIg) as was also found in our patient, suggesting that cross-reacting antibodies from a previous dengue infection may be responsible for the illness.

Thus, in conclusion, one should rule out dengue-associated GBS in areas endemic for dengue whether, or not they have other features of the disease.

References


Dengue presenting as Guillain Barre Syndrome


Dengue is the commonest arboviral infection that affects mankind. Infection by any of the four serological types of dengue viruses (DENV-1 to 4) cause dengue fever (DF), dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS). In recent years, there have been reports of neurological complications associated with dengue in Asia and South America, though their frequency still remains unknown. The encephalopathy associated with dengue infection has been postulated to be due to prolonged DHF with fluid extravasation, cerebral oedema, hyponatremia, liver failure, renal failure and a possible direct neurotropic effect of dengue virus. Though central nervous system (CNS) manifestations have been reported in adults, reports in children are rare and have only been reported in three studies, and none from India. We studied a 10-year-old girl who presented with brainstem encephalitis, thrombocytopenia, elevated liver transaminases and positive dengue IgM with complete resolution of her symptoms within three days of the onset of the disease.

Case report

A 10-year-old girl born of non-consanguineous marriage presented in monsoons with fever since eight days, projectile vomiting since five days, altered sensorium, inability to stand and squint since day 1. Her younger brother was also admitted with fever and was diagnosed to have dengue. She had no convulsions. On examination, she was conscious and oriented and had meningeal signs with positive Macewan’s sign. Her vital parameters were normal. Fundus showed papilledema. She had right lateral palsy and tone and power were normal. Deep tendon reflexes were brisk and right planter was extensor. She had ataxia with intention tremors. Other systemic examination was normal. Thus, she was suspected to have brainstem encephalitis or posterior fossa tumor with raised ICT. Her haemogram revealed thrombocytopenia (platelet 84 000/cumm) with normal haemoglobin and WBC count. Liver transaminases were deranged (SGOT = 121 IU/L), SGPT = 60 IU/L); serum electrolytes, renal function tests and blood sugar was normal. MRI brain did not show any space-occupying lesion and showed only inflammatory changes in right maxillary, ethmoid and sphenoid sinuses. With MRI ruling out posterior fossa tumor, cerebrospinal fluid (CSF) analysis was done which showed aseptic meningitic picture (3 polymorphs, 19 lymphocytes/cumm; 52 mg/dl – sugar; proteins = 10.3 mg %). CSF culture was negative. In view of thrombocytopenia, deranged liver enzymes and meningitis and her brother suffering from dengue, her dengue IgM
by capture ELISA was done, which was positive {1.2 OD units (positive = > 0.9 OD units)}. Blood culture and leptospira IgM ELISA were negative. Thus, this child was diagnosed as a case of dengue brainstem encephalitis. She was treated with Mannitol and all her symptoms resolved within three days. Her repeat platelet count and liver transaminases were normal. Thus, here is a case of dengue presenting as brainstem encephalitis.

**Discussion**

Dengue virus is a member of the *Flaviviridae* group of viruses, which include a number of neurotropic viruses such as Japanese encephalitis virus, St. Louis encephalitis virus and tick-borne encephalitis virus. In recent years, we and others have reported neurological manifestations in patients with DHF and DSS characterized by depression of consciousness with normal CSF analysis[1,10] and complete recovery in survivors. In fact, in a study published by us, we found that almost 48.7% of patients had altered sensorium on presentation with higher predominance in the DSS group as compared to the DHF group (77.8% vs 25%, P = 0.0035). However, most of the patients had additional features such as bleeding manifestations, hepatomegaly and serositis. Isolated CNS involvement is a rarity as seen in our patient.

The encephalopathy was thought to be due to cerebral oedema, anoxia, haemorrhage, hyponatremia, hepatic failure, microcapillary haemorrhage and release of toxic substances.[1] However, recent reports have demonstrated a possible direct neutropic effect of dengue virus[9] and localized invasion of the CNS. Similar localized CNS manifestation in the form of brainstem encephalitis with raised intracranial tension and CNS leukocorhria was seen in our patient. A case report by Lum et al.[4] has isolated dengue virus (DENV-2 and 3) from the CSF of affected patients, suggesting direct invasion of the brain and neurovirulent properties of the dengue virus.

Cam et al.[11] have determined that patients with dengue encephalitis have significantly elevated liver enzymes with cerebral oedema. Similar findings were noted in our patient where the child had elevated liver transaminases and complete CNS recovery with Mannitol. In fact, in our patient, the presence of thrombocytopenia with history of her brother suffering from dengue made us suspect dengue encephalitis. In a previous study, we had found that 92.3% of patients affected by dengue had thrombocytopenia, and this combination of thrombocytopenia, elevated liver transaminases and fever is very characteristic of dengue in Mumbai.[10]

Thus, it would be wiser to investigate patients with encephalitis and encephalopathy in dengue-endemic areas for dengue infection, irrespective of the fact whether they have other features of the disease or not.

In conclusion, it can be stated that dengue encephalitis is rare but neutropism of the virus is known. It can masquerade as other types of viral encephalitis, but its clinical course and outcome is usually favourable.

**References**


Short note

Dengue presenting as viral myocarditis

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Dengue fever (DF) is endemic in India and causes dengue fever, its severe form dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). Hypotension due to intravascular depletion due to capillary leak is common in DHF and DSS. However, myocardial dysfunction is also seen with DHF/DSS and could be responsible for hypotension and shock. Cardiac involvement in the form of decreased left ventricular performance and arrhythmias are known. However, isolated myocarditis has rarely been seen with dengue virus. We report an 11-year-old boy who presented with viral myocarditis due to dengue infection, with complete recovery within three months.

Case report

An eleven-year-old boy born of non-consanguineous marriage presented with vomiting since two days and fever since day one. He had no skin rash or bleeding from any site. On examination, he had tachycardia (heart rate = 110/min) with hypotension (BP = 85/50 mm of Hg) with prolonged capillary refill and bilateral basal crepitations. There were no signs of dehydration. Other systemic examination was normal. He was immediately treated with Dobutamine and IV fluids. He was suspected to have viral myocarditis with cardiogenic shock. His investigations showed lymphopenia with thrombocytopenia (haemoglobin = 12.9 gm/dl, WBC = 7200/cumm, absolute lymphocyte count = 720/cumm, platelet count = 127,000/cumm) with absent C-reactive protein and no growth on blood culture. His serum CPK levels were elevated (344 IU/L) and CPK-MB fraction was also elevated (71 units/L (normal = upto 25 units/L)). Serum LDH was also elevated (972 IU/L). His renal function tests, liver function tests and blood sugar were normal. Echocardiography showed left ventricular dilatation with systolic dysfunction and fractional shortening of 22%. His dengue IgM by ELISA was positive {0.95 AI (Positive = > 0.9 AI)} and leptospira IgM and peripheral smear for malaria were negative. He was thus diagnosed as a case of dengue myocarditis. Intravenous immunoglobulin (2 gm/kg) was given following which his left ventricular functions improved with fractional shortening of 31% and ejection fraction of 62%. He was gradually tapered off the ionotropic support and discharged. On follow up after three months, he was asymptomatic and a repeat echocardiography showed normal left ventricular dimensions and functions with fractional shortening of 37% and ejection fraction of 74%.

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Discussion

Viral myocarditis may lead to cardiogenic shock due to fulminant cardiac failure, recurrent wheezy episodes (mistaken as bronchial asthma), bronchiolitis and rhythm disturbance. Serum enzymes such as SGOT, CPK, LDH are elevated and echocardiography shows left ventricular dilatation with global dyskinesia. Our patient also presented with cardiogenic shock due to cardiac failure, had elevated CPK and LDH, and echocardiography showed left ventricular dilatation with systolic dysfunction confirming the diagnosis of myocarditis. Viral myocarditis is known to occur commonly with Coxsackie virus B1 and B4 and rarely with dengue virus. Our patient had a positive dengue IgM test, confirming the diagnosis of dengue myocarditis. Cardiac dysfunction in the form of reduced ejection fraction and decreased end-diastolic volume are noted in patients with dengue. Similar echocardiographic findings were noted in our patient. Viral myocarditis may respond favourably to routine anti-failure measures, steroid therapy and intravenous immunoglobulins (IVIG), with complete reversal of echocardiographic findings within months. Similarly, our patient had a favourable response to IVIG and anti-failure measures and had complete recovery after three months of the onset of symptoms.

In conclusion, viral myocarditis may be a manifestation of dengue, though relatively uncommon as an isolated feature, and its response to therapy is favourable.

References


Defrost-water-collection trays of refrigerators –
A potential breeding habitat of *Aedes aegypti* in dengue- and chickungunya-infested areas of southern India

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Arthropod-borne viral diseases like dengue fever (DF) and its severe form, dengue haemorrhagic fever (DHF), and chikungunya fever are known to occur throughout the tropics the world over. These diseases are increasing alarmingly in the South-East Asian countries. For the control of dengue outbreaks, management of *Aedes aegypti*, the vector species, through source reduction remains the mainstay. Hence, an assessment of the breeding sources of this mosquito is essential in order to devise and develop a suitable control strategy.

*Ae. aegypti* is mainly a domestic species of urban areas, but it has now spread to rural, suburban and urban areas as well. This species prefers to breed in man-made containers holding stored water or rainwater in domestic and peri-domestic environments.

During epidemic investigations in Puducherry (earlier known as Pondicherry) and Cuddalore during 2004-05, a rare phenomenon of *Ae. aegypti* breeding was observed in the defrost trays of refrigerators in both these towns. To understand the importance of this habitat in the proliferation of this vector mosquito during different seasons, a total of three surveys were carried out: one each during the south-west monsoon season (June-August 2004), north-east monsoon period (October-December 2004) and during summer (March-May 2005). In houses ranging from 746–792, the number of refrigerators examined ranged from 29–335. Water was emptied into an enamel tray employing either a siphon or pipette and absolute count of larvae and pupae, if any, was made, brought to laboratory, reared to adults and identified to species, following a standard key. The breeding of *Aedes* mosquitoes was found in 39 trays (1.6–8.4%) in Puducherry and 8 trays (4.8–6.9%) in Cuddalore (Table). *Aedes aegypti* was the only species obtained from the pupae collected from defrost-water-collection trays in refrigerators from both the areas. The larvae and pupae recorded were significantly high (p > 0.05) during the summer period in both the towns as compared to the other two seasons.

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It is evident that the defrost water-collection trays in refrigerators inside houses play an important role, as an alternative source for breeding in the absence of the preferred sites during summer. Since these are invisible breeding sites, these are easily overlooked. This highlights the need for creating awareness among communities about this important breeding site of *Ae. aegypti*. Emptying the water content in these trays and destroying the eggs, which tolerate desiccation, by scrubbing the trays at weekly intervals could reduce the foci, which augment the population under favourable conditions.

### Acknowledgements

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### References


The global resurgence of dengue in otherwise naive locations has been associated with concurrent dissemination of identical vector-borne viral diseases. Concurrent infection by dengue virus (DENV) and chikungunya virus (CHIKV) has been known for several decades.[1] Nevertheless, recent global CHIKV dissemination[2] or its local re-emergence[3] after a gap has been intriguing. Increased intercontinental travel has blown up the ghost of the traditional endemic foci of CHIKV and has resulted in CHIKV patients being found in the United States.[4] Moreover, there could even be coincidental episodes of the Japanese encephalitis virus (JEV) infection. During the early 1940s, there were dengue outbreaks in Guam, followed, during 1947, by the concurrent epidemics of mumps virus and JEV. Serological investigations in children aged 10 years or less during 1953 had revealed neutralizing antibody to JEV, and DENV-1 and -2 among those born before 1947.[5]

In India, during 1997, concurrent infection was detected in Haryana state in the north of the country. In a follow-up study of a dengue outbreak, out of 30 serum samples collected, eight cases were found positive for dengue IgM antibodies, two were positive for all the three infections, viz. DENV, JEV and WNV, while one sample was positive for two infections, viz. JEV and West Nile virus (WNV).[6]

There has been no chemotherapy available for patients with JEV, DENV, CHIKV or WNV. Such cases require appropriate clinical management and public health response. During the initial stage of illness the clinical presentations are ambiguous and are accompanied by viremia. These cases are highly infectious with their blood teeming with viral RNA. If bitten by the mosquito vector, they would contribute to disease dissemination. Moreover, they are likely to be offered empirical doses of antibiotics by the clinicians. Any point-of-care indication about JEV, DENV, CHIKV or WNV would be imperative from the clinical and public health perspective. That would necessitate initiating appropriate anti-vector measures attuned to vector biology. Vectors transmitting JE and dengue/chikungunya/West Nile fever would be controlled by entirely different strategies.

A commercial diagnostic kit for the field diagnosis of Japanese encephalitis (JE) antiviral IgM has been evaluated recently.[7] Rather than a multi-step, time-consuming format, the on-the-spot point-of-care diagnostic would address the ground realities in the JE-endemic areas. Without prejudice to the conventional enzyme-
linked immunosorbent assay (ELISA), a simpler on-the-spot diagnostic would assist health care providers better. Obviously, immunoglobulin M (IgM)-based assays would be adequate for both JE and chikungunya. Nevertheless, for dengue, additional markers would be essential. The immunological response during the secondary or tertiary dengue episodes is secondary rather than a primary one. The initial immunological response would be towards IgG production rather than IgM. Dengue non-structural antigen, NS1, has been reported to be better than IgM in cases of primary or secondary infections.\(^8\) Immunochromatographic assay kits for a point-of-care dengue IgG and IgM detection have been popular for a while, namely, the Panbio Dengue Duo IgM and IgG Rapid Strip test, and the Bio-Check Plus Dengue G/M Cassette Test (Brittney). Recent introduction of a rapid, immunochromatographic kit for chikungunya has been a positive feature.\(^9\)

International organizations including the World Health Organization, the Programme for Appropriate Technology in Health (PATH) and the Centers for Disease Control and Prevention (CDC) would be obliged to encourage standardization of a concurrent point-of-care diagnostic for JE, dengue, chikungunya and West Nile fever. A multifaceted but cost-effective rapid assay format would be a valuable armour for clinicians and public health personnel to diagnose dengue, chikungunya, Japanese encephalitis and West Nile fever.

References


Role of helplines for dissemination of information during an outbreak of dengue fever in Delhi, India, in 2006: An experience

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Outbreaks of dengue fever (DF) and dengue haemorrhagic fever (DHF) have been reported in various parts of India during the last four decades. Delhi has been endemic for DF/DHF for quite some time and still maintains high vulnerability due to a large number of internal migrants and international tourists visiting the Capital and the extensive breeding potential the city provides to Aedes aegypti mosquitoes. Delhi recorded epidemics of dengue fever during 1967, 1970, 1982, 1988, 1991, 1992 and 1996. During the 1988 outbreak of DF/DHF, about 33% mortality was reported among children admitted in hospitals, while during 1996, 10,252 cases were hospitalized and 423 deaths were recorded. This epidemic peaked in September when an Aedes aegypti larval house index (HI) of 43.70% was recorded.

In 2001, there were 3306 cases with 53 deaths; in 2002, the number was 1926 cases and 33 deaths. In 2003, there was a large outbreak with 12,754 cases and 215 deaths. The 2006 outbreak started in August and peaked in October. As of end-November 2006, it was estimated that 10,344 cases and 162 deaths due to severe forms of dengue (i.e. DHF/DSS) had taken place.

As is generally the case, there was widespread panic and apprehension in the general public about the outbreak. Anybody having fever rushed to a health facility or the local private practitioner thinking that the fever may be due to dengue. Reports of chikungunya fever from the states of Tamil Nadu and Kerala in south India made the situation worse. A few cases of chikungunya were reported in Delhi too.

Though some public information advertisements issued by the Municipal Corporation of Delhi, the New Delhi Municipal Council and the Ministry of Health and Family Welfare appeared in the national and regional dailies and on radio and television for dissemination of information on dengue fever and chikungunya fever and their prevention and management, there was a clear gap in meeting these information needs – especially on the management of fever. As a result, a large attendance of patients with fever and suspected dengue fever was recorded at major hospitals of the city, particularly the All India Institute of Medical Sciences (AIIMS) which bore the brunt of this patient load.

AIIMS is considered to be a premier medical institute in India. Though envisaged to be a tertiary care/referral hospital when it was established in 1956, but owing to its growing reputation for providing quality health
Role of helplines during the 2006 Delhi outbreak


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care, it has been attracting a large number of patients in its outpatient department (OPD) and Emergency services. For example, in 2005, the attendance in its OPD was a whopping 16,304,799 patients.\[8\] This included a large percentage of patients who don’t qualify to be called as “referred patients”.

In the dengue outbreak of 2006, even though the Government of Delhi had made arrangements for the management of fever/suspected dengue cases in about 33 hospitals of the city, a large number of patients – about 1000 to 1200 per day – reported at AIIMS starting from the first week of October to mid-November,\[7\] stretching its human resources and infrastructural facilities to the maximum.

One important method of containment of dengue outbreak was considered to be dissemination of correct information to the general public on the preventive aspects of these diseases as well as on the management of fevers. Allaying the fear and panic setting in the general public was the real need. AIIMS took several important steps in this direction. Apart from distributing 18,000 information booklets, 10,000 handbills and pamphlets and putting up banners, it started two helplines to augment public education efforts:

- **Telephone helpline:** It was a manned telephone service that functioned from 10 AM to 10 PM every day – including holidays. A trained public health educator, supported by a qualified medical doctor and a public health specialist, manned this service. This helpline (Phone no. 011-26589712) was subsequently converted into a 24-hour helpline in 2007.

- **Internet (E-mail) helpline:** To help those who are net savvy, an Internet (E-mail) helpline (dengueprevention@gmail.com) was also started. Questions received were responded to within a time frame of 6-8 hours.

**Observations**

Both these services were operated for six weeks starting from the first week of October 2006 to the second week of November 2006. By mid-November, the outbreak was almost over. Some of the highlights of these helplines are as follows:

- **No. of calls/queries received:** During the six weeks of the operations, the telephone helpline received 378 calls, while 79 queries were received on the Internet.

- **Geographical distribution of the callers:** 263 (69.5%) of the callers were from the Capital and 98 (26%) were from outside Delhi. Rest of the callers did not reveal their place of residence. Residents of south and west Delhi dominated (50%) among those who called from Delhi. The details are given in Table 1.

**Table 1: Geographical distribution of telephone callers**

<table>
<thead>
<tr>
<th>Area</th>
<th>No. of callers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Delhi</td>
<td>84(22.22%)</td>
</tr>
<tr>
<td>West Delhi</td>
<td>105(27.77%)</td>
</tr>
<tr>
<td>East Delhi</td>
<td>46(12.16%)</td>
</tr>
<tr>
<td>North Delhi</td>
<td>19(5.02%)</td>
</tr>
<tr>
<td>Central Delhi</td>
<td>9(2.38%)</td>
</tr>
<tr>
<td>Outside of Delhi</td>
<td>98(25.92%)</td>
</tr>
<tr>
<td>Didn’t specify</td>
<td>17(4.49%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>378(100%)</strong></td>
</tr>
</tbody>
</table>
Role of helplines during the 2006 Delhi outbreak

Table 2: Frequently asked questions*

<table>
<thead>
<tr>
<th>Type of question asked</th>
<th>Telephone helpline</th>
<th>Internet (E-mail) helpline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advice about management of someone with fever in the family</td>
<td>200(52.91%)</td>
<td>45(56.96%)</td>
</tr>
<tr>
<td>What is the treatment of dengue?</td>
<td>117(30.9%)</td>
<td>26(32.91%)</td>
</tr>
<tr>
<td>What are the signs and symptoms of dengue?</td>
<td>57(15.07%)</td>
<td>25(31.64%)</td>
</tr>
<tr>
<td>Blood tests for diagnosis of dengue?</td>
<td>22(5.82%)</td>
<td>15(18.98%)</td>
</tr>
<tr>
<td>How to take care of a patient with fever?</td>
<td>35(9.25%)</td>
<td>10(12.65%)</td>
</tr>
<tr>
<td>Differences between dengue and chikungunya</td>
<td>12(3.17%)</td>
<td>10(12.65%)</td>
</tr>
<tr>
<td>General queries about the outbreak</td>
<td>34(8.99%)</td>
<td>9(11.39%)</td>
</tr>
<tr>
<td>Prevention</td>
<td>57(15.0%)</td>
<td>24(30.37%)</td>
</tr>
<tr>
<td>Cause</td>
<td>17(4.4%)</td>
<td>10(12.65%)</td>
</tr>
</tbody>
</table>

\*Multiple questions

- **Frequently asked questions**: On the telephone helpline as well as the E-mail helpline, the most frequently asked questions pertained to seeking guidance and advice about someone in the family who had fever. About 32.9% of the E-mail helpline users and about 30.9% of the telephone helpline users wanted to know about treatment of DF/DHF. About 30.3% of the E-mail helpline users and 15% of the telephone helpline users were keen to know about the prevention methods of these diseases. The rest of the helpline users wanted to know about other aspects of these diseases/outbreak (Table 2).

- **Frequency of calls**: As can be seen from the Figure, almost 51%(193) of the calls were received during the first week of the starting of the service. This was also the time when the outbreak was reaching its peak. As the weeks passed by, the number of calls gradually declined.

- **Source of information about the telephone helpline**: It was found that television played a dominant role in making people aware about the availability of the helpline and the phone number. 144(38%) persons came to know about it from various television channels. For 105(27.7%) persons, the source of information was newspapers, while for others the sources were: banners at AIIMS 25(6.6%), radio 45(11.9%), word of mouth 30(7.9%) and the city’s telephone system Mahanagar Telephone Nigam Limited (MTNL) phone enquiry 29(7.6%) (Table 3).

**Figure**: Week-wise distribution of telephone callers

![Week-wise distribution of telephone callers](image-url)


**Table 3: Source of information about the telephone helpline (first response)**

<table>
<thead>
<tr>
<th>Source</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Television</td>
<td>144 (38.0%)</td>
</tr>
<tr>
<td>Newspapers</td>
<td>105 (27.7%)</td>
</tr>
<tr>
<td>MTNL phone enquiry</td>
<td>29 (7.6%)</td>
</tr>
<tr>
<td>Banners at AIIMS</td>
<td>25 (6.6%)</td>
</tr>
<tr>
<td>Word of mouth</td>
<td>30 (7.9%)</td>
</tr>
<tr>
<td>Radio</td>
<td>45 (11.9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>378 (100%)</strong></td>
</tr>
</tbody>
</table>

**Discussion**

It is evident that these types of helplines do fulfill a felt need of the community in a crisis situation. One of the major problems in an outbreak of a communicable disease that can be fatal is the panic and apprehension that sets in in the general populace – mainly due to lack of adequate and easily available and technically correct information. As most of the helpline-users wanted to know about “What to do and where to go because someone in their family had fever”, it is important that all available means of mass communication should be effectively utilized by health care managers to provide correct information. Helplines, especially on telephone, can be very effective because of their easy, personalized and reassuring approach. This can supplement other methods of disseminating information effectively. Hence, such helplines must be operated in crisis situations. Local language should be the mode of communication on this type of service.

**References**


[7] All India Institute of Medical Sciences. Press releases on dengue updates from AIIMS from October 2-18 October 2006 and published in the Times of India and other dailies from New Delhi.

The consultation on integrated vector management (IVM) took place at the headquarters of the World Health Organization (WHO) in Geneva, Switzerland, from 1 to 4 May 2007. Its purpose was to bring together experts in IVM from country, regional and global levels to advance the development and promotion of the IVM approach, as set out in the Global strategic framework for integrated vector management,1 by reviewing current status and developing a practical strategic plan for the next three years.

The four key recommendations of the consultation were:

(1) In order to fully embed IVM in all control programmes dealing with vector-borne diseases, concerted efforts should be made, through advocacy and social mobilization, to articulate the need for and benefits of IVM and to mobilize resources for implementation.

(2) The current human resource and infrastructure base for IVM is inadequate to meet increasing demands. This must be addressed urgently, through capacity-building, and both needs-based training and institutional development, to ensure those trained receive continued mentoring and support, and can function in systems where their skills are most effectively applied.

(3) Action must be evidence-based, so it is essential to intensify applied research and monitoring and evaluation to assess the impact and performance of vector control, both to refine implementation of IVM and to provide data for advocacy.

(4) Develop an institutional framework to promote, further develop, implement and scale up IVM, including national legislative frameworks.

The main conclusion of the meeting was that now is the time to tap the preventive power of vector control, given the serious risks of increasing transmission of vector-borne diseases related to climate change, population movement and environmental degradation, and the major opportunities for financial support.

The next steps will be to promote and implement the strategic plan, propose WHO appoint a panel of experts on IVM to guide its development, organize a follow-up meeting with wider representation of other sectors and disease control programmes and develop the IVM concept through the homepage of the VEM website.

Reference

Dengue is the most rapidly spreading vector borne disease. An estimated 50 million dengue infections occur annually and approximately 2.5 billion people live in dengue endemic countries. Because of the rapidly increasing public health importance of this disease, in 1999 dengue was incorporated in the portfolio of the UNICEF, UNDP, World Bank, WHO Special Programme for Research and Training in Tropical Diseases (TDR). The 2002 World Health Assembly Resolution WHA55.17 urged greater commitment to dengue among Member States and WHO; of particular significance is the 2005 Revision of the International Health Regulations (WHA58.3), which includes dengue as an example of a disease that may constitute a public health emergency of international concern.

It was against this background that the Dengue Scientific Working Group of 60 experts from 20 countries including WHO staff from four Regions and Headquarters met in Geneva in October 2006 to review existing knowledge on dengue and establish priorities for future dengue research aimed at improving dengue treatment, prevention and control. The goal of the Scientific Working Group was to outline a research agenda by identifying bottlenecks and making detailed and specific research recommendations. The SWG wanted to identify areas of research that could lead to tangible benefits for people in disease endemic countries within the coming years as well as outline a strategic vision for applied and basic research from which benefits would be felt in the medium to long term.

As a result of major demographic changes, rapid urbanization on a massive scale, global travel and environmental change, the world, particularly the tropical world, faces enormous future challenges from emerging infectious diseases. Dengue epitomizes these challenges. In the early years of the 21st Century we are collectively failing to meet the challenge posed by dengue as the disease spreads unabated and almost 40% of the world’s population now lives at risk of contracting the disease. There is currently no specific clinically useful diagnostic test, no drugs, and no vaccine, and we have failed to widely or effectively implement existing vector control and clinical management measures that we know would help to reduce the vector population and reduce case fatality rates. Yet there has never been a more optimistic time to be involved in dengue and dengue research, and interest in the disease has attracted a new generation of talented and committed clinicians and scientists. Modern science, from clinical medicine to basic research on pathophysiology, drug and vaccine discovery, through to the social and behavioural sciences and vector biology and control, offers a unique
opportunity to make a tangible and substantial impact on dengue over the next decade. But in order to achieve what is possible, a paradigm shift is required in our current approach. The dengue research community needs to: push for much greater implementation of existing knowledge to reduce case fatality rates, extend basic and clinical research to understand the underlying pathophysiology, aid diagnostics and drug discovery and further improve clinical outcome, speed up the development of vaccine candidates including moving as quickly as possible to efficacy trials, and gather evidence for implementing best practices for control of the vector.

All of this is possible in the next ten years. But to achieve this, dengue needs a much stronger voice within dengue endemic countries and within the global public health community to persuade society, funding agencies and policy-makers of the importance of the disease. We are at a critical epidemiological juncture in infectious, particularly viral, emerging diseases at the start of the 21st Century, and in many ways dengue serves as a model for how we might meet that challenge. The lessons learned from dengue will have implications for a number of other diseases and our approach to their control. The implementation of the best of existing knowledge and practice supplemented by future research applied in an integrated, holistic fashion can be expected to significantly change the lives of individuals living in dengue-endemic countries in the coming years. The Scientific Working Group hopes this research agenda will help provide a strategic plan for how we might collectively achieve the aims of reducing morbidity and mortality based on better understanding of the pathophysiology associated with dengue, on implementation strategy, and on reduction of virus transmission.

Global dengue research agenda

The priority dengue research areas are organized around four major research streams, which will provide evidence and information for policy-makers and control programmes and lead to more cost-effective strategies that will reverse the epidemiological trend.

Stream 1: Research related to reducing disease severity and case fatality

Optimization of clinical management

An efficient out-patient system and clinical and laboratory indicators of early dengue virus infection, plasma leakage and shock, as well as an effective and safe method of managing severe haemorrhage, dengue in pregnancy, and patients with co-morbidity, need to be validated in order to scale up the use of improved treatment guidelines.

It is recommended to analyse:

- New methods and guidelines for triage and out-patient care.
- The validity, role and accessibility of available and new diagnostics.
- The predictive value of prognostic markers (host and viral, non-invasive measurement of vascular leakage).
- Standardized approaches to determining and documenting severe disease and response to treatment.
- Best practices for the treatment of dengue including early treatment to reduce severity, treatment of
established shock, and effective and safe management of haemorrhage.

- The impact of co-morbidities on disease severity, and the effect of pregnancy.
- The causes of dengue deaths (including treatment failures).

**Process and impact evaluation of staff training**

Training programmes in case management can help to rapidly reduce case fatality. However, training has to be standardized and adapted to the prevailing local health care system and best practice has to be identified and implemented in dengue-endemic countries.

It is recommended to analyse:

- The process and impact of existing/future training programmes.

**Critical issues in dengue pathogenesis**

A better understanding of dengue pathogenesis will provide a foundation for future rational clinical interventions. In particular we need to understand the changes underlying: endothelial permeability in plasma leakage, dengue virus diversity, and immune response to dengue viruses.

It is recommended to analyse:

- The physiological and molecular mechanisms leading to vascular leak and haemorrhage.
- The host genetic factors associated with dengue severity.
- The dengue viral factors and antigenic subtypes related to tropism, epidemic potential, and virulence.
- The mechanisms of antibody-mediated enhancement and protection.

- The mechanisms of virus entry and cellular/tissue tropism.
- T and B cell responses and their relation to immunopathology and protection.
- Genetic predisposition to dengue.

**Stream 2: Research related to transmission control through improved vector management**

**Development and evaluation of vector control tools and strategies**

The effectiveness of powerful vector control tools has been compromised by issues of delivery, coverage and acceptability. Promising new tools and approaches need to be evaluated.

It is recommended to analyse:

- The efficacy of new vector control tools and strategies in different contexts.
- Combinations of new and/or existing tools in different contexts.
- The scaling up of successful pilot projects to state or national level.

**Surveillance and response**

Strengthening of surveillance systems through development and validation of reliable risk indicators and the application of information technology is needed for improved decision-making.

It is recommended to analyse:

- Improved methods and their application/standardization in operational contexts.
- The development and utilization of early warning and response systems.
Stream 3: Research related to primary and secondary prevention

Vaccines

Vaccines offer the greatest hope for dengue prevention and there are several candidates in clinical development. The identification of vaccine components that are suitably safe and immunogenic, and of immune correlates of protection, should accelerate successful vaccine development and regulatory approval.

It is recommended to analyse:

- New vaccine candidates, adjuvants, and vaccination strategies.
- Correlates of protective immunity for use as an endpoint in vaccine trials.
- Immune responses in vaccine trials and natural infections.
- Prospects for phase 3 and 4 vaccine evaluation trials in multiple field sites.
- Issues associated with future vaccine usage and coverage, including cost-effectiveness studies of implementation.

Drugs

Anti-dengue drugs may have prophylactic (e.g. outbreak prevention) and therapeutic (e.g. prevention of severe disease) uses, including potential for impact on incidence and severity of ensuing disease. Anti-viral drug discovery for dengue has accelerated in recent years along with our knowledge of ‘drugable’ targets in the virus.

It is recommended to analyse:

- Viral-encoded proteins for drug, diagnostics and vaccine design.
- New (including natural) products or existing licensed drugs.

Stream 4: Health policy research contributing to adequate public health response

There is a contradiction between the high priority afforded to dengue at political level and the low level of resources allocated to dengue prevention and control programme activities. Health policy research will facilitate a redress of this imbalance.

It is recommended to analyse:

- Tools for rational decision-making and adequate prioritization of dengue, such as studies of dengue burden and costs of illness.
- Factors leading to success or failure of national programmes.
- Decision-making that results in declaration of state of emergency.
- The importance and burden of dengue in less studied regions (e.g. Africa).

Conclusion

The Scientific Working Group hopes this research agenda will help provide a strategic plan for how we might collectively achieve the aims of reducing dengue morbidity and mortality and its negative socioeconomic impact. Donors and the research community are encouraged to take part in this major programme and to contribute through timely information to the database TDR is establishing for keeping track of research activities and relevant findings.
Outbreak and spread of chikungunya

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http://www.who.int/wer

Chikungunya is a mosquito-borne disease first described during an outbreak in the southern part of the United Republic of Tanzania in 1952. The name chikungunya derives from a root verb in the Kimakonde language meaning “to become contorted” and it describes the stooped appearance of sufferers with arthralgia. The causative agent is an alphavirus of the family Togaviridae. Since the first description of chikungunya, there have been numerous outbreaks in Africa, India and South-East Asia, the principal vectors being *Aedes* mosquitoes, of which *Aedes albopictus* and *Ae. aegypti* are the most important. Chikungunya often produces a mild illness that may be confused with dengue, since symptoms include fever, headache, arthralgia, myalgia and rash. Although serious complications are uncommon, arthralgia can be debilitating and may persist for months or even years after infection; in elderly people, chikungunya can be a contributing factor to death. In immunologically naive populations, where mosquito vectors are numerous, epidemic outbreaks may occur affecting many thousands of people. Outside of epidemics and wherever serological surveillance is lacking, the diagnosis of chikungunya may easily be missed. Chikungunya circulated in West and East Africa at relatively low levels until 1999–2000 when around 50 000 people became infected during an outbreak in the Democratic Republic of the Congo. Evidence from India suggested that outbreaks during the 1960s, were followed by relatively little transmission after 1973. Chikungunya outbreaks are often separated by periods of >10 years when infection is not apparent. For example, in Indonesia, chikungunya occurred sporadically until 1985 after which there were no reports until a series of outbreaks between 2001 and 2007.

Recent outbreaks, 2001–2007

Starting in February 2005, a major outbreak occurred among islands in the western part of the Indian Ocean, affecting the Comoros, Madagascar, Mayotte, Mauritius, La Réunion and the Seychelles (Figure). In La Réunion, by June 2006 there had been an estimated 266 000 cases, accounting for roughly one third of the population. Chikungunya continued to circulate in La Réunion in 2007, albeit at a much reduced level. The major vector in La Réunion is *Ae. albopictus*, which appears to have displaced *Ae. aegypti* on much of the island as it has also in some other regions. During this period, there were an estimated 9000 cases of chikungunya in the Seychelles, 7290 in Mayotte and about 6000 in Mauritius. Associated with this outbreak in the Indian Ocean islands, there were a large number of imported cases of chikungunya in Europe, occurring in returning tourists and visitors,
Outbreak and spread of chikungunya

particularly those returning from La Réunion. Metropolitan France experienced the largest number of cases: between April 2005 and August 2006, there were 808 imported cases of chikungunya confirmed by serology. The temporal change in numbers reflected the rise and decline of the epidemic in La Réunion, with a peak of 178 cases in March 2006. Elsewhere in Europe, cases were reported from Germany, Italy, Norway and Spain.

*Ae. albopictus* has been inadvertently introduced into several European countries during the past 30 years; these countries include Albania, Belgium, Bosnia, Croatia, France, Greece, Italy, Montenegro, the Netherlands, Serbia, Slovenia, Spain and Switzerland. Although *Ae. albopictus* occurs in the departments of Alpes-Maritimes and Var in France, where cases of chikungunya that originated in La Réunion have been diagnosed, there has been no evidence of local transmission.

During 2006, there was a large outbreak of chikungunya in India, with 1.39 million officially reported cases spread over 16 states; attack rates were estimated at 45% in some areas. The outbreak was first noticed in Andhra Pradesh and it subsequently spread to Tamil Nadu. Thereafter, Kerala and Karnataka were affected; subsequently, the outbreak spread northwards as far as Delhi. During 2007, up until 12 October, a further 37,683 cases had been reported by national authorities, largely in areas not involved in the previous year’s epidemic. In India, the persistent and disabling arthralgia following infection has been of particular concern. In some areas, the outbreak was associated with high densities of *Ae. albopictus*, but *Ae. aegypti* is thought to be the major vector elsewhere. During 2006, there were also outbreaks in the Andaman and Nicobar islands, Malaysia, and by November 2006, chikungunya had appeared in Sri Lanka. Between January 2001 and April 2007, Indonesia reported 15,207 chikungunya cases from 7 provinces, with a peak in 2003. During 2006, there were 9 imported cases of chikungunya in the Caribbean, involving Martinique, French Guyana and Guadeloupe; these mainly occurred in travellers.

**Figure:** *Geographical distribution of chikungunya cases 2001–2007 (data are presented as reporting period followed by the estimated number of cases, where data are available)*
Outbreak and spread of chikungunya

from the Indian Ocean islands. The Caribbean is an area of active dengue transmission, the vectors of which are also vectors of chikungunya, hence the region is vulnerable to local transmission. In 2006, in the United States, 37 cases of chikungunya were confirmed in returning travellers, 32 from India, 3 from Sri Lanka, 1 from La Réunion and 1 from Zimbabwe. In 2006, in the United States, 35 cases of chikungunya were confirmed in returning travellers, 2 returning from La Réunion and 33 from India. The United States is also vulnerable to local transmission owing to the wide distribution of *Ae. albopictus*. Large areas of Central and South America are similarly vulnerable owing to the presence of abundant populations of both *Ae. aegypti* and *Ae. albopictus*.

In 2007, there was an outbreak of chikungunya in Gabon, mainly affecting Libreville. Symptoms included headache, severe arthralgia and skin rash. Up until 24 June, a total of 17 618 cases had been reported, with 808 hospitalizations. During a mosquito survey in Libreville in November 2006, *Ae. albopictus* was found for the first time in Gabon, and virus was subsequently isolated from field populations of this species collected at the time of the outbreak. *Ae. aegypti* is also common in the city, but none of the specimens collected at the same time were positive for chikungunya virus.

In August 2007, the first cases of indigenous transmission of chikungunya in Europe were reported from a largely rural area in the provinces of Ravenna and Forlì-Cesena in the Emilia-Romagna region of north-east Italy. *Ae. albopictus* is widespread and numerous within much of Italy. Between 15 June and 21 September 2007, 292 suspected cases of chikungunya were identified in the region through a programme of active surveillance; many have been laboratory-confirmed. The index case is presumed to be a person from the region who travelled to a chikungunya-affected area of Kerala (India) and returned to Italy in mid-June, developing symptoms shortly thereafter. The peak of the outbreak occurred in the third week of August. Epidemiological data suggest that transmission took place at 4 different localities in Emilia-Romagna, and the virus has been isolated from local *Ae. albopictus* mosquitoes.

It has been confirmed that European *Ae. albopictus* are susceptible to infection with chikungunya virus from the Indian Ocean outbreak. Samples of this mosquito, collected as eggs from the Alpes-Maritimes, France, have been reared in the laboratory and the adult females experimentally infected by feeding on blood containing a strain of chikungunya virus derived from La Réunion. When *Ae. albopictus* were examined 12–14 days after engorgement, 27/35 (77%) showed evidence of infection in the head. Such a high infection rate supports the view that this strain of the virus is well adapted to transmission by this mosquito species. In the same experiments, 33/49 (67%) *Ae. detritus* became infected. This species commonly bites humans and has a wide distribution in the Palaearctic Region, breeding in coastal and inland saline water. *Ae. capsius*, another species from the Palaearctic Region found in brackish water, showed an infection rate of 4/16 (25%). A single female *Ae. vittatus* survived the incubation period and was found to be infected. This study raises the possibility that these other mosquito species may play a role in transmission during an outbreak of chikungunya.

Genotyping of viral isolates has provided useful information on the possible origins of the outbreak in the Indian Ocean islands as well as evidence for the circulation in that outbreak of viruses with a high evolutionary potential. During outbreaks, arboviruses such as chikungunya diversify due to mutation, leading to several identifiable strains. Chikungunya virus has its origins in continental Africa and is related to the virus responsible for o’nyong-nyong. The
Outbreak and spread of chikungunya

Chikungunya virus circulating in the outbreak in the Indian Ocean islands represents a distinct clade within the East-Central-South African phylogroup, compatible with its recent origin in the African region. However, evidence from genotyping suggests that some time after the virus reached La Réunion, before transmission rates rose steeply, a mutation occurred that enabled more effective transmission by *Ae. albopictus*. If this hypothesis were confirmed, it could help explain the rapid spread and dissemination of chikungunya after it reached La Réunion. The viral genotype involved in the large outbreak in India is from the same lineage as that seen in La Réunion, whereas previous outbreaks in India have involved an Asian genotype of the virus.

Although the 2 major mosquito species, *Ae. aegypti* and *Ae. albopictus*, have been implicated in the larger outbreaks of chikungunya, within Africa, several other mosquito vectors have been implicated, including species of the *Ae. furcifer-taylori* group and *Ae. luteocephalus*. In this region, various primates have been implicated as reservoir hosts. Elsewhere in Africa, little is known of the possible role animals have as reservoirs or of other mosquitoes as vectors.

Whereas *Ae. aegypti* is confined to the tropics and subtropics (approximately within the 10° isotherms), *Ae. albopictus* also occurs extensively in temperate and even cold-temperate regions, thereby extending the geographical range over which chikungunya transmission may occur. *Ae. albopictus* exploits a wider range of breeding sites than *Ae. aegypti*. These include bromeliads, coconut husks, cocoa pods, bamboo stumps, tree holes and rock pools in addition to outdoor artificial containers, including vehicle tyres, various discarded containers and saucers beneath plant pots. This diversity of larval habitats explains the abundance of *Ae. albopictus* in rural as well as periurban areas and in shady city parks. *Ae. aegypti* is more closely associated with human habitation and exploits indoor breeding sites, including flower vases, water storage vessels and concrete water tanks in bathrooms, as well as the same artificial habitats outdoors as *Ae. albopictus*.

**Control measures**

A key factor in the increasing geographical range and prevalence of *Ae. albopictus* has been the globalization of trade, particularly the transportation of used tyres between countries and continents. Both *Ae. aegypti* and *Ae. albopictus* have drought-resistant eggs that remain viable over periods of several weeks, enabling them to survive lengthy journeys with sea-borne freight, and on aircraft or long-distance road transport. For these reasons, some countries have introduced a ban on the importation of used tyres. Both mosquito species are competent vectors of other arboviruses, including dengue, so there are wider public health concerns about the accidental transfer of these species between countries and continents.

It will be impossible to eliminate *Ae. albopictus* with current technologies in areas where it has become well established (the Americas, Cameroon, Italy and Nigeria). In such regions, efforts should concentrate on reducing vector density in order to reduce the risk of transmission. Where a newly introduced *Ae. albopictus* population is detected sufficiently early, concerted efforts, including the use of insecticide applications, are required to prevent this species becoming established; there are examples of the successful use of this approach. In areas at high risk for the introduction of *Ae. albopictus*, good vector surveillance is required since there is only a short window of opportunity to eliminate new foci before they become established.
Chikungunya poses considerable problems for public health authorities once an outbreak has commenced. The major environmental measures used to reduce sources of mosquito breeding may not be fully implemented within the timescale of an outbreak. Few public health programmes are up to the task of responding to large epidemics of arboviruses, but those countries with good routine programmes of vector surveillance and control are better able to mitigate the effects of an outbreak.

Measures to reduce the number of water-containing receptacles may contribute significantly to a reduction in emergent mosquitoes, but such action needs to be based on sound knowledge of the ecology of the immature stages of the mosquito in that specific location. During an arbovirus outbreak, a reduction in transmission may require the application of insecticides as space sprays, as residual applications on surfaces in and around container habitats where mosquitoes alight, and as larvicides to kill the immature stages. Source reduction will be particularly difficult in rural areas where numerous natural, as well as man-made, breeding sites may occur. Nevertheless, reducing the number of containers in and around the home is to be encouraged, and the most important health messages for the public relate to actions that can be taken to reduce the number of habitats.

In urban areas, used-tyre dumps enable large-scale breeding, particularly of Ae. albopictus, and require focused control measures, including the application of insecticides. In the longer term, investment in recycling programmes for tyres should be encouraged. In hospitals and other health-care settings where chikungunya (and dengue) patients may be treated, vector control measures should be routinely applied and intensified during outbreaks. Viraemic patients should be nursed in screened wards or under mosquito nets. The potential risks of transmission through donation of blood or organs should also be considered.

**Personal protection**

For individual protection during outbreaks of chikungunya, clothing that minimizes skin exposure to the day-biting vectors is advised. Repellents may be applied to exposed skin or to clothing. Repellents should contain DEET (N, N-diethyl- 3-methylbenzamide), IR3535 (3-[N-acetyl-N-butyl]-aminopropionic acid ethyl ester) or icaridin (1-piperidinecarboxylic acid, 2-[2-hydroxyethyl]-1-methylpropylester). They should be applied in strict accordance with label instructions. Insecticide-treated mosquito nets afford good protection for those who sleep during the day, particularly young children. Where indoor biting occurs, mosquito coils or other insecticide vaporizers may also reduce biting.

**Surveillance**

Accurate data on the extent of infection in the human population requires the use of immunological testing, such as enzyme-linked immunosorbent assays (ELISA) for the detection of immunoglobulin M and immunoglobulin G anti-chikungunya antibodies. Various reverse transcriptase–polymerase chain reaction (RT-PCR) methods are available; they are of variable sensitivity and some will detect very low levels of viral transcripts. Some are suited to clinical diagnosis and others to surveys. RT-PCR products from clinical samples may also be used for genotyping of the virus, allowing comparison with virus from various geographical sources.

In areas of particular risk, routine vector surveillance is essential for effective vector control, as identified above.
Multicountry study of *Aedes aegypti* pupal productivity survey methodology: Findings and recommendations*

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**Rationale**

Dengue fever and the potentially lethal haemorrhagic form of dengue are a fast growing public health problem worldwide (50 million cases with 500 000 manifesting shock and/or haemorrhage each year), and particularly important in the Americas and Asia. The only known strategy for reducing dengue transmission is to reduce the vector population, which is achieved through interventions in domestic and peri-domestic water containers. However, little is known about the efficacy of interventions targeted to specific classes of water-holding container, and there is uncertainty about the best indicators to use for measuring the success of interventions on vector populations.

TDR financed a multicountry study involving nine Latin American, Asian, and African countries, based on the rationale that certain water containers are particularly productive of the dengue vector, *Aedes aegypti* (L.), and that, therefore, an intervention targeted to these containers would bring vector densities below the threshold for epidemic transmission. This pupal/demographic survey method was described recently.¹

The objective of this multicountry study was to evaluate the practicality of the survey method and whether it can consistently identify and classify particularly productive classes of container, and so provide guidance on development of targeted control strategies. As will be discussed in greater detail, containers may be classified on any number of criteria such as descriptive name, volume, use, abandonment, location indoors or out, etc. The results from this study will have important consequences for the development of improved dengue control strategies.

It is important to emphasize that operationally, pupal/demographic surveys are expected to be conducted infrequently, perhaps only at the beginning of a process to identify the epidemiologically important container classes to target. When targeted interventions via community participation methods are under way, the primary survey goal would be simply to determine the actual amount of intervention in place without larvae or pupae being counted.

This document summarizes the large amount of data created by the nine study teams.

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¹ For complete details, please access the original document TDR/IRM/DEN/06.1
with the goal of serving as a reference text for other researchers as well as for control programmes which are expected to adopt the targeted intervention technique. The document reflects the fact that today, most dengue control efforts are based on suppression of *Ae. aegypti* and not on eradication; these efforts would benefit from answers to the following questions:

- How much suppression is adequate to be prophylactic for dengue outbreaks in a particular location (Table 2)?
- How do we monitor the degree of suppression achieved in ongoing programmes?

Some have recently been advocating an additional question:

- Given that the epidemiological importance of a particular class of container is the product of the average adult productivity and the abundance of that class of container in the environment, how do we select subsets of containers for intervention such that we minimize labour and costs while maximizing reduction of adults?

And finally,

- In developing a targeted strategy, how many households must be surveyed to be confident that the important container classes have been identified?

Answering the first question involves an understanding of transmission thresholds – what they represent and how they are estimated. Thresholds are a function of many factors, but a key factor is the ratio of the numbers of adult *Ae. aegypti* and people, hence the need for a survey method that permits estimation of this important variable. Because pupae can be counted and are highly correlated with the standing crop of adults, a survey method involving counting pupae and people has been developed. Mechanistic and mathematical models of dengue transmission have been developed that allow estimation of transmission thresholds in terms of *Ae. aegypti* pupae per person as a function of temperature and herd immunity; it is only this statistic that can provide estimates of how much reduction is necessary to be prophylactic for dengue outbreaks (Table 2). The practical challenges of obtaining this statistic and its usefulness in developing targeted strategies are the subject of this paper.

Several authors have recently made the case that use of the traditional *Stegomyia* indices as epidemiologic indicators of dengue transmission risk should be abandoned as they have a number of serious shortcomings. The argument is that a pupal and demographic survey that provides an estimate of the number of pupae per person in a community by class of container, e.g. drums, flower vases and pots, cisterns, abandoned containers, discarded tyres, is more appropriate for assessing risk and directing control operations. The ratio of pupae per person is used for several reasons:

- Unlike any of the other mosquito life-cycle stages, it is possible to actually count the number of *Ae. aegypti* pupae in most domestic environments (for exceptions, see summary of the Viet Nam study below).
- Container-inhabiting *Stegomyia* pupae are easily and inexpensively separated from other genera and identified to species as pupae or adults.
- Because pupal mortality is slight and well-characterized, the number of pupae is highly correlated with the number of adults.
The statistic of pupae per person can be related to transmission risk and provide target levels of reduction required in control efforts.[2]

The survey method being evaluated in these studies is called the pupal/demographic survey, so named because we count both pupae and the number of people associated with them. In practice, such a survey involves visiting 100 or more residences (see section on survey size requirements below), usually by two inspectors equipped with nothing more than a few litres of clean water, a sieve,* some largemouth pipettes, a white enamel pan, and small shell vials. The inspectors request permission to examine the water-holding containers in a house, and enquire as to the number of people living in the house (or sleeping there the previous night). With permission, they proceed to strain the contents of each container at the location, re-suspending the sieved contents in a small amount of clean water in the enamel pan, from where the pupae from each container are pipetted into a labelled vial. If there are other container-inhabiting species in the area besides *Ae. aegypti*, the contents of each vial are transferred to small cups covered with bridal veil secured with a rubber band; these are held in the lab until the adults emerge, when they can be identified. As a minimum, the data recorded for each container include the type of container (drum, bottle, etc.), location (indoors, outdoors, under vegetation, etc.), method of filling (manually, by rain, roof runoff, etc.), and the number of pupae by species. These inputs are used to develop a classification scheme (see container classification section below). Data are usually summarized by container class on a spreadsheet; for each container class found, simply add up the number of pupae associated with that type in the survey, and divide by the sum of the number of people living in the residences inspected. Sorting the list of container classes by their associated number of pupae per person quickly identifies the productive and non-productive classes of container.

While exhaustive counts of pupae in every container are certainly more labour intensive than simply noting whether a container has larvae and/or pupae (as in the various traditional *Stegomyia* indices), the pupal/demographic survey permits us to know the number of pupae per person associated with the various classes of container in the environment. This statistic, *Ae. aegypti* pupae per person by class of container, is not an index after the fashion of the *Stegomyia* indices, rather it is the epidemiologically significant statistic, the ratio of hosts and vectors; published transmission thresholds permit us to appreciate this statistic in the context of estimates of herd immunity and ambient temperatures.[2] What each of the studies summarized below demonstrates is that some classes of container are more important by virtue of their production of *Ae. aegypti* adults than others, and that this leads logically to the development of targeted interventions. An earlier publication provides a discussion of the utility of other indices for dengue risk assessment and control.[2]

The objectives of this document are to:

- Summarize the key findings from the nine study sites of the multicountry project on pupal/demographic survey methodology.
- Outline the prospects and limitations of this survey methodology.
- Draw conclusions and propose further research and implications for dengue prevention and control programmes.

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*USA standard sieve series, number 30 sieve (equivalent to ASTM designation E11, 600 μm [0.0243"] opening)*
Key findings of the multicountry study

- The pupal/demographic survey identifies epidemiologically important and unimportant types of container
- Containers and sites can be classified using different schema
- Distribution of *Ae. aegypti* pupae per container is strongly clumped
- A potentially important class of container, the rare but extremely productive container
- Survey size requirements to develop a targeted control strategy

Prospects and limitations of the pupal productivity survey methodology

- Results are useful in developing targeted source reduction/control strategy for dengue
- Difficulty of surveying large containers

Conclusions, further research, and implications for dengue prevention and control programmes

Conclusions

The studies have confirmed that the pupal/demographic survey provides the necessary data for developing targeted strategies. The method certainly requires more labour than do the traditional *Stegomyia* indices, but on a cost/benefit analysis, the traditional measures fail because, regardless of cost, they cannot inform targeted strategy nor can they be used for transmission risk assessment. Unpublished longterm (>5 years) and repeated longitudinal pupal/demographic surveys in Iquitos, Peru,[3] and Yogyakarta, Indonesia, indicate that pupal production by container type is remarkably stable over time and suggest, as stated above, that only an initial, high quality survey may be required.

Calibrated sweep net surveys may provide a method for estimating absolute numbers of pupae for some types of container. However, calibration factors determined in one location should be used with great caution in another area, where subtle differences in climate, elevation, methods of handling water, etc., may influence productivity, and hence accuracy.

Further research

Evaluate dengue control strategies using transmission thresholds and pupal/demographic survey methodology

Since the appearance of dengue haemorrhagic fever in South-East Asia in the 1960s and its subsequent spread to the Americas and Oceania, several control strategies have been advocated. Ultra low volume (ULV) spray applications applied through national control programmes were given prominence for several decades. While it was documented that the sprays certainly killed adult mosquitoes, their use in controlling epidemics was harder to demonstrate. Transitory but infrequent depressions in adult mosquito survival simply could not suppress dengue transmission in endemic locations. As appreciation of the inadequacies of ULV grew in the public health community, the focus of control efforts turned to involving affected communities, and much was made of community participation for the purpose of source reduction and/or control. Yet experience, with the exception of the two vertical and national programmes of Singapore and Cuba, has been disappointing. Some thought the
problem lay with an inadequate understanding on the part of the communities and that, with time and education, dengue control could be achieved. Others suggested that another factor was at work—that the public was in fact conducting a cost–benefit analysis with regard to dengue—and simply concluded that preventing mosquito breeding in all containers every day indefinitely was, as indicated by practice, simply not worthwhile from a labour/time perspective. The promise of targeted source reduction and control, building on the significant accomplishments to date in the area of community participation, lies with reducing the labour and costs in the cost–benefit calculation. With few exceptions, evaluations of community-based interventions were based on sociological evidence such as an increase in knowledge, change in practice, etc., and not on measurements of transmission or disease prevalence. Perhaps this is understandable in light of the time and effort required to change human behaviour with regard to cigarettes, seat belts, etc.

In light of the foregoing comments, the chief research recommendation of this summary is to conduct targeted interventions with the dependent variable being some measure of transmission. Studies with significant funding can measure transmission directly using longitudinal serosurveys of infants and adolescents using the prevalence of anti-dengue IgM as a proxy; other studies will have to rely on case data. In addition, evaluations of interventions should monitor the actual impact on pupal production in the targeted containers.** If a targeted intervention effort fails, it is critical to know whether failure is attributable to inadequate control in targeted containers or if the problem lies with the notions of targeted intervention and transmission threshold.

Estimate the amount of suppression required in interventions using transmission thresholds

Mechanistic models of the dynamics of dengue have been used to estimate transmission thresholds (the number of Ae. aegypti pupae per person) as a function of pre-existing antibody levels in the human population (herd immunity) and ambient air temperature (Table 1). The threshold, an epidemiological concept central to infectious disease control, is simply the breaking or tipping point; at levels below threshold, infection rates and viral persistence decline in the human population. Threshold levels were estimated to range between 0.5 and 1.5 Ae. aegypti pupae per person for an ambient air temperature of 28 °C and initial seroprevalence of 0% to 67%. The concept of transmission threshold has given us a new and hopefully useful tool for monitoring targets for source reduction/control efforts. Moreover, in terms of risk assessment, transmission thresholds provide estimates of the level of elimination or control necessary to preclude transmission (Table 2). In targeted interventions, attempts should be made to reduce the number of Ae. aegypti pupae per person to below the threshold estimate. Note in table 2 that several locations would require >70% reduction in adult production to be prophylactic.

It goes without saying that we see exceeding the threshold as being a necessary but not sufficient cause of transmission. The table of transmission thresholds (Table 1) takes into account the degree of susceptibility in the human population (and indicates the possible

**A current evaluation in Yogyakarta, Indonesia, using pyriproxyfen to prevent adult emergence involves: weekly visual inspection of targeted containers for the presence or absence of the compound; laboratory bio-assay of water collected from treated containers in the field; and laboratory observation of field-collected pupae for emergence success and deformities. Transmission is monitored using: case data, and seroprevalence of IgM in children and infants measured every three months.
Table 1: Transmission thresholds in terms of Ae. aegypti pupae per person as a function of ambient temperature and prevalence of dengue antibody. This table shows the estimated number of Ae. aegypti pupae per person required to result in a 0% or greater rise in seroprevalence of dengue antibody during the course of a year resulting from monthly viral introductions of a single viraemic individual[2]

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Transmission threshold by initial seroprevalence of antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>22</td>
<td>7.13</td>
</tr>
<tr>
<td>24</td>
<td>2.20</td>
</tr>
<tr>
<td>26</td>
<td>1.05</td>
</tr>
<tr>
<td>28</td>
<td>0.42</td>
</tr>
<tr>
<td>30</td>
<td>0.10</td>
</tr>
<tr>
<td>32</td>
<td>0.06</td>
</tr>
</tbody>
</table>

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consequences of inadequately knowing the herd immunity level). But transmission thresholds obviously make no statement about the presence or type of viruses that may or may not be circulating or introduced. Transmission thresholds are useful for risk assessment and risk reduction. In the absence of virus and a control programme, they speak of receptivity to virus; in the endemic situation, they provide targets and end points for targeted source reduction/control programmes. In targeted interventions, account should be taken of seasonal variations in transmission threshold due to temperature fluctuation, and strategies should provide sufficient reduction in Ae. aegypti production to suppress transmission. In dengue endemic areas where herd immunity levels (prevalence of anti-dengue IgG by serotype) are unknown, conservative estimates should be used, e.g. 33%, to estimate the target level of reduction in number of pupae per person.

The Cuban team noted that two measures, the number of Ae. aegypti pupae per person and the number of pupae per hectare, were highly correlated (89%); they suggested that ‘pupae per person’ does not require documentation of human density and is, therefore, perhaps the more desirable statistic. It is important to note, however, that ‘pupae per hectare’ (an index) cannot be related to risk of transmission because, among other factors such as temperature and herd immunity, dengue infection is a function of the ratio of vector and host abundance, i.e. of pupae per person. The difference in cost is minimal, but only ‘pupae per person’ permits estimation of the degree of reduction necessary to effect control (Tables 1 and 2).[2]

High correlation between pupae per person and pupae per hectare was also noted in the Peru study, and in Iquitos it was difficult to estimate the number of people per house with much confidence.

Continue evaluation of calibrated sweep nets

Based on the experiences of some study groups, obtaining absolute pupal counts in some types of large container is a significant problem, suggesting that efforts to evaluate the calibrated sweep net method are warranted. The Viet Nam team was able to identify the same container types to be targeted as identified by the pupal/demographic survey method. If the linear relationship between abundance of late instars and pupae is consistent, it may be possible to use sweep net counts to estimate pupal abundance and evaluate strategies in terms of reduction in pupae per person.
Develop better classification schemes for containers, and methods to locate rare but especially productive containers at house and block scales

When rare but especially productive containers (REPCs) are encountered, priority should be given to:

- elucidating the factors responsible for the anomalous production
- identifying the common features among REPCs discovered during the survey (location, association with vegetation, type of container with respect to material, use/non-use, etc.)
- developing a classification scheme that includes factors to facilitate the locating of REPCs without exhaustive survey.

The fact that REPCs are infrequently found, that they appear as anomalies and are not typical, should not mean serious consideration is not given to this potentially important class of container. Rare they may be, but most reasonably sized surveys discover one or more REPCs often accounting for a significant proportion of production. In addition to discovering common factors associated with REPCs which might be useful in developing a search strategy targeting them, another important question is whether their production is stable over time. If it is stable (the likely case) then we should probably always include an REPC class in our surveys to keep us mindful of their existence and importance, helping us to think in terms of answering the three points above.

The container classification schemes developed in the studies in Peru, Puerto Rico, and other countries, involving the concepts of abandonment, location with respect to vegetation or placement, etc., are very good examples of the utility of thinking in terms of new covariates of production and should be studied and applied to local surveys. Rapid location of especially productive classes of container could serve to further reduce the labour associated with targeted strategies.

Implications for dengue prevention and control programmes

The results of these studies are encouraging—they suggest that control of transmission may be possible with the treatment or elimination of only a small portion of the large number of classes of water-holding container found in the environment. Each study identified the important classes of container to be controlled, and also the classes that could safely be ignored. Commonly, the more productive classes were larger containers that were stable through time, a feature that may make them more amenable to control with biological control agents or chemicals. Given that all control programmes are resource limited, this new and quantitative understanding of productivity can lead to targeted interventions that may be sustainable because control that relies on targeted strategy requires substantially less labour. A TDR supported multicentre study on the cost-effectiveness of targeted vs. non-targeted interventions will provide answers to this question.

Reducing the number of breeding containers will not cause an increase in productivity of the remaining containers

A common concern regarding targeted strategies is the notion that, immediately following the elimination of one or more classes of container, more oviposition, and hence more adult...
### Table 2: Comparison of observed numbers of Ae. aegypti pupae per person in various dengue-endemic or dengue-receptive locations with estimated transmission thresholds based on average summertime temperatures and an initial seroprevalence of %. The column ‘% control’ is the degree of reduction in pupae per person necessary to reduce the observed field level to that of the threshold.

<table>
<thead>
<tr>
<th>Location</th>
<th>Temp (°C)</th>
<th>Pupae per person</th>
<th>Threshold</th>
<th>Ratio</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reynosa, Mexico</td>
<td>29.4</td>
<td>2.75</td>
<td>0.26</td>
<td>10.4</td>
<td>90</td>
</tr>
<tr>
<td>Mayaguez, Puerto Rico</td>
<td>26.6</td>
<td>1.73</td>
<td>1.05</td>
<td>1.7</td>
<td>40</td>
</tr>
<tr>
<td>Trinidad (20 sites)</td>
<td>27.0</td>
<td>22.78</td>
<td>0.86</td>
<td>26.4</td>
<td>96</td>
</tr>
<tr>
<td>El Progreso, Honduras</td>
<td>29.1</td>
<td>0.34</td>
<td>0.31</td>
<td>1.1</td>
<td>10</td>
</tr>
<tr>
<td>San Juan, Puerto Rico</td>
<td>27.8</td>
<td>2.75</td>
<td>0.58</td>
<td>4.7</td>
<td>79</td>
</tr>
<tr>
<td>Bangkok, Thailand</td>
<td>29.2</td>
<td>1.69</td>
<td>0.29</td>
<td>5.8</td>
<td>83</td>
</tr>
</tbody>
</table>

- **Temp** refers to average temperature during the months of June–August or December–February in locations above and below the equator, respectively.
- **Pupae per person** refers to the average number of Ae. aegypti pupae per person observed in the survey.
- **Threshold** refers to the estimated number of Ae. aegypti pupae per person that will preclude epidemic transmission. These estimates were derived from dengue transmission models. Because the models are stochastic, around this tipping point or transmission threshold, multiple model runs under the same conditions typically have little or no subsequent transmission following the introduction of a single viraemic individual times per year. The values shown in the table are based on thresholds assuming an initial seroprevalence of %.
- **Ratio** is the ratio of observed pupae per person and the estimated temperature—and seroprevalence-specific threshold.
- **% Control** is the degree of reduction in pupae per person necessary to reduce the observed field level to that of the threshold.
- Unpublished studies conducted by Focks in collaboration with others. Surveys in Puerto Rico and Mexico were limited and preliminary.
- Observed range: 0.4–6.4 pupae per person; the island-wide average is used for calculation.

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production, will occur in the remaining containers. However, adult production is in equilibrium with, and is a function of, the rate at which food arrives (or is produced) in the container; adult production is not a function of, nor is it limited by, the rate of oviposition. In the population dynamics of Ae. aegypti there is a surfeit of eggs and a paucity of food.[4] Consider a location with ten containers, each producing an average of two adults per day. If we assume an even sex ratio and an average lifespan of about 10 days, these 10 containers would be associated with a standing crop of 100 females (10 days x 10 containers x 1 new female per day per container). Further, lets assume an average fecundity of 25 eggs per day per female. Expected daily oviposition at our location would be 2500, or 250 eggs per container—each day 250 eggs are laid, yet each day only 2 adults emerge from each container. The high rate of mortality between oviposition and emergence is due to a number of factors including egg predation and density-dependent larval mortality; there are simply more eggs laid than the larval environment can support. As there are no density-dependent survival factors in the
adult stage, elimination of 25% or 50% of adult production results in a similar reduction in the standing crop of adults.[5]

In a targeted source reduction campaign, in addition to removing the waterholding containers, the associated *Ae. aegypti* larval food is removed from the environment and productivity of the environment goes down, as does the standing crop of adults. Any transient rise in oviposition in untreated containers is met with increased density-dependent larval mortality as productivity is a function of food availability—recall the large ratio of daily oviposition and emergence. In contrast, in an intervention involving larvicides, biological control agents, or insect growth regulators, neither the number of oviposition sites nor the amount of food declines. Our treatment has eliminated the production in some containers, thereby lowering the standing crop of adults and oviposition. One might wonder if productivity would go down in untreated containers, oviposition rates in them being somewhat lower, as a lower number of females is associated with the same number of containers. Simulation and field studies in Kenya suggest that until adult populations are reduced by >40%, container productivity will not be limited by lowered oviposition rates because food-based density-dependent larval mortality decreases.[4]

References


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.

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The endemicity of dengue fever and dengue haemorrhagic fever (DHF) in countries of the South-East Asia (SEA) and the Western Pacific regions continues to rise. During 2007, in the SEA Region, Indonesia, Thailand and Myanmar contributed 62.89%, 25.14% and 6.10% dengue cases respectively, both in morbidity and mortality. When compared with 2006, the number of cases in all three countries showed an increasing trend. Nepal which reported 25 cases for the first time in 2006 reported only three cases in 2007. DENV-2 was found to be circulating in Nepal. In view of this scenario, WHO developed the biregional Asia-Pacific Strategic Plan (2008-2015) for Prevention and Control of Dengue in 2007. The thrust of this plan will be to strengthen systems in countries to predict and prevent epidemics, improve early recognition and management of cases, support prevention of dengue through integrated vector management and on community participation and research.

The WHO South-East Asia Regional Office, in collaboration with the Western Pacific Regional Office, jointly publish the annual *Dengue Bulletin*. The current Volume 31 (2007) of Dengue Bulletin includes contributions from WHO’s SEA Region (5); the Western Pacific Region (8); and the American Region (4).