Acknowledgements

Editor, Dengue Bulletin, WHO/SEARO, gratefully thanks the following for peer reviewing manuscripts submitted for publication.

In-house Review:

Nand L. Kalra: Reviewed the manuscripts in respect of format check, content, conclusions drawn, including condensation of tabular and illustrative materials for clear, concise and focused presentation and bibliographic references. He was also involved in the final stages of printing of the Bulletin.

Peer Reviewers

1. Dana A. Focks
   John W. Hock Company
   P.O. Box 12852
   Gainesville, FL 32604
   USA
   E-mail: DAFocks@JohnWHock.com

2. Duane J. Gubler
   Director
   Asia-Pacific Institute of Tropical Medicine and Infectious Diseases
   Leahi Hospital
   3675 Kilauea Ave
   Honolulu, Hawaii 96816
   USA
   E-mail: dgubler@hawaii.edu

3. Oon Chong Teik
   Tropical Medicine & Infectious Diseases
   Mt Elizabeth Hospital
   Singapore 228510
   E-mail: oonchongteik@hotmail.com
4. Scott Halstead  
Uniformed Services University of the Health Sciences  
Bethesda, Maryland  
USA  
E-mail: halsteads@erols.com

5. Siripen Kalyanarooj  
WHO Collaborating Centre for Case Management of Dengue/DHF/DSS  
Queen Sirikit National Institute of Child Health (Children’s Hospital)  
Bangkok  
Thailand  
E-mail: sirip@health.moph.go.th

6. Suchitra Nimmannitya  
WHO Collaborating Centre for Case Management of Dengue/DHF/DSS  
Queen Sirikit National Institute of Child Health (Children’s Hospital)  
Bangkok  
Thailand  
E-mail: sujitran@health.moph.go.th

7. Kevin Palmer  
Regional Office for the Western Pacific  
P.O. Box No. 2932  
12115 Manila  
Philippines  
E-mail: palmerk@wpro.who.int

8. Michael Nathan  
World Health Organization  
Headquarters  
20 Avenue Appia  
1211 Geneva 27  
Switzerland  
E-mail: nathanm@who.int

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.
From the Editor’s Desk

Over the decades dengue/dengue haemorrhagic fever has emerged as a global public health problem with countries in Asia and the Pacific sharing more than 70% of the disease burden. In some of these countries, DHF is gaining hyper-endemicity causing deaths among children. During 2004, Indonesia reported a major dengue outbreak encompassing Central Java, Sumatra and some outer islands. Till the end of July 2004, 69,017 cases of DF/DHF and 770 deaths were registered by Indonesian health authorities. During this epidemic DEN-3 was the predominant serotype.

Sri Lanka also reported a major outbreak with 12,400 cases and 71 deaths as of 23 August 2004. A majority of the cases were reported from five cities: Colombo, Kandy, Gampaha, Kalutara and Kurunegala.

In the South-East Asia Region, Bhutan and Nepal continued to enjoy dengue-free status till 2003 because of their sub-mountainous location. However, during August 2004, Bhutan recorded the first-ever outbreak of DF/DHF in Phuntsholing (population 27,000), a border town with India. During this outbreak a total of 2,544 DF/DHF cases with no deaths were reported. More than 93% of those affected were persons above 5 years of age. This sent a strong signal to the adjoining DF-free north-eastern part of India and Nepal to take appropriate preventive action.

DengueNet, the WHO Global Surveillance System for management of epidemiological and virological surveillance data for early detection, planning and response, was launched in the South-East Asia and Western Pacific countries during 2004. Each country identified institutions which would participate in the programme.

The current Volume 28 (2004) of the Dengue Bulletin includes contributions from the South-East Asia Region (13), the Western Pacific Region (7), the American Region (5) and the European Region (4).

A supplement, featuring experiences from different countries in social mobilization and communication for dengue prevention and control, is also being issued along with this volume.

We now invite contributions for Volume 29 (2005). The deadline for the receipt of contributions is 30 June 2005. Contributors are requested to follow the instructions carefully while preparing the manuscript. Contributions accompanied by computer diskettes using MS Word for Windows should be sent to the Editor, Dengue Bulletin, WHO/SEARO, Mahatma Gandhi Road, IP Estate, Ring Road, New Delhi-110 002, India, or by e-mail as a file attachment to the Editor at dengue@whosea.org. Readers desirous of obtaining copies of the Dengue Bulletin may contact the respective WHO Regional Offices in New Delhi or Manila or the WHO Country Representative in their country of residence.

Dr Chusak Prasittisuk
Regional Adviser
Vector-borne Disease Control
World Health Organization
Regional Office for South-East Asia
New Delhi, India
Contents

1. Annual Changes of Predominant Dengue Virus Serotypes in Six Regional Hospitals in Thailand from 1999 to 2002
   Surapee Anantapreecha, Sumalee Chanama, Atchareeya A-nuegonpipat, Sirirat Naemkhunthot, Areerat Sa-ngasang, Pathom Sawanpanyalert and Ichiro Kurane

2. A Retrospective Study of the 1996 DEN -1 Epidemic in Trinidad: Demographic and Clinical Features
   T.U. Brown, K. Babb, M. Nimrod, C.V.F. Carrington, R.A. Salas and M.A. Monteil

   Maria Lucia F. Penna


   Wutjanun Muttitanon, Pongpan Kongthong, Chusak Kongkanon, Sutee Yoksan, Narong Nitapattana, Jean Paul Gonzalez and Philippe Barbazan

   Nand Lal Kalra and Chusak Prasittisuk

7. Autoimmunity in Dengue Virus Infection
   Chiou-Feng Lin, Huan-Yao Lei, Ching-Chuan Liu, Hsiao-Sheng Liu, Trai-Ming Yeh, Shun-Hua Chen and Yee-Shin Lin

8. Inhibition of the NS2B-NS3 Protease – Towards a Causative Therapy for Dengue Virus Diseases
   Gerd Katzenmeier
9. Prognostic Factors of Clinical Outcome in Non-Paediatric Patients with Dengue Haemorrhagic Fever/Dengue Shock Syndrome
   Jaime R. Torres, José M. Torres-Viera, Hipólito García, José R. Silva, Yasmín Baddour, Angel Bajares and Julio Castro M.

    Prasonk Witayathawornwong

11. A New Tool for the Diagnosis and Molecular Surveillance of Dengue Infections in Clinical Samples
    C. Domingo, G. Palacios, M. Niedrig, M. Cabrerizo, O. Jabado, N. Reyes, W.I. Lipkin and A. Tenorio

12. Clinical and Laboratory Observations Associated with the 2000 Dengue Outbreak in Dhaka, Bangladesh

13. Current Status of Dengue Diagnosis at the Center for Disease Control, Taiwan
    Pei-Yun Shu, Shu-Fen Chang, Yi-Yun Yueh, Ling Chow, Li-Jung Chien, Yu-Chung Kuo, Chien-Lin Su, Tsai-Ling Liao, Ting Hsiang Lin and Jyh-Hsiung Huang

14. Detection of Dengue Virus Serotype-specific IgM by IgM Capture ELISA in the Presence of Sodium thiocyanate (NaSCN)
    Masaru Nawa, Tomohiko Takasaki, Mikako Ito, Ichiro Kurane and Toshitaka Akatsuka

15. Genetic Influences on Dengue Virus Infections
    J.F.P. Wagenaar, A.T.A. Mairuhu and E.C.M. van Gorp

16. Identification and Phylogenetic Analysis of DEN-1 Virus Isolated in Guangzhou, China, in 2002
    Jun-lei Zhang, Rui Jian, Ying-jie Wan, Tao Peng and Jing An
17. Induction of Cytotoxic T Lymphocytes by Immunization with Dengue Virus – Derived, Modified Epitope Peptide, Using Dendritic Cells as a Peptide Delivery System
   Yoshiki Fujii, Hideyuki Masaki, Takanori Tomura, Kiyohiro Irimajiri and Ichiro Kurane

18. Molecular Characterization of Brazilian Dengue Viruses
   Marize Pereira Miagostovich, Flávia Barreto dos Santos and Rita Maria Ribeiro Nogueira

19. Unusual Emergence of Guate98-like Molecular Subtype of DEN-3 during 2003 Dengue Outbreak in Delhi
   Manoj Kumar, S.T. Pasha, Veena Mittal, D.S. Rawat, Subhash Chandra Arya, Nirmala Agarwal, Depesh Bhattacharya, Shiv Lal and Arvind Rai

20. The Animal Models for Dengue Virus Infection
   Tao Peng, Junlei Zhang and Jing An

21. Philippine Species of Mesocyclops (Crustacea: Copepoda) as a Biological Control Agent of Aedes aegypti (Linnaeus)
   Cecilia Mejica Panogadia-Reyes, Estrella Irlandez Cruz and Soledad Lopez Bautista

22. Susceptibility of Aedes aegypti to Insecticides in Vietnam
   Vu Duc Huong, Nguyen Thi Bach Ngoc, Do Thi Hien and Nguyen Thi Bich Lien

23. Ovipositioning Behaviour of Aedes aegypti in Different Concentrations of Latex of Calotropis procera: Studies on Refractory Behaviour and its Sustenance across Gonotrophic Cycles
   Manju Singhi, Vinod Joshi, R.C. Sharma and Keerti Sharma

24. Community-based Assessment of Dengue-related Knowledge among Caregivers
   Khynn Than Win, Sian Za Nang and Aye Min

25. Students' Perceptions about Mosquito Larval Control in a Dengue-Endemic Philippine City
   Jeffrey L. Lennon
Contents

Short Notes

1. Sero-surveillance in Delhi, India – An Early Warning Signal for Timely Detection of Dengue Outbreaks
   D. Bhattacharya, Veena Mittal, M. Bhardwaj, Mala Chhabra, R.L. Ichhpujani and Shiv Lal
   207

2. Detection of Dengue Virus in Wild Caught Aedes albopictus (Skuse) around Kozhikode Airport, Malappuram District, Kerala, India
   B.P. Das, L. Kabilan, S.N. Sharma, S. Lal, K. Regu and V.K. Saxena
   210

3. Entomological Investigations for DF/DHF in Alwar District, Rajasthan, India
   Kalpana Baruah, Avdhesh Kumar and V.R. Meena
   213

4. Essentiality of Source Reduction in both Key and Amplification Breeding Containers of Aedes aegypti for Control of DF/DHF in Delhi, India
   B.N. Nagpal, Aruna Srivastava, M.A. Ansari and A.P. Dash
   216

5. Breeding of Dengue Vector Aedes aegypti (Linnaeus) in Rural Thar Desert, North-western Rajasthan, India
   B.K. Tyagi and J. Hiriyan
   220

Book Reviews

1. A Review of Entomological Sampling Methods and Indicators for Dengue Vectors
   223

2. DengueNet in India
   224

3. WHO/WPRO/SEARO Meeting on DengueNet Implementation in South-East Asia and the Western Pacific, Kuala Lumpur, 11-13 December 2003
   226

Instructions to Contributors

232
Annual Changes of Predominant Dengue Virus Serotypes in Six Regional Hospitals in Thailand from 1999 to 2002


*National Institute of Health, Department of Medical Sciences, Ministry of Public Health, 88/7, Tivanond Road, Muang, Nonthaburi, 11000, Thailand
**Department of Virology I, National Institute of Infectious Diseases Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Abstract

A virological study was conducted in six hospitals spread across Thailand from 1999 to 2002. All four dengue serotypes were identified, of which DEN-1 was the most predominant. The predominant dengue serotypes changed every 1-2 years in three of the six hospitals and the predominant serotypes were different in different hospitals. DEN-1 was predominant in two hospitals during three years of the study period (2000-2002). DEN-4 was not isolated, or accounted for only a small percentage of the total isolates, except in one hospital in 2002.

Keywords: Dengue virus, dengue virus serotypes, Thailand.

Introduction

The dengue virus consists of four serotypes: DEN-1, DEN-2, DEN-3 and DEN-4. The clinical manifestations range from undifferentiated febrile illness to classic dengue fever (DF), dengue haemorrhagic fever (DHF) and to dengue shock syndrome (DSS)[1-3]. Dengue virus infections are now important public health problems in many tropical and subtropical areas in the world. In Thailand, a dengue outbreak first occurred in Bangkok in 1958. It then occurred in a pattern of 2-year cycle, and subsequently in irregular cycles as the disease spread throughout the country. The largest outbreak was reported in 1987, with 174,284 reported cases, an incidence rate of 325 cases per 100,000 population. It was followed by another outbreak in 2001, which reported an incidence rate of 224 cases per 100,000 population[4,5]. In 2002, 114,800 dengue cases were reported with an incidence rate of 183 cases per 100,000 population. It is important to determine which of the four serotypes causes the
dengue epidemic each year, and whether the dominant serotype is the same or different in each region in the country. In the present study, we analysed the predominant dengue serotypes at the six regional hospitals located in the four regions of Thailand – north, north-east, central and south regions – in each year from 1999 to 2002.

Materials and methods

Collection of blood specimens
A total of 5,160 and 3,619 blood specimens were collected at acute and convalescent stages, respectively, from suspected dengue cases who visited Lampang Hospital in Lampang (north), Maharat Nakhon Ratchasima Hospital in Nakhon Ratchasima (north-east), Pathum Thani Hospital in Pathum Thani, Chareonkrung Pracharak Hospital in Bangkok, Ratchaburi Hospital in Ratchaburi (central) and Hadyai Hospital in Songkhla (south) from 1999 to 2002 (Figure 1). Blood specimens were drawn into tubes with ethylene diamine tetraacetate (EDTA) anticoagulant and centrifuged. The number of specimens ranged from 1 to 3 per patient. The first samples were collected on the day of hospitalization and the second samples were collected on the day of discharge. Third samples, if any, were collected between 10-14 days after the onset of symptoms. Plasma and buffy coat were separated at each hospital, kept in liquid nitrogen and then transported to the Arbovirus Section of the National Institute of Health, Nonthaburi. Specimens were kept at -70 °C for virus isolation.

Virus isolation and determination of dengue virus serotypes
Ten microlitres of acute phase-buffy coat samples were inoculated onto monolayer of C6/36 cells in a 24-well plate with rocking for 90 minutes at room temperature[6]. The inocula were discarded and replaced by Leibovitz 15 medium (L-15, Gibco BRL) containing 1% heat-inactivated fetal bovine serum. After 7 days of incubation at 28 °C, the cultured media were collected, the infected cells were
stained by IFA, and dengue virus serotypes were determined by IFA. DEN-1 (16007 strain), DEN-2 (16681 strain), DEN-3 (16562 strain) and DEN-4 (1032 strain) were used as virus controls in IFA.

**Results and discussion**

The numbers of samples tested and found positive for virus isolation are shown in Table 1.

<table>
<thead>
<tr>
<th>Year</th>
<th>Hospital</th>
<th>Acute sample</th>
<th>Convalescent sample</th>
<th>Virus isolation positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>Lampang</td>
<td>128</td>
<td>108</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>26</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Chareonkrug Pracharak</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>35</td>
<td>11</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Hadyai</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2000</td>
<td>Lampang</td>
<td>35</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>22</td>
<td>17</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>25</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Chareonkrug Pracharak</td>
<td>15</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>438</td>
<td>419</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>Hadyai</td>
<td>113</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>2001</td>
<td>Lampang</td>
<td>262</td>
<td>238</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>358</td>
<td>251</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>265</td>
<td>170</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>Chareonkrug Pracharak</td>
<td>224</td>
<td>216</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>389</td>
<td>363</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td>Hadyai</td>
<td>450</td>
<td>231</td>
<td>276</td>
</tr>
<tr>
<td>2002</td>
<td>Lampang</td>
<td>339</td>
<td>236</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>930</td>
<td>600</td>
<td>292</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>154</td>
<td>73</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Chareonkrug Pracharak</td>
<td>135</td>
<td>128</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>490</td>
<td>346</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Hadyai</td>
<td>326</td>
<td>103</td>
<td>77</td>
</tr>
</tbody>
</table>

All the four dengue serotypes were detected in the six hospitals during this study. When the total numbers of isolates were analysed, DEN-1 was the predominant serotype (48%), followed by DEN-2 (27%), DEN-3 (20%) and DEN-4 (5%). The predominant dengue virus serotypes in each hospital were analysed when more than nine isolates were obtained in a year. In the Lampang Hospital, the predominant serotypes were DEN-2 (44%) in 1999, DEN-1 (86%) in 2000, DEN-1
The predominant serotypes were compared among six hospitals in each of the study years (Table 2). In 1999 when data were analysed for two hospitals (Lampang and Ratchaburi), DEN-2 was predominant in one hospital (Lampang), and DEN-1 and DEN-2 were equally predominant in the other hospital (Ratchaburi). In 2000 when data were analysed for four hospitals (Lampang, Chareonkrung Pracharak, Ratchaburi and Hadyai), DEN-1 and DEN-3 were predominant in three hospitals (Lampang, Chareonkrung Pracharak and Hadyai) and one hospital (Ratchaburi), respectively. In 2001, DEN-1 was predominant in five hospitals (Lampang, Maharat Nakhon Ratchasima, Chareonkrung Pracharak, Ratchaburi and Hadyai) and DEN-1 and DEN-2 were equally predominant in one hospital (Pathum Thani). In 2002, DEN-1 and DEN-2 were predominant in four hospitals (Maharat Nakhon Ratchasima, Pathum Thani, Chareonkrung Pracharak and Hadyai) and two hospitals (Lampang and Ratchaburi), respectively. These results suggest that DEN-1 was the most predominant serotype from 1999 to 2002 in Thailand; however, the predominant serotypes were different in different hospitals.

The results of the present study are generally consistent with those previously reported. The analysis of dengue virus isolates at Bangkok Children’s Hospital from 1973 to 1999 showed changes in dengue virus serotypes from year to year [9]. Although our study leads to a similar conclusion, the study was conducted in six regional hospitals throughout the country and not only in central Bangkok.

Each dengue serotype may possess unique characteristics in a dengue epidemic and disease severity. There were associations between DEN-1, DEN-2 and DEN-3 and moderately severe dengue epidemic years (1984-85, 1989-90, 1997), and between DEN-3 and severe dengue epidemic years (1987 and 1998)[9]. In that sense, it is important to collect more information on the predominant serotypes and levels of epidemics. Although the present study generated information on isolates only from the patients who visited the six hospitals, they are located far away from each other in four regions of Thailand: north, north-east, central and south. It is thus assumed that the results of this study demonstrate, to a certain extent, the general features of dengue epidemics in Thailand.
### Table 2. Proportion of each of the four dengue serotypes determined by virus isolation in the six hospitals

<table>
<thead>
<tr>
<th>Year</th>
<th>Hospital</th>
<th>Total</th>
<th>DEN-1 (%)</th>
<th>DEN-2 (%)</th>
<th>DEN-3 (%)</th>
<th>DEN-4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>Lampang</td>
<td>74</td>
<td>31 (42)</td>
<td>33 (44)</td>
<td>8 (11)</td>
<td>2 (3)</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>6</td>
<td>4 (67)</td>
<td>2 (33)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Chareonkrung Pracharak</td>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>31</td>
<td>10 (32)</td>
<td>10 (32)</td>
<td>8 (26)</td>
<td>3 (10)</td>
</tr>
<tr>
<td></td>
<td>Hadai</td>
<td>0</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2000</td>
<td>Lampang</td>
<td>15</td>
<td>13 (86)</td>
<td>1 (7)</td>
<td>0 (0)</td>
<td>1 (7)</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>3</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>4</td>
<td>1 (25)</td>
<td>2 (50)</td>
<td>1 (25)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Chareonkrung Pracharak</td>
<td>10</td>
<td>5 (50)</td>
<td>4 (40)</td>
<td>1 (10)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>238</td>
<td>99 (42)</td>
<td>9 (4)</td>
<td>125 (52)</td>
<td>5 (2)</td>
</tr>
<tr>
<td></td>
<td>Hadai</td>
<td>22</td>
<td>21 (95)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2001</td>
<td>Lampang</td>
<td>150</td>
<td>85 (57)</td>
<td>35 (23)</td>
<td>26 (19)</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>176</td>
<td>83 (47)</td>
<td>68 (39)</td>
<td>16 (9)</td>
<td>9 (5)</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>143</td>
<td>58 (41)</td>
<td>59 (41)</td>
<td>19 (13)</td>
<td>7 (5)</td>
</tr>
<tr>
<td></td>
<td>Chareonkrung Pracharak</td>
<td>99</td>
<td>60 (61)</td>
<td>25 (25)</td>
<td>11 (11)</td>
<td>3 (3)</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>256</td>
<td>98 (38)</td>
<td>43 (17)</td>
<td>96 (37)</td>
<td>19 (7)</td>
</tr>
<tr>
<td></td>
<td>Hadai</td>
<td>276</td>
<td>219 (79)</td>
<td>4 (2)</td>
<td>39 (14)</td>
<td>14 (5)</td>
</tr>
<tr>
<td>2002</td>
<td>Lampang</td>
<td>95</td>
<td>10 (11)</td>
<td>75 (79)</td>
<td>8 (8)</td>
<td>2 (2)</td>
</tr>
<tr>
<td></td>
<td>Maharat Nakhon Ratchasima</td>
<td>292</td>
<td>166 (57)</td>
<td>70 (24)</td>
<td>49 (17)</td>
<td>7 (2)</td>
</tr>
<tr>
<td></td>
<td>Pathum Thani</td>
<td>32</td>
<td>15 (47)</td>
<td>8 (25)</td>
<td>7 (22)</td>
<td>2 (6)</td>
</tr>
<tr>
<td></td>
<td>Chareonkrung Pracharak</td>
<td>65</td>
<td>42 (65)</td>
<td>16 (25)</td>
<td>3 (5)</td>
<td>4 (5)</td>
</tr>
<tr>
<td></td>
<td>Ratchaburi</td>
<td>170</td>
<td>21 (12)</td>
<td>126 (74)</td>
<td>12 (7)</td>
<td>11 (7)</td>
</tr>
<tr>
<td></td>
<td>Hadai</td>
<td>77</td>
<td>34 (44)</td>
<td>13 (17)</td>
<td>13 (17)</td>
<td>17 (22)</td>
</tr>
</tbody>
</table>

### Acknowledgements

We thank Dr Piyaporn Bowonkiritakachorn and Ms Souvapa Pongsathaporn of Chareonkrung Pracharak Hospital, Dr Vathana Sriruksit and Mr Suthichai Pongphot of Pathum Thani Hospital, Dr Paiboon Vechapanich and Mr Prayuth Kaemwong of Maharat Nakhon Ratchasima Hospital, Dr Wilaiwan Gulponkam, Dr Aroonrat Suwanarat and Mr Somchai Niyomthai of Lampang Hospital and Dr Vitaya Jiwariyaves and Ms Vanna Pengruangrojanachai of Ratchaburi Hospital.
Dr Suda Chubuppakarn and Ms Raruay Jitsakulchaidej of Hadyai Hospital, and other doctors, nurses and laboratory staff for assisting us with the collection of samples.

This work was partly supported by grants from the Department of Medical Sciences, Ministry of Public Health, Thailand, and the Japan Health Science Foundation.

References


A Retrospective Study of the 1996 DEN-1 Epidemic in Trinidad: Demographic and Clinical Features

T.U. Brown*, K. Babb*, M. Nimrod*, C.V.F. Carrington*, R.A. Salas** and M.A. Monteil*

*Faculty of Medical Science, University of the West Indies, St. Augustine, Trinidad
**Caribbean Epidemiology Centre, Port-of-Spain, Trinidad

Abstract

A retrospective analysis of the 1996 DEN-1 epidemic in Trinidad was undertaken to better understand the clinical and demographic expression of dengue infection in the island during one of the larger epidemics in the past 10 years and following the reintroduction of DEN-1 into the island in 1991 after a gap of 14 years. A total of 393 laboratory-confirmed cases were identified. Of these, notes for 157 patients were available for analysis. The epidemic was island-wide, though most cases occurred in the most densely populated county of St. George. There was a slight predominance of females (51.6%) among the cases, and while all age groups were affected, older children and adults comprised the majority. South Asians among the population predominated. Overall, 27 clinical symptoms were reported. The most common were: fever (98.7%), generalized pain (96.2%) and anorexia (63.1%). Rash, arthralgia, retro-orbital pain and haemorrhage (all mentioned in the WHO clinical description for dengue fever) were reported in <50% of cases. Gastrointestinal symptoms were also very common and occurred in over two-thirds of cases at presentation. Bleeding manifestations were reported in 30% of patients and commonly involved the gastrointestinal tract. Features of DHF were noted in only six (4%) patients and there was one fatality. Deficiencies in documented clinical and laboratory monitoring of patients, coupled with a lack of population-specific laboratory reference ranges, may contribute to under-diagnosis of DHF in Trinidad.

Keywords: Dengue, DHF, demographic analysis, clinical analysis, Trinidad.

Introduction

The dengue viruses (DEN), of the Flaviviridae family, are mosquito-transmitted viruses that can cause dengue fever (DF). DF is an acute febrile illness characterized by intense headaches, retro-orbital pain, myalgia, arthralgia, anorexia and rash. Additionally, in a minority of cases, a severe and potentially fatal form of dengue infection known as dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) is manifested, primarily through increased vascular permeability and shock. Dengue exists as four distinct serotypes, DEN-1-4. Infection with one serotype gives life-long immunity for that virus but not to the others. Data suggest that secondary infection with a
serotype different from the primary infection enhances the risk of developing DHF/DSS\cite{1,2}. The pathological processes that lead to DHF/DSS remain poorly elucidated, but epidemiological studies have highlighted viral and host factors that are associated with the development of DHF/DSS\cite{3}.

An estimated 100 million people across the globe contract dengue annually with about 250,000 persons developing DHF/DSS\cite{4}. In the past 20 years, DF and DHF/DSS have become significant public health problems in the Caribbean and in Latin America. The discontinuation of the Pan American Health Organization (PAHO)-initiated Aedes aegypti eradication programmes in the 1970s caused a re-introduction of the mosquito vector into virgin areas and a resurgence of dengue outbreaks throughout the region. Furthermore, prior to the 1980s, dengue outbreaks in the Americas were caused by single serotypes and were geographically restricted and largely self-limiting, with no reports of DHF/DSS\cite{5}. The first cases of DHF/DSS documented in this region were identified during the Cuban DEN-2 epidemic in 1981\cite{6} and the pattern of the disease has changed dramatically since then\cite{5}. Given the strong evidence linking the disease severity with secondary heterologous infection\cite{7-9}, increased hyperendemicity is widely thought to be one of the most significant factors contributing to increases in the disease severity\cite{3} in the region where all the four serotypes are now present.

The first dengue outbreak in over 20 years in the island of Trinidad was reported in 1977. That outbreak caused by DEN-1 resulted in a few DF cases with no reported case of DHF/DSS\cite{10}. Since then, increasingly larger outbreaks have been documented throughout the 1980s and the 1990s, caused by DEN-2 and DEN-4. The first cases of DHF/DSS were reported during the 1993 DEN-1 epidemic\cite{11}, and this was followed in 1996 by yet another DEN-1 outbreak which was characterized by a larger number of reported and confirmed cases of DHF/DSS\cite{12}.

We present here a retrospective analysis of the 1996 DEN-1 epidemic in Trinidad. This study was done, firstly, to observe the clinical and demographic expression of the dengue infection in the Trinidad population during one of the largest epidemics of the past 10 years. In particular, we wished to assess the ethnic distribution of the disease since there had been previous anecdotal reports of a more severe disease in persons of South Asian ancestry. Secondly, the study was done to gain an understanding of the clinical and laboratory infrastructure for dengue in Trinidad and Tobago as part of the development of our ongoing dengue-related clinical research in the island.

**Methods**

A retrospective analysis of the clinical and demographic features of patients with laboratory-confirmed dengue from the 1996 DEN-1 epidemic in Trinidad, West Indies, was conducted. Patients were identified from the laboratory database of The Caribbean Epidemiology Centre in Trinidad (CAREC). The CAREC is a subsidiary of the Pan American Health Organization (PAHO) and offers infectious disease testing of sera from patients with suspected dengue infections. Since 1975, CAREC has systematically conducted the isolation and
typing of dengue viruses in support of viral surveillance programmes[12].

A total of 1,200 samples from symptomatic suspected dengue cases in Trinidad and Tobago were sent to CAREC in 1996 for laboratory confirmation, of which 393 were found positive. We identified these confirmed cases of dengue infection and the medical institutions from which the samples had originated. The demographic, clinical and laboratory information related to each sample was collected from the respective medical records of each patient.

Data were entered into a computerized Excel database (Microsoft® Excel 2000) and analysed according to demographic groupings such as ethnicity, county of residence, age and gender and by frequencies of clinical symptoms. The incidence rates per 10,000 persons were calculated using the 1990 census data[13]. Proportional data were tested using the Chi square test and statistical significance was established at $P<0.05$. All statistical analyses of the demographic and clinical data were done using SigmaStat® Statistical Software for Windows Version 2.03 (Copyright® 1992-1997 SPSS Inc.). Ethical approval for this project was obtained from the Ethics Committee of the Faculty of Medical Sciences, University of the West Indies, St. Augustine.

Results

The names of the 393 laboratory-confirmed dengue patients were obtained from the CAREC database for 1996. Of these, 258 (66%) could be traced to the source institution or the treating doctor. Clinical records were obtained for a total of 157 patients (40%). Information was incomplete in 60 (15%) of these cases. The following is an analysis of the clinical and demographic data available from the records of the 157 dengue-confirmed cases.

Geographical and temporal distribution of dengue virus infections

One hundred and fifty-four patients' addresses were available. The estimated county-specific incidence rates (per 10,000 population) based on these addresses ranged from 0.60 to 1.87, with the extreme values occurring in the rural counties of Nariva/Mayaro and St. Andrews/St. David respectively (Figure 1). The highest proportion of these 154 confirmed cases (46%) occurred in the most densely populated county of St. George, in which the capital, Port-of-Spain, is situated. There was no significant difference in the rates of infection among all the counties ($P=0.513$; power of test with $\alpha=0.05$; $\beta=0.301$).

A review of the admission dates to health care facilities for 136 dengue-confirmed patients showed that there was a gradual increase in the number of cases from April to June, followed by a large rise in July (29 cases) to a maximum in the month of August (51 cases). In 1996, the rainfall pattern (Figure 2) consisted of a large increase in the rainfall (171.7 mm) between April and May, followed by peak precipitation in June (333.1 mm). A comparison of dengue admissions with the monthly precipitation suggests a direct relationship between the rainfall and the number of cases reported, with a two-month lag between the peak rainfall and the peak number of admissions.
A Retrospective Study of the 1996 DEN-1 Epidemic in Trinidad: Demographic and Clinical Features

Figure 1. Map of Trinidad showing county-specific incidence rates (per 10,000 persons) for dengue infections in 1996 (values in lower half of boxes) based on addresses from 154 confirmed cases. Values in the upper half of boxes show the proportion of the 157 cases found in each county. County divisions are according to the Regional Health Authorities of Trinidad & Tobago.

Figure 2. Monthly admissions of 136 laboratory confirmed dengue cases and precipitation levels for the year 1996. Rainfall data obtained from the records of the Trinidad & Tobago Meteorological Office, Piarco, Trinidad.
Incidence rates of dengue infections in relation to gender, age and ethnicity

Gender and age data were available for all 157 cases. There was a slight female predominance of 51.6%.

The highest proportion of the cases occurred in the 30-34-year age group (15.9% of the cases), followed by 59 and 10-14-year olds (14.0% and 14.6% respectively) (Figure 3). The differences among the groups were found to be highly significant (P<0.001; power of test with \( \alpha = 0.05 \): 0.998). The estimated incidence rate in children (≤15 yrs) was not significantly different from that in adults (>15 yrs). An analysis of the estimated incidence rates by county showed that there was variation in the age groups with the highest incidence rates for each county. In three counties, the highest estimated incidence rates occurred in persons 40 years and over; Caroni (50-59 yrs) (2.9%), Victoria (40-49 yrs) (2.09%) and St. Andrews/St. David (40-49 yrs) (3.65%). However, estimated highest incidence rates were observed in younger patients in the counties of St George (0-9 yrs) (1.89%) and St. Patrick (10-19 yrs) (2.46%).

Figure 3. Age distribution of 157 confirmed dengue cases
Self-reported ethnicity was available for 136 patients. Of these, 50% were South Asians, 35% were Africans, 11% were mixed, and 4% were Chinese and Europeans. As seen in Figure 4, a comparison of the incidence rates by ethnicity for the whole sample and by county showed that the incidence rates of dengue infection were the greatest among South Asians for the group as a whole and in three of the four counties. A statistically significant difference was achieved in the county, St. Patrick, where the number of South Asian patients was higher than the African patients ($P=0.005$; power of test with $\alpha=0.05$: 0.807), and in the whole group where the incidence rate for South Asians was significantly higher than the mixed population ($P=0.016$; power of test with $\alpha=0.05$: 0.70).

Figure 4. Incidence rates (per 10,000 persons) for 136 confirmed dengue infection patients by ethnicity for whole population and individual counties. County Victoria was excluded since ethnicity was reported in <50% of cases in that county.

Summary of clinical and haemorrhagic manifestations
Overall, 24 clinical symptoms were reported in the medical records retrieved. The frequency of these symptoms at presentation is shown in Figure 5. Multiple symptoms at the time of admission were generally reported. In this cohort, gastrointestinal (GIT) complaints (nausea, vomiting, diarrhoea and anorexia) were commonly reported (66.2%). Symptoms highlighted in black indicate the symptoms listed in the WHO clinical description of dengue fever\cite{14}. Four of these symptoms, arthralgia, retro-orbital (R/O) pain, rash and haemorrhage, were recorded in <50% of cases.
In 34 patients (21.7%), haemorrhagic manifestations (HM) other than petechiae were reported. The reported haemorrhagic symptoms and their frequency of occurrence are illustrated in Figure 6. More than one form of bleeding was noted in some patients. In keeping with the high frequency of gastrointestinal complaints reported, GIT bleeding including bloody stool and rectal bleeding occurred in 50% of the patients.

Six cases (approximately 4% of the 157 cases) could be defined as of DHF based on the WHO criteria for the diagnosis of DHF/DSS [14]. Of these, five were female; five were South Asians and one was mixed; four were children and one case (29-year old, South Asian female) died.
Figure 6. **Type and frequency of haemorrhagic symptoms among 34 dengue confirmed patients who presented with haemorrhagic manifestations. Symptom highlighted in black is part of the WHO clinical description for DHF**

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bloodstained sputum</td>
<td>2.9%</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>2.9%</td>
</tr>
<tr>
<td>Heavy menstrual flow</td>
<td>5.9%</td>
</tr>
<tr>
<td>Not specified</td>
<td>5.9%</td>
</tr>
<tr>
<td>Nasal bleeding</td>
<td>11.8%</td>
</tr>
<tr>
<td>Urinary tract bleeding</td>
<td>14.7%</td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>29.4%</td>
</tr>
<tr>
<td>Gingival bleeding</td>
<td>29.4%</td>
</tr>
<tr>
<td>Gastrointestinal bleeding</td>
<td>29.4%</td>
</tr>
</tbody>
</table>

**Discussion**

We reviewed the clinical and demographic features of 157 dengue-confirmed patients from a DEN-1 epidemic in Trinidad. This epidemic followed the re-introduction of this serotype into the island in 1991 after an absence of 14 years and the first documentation of DHF in the island in 1993. Approximately 1,200 samples from suspected symptomatic patients were sent for laboratory confirmation and, of these, 393 (approx. 33%) proved to be positive. Clinical and demographic data reported here are from 40% of the confirmed cases from that outbreak. The outbreak was island-wide with the estimated incidence rates being the highest in rural communities, though most cases occurred in the most densely populated county of St. George. There was a slight predominance of females and the infection occurred in all age groups. The estimated incidence rates were generally higher in patients of South Asian ethnicity. Gastrointestinal symptoms were the most common clinical manifestations at presentation. Haemorrhagic manifestations, when present, also commonly involved GIT.

Six patients met the clinical and laboratory criteria for DHF and, of these, one died.

However, the 157 (40%) available records closely reflected the distribution of patients from public and private hospitals, private doctors and health centres seen for the 258 names that were traced to their respective sample sources. In the sample of 258 patients, public hospitals accounted for
71%, private hospitals 16.7%, and health centres and general practitioners 6.2% each. In the sample of 157 patients for whom medical records were obtained, public hospitals accounted for 67%, private hospitals 17.2%, health centres 6.4% and general practitioners 10%. Over 80% of the patients were seen in hospitals.

The majority of cases (46%) in the sample lived in the most densely populated county in Trinidad, St. George's where 36.5% of the total population of Trinidad resides and where the Capital, Port-of-Spain (POS), and its environs are located. Dengue, as a mosquito-borne infection, would be expected to spread rapidly in populous areas, so the presence of a large proportion of patients in and around POS is consistent with the experience elsewhere of dengue infection as an urban phenomenon. Interestingly, our estimates of county-specific dengue incidence (based on the sample of 157 patients) yielded rates that did not differ significantly between rural and urban counties in Trinidad. This suggests that despite the variation in population density, DEN-1 infection was fairly evenly spread across the island. The island of Trinidad is merely 4,828 km² and the similarity of the incidence rates across the island may reflect a combination of a DEN-1 naïve population and easy accessibility to all parts of the island.

The DEN-1 1996 epidemic occurred in the rainy season with most admissions taking place in July and August, 1-2 months after the peak rainfall for that year in June. Rainfall is considered to be a risk factor for the development of dengue outbreaks, especially where there is improper storage of water or the presence of receptacles in the domestic environment such as discarded plastic containers in which water can collect. The main mosquito vector for dengue viruses in the Caribbean and Latin America is Aedes aegypti, which breeds primarily in relatively clean, stagnant domestic water containers. Local research has shown that important breeding sites for Aedes in Trinidad and Tobago include outdoor drums, water tanks, tyres and small discarded bottles and cans, which get filled easily with stagnant fresh water during the rainy season. The lag between the peak rainfall and the number of confirmed dengue cases might reflect the period of the mosquito-vector population expansion.

All age groups were affected but over 63% of them were adults (>15 years, the cut off age for paediatric patients in Trinidad). Since DEN-1 had been reintroduced in Trinidad in 1991, it is expected that children aged 5 years or less would constitute a susceptible population. Infection in this group would be primary in which the majority would be asymptomatic. This may account for children aged 0-4 years comprising only 8% of the sample. Despite the 1992/93 DEN-1 outbreak in which over 3,060 cases were reported, there were still many adults in 1996 who were susceptible to DEN-1. In total, there were 3,588 reported cases that year. However, based on the CAREC experience, only 33% of the clinically suspected dengue cases at that time were actually confirmed by laboratory tests, suggesting thereby that even during that outbreak, many cases with clinical features consistent with dengue may in fact not have been of dengue infection. Clinical symptoms of dengue are known to overlap with many other infections such as measles, hepatitis A, rubella and leptospirosis.
The high proportion of cases in the 30-34, 5-9 and 10-14-year age groups suggests the possibility of spread between children and parents. In Trinidad and Tobago, 25% of the heads of households are 30-39 years of age with an average of 1.99 children per household\(^{[13]}\). Trinidadian women often start their families in late teenage or early twenties, so parents aged 30-34 years will have children in the age groups 5-9 or 10-14 years.

The racial composition of this sample differs from that reported for the general population, which is composed of South Asians (40.3%), Africans (39.6%), mixed (18.4%) and the rest (1.6\%)\(^{[13]}\). Thus, there is an overrepresentation of South Asians (50%) in our sample with fewer persons of African origin (35%) and of mixed race (11%). Since hospitalized patients account for 84% of the study population, it may reflect an increased number of South Asians being hospitalized for dengue infection as compared with other racial groups. Teelucksingh\(^{[20]}\) has previously reported a higher incidence of more severe dengue infection and mortality in South Asian people (colloquially referred to as East Indians) in Trinidad.

These data cannot exclude differences in the environmental factors that might also increase the exposure of South Asian people to more mosquito bites. South Asians have traditionally been the predominant racial group in agriculture and animal rearing in Trinidad. In rural communities on the island there is relatively poor piped water supply, resulting in more water storage, thus providing ideal domestic breeding sites for Aedes aegypti\(^{[16]}\). Therefore, farmers in rural areas of Trinidad may be at an increased risk of mosquito bites and consequent dengue infection. The tendency towards an increased incidence in South Asians is interesting and merits further analysis with larger cohorts of dengue-confirmed cases.

The range of symptoms reported in the 157 Trinidadian dengue-confirmed patients is similar to those described for patients with acute dengue in other parts of the world\(^{[18]}\). Recent-onset fever and generalized body pains occurred in almost all patients. Headache, anorexia and myalgia, which often occur in the prodrome, were noted in 60% or more of the cases. Gastrointestinal and upper respiratory symptoms were also common complaints at presentation. Gastrointestinal symptoms have been described as predominant clinical manifestations in epidemics in which there is an adult-susceptible population\(^{[21]}\). The 1996 epidemic was almost wholly due to DEN-1 and the bulk of the study population comprised older children and adults.

The symptoms of classic dengue as described by WHO occurred in fewer than expected numbers of patients. For example, retro-orbital pain and arthralgia were reported in 32% of patients and rash only noted in 27%. While this may be due to a true low prevalence of these symptoms in the study population, the cultural expression of symptomatology may also contribute to a falsely low detection of these symptoms. For example, in Trinidad, all pains in the head region, including orbital pain, are commonly referred to as “headache”. Similarly, joint pains may often be included in “muscle” pain or be part of a more generalized “body pains”. The rash, which is usually transient, may be easily missed and even more so in dark-skin complexions.

Haemorrhagic manifestations, including petechiae, were noted in 51 (32.5\%) patients. This is consistent with other reports
of the prevalence of haemorrhage in dengue fever[18]. In keeping with the global experience, the bleeding manifestations occurred at many sites. Despite the bleeding manifestations in almost a third of the patients, the WHO criteria for DHF were fulfilled only by 6 (4%) patients[14]. Of these, five were female and one was male, five were South Asian and one was of mixed ethnicity and four were children and two were adults. One patient, a 29-year-old female of South Asian ethnicity died. The female gender and children were more prone to the development of DHF/DSS in dengue-endemic areas[3]. While the 1996 epidemic was predominantly DEN-1, DEN-2 was already endemic in the island[12]. Further, the predominance of persons of South Asian ancestry in this group was also consistent with the observation that certain races were more susceptible to DHF/DSS than others.

The number of cases of DHF/DSS may have been underestimated. One of the essential WHO criteria for establishing the diagnosis of DHF/DSS is the proof of increased vascular leakage such as an increase of at least 20% in average haematocrit for age and sex; a drop in haematocrit of 20% or more following treatment, or clinical evidence of the same such as pleural effusion, ascites or hypoproteinaemia[14]. However, since the plasma leakage can be transient and only evident by laboratory testing in milder forms of DHF cases (e.g. Grade 1 and 2 DHF), it is possible to miss patients with these forms of DHF if clinical monitoring of the haematocrit levels or tests such as lateral decubitus chest X-rays to detect small pleural effusions are not performed. The frequency of chest Xrays and serial blood tests in this cohort of patients varied with the hospitals that were visited. For example, chest Xrays were performed in fewer than 10% of the patients admitted to private hospitals but in over 60% of the patients in one public hospital. Serial blood tests were performed in approximately 20% of dengue cases in private hospitals but in 40-80% of patients admitted to three public institutions[22].

Furthermore, there are no locally developed reference ranges for haematocrit by age and sex in use in many laboratories in Trinidad where the reference ranges developed in primarily the Caucasian populations have been adopted for use. Moreover, haemoglobinopathies such as thalassaemia and sickle cell traits are not uncommon among the Trinidadian population and these persons tend to have normal haemtocrit levels below the reference ranges in use. Consequently, such patients presenting with 20% or more increase in haemconcentration may fall within the adopted reference range, and if there is no repeat of haematocrit concentration in the convalescent period, evidence of haemoconcentration would have been completely missed and the patient wrongly diagnosed as DF instead of a mild form of DHF[22].

In summary, the DEN-1 epidemic in Trinidad in 1996 was characterized by a large number of clinically suspected but unconfirmed cases of dengue infection. Laboratory analysis of a third of all reported cases revealed that most of these cases were not of dengue. Furthermore, the variability in the data available from clinical records, wherever these could be found, resulted in less than optimal information retrieval for further analysis. From the available data set, the Trinidadian dengue-infected patients were mainly older children and adults from
rural and urban communities. South Asians were predominant. A wide range of clinical manifestations was recorded, but the most common were gastrointestinal. Bleeding manifestations occurred in over 30% of DF cases but features of DHF were noted in only 4%. Improvement in clinical diagnosis and record-keeping is urgently required to underpin future clinical research in Trinidad.

References


Ecological Study of Rio de Janeiro City
dEN-3 Epidemic, 2001-2002

Maria Lucia F. Penna*

Escola Nacional de Saude Publica, Fundação Oswaldo Cruz, Rua Leopoldo Bulhões 1480, DENSP, 21041-210 Rio de Janeiro, RJ, Brazil

Abstract
Dengue virus serotype 3 was introduced in the Rio de Janeiro metropolitan area in January 2001 which produced a large epidemic during 2001 and 2002. This study looks into the relationship between the urban socioeconomic organization and the dengue attack rate during the epidemic in Rio. This study uses secondary data published in the data website of the city administration, including variables related to sanitation, use of the city area, tax collection, population density, education, income and life expectancy. The model includes as predictors the proportion of households with a well, the proportion of the available area in the city used for commerce and services in general, the proportion of city taxes collected from industries, the mean per capita income and the residential area per inhabitant, all with a statistical significant level of less than 0.05. The model explains 71% of the attack rate variance. The variables included in the model indicated that dengue distribution in the city was related to people’s socioeconomic status and the city organization. Variables that correlate with the movement of people across towns have emerged as the most significant.

Keywords: Dengue virus, socioeconomic status, urban organization, Rio de Janeiro.

Introduction
After decades of freedom from dengue virus infection, Brazil experienced an outbreak of DEN-1 and DEN-4 in areas close to the borders with Venezuela in 1981-82. Intensive vector control measures successfully controlled this outbreak and checked its spread to other areas in the country[1]. In 1986, DEN-1 was introduced in the Rio de Janeiro metropolitan area, which spread to other areas, causing a massive epidemic that later covered the entire country. DEN-2 and DEN-3 were also introduced in the country in 1990 and 2001, respectively[2]. Once introduced in the Rio de Janeiro metropolitan area, DEN-3 caused a large epidemic during 2001 and 2002. In 2003, DEN-1, DEN-2 and DEN-3 were in circulation in all except the two southern states of the country, with 341,092 cases reported (Figure 1)[3].

...
since the 1960s have resulted in overcrowded cities with multiple deficiencies, particularly in housing and basic sanitation. The strategy that resulted in the eradication of Aedes aegypti in the 70s is no longer applicable to the reality of the social, demographic, economic and political situation in South American countries[4-6].

Figure. Circulation of dengue virus in the Brazilian states, 2003

Predicting the risk of dengue correctly based on sociocultural factors has been the goal of many authors[7,8], but the dynamics of dengue transmission in urban settings are still poorly understood[9]. The understanding of the virus transmission dynamics requires a theoretical framework that includes individuals, households and behavioural risk factors as well as the administrative aspects of city management services, use of city space and movement of people across the metropolis. This understanding can help control measures to be more effective with responsibilities divided between the government and communities as per priorities established on the basis of reliable data.

The present study looked into the relationship between the urban socioeconomic organization and the dengue attack rate during the DEN-3 epidemic in Rio de Janeiro city in 2001-2002. It is mainly an exploratory study, based on available secondary data, that is likely to
throw some light on how urban settings contribute to dengue transmission.

**Methods**

**Description of study area**
Rio de Janeiro city is located at -22°54'23" south latitude and -43°10'21" west longitude, at the seashore, with an urban area of 1,255 km², including inland and continental waters. The municipal area was divided into 26 administrative regions (ARs) in 1999 which were used as spatial units for analysis. The climate is tropical, hot and humid, with local variations due to altitude differences, vegetation and proximity to the sea. The mean annual temperature is 22 °C, with high daily means in summer (30 to 32 °C). Rainfall is 1,200 to 1,800 mm per year, concentrated in summer from December to March. The city has the lowest rate of population growth among the Brazilian capital cities, 6.9% between 1991 and 2000.

**Database**
This study used secondary data published in the website of the city administration[10], including the proportion of households belonging to the sewage collection system (SEWAGE), the proportion of households belonging to the water supply system (WATER), the proportion of households with a well (WELL), the proportion of households with waste collection by the city authority (WASTE), the number of inhabitants per household (INH/HOUSE), the area of residential properties (square metres) per inhabitant (M²/INH), the proportion of households in slums (SLUMS), the proportion of the total area built for residence (HOUSE), for industry (INDUSTRY), for commerce or service (COMMERCE); the proportion of unused areas (UNUSED); the proportion of the total area of parks and squares (PARKS); the amount of city tax collected per inhabitant (TAX); the proportion of city tax of commercial origin (COMTAX), industrial origin (INDTAX) and service origin (SERVTAX); the mean per capita income (INCOME); the life expectancy (LE); the proportion of literacy among inhabitants (LITERACY); and the proportion of children aged 7 to 15 years attending school (SCHOOL) as independent variables. All proportions were presented as percentages and income and taxes as 1,000 reais (Brazilian currency). The independent variable was the attack rate for DEN-3 epidemic, calculated by the number of reported dengue cases during 2001-2002 divided by the population estimate for January 2001, per 100,000 inhabitants.

**Statistical methods**
A multiple regression model was adjusted to the data using stepwise forward approach, with F to enter = 1 and F to leave = 0.95, and the author interference to limit the number of steps in order to prevent a saturated model. The independent variable was given a log transformation. The software used was Statistica, from Statsoft[11].

**Results**
Table 1 shows the correlation coefficients between the independent variable and all those variables presented in the model, along with their range. The model included as predictors the proportion of households
with a well, the proportion of the available area used for commerce and services, the proportion of city taxes collected from industries, the mean per capita income and the built household area per inhabitant (Table 2), all with a statistical significant level less than 0.05. The model explains 71% of the attack rate variance. Table 3 shows the partial correlation coefficients for the variables included in the model, which is a measure of the association of each variable when the other variables are controlled for, making it possible to rank their effect. The residues fitted well into a normal distribution and presented no correlation with the predictors.

Table 1. Descriptive statistics and correlation coefficient with log (attack rate)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WASTE</td>
<td>1</td>
<td>0.93</td>
<td>1</td>
<td>0.09</td>
</tr>
<tr>
<td>SWAGE</td>
<td>1</td>
<td>0.30</td>
<td>1</td>
<td>0.09</td>
</tr>
<tr>
<td>WATER</td>
<td>1</td>
<td>0.88</td>
<td>1</td>
<td>-0.30</td>
</tr>
<tr>
<td>WELL</td>
<td>0</td>
<td>0.00</td>
<td>0</td>
<td>0.39</td>
</tr>
<tr>
<td>M²/INH</td>
<td>24</td>
<td>5.56</td>
<td>56</td>
<td>0.20</td>
</tr>
<tr>
<td>SLAM</td>
<td>15</td>
<td>0.00</td>
<td>41</td>
<td>0.04</td>
</tr>
<tr>
<td>INH/HOUSE</td>
<td>3</td>
<td>2.36</td>
<td>4</td>
<td>-0.05</td>
</tr>
<tr>
<td>LE</td>
<td>72</td>
<td>65.99</td>
<td>78</td>
<td>-0.01</td>
</tr>
<tr>
<td>LITERACY</td>
<td>96</td>
<td>90.74</td>
<td>99</td>
<td>-0.38</td>
</tr>
<tr>
<td>SCHOOL</td>
<td>90</td>
<td>67.66</td>
<td>113</td>
<td>-0.36</td>
</tr>
<tr>
<td>INCOME</td>
<td>670</td>
<td>212.21</td>
<td>2229</td>
<td>-0.13</td>
</tr>
<tr>
<td>HOUSE</td>
<td>69</td>
<td>9.89</td>
<td>89</td>
<td>-0.26</td>
</tr>
<tr>
<td>COMMERCE</td>
<td>12</td>
<td>3.40</td>
<td>39</td>
<td>0.27</td>
</tr>
<tr>
<td>INDUSTRY</td>
<td>7</td>
<td>0.11</td>
<td>21</td>
<td>0.01</td>
</tr>
<tr>
<td>UNUSED</td>
<td>981</td>
<td>0.99</td>
<td>19729</td>
<td>0.02</td>
</tr>
<tr>
<td>PARKS</td>
<td>2</td>
<td>0.00</td>
<td>25</td>
<td>-0.02</td>
</tr>
<tr>
<td>COMTAX</td>
<td>0</td>
<td>0.00</td>
<td>3</td>
<td>-0.20</td>
</tr>
<tr>
<td>INDTAX</td>
<td>0</td>
<td>0.00</td>
<td>1</td>
<td>0.30</td>
</tr>
<tr>
<td>SERVTAX</td>
<td>99</td>
<td>94.30</td>
<td>100</td>
<td>0.03</td>
</tr>
<tr>
<td>TAX</td>
<td>396</td>
<td>2.78</td>
<td>7157</td>
<td>0.19</td>
</tr>
</tbody>
</table>
Table 2. Regression summary
(Standard regression coefficient, regression coefficient, t and P value)

<table>
<thead>
<tr>
<th></th>
<th>Beta</th>
<th>Standard error of Beta</th>
<th>B</th>
<th>Standard error of B</th>
<th>t (20)</th>
<th>P level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>6.360585</td>
<td>0.184526</td>
<td>6.40582</td>
<td>0.184526</td>
<td>34.46979</td>
<td>0.000000</td>
</tr>
<tr>
<td>WHELL</td>
<td>0.33653</td>
<td>0.145309</td>
<td>0.33653</td>
<td>0.145309</td>
<td>2.31596</td>
<td>0.013194</td>
</tr>
<tr>
<td>COMMERCE</td>
<td>0.34087</td>
<td>0.136198</td>
<td>0.34087</td>
<td>0.136198</td>
<td>3.299001</td>
<td>0.009638</td>
</tr>
<tr>
<td>INDTAX</td>
<td>0.46826</td>
<td>0.126483</td>
<td>0.46826</td>
<td>0.126483</td>
<td>3.70211</td>
<td>0.000141</td>
</tr>
<tr>
<td>INCOME</td>
<td>-1.06086</td>
<td>0.273221</td>
<td>-1.06086</td>
<td>0.273221</td>
<td>-3.88279</td>
<td>0.000925</td>
</tr>
<tr>
<td>M²/INH</td>
<td>1.12201</td>
<td>0.294558</td>
<td>0.12201</td>
<td>0.294558</td>
<td>3.80912</td>
<td>0.001099</td>
</tr>
</tbody>
</table>

R = 0.84282066; R² = 0.71034667; F(5,20) = 9.8096; P < 0.00007; Standard error of estimate = 0.35169

Table 3. Partial and semi-partial correlation

<table>
<thead>
<tr>
<th></th>
<th>Beta in</th>
<th>Partial correlation</th>
<th>Semi-partial correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>WELL</td>
<td>0.33653</td>
<td>0.459859</td>
<td>0.278711</td>
</tr>
<tr>
<td>COMMERCE</td>
<td>0.34087</td>
<td>0.488354</td>
<td>0.301187</td>
</tr>
<tr>
<td>INDTAX</td>
<td>0.46826</td>
<td>0.637673</td>
<td>0.445527</td>
</tr>
<tr>
<td>INCOME</td>
<td>-1.06086</td>
<td>-0.655600</td>
<td>-0.467271</td>
</tr>
<tr>
<td>M²/INH</td>
<td>1.12201</td>
<td>0.648419</td>
<td>0.458405</td>
</tr>
</tbody>
</table>

Discussion

The exploratory aspect of this study justifies the presentation of a high number of urban and socioeconomic variables in the model. These variables present high covariance that induces the use of the forward stepwise method. To avoid a saturated model, the author restrained the number of steps and assured that only significant variables were kept in the model by using a relatively high value of F to leave. The rupture of the homocedasticy assumption due to the nature of data (proportions) was successfully dealt with by the logarithmic transformation of the attack rate[12], as shown by the residual analysis.

The proportion of households with a well is really a proxy variable of the discontinuity of water supply. The proportion of households that have access to the city’s water supply network is 95.07% for the entire city, indicating a high coverage of the city’s water supply network. But the water supply is not equally effective in all the administrative regions (ARs), with some areas having chronic problems, mainly discontinuous supply. The presence of a
well, although small, only in 1.12% of the households (maximum of 11.85% in Paqueta AR and 11.06% in Guaratiba AR) is a proxy variable for the discontinuity problem, for it is a solution that involves financial expenses which is only justified in the face of an important and lasting problem of supply. These results point to the fact that water supply is an important issue in dengue control, but only in the context of lack of or irregular water supply where the population is forced to resort to storage, which creates breeding sites for the vector and not in the usual context of adequate supply as suggested by other authors[13].

The model included mean per capita income as a protective factor meaning that low socioeconomic status of residents of an AR is a risk factor for dengue transmission. A study in Brazil found no association between the socioeconomic status and dengue risk at the individual level[14] and suggested that the previous finding of such an association at the aggregate level[15,16] was a fallacy. However, it should be noted that studies that focus on factors at individual level are insufficient to address the ecological links in the causal chain. Ecological studies may be, as pointed out by Koopman and Languini[17], the only way to study risk factors for infectious diseases. The risk of dengue virus infection is not dependent on the physiological characteristics of individuals, but on the environmental characteristics of the area where the group of individuals live. This includes other individuals, the natural environment and the way it is transformed by humans. The mean per capita income has to be interpreted as an environmental measure concerning the area and not as an aggregate measure of individuals, because it impacts the neighbouring households as well as the nearby public areas, thus affecting the presence of potential breeding sites for the vector. This discussion reinforces the relevance of multi-level analysis in the evaluation of dengue risk factors.

The inclusion in the model of the proportion of the area used for commerce and services and the proportion of city taxes paid by industry shows that urban organization plays an important role in the distribution of dengue. These two variables are proxy variables for the movement of people across ARs. The presence of commerce and general services was represented by the proportion of the area and the presence of industry as the proportion of city taxes, because those are the variables correlated to the intensity of the movement of people. The luxury commerce and services may collect more taxes than the popular commerce and services, but the areas of popular commerce and services have a much bigger flow of people. On the other hand, the relative size of the industrial area is related to the type of industry, as for instance big industrial storage areas, and not to the production or the number of workers. Industries that collect more taxes have a larger production and are more likely to have a higher number of workers. Other authors[18] indicate that the probability of being reached by a new dengue virus is correlated to the intensity of communication among people and the density of traffic and the road network. It is important to note that the diffusion of the epidemic by proximity may be less important than the diffusion caused by the circulation of people in central areas in big cities, which is supported by the present finding. This area initially imports infected individuals from the initial foci of the virus where it is newly introduced, resulting in higher local transmission, which, in turn,
results in exportation of infected individuals to other areas, reinforcing the epidemic all over the city. This fact clearly indicates the priority of mosquito control in areas with high levels of population mobility, such as the Rio de Janeiro city central area.

The model also included the area of residential property per inhabitant as a risk factor for dengue. This variable is closely correlated with the socioeconomic status represented by the mean per capita income \((R=0.864605)\) that is controlled in the model, meaning that, for the same socioeconomic status, a bigger area of residential property per inhabitant results in bigger dengue risk. This is possibly due to the existence of empty spaces in properties, such as backyards, thus creating greater opportunities for the existence of the vector breeding sites. This hypothesis has to be investigated for its importance in the selection of priorities for vector control in private residences as well as in defining the contents of educational interventions.

The results of the present study allow the establishment of priorities in vector control and educational interventions, highlighting the importance of the flow of people across cities and its significance in dengue epidemics.

**Conclusion**

The current efforts to control dengue demand a more comprehensive approach, including health education, community participation, garbage disposal and proper urban planning, besides chemical and biological mosquito control measures. In order to improve the efficiency of control efforts, these activities have to concentrate on areas and populations at higher risk, which implies early identification of higher incidence periods and areas and their characteristics\(^6\).

In Rio de Janeiro, emphasis has been placed on bringing about behavioural changes in the communities to make an impact on the determinants and risk factors of dengue through educational interventions. Health authorities and the press attributed the main responsibility of the problem to public behaviour, not clearly distinguishing between the responsibilities of the government and that of the private citizen\(^6\).

**References**


Sero-epidemiological and Virological Investigation of Dengue Infection in Oaxaca, Mexico, during 2000-2001

A. Cisneros-Solano*, M.M.B. Moreno-Altamirano**, U. Martínez-Soriano***, F. Jimenez-Rojas†, A. Díaz-Badillo‡ and M.L. Muñoz†

*Escuela Nacional de Medicina y Homeopatía
**Escuela Nacional de Ciencias Biológicas-Instituto Politécnico Nacional
***Universidad Autónoma “Benito Juárez” de Oaxaca
†Lab. Est. Salud Pública de Oaxaca
‡Centro de Investigación y Estudios Avanzados-IPN

Abstract

A sero-epidemiological-cum-virological investigation was carried out in Oaxaca, Mexico, during 2000-2001 to assess the incidence of dengue infection and the circulating viruses.

A total of 200 serum samples reportedly from dengue patients, based on clinical diagnosis, were collected from Oaxaca’s Central Laboratory of Public Health (in the capital city of the state of Oaxaca). The samples were initially collected from ten regional health centres located across Oaxaca. The sample population for the study included both sexes and age groups with clinical signs compatible with dengue infection. All samples were tested for the presence of dengue virus, mainly by MAC-ELISA and RT-PCR.

Ninety-four out of 100 serum samples suspected of dengue were confirmed to be positive. Thirty-two were found positive by MAC-ELISA and 58 were positive by RT-PCR. In addition, the RT-PCR analysis showed that the prevalent serotype in the localities in the study area was DEN-2. However, one isolate of DEN-1 and another of DEN-4 were also detected. The number of infected females was higher than that of infected males and the most affected age group was of people aged under 35 years. The study also highlighted that the sensitivity and specificity of diagnostic tools were crucial for epidemiological studies.

Keywords: Serodiagnosis, MAC-ELISA, RT-PCR, DEN-2, Oaxaca, Mexico.

Introduction

In recent years, dengue fever (DF) / dengue haemorrhagic fever (DHF) has emerged as major health problem in Mexico. In 1960, the Aedes aegypti mosquito was eradicated but it reappeared in 1965[1,2]. As pointed out by Gubler[3], factors such as demographic and social changes are responsible for the re-emergence of dengue. Mexico is considered an endemic country for dengue and it is reported that major epidemics of DEN-1 occurred on the eastern coast of Mexico during 1979-1980. In 1984-1985,
Dengue was diagnosed in 25 of the 32 states of Mexico. By then, DEN-1, DEN-2 and DEN-4 were present in the country, and in 1995, DEN-3 was circulating as well. Several cases of DHF were also confirmed\[4\]. In subsequent years dengue achieved endemicity in the country.

As per the records of the Mexican Health Office\[5\] (Secretaría de Salud, SS), a higher number of dengue cases were recorded in the states of Nuevo León, Tamaulipas, Veracruz and Oaxaca during 1998-2001.

Oaxaca is located in the subtropical region of Mexico at about 1,600 metres above sea level (Figure 1A). There is high demographic pressure and migration to different urban zones is common, resulting in the establishment of scattered human settlements with deficient public services. All these factors contributed to the propagation of the Aedes aegypti mosquito, resulting in dengue outbreaks every year in most of Oaxaca’s communities\[6\].

Figure 1. O axaca, Mexico
(A) United States of México. O axaca is located in the west coast
(B) O axaca is divided by the health authorities in six jurisdictions (I-VI)
To assess the dengue situation, epidemiological studies were undertaken to make an estimate of the incidence of dengue virus infection and the circulating serotypes in some selected endemic areas of Oaxaca during 2000-2001.

Materials and methods

Population study

The state of Oaxaca is located on the west coast of Mexico. The Mexican Health Office has divided it into six jurisdictions: (I) Central Valleys, (II) Tehuantepec isthmus, (III) Tuxtepec, (IV) The Coast, (V) The Mixteca, and (VI) The Sierra. The presence of dengue virus has been registered in all six jurisdictions (Figure 1B). This study was carried out in ten municipalities distributed in five jurisdictions in the state of Oaxaca. The study population included both sexes and all age groups. Two population groups were included in this study: one group consisting of 200 serum specimens from patients manifesting signs and symptoms of dengue infection, and another group of 50 serum samples from healthy controls, all from the same jurisdictions.

Sample collection and diagnosis of dengue

Human sera were obtained from 200 patients presenting clinical manifestations of dengue and tested for anti-dengue IgM antibodies. Serum samples were collected by venipuncture, using Vacutainer tubes (Becton-Dickinson). The clinical samples corresponded with dengue cases reported during 2000-2001. Dengue-infected samples were obtained during the first five days of the onset of fever and were processed for anti-dengue IgM detection using IgM capture ELISA (MAC-ELISA) as described by Vorndam et al. Samples from healthy donors were obtained at about the same time.

As a routine practice and with the idea of recording epidemic data, the suspected dengue samples already clinically diagnosed in community health centres were sent to the Central Laboratories in the city of Oaxaca (Laboratorio Estatal de Salud Pública del estado de Oaxaca, Secretaría de Salud). In this laboratory, the presence of dengue virus was confirmed by MAC-ELISA and RT-PCR.

Dengue virus isolates

Aedes albopictus C6/36 cells were grown in 48-well tissue culture plates as described by Igarashi. Briefly, 2x10^5 cells were plated in 1 ml of minimum essential medium (Gibco-BRL, Grand Island, N.Y.) supplemented with 7% fetal bovine serum (Sigma Chemical Co., St. Louis, Mo) and 1% glutamine, vitamins and nonessential amino acids. After 24 hours of culture, 100 µl of every sera diluted 1:10 was added to the corresponding well. The mixture was then gently shaken and incubated for 60 minutes at room temperature. Cells were then washed with serum-free medium and cultured at 28 °C with complete medium for at least 10 days. Cells were harvested for RT-PCR diagnosis.

RNA extraction

Total RNA was extracted either from 100 µl of serum or from cultured cells by using Trizol LS (GIBCO BRL, Gaithersburg, MD.) according to the manufacturers’
recommendations. Ethanol-precipitated RNA was recovered by centrifugation and air-dried. The RNA pellet was re-suspended in 50 µl of Diethyl-pyrocarbonate (Sigma)-treated water (DEPC water) and used as a template for RT-PCR.

**RT-PCR**

Synthetic oligonucleotide primer pairs were designed based on published sequence data for each of the four serotypes of dengue[10,11]. Four fragments of an expected size of 482 bp (DEN-1), 392 bp (DEN-4), 290 pb (DEN-3) and 119 bp (DEN-2) were obtained by using the SuperScrip™ One Step RT-PCR kit in conjunction with Platinum® Taq polymerase (Invitrogen, Life Technologies). A mixture of 5 µl of RNA, 25 µM of sense and anti-sense PCR primers, and DEPC water to a total volume of 50 µl was incubated at 85 °C for 5 minutes and then chilled on ice. The tubes-reaction mixture containing 2X PCR buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO₄ and Super Script™ RT/platinum® Taq Mix, as recommended by the manufacturer (Invitrogen, Life Technologies), was added to the RNA and primers-containing tube. The reverse transcription reaction was performed at 50 °C for 30 minutes. Thermocycling began with a hot start at 94 °C for 2 minutes followed by 40 cycles of annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute and denaturing at 94 °C for 15 seconds.

The PCR conditions for serotype assessment were as follows: 40 cycles of denaturing at 94 °C for 30 seconds, annealing at 55 °C for 1 minute, and extension at 72 °C for 1 minute and, a final extension at 72 °C for 7 minutes. The reaction mixtures were electrophoresed and visualised under UV light after ethidium bromide staining of the gels.

**Results**

**Diagnosis of the samples by MAC-ELISA**

Two hundred serum samples initially reported as suspected positive for dengue, based on clinical reports from the hospital where patients were hospitalised, were submitted for diagnosis based on anti-dengue IgM antibodies detection by MAC-ELISA. From these, only 34 samples were positive for IgM antibodies*. As expected, the 50 negative-control samples resulted negative for anti-dengue IgM antibodies (Table 1).

**Diagnosis by RT-PCR**

Once the serum samples were tested for anti-dengue IgM antibodies, the results were confirmed by RT-PCR. In this case, only 25 samples from healthy donors were tested. By this method 58 samples proved to be positive for dengue, i.e. 24 more than by MAC-ELISA. Interestingly, all samples positive for MAC-ELISA were also positive by RT-PCR. Those samples showing positivity for DEN by RT-PCR were further tested for the four serotypes (DEN-1, -2, -3 and -4). It was found that the main circulating serotype in Oaxaca during 2000-2001 was DEN-2. Two other serotypes (DEN-1 and DEN-4) were also found (only one case each) (Table 1).

* Out of 200 samples, originally sent, only 100 samples were found in good condition for evaluation by MAC-ELISA or RT-PCR. Other samples deteriorated under transportation/storage conditions.
Table 1. Positivity for dengue by MAC-ELISA and RT-PCR
From one hundred samples tested, from patients with clinical diagnosis of dengue, 36% proved positive by MAC-ELISA and 61% by RT-PCR. DEN-2 was the prevailing circulating serotype.

<table>
<thead>
<tr>
<th>Date of collection</th>
<th>Jurisdiction</th>
<th>Locality</th>
<th>Number of cases</th>
<th>MAC-ELISA</th>
<th>RT-PCR</th>
<th>Serotype</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nov 2000</td>
<td>II</td>
<td>Salina Cruz</td>
<td>18</td>
<td>13+/5-</td>
<td>9+/9-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Nov 2000</td>
<td>III</td>
<td>Tuxtepec</td>
<td>9</td>
<td>2+/7-</td>
<td>5+/4-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Nov 2000</td>
<td>II</td>
<td>Tehuantepec</td>
<td>10</td>
<td>4+/6-</td>
<td>5+/5-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Nov 2000</td>
<td>II</td>
<td>Juchitan</td>
<td>3</td>
<td>2+/1-</td>
<td>2+/1-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
</tbody>
</table>

**Total cases** 24+/24- 26+/22-

<table>
<thead>
<tr>
<th>Date of collection</th>
<th>Jurisdiction</th>
<th>Locality</th>
<th>Number of cases</th>
<th>MAC-ELISA</th>
<th>RT-PCR</th>
<th>Serotype</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun-Nov 2001</td>
<td>I</td>
<td>Oaxaca</td>
<td>7</td>
<td>2+/5-</td>
<td>7+/0-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Apr 2001</td>
<td>II</td>
<td>Juchitan</td>
<td>2</td>
<td>0+/2-</td>
<td>2+/0-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>May 2001</td>
<td>II</td>
<td>Salina Cruz</td>
<td>1</td>
<td>0+/1-</td>
<td>1+/0-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Feb 2001</td>
<td>III</td>
<td>Tuxtepec</td>
<td>7</td>
<td>2+/5-</td>
<td>2+/5-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Feb 2001</td>
<td>V</td>
<td>Tonalá</td>
<td>2</td>
<td>0+/2-</td>
<td>2+/0-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
<tr>
<td>Feb 2001</td>
<td>V</td>
<td>Huajuapan</td>
<td>6</td>
<td>0+/6-</td>
<td>6+/0-</td>
<td>DEN-2</td>
<td>DEN-2</td>
</tr>
</tbody>
</table>

**Total cases** 10+/36- 32+/14-

Table 2. Distribution of dengue cases by age and sex

<table>
<thead>
<tr>
<th>Age (in years)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2-4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>5-9</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>10-14</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>15-20</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>21-30</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>&gt;30</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>20 (38%)</td>
<td>33 (62%)</td>
</tr>
</tbody>
</table>

Prevalence of infection by age and sex
An analysis by age and sex revealed a higher prevalence (61%) of infection in females than in males (39%) and that the most affected group of people was the under-35-year-olds (Table 2).

Discussion
The Aedes aegypti mosquito’s adaptability to changing environmental conditions has contributed significantly to the increase in dengue epidemics in the world. Mexico is considered an endemic country where the...
four serotypes of the dengue virus are in circulation. This study provides some insight into the dengue epidemic situation in Oaxaca state, Mexico, in an attempt to contribute to the prevention and control of outbreaks of DF/DHF.

From the 200 serum samples collected from suspected dengue patients initially considered for the study, only 100 could be used. The remaining 100 samples were presumably subjected to non-appropriate storage conditions. From the 100 suspected samples tested, nearly 100% proved to be positive for dengue (36% by MAC-ELISA and 61% by RT-PCR). However, the data reported here could be an underestimation considering the several factors that could influence the laboratory determination outcome, such as sample handling and the diagnosis systems performed at local hospitals. In some localities of Oaxaca, the diagnosis for dengue was being simultaneously carried out with the diagnosis for rubella and toxoplasma in a monoclonal antibodies-based multiplex assay. In rural communities, however, only the presence of anti-dengue IgM antibodies was tested.

In this regard, it is possible that some patients presenting an early secondary infection in the absence of strong clinical manifestations had undetectable levels of anti-dengue IgM antibodies, since IgG is the prevalent Ig isotype at this stage of the infection. For these cases, it would be necessary to consider some other diagnosis techniques such as virus isolation or RT-PCR. Unfortunately, these are difficult to carry out in rural hospitals due to high costs and lack of suitably trained personnel.

Additional effort is needed to ensure appropriate sample collection, handling and storage in order to send them to the reference laboratory for adequate diagnosis. It is worth noting that in several Mexican states, health authorities are working on vector control as well as on facilities for sample collections to be sent to the Instituto de Referencia Epidemiológica (InDRE) in Mexico City for a proper diagnosis. It is still, however, a long way for good quality medical care to reach most Mexicans.

This report shows that by MAC-ELISA, 36% of the tested samples were found positive for dengue, whereas by RT-PCR up to 64% of the samples proved to be positive. Although the sensitivity and specificity reported for MAC-ELISA is reported to be good enough for a diagnosis system, it is likely that as a result of inadequate handling and storage conditions, some samples reported as negative could in fact be positive for dengue when tested by RT-PCR. No false positive results were found. This raises the question as to how many laboratory assays must be carried out on a suspected dengue sample before reporting it as negative.

The use of RT-PCR makes it possible to identify the dengue serotype involved; in this regard this study shows that in Oaxaca, Mexico, the prevalent serotype of dengue virus was DEN-2, although isolated cases of DEN-1 and DEN-4 infections were also found. Some other local reports had also mentioned the presence of DEN-3 and several cases of DHF.

Acknowledgements

We thank Dr. F. Javier Sánchez-García for critically reviewing the manuscript. M.M. B.M. is an EDI/IPN fellow.
References


Spatial and Temporal Dynamics of Dengue Haemorrhagic Fever Epidemics, Nakhon Pathom Province, Thailand, 1997-2001

Wutjanun Muttitanon*, Pongpan Kongthong**, Chusak Kongkanon**, Sutee Yoksan***, Narong Nitapattana***, Jean Paul Gonzalez† and Philippe Barbazan‡

*Asian Journal of Geoinformatics; Space Technology Application and Research Program, Asian Institute of Technology, P.O. Box 4, Klong Luang, Pathumthani, Thailand
**Department of Geography, Faculty of Education, Ramkhamhaeng University, Bangkok 10110, Thailand
***Center for Vaccine Development (CVD), Institute of Science and Technology for Research and Development, Mahidol University, Nakhon Pathom 73170, Thailand
†Research Center for Emerging Viral Diseases (RCEVD) – IRD – Center for Vaccine Development, Institute of Science and Technology for Research and Development, Mahidol University, Nakhon Pathom 73170, Thailand
‡Institut de Recherche pour le Développement (IRD) Ur034, 213 rue La Fayette, 75480, Paris cedex 10, France

Abstract
Several environmental factors modulate the distribution of dengue fever (DF), such as climate, density of vector and human populations in urban areas and distribution of herd immunity. In order to identify geographical variables involved in the spread of a DHF process, a Geographic Information System (GIS) has been built to create links between geo-referenced data including medical records and socioeconomic and environmental data. Applied to a retrospective analytical study of DHF epidemics in Nakhon Pathom province (1997-2001), the GIS allowed a mapping of spatial variations of DHF incidence, the recognition of different temporal incidence patterns and the quantification of the dispersal of outbreaks among defined spatial units. The analysis showed that the diffusion process of these epidemics was of a contagious type as the distance between epidemic areas (sub-districts) was significantly lower than the average distance between every sub-district. This result indicates that these epidemics were likely to be due to the spread of a new or rare virus serotype, from its emergence location in the province to areas with a sufficient density of vectors and a similar limited immune protection against this serotype.

Keywords: Dengue haemorrhagic fever, dengue virus, transmission, Geographic Information System, spatial analysis.

* E-mail: fnpbb@diamond.mahidol.ac.th Tel./Fax: (66) 2 441 01 89

Introduction

Dengue fever (DF) is a viral disease with a worldwide distribution in all tropical areas. It is caused by the dengue virus (genus Flavivirus, family Flaviviridae) which presents four antigenic forms or serotypes: DEN-1, DEN-2, DEN-3 and DEN-4. In Thailand, Dengue haemorrhagic fever (DHF) a severe form of dengue fever has been endemic since 1958, with a cumulative total of 1,369,542 cases till date[1]. Epidemics occur with a periodicity of between two and four years; these epidemics are of significant concern for the public health authorities. In most of the areas where serotype identifications were performed, two or three serotypes were found to be co-circulating[2].

The dengue virus is an arbovirus (arthropod-borne virus) transmitted by the mosquito Aedes aegypti (L.). Control of the spread of the disease focuses on vector control strategies based mainly on the elimination of potential breeding sites[3]. A major attribute of the virus transmission is its anthropophilic behaviour, as females mainly bite humans and lay eggs in man-made containers near houses (for example, water jars, cans, used tyres). The short flight range of the vector, less than 1 km, contributes to the limited spread of the disease by an infected female. Most of the infections by dengue viruses are not severe and present symptomatically, allowing infected patients to maintain normal activities.

Two types of viral spread can be described: (i) the diffusion of human infections as a function of the spatial distribution of houses and the limited flight range of infectious or infected Aedes aegypti females (intra-communal, contagious/continuous); and (ii) inter-communal dispersion, largely a function of the stochastic movement of incubating/infectious humans and the transport via vehicles of virus-positive females[4].

The understanding of the mechanism of the inter-community spread of DHF during epidemic periods is a primary factor likely to lead to an evaluation of the risk of virus transmission and disease dispersal[5]. Moreover, it would provide some guidance on the distance from the spatial origin of an epidemic at which preventive control measures should be applied.

At a monthly time-scale, the main geographical factors involved in dengue transmission (urbanization, demography, cultural and social characteristics) are stable[6]. A change in the pattern of monthly DHF transmission, such as the emergence of epidemics in an endemic area, should then rather be related to factors evolving with time: climate, density of vectors, emergence of a new or rare virus serotype, each type of factor inducing a specific pattern of diffusion of the disease[7,8]. The emergence of a new serotype in a given population is likely to exhibit particular spatial characteristics. The outbreak would begin where the serotype first arrived and then move to places where a low specific herd immunity (towards this serotype) and a sufficient density of mosquito allow a high level of transmission. The spread of a new serotype is then likely to follow the main model of contagious diffusion described for the spread of other types of moving phenomena[9]. Applied to the diffusion of an infectious disease, it means that the probability for an area to be reached by a contagious disease will be inversely correlated to the distance to the formerly contaminated areas, leading to clusters of epidemic areas.
In order to test the validity of this model in the frame of dengue dispersal, a study was conducted to describe the spread of significantly higher levels of incidence rate (of epidemic significance) among sub-districts. The study, done in a province of Thailand, covering the period 1997-2001, included two DHF epidemics.

Materials and methods

Data collection

Data on clinically diagnosed DHF cases were recorded at the Ministry of Public Health, the demographic data were provided by the Administrative Department of the Ministry of Interior, and the geographical maps by the Royal Thai Survey Department. DHF cases were defined according to WHO criteria[10].

Population and study area

Nakhon Pathom province is a part of the central plain region in Thailand encompassing the latitude of 13° 38'45.6“ N to 14° 10'37.2“ N and the longitude of 99° 51'10.8“ E to 100° 17'6“ E. It covers 2,164 sq km, has a population of 774,276 inhabitants and includes 7 districts and 106 sub-districts (Figure 1a). The population density ranges from 153 to 623 inhabitants/sq km. The average surface area of sub-districts is 20.4 sq. km. The provincial health department reported 14,079 DHF cases during 1983-2001; two DHF epidemics occurred in 1997-1998 and 2000-2001 (Figure 2).

Figure 1. District scale approach: (i) Administrative limits of districts and sub-districts; density of population; main roads; (ii) Average incidence observed before the epidemics (cases/100,000 people), January 1992 - June 1997; (iii) Ratio of the incidence during the first three months of the DHF epidemic compared to the average incidence from January 1992 - June 1997
Method of analysis

The study aimed to describe the spatial-temporal dynamics at a monthly time-scale of a DHF epidemic among Nakhon Pathom’s 106 sub-districts considered as the spatial units. As a first step, epidemics were defined at the province level as periods of time (at least two consecutive months) when the incidence is higher than the average, plus one standard deviation of the monthly incidence of each month (i.e. January, February, etc.). The average was calculated over the entire 1983-2001 period.[11]

During these epidemic months (EMs), epidemic sub-districts (ESDs) were those where the monthly incidence was significantly higher than in other sub-districts. The threshold for a significantly higher incidence was leveled at the average monthly incidence (per 100,000 inhabitants) plus one standard deviation, observed among every sub-district during that EM.

In a contagious model for an infectious disease, the spatial entities close to an infected one were assumed to be more at risk to become infected than the distant ones. Applied to the diffusion of an epidemic phenomenon, it meant that the distance between the new epidemic sub-districts and the former ones (observed distance) should be shorter than the average (expected) distance between all the sub-districts. The distance between sub-districts was defined as the Euclidian distance between their centroids. The expected distance was the average distance between each ESD and every other sub-district. The observed distance was the average distance between each ESD and every other ESD, during the same month (cluster study), or from one month to the next (spread study).

\( H_0 \) (null hypothesis) = the average observed distance (between ESD) was not different from the average expected distances.

\( H_1 = \) average observed distance < average expected distance.
The Z test was used to compare the average distances.

The method was applied to the study of two phenomena: (i) the occurrence of clusters of ESD during one month; and (ii) the spread of the epidemic among sub-districts from one month to the next. A cluster is defined here as an aggregation of ESD (during one EM) of sufficient size and concentration to be unlikely to have occurred by chance, i.e. if the average distance (between these ESD) is shorter than the average distance between all sub-districts. The spread of the epidemic is based on the comparison of observed and expected distances during two consecutive EMs, i.e. the average distance between ESD during one epidemic month (EM_m) and ESD during the next epidemic month (EM_{m+1}), versus the average distance between ESD during EM_m and every sub-district during EM_{m+1}.

The (discrete) distance, at which an epidemic can spread in one month, was estimated by summing the number of ESD centroids during EM_{m+1} observed inside circles centred on each ESD during EM_m and drowned at 5 km; 10 km; 15 km; 20 km and out of 20 km. This number is compared to the number of sub-districts centroids distributed in these surfaces to build a relative risk index.

Relative risk index = \frac{\text{number of ESD in a circle}}{\text{total number of ESD}} \times \frac{\text{number of sub-districts in a circle}}{\text{total number of sub-districts}}

Results

At the district scale, the DHF incidence was higher in the central-west part of the province. The epidemic broke out in the northern district with a medium density of population (Figures 1b and 1c). At the sub-district scale, the maximum DHF incidence rate reached 540 cases per 100,000 inhabitants in July 1997.

Nineteen EMs were identified in Nakhon Pathom province from January 1997 to August 2001 (Table); the number of EMs in one sub-district ranged from 0 month (in 27 sub-districts) to a maximum of 11 months.

Table. Chronological distribution of epidemic months from January 1997 to August 2001 in Nakhon Pathom province, Thailand

<table>
<thead>
<tr>
<th>Year</th>
<th>Jan</th>
<th>Feb</th>
<th>Mar</th>
<th>Apr</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>Oct</th>
<th>Nov</th>
<th>Dec</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1998</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[\text{= Epidemic month}\]
A total of 49 ESDs were identified during the first outbreak (1997-1998) and 61 during the second outbreak (2000-2001); 31 sub-districts were epidemic-affected during the two outbreaks. The probability of one sub-district being epidemic-affected during the first outbreak to be epidemic-affected during the second outbreak as well is not significantly different from a random distribution ($P < 0.05$).

Cluster study: During EM, 78.67% of the average observed distances were significantly lower ($P < 0.05$) than the average expected distance, characterizing the occurrence of clusters of ESDs according to the $H_1$ hypothesis.

Spread study: The distance observed between ESDs during EM$_m$ and ESDs during EM$_{m+1}$ was significantly smaller than the expected distance (Figure 3), characterizing the contagious spread of DHF among ESDs according to the $H_1$ hypothesis.

As a consequence, the distribution of ESDs during EM$_{m+1}$ in surfaces drowned round ESD during EM$_m$ showed a significant ($P < 0.05$) aggregation within the first two circles (5 and 10 km).

**Discussion**

The method used for the identification of an epidemic month in the province[11] allowed a precise framing of epidemics, defined as periods during which the incidence was...
significantly higher than the observed average over the complete time series of data (19 years). Meanwhile, this method could not be used directly at the sub-district scale because of the lack of long-time data series on the incidence at this scale. Moreover, the variance of the DHF incidence in most of the sub-districts was very high because of the low values of incidence often recorded (during the study period a null monthly incidence was reported in 66% of the 5,936 months X sub-districts). Similarly, the village scale (from 3 to 24 villages per sub-district) could not be used as the spatial unit as the addresses of patients were often consistent only at the sub-district scale and many students and pupils did not live in their village.

We assumed in this study that sub-districts could be considered as homogeneous small areas and that human displacements were sufficient to produce a homogenization of the population, allowing consideration of the sub-district as a unit towards DHF transmission. An ‘epidemic’ pattern can then be identified in any sub-district, whatever its density of population: the distribution of ESD was not correlated to the density of population (Pearson’s correlation = -0.24, P = 0.71). Moreover, we used the incidence rate per 100,000 inhabitants to reduce the bias related to the size of the population.

The geographical heterogeneity of the environment, e.g. the density of urbanization or the road network, could also be at the origin of clusters of ESDs. Meanwhile, after the two epidemics, ESDs were found to be uniformly distributed over the entire province, and the spatial distribution of all sub-districts having been epidemic-affected at least during one month (67% of the sub-districts) was not significantly different from the spatial distribution of all sub-districts (average distances not different, P= 0.95). Meanwhile, the results implied a high degree of spatial auto-correlation, meaning that neighbouring sub-districts shared similar characteristics, such as the level of immunity for the different serotypes (due to a similar epidemiological history) or the density of the vector.

As shown in Figure 3, the observed distances are smaller than the expected ones, but exhibit similar monthly variations. This was mainly because of a border effect, the propagation in sub-districts located in neighbouring provinces not being taken into account. During the months where ESDs were located on the periphery of the province, the average distance to other sub-districts was larger than during the months where ESDs were located near the centre of the province, as several neighbouring sub-districts located in other provinces (epidemic or not) were ‘missing’ in the calculation. The absolute level of expected and observed distances was then directly dependent on the location of the ESD in the province.

The spread of the epidemic between sub-districts followed Hagerstrand’s model that has been used to describe many types of phenomena, such as the spread of new ideas[9] or the waves of innovation which lose their ‘energy’ when the distance from the source increases[12]. In public health research it has been applied to infectious influenza[13]. Applied to the DHF epidemic

in Nakhon Pathom, it means that during the epidemic periods the ESDs were the origin of the emergence of epidemics in neighbouring sub-districts during the next month. The probability of this emergence at \( m+1 \) significantly decreased with the distance from the former ESD. This model is of a contagious type and may be opposed to a random or homogeneous model. In the homogeneous models the occurrence of an epidemic could be due to a global phenomenon, such as an increase in temperature, which should have been observed in any sub-district, leading to a random distribution of ESD\([14]\) and an observed distance not different from the expected distance.

Inside human communities (villages) it has been shown that the spread of DHF viruses from one house to neighbouring houses due to the displacement of infected vectors or hosts follows a pattern similar to what we have described between sub-districts\([15]\). Meanwhile, among communities separated by several kilometers, the spread of viruses cannot be due to the active dispersal of mosquitoes or to their transport by car, which is much more rare than the displacement of infected hosts. More than 80% of infections by dengue virus are unapparent or not severe, allowing healthy carriers to travel. The presence of sufficient densities of vectors in destination communities is also necessary to allow the transmission of the virus after it has been imported.

The contagious distribution and spread of the two DHF epidemics among the sub-districts strongly suggests that they were due to the emergence of a new or rare serotype. DHF is endemic in Thailand and the different serotypes are largely distributed, as at least two or three serotypes are generally found at the same time in the same area\([2,16]\). Meanwhile, during epidemic periods the relative prevalence of the serotypes varies, as the 2000 epidemic in Bangkok that was due mainly to the serotypes DEN-1 and DEN-2 (each reaching 42% of total isolations), whereas the 1994 epidemic was due mainly to the rise in DEN-4 (36%). But as serology and isolation of viruses are rarely performed, the emergence of a DHF epidemic cannot be forecast by using these methods. Indirect methods, such as the statistical identification of epidemic months, are then necessary to identify early the emergence of DHF epidemics.

The epidemiology of DHF in Thailand is changing\([17]\). This approach of the displacement of epidemics is likely to contribute to the localization of the origins of outbreaks and the delineation of areas at risk during epidemics, as well as to help public health authorities to focus vector control activities on selected areas.

**Acknowledgements**

The study and the preparation of this paper was supported by the Institut de Recherche pour le Développement (IRD)-Ur034, France, by a fellowship to the Center for Vaccine Development, Institute of Science and Technology for Research and Development, Mahidol University, Thailand, and by the Department for Technical and Economic Cooperation, Thailand. We thank Professor Nath Bramapavarati for his constant support.
Spatial and Temporal Dynamics of DHF Epidemics in Thailand

References


Sporadic Prevalence of DF/DHF in the Nilgiri and Cardamom Hills of Western Ghats in South India: Is it a Seeding from Sylvatic Dengue Cycle - A Hypothesis

Nand Lal Kalra* and Chusak Prasittisuk**

* A-38, Swasthaya Vihar, Vikas Marg, Delhi - 110 092
** Regional Office for South-East Asia, World Health Organization, New Delhi, India

Abstract

The Western Ghats of south India, encompassing the Nilgiri and Cardamom hills, are the wettest region of the country. Hills rising up to 3,000 metres receive over 200 cm of rainfall from both the south-west monsoon (June to September) and north-eastern monsoon (October to January). The eastern slopes of the Nilgiri Hills (200-500 metres) are bounded by Coimbatore and Erode districts of Tamil Nadu, whereas the western slopes of the Nilgiri and Cardamom hills are in the state of Kerala. The countryside has rich forests of teak and sandalwood, interspersed by groves of coconut, rubber, pepper, cardamom and banana plantations. Apart from the rich flora, monkeys (*Macaca radiata*) maintain a strong association in orchards with humans competing for food.

The emergence of DF/DHF in this hilly region is a recent occurrence. An epidemiological team from the National Institute of Communicable Diseases (NICD) investigated the first-ever reported outbreak in Coimbatore in 1998. In all, 20 serological positive (IgM) cases were recorded by the city corporation. Five cases came from urban towns and 15 cases from rural areas of the two districts of Coimbatore and Erode. Rural cases were scattered in distantly located villages. Pyramid characterization and clustering of cases was conspicuously absent. No attempt was made to link urban cases to central/peripheral wards, nor the history of movement of patients two weeks prior to the onset of fever was investigated. No increase in fever rate was observed. DF cases did not show any relationship with presence/absence of *Aedes* breeding. *Aedes aegypti* detected in urban centres failed to amplify the infection.

Kerala state also reported 116 cases in 1997, from Kottayam district. Out of these, 14 cases were confirmed serologically. After a lull of 4 years, 70 probable cases out of 877 were reported from the four districts famous for rubber plantations. Entomological investigations recorded only *Aedes albopictus* in these areas. Considering the high experimental susceptibility of both species of monkeys, viz. *Macaca mulatta* and *Macaca radiata* to yellow fever virus, detection of dengue antigen in field collected *Aedes albopictus* in Kozhikode (Kerala) and the evidence of transovarian transmission in *Aedes albopictus* reared from soils of tree holes at Jodhpur - Rajasthan (western India) lend support to the hypothesis that DF in the Western Ghats of South India exists as an enzootic monkey - *Aedes albopictus* - monkey cycle and causes epizootics among rural human population either during periodic amplification of the enzootic cycle or as occupational hazards to the people working in orchards.

Keywords: DF/DHF, sporadic cases, *Macaca radiata*, dengue antigen in *Aedes albopictus*, transovarial transmission, sylvatic cycle, Nilgiri and Cardamom hills, south India.

E-mail: chusakp@whosea.org
Introduction

The Western Ghats of south India encompass two southern states i.e. Tamil Nadu and Kerala (Figure). The crests of the Nilgiri and Cardamom Hills (rising up to 3,000 metres altitude) separate these two states. The eastern slopes of the Nilgiri Hills have a gentle slope and include two important district towns of Tamil Nadu, viz. Erode and Coimbatore, situated at an altitude varying between 200 to 500 metres; each with over one million population. This part of the region receives rains from both the south-west and north-east monsoon. The south-west monsoons become weak, being on the leeward side of the hills, but maximum rains come from the north-east monsoon, the total being <100 cm. The forests are tropical monsoon, which are famous for teak and sandalwood, with patches of arecanut palms[1].

The western side of the Cardamon hills encompasses the state of Kerala. Strong winds of the south-west monsoon lead to the formation of heavy sand dunes in coastal areas and the rain water coming from the steep hills results in formation of shallow lagoons, all along the coast, at places connected to the sea. These lagoons are connected by canals. These backwaters are the characteristics of Kerala state. The banks of these backwaters and sand dunes are dotted with coconut trees[1].

The Region receives heavy rains (>200 cm) during the south-west monsoon. Hence, the whole region is very wet and supports luxuriant growth. Large tracts of forests have been cleared for raising cash crops, viz. arecanut palms, rubber, banana, pepper and cardamom plantations[1].

Dengue transmission cycles

Transmission of dengue viruses occurs in two cycles, viz. enzootic and epidemic cycles. The enzootic cycle is a primitive sylvatic cycle maintained by lower primates (monkeys) and canopy dwelling Aedes mosquitoes, as reported from South-Asia[2], Africa[3] and Sri
Lanka[4]. Current epidemiological evidence suggests that these viruses do not regularly move out of the forests to urban centres but at times are involved in an epidemic cycle in small rural villages or islands[3]. A number of Aedes species may act as reservoirs[2].

The epidemic cycle is confined to large urban centres. The viruses are maintained in the Aedes aegypti – human – Aedes aegypti cycles with periodic/cyclic epidemics. Generally all serotypes circulate and give rise to hyperendemicity. Virus is maintained either transovarially by the vectors or by continuous low-grade transmission in susceptible hosts added to the population. DF/DHF in urban cycles is characterized by ‘iceberg’ or ‘pyramid’ phenomenon. At the base most of the cases are symptom-less, followed in increasing rarity, by undifferentiated fever, DF, DHF or DSS[5]. Occurrence of multiple cases in a single household or clustering of cases in a locality is yet another characteristic of this disease[2].

DF/DHF in India

In recent years the first outbreak of DF/DHF was reported from Kolkata (earlier known as Calcutta) in 1963[6]. Since then, more than 60 outbreaks have been reported from all over the country[7]. Aedes aegypti, invariably has been found to be associated with these epidemics. All the four serotypes, DEN-1, 2, 3 and 4, are now circulating in the country.

DF/DHF in Nilgiri/Cardamom hilly areas of south India

Since 1996-97, there have been reports of sporadic occurrence of DF/DHF cases in Tamil Nadu and Kerala.

DF in hilly regions of Tamil Nadu

During 1998, a team from the National Institute of Communicable Diseases (NICD), Delhi, investigated the first-ever reported outbreak in Coimbatore[8], a town situated at an altitude varying from 300 to 500 metres on the eastern slopes of the Nilgiri Hills. The epidemiological characteristics of the outbreak are summed up below:

• In all, 20 serologically positive and compatible to DF/DHF cases were reported. Eighty percent (16/20) were children below 10 years and two patients aged 16 and 5 died of DHF.
• Fourteen cases were males.
• Seventeen cases came from Coimbatore district and three cases from rural areas of adjoining Erode district.
• Only five cases came from urban Coimbatore town and the rest (12 cases) were from rural areas of Coimbatore district.
• Rural cases were scattered in distantly located villages. Clustering of cases and pyramid phenomenon was conspicuously absent. No attempt was made to link urban cases to central or peripheral wards/zones nor the movement history of patients two weeks prior to the onset of fever was investigated.
• No relationship could be established between outbreak and increased fever rate.
• Eighty-nine percent of blood samples from healthy contact persons from urban and rural areas showed dengue virus IgG antibodies.
• Entomological investigation recorded *Aedes aegypti* in all areas surveyed in urban and rural areas, but failed to amplify the infection.

• During the 2003 outbreak in Coimbatore town 23 cases of DF were recorded. Distribution once again followed the same pattern, i.e. 5 cases from urban towns and 18 cases from rural areas (Source: VBDC, New Delhi)

## DF in Kerala

### Epidemiological data

As per investigations undertaken by the Centre for Research in Medical Entomology (CRME)[9], the state of Kerala started reporting DF for the first time in 1997. Distribution of DF cases are included in Table 1.

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of suspected cases</th>
<th>No. serologically positive</th>
<th>Deaths</th>
<th>Districts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>116</td>
<td>14</td>
<td>4</td>
<td>Kottayam</td>
</tr>
<tr>
<td>1998</td>
<td>67</td>
<td>0</td>
<td>13</td>
<td>Kottayam</td>
</tr>
<tr>
<td>1999</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>Kottayam</td>
</tr>
<tr>
<td>2000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Kottayam</td>
</tr>
<tr>
<td>2001</td>
<td>877</td>
<td>70</td>
<td>1</td>
<td>4 districts*</td>
</tr>
</tbody>
</table>

* Four districts included Kottayam, Idukki, Ernakulam and Thiruvananthapuram—famous for rubber plantations

Table 2: Results of *Aedes* survey of 4 districts of Kerala state during 2001-2003

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of district</th>
<th>House index</th>
<th>Container index</th>
<th>Breteau index</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. ex.</td>
<td>No. + ve</td>
<td>%</td>
<td>No. ex.</td>
</tr>
<tr>
<td>1.</td>
<td>Alappuzha</td>
<td>150</td>
<td>40</td>
<td>26.7</td>
<td>201</td>
</tr>
<tr>
<td>2.</td>
<td>Ernakulam</td>
<td>24</td>
<td>10</td>
<td>41.7</td>
<td>54</td>
</tr>
<tr>
<td>3.</td>
<td>Kottayam</td>
<td>70</td>
<td>24</td>
<td>34.2</td>
<td>125</td>
</tr>
<tr>
<td>4.</td>
<td>Kozhikode</td>
<td>3,311</td>
<td>895</td>
<td>26.23</td>
<td>17,912</td>
</tr>
</tbody>
</table>

*Pool rearing of larval breeding collected from all localities indicated *Aedes albopictus* as the major species. *Aedes aegypti* was encountered in very few places and in scanty numbers (Source: NICD, Delhi, 2004)
**Entomological data**

Entomological investigation initiated by a CRME team in 2 localities of Kottayam district yielded only Aedes albopictus, and Aedes aegypti was not detected. Sylvan environment of rubber plantations was detected as the unique habitat of the Aedes albopictus\(^{[10,11]}\).

Results of yet another entomological study carried out by the National Institute of Communicable Diseases (NICD) field station located at Kozhikode, in DF affected districts during 2001-2003, are included in Table 2.

**Antigen detection of dengue virus**

During May 2004, a pool of landing collection of Aedes albopictus (dessicated), collected from the fringe of forested villages, 600 metres away from Kozhikode (earlier known as Calicut) International Airport, yielded dengue antigen (processed at CRME, Madurai)\(^{[12]}\).

**Vertical transmission by Aedes albopictus**

A recent study has been carried out at the Desert Medicine Research Centre (DMRC), Jodhpur (Rajasthan), an institution under the Indian Council of Medical Research, on possible existence of Aedes albopictus - monkey - Aedes albopictus cycle. The highlights of the study included that:

- In a desert ecosystem, both Aedes aegypti and Aedes albopictus breed in tree holes in zoo and monumental parks, harbouring monkeys, outside the city limits.
- Viable eggs retrieved from the soil of tree holes were reared to adults. Aedes albopictus, when subjected to IFA test, showed the presence of dengue antigen, thereby confirming the transovarial cycle of the virus\(^{[13]}\).

**Hypothesis**

Occurrence of DF cases in peripheral and rural areas of Coimbatore and Erode districts in Tamil Nadu and non-amplification of infection by Aedes aegypti and sporadic occurrence of DF cases in Kerala in the absence of Aedes aegypti points out to either spillover of enzootic foci of dengue during periodic amplification of the sylvatic cycles or occupational hazards in the presence of vertical transmission as evidenced by the Kozhikode and Jodhpur studies.

- Both the Nilgiri and Cardamom hills are infested with Macaca radiata, the bonnet monkeys. Enzootic cycle of simian malaria caused by Plasmodium cynomolgi and Plasmodium inui, transmitted by Anopheles elegans (Anopheles dirus group)\(^{[14,15]}\), has been detected in the Nilgiri hills. Whereas in Kerala, a similar simian foci has been detected at Nilambur district of the western slope in Macaca radiata, while in Alappuzha (district in the central plains, earlier known as Alleppey) monkeys were found negative for lack of Anopheles elegans population\(^{[16]}\).
- Both the monkey species, viz. Macaca mulatta and Macaca radiata have been found to be highly susceptible to yellow fever virus (flaviviruses) under experimental conditions\(^{[17]}\). This lends support to the susceptibility of these monkeys to dengue virus as well.
Lack of vectorial competence of *Aedes albopictus* in the amplification of urban dengue epidemic has recently been demonstrated during the investigation of the first-ever DF outbreak at Phuentsholing, Bhutan in 2004\(^{(18)}\). Entomological investigations revealed that *Aedes aegypti* occupied domestic habitats breeding primarily in storage containers inside houses, while *Aedes albopictus* bred in tree holes, 55-gallon drums and used tyres in the extra-domestic habitats. The overlapping zone was the peridomestic areas where both species shared breeding in flower vases/trash. A large-scale source reduction/larvicidal campaign supported by deltamethrin fogging in residential areas largely eliminated *Aedes aegypti* and the cases came down to single digits within a month, while *Aedes albopictus* still maintained high larval indices.

Rudnik and Lim\(^{(19)}\), while working in Malaysia, isolated DEN-1, 2 and 3 viruses from monkeys and also proposed that the rural dengue vector - *Aedes albopictus*, may introduce sylvatic virus into the human population.

Studies in Sri Lanka proved that dengue virus causes epizootics among macaques, rather than being enzootic as observed elsewhere\(^{(4)}\).

Gubler\(^{(20)}\), proposed that at some point in the past, probably with the clearing of the forests and development of human settlements, dengue viruses moved out of the jungles and into a rural environment where they were, and still are transmitted to humans by peri-domestic mosquitoes such as *Aedes albopictus*.

In view of the aforesaid, the land use in the Nilgiri hills of Tamil Nadu and in the Cardamom hills in Kerala is under pressure of deforestation to be replaced with cash crops. This has brought monkey populations much closer to human settlements. Therefore, there is a need for indepth sero-epidemiological and entomological studies with backup of virology support using molecular tools for genomic sequencing of viruses obtained from simian and human sources. Validation of the hypothesis is of great epidemiological significance as it would require radical changes in developing vector control strategies for *Aedes albopictus*-transmitted DF.

**Acknowledgements**

The author gratefully acknowledges Dr. Duane J. Gubler for critically reviewing the manuscript.

**References**

A Suspected Sylcatic Cycle in the Nilgiri and Cardamom Hills in South India


Autoimmunity in Dengue Virus Infection

Chiou-Feng Lin*, Huan-Yao Lei*, Ching-Chuan Liu**, Hsiao-Sheng Liu*, Trai-Ming Yeh***, Shun-Hua Chen* and Yee-Shin Lin*

*Department of Microbiology and Immunology, National Cheng Kung University Medical College, Tainan, Taiwan
**Department of Paediatrics, National Cheng Kung University Medical College, Tainan, Taiwan
***Department of Medical Technology, National Cheng Kung University Medical College, Tainan, Taiwan

Abstract

Dengue haemorrhagic fever (DHF) is a complicated disease associated with viral and immune pathogenesis. There is still no effective vaccine to prevent the progression of DHF because of its undefined pathogenic mechanisms. The generation of autoimmunity in dengue virus (DEN) infection has been implicated in dengue pathogenesis. Based on our previous studies showing antibodies (Abs) against DEN nonstructural protein 1 (NS1) cross-reacted with human platelets and endothelial cells, a mechanism of molecular mimicry may contribute to autoantibody (autoAb) production. Here, the generation of autoAbs against human endothelial cells in patients infected with different DEN serotypes is shown. The levels of autoAbs present in different disease stages of DHF and the induction of endothelial cell apoptosis by patient sera were also determined. The results suggest that autoimmune responses are implicated in dengue disease pathogenesis and cause concern in vaccine development.

Keywords: Dengue haemorrhagic fever, dengue virus serotype, autoimmunity, autoantibody, endothelial cells.

Introduction

Infection with dengue virus (DEN) causes dengue fever (DF) - an important arthropod-borne viral disease in terms of morbidity and mortality[1,2] and may result in severe dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS). Globally, about 2.5 billion people are at risk of the infection[3]. A recent dengue outbreak in Indonesia led to a 1.1% case-fatality rate in 58,301 cases by April 2004[4]. All four DEN serotypes were present in this outbreak.

Presently, the severity of dengue disease is primarily predicted according to the effect of antibody-dependent enhancement (ADE) in different serotype cross-infections[2,5,6]. In order to effectively control the progression of the disease, development of an effective vaccine against DEN infection is needed. There are several vaccine candidates undergoing clinical trials[7-10]. Nevertheless, the role that antibodies (Abs) may play in increasing the severity of dengue infections[2,6,10] remains a matter of concern in vaccine development.

* E-mail: yslin1@mail.ncku.edu.tw
In addition to the ADE of DEN infection, autoantibody (autoAb) production may also be involved in dengue disease[11-15]. We demonstrated that the autoAbs generated in DEN infection induced endothelial cell damage[13] and inflammatory activation (in press). A mechanism of molecular mimicry in which Abs directed against DEN nonstructural protein 1 (NS1) is, at least in part, responsible for the autoimmunity. The relationships of the autoAb levels with dengue serotypes and disease severity are examined in this study.

Materials and methods

Patient sera
DEN-2 and DEN-3 patient sera were collected during the outbreaks in southern Taiwan from 1997 to January 1999[16]. DEN-4 patient sera were obtained from the Department of Dengue Hemorrhagic Fever, Children’s Hospital No. 1, Ho Chi Minh City, Viet Nam. The disease severity was based on the WHO definition[3]. Normal control sera from five healthy individuals were used as background.

Cell cultures
Human umbilical cord vein endothelial cells (HUVEC) were cultured in modified M-199 medium as described previously[13]. For experiments, 1,000 U/ml trypsin and 0.5 mM ethylenediaminetetraacetic acid (EDTA) were used to detach cells.

Binding activity detection
After detachment, cells were suspended at 5×10^5 for flow cytometry. The cells were washed briefly with phosphate-buffered saline (PBS) and fixed with 1% formaldehyde in PBS at room temperature for 10 minutes, then washed again with PBS. Patient sera were 1:25 diluted and incubated with cells at 4°C for 1 hour. After being washed three times with PBS, the cells were incubated with 20 µl of fluorescein isothiocyanate (FITC)-conjugated anti-human IgG or IgM (PharMingen, San Diego, CA) at 4°C for 1 hour. The binding activity of Abs to cells was analysed using flow cytometry (FACScan; BD Biosciences, San Jose, CA) with excitation set at 488 nm.

Cell death detection
For cell viability determination, cells were stained with eosin Y and counted using light microscopy. Apoptosis-induced DNA strand breaks were analysed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) reaction using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA). After incubation with patient sera for 24 hours, endothelial cells (1×10^5) were fixed and stained according to the manufacturer’s instructions, and then analysed using flow cytometry.

Statistical analysis
The statistical difference was analysed using unpaired Student's t-tests in SigmaPlot version 4.0 for Windows (Cytel Software Corporation, Cambridge, MA).

Results

Generation of autoAbs in dengue patients infected with different serotypes and at different disease stages
Our previous studies demonstrated the presence of anti-platelet and anti-endothelial cell autoAbs in dengue patient sera[12,13]. The levels of these autoAbs were

---

1 deoxyuridine triphosphate
Autoimmunity in Dengue Virus Infection

higher in DHF/DSS than in DF patient sera. The dysfunction of platelets and endothelial cells caused by the autoAbs was also shown. The cross-reactivity of patient sera with endothelial cells was the highest in the acute stage (3-7 days after fever onset) and subsequently decreased in the convalescent (1-3 weeks after acute phase) and later (8-9 months) stages. In our previous study, patient sera were collected from an outbreak of DEN-3 infection. In this study, we further examined the autoAb levels produced by patients infected with different DEN serotypes and the relationship between the autoAb levels and disease severity. The results showed that the levels of anti-endothelial cell Abs, as determined by both the percentages of endothelial cells reactive with patient sera IgM or IgG and the mean fluorescence intensity, were similar in patients infected with DEN-2, 3 or 4 (Table 1). There was no significant difference between different serotype infections. The levels of autoAbs were higher in DHF/DSS than in DF patient sera. In addition, the levels of IgM isotype of autoAbs were higher than those of IgG. The DEN-1 serotype was not tested because we had no DEN-1-infected patient sera. We next investigated the endothelial cell cross-reactivity of DHF patient sera at different disease grades. DHF patient sera collected from Grades I to IV with DEN-4 infection, according to the WHO definition, were tested. There was no significant difference between the four grades of DHF in both anti-endothelial cell IgM and IgG (Table 1). Due to our limited sample sizes of patient sera, especially of Grades I and IV, we were unable to determine whether there was any correlation of autoAbs with disease severity.

Table 1. Anti-endothelial cell IgM/IgG levels in the sera of dengue patients infected with different dengue serotypes and at different disease grades

<table>
<thead>
<tr>
<th>% of endothelial cells reactive with patient sera</th>
<th>Mean fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM Mean (SD)</td>
</tr>
<tr>
<td>Normal (n=5)</td>
<td>4.7 (0.5) 2.6 (0.6)</td>
</tr>
<tr>
<td>DEN-2 infection</td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>38.6 (1.4)*** 14.8 (5.1)*</td>
</tr>
<tr>
<td>DEN-3 infection</td>
<td></td>
</tr>
<tr>
<td>DF (n=6)</td>
<td>35.1 (3.7)*** 12.7 (2.1)*</td>
</tr>
<tr>
<td>DHF/DSS (n=5)</td>
<td>54.6 (4.4)*** 23.7 (1.9)**</td>
</tr>
<tr>
<td>DEN-4 infection</td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>35.9 (1.6)*** 15.1 (3.5)*</td>
</tr>
<tr>
<td>DHF (n=36)</td>
<td>50.5 (9.5)*** 24.9 (8.3)**</td>
</tr>
<tr>
<td>Grade I (n=1)</td>
<td>66.9 17.8</td>
</tr>
<tr>
<td>Grade II (n=26)</td>
<td>51.7 (9.0)*** 25.5 (7.7)*</td>
</tr>
<tr>
<td>Grade III (n=8)</td>
<td>45.4 (8.6)*** 24.7 (10.6)*</td>
</tr>
<tr>
<td>Grade IV (n=1)</td>
<td>42.2 17.9</td>
</tr>
</tbody>
</table>

Student’s t-tests: *P<0.05 vs Normal; **P<0.01 vs Normal; ***P<0.001 vs Normal.
Induction of endothelial cell apoptosis by sera of dengue patients infected with different serotypes

Anti-endothelial cell autoAbs caused cell damage which was characterized by apoptosis\[13\]. The ability of patient sera with different DEN serotype infections to induce endothelial cell apoptosis was tested. HUVEC were treated with a 1:25 dilution of dengue patient or healthy-control sera for 24 hours, and cell apoptosis was measured using TUNEL reaction followed by flow cytometric analysis. The histogram and the percentages of apoptotic cells from one set of duplicate cultures are shown in the Figure below. The results indicated that cell apoptosis was induced by all patient sera and the cells underwent a higher percentage of apoptosis when induced by DHF patient sera than by DF patient sera. Healthy-control sera showed only the background level. Cell viability detected using eosin Y staining showed an inverse relationship with the percentages of apoptosis (Table 2). There was no significant difference in endothelial cell apoptosis induced by patient sera with different serotype infections.

Figure. Dengue patient sera induced endothelial cell apoptosis

![Graph showing apoptosis percentages for different DEN serotypes](image)
### Table 2. Endothelial cell apoptosis induced by sera of dengue patients infected with different dengue serotypes

<table>
<thead>
<tr>
<th></th>
<th>% of cell viability</th>
<th>% of endothelial cell apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n=5)</td>
<td>94.1 (3.5)</td>
<td>6.9 (2.8)</td>
</tr>
<tr>
<td><strong>DEN-2 infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>75.2 (5.4)**</td>
<td>21.9 (5.6)**</td>
</tr>
<tr>
<td><strong>DEN-3 infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=6)</td>
<td>81.7 (5.1)**</td>
<td>19.5 (7.1)**</td>
</tr>
<tr>
<td>DHF/DSS (n=5)</td>
<td>66.6 (10.8)**</td>
<td>29.7 (9.3)**</td>
</tr>
<tr>
<td><strong>DEN-4 infection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (n=5)</td>
<td>72.0 (9.5)**</td>
<td>22.0 (5.6)**</td>
</tr>
<tr>
<td>DHF (n=5)</td>
<td>63.3 (15.1)**</td>
<td>37.2 (5.5)**</td>
</tr>
</tbody>
</table>

Student’s t-tests: **P < 0.01 vs Normal; ***P < 0.001 vs Normal.

### Discussion

DHF is a life-threatening disease with poorly defined pathogenic mechanisms\[^{1,2,5,10}\]. An ADE effect in different serotype cross-infection is frequent\[^{2,5,6}\]. Patients may develop severe complications of progressive DHF. Vascular leakage and haemorrhagic diathesis are the hallmarks in DHF patients. A number of studies have demonstrated abnormal immune responses caused by DEN infection, including cytokine and chemokine production, complement activation and immune cell activation\[^{10,11,17-20}\]. In addition, autoimmune responses may be involved in DHF pathogenesis\[^{12-13,22}\]. Dengue patients produced Abs which cross-reacted with human platelets and endothelial cells\[^{12,13}\]. Anti-NS1 produced after DEN infection may, at least in part, account for the cross-reactivity of patient sera with endothelial cells. In this study, we further showed that the levels of anti-endothelial cell Abs were similar in patients infected with different DEN serotypes. The percentages of endothelial cells reactive with DHF/DSS patient sera were higher than those with DF patient sera. However, there was no difference in anti-endothelial cell Ab levels at different DHF disease grades. The sample size of patient sera needs to be increased to gain an insight into the role of anti-endothelial cell Abs in DHF pathogenesis. These autoAbs exerted similar effects in the induction of endothelial cell apoptosis of patients infected with different DEN serotypes.

In dengue pathology, various cytokines and chemokines including TNF-α, IL-6, IL-8, and RANTES\[^{1}\] have been detected in patient sera with DHF/DSS and in DEN-infected endothelial cell culture supernatants\[^{17,18,20}\]. Our recent studies also demonstrate that anti-NS1 Abs can stimulate cytokine and chemokine production (in press). Therefore,

[^1]: regulated upon activation normal T cell expressed and secreted
both immune activation and apoptosis occur in endothelial cells after stimulation by autoAbs.

There are no dengue vaccines available. Yet, several potential vaccines, including life-attenuated whole DEN and DNA vaccines, are undergoing clinical trials\(^7\)\(^{-10}\). It is hoped that a fusion or a chimera dengue vaccine will be developed to provide protection against all serotypes of DEN infection. In addition, DEN NS1 protein used as a vaccine candidate in mice showed resistance to fatal DEN encephalitis\(^{22}\). Passive administration of anti-NS1 Abs also conferred protection in mice when challenged with lethal doses of DEN\(^{23}\). However, these previous studies only monitored the survival rates of mice but did not examine the potential histopathological effects. Studies by Falconar\(^{21}\) showed the cross-reactivity of anti-NS1 to host antigens and cells and a haemorrhage-like hallmark in mice. This, taken together with our findings, suggests that a potential pathogenic effect of DEN NS1 vaccine should be taken into consideration. The possible approaches include gene modifications of DEN NS1 to truncate or mutate the epitopes that may cause the pathogenic effects.

**Acknowledgement**

We thank Dr N.T. Hung from the Department of Dengue Haemorrhagic Fever, Children’s Hospital No. 1, Ho Chi Minh City, Viet Nam for providing the DEN-4 patient sera. The authors acknowledge the editorial assistance of Bill Franke. This work was supported by grant NSC92-3112-B006-003 from the National Science Council, Taiwan.

**References**


Autoimmunity in Dengue Virus Infection


Inhibition of the NS2B-NS3 Protease - Towards a Causative Therapy for Dengue Virus Diseases

Gerd Katzenmeier

Laboratory of Molecular Virology, Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Phutthamonthon No. 4 Rd., Nakornpathom 73170, Thailand

Abstract

The high impact of diseases caused by dengue viruses on global health is now reflected in an increased interest in the identification of drug targets and the rationale-based development of antiviral inhibitors which are suitable for a causative treatment of severe forms of dengue virus infections – dengue haemorrhagic fever and dengue shock syndrome. A promising target for the design of specific inhibitors is the dengue virus NS3 serine protease which – in the complex with the small activator protein NS2B – catalyses processing of the viral polyprotein at a number of sites in the nonstructural region. The NS3 protease is an indispensable component of the viral replication machinery and inhibition of this protein offers the prospect of eventually preventing dengue viruses from replication and maturation. After nearly a decade of mainly genetic analysis of flaviviral replication, recent studies have contributed substantial biochemical information on polyprotein processing including the 3-dimensional structure of the dengue virus NS3 protease domain, the mechanism of co-factor-dependent activation and sensitive in vitro assays which are needed for studies on substrate specificity and the development of high-throughput assays for inhibitor screening. This review discusses recent biochemical findings which are relevant to the design of potential inhibitors directed against the dengue virus NS3 protease.

Keywords: Dengue virus, NS2B/NS3, polyprotein, protease, inhibitor, treatment.

Viral polyprotein processing

Dengue viruses, members of the Flaviviridae family, possess single-stranded, positive sense RNA genomes and generate mature viral proteins by co- and post-translational proteolytic processing of a polyprotein precursor catalysed by host cell and virus-encoded proteases [for review see refs. 1, 2 and references herein]. The genomic RNA of dengue virus serotype 2 contains 10,723 nucleotides and encodes a single polyprotein precursor of 3,391 amino acid residues[3]. Individual viral proteins are arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Proteolytic cleavages in the N-terminal region of the viral polyprotein are mediated by a host signal peptidase and yields three structural proteins C, prM and E, which constitute the virion[4]. Before the virion exits the cell, prM is cleaved by a cellular furin-type protease.
prohormone convertase in the post-Golgi acidic compartment to yield the M protein. Cleavages at the NS1/NS2A and NS4A/NS4B junctions are catalysed by a signalase bound to the membranes of the ER \cite{6,7}. Proteolytic cleavages in the nonstructural region of the polyprotein are mediated by a heterodimeric complex of NS3 with the activator protein NS2B which catalyses in cis (intramolecular) cleavages at the NS2A/NS2B and NS2B/NS3 sites and in trans (intermolecular) cleavages at the NS3/NS4A and NS4B/NS5 polyprotein junctions \cite{8-10}. Additional cleavages mediated by the NS3 protease within the C, NS2A, NS4A and within a conserved C-terminal portion of NS3 itself have been described in the literature \cite{11-13}.

Figure 1. The 3-dimensional structure of the dengue virus NS3 protease (shown here is a superimposition of a ribbon-presentation of the Cα trace on a space-filling surface model. Residues of the catalytic triad (His51, Asp75, Ser135) are shown as sticks. The figure was generated by Deepview Swiss-Pdb Viewer).

Cleavage sites recognized by the NS2B/NS3 protease consist of ‘dibasic’ residues Lys-Arg, Arg-Arg and Arg-Lys at the (nonprime) P1 and P2 positions of the cleavage site sequence followed by short chain residues such as Gly, Ala and Ser at the (prime) P1’ position. The “non-canonical” NS2B/NS3 site contains a Gln residue at the P2 position (Figure 1).

The NS3 protease domain

The existence of a trypsin-like serine protease domain in the N-terminal region of the flaviviral NS3 proteins was originally predicted by sequence comparisons between cellular and virus-encoded proteases \cite{14}. The NS2B-NS3 endopeptidases of the Flavivirus genus which at present comprises at least 68 known members, are now commonly designated as flavirin (EC 3.4.21.91) \cite{15,16}. The dengue virus 69 kDa NS3 protein is a multifunctional protein with a serine protease domain located within the N-terminal 167 amino acid residues \cite{17} and activities of a nucleoside triphosphatase (NTPase) and RNA helicase in the C-terminal moiety \cite{18}. A catalytic triad consisting of residues His51, Asp75 and Ser135 was identified by site-directed mutagenesis experiments and replacement of the catalytic serine by alanine resulted in an enzymatically inactive NS3 protein \cite{19}. The NS3 protease is an essential component for maturation of the virus and viable virus was never recovered from infectious cDNA clones carrying mutations in the NS3 sequence which abolished protease activity \cite{20}. Interaction of the helicase portion of NS3 with the viral RNA-dependent RNA polymerase NS5 may promote the association of the viral replicase complex to the membranes of the ER \cite{21}. 
The 3-dimensional structure of the N-terminal 185 residues of the dengue virus NS3 protease domain (NS3pro) was resolved at a resolution of 2.1 Å[22]. The overall folding of the protein resembles the 6-stranded β-barrel conformation typical for chymotrypsin-like serine proteases. Interestingly, the structure of the dengue virus NS3 protease is closer to that of the hepatitis C virus NS3/NS4A co-complex than to the unliganded HCV NS3 protease, an observation which is suggestive of major structural differences in the co-factor-dependent activation mechanism of the two proteases[23]. The substrate binding site of NS3pro is relatively shallow and contourless and specific enzyme-substrate interactions were not predicted to extend beyond the P2 and P2’ positions of the substrate peptide in the absence of the NS2B co-factor[22] (Figure 2).

The NS2B co-factor

The presence of a small activating protein or co-factor is a prerequisite for optimal activity of the flaviviral NS3 proteases with their natural polyprotein substrates. Although the dengue virus NS3 protease exhibits NS2B-independent activity with model substrates for serine proteases, enzymatic cleavage of dibasic peptides is markedly enhanced with the NS2B-NS3 co-complex and the presence of the NS2B activation sequence is indispensable for the cleavage of polyprotein substrates in vitro[24]. The initial characterization of the co-factor requirement for the dengue virus NS3 protease had revealed that the minimal region necessary for protease activation was located in a 40-residue hydrophilic segment of NS2B[23]. The hydrophobic flanking regions of the 14 kDa NS2B protein are likely to be involved in targeting the protease complex to the membranes of the ER where genome replication occurs. Fusion of the NS2B core sequence to the NS3 protease domain yielded a catalytically active NS2B( H)-NS3p protein, which, upon expression in E. coli and subsequent refolding, displayed autoproteolytic processing at the NS2B/NS3 site conducive to the formation of a non-covalent adduct[24]. Incorporation of a flexible nonamer linker, Gly₄-Ser-Gly₄, between the NS2B core
segment and the protease domain resulted in a cleavage-resistant protease with optimized enzymatic activity against hexapeptide substrates representing native polyprotein cleavage junctions\textsuperscript{[26]}. A recombinant construct representing the full-length NS2B co-factor linked to the NS3 protease domain was enzymatically active with peptide substrates derived from the polyprotein; however, this protein was completely resistant to proteolytic self-cleavage\textsuperscript{[27,28]}.

An essential requirement for the correct association of the co-factor with the protease is the presence of hydrophobic residues which act as ‘anchor’ for the protease - co-factor interaction. Recently, based on mutagenesis experiments and sequence comparisons of known flaviviral co-factors, the “Φ₃Φ” - motif was proposed as the common structural element involved in co-factor binding to the protease\textsuperscript{[29]}. The “Φ₃Φ” - motif is comprised of two bulky hydrophobic residues separated by three unspecified residues and it was hypothesized that additional residues located at the N-terminus of the activation sequence would contribute to the stringent specificity of the protease for the polyprotein substrate. A mutagenesis study with the dengue virus NS2B co-factor had revealed that substitutions of the “Φ” - residues (Leu75 and Ile79 in NS2B of dengue virus type 2) by alanine resulted in preponderant effects on the catalytic activity of the NS3 protease rather than on substrate binding [P. Niyomrattanakit, unpublished data]. A single residue in the N-terminal region of NS2B, Trp62, was critical for protease activation and replacement of this residue yielded a NS3 protease which was catalytically inactive in autoproteolysis and reaction with the synthetic substrate peptide GRR-AMC.

For the HCV NS3-NS4A protease complex, large structural rearrangements leading to a catalytic triad, which is conformationally optimized for proton shuttle during catalysis, were observed as a result of co-factor binding\textsuperscript{[30]}. No 3-dimensional structure is available for the dengue virus NS2B-NS3 co-complex and the precise mechanism of co-factor-dependent activation is not fully elucidated as yet. In particular, it is an open question as to whether binding of the substrate contributes to the formation of an ‘induced fit’ conformation as observed with the HCV NS3 protease\textsuperscript{[31,32]}.

**Substrate specificity**

So far, only very limited efforts have been undertaken to analyse the precise substrate requirements and determinants of cleavage efficiency for the dengue virus NS3 protease. This is surprising in the light of the fact that development of inhibitors against serine proteases usually starts with optimal peptide substrates derived from the nonprime side wherein the scissile amide bond is replaced by an electrophile which reacts with the catalytic serine residue.

The NS3 protease reacts with small model substrates for serine proteases such as N-α-benzoyl-L-arginine-p-nitroanilide (BAPA) and activity of the unliganded NS3 protease towards this substrate is higher than that of the NS2B-NS3 co-complex\textsuperscript{[24]}, a finding which suggests that substrate recognition in the complex requires additional interactions extending beyond the P1 side for optimal activity. A number of fluorogenic tripeptides containing dibasic residues at the P1 and P2
positions are cleaved by NS2B(H)-NS3pro protease and the best substrate identified in these experiments was GRR-AMC, which had a $K_m$ value of 180 $\mu$M, a $k_{cat}$ value of 0.031 s$^{-1}$ and a catalytic efficiency expressed as $k_{cat}/K_m$ of 172 s$^{-1}$ M$^{-1}$ [24].

Chromogenic p-nitroanilide peptides representing hexameric sequences of the native polyprotein cleavage junctions were tested in a photometric assay and the most efficiently cleaved substrate derived from the NS4B/NS5 site (Ac-TTSTRR-pNA) had a $K_m$ of 346 $\mu$M, $k_{cat}$ of 0.095 s$^{-1}$ and a $k_{cat}/K_m$ of 275 s$^{-1}$ M$^{-1}$ [26].

Recently, we have shown by a HPLC-based assay with fluorometric detection that the NS2B-NS3pro protease incorporating a full-length NS2B co-factor could cleave N-terminally dansylated 12mer peptides mimicking native polyprotein junctions in the absence of microsomal membranes[28]. However, this protein was completely inactive in autocleavage and the efficiency of this recombinant protease with the peptide substrate was markedly reduced when compared to constructs incorporating the 40-residue NS2B core sequence, likely due to structural distortions induced by the flanking regions of NS2B and inefficient activation of the protease.

Currently, the detailed study on substrate specificity of the dengue virus NS3 protease is in progress, which uses combinatorial libraries of internally quenched fluorogenic peptides labelled with the aminobenzoyl/p-nitrotyrosine reporter pair. For a substrate peptide based on the NS3/NS4A cleavage site, Abz-AAGRKR?SLTY(NO$_2$)R-NH$_2$ (? denotes the cleavage site), a $K_m$ value of 141 $\mu$M, a $k_{cat}$ of 0.18 s$^{-1}$ and a $k_{cat}/K_m$ of 1262 s$^{-1}$ M$^{-1}$ was found, which is approximately 10-fold better than the best commercially available substrate tested so far, GRR-AMC [J. Wikberg, unpublished data]. In the near future, these investigations will likely lead to the identification of NS3 substrates with optimized sequence length and improved binding affinities, which can be applied as sensitive probes for enzyme activity in high-throughput inhibitor screenings.

**Perspectives for inhibitor development**

Principally, every step of viral morphogenesis, from cell-entry, uncoating, replication and assembly of new virus particles, is a potential target for antiviral inhibitors. However, the molecular events in the infectious cycle of flaviviruses such as dengue virus are characterized only to a very limited extent, making the design of specific inhibitors an adventurous task. In contrast, proteases and their inhibitors have been intensively studied because of their potential for the development of selective antiviral compounds. Although tremendous progress in the field is indicated by the design and clinical use of antiviral drugs against HIV (AIDS) and hepatitis C virus proteases, the potential of dengue and related flaviviral proteases for inhibitor discovery is largely unexploited. In response to the global problem of the dengue virus epidemics, considerable efforts are now being devoted to the development of drugs which will eventually be suitable for a chemotherapeutic intervention in acute dengue diseases, not only by academic institutions but also by pharmaceutical companies (for example see http://www.nitd.novartis.com).
In a first step towards a rational inhibitor design for the dengue virus NS3 serine protease, inhibition by synthetic peptides mimicking uncleavable transition state isosteres of the P6-P2' residues of the native polyprotein sites, was demonstrated\cite{26}. The peptides with an α-keto amide in place of the scissile amide bond acted as competitive inhibitors of the NS3 protease with \( K_i \) values in the micromolar range. Replacement of the P1’-P2’ residues by a carboxyl-terminal aldehyde in the NS3/NS4A-derived peptide (Ac-FAAGRR-CHO) yielded a competitive inhibitor with a \( K_i \) of 16 \( \mu \)M. For the dengue virus protease, the hexapeptides displayed \( K_i \) values which were only 2- to 6-fold lower than the \( K_m \) value for the corresponding substrate, a feature which discriminates dengue virus NS3 from the HCV protease, where product inhibitors had binding affinities which were one order of magnitude lower than those for the substrates\cite{33,34}.

Product inhibition of the HCV NS3 protease by cleavage-site derived peptides led to the discovery of very potent inhibitors of this enzyme with IC\(_{50}\) - values in the nanomolar range by cyclic optimization of the inhibitor structures\cite{33,34}. Recently, we have shown that peptides representing non-prime-side residues of the dengue virus NS3 protease act as competitive inhibitors of the enzyme, whereas prime-side peptides appeared to have negligible effects on enzyme inhibition at concentrations >1.0 mM. (S. Chanprapaph, unpublished data). \( K_i \) - values for hexapeptides derived from all 4 dengue virus cleavage sites were in the low micromolar range and the best inhibitor was based on the NS2A/NS2B site (Ac-RTSKKR-CO\( \text{NH}_2 \)) and gave a \( K_i \) of 12 \( \mu \)M. In analogy to the HCV NS3 protease, these findings suggest the existence of a high-affinity binding site in the non-prime region of the enzyme and offer the prospect of developing effective inhibitors against the dengue virus protease by combinatorial optimization based on the structure(s) of native polyprotein cleavage site peptides. However, in general, peptide-based inhibitors exhibit poor pharmacokinetic properties and usually the conversion of these structures into less “peptide-like” compounds (“peptidomimetics”) is required to generate drug-like entities.

Inhibitor discovery for the dengue virus NS3 protease is currently limited by the lack of a 3-dimensional structure for the NS2B-NS3 co-complex; however, it can be expected that crystallographic studies and NMR-experiments will provide more insight into the structure and catalytic mechanism of the enzyme in the near future. In addition, powerful computer modelling approaches exist which may help to obtain information required for a rational drug design even in the absence of crystallographic structures.

Proteochemometric modelling (PCM) is currently explored as a tool to analyse the structural and physicochemical properties which are necessary for the interaction of potential inhibitors with the dengue virus NS3 protease target structure\cite{35}. Preliminary data obtained by this approach suggest that the proteochemometric models are valid and useful for the accelerated design of novel inhibitors. This approach does not only circumvent the traditional erratic drug discovery process with its high attrition rates, but also allows to incorporate potential resistance of the target and the development of ‘drug resistance - resistant’ compounds as an initial consideration in the design process. The existence of large conformational
Inhibition of NS2B/NS3 Protease

ensembles is particularly challenging in the case of rapidly mutating viral enzymes, where a design against a moveable target would require large sets of corresponding inhibitors. In the future, these problems are likely to be addressed by ‘shotgun approaches’ to the structure of enzyme-inhibitor complexes and the identification of hot spots of ‘interaction flexibility’ by the use of fast, high-resolution methods such as NMR. Detailed accounts on this strategy are given in Reference 36.

Conclusions

Substantial progress has been made over the past few years in the biochemical characterization of the dengue virus NS2B-NS3 two-component protease. The data, which are available now, make the dengue virus NS3 protease a valid molecular target for the development of antiviral compounds. A large repertoire of powerful methods for inhibitor development and evaluation exists which includes state-of-the-art technology in organic synthesis and computer-aided molecular design. Although there is no suitable animal model available for dengue virus diseases, initial screening of potential antiviral compounds would be facilitated by well-established insect and mammalian cell culture systems which are useful to monitor the effects of anti-NS3 inhibitors on the propagation of the virus.

Moreover, alternative drug targets which are present on the dengue virus NS3 protein can be exploited for inhibitor development. These include the binding site of the NS2B co-factor to the NS3 protease, the NS3 NTPase / helicase portion and the interaction surface of NS3 with the NS5 replicase. The presence of multiple biomedical targets in the dengue virus polyprotein would even make a therapy feasible, which uses combinations of different inhibitors and therefore could minimize the risk of rapid resistance development.

It can also be expected that progress for inhibitors against the dengue virus protease will be of large benefit for drug design against related human pathogens of the flavivirus complex such as yellow fever virus, Japanese encephalitis virus and West Nile virus.

However, in order to bring an effective anti-dengue drug from the ‘bench to the bedside’, several questions and limitations need to be addressed. These include the evaluation of prognostic markers for disease severity, the pathobiology of dengue haemorrhagic fever and shock syndrome, the problem of selectivity against pharmacologically relevant human proteases such as furin and the risk of adverse effects. Potential complications may also arise from the presence of four related dengue serotypes in the case that their NS3 proteases show marked differences in their inhibition profiles. Intensive efforts and sustained multidisciplinary research is required in the future to cope with the challenging task of a causative treatment for dengue virus diseases.

Acknowledgements

We thank Dr J. Wikberg, Department of Pharmaceutical Biosciences, Uppsala University, Sweden, for intensive discussions and access to data prior to publication. This work was supported by grants from the Thailand Research Fund (TRF).
References


[16] Bazan JF, Fletterick RJ and Rice CM. Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. Proc Natl Acad Sci USA, 1990, 87: 8898-8902.

Inhibition of NS2B/NS3 Protease


Prognostic Factors of Clinical Outcome in Non-Paediatric Patients with Dengue Haemorrhagic Fever/Dengue Shock Syndrome


*Tropical Medicine Institute, Infectious Diseases Section, Universidad Central de Venezuela
**Clínica Santa Sofía, Caracas, Venezuela

Abstract

A total of 112 adults with dengue haemorrhagic fever (DHF) admitted at Clínica Santa Sofía, Caracas, Venezuela, were studied during June 1998–June 2001. Capillary leakage (CL) occurred in 28.8% cases, 21.6% experienced bleeding, 9.2% developed pleural effusion (PE) and 9.2% developed acute acalculous cholecystitis (AAC). High correlation was noticed between the length of illness prior to admission (IPA) and length of hospitalization (LH) and levels of Hct, Hb and leukocytes. Significant differences were seen in the length of IPA, LH and level of platelets for patients with or without bleeding (P<0.05) or CL (P<0.005), and in LH for patients with or without PE (P<0.005), or CL (P<0.05). Patients with AAC reached higher leukocyte counts (P<0.05). ANOVA showed an association between IPA and LH, between either of them and levels of Hb, Hct or leukocytes, and between platelets levels and PE, CL or bleeding. The length of IPA and degree of alteration of Hb, Hct, leukocytes and platelets predicted a more severe course in adults with DHF.

Keywords: Dengue, dengue haemorrhagic fever, adults, prognostic factors.

Introduction

Over the last decade, dengue fever has dramatically spread in virtually all Latin American and Caribbean countries which are infested with Aedes aegypti. During this period, the number of cases reported every year in these countries jumped from 250,000 to more than 750,000[1]. Furthermore, recent serological surveys suggest the occurrence of millions of such infections[2]. After its emergence in Cuba in 1981[3], epidemics or sporadic cases of dengue haemorrhagic fever (DHF) have been reported in at least 25 countries in the Americas[4]. Venezuela has recorded large numbers of DHF cases every year, and, in 1995, the country reported the largest outbreak in the region with almost 30,000 dengue cases and 5,000 DHF cases. Although DEN-1, DEN-2 and DEN-4 had been isolated during this epidemic, DEN-2 was the predominant serotype[5].

In contrast with observations made in Asian countries, where DHF is almost completely restricted to young children, in the Americas, older age groups are widely involved[6-8].

---

E-mail: torres@iname.com; Fax: + (58-212) 9876590
The host's immune response appears to be a major factor influencing the type and severity of disease, as sequential infection with different dengue virus serotypes in the presence of non-neutralizing antibodies has been strongly incriminated in the occurrence of DHF/DSS[8-10], and cases of DHF/DSS are seldom documented in patients with primary infection[11-13]. Individual factors, such as age, sex, genetic background and underlying diseases, may also play a role[14].

Since most of the currently available clinical and epidemiological data on DHF/DSS derives from observations on infected children, information is sparse regarding prognostic factors of poor evolution among adult patients. In an attempt to identify potential prognostic factors in this specific setting, a retrospective study was carried out among non-paediatric inpatients with DHF/DSS followed at a single South American private medical institution.

**Materials and methods**

A total of 112 non-paediatric patients (male/female ratio: 64/48; age range: 15-92 years; median 36 years) admitted to Clínica Santa Sofía, Caracas, Venezuela, during a 36-month period from June 1998 to June 2001, who were attended to by the same team of physicians, were included in the study. The endemic dengue transmission season in the country typically extends from May to October, matching the yearly cycle of rains.

All cases fulfilled the diagnostic criteria of DHF/DSS, according to WHO and PAHO definitions: acute febrile illness with evidence of bleeding, thrombocytopenia $<10^5$ per $\mu$L, and evidence of plasma extravasation, such as haemococoncentration (20% increase over base Hct, or 20% decrease after rehydration), polyserositis, or hypoproteinemia, with or without signs of circulatory failure, including narrowing of pulse pressure ($\leq$ 20 mm Hg), hypotension, or shock[15]. The level of severity of the condition was established according to the following scale:

(i) Grade I, fever + nonspecific constitutional symptoms + positive tourniquet test + evidence of haemococoncentration and thrombocytopenia;

(ii) Grade II, all of the above + spontaneous bleeding, usually restricted to the skin ± other sites;

(iii) Grade III, all of the above + circulatory failure manifested by rapid and weak pulse, narrowing of pulse pressure (20 mm Hg or less), or hypotension with the presence of cold, clammy skin, and restlessness or agitation, and

(iv) Grade IV, all of the above + profound shock with undetectable blood pressure and pulse[15].

Clinical evidence of gross capillary leakage (CL) was defined as the occurrence of polyserositis, expressed by any of the following: symptomatic pleural effusions, ascytis, pericardial effusions, gallbladder wall edema and/or acute acalculous cholecystitis.

Acute acalculous cholecystitis (AAC) was diagnosed according to the following criteria: fever; persistent abdominal pain; nausea and vomiting. On physical examination, occurrence of tenderness or muscle rigidity in the right upper abdominal quadrant, epigastrium, or both, and
Murphy's sign. Additional relevant findings were a palpable mass in the region of the gall-bladder, jaundice, and mild elevations in the serum levels of bilirubin, alkaline phosphatase, and/or transaminases. On ultrasound, demonstration of an enlarged gall-bladder with thickened wall (≥ 6 mm) and pericholecystic fluid appearing as a halo, or the presence of a diffuse, homogeneous, non-shadowing, medium level echogenicity within the gall-bladder lumen, were all considered 'positive' findings[16].

Either specific IgM or IgG seroconversion over a period of 15 days was documented by means of a rapid commercial qualitative immunochromatographic test (PanBio Dengue®, Windsor, Australia).

Statistical analysis was performed using a StatSoft, Inc. (1995), STATISTICA for Windows, Computer programme manual, Tulsa, OK. Either Student's t-test for the comparison of means from independent samples of unknown variances, Pearson's correlation analysis, univariate and multivariate logistic regression analysis was performed with CAA, vascular leakage or bleeding as main outcomes, by means of a forward stepwise independent variable entry in the final model. All calculations were two-tailed, and 0.05 significant criteria were used. ANOVA analysis of variables was performed as required.

**Results**

Out of 112 patients, 71 (63.4%) developed DHF grade I, 23 (20.5%) had DHF grade II, and 18 (16.1%) had either DHF grade III or grade IV. In 37 patients (33%), clinical signs of vascular leakage were evident, 25 (22.3%) experienced moderate to severe bleeding, and 14 (12.5%) developed pleural effusion on plain chest X-ray films. AAC ensued in 14 (12.5%) cases. No deaths occurred (Table 1).

**Table 1. Degree of severity of DHF and clinical complications in 112 non-paediatric Venezuelan patients**

<table>
<thead>
<tr>
<th>Severity of disease and clinical complication</th>
<th>Number of cases</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHF grade I</td>
<td>71</td>
<td>63.4.9</td>
</tr>
<tr>
<td>DHF grade II</td>
<td>23</td>
<td>20.5</td>
</tr>
<tr>
<td>DHF grade III or IV</td>
<td>18</td>
<td>16.1</td>
</tr>
<tr>
<td>Bleeding</td>
<td>25</td>
<td>22.3</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>14</td>
<td>12.5</td>
</tr>
<tr>
<td>Acute calculus cholecystitis</td>
<td>14</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Pearson’s correlation analysis results according to clinical complications are depicted in Table 2. A significant correlation was found between the levels of Hb and Hct ($r=0.762; P<0.0001$), and between the level of platelets and Hb ($r=-0.280; P<0.01$). Correlation was also observed between the number of days of illness prior to admission (IPA) and the length of hospitalization ($r=-0.233; P<0.05$), as well as between the length of hospitalization (LH) and the degree of alteration in platelets level ($r=-0.198; P<0.05$). A higher correlation was seen between the length of hospitalization and the number of days of IPA ($r=-0.426; P<0.005$) for patients with dengue grade II or higher, or those with bleeding ($r=-0.427, P<0.005$).
Significant differences were seen in the length of IPA (mean 4.32 days vs. 3.60 days; \( P < 0.05 \)), and the minimum level of platelets (mean 43,000 per \( \mu L \) vs. 65,816 per \( \mu L \); \( P < 0.05 \)) for patients with or without clinical bleeding, as well as those with or without dengue type 2 (mean 4.43 vs. 3.56 days, and 48,870 vs. 70,800 per \( \mu L \); \( P < 0.05 \), respectively). Significant differences were also found in the length of hospitalization (mean 5.77 days vs 3.61 days; \( P < 0.005 \)) for patients with or without pleural effusion, as well as for those with or without clinical vascular leakage (mean 4.85 days vs. 3.53 days; \( P < 0.005 \)). The length of IPA was longer (mean 4.35 days vs. 3.26 days; \( P < 0.05 \)), the length of hospitalization was longer (mean 4.85 days vs. 3.61 days; \( P < 0.001 \), the serum levels of alkaline phosphatase were higher (mean 183 \( \mu L \) vs. 91 \( \mu L \); \( P < 0.05 \)), and the minimum level of leukocytes was higher (mean 12,890 per \( \mu L \) vs. 5,026 per \( \mu L \); \( P < 0.001 \)) in patients developing vascular leakage. Patients with AAC exhibited a significantly longer hospital stay (4.71 days vs. 3.71 days; \( P < 0.05 \)), and a higher mean level of peripheral blood leukocytes (15,986 x \( mm^3 \) vs. 5,071 x \( mm^3 \); \( P < 0.001 \)).

Table 2. Significant differences in disease outcome in 112 non-paediatric Venezuelan patients with dengue haemorrhagic fever according to type of clinical complication

<table>
<thead>
<tr>
<th>Clinical complication</th>
<th>Variable</th>
<th>Mean value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding</td>
<td>Illness prior to admission</td>
<td>4.66 days 3.70 days</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td></td>
<td>Minimum platelet level</td>
<td>43,619 per ( \mu L ) 61,128 per ( \mu L )</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>Length of hospitalization</td>
<td>5.0 days 3.6 days</td>
<td>( P &lt; 0.005 )</td>
</tr>
<tr>
<td>Signs of capillary leakage</td>
<td>Length of hospitalization</td>
<td>4.24 days 3.53 days</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td></td>
<td>Illness prior to admission</td>
<td>4.71 days 3.56 days</td>
<td>( P &lt; 0.001 )</td>
</tr>
<tr>
<td></td>
<td>Minimum platelet level</td>
<td>45,178 per ( \mu L ) 68,784 per ( \mu L )</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>AAC</td>
<td>Peripheral blood leukocytes</td>
<td>11,300 per ( \mu L ) 3,418 per ( \mu L )</td>
<td>( P &lt; 0.05 )</td>
</tr>
</tbody>
</table>

Both one-way and multivariate logistic regression analysis revealed that the only variables significantly associated with bleeding were illness severity on admission according to WHO scale (\( OR = 5.3; P < 0.001 \)), and length of IPA (\( OR = 1.7; P < 0.001 \)).
P<0.05). Vascular leakage was also associated with illness severity on admission in the multivariate regression analysis (OR=14.3; P<0.005), as well as with the length of hospitalization (OR=1.79; P=0.001), illness severity on admission (OR=26.5; P=0.001), leukocytes level (OR=1.33; P=0.001), and a prolonged aPTTA† (OR=18.9; P=0.001), in the univariate regression analysis. Of note was the finding that whereas patients with bleeding did not remain hospitalized longer, those with vascular leakage did (OR=1.79; P=0.001). Statistically significant results for the one-way and multivariate logistic regression analysis are summarized in Tables 3, 4 and 5.

### Table 3. Logistic regression analysis of clinical variables associated significantly with capillary leakage in 112 non-paediatric Venezuelan patients with dengue haemorrhagic fever

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>P</td>
</tr>
<tr>
<td>Degree of severity</td>
<td>26.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Length of hospitalization</td>
<td>1.79</td>
<td>0.001</td>
</tr>
<tr>
<td>Blood leucocytes level</td>
<td>1.33</td>
<td>0.001</td>
</tr>
<tr>
<td>Abnormal PTT</td>
<td>18.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Bleeding</td>
<td>3.51</td>
<td>0.015</td>
</tr>
</tbody>
</table>

OR = odds ratio  
Blank cells indicate values that should not be included since they are not significant

† Activated partial thromboplastin time

### Table 4. Logistic regression analysis of clinical variables associated significantly with bleeding in 112 Venezuelan patients with dengue haemorrhagic fever

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>P</td>
</tr>
<tr>
<td>Degree of severity</td>
<td>5.85</td>
<td>0.001</td>
</tr>
<tr>
<td>Length of illness prior to admission</td>
<td>1.37</td>
<td>0.032</td>
</tr>
<tr>
<td>Platelets (nadir)</td>
<td>0.99</td>
<td>0.015</td>
</tr>
<tr>
<td>Platelets &lt;50,000</td>
<td>3.83</td>
<td>0.005</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>6.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

OR = odds ratio  
Blank cells indicate values that should not be included since they are not significant

### Table 5. Logistic regression analysis of clinical variables significantly associated with acute achalculus cholecystitis (AAC) in 112 non-paediatric Venezuelan patients with dengue haemorrhagic fever

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>P</td>
</tr>
<tr>
<td>Degree of severity</td>
<td>50.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Blood leucocytes level</td>
<td>1.58</td>
<td>0.001</td>
</tr>
<tr>
<td>Abnormal PTT</td>
<td>1.81</td>
<td>0.001</td>
</tr>
<tr>
<td>Pleural effusion</td>
<td>9.75</td>
<td>0.001</td>
</tr>
</tbody>
</table>

OR = odds ratio  
Blank cells indicate values that should not be included since they are not significant

### Discussion

The age distribution for DHF cases in the Americas differs from that observed in South-East Asia[1,3,6,7,17,18], where young
children continue to be the age group almost exclusively affected. In contrast, an age range of 31 to 45 years has been reported for Brazilian patients with DHF/DSS, while in Puerto Rico, the mean age of the patients reported in 1990-91 was 38 years. Furthermore, during the outbreaks in Cuba in 1981 and in Venezuela in 1989, about one third of the deaths were among patients older than 14 years, and in the 1997 Cuban outbreak, all registered deaths were seen among adults.

The main pathogenic feature of dengue is an increase in vascular permeability leading to loss of plasma from blood vessels, which causes haemoconcentration, low blood pressure and shock. This may also be accompanied by haemostatic abnormalities such as thrombocytopenia, vascular changes and coagulopathy.

The clinical spectrum of dengue virus infection may range from an asymptomatic infection to a severe and rapidly fatal disease. The most severe end of the spectrum of dengue virus infection in children is represented by dengue shock syndrome (DSS). Adults seem less likely than children to suffer from DSS. Indeed, in a retrospective study of 108 adult Malaysians with DHF, the overall morbidity was significant (29.4%) but the case-fatality rate remained low (2.0%).

Haemorrhagic manifestations in DHF usually consist of petechiae, ecchymoses, easy bruising and bleeding from venipuncture sites. Epistaxis, gum bleeding and gastrointestinal haemorrhage are less common. If improperly treated, shock leads to metabolic acidosis, severe generalized bleeding and, eventually, death. Unlike children, many adult patients show severe bleeding of the gastrointestinal (GI), or of other sites, preceding the shock, which may be severe enough to cause death.

Relevant laboratory findings in DHF cases include thrombocytopenia, haemoconcentration and hypoproteinaemia. A drop in platelet count to below 100,000 per µL and an increase of 20% or more in the haematocrit, both resulting from increased vascular permeability, are consistent findings. Other signs of plasma leakage include pleural effusion, ascites and hypoproteinaemia. Leukopenia and leukocytosis are common, and aminotransferases are usually elevated. Thrombin, prothrombin and partial thromboplastin times are often prolonged. Fibrinogen levels decrease and fibrin degradation products may increase. In patients with DSS, the severity of laboratory abnormalities described for DHF tends to be worse. Dilutional hyponatremia and hypoproteinaemia correlate with disease severity.

In the current series, patients with longer length of hospitalization exhibited significantly lower levels of platelets, as well as a higher tendency to severe capillary vascular leakage (CVL). Overall, a shorter duration of IPA associated surprisingly with a longer length of hospitalization, probably reflecting the fact that more severely ill patients tended to seek medical attention earlier. Nevertheless, patients with dengue type II, as well as those with evidence of CVL, exhibited significantly more protracted IPA. The occurrence of the latter two conditions most likely reflect the delay in

---

CVL may induce many other clinical manifestations and complications besides clinical bleeding, such as pleural effusion, ascitis, joint swelling, etc.
initiating a proper and adequate fluid-replacement treatment, which is the key to treating DHF in order to compensate for the loss of plasma from blood vessels due to increased vascular permeability\cite{1,15}.

Of note is the finding that a considerable percentage of the cases (12.5\%) developed AAC. Recent data suggest that in children with DHF, a gall-bladder wall thickening \( \geq 5 \) mm on ultrasonography correlates with a higher risk of hypovolemic shock\cite{26}. However, despite a few scattered reports of AAC complicating adult DHF patients\cite{27-30}, little information exists in medical literature on the pathological and clinical implications of this newly recognized condition. It is worth mentioning that while our nine patients with AAC exhibited a significantly increased level of peripheral blood leukocytes during hospitalization, their clinical outcome in terms of IPA, length of hospitalization or occurrence of other life-threatening complications, did not differ from that of patients without AAC. Details of the clinical aspects and imaging techniques findings for this set of patients will be discussed elsewhere.

Viral, serological and genetic factors may influence virulence. Molecular studies have identified genetic variation among all 4 dengue virus serotypes\cite{31-34}. Of note here is the finding that DEN-2 strains associated with grade II DHF or DSS grow to higher titers in peripheral blood leukocytes than do DEN-2 strains isolated from mildly ill patients\cite{35,36}. Although characterization of the viral serotypes involved in these patients was not performed, DEN-1, 2 and 4 all circulated in Venezuela during the period when the cases occurred, DEN-2 being the most prevalent one\cite{5}.

In conclusion, the number of days of IPA, the length of hospitalization and the degree of alteration in the level of Hb, Hct, leukocytes and platelets were all predictors of a more severe and complicated course in adult patients with DHF/DSS. The onset of leukocytosis must suggest the occurrence of inflammatory complications such as AAC. DHF/DSS in the Americas continues to occur in a significant number of adults, but it is not clear whether this relates with the genetic background of the populations, the epidemiological events or, else, with other unknown factors.

References


Prognostic Factors in Adult Patients with Haemorrhagic Dengue


Dengue Haemorrhagic Fever with Encephalopathy/Fatality at Petchabun Hospital: A three-year Prospective Study (1999-2002)
Prasonk Witayathawornwong
Department of Paediatrics, Petchabun Hospital, Petchabun Province, Thailand

Abstract
During the three-year period from 1 May 1999 to 30 April 2002, there were 1,465 cases of dengue haemorrhagic fever (DHF) admitted to the Department of Paediatrics, Petchabun Hospital. The male to female ratio was 722:743 (1:1.03). Their ages ranged from 80 days to 15 years with a median of 9 years. Thirty-two patients (2.2%) were under one year of age with a median of 8 months, and all except two had primary dengue infection.

There were 34 DHF patients with encephalopathy (2.3% of all DHF cases). The male to female ratio was 17:17 (1:1). The median age was 8 years and 11 months (range 10 months to 13 years). Thirty patients (88.2%) were older than 5 years. Thirty and four patients respectively developed encephalopathy in shock and convalescent stages. All 17 fatal cases (1.16% of the total DHF cases, male:female = 8:9) had both prolonged shock and massive gastrointestinal haemorrhage since admission. About 64.7%, 76.4% and 58.8% of the seventeen non-fatal cases (male:female = 9:8) had gastrointestinal haemorrhage, shock state and massive fluid overload since admission respectively. The risk factors for encephalopathy included prolonged shock, severe gastrointestinal haemorrhage, severe hepatic dysfunction and prior fluid overload.

Keywords: Dengue haemorrhagic fever, encephalopathy, fatality, gastrointestinal haemorrhage, shock, fluid overload, hepatic dysfunction.

Introduction
The major pathophysiological hallmarks in dengue haemorrhagic fever (DHF) are leakage and abnormal haemostasis that leads to hypovolemic shock and/or haemorrhage. Generally, vital organs are not primarily involved in DHF but unusual manifestations, mainly the involvement of the central nervous system (CNS) and severe hepatic dysfunction, are increasingly being detected[14]. The incidence of CNS involvement in dengue infection was about 0.88-5.4%[7-11] first reported in 1976[12]. Although dengue encephalitis existed as evident from the direct dengue viral invasion[13-17], the more common conditions were encephalopathy secondary to fluid extravasation, cerebral oedema, hypoperfusion, haemorrhage, hyponatremia, liver failure and renal failure[2,18-19]. The treatment of DHF with CNS involvement is supportive and symptomatic. Early detection and proper fluid management of DHF should be done to prevent any risk factors.
Materials and methods

There were 1,465 cases of DHF admitted to the Department of Paediatrics, Petchabun Hospital, Petchabun province, Thailand, from 1 May 1999 to 30 April 2002. The diagnosis methods used followed the WHO criteria\(^{20}\) and about 82% of cases were serologically confirmed using either enzyme-linked immunosorbent assay or haemagglutination inhibition tests. The treatment consisted of general measures (closely observed vital signs, general appearance and serially recorded haematocrit in 24-48 hours after fever, no medication except antacid for moderate to severe abdominal pain) and fluid therapy (minimal amount to normalize vital signs and haematocrit: 5% dextrose saline for infants, 5% dextrose Ringer acetate for older patients, Dextran-40 in normal saline for impeding or fluid overload and fresh whole blood or packed red cells for significant bleeding, platelet concentrate and plasma not used).

Patients with encephalopathy (drowsy, stuporous, comatose and convulsion) were closely observed for blood sugar or dextrostix every six hours. Serum transaminase was done once a day until recovery. Vitamin K\(_1\) and 10% calcium gluconate were administered for three days. Lumbar puncture was performed cautiously if there was no other risk.

The statistical analysis included the percentage, mean, standard deviation and range for demographic data and Student’s t and chi-square tests for comparing non-categorized and categorized variables, respectively.

Results

Of the 1,465 cases of DHF admitted to the Department of Paediatrics, Petchabun Hospital, from 1 May 1999 to 30 April 2002. The male to female ratio was 722:743 (1:1.03). The patients’ age ranged from 80 days to 15 years with the median of 9 years. The highest incidence was in the 5-10-year age group (57%) and the second highest was in the 10-15-year age group (26.2%). Thirty-two patients (2.2%) were under one year of age with the median of 8 months, and all of them except two had primary dengue infection. Eighty-seven per cent of all cases were found in the rainy season during May-October. The patients were from all 11 districts in the Petchabun province, 84% from the central district alone. There were DHF patients from all villages (178) of all the subdistricts (17) of the central district.

There were 34 DHF patients with encephalopathy (2.3%). The male to female ratio was 17:17 (1:1). The median age was 8 years 11 months (range 10 months to 13 years). Thirty patients (88.2%) were older than 5 years. Thirty and four patients developed encephalopathy in the shock and convalescent stages respectively. There were 7, 18 and 9 patients with encephalopathy stage II (drowsy), stage III (stuporous) and stage IV (comatose) respectively. Twenty-eight patients (82.3%) were referred from district hospitals and 17 patients (50%) had a massive fluid overload. Lumbar puncture was performed in 2 non-fatal cases with normal findings. Their serology indicated secondary dengue response.

About 17 fatal cases (1.6% of total DHF cases, male:female = 8:9 , median age 8 years and 4 months, range 10 months to 12
DHF with Encephalopathy/Fatality at Petchabun Hospital, Thailand (1999-2002)

years) had the median duration of 13 hours for deaths (range 1-26 hours, mean±SD = 13.4±8.4 hours). Sixteen of the 17 cases (94.1%) were dead within 24 hours. Profound shock and massive gastrointestinal haemorrhage since admission were detected in all patients. Hepatic failure, comatose stage and massive fluid overload were detected since admission in 12 (70.6%), 9 (52.9%) and 7 (41.1%) cases respectively. There was no history of taking acetaminophen more than 60 mg per kg/day. Aspirin and non-steroidal anti-inflammatory drug (NSAID), Ibuprofen, were taken by each patient for many days during the febrile stage. Convulsion was detected in 4 cases (Tables 1 and 2).

Table 1. Clinical manifestations of 17 fatal cases

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>TDF/DI</th>
<th>L/S (cm)</th>
<th>DHF grade</th>
<th>Haemorrhage manifestations</th>
<th>Encephalopathy manifestations</th>
<th>Fluid overload</th>
<th>Associated diseases</th>
<th>Referral*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1NW</td>
<td>1.3</td>
<td>F</td>
<td>8</td>
<td>3/3</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>+</td>
<td>Diarrhea convulsion</td>
<td>+</td>
</tr>
<tr>
<td>2SS</td>
<td>11.7</td>
<td>F</td>
<td>42</td>
<td>6/7</td>
<td>2/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3TR</td>
<td>13</td>
<td>M</td>
<td>37</td>
<td>4/3</td>
<td>2/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>+</td>
<td>ASA</td>
<td>+</td>
</tr>
<tr>
<td>4KB</td>
<td>6</td>
<td>F</td>
<td>13</td>
<td>3/3</td>
<td>10/4</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>-</td>
<td>Thalasemia convulsion</td>
<td>+</td>
</tr>
<tr>
<td>5SM</td>
<td>7</td>
<td>M</td>
<td>20</td>
<td>5/6</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>-</td>
<td>Pneumonia G6PDdef</td>
<td>+</td>
</tr>
<tr>
<td>6KC</td>
<td>7</td>
<td>F</td>
<td>19</td>
<td>4/4</td>
<td>2/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7UA</td>
<td>2.11</td>
<td>F</td>
<td>21</td>
<td>7/7</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>+</td>
<td>NSAID convulsion</td>
<td>+</td>
</tr>
<tr>
<td>8WT</td>
<td>10</td>
<td>M</td>
<td>50</td>
<td>5/5</td>
<td>2/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9KF</td>
<td>6-36.3</td>
<td>M</td>
<td>40</td>
<td>5/6</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10RM</td>
<td>0.10</td>
<td>F</td>
<td>7</td>
<td>4/4</td>
<td>4/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11JP</td>
<td>10</td>
<td>F</td>
<td>31</td>
<td>4/5</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>+</td>
<td>ARDS, DIC</td>
<td>+</td>
</tr>
<tr>
<td>12DP</td>
<td>9.0</td>
<td>M</td>
<td>17</td>
<td>5/6</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13BP</td>
<td>9.5</td>
<td>F</td>
<td>23</td>
<td>4/4</td>
<td>5/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14NO</td>
<td>8.4</td>
<td>F</td>
<td>24</td>
<td>6/7</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15TW</td>
<td>8.10</td>
<td>M</td>
<td>20</td>
<td>5/6</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Drowsy/s</td>
<td>-</td>
<td>Hypoglycemia convulsion</td>
<td>+</td>
</tr>
<tr>
<td>16WK</td>
<td>10</td>
<td>M</td>
<td>35</td>
<td>4/4</td>
<td>3/-</td>
<td>4</td>
<td>GIH</td>
<td>Comatose/s</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>17PL</td>
<td>12</td>
<td>M</td>
<td>45</td>
<td>5/6</td>
<td>2/-</td>
<td>4</td>
<td>GIH</td>
<td>Stuporous/s</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

TDF = total duration of fever; DI = duration of illness
GIH = gastrointestinal haemorrhage; S = shock stage; kg = kilograms
1° = first, second degree protein energy malnutrition
* or prior medications especially excessive fluid replacement
ARDS = Adult respiratory distress syndrome
DIC = Disseminated intravascular coagulation
NSAID = Non-steroidal anti inflammatory drug
L/S = liver/spleen
There were 17 non-fatal cases (male:female = 9:8; median age = 9 years, range = 3-12 years). Massive gastrointestinal haemorrhage, shock and massive fluid overload were detected since admission in 11 (64.7%), 13 (grade 3-9, grade 4-4, 76.4%) and 10 (58.8%) cases respectively. There were 5 cases with acute renal failure and one with liver failure since admission. Fifteen of the 17 cases were referred from district hospitals. Haemodialysis and plasmapheresis were done in 3 renal failure cases and 2 cases with only supportive treatment. All 17 cases recovered completely without neurological sequelae. The average (mean ± SD) hospitalization duration and recovery period from encephalopathy were 8.3 ± 5.31 days and 5.1 ± 3.7 days respectively (Tables 3, 4).
Table 3. Clinical manifestations of 17 non-fatal encephalopathy cases

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (y.m)</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>TDI/DI</th>
<th>L/S (cm)</th>
<th>DHF grade</th>
<th>Hemato manifestations</th>
<th>Enceph signs/onset</th>
<th>Fluid overload</th>
<th>Associated diseases</th>
<th>Referral</th>
</tr>
</thead>
<tbody>
<tr>
<td>1YB</td>
<td>9 M</td>
<td>25</td>
<td>4/4</td>
<td>3/-</td>
<td>4 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>+ ARF</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2PS</td>
<td>4.4 F</td>
<td>17</td>
<td>5/3</td>
<td>3/-</td>
<td>4 Petichia</td>
<td>Stuporous/s</td>
<td>- Pneumonia</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3ST</td>
<td>10.3 F</td>
<td>29</td>
<td>6/6</td>
<td>3/-</td>
<td>3 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4SJ</td>
<td>6 M</td>
<td>15</td>
<td>7/7</td>
<td>3/-</td>
<td>3 GIH</td>
<td>+</td>
<td>Stuporous/s</td>
<td>+ Pneumonia, Tracheitis</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5CP</td>
<td>5.9 M</td>
<td>17</td>
<td>7/7</td>
<td>1/-</td>
<td>2 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6WD</td>
<td>9.9 F</td>
<td>43</td>
<td>5/5</td>
<td>1P</td>
<td>3 GIH</td>
<td>+</td>
<td>Stuporous/s</td>
<td>+ ARF</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7WB</td>
<td>6.6 M</td>
<td>18</td>
<td>3/3</td>
<td>3/-</td>
<td>3 Petichia</td>
<td>Stuporous/s</td>
<td>+ -</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8SS</td>
<td>9 F</td>
<td>41.5</td>
<td>5/5</td>
<td>2/-</td>
<td>3 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>- Hypoglycemia, ARF</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9NS</td>
<td>11 F</td>
<td>30</td>
<td>4/7</td>
<td>4/-</td>
<td>2 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>- ARF</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10PP</td>
<td>11 M</td>
<td>34</td>
<td>5/5</td>
<td>2/-</td>
<td>4 GIH</td>
<td>+</td>
<td>Stuporous/s</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11Sma</td>
<td>9.11 M</td>
<td>28</td>
<td>5/5</td>
<td>5/-</td>
<td>3 GIH</td>
<td>+</td>
<td>Drowsiness/s</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12WL</td>
<td>6.1 F</td>
<td>19</td>
<td>5/5</td>
<td>8/6</td>
<td>3 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>+ Thalasemia, ARDS, pneumonia</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13ThP</td>
<td>7.9 M</td>
<td>22</td>
<td>5/5</td>
<td>4/-</td>
<td>2 GIH</td>
<td>-</td>
<td>Stuporous/s</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14SL</td>
<td>5.9 M</td>
<td>25</td>
<td>5/5</td>
<td>5/-</td>
<td>3 Petichia</td>
<td>Stuporous/s</td>
<td>+ ARF</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15KN</td>
<td>11.11 M</td>
<td>23</td>
<td>5/5</td>
<td>1/-</td>
<td>4 Petichia</td>
<td>Drowsiness/s</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16MK</td>
<td>10.4 M</td>
<td>49</td>
<td>6/6</td>
<td>2/-</td>
<td>3 Petichia</td>
<td>Drowsiness/s</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17WTh</td>
<td>3 F</td>
<td>13.5</td>
<td>5/7</td>
<td>2/-</td>
<td>2 Petichia</td>
<td>Drowsiness/s</td>
<td>+ Rhinitis</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1,2 = first, second degree protein energy malnutrition
ARF = acute renal failure; ARDS = Adult respiratory distress syndrome
GIH = gastrointestinal haemorrhage; JP = just palpable

Table 4. Laboratory investigations of 17 non-fatal encephalopathy cases

<table>
<thead>
<tr>
<th>Patients</th>
<th>Platelets/Hct (max-min)</th>
<th>AST/ALT (IU/L)</th>
<th>PT/PTT* (sec)</th>
<th>Sodium (mEq/L)</th>
<th>DHF serology</th>
<th>Duration**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1YB</td>
<td>62,000(51-30)</td>
<td>9,420/2,239</td>
<td>23.4/&gt;120</td>
<td>128 SDI</td>
<td>13/7</td>
<td></td>
</tr>
<tr>
<td>2PS</td>
<td>17,000(50-26)</td>
<td>2,023/458</td>
<td>14.4/59.9</td>
<td>135 SDI</td>
<td>9/5</td>
<td></td>
</tr>
<tr>
<td>3ST</td>
<td>16,000(50-38)</td>
<td>1,098/452</td>
<td>14.7/121.6</td>
<td>130 SDI</td>
<td>4/2</td>
<td></td>
</tr>
<tr>
<td>4SJ</td>
<td>90,000(46-34)</td>
<td>255/148</td>
<td>16.8/48.5</td>
<td>135 SDI</td>
<td>17/10</td>
<td></td>
</tr>
<tr>
<td>5CP</td>
<td>44,000(46-34)</td>
<td>160/110</td>
<td>18.4/64.5</td>
<td>140.3 SDI</td>
<td>13/10</td>
<td></td>
</tr>
<tr>
<td>6WD</td>
<td>36,000(41-30)</td>
<td>13,895/5,200</td>
<td>24.6/77.6</td>
<td>127 SDI</td>
<td>11/7</td>
<td></td>
</tr>
<tr>
<td>7WB</td>
<td>4,000(58-34)</td>
<td>2,128/918</td>
<td>15.1/67.8</td>
<td>128 SDI</td>
<td>6/3</td>
<td></td>
</tr>
<tr>
<td>8SS</td>
<td>20,000(48-32)</td>
<td>14,580/5,852</td>
<td>16.4/56.0</td>
<td>134 SDI</td>
<td>107/10</td>
<td></td>
</tr>
<tr>
<td>9SN</td>
<td>68,000(49-27)</td>
<td>6,987/3,789</td>
<td>32.5/57.2</td>
<td>138 SDI</td>
<td>7/3</td>
<td></td>
</tr>
<tr>
<td>10PP</td>
<td>21,000(42-27)</td>
<td>3,810/1,935</td>
<td>15.3/52.5</td>
<td>129 SDI</td>
<td>4/2</td>
<td></td>
</tr>
<tr>
<td>11Sma</td>
<td>34,000(48-33)</td>
<td>2,250/448</td>
<td>14.9/62.9</td>
<td>128 SDI</td>
<td>6/3</td>
<td></td>
</tr>
<tr>
<td>12WL</td>
<td>20,000(42-27)</td>
<td>3,436/1,841</td>
<td>15.0/89.3</td>
<td>143 SDI</td>
<td>21/15</td>
<td></td>
</tr>
<tr>
<td>13ThP</td>
<td>56,000(42-30)</td>
<td>1,315/549</td>
<td>12.4/61.5</td>
<td>134 SDI</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>14SL</td>
<td>45,000(56-33)</td>
<td>4,746/1,470</td>
<td>15.9/113.3</td>
<td>131.6 SDI</td>
<td>2/2</td>
<td></td>
</tr>
<tr>
<td>15KN</td>
<td>40,000(49-32)</td>
<td>8,150/4,450</td>
<td>16/72.2</td>
<td>137 SDI</td>
<td>7/4</td>
<td></td>
</tr>
<tr>
<td>16MK</td>
<td>3,000(51-35)</td>
<td>2,996/1,482</td>
<td>13.8/64.0</td>
<td>132.8 SDI</td>
<td>5/3</td>
<td></td>
</tr>
<tr>
<td>17WTh</td>
<td>60,000(32-30)</td>
<td>275/599</td>
<td>15.4/66.7</td>
<td>128 SDI</td>
<td>4/3</td>
<td></td>
</tr>
</tbody>
</table>

* normal: (10-14) / (23-35) sec
** duration in hospital/consciousness change (days)
A comparison between the clinical manifestations and laboratory investigations of the fatal and non-fatal cases, usual manifestations and encephalopathy cases are shown in Tables 5, 6 and 7 respectively.

Table 5. Comparison between clinical manifestations and laboratory investigations of fatal and non-fatal cases

<table>
<thead>
<tr>
<th>Clinical manifestations or laboratory investigations*</th>
<th>Fatal cases (n=17)</th>
<th>Non-fatal cases</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shock (grade IV)</td>
<td>17</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Internal bleeding</td>
<td>17</td>
<td>11</td>
<td>0.007</td>
</tr>
<tr>
<td>Onset of encephalopathy in shock stage</td>
<td>17</td>
<td>13</td>
<td>0.035</td>
</tr>
<tr>
<td>Coma since admission</td>
<td>9</td>
<td>0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets (/cubic millimeters)**</td>
<td>29.33 (26.662.1)</td>
<td>37,411.7 (24,153.3)</td>
<td>0.362</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>4,641.4 (5082.7)</td>
<td>4,560.7 (4,454.8)</td>
<td>0.961</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>2,108.8 (2,655.1)</td>
<td>1,878.8 (1,838.6)</td>
<td>0.771</td>
</tr>
<tr>
<td>PT in sec</td>
<td>25.5 (11.1)</td>
<td>17.3 (5.3)</td>
<td>0.011</td>
</tr>
<tr>
<td>PTT in sec</td>
<td>146.1 (98.8)</td>
<td>75.9 (25.5)</td>
<td>0.011</td>
</tr>
<tr>
<td>Serum sodium (mEq/L)</td>
<td>133.9 (3.3)</td>
<td>132.8 (4.8)</td>
<td>0.471</td>
</tr>
</tbody>
</table>

* mean (standard deviation, SD)
** minimum value
PT= Prothrombin time
PTT= Partial thromboplastin time

Table 6. Comparison between clinical manifestations of usual manifestations and encephalopathy cases

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Usual manifestations (n=1431)</th>
<th>Encephalopathy (n=34)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &lt; 1 yr</td>
<td>31 (2.1%)</td>
<td>1 (2.9%)</td>
<td>0.775</td>
</tr>
<tr>
<td>1 - 4 yr</td>
<td>210 (14.6%)</td>
<td>4 (11.7%)</td>
<td>0.661</td>
</tr>
<tr>
<td>5 - 10 yr</td>
<td>812 (56.7%)</td>
<td>23 (67.6%)</td>
<td>0.219</td>
</tr>
<tr>
<td>10 - 15 yr</td>
<td>378 (26.4%)</td>
<td>6 (17.6%)</td>
<td>0.252</td>
</tr>
<tr>
<td>Female</td>
<td>726 (50.7%)</td>
<td>17 (50.0%)</td>
<td>&gt; 0.993</td>
</tr>
<tr>
<td>Shock</td>
<td>332 (23.2) (grade 3 = 330, 4 = 2)</td>
<td>30 (88.2%) (grade 3 = 9, 4 = 21)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Referal or prior medications</td>
<td>266 (18.6%)</td>
<td>28 (82.3%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Internal bleeding</td>
<td>130 (9.1%)</td>
<td>28 (82.3%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Malnutrition[21]</td>
<td>716 (50.0%) (1°= 511, 2°= 192, 3°= 13)</td>
<td>12 (35.3%) (1°= 10, 2°= 2)</td>
<td>0.091</td>
</tr>
<tr>
<td>Death</td>
<td>-</td>
<td>17 (50%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Duration in hospital (days): mean (standard deviation)</td>
<td>3.4 (1.38)</td>
<td>83 (5.31)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

* only non fatal cases
Table 7. Comparison between laboratory investigations of usual and encephalopathy cases

<table>
<thead>
<tr>
<th>Laboratory data*</th>
<th>Usual manifestations (n=1431)</th>
<th>Encephalopathy (n=34)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/L)</td>
<td>257.3 (250.6)</td>
<td>4601.0 (4748.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>119.5 (138.1)</td>
<td>1993.8 (2251.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets (minimum)</td>
<td>70697.9 (2975.7)</td>
<td>33372.8 (2538315)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PT (10-14 sec)</td>
<td>11.2 (1.1)</td>
<td>21.4 (9.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PTT (23-35 sec)</td>
<td>51.9 (13.4)</td>
<td>111.0 (79.5)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* mean (standard deviation, SD)

Discussion

DHF patients from all the 11 districts in the Petchabun province were included in this study. The minors were referred from 10 other districts. The adults were from the 178 villages in all 17 subdistricts of the central district. This epidemic was similar to the previous dengue epidemic in 1997[22]. This data implied that the dengue virus had spread nationwide. The percentage of DHF patients with CNS involvement in this study was 2.3%, the same as in the previous study[22] as well as in other studies[7-11]. Female patients were reported to be more severely affected and accounted for more fatalities than male patients but without any significant difference[18,23-25]. In this study, the number of both sexes was equal and both had usual manifestations and encephalopathy cases. Young patients, especially those less than 1 year of age, had the tendency to be more severely and fatally affected[18,23-25]. Age itself was not a risk factor of disease severity in this study. Although there was one fatal case aged 10 months (10 RM), 85% of those who died were older than 5 years. Most of the DHF/DSS patients were well-nourished but patients with CNS involvement were more undernourished without significant difference[24,26]. In this study, patients with usual manifestations and encephalopathy were underweight[21] by about 50% and 35.3% respectively. Patients with encephalopathy were mainly in stage III[7,8,18,27] (88.2%) initially developed in shock stage (52.9%), more than in febrile or convalescent stage[7,8,28]. Seizure in DHF with encephalopathy had been reported in about 18.8-100%[7-9,13,18,24,27,29,30]. In this study seizure was detected in 4 out of 34 patients (11.7%). Two patients (1NW, 7UA), under 5 years, developed seizure in afebrile stage, indicating that the seizure might have had a specific primary cause[10]. However, some children had possible confounding factors such as hyponatremia (3 patients, 1NW, 4KB, 7UA), hypoglycemia (15TW), and liver failure (3 patients, 1NW, 7UA, 15TW). Other factors could be a history of previous febrile seizure, co-infection[20] and drug ingestion.

The causes or factors contributing to CNS manifestations included the following: direct CNS infection – a rare entity, CNS
bleeding\cite{6,18} and severe hepatic dysfunction. Lumbar puncture was done in only two patients (5CP, 15KN) in the convalescent stage with normal findings. Direct CNS infection could not be evaluated in this study. There was massive gastrointestinal haemorrhage in 28 out of 34 cases (82.3%). Acute liver failure could be the direct or indirect cause of encephalopathy\cite{31,32} and an important cause of death in DHF\cite{33}. Twelve of the 17 fatal cases (70.5%) had this severe condition. Severe hepatic dysfunction could be due to profound shock, massive gastrointestinal haemorrhage or immune complex mechanism\cite{34}. The liver pathology in profound shock stage might be centrilobular hepatocellular necrosis\cite{35} or extensive necrosis of hepatocytes usually in a massive or submassive distribution\cite{33}. Massive gastrointestinal haemorrhage might cause hepatic dysfunction and vice versa. Immune complexes, detected in 80% of DHF patients\cite{36}, might be deposited in hepatocytes and then destroyed as in hepatitis B virus infection\cite{34}. Excessive use of hepatotoxic drugs such as acetaminophen, an antiemetic drug, might interfere with liver function. There was no history of excessive use of such drugs in this study. Two fatal cases with liver failure and massive gastrointestinal haemorrhage had taken aspirin (3TR) and non-steroidal anti-inflammatory drug, Ibuprofen (7UA), for many days during the febrile stage. Both drugs aggravated the gastrointestinal haemorrhage and aspirin could have induced Reye syndrome\cite{37}.

Thalassemia, of which two cases with a large liver and spleen were included in this study (4KB, 12WL), was a risk factor for hepatic failure\cite{18}. Acute renal failure (ARF) was a rare complication of DHF but could occur in severe cases with prolonged shock, DIC and also hepatorenal syndrome\cite{37}. There were five patients with ARF in this study. All of them recovered completely, three (6WD, 8SSr, 14SL) with haemodialysis and plasmapheresis and two (1YB, 9SN) with only supportive and symptomatic therapy. Hyponatremia was a factor in CNS involvement. Serum sodium of encephalopathy patients in this study was slightly low. It was due to excessive hypotonic solution replacement. The risk factors of encephalopathy in this study were profound shock, massive gastrointestinal haemorrhage, excessive fluid overload and severe hepatic dysfunction. Among the fatal and non-fatal encephalopathy cases, laboratory investigations except for coagulogram, were not significantly different. But fatal cases had more severe shock conditions, gastrointestinal haemorrhage and onset and depth of encephalopathy (Table 5).

**Conclusion**

In conclusion, encephalopathy in DHF was a severe complication with high mortality, although neurological sequelae in recovered patients were rare. Prevention should be attempted by early diagnosis and proper management of fluid therapy.

**Acknowledgements**

The author thanks officials of the Regional Medical Science Centre, Phitsanuloke, Thailand, for DHF serology testing and all nursing staff and workers of the Paediatrics Department of Petchabun Hospital for taking good care of the patients.
References


DHF with Encephalopathy/Fatality at Petchabun Hospital, Thailand (1999-2002)


A New Tool for the Diagnosis and Molecular Surveillance of Dengue Infections in Clinical Samples


*Laboratorio de Arbovirus y Enfermedades Viricas Importadas, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Carretera de Pozuelo Km 2 (28220 Majadahonda), Madrid, Spain
**Jerome L and Dawn Greene Infectious Disease Laboratory, Columbia University, New York, USA
***Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany

Abstract

Dengue fever and dengue haemorrhagic fever are amongst the most important challenges in tropical diseases due to their expanding geographical distribution, increasing outbreak frequency, hyperendemicity and evolution of virulence.

Here, the use of a RT-nested PCR for both the diagnosis and genetic characterization of dengue infections in clinical samples is described.

Keywords: Dengue, dengue haemorrhagic fever, diagnosis, molecular epidemiology, surveillance, glycoprotein E gene, NS1 gene.

Introduction

Dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are considered to be the most important arthropod-borne viral diseases due to the high rates of morbidity and mortality caused by them. Over 2.5 billion people are at risk of the infection and more than 100 countries are home to endemic dengue transmission with an increasing incidence of DHF cases[1-3], making dengue an archetypal “emerging” disease. International travel, urbanization, overpopulation, crowding, poverty and a weak public health infrastructure in most endemic areas are the likely factors contributing to the surge in new cases[3]. A major concern is the potential spread of dengue fever into the United States of America and Europe due to climate warming and the spread of its vector.

Travellers to areas where dengue is endemic are a potential source of the spread. Most infections manifest as a mild febrile illness during travel that coincides with the peak of viral shedding and risk for transmission. Imported dengue virus infections have been reported in several non-endemic countries; and dengue virus infection is one of the most frequent causes

---

#E-mail: cdomingo@isciii.es Phone: (+34) 918223954, Fax: (+34) 915097966
of febrile illness in tourists and people working in dengue-endemic areas\cite{5,6,7,8}. As early symptoms of DF mimic those of other diseases such as malaria or leptospirosis, rapid laboratory diagnosis is important for proper patient care. Dengue fever can be diagnosed by virus isolation, genome and antigen detection, or serological studies. Samples obtained for the detection of dengue virus by cell-culture require proper handling of the sample for viral viability. Serology, even for anti-dengue IgM antibodies, is feasible only after 5 days following the onset of the symptoms. Thus, molecular techniques fulfil an important role in the diagnosis of dengue infection during its early stages.

The characterization of circulating dengue virus serotypes is important in surveillance, since the introduction of a new variant to areas affected by pre-existing serotypes constitutes a risk factor for DHF/DSS\cite{9}. By defining intra-serotypic genetic variation, the global distribution and spread of virus strains can be mapped and followed up\cite{10-13}, and the genetic differences associated with disease severity can be identified\cite{10,14-16}. Moreover, recent epidemiological analyses suggest that the more virulent genotypes are now displacing those of lower epidemiological impact\cite{17}, resulting in dengue outbreaks\cite{18}. In this context, a methodology for real-time, worldwide surveillance is needed to track dengue strains and help anticipate changes in the epidemiology of the infection.

Here we report the amplification and analysis of a genomic interval spanning the E/NS1 junction of the dengue genome for the detection and typing of all four dengue virus serotypes in clinical specimens. This sensitive, specific and rapid alternative assay requires only a single acute phase serum sample.

Materials and methods

Virus isolates and clinical samples

Viral RNAs were provided by the National Collection of Pathogenic Viruses (Porton Down, Salisbury, UK): serotype 1 dengue virus (DEN-1; strain Hawaii), serotype 2 dengue virus (DEN-2; strain New Guinea C), serotype 3 dengue virus (DEN-3; strain H87), and serotype 4 dengue virus (DEN-4; strain H241); the RNAs from prototype strains of Japanese encephalitis (JEV), yellow fever (YFV), tick-borne encephalitis (TBEV), Murray Valley encephalitis (MVEV) and St. Louis encephalitis (SLEV) viruses were used to check the specificity of the dengue virus assay. Serial dilutions of this genome material were prepared to obtain the standards to assess the sensitivity of the assay.

Viremic human sera samples were obtained from patients with a clinical diagnosis of dengue infection (Sera 1794F02; 13VI02; 366VI03; 438VI03). These were travellers who presented at the Spanish Tropical Medicine Units with dengue-compatible symptomatology on their return from the Dominican Republic, India, Indonesia and Nicaragua, respectively, and suffered from classical DF as defined by the WHO criteria\cite{19}.

Selection and synthesis of oligonucleotide primers

A RT-nested PCR protocol was developed for the detection of the four serotypes of dengue virus in clinical samples. Dengue virus primers for amplification and/or sequencing (Table) were designed based on dengue virus sequences in the public sequence databases, using a computer-assisted analysis (MACAW version 32 software, 1995, NCBI, Maryland) to determine consensus sequences. The Table
shows the sequences and the respective primer positions in the prototype strains of the four dengue serotypes. To address the natural variability of dengue viruses, mixtures of degenerated primers were used to enable hybridization with all known serotypes.

RT-nested PCR

Using purified dengue virus RNA as a template, relevant aspects of the RT-PCR and nested PCR assay (Mg²⁺ concentration, primers, RT temperature, number of cycles, annealing temperatures) were initially optimized to achieve the greatest sensitivity. A PTC-200 Peltier thermal cycler (MJ Research) was used throughout. 5 µl of viral RNA solution were added to 45 µl of a medium compatible with both the reverse transcription and PCR amplification steps (QIAGEN® OneStep RT-PCR kit). The RT-PCR mix contained 1×OneStep RT-PCR buffer, 400 mM of each dNTP, 20 pmol of each sense or antisense degenerated primer (S1871DEN1, 1871DEN2, 1871DEN3, 1871DEN4, AS2622DEN1, AS2622DEN2, AS2622DEN3, AS2622DEN4) and an optimized combination of Omniscript and Sensiscript reverse transcriptases and HotStar Taq DNA polymerase. The RT-PCR reactions were carried out using an initial reverse transcription step at 41 °C for 45 minutes followed by a denaturation and Hot Star Taq polymerase activation step (94 °C, 15 minutes) and 40 cycles of denaturation (94 °C, 30 seconds), primer annealing (55 °C, 1 minute), and primer extension (72 °C, 30 seconds) and a further extension step at 72 °C for 5 minutes.

Dengue virus sequence database

A dengue sequence database was constructed by extracting sequences from the NCBI GenBank. Each sequence was identified by name, place, date and serotype. Previously described genotypes were taken from the references listed: dengue strains genotypes were noted as described by Rico-Hesse for DEN-1, 3 and 4[17] and by Twiddy et al. for dengue virus type 2[20]. A manual search was employed for all the sequences in GenBank encompassing the targets of selected primers. Next, we used BUSSUB, a new tool developed at the Bioinformatics Unit of the Institute of Health Carlos III[21]. This software simplifies and boosts the process of retrieving sequences contained between two given flanking regions, improving the final results of a search. Genetic characterization was performed on a total data set of 113 DEN-1, 191 DEN-2, 102 DEN-3 and 153 DEN-4 sequences.

Sequence analysis of amplified products

Original sequence data were first analysed by the CHROMAS software (version 1.3; McCarthy 1996; School of Biomolecular and Biomedical Science, Faculty of Science and Technology, Griffith University, Brisbane, Queensland, Australia); forward and reverse sequence data of each sample were aligned using the programme EDITSEQ (DNASTAR

Dengue Bulletin - Vol 28, 2004
Inc. Software, Madison, Wisconsin, USA). The consensus sequence was compared and aligned to other samples or DNA database sequences using the programme CLUSTAL X, version 1.83. Programmes from the MEGA package were used to produce phylogenetic trees using NJ as the method to reconstruct the phylogeny and Kimura-2p as nucleotide substitution calculation method. The statistical significance of a particular tree topology was evaluated by bootstrap re-sampling of the sequences 1,000 times. Published sequences used in the comparisons were obtained from the GenBank databases. Pair-wise comparisons of the dengue virus database were done by global alignment using the Needleman–Wunsch algorithm using the implementation from EMBOSS, the European Molecular Biology Open Software Suite. Z-Scores were calculated to test the significance of each pair-wise alignment by Monte Carlo simulation on the shuffled sequences. Statistical analysis was conducted with the SPSS statistical package (SPSS Software, Chicago, IL).

### Results

#### Design of the primers

The E/NS1 region of the genome was chosen for the development of a RT-nested PCR. The primers selected specifically amplify the four dengue viruses with no cross reactivity to other members of the flavivirus family. A mix of degenerate primers representing each serotype was used to ensure coverage for the highly variable dengue serotypes (Table).

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Sequence†</th>
<th>Genome position‡</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1871DEN1</td>
<td>5’TGGCTGAGACCCARCATGNNAC-3’</td>
<td>1869 to 1890</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>S1871DEN2</td>
<td>5’TAGCAGAACCACARCATGNNAC-3’</td>
<td>1871 to 1889</td>
<td></td>
</tr>
<tr>
<td>S1871DEN3</td>
<td>5’TCCTGAAACGCARCATGNNAC-3’</td>
<td>1863 to 1884</td>
<td></td>
</tr>
<tr>
<td>S1871DEN4</td>
<td>5’TGGCAGAACCACARCYGGNAC-3’</td>
<td>1873 to 1894</td>
<td></td>
</tr>
<tr>
<td>AS2622DEN1</td>
<td>5’CAATTCATTTGATATTTGYTTCCAC-3’</td>
<td>2620 to 2644</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>AS2622DEN2</td>
<td>5’CAATTCTGGTGTTAATTGYTTCCAC-3’</td>
<td>2622 to 2646</td>
<td></td>
</tr>
<tr>
<td>AS2622DEN3</td>
<td>5’CAGTTCCATRAGCTATTGYTTCCAC-3’</td>
<td>2614 to 2638</td>
<td></td>
</tr>
<tr>
<td>AS2622DEN4</td>
<td>5’TAGCTCGTTGGTTAATTGYTTCCAC-3’</td>
<td>2624 to 2648</td>
<td></td>
</tr>
<tr>
<td>S2176DEN1</td>
<td>5’ATCCTGGGAGACACYGCNTGGG-3’</td>
<td>2174 to 2195</td>
<td>Nested</td>
</tr>
<tr>
<td>S2176DEN2</td>
<td>5’ATTTTRGTTACAGCGNTGGG-3’</td>
<td>2176 to 2197</td>
<td></td>
</tr>
<tr>
<td>S2176DEN3</td>
<td>5’ATCTTGAGAGACACAGCTGGG-3’</td>
<td>2168 to 2189</td>
<td></td>
</tr>
<tr>
<td>S2176DEN4</td>
<td>5’ATCTCAGGAGAACAGCNTGGG-3’</td>
<td>2178 to 2199</td>
<td></td>
</tr>
<tr>
<td>AS2504DEN</td>
<td>5’TGAAAYTTRATYGCTCTGTC-3’</td>
<td>2506 to 2527</td>
<td>Nested</td>
</tr>
</tbody>
</table>

*Primers names beginning with “S” indicate a genome (plus)-sense orientation; names beginning with “AS” indicate a complementary sense orientation.

†The genome positions are given according to each dengue virus serotype prototype strain (DEN-1: strain Mochizuki, DEN-2: strain Jamaica N-109, DEN-3: strain H87, DEN-4: strain 814669)

‡Degenerate positions: N:A/C/g/T, R:A/g, Y:T/C
The specificity of the RT-nested PCR was determined by analysing serial dilutions of RNA from related flavivirus (JEV, MVEV, SLEV, TBEV, WNV, YFV) and no amplification was obtained (data not shown).

The amplification was successful with both commercial RNA and serum samples for all dengue virus serotypes as shown (Figure 1), yielding a distinct DNA product of the expected size (328-pb) in agarose gels.

Figure 1. Amplification products obtained through PCR analysis of sera from subjects with acute dengue virus infection

[Arrow indicates 328 bp E/NS1 products. 1% agarose gel. MWM: Molecular weight markers; 438VI03 (DEN-1); 13VI02 (DEN-2); 1794F02 (DEN-3); 366VI03 (DEN-4)]

One hundred and sixty-four serum samples from cases of febrile illness associated with travel were tested with the assay. Thirty-seven cases were diagnosed as of classical dengue fever by the WHO criteria[19]. Sixteen of these cases were found positive by using our E/NS1 assay. Convalescent sera were available for 13 of these cases; all were later confirmed to be seroconvert. All serum samples found positive by RT-nested PCR were collected in the first week after the onset of the symptoms.

Sequence analysis results

The phylogenetic trees obtained by the analysis of the representative strains of the four serotypes and unknown sample sequences allowed rapid differentiation of the corresponding serotype (Figure 2).

Pair-wise sequence analysis using Needleman Wunsch global alignment was carried out on the 220bp sequence where a higher amount of sequences were available for comparison. As expected, comparisons between serotypes showed a low sequence similarity and could be easily grouped. An all-against-all sequence comparison was done within each serotype to evaluate the possibility of using sequence similarity to classify genotypes. Significant sequence similarity was observed when comparing sequences within the same genotype. This was evaluated by an analysis of variance between groups (ANOVA), comparing the scores of sequence comparisons within genotypes to comparisons between genotypes. Each group was significant to the P<0.001 level. Genotypes with only one member sequence were excluded from the analysis.
Figure 2. **Phylogenetic tree constructed with the E/NS1 fragment which identifies the four dengue serotypes**

[Phylogenetic analysis was performed using the Kimura-two parameter model as a model of nucleotide substitution and using the neighbor joining method to reconstruct the phylogenetic tree (MEGA version 2.1 software package)]

```
DENGUE SEROTYPE 1

YellowFever
WestNile
JVE

DENGUE SEROTYPE 2

DENGUE SEROTYPE 3

DENGUE SEROTYPE 4

Sequences that had no known genotype were classified with respect to the group to which they were most similar. To verify the utility of this method, a phylogenetic tree was built in parallel with the unknown and characterized sequences. Bootstrap values in the 220bp region were too low to generate a full taxonomy tree, but did fully differentiate the genotypes (data not shown). Even with this simple method, all unknowns were classified correctly into their genotypic group (Figure 3 illustrates one example result for each serotype, compared to known sequences).
Figure 3. Pair-wise analysis of four dengue strains detected by PCR amplification of 328 bp E/NS1 products from patient sera. Samples are (a) 438VI03, DEN-1 AMERICAN-AFRICAN genotype, (b) 13VI02, DEN-2 INDIAN genotype, (c) 1794F02, DEN-3 COSMOPOLITAN genotype, and (d) 366VI03, INDONESIAN DEN-4 genotype.

**Discussion**

The efficient worldwide control of dengue virus requires the definition of sources of epidemic viruses and the precise identification of virus genotypes. A key objective of DF and DHF surveillance programmes is early detection of outbreaks to permit the implementation of control measures. DHF outbreaks can be anticipated by monitoring the emergence of new genotypes in a region. The need for surveillance is warranted, since air travellers can quickly move viruses from an endemic area to a receptive area. Dengue virus surveillance should be implemented in endemic and non-endemic areas to aid governments and healthcare workers in planning for potential outbreak situations. The advent of a simple and accurate method for diagnosis and surveillance could improve the establishment of these programmes in developing countries affected by the disease, and in non-endemic areas where dengue is a travel-acquired infection.

The RT-nested PCR described here allows rapid direct diagnosis of acute dengue infection in laboratories without BSL-3 (bio-safety level 3) facilities.

Pair-wise comparison to classify sequences has been used for enteroviruses and potyvirus[26-28]. Multiple alignment and
rigorous phylogenetic methods are preferable to establish exact lineages of sequence strains and discover recombination events. Pair-wise comparisons can substitute if only a high level of taxonomic classification is desired. Our method allows classification of dengue genotypes using the sequence of the 220bp region amplified by the PCR assay. The advantage of pair-wise comparison for classification is its speed, simplicity and availability. The database and classification scheme provides a repository for sequences, complementing efforts in tracking dengue genotype distribution. A website could be deployed wherein clinical laboratories post their sequences, location and circumstances of isolation. This would allow rapid centralized analysis detailing the genotype, date and location of the most similar sequence isolate in the database. New genotypes could be rapidly identified by failure to relate them to a described group.

Acknowledgements

This investigation received financial support from the Instituto de Salud Carlos III (ISCIII) through research project grants (MPI 1194/02 and C03/04). G. Palacios and WI Lipkin were supported by the Ellison Medical Foundation and the National Institutes of Health (AI 51292 and U54 AI57158-Lipkin). C. Domingo was contracted by an agreement between the Public Health Division of the Spanish Ministry of Health (DGSP-MSC) and the Instituto de Salud Carlos III (ISCIII) for the development of the Haemorrhagic Viral Fevers Surveillance and Control Programme. The authors thank Dr J. Gascón, Dr R. López-Vélez and Dr S. Puente and the many scientists who contributed dengue-infected patient samples for this work. The authors are grateful to Dr J.E. Mejia for assisting in manuscript preparation.

References


Clinical and Laboratory Observations Associated with the 2000 Dengue Outbreak in Dhaka, Bangladesh


*Department of Virology, Dhaka Medical College, Dhaka, Bangladesh
**Department of Virology, Bangabandhu Sheikh Mujib Medical University, Shahbag, Dhaka, Bangladesh
***Shahid Suhrawardi Hospital, Dhaka Bangladesh

Abstract

A large outbreak of dengue fever (DF)/dengue haemorrhagic fever (DHF) occurred in Dhaka city, Bangladesh, in 2000. The present study was conducted on 105 clinically-suspected cases of DF to confirm the diagnosis, determine the major clinical manifestations and correlate the haemorrhagic manifestations with different dengue serotypes circulating during the outbreak. A total of 97 cases were positive for anti-dengue IgM and were considered as recent dengue infection; 52.6% patients had secondary and 47.4% had primary dengue infection. According to WHO case-definition, 79 cases were classified as DF, 17 as DHF and 1 as DSS. Among the 18 DHF/DSS cases, 14 had secondary and 4 had primary type of antibody response. The mean age of the dengue patients was 29.2±12.9 years and most of them (37.1%) belonged to the 20-29-year age group. All the clinically-suspected patients had fever ranging from 100-104 °F, but the secondary dengue fever patients had higher (101.6±1.4 °F) mean body temperature. Common complaints included myalgia (84.5%), headache (82.5%), arthralgia (68.0%), lethargy (80.4%) and retro-orbital pain (49.5%). Rash, especially maculopapular type, was significantly higher in primary infection (P<0.01), while hepatomegaly was higher in secondary infections (P<0.01). Haemorrhagic manifestations were observed both in primary and secondary dengue patients and were mostly associated with serotypes DEN-3. In 22.7% of cases, the platelet count was <1x10^5/mm^3 and was associated more with secondary infection. Haematocrit more than 45% was found in 16.5% patients and a significantly higher association was detected among the secondary dengue fever patients (P=0.02). Although all 4 dengue serotypes were prevalent during the outbreak, DEN-3 was the predominant serotype (70.5%) and was associated with more severe clinical manifestations.

Keywords: Primary dengue fever, secondary dengue fever, dengue haemorrhagic fever, serotypes, Bangladesh.

# E-mail: msakib@dhaka.net
Introduction

Dengue was first reported in Bangladesh in 1964 and the outbreak came to be known as ‘Dacca Fever’[1]. For a long period after that, dengue cases remained undetected. A few cases were reported in 1999 before a large outbreak occurred in 2000, during which 5,551 cases and 93 dengue-related deaths were reported[2,3]. Cases reported during the outbreaks were mostly diagnosed clinically, except for a few serologically diagnosed cases[4].

Several studies have observed that sequential or secondary dengue infections are more likely to produce a severe form of the disease. DHF and DSS occur mostly in persons with pre-existing dengue antibodies acquired actively or passively[5-7].

Detection of anti-dengue IgM indicates the diagnosis of recent dengue infection in both primary and secondary cases. Anti-dengue IgM develops earlier than IgG in primary infection and is usually detectable by day 5 of illness and wanes after 1-2 months[8]. The ratio of IgM and IgG antibodies determined by ELISA is useful for distinguishing primary and secondary infections[8,9]. In primary infection, the IgM/IgG ratio generally exceeds 1.8 OD units in acute or convalescent sera[8]. Detection of an early and excess of IgG characterizes secondary infection. In the present study, dengue patients were classified into primary and secondary cases on the basis of this concept and it was carried out to confirm the clinical diagnosis, correlate the clinical manifestations with laboratory findings and establish the association of haemorrhagic manifestations with different dengue virus serotypes responsible for the outbreak of 2000.

Materials and methods

The study was conducted on clinically suspected cases of dengue fever attending the inpatient and outpatient departments of Medicine and Paediatrics, Bangabandhu Sheikh Mujib Medical University (BSMMU) Hospital, and patients admitted at the Dhaka Medical College Hospital during June – December 2000. Some patients seeking diagnostic facilities at the Department of Virology, BSMMU, were also included in the study.

According to specific inclusion criteria, 105 clinically suspected patients with fever (presenting within 5 days of onset with body temperature above 100 °F at the time of blood sample collection) and fulfilling the case-definition criteria of dengue fever (DF) and dengue haemorrhagic fever (DHF) of WHO[10] were enrolled in the study. The exclusion criteria defined cases of febrile illness of more than 5 days and/or with definite sources of infection, chronic illnesses like tuberculosis, bronchial asthma, congenital heart disease, renal failure, history of bleeding tendency since birth and patients who refused to give two blood samples.

Clinical data were collected through interviewing the patients or their attendants and meticulous physical examination of the patients conducted by a doctor. The tourniquet test was performed in all patients by conventional method[11]. Hepatomegaly and ascites were ascertained by physical examination and on reports of ultrasonography and X-rays. 5 ml of venous blood was collected aseptically from all patients during both the early and convalescent stages of fever irrespective of age and sex. This was processed and stored appropriately for virus isolation, antibody assay, platelet count and haematocrit.
estimation. Detection of IgM and IgG anti-dengue antibody, isolation of dengue viruses by mosquito inoculation technique and serotyping of the isolated viruses were done as described previously. Patients were classified into DF and DHF or DSS according to WHO, 1997.

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

Results
Of the 105 clinically suspected dengue patients, 39 (37.1%) were positive for either anti-dengue IgM or IgM and IgG antibodies and 38 (36.2%) patients developed only anti-dengue IgG in acute stage by ELISA. When these patients were tested in the convalescent stage, 21 (20%) were positive for only IgM and 76 (72.4%) were positive for both IgM and IgG antibodies. Eight patients did not develop anti-dengue IgM and IgG in either of the specimens (Table 1) and were also negative on virus isolation. Thus, a total of 97 (92.4%) cases were diagnosed serologically as current dengue infection, while 8 (9.8%) cases were considered as non-dengue febrile illness and were excluded from further analysis. Of the 97 dengue patients, 46 (47.4%) had primary and 51 (52.6%) had secondary infection depending on anti-dengue IgM and IgG antibody level of acute and convalescent stage sera (Table 2). Among them, 17 patients developed DHF and one developed DSS. Of the 18 DHF/DSS patients, 14 had secondary type of infection and 4 had primary infection.

Table 1. IgM and IgG dengue antibody positive cases by ELISA

<table>
<thead>
<tr>
<th>Time of serum collection</th>
<th>Only IgM antibody positive cases n (%)</th>
<th>Only IgG antibody positive cases n (%)</th>
<th>Both IgM and IgG antibody positive cases n (%)</th>
<th>IgM and IgG antibody negative cases n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute stage serum (within 5 days of fever)</td>
<td>21 (20.0)*</td>
<td>38 (36.2)</td>
<td>18 (17.1)*</td>
<td>28 (26.7)</td>
<td>105 (100)</td>
</tr>
<tr>
<td>Convalescence stage serum (within 14-21 days of fever)</td>
<td>21 (20.0)**</td>
<td>00 (0.0)</td>
<td>76 (72.4)**</td>
<td>8 (7.6)**</td>
<td>105 (100)</td>
</tr>
</tbody>
</table>

* Total number of dengue infection cases detected in acute stage serum (21 + 18) = 39 (37.1%).
**Total number of dengue infected cases detected in convalescence serum (21 + 76) = 97 (92.4%).
***Non-dengue febrile illness cases = 8 (7.6%).
Figures in parenthesis indicate percentage.
### Table 2. Age distribution of dengue patients by primary and secondary dengue infection

<table>
<thead>
<tr>
<th>Age in years</th>
<th>Primary infection n (%)</th>
<th>Secondary infection n (%)</th>
<th>Total n</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>3 (42.9)</td>
<td>4 (57.1)</td>
<td>7</td>
</tr>
<tr>
<td>10-19</td>
<td>4 (44.4)</td>
<td>5 (55.6)</td>
<td>9</td>
</tr>
<tr>
<td>20-29</td>
<td>20 (55.6)</td>
<td>16 (44.4)</td>
<td>36</td>
</tr>
<tr>
<td>30-39</td>
<td>11 (45.8)</td>
<td>13 (54.2)</td>
<td>24</td>
</tr>
<tr>
<td>40-49</td>
<td>7 (53.8)</td>
<td>6 (46.2)</td>
<td>13</td>
</tr>
<tr>
<td>&gt;50</td>
<td>1 (12.5)</td>
<td>7 (87.5)</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>46 (47.4)</td>
<td>51 (52.6)</td>
<td>97 (100.0)</td>
</tr>
</tbody>
</table>

*Mean±SD 27.5±11.0 30.7±14.4 29.2±12.9

P value 0.214 (unpaired student’s ‘t’ test)

* Mean of age

Figures in parenthesis indicate percentage

The involvement of all age groups, especially an adult predominance, was observed. The mean age of the dengue patients was 29.2±12.9 years and most belonged to the 20-29-year age group. The mean age of primary dengue fever patients was 27.5±11.7 years and that of secondary patients was 30.7±14.4 years.

Dengue viruses were isolated from 44 of the 97 dengue patients. The rate of dengue virus isolation was significantly higher (68.2% vs 31.2%) among primary than secondary infection patients (P=0.018). The isolation rate decreased gradually with the increasing duration of fever (see Figure).

**Figure. Isolation rate of dengue virus between primary and secondary dengue patients by day of fever**

![Isolation rate of dengue virus between primary and secondary dengue patients by day of fever](image)

(P = 0.018)

P value reached from Z test
The distribution of clinical manifestations in dengue cases is given in Table 3. The mean body temperature of the dengue patients was 101.5±1.4 °F but there was no significant difference in the mean body temperature between primary and secondary DF patients. Other common symptoms included myalgia (84.5%), headache (82.5%), arthralgia (68%), lethargy (80.4%) and retro-orbital pain (49.5%). Patients with primary dengue infection presented more commonly with headache, arthralgia and retro-orbital pain, whereas lethargy was commonly associated with secondary dengue infection.

The most common presenting sign was rash, especially maculopapular type, and its association was significantly higher with primary DF cases (P=0.016). Ascitis was observed in 5 (9.8%) cases; all had secondary DF. Hepatomegaly was present in 13 (13.4%) patients and its association was significantly higher (P=0.002) in secondary DF patients. Abdominal pain (6.2%) and lymphadenopathy (4.1%) was less frequent among our study patients though abdominal pain was more common (7.8%) in secondary DF patients.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Types of dengue</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary infection n=46</td>
<td>Secondary infection n=51</td>
<td>Total n=97</td>
<td>P-value</td>
</tr>
<tr>
<td>Mean temperature (°F)</td>
<td>101.4±1.4</td>
<td>101.6±1.4</td>
<td>101.5±1.4</td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>41 (89.1)</td>
<td>39 (76.5)</td>
<td>80 (82.5)</td>
<td></td>
</tr>
<tr>
<td>Arthralgia</td>
<td>34 (73.9)</td>
<td>32 (62.7)</td>
<td>66 (68.0)</td>
<td></td>
</tr>
<tr>
<td>Retro-orbital pain</td>
<td>25 (54.3)</td>
<td>23 (45.1)</td>
<td>48 (49.5)</td>
<td></td>
</tr>
<tr>
<td>Myalgia</td>
<td>39 (84.8)</td>
<td>43 (84.3)</td>
<td>82 (84.5)</td>
<td></td>
</tr>
<tr>
<td>Lethargy</td>
<td>36 (78.3)</td>
<td>42 (82.4)</td>
<td>78 (80.4)</td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td>19 (41.3)</td>
<td>13 (25.5)</td>
<td>32 (32.9)</td>
<td></td>
</tr>
<tr>
<td>Maculopapular</td>
<td>17 (36.9)</td>
<td>8 (15.7)</td>
<td>25 (25.8)</td>
<td>0.016⁺</td>
</tr>
<tr>
<td>Petechial</td>
<td>2 (4.3)</td>
<td>5 (9.8)</td>
<td>7 (7.2)</td>
<td></td>
</tr>
<tr>
<td>Anorexia, nausea and vomiting</td>
<td>16 (34.8)</td>
<td>19 (37.3)</td>
<td>35 (36.1)</td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>2 (4.3)</td>
<td>4 (7.8)</td>
<td>6 (6.2)</td>
<td></td>
</tr>
<tr>
<td>Ascitis</td>
<td>0 (0.0)</td>
<td>5 (9.8)</td>
<td>5 (5.1)</td>
<td></td>
</tr>
<tr>
<td>Enlarged lymph node</td>
<td>2 (4.3)</td>
<td>2 (3.9)</td>
<td>4 (4.1)</td>
<td></td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>1 (2.2)</td>
<td>12 (23.5)</td>
<td>13 (13.4)</td>
<td>0.002⁺</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage
P-value reached from chi-square analysis
Haemorrhagic manifestations in primary and secondary dengue infections are indicated in Table 4. The most common sign of bleeding manifestation, i.e. a positive tourniquet test, was observed in 18 (18.6%) patients. Petechiae 15 (15.5%), purpura 12 (12.4%), gum bleeding 12 (12.4%) and haematemesis/melaena 11 (11.3%) were the other bleeding manifestations. Besides these, conjunctival bleeding, haematuria and per rectal bleeding also occurred in a small number of patients.

**Table 4.** Haemorrhagic manifestations among primary and secondary dengue cases

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Types of dengue</th>
<th>Total n=97</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary infection n=46</td>
<td>Secondary infection n=51</td>
</tr>
<tr>
<td>Positive tourniquet test</td>
<td>7 (15.2)</td>
<td>11 (21.6)</td>
</tr>
<tr>
<td>Petechiae</td>
<td>8 (17.4)</td>
<td>7 (13.7)</td>
</tr>
<tr>
<td>Purpura</td>
<td>5 (10.9)</td>
<td>7 (13.7)</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>1 (2.2)</td>
<td>2 (3.9)</td>
</tr>
<tr>
<td>Haematemesis/melaena</td>
<td>8 (17.4)</td>
<td>3 (5.9)</td>
</tr>
<tr>
<td>Gum bleeding</td>
<td>5 (10.9)</td>
<td>7 (13.7)</td>
</tr>
<tr>
<td>Per vaginal bleeding</td>
<td>2 (4.3)</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>Conjunctival bleeding</td>
<td>1 (2.2)</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Haematuria</td>
<td>1 (2.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Per rectal bleeding</td>
<td>0 (0.0)</td>
<td>2 (3.9)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage

The haematological features in primary and secondary dengue infections are given in Table 5. Platelet counts of <1x10^5/mm^3 was detected in 22 (22.7%) patients and was more frequently 16 (31.4%) associated with secondary DF (Table 5). Haematocrit value of >45% was observed in 16 (16.5%) patients and a significantly higher association of >45% haematocrit level was detected among secondary DF patients (P=0.02).

Haemorrhagic manifestations were mostly associated with DEN-3 and DEN-4 infections; only one patient with DEN-1 infection had per rectal bleeding (Table 6).
Table 5. Haematological features in primary and secondary dengue infection

<table>
<thead>
<tr>
<th>Haematology</th>
<th>Types of dengue infection</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary infection n= 46</td>
<td>Secondary infection n= 51</td>
<td>Total n= 97</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td>Platelets/cu mm</td>
<td>6(13.1)</td>
<td>16(31.4)</td>
<td>22(22.7)</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td>&lt;1×10^5</td>
<td>40(86.9)</td>
<td>35(68.6)</td>
<td>75(77.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;45%</td>
<td>3(6.5)</td>
<td>13(25.5)</td>
<td>16(16.5)</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>&lt;45%</td>
<td>43(93.5)</td>
<td>38(74.5)</td>
<td>81(83.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage
P-value reached from chi-square analysis

Table 6. Haemorrhagic manifestations in different serotypes of dengue virus infections

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Serotypes of dengue viruses</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEN-1 (n= 6)</td>
<td>DEN-2 (n= 3)</td>
<td>DEN-3 (n= 31)</td>
<td>DEN-4 (n= 4)</td>
</tr>
<tr>
<td>Positive tourniquet test</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>6 (19.4)</td>
<td>3 (75.0)</td>
</tr>
<tr>
<td>Petechiae</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (3.2)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Purpura</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (9.7)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Epistaxis</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (3.2)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Haematemesis/melaena</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>4 (12.9)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Gum bleeding</td>
<td>0 (0.0)</td>
<td>1 (33.3)</td>
<td>4 (12.9)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Haematuria</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (3.2)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Per rectal bleeding</td>
<td>1 (16.7)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (25.0)</td>
</tr>
</tbody>
</table>

Figures in parenthesis indicate percentage

Discussion

The secondary dengue infection is usually associated with more severe manifestations than the primary infection. In our study, out of the 97 dengue cases, 46 (47.4%) had primary and 51 (52.6%) had secondary dengue infection. The presence of secondary cases indicated that DF was present in the area and perhaps a low-grade transmission was continuing during the previous years. Thus, it may be speculated that a considerable proportion of cases, which were diagnosed as “viral flu” in the
past, may have been due to dengue virus infection. On serological test in acute stage, only 39 (37.1%) cases were found to be positive for dengue antibody. However, when the serum from the patients was tested again in the convalescence stage, 97 (92.4%) cases were found to be positive, indicating that they had recent dengue virus infection (Table 1). Thus, the detection of a significant number of cases could have been missed if the tests were not done again at the convalescence stage. A second test should therefore be considered at the convalescence stage to delineate the actual numbers of infection due to dengue.

Our study indicates the involvement of all age groups of the population with adult predominance. This finding is not consistent with the epidemiological data from other endemic countries in Asia where young children were affected\[14,15\]. However, adults may also be the major victims of DHF as reported in different epidemics where dengue was endemic\[16,17,18\]. No significant age or sex difference among patients was observed between primary and secondary infections in the present study. Similar observations were also reported from an outbreak in Fiji\[11\].

Anorexia, nausea, vomiting, abdominal pain and ascites were associated more with secondary than primary dengue infections (Table 3). In the present study, a statistically significant (P=0.01) association of rash (32.9%) among primary dengue infection cases was demonstrated. The predominant type of rash in primary infection was macular or maculopapular whereas petechial rash was found frequently in secondary infection. Other studies also reported similar association of rash in DF\[11,19\]. Petechiae were frequently found in DHF in Cambodian\[20\] and Thai children\[21\], while it was less frequent in patients in our study. In the present study, statistically significant (P=0.002) hepatomegaly (13.4%) in secondary dengue infection was noted. Hepatomegaly was also a common clinical finding in several other studies\[22-28\]. Acute abdominal pain, which is considered as an early sign of shock in DF/DHF\[19\], was present in only 6 (6.2%) patients in the present study. This is similar to a study from Lucknow, India, where abdominal pain was found in only 5% of cases in an epidemic of DHF\[19\]. In some studies, acute abdominal pain was strongly correlated with DHF and DSS\[20,24,25\]. In the present study, ascites, a sign of plasma leakage, was present in only 5 (9.8%) secondary infection cases and was in agreement with the findings of other studies\[26,28\].

The positive tourniquet test, which reflects capillary fragility and is used as a guiding test for detecting dengue illness\[27\], was found in only 18.6% cases in the present study. There was no statistically significant difference in test positivity between primary and secondary DF patients although it was more frequently positive in secondary DF. In most studies, comparing DHF with classic DF, either a higher incidence of tourniquet test positivity in DHF\[23,26,28\] or no difference between the two groups\[24,28\], was reported. These variable findings are probably because the pathogenesis of tourniquet positivity and other bleeding manifestations in dengue cases are different. Moreover, the defects which lead to increase in capillary fragility may also predispose to the development of shock, thus indicating a better correlation of the test to shock rather than to haemorrhage.
In our study, platelet counts of $<1 \times 10^5/mm^3$ were observed among 22 patients and were associated more with secondary than primary infections (Table 4). Lack of correlation between a moderately low platelet count and bleeding manifestation in dengue patients has been noted by other investigators\[8\]. The bleeding in dengue is probably due to other factors with or without the additive effect of thrombocytopenia. Haemoconcentration, the evidence of plasma leakage and shock, was observed in 16 patients with secondary infection and the association was statistically significant ($P=0.02$). Increased haematocrit was found in 95.5% patients with shock in contrast to 31.7% cases without shock\[21\].

In the present study, the virus isolation rate was significantly higher in patients with primary (68.2%) than secondary (31.2%) infections ($P=0.018$). Similar results have been reported in a study from Fiji\[11\]. In Bantal, Indonesia, the isolation rate was 100% among patients with primary dengue infection and 57% with secondary dengue infection\[30\]. However, a study from Thailand reported a high rate of virus isolation in both primary (98%) as well as secondary (93%) infections\[31\]. The reason for a higher isolation rate in secondary infection was probably because blood samples were collected at an early febrile phase (within 72 hours). In our study, the isolation rate was also high in both primary (100%) and secondary (68%) infections in early febrile period, i.e. on day 1 of fever. Thereafter, the rate of isolation was always higher in primary than in secondary dengue infection in the subsequent days of fever (Figure).

In conclusion, the clinical diagnosis is not very reliable in dengue infection as there is a wide variation in the presence of various symptoms, which may misguide the physician regarding the severity of the disease. Therefore, the use of clinical case-definition results in inaccuracies. Thus, in order to identify the cases accurately, one should take the help of a simple and cheap diagnostic test that is able to diagnose accurately in the laboratory. In this context ELISA is clearly very promising for the confirmation of clinical diagnosis and to differentiate between primary and secondary infections. This will guide the clinician to take prompt and meticulous clinical management and early hospitalization of severe cases. Indeed, it is hoped that accurate laboratory diagnosis will not only reduce the morbidity and mortality but will also reduce the economic burden of the patient and the government.

Acknowledgements

We express our sincere thanks to Dr Vorndam Vance, CDC Dengue Lab, San Juan, Puerto Rico, USA, and Dr Ichiro Kurane, Arbovirus Research Laboratory, NIID, Japan, for their kind support in providing cell line and anti-dengue monoclonal antibodies. We are also grateful to the patients who participated in the study. We would like to thank Mr Tauhid Uddin Ahmed, Ex-PSO, IEDCR, Bangladesh, and Noor-e-Jannat for providing mosquito colony and rearing techniques. We would also like to thank Dr Bijon Kumar Sil, Ex-Senior Scientific Officer, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh. We also express our gratitude to the Department of Medicine and Paediatrics, BSMMU, and Dhaka Medical College, Bangladesh, for their support in the study.
References


Clinical and Laboratory Observations during the 2000 Dengue Outbreak in Dhaka


Current Status of Dengue Diagnosis at the Center for Disease Control, Taiwan

Pei-Yun Shu, Shu-Fen Chang, Yi-Yun Yueh, Ling Chow, Li-Jung Chien, Yu-Chung Kuo, Chien-Lin Su, Tsai-Ling Liao, Ting-Hsiang Lin and Jyh-Hsiung Huang

Center for Research and Diagnostics, Center for Disease Control, Department of Health, 161, Kun-Yang Street, Taipei, Taiwan

Abstract

A national-level diagnostic laboratory has been set up in Taiwan for routine diagnosis of reported cases of dengue fever (DF)/dengue haemorrhagic fever (DHF), Japanese encephalitis (JE) and yellow fever (YF). The facilities include serological diagnosis, virus isolation by cell culture, molecular diagnosis and molecular tools for epidemiological investigations. To detect and differentiate dengue, JE and YF virus infections, a differential diagnostic system has been developed. For acute-phase sera, virus isolation by cell culture and real-time one-step reverse transcription-polymerase chain reaction (RT-PCR) has been established. For all of the serum samples reported, serological diagnosis of specific antibodies based on envelope and membrane (E/M)-specific capture IgM and IgG enzyme-linked immunosorbent assay (ELISA) are performed. In this report, a case study from Taiwan has been presented with the analysis of 959 serum samples (including some paired sera) collected between day 1-30 of illness from 799 confirmed dengue cases reported in 2002. The results demonstrated that 94.5% of acute-phase serum samples of confirmed dengue cases could be identified as positive or probable with the combined use of real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA. Furthermore, a nonstructural protein NS1 serotype-specific indirect IgG ELISA has been developed and used to analyse dengue NS1-specific IgG antibodies. Both E/M-specific capture IgM and IgG ELISA and the NS1 serotype-specific indirect IgG ELISA have been used to detect and differentiate primary and secondary dengue virus infections. In addition, the NS1 serotype-specific indirect IgG ELISA has the potential of replacing the plaque-reduction neutralization test (PRNT) and is being used for a large-scale seroepidemiological study.

Keywords: Dengue virus, virus isolation, real-time one-step RT-PCR, E/M-specific capture IgM and IgG ELISA, NS1 serotype-specific indirect IgG ELISA.

E-mail: jhhuang@cdc.gov.tw Tel.: 886-2-26531374, Fax: 886-2-27883992

Introduction

The dengue viruses cause a broad spectrum of illness ranging from inapparent infection, mild undifferentiated fever and classic dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), resulting in high morbidity and mortality. The diagnosis of dengue virus infection based on clinical syndromes is not reliable, and a confirmation of the infection should rely on laboratory diagnosis with the detection of the specific virus, viral antigen, genomic sequence and/or antibodies.

A rapid, simple, sensitive and specific assay system to detect the virus in the acute-phase serum is essential to improve the clinical treatment, etiological investigation and disease control of dengue virus infection. Among the various assays for virus detection, virus isolation by cell culture and dengue virus antigen detection by ELISA suffer from some disadvantages - while the former needs a longer time, the latter has low sensitivity. However, recent advances in molecular diagnosis have demonstrated that various RT-PCR protocols can be reliably used to detect the viral genomic sequence with high sensitivity and specificity. More recently, several investigators have reported real-time RT-PCR assays for the detection of dengue virus in acute-phase serum samples. The real-time RT-PCR assay has many advantages over the conventional RT-PCR methods, which include rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardization.

For convalescent sera, detection of specific IgM and IgG antibodies based on haemagglutination inhibition (HI) test and E/M-specific capture IgM and IgG ELISA are the two most commonly used serological techniques for the routine diagnosis of flavivirus infection. The serodiagnosis of flavivirus is rather complicated due to the high cross-reactivity of IgG antibodies to homologous and heterologous viruses. We have attempted to set up an ELISA system that can be easily and reliably used to detect and differentiate various flavivirus infections. To accomplish this goal, three different forms of ELISA were developed including: (i) E/M-specific capture IgM and IgG ELISA; (ii) E/M-specific antigen-coated indirect IgM and IgG ELISA; and (iii) NS1 serotype-specific indirect IgG ELISA.

Case study of 2002 outbreak of DEN-2 in southern Taiwan

We present here a case study of the analysis of biological material obtained during a DEN-2 outbreak in 2002 in southern Taiwan, by utilizing the facilities available in the national diagnostic laboratory.

A major DEN-2 epidemic occurred in southern Taiwan, affecting Kaohsiung city, Kaohsiung county and Pingtung county between October 2001 and December 2002, with more than 5,000 confirmed cases. Among these, 227 cases were classified as of DHF with 21 deaths. This outbreak was a repeat of the 1987-1988 DEN-1 epidemic in many aspects. In this report, we present the results of a total of 959 acute- and convalescent-phase sera collected from 799 confirmed dengue patients reported to the Kun-Yang office of the Center for Disease Control (CDC), Taiwan, 2002.
Materials and methods

Human serum samples
The serum samples used in this study were collected from the confirmed cases of dengue patients reported to the Arbovirus Laboratory in the Kun-Yang office, CDC, Department of Health, 2002. A total of 959 acute- and convalescent-phase sera collected from 799 confirmed dengue patients were analysed. Most of these serum samples were from the major DEN-2 outbreak, together with a few serum samples from imported cases contracted during travel to the neighbouring South-East Asian countries.

Case definitions
A confirmed case of dengue virus infection was defined as febrile illness associated with: (i) the isolation of dengue virus; (ii) positive test of real-time one-step RT-PCR; (iii) positive seroconversion or ≥ four-fold increase in dengue-specific IgM or IgG antibody from appropriately timed paired serum; or (iv) high-titer dengue-specific IgM and IgG antibody in a single serum specimen where cross-reaction to Japanese encephalitis (JE) had been excluded. Sera collected during day 1-7 after the onset of symptoms are referred to as acute-phase sera. Early and late convalescent sera refer to the specimens collected during day 8-13 and day 14-30, respectively.

Virus isolation by cell culture and virus antigen preparation
The isolation of dengue virus by cell culture and virus antigen preparation from culture supernatants of DEN-1, DEN-2, DEN-3, DEN-4 or JE-virus infected Vero cells were performed as previously described[7,9]. 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.

One-Step SYBR Green I Real-Time RT-PCR
One-step SYBR Green I real-time RT-PCR for dengue virus was performed in the Mx4000™ quantitative PCR system (Stratagene) as recently described[7]. Briefly, a set of flavivirus- (in the NS5 gene region), dengue- and serotype-specific primer pairs (in the core gene region) was selected and used for analysis. To assure the specificity of amplicons produced from SYBR Green I real-time RT-PCR in daily routine screening, both flavivirus- and dengue-specific primer pairs were used for each of the serum samples tested. Serum samples found positive for initial screening were then tested for serotype by each of the four serotype-specific primer pairs.

ELISA

E/M-specific capture IgM and IgG ELISA
A modified E/M-specific capture IgM and IgG ELISA was performed to measure the dengue-specific IgM and IgG antibodies as recently described[13]. Briefly, each microtiter 8 wells strip was coated with 5 µg/ml, 100 µl/well of affinity purified goat anti-human IgM (µ-specific) or IgG (γ-specific) antibodies, followed by incubation with 1:100 diluted serum, incubation with cocktail contained 1:3 diluted pooled virus antigens from culture supernatants of DEN-1, DEN-2, DEN-3 or DEN-4 infected Vero cells.
and 1 µg/ml mAb D56.3, incubation with 1:1,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (γ-specific). The enzyme activity was developed with the substrate p-nitrophenyl-phosphate and optical density (OD) was taken 30 minutes later. For routine screening, culture supernatant from JE virus-infected Vero cells was used as negative control antigen due to the limited cross-reactivity between dengue- and JE-specific IgM antibodies measured by E/M-specific capture IgM ELISA. This was in contrast to the high cross-reactivity of dengue- and JE-specific IgG antibodies among dengue patients with secondary infection.

**E/M-specific antigen-coated indirect IgM and IgG ELISA**

E/M-specific antigen-coated indirect IgM and IgG ELISA were performed as previously described\[10\]. Two-fold serial dilutions of appropriately timed paired sera diluted from 1:100 to 1:12,800 were analysed to determine whether ≥ four fold increase of dengue-specific IgM or IgG antibody could be found. This assay has the advantage of better sensitivity in the detection of IgM and IgG antibody increase than E/M-specific capture IgM and IgG ELISA.

**NS1 serotype-specific indirect IgG ELISA**

NS1 serotype-specific indirect IgG ELISA was performed as previously described\[9,13\]. Briefly, each microtiter 8 wells strip was coated with 5 µg/ml, 100 µl/well of mAbs D2/8-1, followed by incubation with 1:3 diluted NS1-containing culture supernatants of DEN-1, DEN-2, DEN-3, DEN-4 or JE viruses-infected Vero cells, incubation of serum samples at a 1:50 dilution, incubation with goat anti-human IgG conjugated to alkaline phosphatase. The enzyme activity was developed and OD was taken 30 minutes later.

**Data analysis**

For E/M-specific capture IgM and IgG ELISA, primary dengue virus infection was defined if the IgM:IgG OD ratio was ≥1.2, or secondary if the OD ratio was <1.2. For those sera with positive NS1-specific IgG antibody response, NS1 serotyping was calculated by the ratio of the highest OD value and the second highest OD value read from the four dengue serotypes. Positive serotype-specificity is defined if the OD ratio is ≥1.2 and negative serotype-specificity is defined if the OD ratio is <1.2. Based on NS1 serotype-specific indirect IgG ELISA, primary dengue virus infection was defined if: (i) negative NS1-specific IgG antibody response was found for sera collected between day 1 and 14 of illness, or (ii) positive serotype-specificity for sera collected ≥9 days of illness. Secondary dengue virus infection was defined if: (i) positive NS1-specific IgG antibody response was found for sera collected between day 1 and 8 of illness, or (ii) positive NS1-specific IgG antibody response and negative serotype-specificity was found any time after the onset of infection.

**Results**

**Dengue surveillance system and laboratory diagnosis in Taiwan**

Taiwan has an integrated programme for dengue surveillance and control. The dengue prevention and control centre is a
mission-oriented structure jointly sponsored by the Department of Health and the Environment Protection Administration responsible for the planning and execution of dengue control. To assure the effectiveness of dengue surveillance, three report systems are currently associated with the dengue surveillance programme including: (i) hospital-based passive report system; (ii) syndrome report system (under the classification of viral haemorrhagic fever); and (iii) active surveillance system. The Arbovirus Laboratory in Kun-Yang office, Taipei City, CDC, Department of Health, is responsible for the diagnosis of various flaviviruses. In addition, a second dengue diagnostic laboratory was set up in the Fourth Branch, Kaohsiung City, CDC, in July 2002 to provide prompt service to Kaohsiung city, Kaohsiung county and Pingtung county due to the large samples generated by the DEN-2 outbreak. For routine diagnosis, serum samples from the reported cases were sent to the laboratory on a daily basis and tested according to the flow chart shown in Figure 1. The periods of time required to complete these tests were 7 days, 6 hours and 4 hours for virus isolation, real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA, respectively. The results were reported as positive, negative or probable cases. The probable case was referred to ELISA result with only IgM or IgG antibody positive. For negative and probable cases, the convalescent serum samples collected after day 14 of the illness were demanded and tested for the presence of or increase in IgM and/or IgG antibodies.

Figure 1. Flow chart of laboratory diagnosis of dengue virus infection

![Flow chart of laboratory diagnosis of dengue virus infection](chart.png)
Representative results of routine diagnosis measured by virus isolation, real-time one-step RT-PCR, and E/M-specific capture IgM and IgG ELISA

Table 1 shows the representative results of serum samples analysed by virus isolation, real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA. The results provided a good example of the dynamic change of dengue virus and specific IgM and IgG antibodies in the acute- and convalescence-phase sera from patients with primary or secondary dengue virus infection covering all four serotypes. As shown in Figure 1, all of the three assays were performed for acute-phase sera, whereas only E/M-specific capture IgM and IgG ELISA was tested for convalescence-phase sera.

Table 1. Representative results of routine diagnosis of serum samples from reported dengue cases

<table>
<thead>
<tr>
<th>Dengue infection</th>
<th>Dengue serotype</th>
<th>Onset days</th>
<th>Virus isolation</th>
<th>Real-time one-step RT-PCR Ct value</th>
<th>E/M-specific capture IgM and IgG ELISA OD 405 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Flavi-specific</td>
<td>Dengue-specific</td>
<td>Serotype-specific</td>
</tr>
<tr>
<td>Primary infection</td>
<td>DEN-1</td>
<td>3</td>
<td>+</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEN-2</td>
<td>2</td>
<td>+</td>
<td>26</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEN-3</td>
<td>3</td>
<td>+</td>
<td>25</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEN-4</td>
<td>3</td>
<td>-</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary infection</td>
<td>DEN-1</td>
<td>1</td>
<td>+</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEN-2</td>
<td>2</td>
<td>+</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEN-3</td>
<td>1</td>
<td>+</td>
<td>18</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEN-4</td>
<td>7</td>
<td>+</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ = positive
- = negative

Due to the long time needed to isolate virus using the cell culture method, it has limited value in rapid diagnosis. The isolated virus, however, is the key material for the later studies of molecular epidemiology and pathogenesis. The real-time one-step SYBR Green I RT-PCR we developed is a simple, reliable and universal RT-PCR protocol that can be used to systematically detect and differentiate various flavivirus. To assure the specificity of amplicons produced from SYBR Green I real-time RT-PCR in routine screening, both flavivirus- and dengue-specific primer pairs were run (Table 1).
Those serum samples positive for initial screening were then tested for serotype by each of the four serotype-specific primer pairs. The analysis of acute-phase serum samples demonstrated that the one-step SYBR Green RT-PCR was more sensitive to the virus isolation method and could detect two-times more the acute-phase sera with positive dengue-specific IgM and/or IgG antibodies.

The E/M-specific capture IgM and IgG ELISA has several advantages in the detection of dengue-specific IgM and IgG antibodies including: (i) high sensitivity; (ii) high specificity (only for IgM antibody); (iii) analysis of isotype-specific antibody responses; (iv) easy automation to test large amount of serum samples; and (v) differentiation of primary and secondary dengue infections. The results shown in Table 1 demonstrated the low cross-reactivity between dengue- and JE-specific IgM antibody and inverse pattern of IgM:IgG OD ratio of primary and secondary infection.

Therefore, a positive dengue-specific IgM and IgG antibody response can be easily used to detect and differentiate primary and secondary dengue virus infections.

E/M-specific antigen-coated indirect IgM and IgG ELISA for the detection of dengue virus infection

Occasionally, there were acute-phase sera which tested positive with E/M-specific capture IgM or IgG antibody response, but did not show an apparent increase in antibody titers in convalescent sera. Due to the higher sensitivity of E/M-specific indirect IgM and IgG ELISA (especially for IgG antibody), it can be reliably used to determine whether ≥ four-fold increase of dengue-specific IgM or IgG antibody were presented. Figure 2 shows an example where significant dengue-specific IgG antibody increase in a convalescent serum.

Figure 2. E/M-specific antigen-coated indirect IgM and IgG ELISA to detect the increase of dengue-specific IgM and/or IgG antibodies in the pair sera. The serum samples showed a four-fold increase in dengue-specific IgG antibody titer in the convalescent sera.
**Statistical analysis of results of serum samples from confirmed dengue cases reported in 2002**

Table 2 shows the comprehensive analysis of the results of serum samples from confirmed dengue cases reported to Kun-Yang office in 2002. The results of virus isolation, real-time RT-PCR and E/M-specific capture IgM and IgG ELISA were analysed separately or in combination from the sera samples collected on 1-30 day of illness. The positive rate for real-time RT-PCR was 74.7%, 69.5%, 72.3%, 76.6%, 57.7%, 36.3% and 22.2% for day 1-7 of illness, respectively. The positive rate for E/M-specific capture IgM and/or IgG ELISA was 31.6%, 32.6%, 30%, 39%, 52.6%, 87.5% and 80% for day 1-7 of illness, respectively. Thus, the combined results of real-time RT-PCR and E/M-specific capture IgM and IgG ELISA (IgM and/or IgG positive) could detect an average 94.5% (89.7% to 97.5%) of acute-phase serum samples of confirmed dengue cases. The results also showed that the real-time RT-PCR was more sensitive than virus isolation although very few sera, which were virus-isolation positive, were missed by real-time RT-PCR.

**Table 2. Statistical analysis of results of serum samples from confirmed dengue cases reported in 2002**

<table>
<thead>
<tr>
<th>Assays</th>
<th>Days after onset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total serum no. tested</td>
<td>95</td>
</tr>
<tr>
<td>V.I. + (Virus isolation)</td>
<td>53</td>
</tr>
<tr>
<td>% of V.I. +</td>
<td>55.8</td>
</tr>
<tr>
<td>RT-PCR + and V.I. +</td>
<td>52</td>
</tr>
<tr>
<td>RT-PCR + or V.I. +</td>
<td>72</td>
</tr>
<tr>
<td>RT-PCR + (Real-time)</td>
<td>71</td>
</tr>
<tr>
<td>% of RT-PCR +</td>
<td>74.7</td>
</tr>
<tr>
<td>RT-PCR + (Real-time)</td>
<td>24</td>
</tr>
<tr>
<td>ELISA + (E/M-specific capture IgM or IgG*)</td>
<td>20</td>
</tr>
<tr>
<td>Probable (ELISA + IgM or IgG*)</td>
<td>10</td>
</tr>
<tr>
<td>% of IgM + and/or IgG</td>
<td>31.6</td>
</tr>
<tr>
<td>RT-PCR +, V.I., ELISA IgM or IgG</td>
<td>3</td>
</tr>
</tbody>
</table>

Dengue Diagnosis in CDC, Taiwan

114 Dengue Bulletin – Vol 28, 2004
### Dengue Diagnosis in CDC, Taiwan

<table>
<thead>
<tr>
<th>Assays</th>
<th>Days after onset</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>RT-PCR+ and IgM+ IgG+</td>
<td>3</td>
</tr>
<tr>
<td>RT-PCR+ and IgM+ IgG-</td>
<td>6</td>
</tr>
<tr>
<td>RT-PCR+ and IgM IgG+</td>
<td>1</td>
</tr>
<tr>
<td>RT-PCR+ and IgM IgG-</td>
<td>61</td>
</tr>
<tr>
<td>RT-PCR and IgM+ IgG+</td>
<td>17</td>
</tr>
<tr>
<td>RT-PCR and IgM+ IgG-</td>
<td>0</td>
</tr>
<tr>
<td>RT-PCR and IgM IgG+</td>
<td>4</td>
</tr>
<tr>
<td>RT-PCR or ELISA+</td>
<td>88</td>
</tr>
<tr>
<td>% (RT-PCR+ or ELISA+ )/ Total serum no. tested</td>
<td>92.6</td>
</tr>
<tr>
<td>RT-PCR+ or ELISA IgM+ and/or IgG+</td>
<td>91</td>
</tr>
<tr>
<td>% (RT-PCR+ or ELISA IgM+ and/or IgG+)/ Total serum no. tested</td>
<td>95.8</td>
</tr>
</tbody>
</table>

**NS1 serotype-specific indirect IgG ELISA in the differentiation of JE, primary and secondary dengue virus infections and for the DEN serotyping of primary infection**

More recently, we have developed a NS1 serotype-specific indirect IgG ELISA in the detection and differentiation of primary and secondary infections. Comparisons of E/M-specific capture IgM and IgG ELISA and NS1 serotype-specific indirect IgG ELISA showed good correlation with 95.90% agreement[^13]. Most importantly, retrospective sero-epidemiological studies on serum samples collected from Liuchiu Hsiang, Pingtung county and Tainan city in southern Taiwan, demonstrated that NS1 serotype-specific indirect IgG ELISA could replace plaque-reduction neutralization test (PRNT) for seroepidemiological study to differentiate JE,

[^13]: Reference number
primary and secondary dengue virus infections and for the DEN serotyping of primary infection[11].

**Discussion**

Recent advances in molecular and serological assays have revolutionized the laboratory diagnosis of flavivirus infection[7,13,14]. Rapid diagnosis of dengue virus infection in the acute-phase sera, which is important for disease control measures and potential treatment, will require very sensitive and specific assays. With the maturation of real-time RT-PCR technique, its routine application to clinical and laboratory diagnosis has now become a reality. For serodiagnosis, E/M-specific capture IgM and IgG ELISA has become the new standard assay for the detection and differentiation of flavivirus infection.

The large DEN-2 epidemic in southern Taiwan, was uncontrolled despite vigorous attempts to contain it by the central and local health governments during October 2001 – December 2002. Although insecticide-resistance was blamed as an important factor for this disaster, other elements including, political, social, environmental, community and human factors were also responsible for this setback. This epidemic was a strong warning to us and suggested that more effective measures should be sought and applied. There is an urgent need to improve the surveillance system and laboratory diagnosis which would help to identify confirmed cases in the acute-phase sera and respond promptly and effectively to control the transmission chain.

Along with the progress of the DEN-2 outbreak in 2002, we have developed and evaluated the real-time RT-PCR method for rapid detection of dengue virus in the acute-phase sera. In this report, we have presented a detailed analysis of a total of 959 acute- and convalescent-phase sera collected from 799 confirmed dengue patients reported to the Kun-Yang office of CDC, Taiwan, in 2002. The results demonstrated that 94.5% of acute-phase serum samples of confirmed dengue cases could be identified as positive or probable with the combined use of real-time one-step RT-PCR and E/M-specific capture IgM and IgG ELISA. The results are very encouraging and suggest that these two assays are well-suited for routine tests for the early diagnosis of dengue virus infection.

**Conclusion**

The real-time RT-PCR assay has many advantages over conventional RT-PCR methods, which include rapidity, quantitative measurement, lower contamination rate, higher sensitivity, higher specificity and easy standardization. Therefore, real-time quantitative assay might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in the acute-phase serum samples.

**Acknowledgements**

We wish to thank Hsiu-Ling Pan, Yun-Yih Chang and Chih-Heng Chen for their expert technical assistance. This work was in part supported by grants DOH91-DC-2007 and DOH91-DC-2016 from the Center for Disease Control, Department of Health, Taiwan.
References


Detection of Dengue Virus Serotype-specific IgM by IgM Capture ELISA in the Presence of Sodium thiocyanate (NaSCN)

Masaru Nawa*, Tomohiko Takasaki**, Mikako Ito**, Ichiro Kurane** and Toshitaka Akatsuka*

*Department of Microbiology, Saitama Medical School, 38, Moroyama, Saitama 350-0495, Japan
**Division of Vector-Borne Viruses, Department of Virology 1, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Abstract

Dengue virus serotype-specific IgM was detected by IgM-capture enzyme-linked immunosorbent assay (IgM-ELISA) in the presence of a chaotropic agent, sodium thiocyanate (NaSCN). NaSCN did not affect the reactions between anti-human IgM and patients' IgM, and between dengue viral antigens and detecting antibody, peroxidase-conjugated flavivirus-specific monoclonal antibody D1-4G2 IgG. Among 18 dengue-confirmed cases, highest IgM responses were detected to infecting serotypes in 14 cases in the presence of 0.5 M of NaSCN. The results indicate that: (i) the protein-denaturing agent, NaSCN, affects antigen-antibody reaction in IgM-ELISA, and enables the differentiation of serotype-specific IgM from cross-reactive IgM; and (ii) IgM responses against the infecting serotypes are higher than those against the other three serotypes in most primary dengue virus infection. In conclusion, the addition of NaSCN to IgM-capture ELISA is useful for highlighting serotype-specific IgM responses in primary dengue virus infections.

Keywords: Dengue, IgM-capture ELISA, serotype-specific IgM response, sodium thiocyanate, NaSCN.

Introduction

Dengue is currently one of the most important arboviral disease in humans. Dengue viruses, belonging to the family Flaviviridae, are comprised of four antigenically cross-reactive serotypes and are responsible for epidemics in tropical and subtropical countries. Since the dengue outbreaks in Osaka, Kobe, Hiroshima and Nagasaki from 1942 to 1945, dengue has not occurred in an epidemic form in Japan[1]. However, imported dengue cases have been reported[2]. Approximately five million Japanese people annually visit countries in tropical and subtropical areas and nearly two million people visit Japan from these areas. Therefore, dengue fever (DF) and dengue haemorrhagic fever (DHF) has become an infectious disease of significance and worthy of more attention from the medical community in Japan.

We have earlier reported the laboratory diagnosis of dengue by reverse transcription polymerase chain reaction (RT-PCR) and IgM-ELISA[3-5]. We demonstrated that IgM-ELISA was a reliable diagnostic method and that IgM responses were generally serotype cross-reactive but often highest against...
infecting virus serotype in most Japanese cases[4]. The serotype specificity of IgM responses in dengue patients has been controversial[6-8]. Burke had reported that serotype-specific IgM responses corresponding to the isolated virus type were detected in primary dengue virus infection[6]. Gubler had reported that in dengue infection, frequent monotypic IgM responses were not correlated with the virus serotype isolated from patients[7]. In 1984, Inouye et al.[9] demonstrated a new technique for the differentiation of antibody avidity after virus infection, i.e. rubella, rota and Japanese encephalitis viruses. They estimated the antibody avidity to viral antigen using a low concentration of a protein-denaturing agent, guanidine hydrochloride, in the diluent of antibody in the ELISA. They concluded that the “stringent immunosorption” technique was useful for investigating the antigenic relationship among closely-related viruses.

In the present study, we examined serotype-specific IgM responses under stringent conditions in the presence of a chaotropic agent, sodium thiocyanate (NaSCN), in the reaction mixture of dengue viral antigens and patients' sera. The development of a simple method to distinguish serotype-specific reaction from cross-reaction will be useful not only for laboratory diagnosis but also for seroepidemiological studies.

Materials and methods

Twenty-eight serum specimens from 18 confirmed Japanese dengue cases were used in the study. Serum samples from 22 Japanese subjects with other illnesses, who had never been to areas where dengue was epidemic-prone or endemic, were used as the control. These sera were obtained for diagnostic purposes in clinics and hospitals in Japan from 2000 to 2002 and sent to the Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan.

Prototype dengue viruses were propagated in the Aedes albopictus mosquito cell clone C6/36, and the infected cell culture supernatants were inactivated by incubation with beta-propiolactone at a final concentration of 0.2% for 30 minutes at 37 °C as previously reported[10]. Tetravalent and four monovalent dengue viral antigens were prepared by the method as previously reported[4,5].

The IgM capture ELISA was carried out according to the method described previously[3-5]. Anti-human IgM (µ chain specific) goat serum and peroxidase-conjugated anti-human IgM were purchased from Sigma-Aldrich, Inc, USA. The IgG fraction of the flavivirus-specific monoclonal antibody, D1-4G2, was prepared by the Protein G affinity chromatography kit (ImmunoPure G IgG Purification kit, PIERCE, USA), and then conjugated with horseradish peroxidase by a commercial kit (EZ-Link Plus Activated Peroxidase kit, PIERCE, USA).

In order to detect the serotype-specific reaction between dengue viral antigen and dengue virus-specific IgM antibody, patient serum was treated with the Protein G affinity chromatography kit described above. The IgG fraction in the serum specimen was removed by adding Protein G beads according to the manufacturer's instruction. Unless otherwise stated, data were presented as the mean of independent two-to-three assays.

Results and discussion

One of the authors has previously reported that the addition of NaSCN to the reaction mixture of ELISA highlights serotype-specific
reaction between crude dengue viral antigen and anti-dengue hyperimmunized mouse sera. The antigen-antibody reaction was affected not only by the concentration of NaSCN but also by the procedures of the NaSCN treatment. A concentration higher than 0.7 M inhibited the reaction nonspecifically, while a concentration lower than 0.3 M had no effect on the discrimination of serotype-specific reaction from the cross-reactive one. According to these results, we decided to use NaSCN in IgM-capture ELISA at the following conditions: (i) NaSCN was included at a final concentration of 0.5 M in 10% normal calf serum-PBS; (ii) peroxidase-conjugated flavivirus-specific monoclonal antibody D1-4G2 (D1-4G2) was diluted in 0.5 M NaSCN; and (iii) viral antigens captured by patients’ IgM were detected by the detection antibody in 0.5 M NaSCN.

Figure 1. The effects of NaSCN treatment on antigen-antibody reaction

(a) Human serum was serially diluted from 1:50 to 1:400 with 10% CS-PBS containing 0.5 M NaSCN, and IgM was captured on anti-human IgM goat serum-coated wells. IgM was detected with peroxidase-conjugated anti-human IgM goat serum. (b) Serially diluted tetravalent dengue viral antigen was captured on the solid phase sensitized with D1-4G2 IgG, and then detected with peroxidase-conjugated D1-4G2 IgG in the presence of 0.5 M NaSCN.
Figure 1 shows the reactions between anti-human IgM and patients' IgM (a), and dengue viral antigen and the detection antibody (b), in the presence of 0.5 M NaSCN in the ELISA. These two reactions were not affected by 0.5 M NaSCN, suggesting that IgM capture and detection of dengue viral antigens were not affected in IgM-capture ELISA.

Figure 2 shows anti-dengue IgM titration curves in the presence or absence of 0.5 M NaSCN in the ELISA. The IgG fraction in the serum specimen was removed by adsorption with the Protein G beads (UltraLink Immobilized Protein G Plus, capacity: ~25 mg IgG/ml of the gel, PIERCE). The levels of cross-reaction between dengue viral antigens and patients' IgM antibody (a) were decreased after the treatment with 0.5 M NaSCN in the ELISA (b): The level of reaction with homologous dengue-1 antigen was less affected. The results suggested that the addition of 0.5 M NaSCN to the reaction mixture of ELISA may highlight a serotype-specific reaction between crude dengue viral antigen and anti-dengue IgM antibody.

**Figure 2.** Titration curves of dengue virus specific IgM antibody in the presence or absence of 0.5 M NaSCN in the ELISA.

A serum specimen obtained from the dengue virus type 1-infected patient was used in the study. The IgG fraction was removed according to the method described in the Materials and Methods section. Protein G-treated serum specimen was serially diluted from 1:20 to 1:2560 in the absence (a) and in presence of 0.5 M NaSCN (b) in 10% CS-PBS, and then reacted on the plates coated with each of 4 dengue viral antigens. Dengue virus specific IgM antibody was detected with 1:500 diluted peroxidase-conjugated anti-human IgM goat serum.
We examined the IgM levels by ELISA with the antigen of 4 dengue virus serotypes. The table below shows the results of 28 serum samples from 18 Japanese dengue patients. The infecting dengue virus serotypes were determined by RT-PCR. The data were presented as the index value according to the method described previously[4]. We defined index values of 2.28 or greater and 26.60 or greater as positive, respectively, in the absence and presence of 0.5 M NaSCN in the ELISA. In all the tested cases, the serotype-specific IgM levels were the highest against the infecting dengue virus serotype than against three other serotypes in the presence of NaSCN. The serotype-specific IgM responses were more highlighted in the presence of NaSCN than in its absence. These data suggest that IgM responses to the infected dengue virus serotype determined by RT-PCR in primary dengue infection were the highest. These results agreed with the report by Burke[6].

### Table. Serodiagnosis of dengue by IgM-ELISA with and without 0.5M NaSCN

<table>
<thead>
<tr>
<th>Patient</th>
<th>RT-PCR</th>
<th>Disease day</th>
<th>Without NaSCN (cut off = 2.28)</th>
<th>With 0.5M NaSCN (cut off = 26.60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D1</td>
<td>D2</td>
<td>D3</td>
<td>D4</td>
</tr>
<tr>
<td>1</td>
<td>D3</td>
<td>4</td>
<td>1.20</td>
<td>1.47</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>8.58</td>
<td>7.73</td>
<td>16.40</td>
</tr>
<tr>
<td>2</td>
<td>D2</td>
<td>7</td>
<td>4.89</td>
<td>52.18</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5.27</td>
<td>37.93</td>
<td>8.87</td>
</tr>
<tr>
<td>3</td>
<td>D3</td>
<td>6</td>
<td>5.34</td>
<td>3.34</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>6.79</td>
<td>7.07</td>
<td>12.38</td>
</tr>
<tr>
<td>4</td>
<td>D3</td>
<td>8</td>
<td>5.61</td>
<td>2.84</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.77</td>
<td>3.16</td>
<td>9.10</td>
</tr>
<tr>
<td>5</td>
<td>D2</td>
<td>6</td>
<td>1.13</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.65</td>
<td>12.31</td>
<td>4.49</td>
</tr>
<tr>
<td>6</td>
<td>D3</td>
<td>6</td>
<td>3.07</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4.06</td>
<td>2.40</td>
<td>9.23</td>
</tr>
<tr>
<td>7</td>
<td>D1</td>
<td>5</td>
<td>2.76</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>13.51</td>
<td>6.65</td>
<td>11.49</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16.91</td>
<td>9.53</td>
<td>13.28</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>24.51</td>
<td>11.49</td>
<td>9.86</td>
</tr>
</tbody>
</table>
Detection of Dengue Virus Serotype-specific IgM by IgM Capture ELISA in the Presence of NaSCN

<table>
<thead>
<tr>
<th>Patient</th>
<th>RT-PCR</th>
<th>Disease day</th>
<th>Without NaSCN (cut off = 2.28)</th>
<th>With 0.5M NaSCN (cut off = 26.60)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>D1</td>
<td>D2</td>
</tr>
<tr>
<td>8</td>
<td>D1</td>
<td>7</td>
<td>1.73</td>
<td>3.89</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>D2</td>
<td>6.95</td>
<td>10.19</td>
</tr>
<tr>
<td>9</td>
<td>D2</td>
<td>5</td>
<td>1.93</td>
<td>3.02</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>D2</td>
<td>ND</td>
<td>0.75</td>
</tr>
<tr>
<td>11</td>
<td>D1</td>
<td>5</td>
<td>1.25</td>
<td>1.28</td>
</tr>
<tr>
<td>12</td>
<td>D1</td>
<td>ND</td>
<td>20.35</td>
<td>15.44</td>
</tr>
<tr>
<td>13</td>
<td>D1</td>
<td>19</td>
<td>18.37</td>
<td>10.69</td>
</tr>
<tr>
<td>14</td>
<td>D1</td>
<td>8</td>
<td>7.55</td>
<td>6.78</td>
</tr>
<tr>
<td>15</td>
<td>D4</td>
<td>19</td>
<td>1.12</td>
<td>1.44</td>
</tr>
<tr>
<td>16</td>
<td>D1</td>
<td>5</td>
<td>1.36</td>
<td>1.13</td>
</tr>
<tr>
<td>17</td>
<td>D1</td>
<td>6</td>
<td>4.74</td>
<td>2.01</td>
</tr>
<tr>
<td>18</td>
<td>D1</td>
<td>15</td>
<td>14.18</td>
<td>8.39</td>
</tr>
</tbody>
</table>

D1 to D4, dengue virus type 1 to 4, respectively. ND, not determined.

* Index values were calculated by the formula $A_{492}$ with the viral antigen/$A_{492}$ with uninfected control antigen.

The chaotropic agent, NaSCN, is known to denature protein structure and inhibit the formation of immune complexes as was exploited for immunoaffinity chromatography[12,13]. There are multiple factors which affect NaSCN’s ability to highlight serotype-specific reaction in comparison with cross-reaction in the ELISA. One of the factors is a characteristic of dengue viral antigens, which are prepared from the infected mosquito cell culture. It was reported that a rapidly sedimenting haemagglutinin (RHA) was cross-reactive and labile, but the soluble complement-fixing antigen (SCF) was serotype-specific and relatively stable against the NaSCN treatment in the ELISA[11,14]. The predominant polypeptides in RHA and SCF fractions represent the envelope glycoprotein and NS1, respectively[11]. Trent et al.[15,16] observed three antigenic determinants on the envelope glycoprotein: (i) flavivirus group-reactive; (ii) complex-specific; and (iii) serotype-specific. Henchal et al.[17] characterized four antigenic determinants on the envelope glycoprotein: (i) flavivirus group-reactive; (ii) dengue complex-specific; (iii) dengue subcomplex-specific; and (iv) dengue serotype-specific.

Brandt et al.[14] reported that the DEN-2 SCF antigen extracted from infected mouse brains was resistant to the treatment with protein denaturing agents. Falconar and Young[18] reported serotype-specific epitopes on NS1. These results suggest that
Detection of Dengue Virus Serotype-specific IgM by IgM Capture ELISA in the Presence of NaSCN

heterologous antigenic determinants on dengue viral antigens contribute to the cross-reactivity among the four dengue serotypes, and that the presence of a chaotropic agent in the reaction mixture induces changes of viral antigens and may decrease the cross-reactive antigenicity.

In conclusion, we demonstrated that the addition of NaSCN to IgM-capture ELISA highlighted the detection of serotype-specific IgM in comparison with serotype cross-reactive IgM. This procedure may be useful for determining infecting dengue virus serotypes, especially in primary dengue virus infection.

Acknowledgement

This work was supported by a grant for research on emerging and re-emerging infectious diseases from the Ministry of Health, Labour and Welfare, Japan.

References


Detection of Dengue Virus Serotype-specific IgM by IgM Capture ELISA in the Presence of NaSCN


Genetic Influences on Dengue Virus Infections

J.F.P. Wagenaar*, A.T.A. Mairuhu and E.C.M. van Gorp

Department of Internal Medicine, Slotervaart Hospital, Louwesweg 6, 1066 EC Amsterdam, The Netherlands

Abstract

Dengue virus infections are an important cause of morbidity and mortality in the tropics, with 100 million people infected annually and an estimated 2.5 billion people at risk. Human infections can be asymptomatic or can manifest as the self-limited febrile dengue fever, or the more severe and life-threatening dengue haemorrhagic fever (DHF). There are several possible reasons why some infected individuals might develop a more severe form of the disease than others. Antibody enhancement and viral virulence have been implicated in the pathogenesis of DHF but host genetic factors may also be relevant and predispose some individuals to DHF. This review discusses the possible involvement of a variety of genetic polymorphisms on the course of dengue virus infections. It has been shown that several common genetic polymorphisms can influence the susceptibility to dengue haemorrhagic fever. Gene polymorphisms concerning human leucocyte antigens, antibody receptors, inflammatory mediators and other factors with immunoregulatory effects are described. The study of genetic polymorphisms might provide important insights into the pathogenesis of a more severe disease and could have an impact on the design of future vaccines.

Keywords: Dengue haemorrhagic fever, genetic polymorphisms.

Introduction

Dengue has become one of the most important arthropod-borne diseases in tropical and subtropical regions of the world. Approximately 100 million cases of dengue infections occur annually, and an estimated 2.5 to 5 billion people are at risk of dengue virus infection[1]. The four serotypes of dengue virus (DEN-1, 2, 3 and 4) are transmitted to humans through the bite of an infective female *Aedes* mosquito and may result in dengue fever (DF), an acute viral infection characterized by fever, rash, headache and muscle and joint pain. Occasionally, dengue virus infections progress to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). These are potentially life-threatening illnesses characterized by haemorrhagic manifestations and the loss of plasma from the vascular compartment, which may give rise to shock in severe cases.

Central in the pathogenesis of DHF and DSS is the loss of endothelial integrity that is believed to be the result of an abnormal immune response and a disturbance in

* E-mail: jfpwagenaar@hotmail.com; Tel.: + 31-(0)20-5124591; Fax: + 31-(0)20-5124783
immune regulation. Elevated levels of several cytokines and chemical mediators, which cause capillary leakage and may lead to shock, have been found in those suffering from DHF and DSS. Replication of dengue viruses occurs primarily in mononuclear phagocytes, which are a major source of tumor necrosis factor (TNF-α) and other vasoactive inflammatory mediators. Several studies have demonstrated that TNF-α and other cytokines that are produced downstream of TNF-α in the inflammatory cascade, e.g. IL-1β, IL-6 and IL-8, are related with disease severity[2-4]. Other inflammatory mediators, like IL-2 and interferon (IFN)-γ, are released from T lymphocytes that are activated during dengue virus infections. The levels of these cytokines are significantly higher in DHF and DSS patients than in DF patients[5,6].

There are several possible reasons why some infected individuals might produce a greater inflammatory response than others. The most favoured hypothesis concerns the antibody-dependent enhancement theory. Several epidemiological studies demonstrate that prior infection with a different viral serotype constitutes the largest risk factor for DHF[7-11]. In vitro studies demonstrated that the presence of anti-dengue antibodies at sub-neutralizing concentrations augment dengue virus infection of Fcγ receptor-positive cells, such as monocytes[12,13]. Based on these epidemiological and laboratory observations, it has been hypothesized that dengue cross-reactive antibodies may increase the number of dengue virus-infected monocytes during secondary infections, and lysis of these dengue virus-infected monocytes may lead to DHF and DSS. Another possibility why some infected individuals might produce a greater inflammatory response is related to viral virulence. Several studies have found that infection with DEN-2 caused more severe disease than other serotypes, suggesting that the virus phenotype influences the outcome[7,11]. In addition, genetic variations within a specific serotype may also account for differences in disease severity, although reports remain scanty[14].

Hyperendemic transmission of multiple DEN serotypes in a Haitian population and the apparent absence of DHF and DSS, in addition to the observation that black people were hospitalized less frequently with DHF and DSS than the whites during epidemics in Cuba, led to the hypothesis that human genetic factors, e.g. gene mutations and gene polymorphisms, may contribute to variable susceptibility[15-17]. Genetic polymorphisms are stable gene variants that usually have minor effects on the regulation or function of proteins. These subtle chances might very well have important consequences for susceptibility to the disease[18]. Several studies have confirmed that some genetic polymorphisms may protect or predispose an individual to DHF and DSS. Understanding the molecular basis for these differences in susceptibility should provide useful insight in the pathogenesis of DHF and DSS and aid in the development of effective therapies and vaccines. This review attempts to describe the current knowledge of the role of genetic influences on dengue virus infections.

**Human leucocyte antigen genes**

The function of the human leukocyte antigens (HLAs), encoded by the major histocompatibility complex (MHC) and whose genes are on chromosome 6, are to
display antigen-proteins to antigen receptors of host T-lymphocytes in order to activate cellular host immune responses. HLA genes show great variability and it could well be that specific polymorphisms seen in human HLA gene regions influence peptide epitope binding\[18\]. A number of studies have looked at the variation in HLA genes and found some of them to be associated with the severity of dengue virus infections (Table).

Table. Effect of HLA and non-HLA alleles on the severity of dengue virus infections

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Class</th>
<th>Effect</th>
<th>Population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA alleles</td>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>Susceptibility</td>
<td>Cubans</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>Susceptibility</td>
<td>Thai</td>
<td>19, 22</td>
<td></td>
</tr>
<tr>
<td>A*0203</td>
<td>Protective</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>A*0207</td>
<td>Susceptibility</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>A24</td>
<td>Susceptibility</td>
<td>Vietnamese</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>A29</td>
<td>Protective</td>
<td>Cubans</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>A33</td>
<td>Protective</td>
<td>Vietnamese</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>B blank</td>
<td>Susceptibility</td>
<td>Cubans/Thai</td>
<td>19, 20</td>
<td></td>
</tr>
<tr>
<td>B13</td>
<td>Protective</td>
<td>Thai</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>B14</td>
<td>Protective</td>
<td>Cubans</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>B44</td>
<td>Protective</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>B46</td>
<td>Susceptibility</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>B51</td>
<td>Susceptibility</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>B52</td>
<td>Protective</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>B62</td>
<td>Protective</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>B76</td>
<td>Protective</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>B77</td>
<td>Protective</td>
<td>Thai</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Class II</td>
<td>DRB1*04</td>
<td>Resistance</td>
<td>Mexicans</td>
<td>29</td>
</tr>
<tr>
<td>Non-HLA alleles</td>
<td>Fc gamma-receptor</td>
<td>Resistance</td>
<td>Vietnamese</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Vitamin D receptor</td>
<td>Resistance</td>
<td>Vietnamese</td>
<td>30</td>
</tr>
</tbody>
</table>

**HLA class I**

HLA class I alleles consist of HLA-A, -B, and -C; its products have a wide distribution and are present on the surface of all nucleated cells and on platelets. Antigens associated with HLA class I products will interact with CD8 cells during an immune response. Polymorphisms in this class I region gene were found to be associated with DHF disease susceptibility. Chiewslip and Paradao were the first to report an association between HLA class I and the severity of dengue virus infection\[19,20\]. In two ethnically and geographically distinct populations evidence was presented suggesting that HLA-
A1, HLA-A2 and HLA-B blank increased in frequency in DHF patients. A negative relationship was found for HLA-B13, HLA-B14 and HLA-A29. However, these studies had a small sample size and additional studies with a larger number of patients were needed. Subsequently, a large case control study in 560 study subjects was performed, which mainly confirmed the observations made by the two previous studies that HLA class I was important[21]. The data demonstrated that polymorphisms in the HLA class I region, particularly of the HLA-A gene, were significantly associated with susceptibility to DHF. Of the 15 alleles studied, two particular alleles were relevant: patients with HLA-A33 were less likely to develop DHF (odds ratio 0.56; 95% confidence interval 0.34-0.39), whereas those with HLA-A24 were at an increased risk to develop DHF (odds ratio 1.54; 95% confidence interval 1.05-2.25). The HLA-B alleles were not associated with DHF disease susceptibility.

Another case control study, in a Thai population, also demonstrated that the HLA-A2 locus serotype was associated with disease susceptibility[22]. HLA-A*0203 was associated with the less severe DF, whereas HLA-A*0207 was associated with susceptibility to the more severe DHF. Interestingly, these associations were only found in immunologically primed persons, but not in immunologically naive patients with primary infection. Dengue virus-specific associations were also observed within the HLA-B5 group of related alleles, whereby molecularly-determined HLA-B51 alleles were associated with the development of DHF after secondary infections. HLA-B51-restricted CTL responses to a variety of viruses have been described, including Hantaan virus which also causes a haemorrhagic fever[23]. HLA-B52 showed a strong association with less severe DF. The reduced frequency of the HLA-B15 group of serotypes, including HLA-B62, B76 and B77, in patients with secondary infections, suggests that they may be protective against developing clinical disease in immunologically primed individuals. By contrast, HLA-B46 that also belongs to the HLA-B15 group of serotypes, was increased in DHF patients with secondary infections. Since HLA-B46 is in strong equilibrium with HLA-A*0207, it is believed that the effect of B46 was likely to be an adjunct to that of A*0207. Finally, HLA-B44 appeared also to be protective against the development of severe disease in patients with secondary dengue virus infections.

**HLA class II**

Class II HLA products consist of HLA-D, -DR, -DP, and -DQ; they have a more limited distribution on B-cells, macrophages, dendritic cells, Langerhans cells and activated T cells. HLA class II alleles have shown to play a role in mycobacterial diseases, and their association with hepatitis clearance is also established[24-26]. HLA-DRB1, which is one of the most polymorphic loci of the HLA complex in the Mexican population[27,28], was studied in Mexican patients suffering from a dengue virus infection[29]. Although the sample size was relatively small, the investigators found that the frequency of HLA-DRB1*04 was lower in DHF patients. Persons homozygous for DRB1*04 were less likely to develop DHF than persons who were DRB1*04 negative (odds ratio 0.28; 95% confidence interval 0.12-0.66), suggesting a protective effect. The envelope protein (E) of the virus is responsible for viral entry into target cells. The immunological determinants of protein E are probably
processed and presented by HLA class II antigens. The HLA-DRB1*04 molecule may present these viral antigens to CD4+ lymphocytes leading to an effective immune response and therefore protection from DHF. These findings are in contrast to the findings of Loke et al., who studied polymorphisms in the HLA-DRB1 gene but did not find an association.

HLA class III

Genes in the class III region encode a number of proteins, including complement proteins (C4A, C4B, C2 and Bf), TNF-α and TNF-β. Loke and colleagues studied promoter polymorphisms in the TNF-α gene but did not find an association. No other studies are reported to have studied HLA class III polymorphisms.

Non-HLA host genetic factors

The number of studies on polymorphisms within non-HLA genes remains low. Loke and colleagues investigated the association between susceptibility to DHF and polymorphic non-HLA alleles: vitamin D receptor (VDR), Fcγ receptor II (FcγRII), Interleukin-4 (IL-4), Interleukin-1 repeat alleles (IL-1RA), and mannose-binding lectin (MBL). Two of the five genes assessed showed evidence of association with altered risk of severe dengue.

Fcγ receptor

The Fcγ receptor is a widely distributed receptor for all subclasses of IgG and is able to mediate antibody dependent enhancement in vitro by binding to virus-IgG complexe. An arginine to histidine substitution at position 131 of the FcγRIIA gene has been associated with meningococcal disease and recurrent respiratory tract infections. It chances the IgG binding affinity of the receptor with reduced opsonisation of IgG2 antibodies causally associated with the arginine variant. Loke et al. found that homozygotes for the arginine variant at position 131 of the FcγRIIA gene may be less susceptible to DHF.

Vitamin D receptor (VDR)

This gene mediates the immuno-regulatory effects of 1,25-dihydroxyvitamin D3, which include activating monocytes, stimulating cellular immune responses and suppressing immunoglobulin production and lymphocyte proliferation. Recently the t allele at position 352 of the VDR gene has been associated with tuberculoid leprosy, enhanced clearance of HBV infection and resistance to pulmonary tuberculosis. Expression of VDR may affect susceptibility to DHF since activated B and T lymphocytes express VDR and 1,25D3 affects monocytes, the main sites of dengue virus infection and replication. The t allele at position 352 of the vitamin D receptor (VDR) gene was associated with resistance to severe dengue, although the exact mechanism needs to be explored.

Interleukin-4 (IL-4)

IL-4, primarily produced by Th2 subset of CD4+ T-cells, regulates B-cell growth, IgG class switching and suppresses Th1-type responses as well. Since this gene affects both antibody responses and inflammatory responses during disease, IL-4 promoter polymorphisms were studied in order to find a relationship in susceptibility to DHF. However, no associations were found in this context.
Interleukin-1 repeat allele (IL-1RA)

IL-1RA was thought to be a good candidate gene as well because IL-1RA is involved in the regulation of IL-1-mediated inflammatory responses by competitive binding to IL-receptors[40]. But no significant difference could be found in the DHF group in addition to the controls[30].

Mannose-binding lectin (MBL)

Several mutations in the MBL gene, which encodes for a protein involved in the activation of the classical complement pathway[41,42], have been associated with a marked reduction in serum MBL levels and MBL-mediated complement activation[43,44]. Polymorphisms in this gene were not proved to have any effect on the susceptibility to DHF. However, this variant allele was relatively low in the observed population, which limits the statistical power of the analysis[30].

Discussion and future perspective

The number of candidate susceptibility and protective genes is expanding rapidly, but what is the use of studying these genes in relation to DHF? Studying host genetic factors will clearly contribute to our understanding of the pathophysiology of dengue virus infections but also of viral infections in general. The finding of a protective association with particular HLA or non-HLA-types may encourage the design of future vaccines, whereas polymorphisms associated with the susceptibility to develop a more severe disease may help to identify certain risk groups in a population. It is therefore of great importance to stimulate the study of the interaction of single and multiple polymorphisms in severe dengue virus infections.

The few studies performed thus far have demonstrated that host genetic factors can be important in susceptibility to DHF. It is most likely that classical HLA class I and class II gene products play a crucial role in determining the severity of dengue virus infections. Two polymorphic non-HLA alleles, the FcγRII receptor and VDR, could also play an important role in susceptibility to DHF. Some polymorphic HLA alleles were observed in several studies, e.g. HLA-A2 in a Thai and Vietnamese population, but differences in susceptibility to DHF were observed[19,21,22]. An explanation for the observed difference may be that a genetic polymorphism is more frequent in a population whereas another is relatively infrequent. Overall, such disease associations warrant further analysis, but also emphasize the need to expand the scope of investigation to other candidate genes within and outside of the HLA region.

References


Genetic Influences on Dengue Virus Infections


Genetic Influences on Dengue Virus Infections


Identification and Phylogenetic Analysis of DEN-1 Virus Isolated in Guangzhou, China, in 2002

Jun-lei Zhang, Rui Jian, Ying-jie Wan, Tao Peng and Jing An*

Department of Microbiology, College of Medicine, Third Military Medical University, Chongqing, 400038, People’s Republic of China

Abstract

Virus was isolated and identified from the serum samples of patients with suspected classical dengue fever (DF) in Guangzhou province, China, in 2002. The serum was incubated with the Aedes albopictus cell line, C6/36, for isolation and 5 of 20 serum samples caused cytopathologic effects on C6/36 cells. A 539-nucleotide (nt) fragment in the NS1 region of the isolated virus genome was amplified using universal primers of 4 serotypes of DEN viruses. By sequencing the primer-extension production and blasting in the GenBank, the isolated strain was the closest to rDEN-1 dalte30 (AY145123), which was an attenuation strain of DEN-1 virus. A 593nt fragment from the Envelope-Nonstructural protein 1 (E/NS1) of these isolates was also sequenced to compare it with published sequences of other DEN-1 viruses. The 240nt from the E/NS1 region of this DEN-1 virus genome was the closest to DEN-1/T14 strain (M32931), which was genotype IV. The phylogenetic analysis of the NS1 (480nt) and E/NS1 (240nt) regions of the gene nucleotide sequences was performed using neighbour-joining methods: 11 strains of DEN-1 virus were divided into three genotypes: I, IV and V, as defined by Rico-Hesse, or Asia, South Pacific and Americas/Africa, as defined by Goncalvez. Based on the sequence information and the definition, the isolated virus (named DEN-1/GZ 2002) strain belonged to DEN-1 and IV or South Pacific genotypes. The suckling mice died on days 10 to 11 after intracerebral inoculation with the isolated virus. TCID50 of the virus was 4.37 log pfu/0.2ml. Small plaques with an unclear edge were seen on Vero cells on days 8 to 9 after infection. Combined with clinical data that thousands of patients only showed DF manifestations, the results suggested that DEN-1/GZ 2002 might be a low-virulence strain.

Keywords: Dengue virus, DEN-1/GZ2002, virulence, phylogenetic analysis.

Introduction

Dengue (DEN) viruses belong to the genus Flavivirus. There are four closely related, but antigenically distinct, virus serotypes (DEN-1, DEN-2, DEN-3 and DEN-4). The viral genome is a positive-sense single-stranded RNA, approximately 11,000 nucleotides long, encoding 10 distinct proteins. The gene order is 5’-C-prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3’, expressed as a single polyprotein that is cleaved by both viral and cellular proteases to form the viral polypeptides. The three 5’ proteins are
three structural proteins: capsid (C), membrane (M) and envelope (E), and the remaining seven are nonstructural (NS) proteins[1]. The DEN viruses cause classical dengue fever (DF) and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). DF is a non-specific viral febrile illness while DHF/DSS is a severe and fatal haemorrhagic disease. Up to 100 million cases of DF occur annually in the world, with some 2 billion people at risk of the infection in tropical and subtropical regions of Africa, Asia and the Americas[2,3].

The pathogenesis of the DEN virus infection is not clear. Although many factors are proposed to be involved in the DEN virus epidemiology and occurrence of DHF/DSS, the evolution of the DEN viral virulence and the spread of DEN viruses in different geographical areas are two important features in the epidemiology and pathogenesis of the disease. Studies have suggested that specific viral structures might contribute to the replication capability in the target cells and to their transmission[4]. Co-circulation of novel DEN genotype, another genotype of the same serotype or different serotypes of DEN viruses in the same area, might lead to the displacement of the native genotype by a new genotype and thus to the occurrence of DHF/DSS[5].

Several studies have shown that the phylogenetic trees obtained from small gene fragment sequences are congruent with the trees obtained from an entire gene sequence, although there might be minor rearrangements in the terminal branches[6-13]. Using a partial sequence from Envelope-Nonstructural protein 1 (E/NS1) junction region, Rico-Hesse defined five genotypes for DEN-1 viruses isolated worldwide: I) America, Africa and South-East Asia; II) Sri Lanka; III) Japan; IV) South-East Asia, the South Pacific, Australia and Mexico; and V) Taiwan and Thailand[14]. Recently, Goncalvez set up the phylogeny of 36 DEN-1 viruses according full E gene sequences. Statistical analyses of the validity of branching patterns (bootstrap) also suggested five classifications: 1) from Asia; 2) from Thailand; 3) from sylvatic/Malaysia; 4) from South Pacific; and 5) from the Americas/Africa, in which three genotypes indicated as 1), 4) and 5) were consistent to I), IV) and V) mentioned by Rico-Hesse[15]. Aviles-G and collaborators sequenced Capsid-pre Membrane (C/prM) and E/NS1 regions of 24 recent isolates of DEN-1 from South America, suggesting that the recent epidemics in Argentina and Paraguay were due to the re-emergence of a previously circulated strain[16]. This indicated that small gene fragment sequences are available for analysis of viral genetic characterization.

Current methods for obtaining DEN virus sequences no longer require a viable virus isolate. In fact, the entire genome sequences can be obtained by enzymatic amplification of viral RNA template in the patient’s blood sample. Unfortunately, there are numerous sequences that contain errors, which have led to serious mistakes in their interpretation. Therefore, it is wise to obtain virus isolates for further genetic characterization[17]. In this study, a viral strain that circulated in Guangzhou, China, in 2002 was isolated first, then identified by amplifying and sequencing 539 nucleotide (nt) in NS1 region of DEN virus genome using universal primers. Also, E/NS1 regions of the isolated virus were sequenced to determine their origin.
Identification and Phylogenetic Analysis of Dengue-1 Virus

Materials and methods

Collection of blood samples
Twenty patients with suspected DEN virus infection admitted to the Eighth People’s Hospital of Guangzhou were enrolled - 12 males and 8 females aged between 23-57 years. The blood samples were collected on day 1 to 11 after the onset of fever. The sera were separated by centrifugation and stored at -70 °C until use.

Viral isolation
Aedes albopictus mosquito cell line, C6/36, was cultured in RPMI 1640 (pH6.8~7.0) containing 2 mM glutamine with 5% CO₂ at 28 °C. 30µl of the serum sample was diluted with 1 ml RPMI 1640 containing 2% FBS and was incubated with overnight C6/36 cells for 1 hour at 28 °C. After removing the serum, the cells were cultured in RPMI 1640 (pH6.8~7.0) containing 2% FBS at 28 °C. The cells were observed daily for cytopathic effect (CPE)\cite{18}. If CPE was not seen initially, the cells were passaged several times to confirm whether there was virus in the serum samples or not.

RNA extraction
Viral RNA was extracted from the culture supernatants of infected C6/36 with ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions.

RT-PCR amplification
Nucleotides from positions 2503~3041 coding a fragment of the NS1 gene were amplified using RT-PCR. The universal primer sequences are: 5’GTG CAC ACA TGG ACA GAA CA 3(forward) and 5’CTT TCT ATC CAA TAA CCC AT 3(reverse). Their combination generated a 539nt fragment. Briefly, 5 µl of the extracted RNA was mixed with 50 pmol (2µl) of reverse primer at 70 °C for 5 minutes and cooled down on ice. Then 13 µl of RT mix containing 4µl of 5X RT XL buffer, 0.5 µl of 10mM dNTP, 6.0 µl of sterile UHQ water, 0.5µl RNase inhibitor and 20 U of AMV reverse transcriptase XL (TaKaRa, Japan) were added. The mixture was incubated at 42 °C for 1 hour for RT reaction, then heated to 95 °C to inactivate AMV and cooled down on ice. PCR was subsequently carried out by adding 24 µl of PCR mix containing 2.5 µl of 10X Taq Polymerase buffer (PROMEGA, USA), 2.5 µl of 25mM MgCl₂, 12.5 pmol each of sense and reverse primers, 0.5 µl of 10mM dNTP, 2U of Taq DNA Polymerase (PROMEGA, USA) and 17.5 µl of sterile UHQ water to the 1st strand synthesis tube containing 1 µl of cDNA. PCR with denaturation at 94 °C for 30 seconds; annealing at 53 °C for 30 seconds; and extension at 72 °C for 1 minute 30 seconds for 30 cycles. The RT-PCR conditions for the amplification of nucleotides from positions 2107~2701 coding for the E/NS1 fragment were similar to those used for NS1 fragment, but with an annealing temperature of 58 °C instead of 53 °C.

DNA purification and sequencing
PCR-amplified DNA products were electrophoresed in 1% agarose gels and stained with 1µg/ml ethidium bromide. The bands of predicted size (539nt for the NS1 fragment and 593nt for E/NS1 fragment) were excised from the gel and purified using
Identification and Phylogenetic Analysis of Dengue-1 Virus

a Golden Beads Product Purification Kit (Songon, CN) according to the manufacturer’s instructions. Purified PCR products were cloned on to pMD18-T Vector (TaKaRa, Japan) and sent to Co. Bioasia, Shanghai, CN, in 15% Glycerol to perform sequencing using an ABI Prism 377 Genetic Analyzer. Amplifying primers were M13-48.

Computer analysis

The multiple sequence alignment programme Clustalx, version 1.8,[19] was used to obtain an optimal nucleotide sequence alignment file. Phylogenetic analysis of nucleotide sequences from the NS1 region and the E/NS1 junction of our isolates were carried out with the neighbor-joining method (NJ)[20], calculating bootstrap confidence intervals of 1,000 replicates. Character state tree-building algorithms (PHYLIP package) were also tested. A strict consensus bootstrap tree was obtained by using the following programmes: (i) SEQBOOT to generate 100 replicas, (ii) DNADIST and NEIGHBOR to acquire the tree of each reiterated data, and (iii) CONSENSE to build a strict consensus bootstrap tree. Phylogenetic trees were drawn using TreeView.[21]

Table 1. Dengue viral sequences from GenBank used in the phylogenetic analysis

<table>
<thead>
<tr>
<th>Virus</th>
<th>Strains</th>
<th>Origin</th>
<th>Year isolated</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN-1</td>
<td>FGA/89</td>
<td>French Guiana</td>
<td>1989</td>
<td>AF226687</td>
</tr>
<tr>
<td>DEN-1</td>
<td>BR/90</td>
<td>Brazil</td>
<td>1990</td>
<td>AF226685</td>
</tr>
<tr>
<td>DEN-1</td>
<td>S275/90</td>
<td>Singapore</td>
<td>1990</td>
<td>M87512</td>
</tr>
<tr>
<td>DEN-1</td>
<td>Abidjan</td>
<td>Ivory Coast</td>
<td>1999</td>
<td>AF298807</td>
</tr>
<tr>
<td>DEN-1</td>
<td>WestPac/74</td>
<td>Nauru</td>
<td>1974</td>
<td>U88535</td>
</tr>
<tr>
<td>DEN-1</td>
<td>A88</td>
<td>Indonesia</td>
<td>1988</td>
<td>AB074761</td>
</tr>
<tr>
<td>DEN-1</td>
<td>GD05/99</td>
<td>Guangdong, CN</td>
<td>1999</td>
<td>AY376738</td>
</tr>
<tr>
<td>DEN-1</td>
<td>GD23/95</td>
<td>Guangdong, CN</td>
<td>1995</td>
<td>AY373427</td>
</tr>
<tr>
<td>DEN-1</td>
<td>GZ/80</td>
<td>Guangzhou, CN</td>
<td>1980</td>
<td>AF350498</td>
</tr>
<tr>
<td>DEN-1</td>
<td>Mochizuki</td>
<td>Japan</td>
<td>1943</td>
<td>AB074706</td>
</tr>
<tr>
<td>DEN-1</td>
<td>Djibouti</td>
<td>Djibouti</td>
<td>1998</td>
<td>AF298808</td>
</tr>
<tr>
<td>DEN-2</td>
<td>TR1751*</td>
<td>Trinidad</td>
<td>1954</td>
<td>M32969</td>
</tr>
</tbody>
</table>

* The full-length gene segment sequence of this viral strain is unavailable in GenBank and the NS1 region in the phylogenetic analysis was sequenced by us.
The resulting unrooted trees were outgrouped to the sequence of DEN-2 (TR1751), which was kindly provided by Dr Oya A (National Institute of Infectious Diseases, Japan) and isolated from a DF patient.\textsuperscript{[22,23]} The RT-PCR amplification of the NS1 region of this virus strain using universal primers and sequencing of amplified products was done as described above.

**Biological character detection**

The virus isolated from the serum samples was propagated in C6/36 cells (MOI = 1) and the titer was determined by the plaque assay using Vero cells monolayer culture under methylcellulose overlay medium. TCID_{50} of the virus was also measured by the plaque assay and calculated using Reed-Muench method.\textsuperscript{[24]} Eighteen suckling mice were intracerebrally (ic) inoculated with 10^4 pfu/mouse of the virus for observing clinical signs and their survival time.

**Results**

**General observation**

After two passages, 5 of the 20 serum specimens caused CPE on C6/36 cells. Infected C6/36 cells became syncytia and cell-cell fusion. Some cells showed necrosis and got detached from the plate at a late stage of the infection (Figure 1). The virus was propagated in C6/36 cells and the highest titer was 5x 10^5 pfu/ml at day 6 post-infection (pi). Using the Reed-Muench method, TCID_{50} of the virus was 4.37 log pfu/0.2ml. Plaque formation on Vero cells was detected on day 8 or 9 after infection and they were small and unclear (Figure 2). After ic inoculation with isolated virus, all of the suckling mice showed kyphoscoliosis and paralysis of hind legs and died on days 10 to 11 after infection.
Comparison of nucleotide sequences

As shown in Figure 3, a 539nt fragment from the NS1 region (positions 2503 to 3041) was amplified using the universal primer by RT-PCR and the amplified products were sequenced to determine the relationships among the 5 isolates and their origin. Comparisons of the NS1 region of the 5 isolates virus strains showed little divergence, so we chose one as the representative strain to compare with the sequences published in GenBank. The nucleotide sequence of this strain was the closest to rDEN-1 dalte30 (AY145123), which was an attenuation strain of DEN-1 virus. According to the full-length gene sequences of rDEN-1 dalte30 (AY145123), we designed the primers to amplify the nucleotides of a fragment from the E/NS1 region (positions 2107 to 2701). The 240nt from the E/NS1 region (position 2309 to 2548) of this DEN-1 virus genome is the closest to DEN-1/T14 strain from Australia isolated in 1981 (M32931), which was genotype IV, their divergence was 2%. Based on sequence information and definition by Rico-Hesse, our isolates belonged to DEN-1 and genotype IV, named DEN-1/GZ2002.

Figure 3. Agarose gel analysis of the cDNA products from RT-PCR of RNA samples isolated from the supernatant of the infected C6/36 cells
(a) After amplification with the universal primer, a band of 539nt for the NS1 fragment was seen. Lanes 2-6: PCR amplified DNA products from different samples respectively; lane 7: blank control; lanes 1 and 8: 1Kb DNA Ladder marker (GeneRuler™)
(b) After amplification with a primer for 593nt fragment of E/NS1 region, an expected band about 593nt was seen (lane 1), Lane 2: 1Kb DNA Ladder marker (GeneRuler™)
**Phylogenetic analysis**

We compared 480nt from the NS1 region (position 2516 to 2995) and 240nt from the E/NS1 region (position 2309 to 2548) of this DEN-1 virus genome with sequences of other published DEN-1 viruses. The phylogenetic analysis of the NS1 (480nt) and E/NS1 (240nt) regions of the gene nucleotide sequences was performed using NJ methods respectively. Two NJ trees were set up and they showed a similar result, except for a little difference in terminal branches (Figure 4). From the NJ trees in our study, 11 strains of the DEN-1 virus were divided into three genotypes coinciding with I, IV and V as defined by Rico-Hesse or Asia, South Pacific and Americas/Africa, as defined by Goncalvez. Our isolated DEN-1/GZ2002 strain belonged to genotype IV or South Pacific genotype. In addition, it was found that an arrangement of the S275/90 strain showed a major difference between the two trees, which supports the previous hypothesis that the S275/90 strain is a recombined strain\[25\].

Figure 4. Phylogenetic relationship of DEN-1/GZ2002 to previously characterized DEN-1 viruses. Two phylogenetic trees were set up using 240nt from the E/NS1 region, position 2309 to 2548 (a) and using 480nt from the NS1 region, position 2516 to 2995 (b) of these DEN-1 virus genomes. The resulting unrooted trees were out-grouped to the sequence of DEN-2 (TR1751).

**Discussion**

It is evident that the DEN virus epidemiology is determined by many factors, including those in the host, the virus, the vector and the environment. DEN virus evolution is also determined by many complex interactions. Some genetic changes that occur during the natural transmission cycles of the DEN virus might affect its
Identification and Phylogenetic Analysis of Dengue-1 Virus

virulence and cause the disease. Therefore, understanding DEN virus variation is especially important to clarify the pathogenesis of DEN virus infection. Although current methods of obtaining DEN virus sequences no longer require viable virus isolates, virus isolates are necessary for further studies of the biological and genetic characteristics[17]. In this study, we isolated DEN-1 virus that circulated in Guangzhou in 2002 and analysed its possible origin and partial biological features.

In an in vitro experiment, the virus caused typical CPE on C6/36 cells such as syncytia and cell-cell fusion, which was continuously shown when the virus passaged several times. Their titer maintained a level about $5 \times 10^5$ pfu/ml during passages. On Vero cells, a small and unclear plaque, a biological marker of attenuation, was seen. The suckling mice survived for 11 days after ic inoculation. Interestingly, the sequences of 539nt from the NS1 were very close to rDEN-1 dalte30 (AY145123), which was an attenuation strain of DEN-1 virus. Combined with clinical data where thousands of patients only showed DF clinical signs, our results indicated that the isolated virus might be a low-virulence strain.

As is known, a comparison of full-length gene segment sequences is impractical, especially when looking at a large number of samples. Recently, several studies have shown that the phylogenetic trees obtained from small gene fragment sequences are highly congruent with those obtained from an entire gene sequence, except for a minor rearrangement in the terminal branches, suggesting thereby that small gene fragment sequences were effective for the study of viral genetic characteristics[6-13]. The E/NS1 junction sequences were a useful indicator in DEN virus evolution and the NS1 sequences were a highly conserved region. In our study, after sequencing E/NS1 (240nt) and the NS1 (480nt) gene region, we analysed the phylogenetic relationship of 11 DEN-1 virus representatives of three genotypes coincidence with I, IV, and V as referred to by Rico-Hesse (1990), or Asia, South Pacific and Americas/Africa, as defined by Goncalvez. This indicated that the isolated virus belonged to genotype IV/South Pacific.

Tolou completed the genome sequence analysis to demonstrate the likelihood of recombination between different strains of DEN-1 virus. The region of 1295~2592nt was considered to be a hot spot for the recombination[25]. We sequenced one region outside (2516~2995nt) and one region inside (2309~2548nt) the possible recombination site. No recombination event occurred in our strains, at least in the regions analysed. However, no virus isolates meeting the stringent criteria for recombination have yet been described and it remains to be determined whether and at what frequency DEN viruses undergo recombination in nature[17].

The evolution of DEN viruses has had a major impact on their virulence for humans and on the epidemiology of the DEN virus around the world. It is necessary that a more complete and systematic survey of DEN-1 samples be undertaken before a link between specific genotypes and the virulence of these viruses can be established.

Acknowledgment

This work was partially supported by grants nos. 30170848 and 30300303 from the National Science Foundation of China (NSFC).
References


Identification and Phylogenetic Analysis of Dengue-1 Virus


Induction of Cytotoxic T Lymphocytes by Immunization with Dengue Virus - Derived, Modified Epitope Peptide, Using Dendritic Cells as a Peptide Delivery System

Yoshiki Fujii*, Hideyuki Masaki**, Takanori Tomura**, Kiyohiro Irimajiri* and Ichiro Kurane***

*Department of Pharmacotherapy, Kinki University School of Pharmaceutical Sciences, Higashi-Osaka, Osaka, Japan
** First Department of Biochemistry, Kinki University School of Medicine, O.saka-Sayama, O.saka 589-8511, Japan
***Department of Virology 1, National Institute of Infectious Diseases, Shinjuku, Tokyo, Japan

Abstract

A single 9-amino acid peptide of the defined murine cytotoxic T lymphocyte (CTL) epitope (named peptide-1), which corresponds to the amino acid residues 298-306 (GYISTRVEM) of NS3 of dengue virus serotypes DEN-2 and 4, was examined for induction of specific CTLs. Immunization of BALB/c mice subcutaneously with the peptide-1 emulsified with complete Freund adjuvant (CFA) did not induce specific CTLs. The peptide-2 (GYISTRVEL), in which the residue (M) at 9th position of the peptide-1 was substituted for L, was prepared. The peptide-2 possessed the complete H-2Kd-binding motif. Intravenous immunization with 5×10^5 dendritic cells (DCs) pulsed with the peptide-2-induced specific CTLs. Furthermore, subcutaneous immunization with the peptide-2 emulsified with CFA-induced CTLs which lysed peptide-1-pulsed target cells as well as peptide-2-pulsed ones. These results indicate that immunization with dengue virus-derived CTL epitope peptide induces specific CTLs, and that DC can be used as a vehicle for the modified epitope peptide.

Keywords: Dengue virus, cytotoxic T lymphocyte, dendritic cell, epitope, peptide, binding motif.

Introduction

Major histocompatibility complex (MHC) class I-restricted, CD8+ cytotoxic T lymphocytes (CTLs) are known to play an essential role in the recovery from viral infection by lysing virus-infected cells[1]. There are two major strategies to induce CTL-mediated protective immunity. One is to have CTL epitope expressed in host cells by infection with live viruses, or by administration of an expression plasmid vector (i.e. DNA vaccine) in which the epitope gene is incorporated. The other is immunization with a defined CTL epitope peptide. The former strategy is more physiological; however, the preparation of immunogen is often difficult and there is a
potential risk that the immunogen may be pathogenic. Immunization with a CTL epitope peptide is relatively easy; however, the epitope varies depending on T cell receptor repertoires and MHC class I haplotypes. Furthermore, the peptide administrated into the body may be degraded or washed away soon. Thus, the peptide is usually less immunogenic for CTL induction, and an appropriate delivery system is necessary for induction of CTLs.\textsuperscript{[2,3]}

Dendritic cells (DCs), which are potent antigen-presenting cells, are postulated to be one of the peptide delivery systems for inducing CTLs.\textsuperscript{[2,4]}

Dengue viruses cause dengue fever and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). Vaccine development against dengue virus infection has not been accomplished yet. It is important to analyse CTL responses elicited by a single epitope in order to understand the role of CTLs in dengue virus infection. In the present study, we employed a single 9-amino acid (a.a.) peptide, in which C-terminal residue was replaced to provide the complete H-2K\textsuperscript{d}-binding motif. This peptide was a derivative of the defined H-2K\textsuperscript{d}-restricted 9-a.a. CTL epitope peptide that corresponds to the residues 298-306 of NS3 of dengue virus types 2 and 4,\textsuperscript{[8]} and we examined whether intravenous immunization with bone marrow-derived DCs pulsed with this peptide elicited specific CTL response.

Materials and methods

Mice

Female BALB/cAJcl mice were purchased from Clea, Japan, and were maintained in the Animal Facility of Kinki University School of Medicine under conventional conditions. Mice were used at the age of 6 to 12 weeks.

Cells

Murine mastcytoma line, P815 (H-2\textsuperscript{a}), was used as target cells in CTL assays. The cells were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) with 5x10\textsuperscript{-5}M 2-mercaptoethanol (2-ME), 100U penicillin, 100µg/ml streptomycin, 10mM HEPES, and 10% heat-inactivated fetal calf serum (Complete medium) at 37 \textdegree{}C in 5% CO\textsubscript{2}.

Peptides

The peptide-1 (GYISTRVEM), which corresponds to the amino acid residues 298-306 of NS3 of dengue virus types 2 and 4, and the peptide-2 (GYISTRVEL) were synthesized with 9-fluorenlymethoxycarbonyl chemistry by Sigma Genosis, Japan. The purity was determined to be 95.0% for the peptide-1 and 96.2% for the peptide-2 by reverse phase HPLC.

Induction of dendritic cells

BALB/c mouse bone marrow cells (9x10\textsuperscript{5}) were cultured in 1ml of AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 20ng/ml mouse GM-CSF (R&D Systems, Minneapolis, MN) in 24-well plate at 37 \textdegree{}C in 5% CO\textsubscript{2}. On days 4 and 6, 50 to 75% volume of the culture medium was changed with the fresh one supplemented with the same amount of GM-CSF. On day 7, cells were harvested, subjected to flow cytometry analysis and used as DCs for immunization.

Flow cytometry analysis

Bone marrow-derived cells (4x10\textsuperscript{5}) were incubated with 1µg of FITC-conjugated anti-mouse I-A\textsuperscript{d}/I-E\textsuperscript{d} antibody (BD PharMingen, San Diego, CA), PE-conjugated anti-mouse
CD86 antibody (BD PharMingen), and biotinylated anti-mouse CD11c (BD PharMingen) in 100μl of phosphate buffered saline (PBS) containing 0.02% NaN₃ (PBS/Na₃) at 4 °C for 20 minutes. Same amounts of FITC-conjugated rat IgG₂a,κ (BD PharMingen), PE-conjugated rat IgG₂a,κ (BD PharMingen) and biotinylated hamster IgG group 1, λ (BD PharMingen) were used as isotype controls. The cells were washed three times with PBS/Na₃ at 4 °C, and then incubated with 0.1μg of streptavidin-conjugated Cy-Chrome™ (BD PharMingen) in 100μl of PBS/Na₃ at 4 °C for 20 minutes. The cells were washed three times, fixed with 1ml of PBS containing 1% paraformaldehyde, and analysed by a FACS Calibur (Becton Dickinson, San Jose, CA) and CELL Quest™ version 3.3 software.

Immunization and CTL induction

Bone marrow cells (10x10⁶) stimulated with GM-CSF for 7 days were incubated in the presence of 10μM peptide-2 in 1ml of AIM-V at 37 °C for 2 hours. The cells were washed two times with RPMI-1640. Peptide-2-pulsed cells (2x10⁶) were injected intravenously into BALB/c mice. Four weeks later, the spleens were collected, minced into single cell suspension, erythrocyte-lysed, and treated with anti-CD4 antibody (BD PharMingen) at the rate of 1μg/1x10⁷ cells and 10% baby rabbit complement (Cederlane, Hornby, Ont, Canada) to deplete CD4-positive cells. Five million cells were then co-cultured with the same number of peptide-2-pulsed, 33Gy X-ray-irradiated syngeneic spleen cells in 2ml of EHA medium (Sigma) supplemented with 100μg/ml nucleic acid precursors, 2mM L-glutamine, 5x10⁻⁸M 2-ME, 100U penicillin, 100μg/ml streptomycin, 10mM HEPES, and 10% fetal calf serum in 24-well plate at 37 °C in 5% CO₂. On day 4, half volume of the medium was replaced with fresh one, and 10 U recombinant mouse IL-2 was added. On day 7, the cells were harvested and used as CTL (cytotoxic T lymphocyte) effector cells. Mice were also immunized by subcutaneous injection with 1 n mole of the peptide emulsified with complete Freund adjuvant (CFA) into two-foot pads. In this immunization protocol, draining lymph node (popliteal lymph nodes) cells were used as the effector cells after stimulation in vitro with the peptide as described above. When mice were immunized with the peptide-1, the cells were stimulated in vitro with peptide-1-pulsed spleen cells.

Cytotoxicity assays

P815 cells (1x10⁶) were pulsed with the peptide at a concentration of 10μM in complete medium at 37 °C for 3 hours. The cells were labelled with 100 μCi of Na₂⁵¹CrO₄ (NEN Life Science Products, Boston, MA) for one hour, then washed three times and suspended in complete medium. Peptide-pulsed, ⁵¹Cr-labelled cells were seeded in 96-well V-bottom plate at 1.5x10³ cells in 100μl of complete medium per well. Effector cells were added to the plate to make various effector/target ratios (E/T ratios) in a total volume of 0.2ml per well, and the plate was incubated at 37 °C in 5% CO₂ for four hours. The supernatant fluids were harvested with a Supernatant Collecting System (Skatoron, Lier, Norway), and ⁵¹Cr content was measured by a gamma counter (Aloka model ARC-300). Maximum ⁵¹Cr release was determined by adding 0.1% Triton X, and spontaneous ⁵¹Cr release was determined with the wells that contained target cells and medium only. Assays were performed in triplicate, and the mean value was used to calculate percent-specific lysis with the following formula: % specific lysis = 100 x [(release with effector cells - spontaneous release) / (maximum release -
spontaneous release). Spontaneous release did not exceed 28.9% of the maximum release.

**Results and discussion**

It is known that mature murine DCs strongly express major histocompatibility complex (MHC) class II antigen, co-stimulatory molecules CD80 and CD86, and CD11c, the α chain of p150/95 β2-integrin[9,10]. We examined the expression of MHC class II antigen I-A^d/I-E^d, CD86, and CD11c on BALB/c mouse bone marrow cells, which were cultured with GM-CSF for seven days, by flow cytometry three colour analysis, and evaluated the purity of DC. As shown in Figure 1a, 25.07% of the bone marrow cells strongly expressed both I-A^d/I-E^d and CD86. The percentage of CD11c-positive cells in these double positive cells was 95.81% (Figures 1b and 1c). In contrast, freshly isolated bone marrow cells did not express these surface molecules (data not shown). These results suggest that bone marrow cells were differentiated into DCs during the culture with GM-CSF for seven days and that DCs accounted for one-fourth of the entire population.

We first attempted to induce specific CTLs by two foot pad immunizations with the peptide-1 (GYISTRVEM) emulsified with CFA. Specific CTL activity was not detected in draining lymph node cells. (Table, Experiment no. 1). We speculated that the inability of the peptide-1 to induce specific CTLs might partly be due to the low binding affinity to H-2K^d MHC class I molecule, because the peptide-1 possesses only one anchor residue (Y) for binding to H-2K^d molecule. We, therefore, prepared the peptide-2 (GYISTRVEL) in which the last residue M of the peptide-1...
was substituted for L in order to provide the complete H-2K<sup>d</sup>-binding motif<sup>[11]</sup>.

It was reported that immunization with virus epitope peptide-pulsed DCs efficiently induced virus-specific CD8<sup>+</sup> CTLs and protective immunity<sup>[5,6]</sup>. We attempted to induce specific CTLs by immunization with peptide-2-pulsed DCs. We intravenously injected 2x10<sup>6</sup> bone marrow cells, which were stimulated with GM-CSF for seven days and pulsed with the peptide-2, into BALB/c mice. One-fourth of the cultured bone marrow cells were DCs and it was reported that intravenous immunization with 1x10<sup>5</sup> to 5x10<sup>5</sup> purified DCs pulsed with peptides induced antiviral immunity<sup>[5]</sup>. Spleen cells from the mice immunized with peptide-2-pulsed DCs lysed peptide-2-pulsed P815 cells in a dose dependent fashion after stimulation in vitro with X-ray-irradiated, peptide-2-pulsed syngeneic spleen cells in the presence of recombinant IL-2 for seven days (Table, Experiment no. 2). This result demonstrates that intravenous immunization with peptide-2-pulsed DCs induced peptide-2-specific CTLs.

### Table. Induction of specific CTLs by immunization with peptide-2-pulsed dendritic cells and the peptide-2 emulsified with complete Freund adjuvant

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Immunization</th>
<th>E/T ratio</th>
<th>% Specific lysis&lt;sup&gt;*&lt;/sup&gt;</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Non-pulsed</td>
<td>Peptide-1-pulsed</td>
<td>Peptide-2-pulsed</td>
</tr>
<tr>
<td>1.</td>
<td>Peptide-1/CFA</td>
<td>20</td>
<td>5.4</td>
<td>7.9</td>
<td>Not done</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>22.9</td>
<td>21.9</td>
<td>Not done</td>
</tr>
<tr>
<td>2.</td>
<td>Peptide-2/CFA</td>
<td>10</td>
<td>6.5</td>
<td>Not done</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>8.4</td>
<td>Not done</td>
<td>55.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>9.9</td>
<td>Not done</td>
<td>65.4</td>
</tr>
<tr>
<td></td>
<td>Peptide-2-pulsed DC</td>
<td>10</td>
<td>20.9</td>
<td>Not done</td>
<td>24.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>18.7</td>
<td>Not done</td>
<td>35.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40</td>
<td>24.0</td>
<td>Not done</td>
<td>44.2</td>
</tr>
<tr>
<td>3.</td>
<td>Peptide-2/CFA</td>
<td>5</td>
<td>12.7</td>
<td>23.0</td>
<td>23.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>20.8</td>
<td>30.4</td>
<td>34.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>28.7</td>
<td>46.3</td>
<td>48.3</td>
</tr>
</tbody>
</table>

<sup>*</sup><sup>51</sup>Cr-labeled P815 mastcytoma (H-2<sup>d</sup>) pulsed with the peptide (10µM, 3hours) were used as target cells.

Specific CTL activity was observed in the draining lymph node cells after immunization with peptide-2/CFA. Interestingly, CTLs induced by peptide-2/CFA demonstrated lower levels of non-specific cytotoxic activity to P815 cells than those induced by peptide-2-pulsed DCs. Peptide-2/CFA-induced CTLs lysed peptide-1-pulsed target cells as well as peptide-2-pulsed ones. (Table, Experiment nos. 2 and 3). These results suggest that the peptide-2 which has the complete binding motif to H-2K<sup>d</sup> molecule can induce specific CTLs when used with CFA, and that peptide-2-specific CTLs also lyse original peptide-1-pulsed target cells. Induction of higher levels of non-specific cytotoxicity by immunization with DCs may be due to the high antigen.
presentation ability of DCs to prime various repertoires of T cells. The other possibility is the difference in the source of lymphocytes. We observed that spleen-derived lymphocytes tended to show higher levels of non-specific lysis than lymph node-derived cells (data not shown). It seems that the draining lymph node cells from mice immunized with the peptide and CFA are a better source of CTLs than the spleen cells from those immunized with peptide-pulsed DCs because of low non-specific cytotoxic activity. The data, however, only suggest how efficiently measurable specific CTLs can be induced in vitro. It is plausible that intravenous immunization with peptide-pulsed DCs induces higher levels of specific CTLs in vivo and protective immunity against viral infections. Moreover, CFA is not accepted for human use. Thus, immunization strategy using peptide-pulsed DCs is still worth investigating for induction of CTL-mediated anti-dengue virus immunity.

References


Molecular Characterization of Brazilian Dengue Viruses

Marize Pereira Miagostovich, Flávia Barreto dos Santos and Rita Maria Ribeiro Nogueira

Laboratory of Flavivirus, Department of Virology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Fiocruz Avenida Brasil 4365 Manguinhos, Rio de Janeiro 21040-360, RJ, Brazil

Abstract

Many countries in Central and South America as well as Brazil have been characterized by a rise in dengue endemicity. Since 1986, dengue infection has gained endemicity in these countries and more than 3 million dengue cases have been reported along with the emergence also of the severe forms of the disease. Once intratypic variations among dengue virus (DEN) serotypes have been associated with the disease severity, the molecular characterization of DEN becomes an indispensable tool for the laboratories performing virological surveillance programmes. In countries endemic for DEN, as in Brazil, the monitoring of DEN activity should be an ongoing programme to detect the eventual introduction of new serotypes/genotypes to curb the impact of the circulating strains. Here, the molecular epidemiological studies performed on Brazilian DEN strains are presented in order to contribute to a better understanding of the dengue epidemiology in the country.

Keywords: Dengue viruses, molecular epidemiology, genotypes, Brazil.

Introduction

The dengue (DEN) virus belongs to the Flaviviridae family, genus Flavivirus, and it has four distinct antigenic serotypes (1 to 4) that cause a spectrum of diseases ranging from asymptomatic, mild, undifferentiated fever and classic dengue fever (DF) to more severe forms known as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)\(^1\)\(^,\)\(^2\).

The DEN virus is a spherical particle of approximately 500 Å in diameter, lipid enveloped that includes one segment of a single-stranded positive sense RNA with \(\sim 11,000\) nucleotides in length. The genomic RNA contains a single long open reading frame (ORF) of over 10,000 nucleotides that encodes a polyprotein precursor of about 3,400 amino acid residues which is co- and post-translationally processed by the host cell and virus-specific protease to yield structural and nonstructural proteins. The coding protein region starts with the sequence for the core (C), precursor of membrane (prM/M) and the envelope (E) structural proteins, followed by a series of seven nonstructural (NS) proteins ordered as follows: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The ORF is flanked by two untranslated regions (5’ and 3’ UTR) and has a type I cap at its 5’ end (m7GpppAmp) and appears to lack a 3’-terminal poly A tract\(^3\)\(^,\)\(^4\).

\(^*\) E-mail: marizepm@ioc.fiocruz.br; Fax: 55-21-2598-4373
Electron micrographs show that the virion is characterized by an electron dense core that consists of an isometric nucleocapsid, made up of a single C (100 amino acids) protein, surrounded by a double lipid layer, whereas both E (495 amino acids) and M (75 amino acids) proteins are associated\[5\]. E-glycoprotein is the major surface protein and as showed by crystallography, the flat elongated dimmer extends parallel to the viral membrane\[6\]. The E protein is associated with a number of biological activities, being the most important antigen with regard to virus biology and immunity\[7\].

The intratypic variation of DEN was demonstrated by the fingerprinting method that determined genetic variants within each serotype and employed the term topotype to define variants representing samples from the same geographical region\[8\]. From 1990 onwards, the molecular analysis by the partial sequencing of the DEN genome gathered the topotypes in genomic groups (genotypes) and became an important tool to determine their genetic variation and to identify risk factors associated with the transmission of particular strains\[9\].

The E gene has been the most commonly surveyed gene in dengue molecular epidemiology\[10-15\], although genes that encode nonstructural proteins and non-coding regions have also been used in the phylogeny studies\[16-22\].

Once intratypic variations among different serotypes were associated with the disease severity, technologies for the molecular characterization of DEN became an indispensable tool for laboratories performing virological surveillance programmes. This paper presents the most relevant results of the molecular characterization of DEN strains isolated in Brazil during the last 18 years since dengue became endemic and surveillance programmes were implemented in the country.

**DEN-1**

The first molecular analysis for DEN Brazilian strains characterization was performed by an analysis on genome fragments from DEN-1 by using restriction endonuclease (RE) enzymes. In this study, restriction fragment heterogeneity by Hae III digestion of cDNA products was used to map the distribution of DEN-1 topotype found in the American region. The strains isolated in the state of Rio de Janeiro from human serum specimens from 1986 to 1994\[23,24\] were grouped in the American (Caribbean) topotype, recognized as the only one circulating in the Americas\[8,25\]. The percentage of the similarity observed among DEN-1 Brazilian strains ranged from 60% to 94%, showing the evolution of those samples since its introduction in the state in 1986.

By the sequencing of 240-nucleotides (nts) spanning the E/NS1 junction (111 nts from the 3' end of E gene and 129 nts from the 5' end of NS1 gene), the DEN-1 Brazilian strains were classified as belonging to genotype I which comprises of strains from the Americas, Africa and South-East Asia\[9\].

The DEN-1 Brazilian strains were also analysed by using one-step amplification with four primers that target regions spanning polymorphic endonuclease restriction specific sites (RSS-PCR) and all of them were grouped into subtype C, which corresponds to the largest genotypic group of DEN-1 described as genotype \[9,26\]. The RSS-PCR has become an alternative tool routinely used,
which has allowed the characterization of DEN strains for molecular epidemiological studies performed in endemic countries, providing rapid identification of viruses currently circulating\cite{27-30}.

Another study performed with the DEN-1 Brazilian strains compared the complete sequences of three strains isolated in 1990, 1997 and 2001. The genome analysis of those strains revealed a remarkable conservation of the structural proteins and 27 amino acids substitutions in the nonstructural genes, and 12 of them in the NS4B-NS5 and nine specific to strains BR/97 and BR/01. Those findings also suggested that recombinant events might have occurred, since some amino acids substitutions were previously identified in DEN-1 strains sequenced so far\cite{31}. The evidence that the genetic diversity of DEN might be generated by recombination among those viruses has been described\cite{32-34}.

DEN-2

The DEN-2 fingerprinting analysis of the American strains showed that this serotype exists in the American continent as two topotypes representing strains from the Caribbean region (Puerto Rico) and from the Americas, India and South Pacific\cite{8}. By the sequencing of the E/NS1 junction those topotypes could be related to genotype I (Native American) and to genotype III (South-East Asian/American), respectively. This latter genotype was introduced into the Americas in 1981, and was responsible for the first DHF/DSS epidemic that occurred in the continent and spread throughout the region over the next two decades\cite{35,36}. The direction of the transmission from South-East Asia to the Americas was demonstrated as well, since DEN-2 from Brazil, Colombia, Mexico and Venezuela have a common progenitor with those from South-East Asia\cite{38}.

In Brazil, the first molecular characterization of DEN-2, introduced in the country in 1990\cite{37,38}, was performed by RE analysis and showed a similarity of 80% with the 1981 Jamaica isolate, suggesting the spread of those viruses from the Caribbean region to South America\cite{39}.

The geographical origin of DEN-2 Brazilian strains was also established by the direct sequencing of cDNA fragments amplified by the polymerase chain reaction of a fragment encoding amino acids 29 to 94 in the E gene. Considering a divergence of 6% between the nucleotide sequences as a cut-off for genotype classification, it was demonstrated that the Brazilian strains belonged to the South-East Asian/American genotype. The comparison of the three DEN-2 strains isolated in Rio de Janeiro, two of them obtained from classic dengue cases and one from a fatal case, did not identify the markers for virulence in the region studied\cite{40}.

The analysis of DEN-2 samples isolated in the states of Rio de Janeiro, Ceará, Bahia, and Alagoas between 1990 and 1995 was performed by the partial sequencing of its 1685 and 2504 encompassing the E gene and demonstrated the spread of this serotype from Rio de Janeiro to other states\cite{39}.

All the characteristics observed in the Brazilian DEN-2 genotype were confirmed by the full-length analysis of the nucleotide and amino acids sequence (GenBank access # AF489932)\cite{40}. The Asian-specific non-conserved amino acid differences, previously described by Leitmeyr et al.\cite{41} as well as additional differences specific to the Brazilian strain were found in E, NS3, and NS5.
genes. Changes in the E protein could affect the immunogenicity or cell entry/tropism, whereas changes in NS3 (helicase/protease) and NS5 (RNA-dependent RNA polymerase) could affect replication efficiency. In addition, differences in the predicted secondary structure of the 5’ and 3’ untranslated regions were found between the South-East Asian/American and native American genotypes; in these regions, the Brazilian isolate was identical to the South-East Asian/American strains in sequence and consequently in the predicted secondary structures. These similarities with the South-East Asian/American genotype were also reported recently for the Martinique 703/98 strain after a complete analysis of the genome.

In Brazil, this DEN-2 genotype was responsible for some clinical features, mainly related to the severity of the disease. In regions where DEN-2 accounted for primary infections, as in the states of Bahia and Espírito Santo, the most common clinical feature consisted of classic fever, with frequent exanthema, pruritus and a few severe cases. However, in other states where DEN-2 circulated after extensive epidemics caused by DEN-1, as in Rio de Janeiro, Ceará, Pernambuco and Rio Grande do Norte, an increase in the number of severe cases was observed. The first DHF/DSS case was reported in Rio de Janeiro after the introduction of DEN-2 in 1990, and it was accompanied by an increasing number of hospital admissions resulting from DEN-2 secondary infections.

By using RSS-PCR we were able to analyse geographically and temporally distinct Brazilian DEN-2 strains encompassing ten years (1990 to 2000). The analysis of the RSS-PCR products showed that all Brazilian strains presented the same pattern, presenting consistent and reproducible amplicons of 582bp and 100bp and, occasionally, extra amplicons of 676 bp or 150 bp. The DEN-2 Brazilian RSS-PCR pattern was consistent; however, it did not match any of the RSS-PCR patterns previously described by Harris et al. Once the method was developed with DEN-2 isolates obtained from 1964-1986, the ongoing evolution of those viruses over the last 15 years could explain the genetic diversity observed. Despite those observations, the sequence of the E/NS1 gene junction (GenBank Access # AF529064 to AF529078) showed that the Brazilian DENV-2 strains still belonged to the South-East Asian/American genotype.

**DEN-3**

Different from the DEN-3 genotype IV (topotype Caribbean) responsible for epidemics in the ‘60s and ‘70s, the DEN-3 re-introduced in the American continent after an absence of 17 years belonged to genotype III (topotype Sri Lanka), represented by strains from Sri Lanka and India, which are associated with DHF/DSS cases in those countries.

By the time of DEN-3 isolation in Brazil, RSS-PCR was extremely valuable which once allowed the rapid characterization of the first strain as subtype C, confirming the introduction and direction of transmission of those viruses from Central to South America.

After the RSS-PCR analysis, the nucleic acid sequencing from positions 278 to 2550 of DEN genome was performed (Access number AY038605) and the parsimony analysis generated a phylogram assigning a
Brazilian DEN-3 strain to genotype III, reconfirming those data\textsuperscript{[13,28]}. The similarity rate of a Brazilian DEN-3 strain to others represented by the same subtype III ranged from 96% to 98% and 98% to 99% for nucleic acid and deduced amino acid sequences, respectively. The comparison of the Brazilian DEN-3 with strains isolated in Guatemala showed a total of 14 nucleic acid substitutions, with one of them resulting in an amino acid change from histidine to arginine\textsuperscript{[28]}.

As a result of several DEN-3 epidemics in Latin American countries, a large number of DEN-3 genome sequences have been recently deposited in the GenBank\textsuperscript{[49-51]}. A phylogenetic study compromising DEN-3 strains isolated in Sri Lanka, East Africa and Latin America confirmed the establishment of the new DEN-3 genotype\textsuperscript{[51]}. According to the author, there are two separate lineages formed within genotype III: Group A consisting of isolates from 1981 to 1989 in Sri Lanka and Group B which was expanded in three distinct clades including isolates from 1989 to 1998 in Sri Lanka, strains isolated in East Africa from 1985 to 1993 and isolates from 1994 in Latin America. The phylogenetic analysis suggested that genotype III was introduced from the Indian subcontinent into East Africa in the 1980s and from Africa into Latin America in 1994, showing a single genotype introduction in the continent and its subsequent diversification\textsuperscript{[52]}. In the same year, Peyrefitte et al.\textsuperscript{[53]} showed a high similarity between the DEN-3 Martinique and the Brazilian strains. Furthermore, the complete genome characterization of the Brazilian DEN-3 sequence (AY679147) strain confirmed an insertion of 11 nts in the 5´ non-coding region of the genome as previously described for the Martinique strain\textsuperscript{[53]}.

The severity of the disease and the occurrence of deaths resulting from primary infections during the DEN-3 epidemic in the state of Rio de Janeiro in 2002 could be explained partially by the virulence of this particular genotype\textsuperscript{[28,54]}. Fatal cases, resulting from primary dengue infections, were previously described\textsuperscript{[55]} before the DEN-3 genotype III introduction in Brazil. However, the highest number of DHF/DSS cases that occurred in the state were due to secondary infection by the South-East Asian/American DEN-2 genotype\textsuperscript{[63]}. Those findings corroborated the previous observations that some DEN strains can be more virulent than others, representing an important risk factor for DHF/DSS and that antibody-dependent enhancement (ADE) itself does not explain all cases of severe disease\textsuperscript{[51,56-58]}. Recently, it was suggested that the more virulent genotypes were now replacing those that had a lower epidemiological impact throughout the world\textsuperscript{[59]}.

Conclusion

In the last few decades, dengue has spread as a pandemic in the American continent, starting in the Caribbean islands and expanding to North, Central and South America\textsuperscript{[60]}. In this context, the dengue epidemiological profile in Brazil has changed from a non-endemic to a hyperendemic one. Since the 1980s when the first DEN strains were isolated, more than 3 million dengue cases and nearly 2,090 DHF/DSS cases have been reported in the country (www.funasa.gov.br)\textsuperscript{[61,62]}. The endemicity of dengue in 25 out of the 27 federative units, the remarkable virulence of the DEN-2 and DEN-3 genotypes and the risk of the introduction of
DEN-4 in the country highlight the alarming Dengue epidemiological picture in Brazil. In this scenario, use of rapid methods for DEN identification and molecular characterization are indispensable tools in the virological surveillance laboratories, mainly due to an obvious need to characterize Dengue genotypes before a major outbreak occurs[26,46,63]. The partial sequencing of DEN strains genome has also been used routinely for DEN molecular epidemiological studies, and it recently characterized the co-infecting genotypes of DEN-1 and DEN-2 in a patient presenting classic Dengue fever in São Paulo[64].

The knowledge of the virus genotype circulating in a particular region has also implications for the potential introduction of vaccines, allowing the evaluation of the genomic relations between the viruses used in vaccine development and the circulating strains. The ability of pre-existing Dengue antibodies to neutralize better certain DEN variants than others has been demonstrated. Some strains may produce a more severe disease, not because of the virulence-inherited properties but because antibodies from a primary infection may enhance infection with one genotype while neutralizing infection with a distinct one[52,65].

Given the limited options available for Dengue control, active surveillance programmes with continuous monitoring of Dengue infection in communities is still one of the strategies available to detect the introduction of new serotypes/genotypes, and, consequently, to prevent the occurrence of epidemics, thus minimizing the impact of the circulating strains.

Acknowledgements

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Oswaldo Cruz (FIOCRUZ), PAPES III – FIOCRUZ. Thanks are also due to the staff of the Laboratory of Flavivirus for their technical assistance.

References

Molecular Characterization of Brazilian Dengue Viruses


Molecular Characterization of Brazilian Dengue Viruses


[54] Nogueira RMR. Personal communication.


Unusual Emergence of Guate98-like Molecular Subtype of DEN-3 during 2003 Dengue Outbreak in Delhi


*National Institute of Communicable Diseases, 22 Shamnath Marg, Delhi - 110 054, India
**Sant Parmanand Hospital, 18 Shamnath Marg, Delhi - 110 054, India

Abstract

With a view to identifying the molecular subtype of the circulating dengue virus responsible for a major outbreak of dengue fever (DF) / dengue haemorrhagic fever (DHF) in and around Delhi during the post-monsoon period in 2003, 32 serum samples were collected from clinically suspected cases. These were subjected to reverse transcription/polymerase chain reaction (RT/PCR) for amplification of 511 bp C-PreM gene region of the dengue virus. Seven specimens, yielding a satisfactory quantum of viral RNA, were subsequently processed for automated nucleotide sequencing. Five of the seven analysed isolates showed close DNA sequence homology with Guate96-98 strains of DEN-3 virus, whereas two turned out to be genotype IV of DEN-2. Earlier, DEN-2 (genotype IV) had been identified as the etiological agent during a major DF/DHF outbreak in Delhi in 1996 and also in 2000. Though DEN-2 continues to prevail, DEN-3, having a close sequence homology with Guate96-98 strains, seems to have entered India for the first time in late 2003, resulting in a major DF/DHF outbreak. How the Guate96-98 strain of DEN-3 entered India remains to be linked epidemiologically.

Keywords: Dengue outbreak, molecular typing, CpreM gene, DEN-3, Delhi.

Introduction

Dengue fever/dengue haemorrhagic fever (DF/DHF) is caused by one or more of the four antigenically-related dengue virus serotypes DEN-1 to DEN-4. It is widespread in tropical and subtropical countries in the world and is a serious cause of morbidity and mortality, threatening about one third of the total human population[1-3]. Many outbreaks and epidemics of DF/DHF have been reported in different parts of India during the past four decades[4-8]. In Delhi alone, a number of outbreaks of dengue virus infection were recorded in 1967, 1970, 1982, 1988 and 1990[9-13]. Again in 1996, a major DHF outbreak, resulting in 10,252 cases with 432 deaths, occurred in and around Delhi. DEN-2 genotype IV was the predominant etiological agent[14,15].

Delhi and its adjoining areas were again struck by a major outbreak of DF/DHF between September and December 2003. The present study was undertaken to unveil the predominant molecular subtype of the dengue virus involved in this outbreak.

a E-mail: manojkumardelhi@yahoo.co.in; Tel./Fax: 91-11-23912960

Dengue Bulletin - Vol 28, 2004 161
Emergence of DEN-3 in Delhi in 2003

Materials and methods

Clinical specimens

A total of 32 serum samples from clinically suspected acute cases of DF/DHF were collected from different hospitals in Delhi between September and December 2003. Most of the serum samples were collected within the first five days of the clinical onset. Samples were transported to the laboratory within six hours of collection and stored at –70 °C until processed. Thirty-two sera are considered adequate to establish the predominant molecular subtype of dengue virus.

Virus RNA isolation

Thirty-two serum samples were subjected to dengue viral RNA isolation. 140µl sera sample was processed for RNA isolation using QIAamp viral RNA Kit (QIAGEN, Germany) using standard kit protocol. Finally, viral RNA was eluted in 30µl nuclease-free water.

Reverse transcription/polymerase chain reaction (RT/PCR)

RT-PCR was carried out using previously reported D1 and D2 primers that were M13 tailed for the convenience of nucleotide sequencing. This primer set is capable of amplifying all the four types of dengue viruses (DEN-1 to DEN-4).

Primer D1 (with M13F tail)

(5′-TGTAACGAGGAGCAGTTCATATGCTGAAACGCGAGAACC-3′)
(M13 forward primer sequence underlined)

Primer D2 (with M13R tail)

(5′-CAGGAAACACAGCTATGACCTTTGACCAAACAGTCAATGCTTCCAGGTTC-3′)
(M13 reverse primer sequence underlined)

Complementary DNA (cDNA) synthesis and gene amplification of 511bp CpreM gene region of the dengue virus was performed using one step GeneAmp RNA Gold RT PCR Kit (Applied Biosystems, USA) with D1 and D2 primers for detection and typing of all the four types of dengue virus⁹⁸. Briefly, 50µl reaction mix containing final concentration of 1X of 5X RT buffer, 1.5mM MgCl₂, 200µM dNTPs, 5mM dithiotheratol and 10pmol of D1 and D2 primers, 10U RNAase inhibitor, 15U of M UlV MultiScribe reverse transcriptase, 2.5U of AmpliTaq Gold DNA polymerase and 5 µl of extracted viral RNA. RT was performed at 42 °C for 20 minutes on GeneAmp 9700 PCR System. Then pre-hold at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, 72 °C for 1 minute and final extension for 72 °C for 10 minutes and hold at 4 °C. Appropriate positive and negative controls were used in RT/PCR. PCR products were electrophoresed on 1.5% agarose gel along with 100bp DNA ladder marker (MBI Fermentas, USA) and were visualized on gel documentation system (Biometra, Germany).

Gene sequencing and phylogenetic analysis

PCR amplicons were purified using Centricon-100 columns (Millipore, USA) and subjected to automated dideoxy chain
termination nucleotide cycle-sequencing using commercial ABI PRISM™ Big Dye Terminator Cycle Sequencing Kit with AmpliTag DNA Polymerase FS, following the manufacturer’s protocol and run on ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, USA). Nucleotide sequences were edited and aligned using Sequence Navigator Software. Subsequently, blast search (http://www.ncbi.nlm.nih.gov/blast) and phylogenetic analysis using DNA Star software, were done to reveal the dengue virus molecular subtype.

**Result and discussion**

During the study, a majority of the subject patients had clinical symptoms of DF and only sporadic cases presented symptoms of DHF/DSS {DF: 24 (75%), DHF: 7 (21.9%) and DSS: 1 (3.1%)}. The samples belonged to all age groups ranging from 5 to 50 years. The male-female ratio was 18:14. The mean platelet count was 77,120, which ranged from 18,000 to 250,000. Most of the samples collected within five days from the onset of the fever were selected for RT-PCR testing.

Out of the 32 serum samples subjected to RT-PCR, only seven yielded amplification of 511bp C-PreM gene region of dengue virus. Lane 1-14 in the Figure included clinical samples, 100bp DNA ladder marker in lane M and negative control in lane Neg. Automated nucleotide sequencing of these seven RT/PCR products revealed two groups of sequences, the first group had five almost identical sequences, while the second group had two similar sequences. All samples were subjected to blast search, which revealed that five isolates had a very close sequence homology (=98%) with Guate98 AB038478 strain of dengue type 3 (DEN-3), while two turned out to be DEN-2 and showed =99% homology with Delhi96 AF047394 strain Delhi 2000 strains (Table). These Cpre M gene sequences were submitted in the Gene Bank wide accession AY 706094-AY706099.

**Result and discussion**

During the study, a majority of the subject patients had clinical symptoms of DF and only sporadic cases presented symptoms of DHF/DSS {DF: 24 (75%), DHF: 7 (21.9%) and DSS: 1 (3.1%)}. The samples belonged to all age groups ranging from 5 to 50 years. The male-female ratio was 18:14. The mean platelet count was 77,120, which ranged from 18,000 to 250,000. Most of the samples collected within five days from the onset of the fever were selected for RT-PCR testing.

Out of the 32 serum samples subjected to RT-PCR, only seven yielded amplification of 511bp C-PreM gene region of dengue virus. Lane 1-14 in the Figure included clinical samples, 100bp DNA ladder marker in lane M and negative control in lane Neg. Automated nucleotide sequencing of these seven RT/PCR products revealed two groups of sequences, the first group had five almost identical sequences, while the second group had two similar sequences. All samples were subjected to blast search, which revealed that five isolates had a very close sequence homology (=98%) with Guate98 AB038478 strain of dengue type 3 (DEN-3), while two turned out to be DEN-2 and showed =99% homology with Delhi96 AF047394 strain Delhi 2000 strains (Table). These Cpre M gene sequences were submitted in the Gene Bank wide accession AY 706094-AY706099.

**Figure. PCR products visualization on 1.5% agarose gel**

Gel picture showing 511bp CpreM gene amplification of dengue virus. 1-14: dengue suspected clinical samples, M: 100bp DNA Ladder marker and Neg: negative control.
Emergence of DEN-3 in Delhi in 2003

Table. Clinical and molecular analysis data of seven samples, yielding RT-PCR positivity for 511bp CpreM gene of dengue virus

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sample ID*</th>
<th>Serum collection</th>
<th>Clinical state</th>
<th>Age/Sex</th>
<th>Platelet count</th>
<th>RT-PCR 511bp</th>
<th>Assigned genotype on blast search</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>04DEL03</td>
<td>22.09.03</td>
<td>DF</td>
<td>30/F</td>
<td>89,000</td>
<td>+ve</td>
<td>DEN-3</td>
</tr>
<tr>
<td>2</td>
<td>06DEL03</td>
<td>24.09.03</td>
<td>DF</td>
<td>20/M</td>
<td>79,000</td>
<td>+ve</td>
<td>DEN-2</td>
</tr>
<tr>
<td>3</td>
<td>10DEL03</td>
<td>07.10.03</td>
<td>DHF</td>
<td>15/M</td>
<td>39,000</td>
<td>+ve</td>
<td>DEN-3</td>
</tr>
<tr>
<td>4</td>
<td>11DEL03</td>
<td>08.10.03</td>
<td>DF</td>
<td>14/F</td>
<td>NA</td>
<td>+ve</td>
<td>DEN-3</td>
</tr>
<tr>
<td>5</td>
<td>16DEL03</td>
<td>01.10.03</td>
<td>DF</td>
<td>34/F</td>
<td>18,000</td>
<td>+ve</td>
<td>DEN-2</td>
</tr>
<tr>
<td>6</td>
<td>19DEL03</td>
<td>04.10.03</td>
<td>DHF</td>
<td>40/M</td>
<td>38,000</td>
<td>+ve</td>
<td>DEN-3</td>
</tr>
<tr>
<td>7</td>
<td>26DEL03</td>
<td>13.10.03</td>
<td>DHF</td>
<td>16/M</td>
<td>36,000</td>
<td>+ve</td>
<td>DEN-3</td>
</tr>
</tbody>
</table>

Out of 32 samples subjected to RT-PCR for 511bp CpreM gene of dengue virus, only 7 turned positive, while 25 did not show any amplification.

All five DEN-3 strains had ≥98% nucleotide sequence homology with Guate98 strains. Both DEN-2 strains had ≥99% nucleotide sequence homology with already circulating Delhi96 and Delhi2000 strains.

NA: Information not available

Several studies have shown that dengue virus infection has been endemic in different parts of India, as documented for over four decades[17,18], and almost all the four known serotypes of dengue virus (DEN-1 to DEN-4) have been reported. The metropolitan city of Delhi witnessed several outbreaks of DF/DHF in 1967, 1970, 1982, 1988,1996 and 2000. DEN-1 and DEN-3 viruses were associated with the 1970 epidemic, DEN-1 and DEN-2 with the 1988 epidemic, while genotype IV of DEN-2 was responsible for the major DHF outbreak in 1996[19]. Our previous findings revealed genotype IV of DEN-2 as the predominant type circulating from 1996 onwards, based on RT-PCR and C-PreM gene sequencing, although cases of DEN-1 were also detected[19-21].

During the present study, the majority of the patients had clinical symptoms of DF and only sporadic cases presented with symptoms of DHF or DSS. The samples referred to our laboratory for molecular characterization were accompanied by IgM serology results. When cross-checked, we found that five RT/PCR-positive samples were IgM-negative, while two RT/PCR-positive samples were dengue IgM-positive. Previous studies had also shown that most, but not all, RT/PCR-positive samples had negative IgM serology. The reason for this is attributed to the fact that the virus had not been recovered from most of the DF/DHF patients beyond the 5th day of the onset of
the symptoms, while detectable levels of dengue-specific IgM antibodies appear after the 4th or the 5th day. Our findings re-established that the ideal time for the collection of samples for molecular test was between days 1-5.

The nucleotide sequence alignment and blast search of the seven RT/PCR-positive samples in the present study, when compared with those of earlier ones, revealed that only ~29% (2/7) belonged to the already prevalent genotype IV of DEN-2; whereas the majority ~71% (5/7) showed close genomic homology (=98%) with GUATE98 AB038478 strain of dengue type 3 (DEN-3) which caused a widespread dengue outbreak in Guatemala in the late Nineties[22]. A similar strain is also reported to have been reintroduced in Martinique (French West Indies)[23] and in Rio de Janeiro, Brazil (unpublished data vide Gene Bank Accession No. AY679147). The first reported evidence of DEN-3 in Delhi was in 1970, based on serotyping, but no genomic data of the 1970 strain of DEN-3 is available. It is difficult to ascertain, after a long gap of 33 years, whether the old 1970 strain of DEN-3 had re-emerged during the current outbreak; or Guate98 DEN-3 strain (prevalent in South American countries) had been introduced for the first time in India.

The changing epidemiology of different subtypes of dengue virus and their co-existence and/or replacement of one type by the other is well documented[24]. During the present outbreak, we found DEN-3 as the predominant type, but it did not seem to completely replace the previously circulating DEN-2. Prior to 1977, co-existence of DEN-2 and DEN-3 in the Americas had also been reported[25]. The first epidemic of DEN-3 in Jamaica and Puerto Rico was witnessed in 1963, which was followed by another epidemic of DEN-3 in Colombia and Puerto Rico in the mid-1970s, and in the Pacific islands in early 1980s[26]. In 1994, a new strain of DEN-3 was introduced in the Americas, causing a major epidemic of DF/DHF in Nicaragua and an outbreak of DF in Panama[27]. But this DEN-3 was genetically different from the DEN-3 strains which circulated in the Americas earlier. Interestingly, in 1994, this DEN-3 genotype was reported to have a close identity with those strains which caused DHF epidemic in some of the South-East Asian countries around the same period[28]. This DEN-3 strain subsequently spread from Asia to Central America and Mexico in 1995 and caused major epidemics. A classic example of the replacement of one type by the other is evident from the fact that, in 1971, DEN-2 was introduced into the Pacific areas followed by a new strain of DEN-1 in 1975 and DEN-4 by 1979, and in early 1980s by yet another new strain of DEN-3[29]. These reports support our current findings that, although DEN-2 (genotype IV) has been predominant in northern India over the past few years, Guate96-98-like DEN-3 strain dominated during the major outbreak of DF in Delhi in 2003.

Acknowledgements

We thank Ms Priyanka, Ms Kamini Singh and Ms Seema George for their technical assistance. The secretarial assistance of Mr A.K. Manchanda, Ms Kiran Bhatt and Ms Sarita Kumar is acknowledged.
References


Emergence of DEN-3 in Delhi in 2003


The Animal Models for Dengue Virus Infection

Tao Peng, Junlei Zhang and Jing An

Department of Microbiology, College of Medicine, Third Military Medical University, Chongqing, 400038, People's Republic of China

Abstract
Currently, the mechanisms involved in the pathogenesis of DHF/DSS remain poorly understood and there is no effective vaccine available to prevent infection with DEN virus. The lack of a reliable small animal model that mimics dengue disease is a major obstacle. In this paper, the development of small animal models such as mice for dengue virus infections is reviewed.

Keywords: Dengue virus, animal model, mice.

Introduction
Dengue (DEN) viruses are mosquito-borne RNA viruses, which belong to the genus Flavivirus (family Flaviviridae), and are grouped into four antigenically distinct types (DEN-1, DEN-2, DEN-3, and DEN-4). Every year, they infect millions of people and can cause a mild-to-debilitating febrile illness (classical dengue fever, DF) or life-threatening syndrome (dengue haemorrhagic fever/dengue shock syndrome, DHF/DSS). In recent years, the geographical range of dengue in tropical and subtropical regions of the world has extended and DHF/DSS is occurring in new areas and with increased incidence[1]. Cardinal signs of DHF/DSS include haemorrhage, abrupt onset of vascular leakage and shock, accompanied by severe thrombocytopenia and massive complement activation. However, the mechanisms involved in the pathogenesis of DHF/DSS remain poorly understood and there is no effective vaccine available to prevent infection with any of the four serotypes of DEN virus. A major technical barrier is the absence of a suitable animal model that mimics DEN disease, including DHF/DSS. So far, there are only three known hosts for DEN virus infections: mosquitoes, humans and lower primates[2]. Although these lower primates infected with wild type DEN viruses develop viremia, they generally manifest only very mild or no clinical signs of disease[3]. Since the appearance of the severe combined immunodeficiency (SCID) mice in 1983[4], efforts have been made to develop new small animal models that may be useful for the development of a future DEN virus vaccine and for studying the pathogenesis of DEN virus infections.

* E-mail: anjing60@yahoo.com.cn; Tel.: +86-23-68752774; Fax: +86-23-65463259
Animal models based on SCID mice

The SCID mice, which do not produce functional T and B cells and lack detectable immunoglobulin (Ig), can support DEN-susceptible human cell lines xenografts, and this system has been employed to study DEN virus infection in vivo. SCID mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID) have been used for studies on the pathogenesis of infection with the human immunodeficiency virus (HIV)[5,6] and for research on treatment of HIV infection[7].

The hu-PBL-SCID mice were firstly evaluated as an animal model for DEN viral infection in 1995[8]. SCID mice were injected intraperitoneally (ip) with hu-PBL for reconstitution and successful engraftment was demonstrated by the presence of a high serum level of human IgG. Hu-PBL-SCID mice were ip-infected with DEN-1 virus. Unfortunately, only 5 of 19 hu-PBL-SCID mice showed sensitivity to DEN-1 virus. It was suggested that the main reason for the low DEN infection rate was a scanty number of appropriate human target cells in the reconstituted mice. Thus, investigators searched for more DEN-susceptible human cell lines to improve the infection rate of the SCID mouse model. One promising candidate was K562 cell, an erythroleukemia cell line. SCID mice were engrafted ip with K562 cells (K562-SCID mice)[9]. After intratumor injection into the peritoneal tumor masses of DEN-2 virus, K562-SCID mice showed neurological signs of paralysis and died at approximately 2 weeks post-infection (pi). In addition to being detected in the tumour masses, high virus titers were detected in the peripheral blood and the brain tissues, indicating that DEN virus had replicated in the infected K562-SCID mice. Other serotypes of DEN viruses were also used to infect the K562-SCID mice, and the mortality rates of the infected mice varied with different challenge strains, suggesting that this animal system might potentially be utilized to define the virulence of various human DEN isolates and to characterize the molecular determinants for such viral virulence. K562-SCID mice were also challenged with DEN-2 virus and received antibody administration at the same time or one day earlier, and the results revealed that these mice exhibited a reduction in mortality and a delay of paralysis onset after DEN virus infection. These results indicated that an in vitro neutralizing antibody also defended K562-SCID mice against DEN-2 virus infection.

Target cells and organs for DEN virus replication in humans remain unclear. Unusual clinical manifestations, mostly cerebral and hepatic symptoms, have become more common in patients with DEN virus infection in recent years[10,11]. The involvement of liver cells in the pathogenesis of DEN virus infection has been indicated by abnormal liver function, pathological findings and detection of viral antigen in hepatocytes and Kupffer's cells at biopsies[12]. It was reported that DEN virus could replicate in a human heptocarcinoma cell line, HepG2, and infectious particles were released into the culture medium[13,14]. Therefore, HepG2 cells were transplanted into SCID mice to develop an animal model for studying the pathogenesis of DEN virus infection[15]. The replication of HepG2 cells in host mice was confirmed by an increase of serum human albumin and propagation of HepG2 cells in the liver. At 7-8 weeks
after transplantation, HepG2-grafted SCID mice were ip-infected with DEN-2 virus. A high titer of the virus was detected in the liver and serum but not in the brain in the early stage of the infection. When the mice showed paralysis, the highest titer of virus was detected in the serum and brain. DEN-2 antigens were also found in HepG2 cells of the liver in the early stage and some neurons of the brain in the late stage. Upon clinical examination, thrombocytopenia, prolonged partial thromboplastin time, increased haematocrit, blood urea nitrogen and tumour necrosis factor a (TNF a) were seen in the paralyzed mice. Moreover, mild haemorrhages in the liver and tarry stool in the small intestine were observed in some mice.

All of above animal models based on SCID mice with transplanted DEN-susceptible human cells mimic some of the aspects of human disease, which may be helpful for studying DEN virus infection, especially in the areas of viral pathogenesis, virus-host interaction and vaccine development against DEN infections. However, it is generally agreed that DHF/DSS is an immune-mediated disease, and since SCID mice are unable to produce the innate immune response, this may impose some limitations on the use of these animal models to extrapolate the situation in human DHF/DSS.

**BALB/c mouse model**

Inbred four-week-old BALB/c mice were found sensitive (haplotype H-2d) to the challenge with dengue virus type 2 (strain P23085)[16]. Mice were ip-infected with a dose of 5 LD50 of the mouse-adapted DEN-2 virus, and the first clinical manifestations such as arching of the back, ruffling of the fur and slowing of activity appeared at end of day 4 pi. The presence of DEN-2 virus in the blood was confirmed on day 2 pi by reverse transcriptase-polymerase chain reaction (RT-PCR). The development of the experimental DEN-2 virus infection in mouse model was accompanied by the virus reproduction in all animals. Within 5 and 6 days pi, all mice showed severe sickness with anorexia and weight loss ending in limb paralysis and 100% mortality rate was noted at 7 days pi. The most impressive changes were seen with TNF-a, which abruptly and steeply increased 24 h before death. Serum levels of interleukin (IL)-1ß, IL-6, IL-10, IL-1 receptor antagonist and soluble TNF receptor I continuously increased during the time of infection. Treating animals with anti-TNF serum reduced the mortality rate down to 40%. This model supports the view that the activation of innate immune response is at least partially responsible for mortality in DEN-2 virus infection, and in line with this concept, anti-TNF treatment significantly reduces the mortality rates. Therefore, inbred 4week-old BALB/c mice are useful models to research the immune activation of host in DEN-2 virus infection.

**Gene knockout mouse (AG129) model**

There is evidence that alpha and beta interferons (IFN-a/ß) and gamma IFN (IFN-?) might be involved in human DEN virus infection[17,18]. In addition, exogenously administered IFN appears to protect mice from DEN virus challenge[19]. This information suggested that mice defective in their IFN response might provide a suitable
model for DEN virus infection. Intraperitoneally administered mouse-adapted DEN-2 virus was uniformly lethal in AG129 mice, which lack IFN-α/β and IFN-γ receptor genes, regardless of age. The mice showed neurological abnormalities, including hind-leg paralysis and blindness at 7 days pi, and died at 12 days after infection. The immunized mice were protected from virus challenge, and the survival time increased following passive transfer of anti-DEN polyclonal antibody. To determine which aspect of the IFN response was critical in protecting these mice from DEN virus infection, animals individually deficient in either IFN-α/β (A129) or IFN-γ (GKO) functions as well as BALB/c controls were subjected to a similar DEN virus challenge. None of these mice exhibited any overt symptoms of illness, indicating that for DEN virus infection, IFN-α, -β, and -γ abnormalities in combination were necessary for the mouse-adapted virus to be lethal when the ip challenge route was used. These results demonstrated that AG129 mice were a promising small animal model for DEN virus vaccine trials.

A/J mouse model

DEN virus infection causes DF and DHF/DSS. No animal model is available that mimics this clinical manifestation. The immunocompetent mouse (A/J strain) was reported as a mouse model for DEN virus infection that resembles the thrombocytopenia manifestation. Intravenous injection of DEN-2 virus into A/J mice induced paraplegia at 2-3 weeks, while the mock-infected controls were normal. Viremia detected by RT-PCR was found transiently at two days but at no other time after infection. Although A/J mice developed paraplegia after virus infection, they recovered after one month. However, there was transient thrombocytopenia at 10-13 days pi. When the mice were re-infected with the same DEN-2 virus two months later, thrombocytopenia was manifested again at 10 days after infection. Anti-platelet antibody was also generated after injection. And there was strain variation in DEN-2 virus infection; the A/J strain was more sensitive than BALB/c or B6 mice. These results show that this DEN-2 virus-infected mouse system accompanied by thrombocytopenia and anti-platelet antibody may be a suitable model to study the pathogenicity, especially immune activation in DEN virus infection. On the other hand, A/J mice had to be inoculated with a large quantity of DEN-2 virus; a dose of less than $1 \times 10^8$ pfu per mouse was not effective in causing paraplegia. Furthermore, viremia was low and transient in A/J mice compared with that in SCID or IFN-deficient AG129 mice. DEN-2 virus could not be isolated from the blood of infected mice; it could only be detected by sensitive RT-PCR in A/J mice.

Conclusion

Although the above-mentioned new small animal models, which mimic some of the aspects of human DEN virus disease, may facilitate not only the study of DEN pathogenesis but also the evaluation of anti-DEN virus as well as vaccine development, there are still drawbacks in each model, especially in mimicking DHF/DSS. Presently, the molecular mechanisms underlying the pathogenesis of DHF/DSS remain unknown, such as the receptors of DEN virus which are still not clear. Even though the use of transgenic animals has been proposed in the quest for an animal model, it is apparent
that one needs to know more about the mechanisms involved in the pathogenesis of DHF/DSS at the molecular level before one can construct a transgenic animal to serve as a model for use in research on the pathogenesis, vaccine development and therapy for DHF/DSS.

Acknowledgment

This work was partially supported by grants nos. 30170848 and 30300303 from the National Science Foundation of China (NSFC).

References


Philippine Species of Mesocyclops (Crustacea: Copepoda) as a Biological Control Agent of Aedes aegypti (Linnaeus)

Cecilia Mejica Panogadia-Reyes*, Estrella Irlandez Cruz** and Soledad Lopez Bautista***

*Department of Biology, Emilio Aguinaldo College, Ermita, Manila, MM, Philippines
**Research Institute for Tropical Medicine, Alabang, Muntinlupa, MM, Philippines
***Department of Medical Technology, Emilio Aguinaldo College, Ermita, Manila, MM, Philippines

Abstract

The predatory capacity of two local populations of Mesocyclops aspericornis (Dayad) and Mesocyclops ogunnus species were evaluated, for the first time in the Philippines, as a biological control agent for Aedes aegypti (L) mosquitoes. Under laboratory conditions, Mesocyclops attacked the mosquito first instar larvae by the tail, side and head. The mean of first instar larvae consumed by M. aspericornis and M. ogunnus were 23.96 and 15.00, respectively. An analysis of the variance showed that there was a highly significant difference between the mean number of first instar mosquito larvae consumed by M. aspericornis and by M. ogunnus, which indicated that the former is a more efficient predator of dengue mosquito larvae.

The results of the small-scale field trials showed that the mean number of surviving larvae in experimental drums was 63.10 and in control drums was 202.95. The Student t-test of means indicated that there was a significant difference between the mean number of surviving larvae in the drums with and without M. aspericornis females were good biological control agents, for they destroyed/consumed about two-thirds of the wild dengue mosquito larvae population.

Keywords: Mesocyclops aspericornis, Mesocyclops ogunnus, biological control agent, Aedes aegypti, Aedes albopictus, Philippines.

Introduction

Copepods feed on paramecium (ciliates protozoans) while naupli feed on Chilomonas spp. The adult planktonic copepods utilize diatoms as the principal food, while predacious adult copepods feed on protozoa, rotifers and several aquatic animals of their own size including small fishes and mosquito larvae. Some adults are able to tear pieces out of the body of their victims with their strong mandibles. In the Philippines, around 14 species of Cyclops have been recorded, of which Mesocyclops aspericornis and M. ogunnus are more common.[1,2]
In Brazil, a study reported that under laboratory conditions, four different strains of *M. aspericornis* showed the potential as biological control agents of *Aedes aegypti* larvae. In Viet Nam, under laboratory conditions, *M. aspericornis* consumed a mean 23.75 L1 and killed a mean 13.43 L1, or a total of 37.18 L1 within 24 hours. *M. ogunnus*, on the other hand, consumed a mean 8.48 L1 and killed a mean 7.54 L1, or a total of 16.02 L1 within 24 hours. In Australia, six species of *Mesocyclops* were evaluated as biological control agents of *Aedes aegypti*.* Of these, *M. aspericornis* was found to be the most effective predator. This study attempted to evaluate, for the first time, the potential of the local population of two species of *Mesocyclops* as biological control agents of *Aedes aegypti* (L) under laboratory and field conditions in the Philippines.

**Methodology**

**Mesocyclops culture**

*Mesocyclops aspericornis* and *M. ogunnus* were raised in laboratory following the techniques adopted by Marten and Thomson. To ensure the establishment of a single species culture, a female with egg sacs was captured and placed in a petri dish for examination under a dissecting microscope before it was transferred to a wide-mouthed beaker containing 100 ml of mixed culture of *Paramecium caudatum* and *Chilomonas* sp. (food of *Mesocyclops*). Populations of *Chilomonas* sp. and *P. caudatum* in the culture bottle were maintained using sterile wheat seeds. Sample specimens from copepods culture were sent to Maria Holynska, Museum and Institute of Zoology, Warsaw, Poland, for identification.

**Laboratory trials**

In a 600 ml beaker, 500 ml filtered tap water with pH 7 was poured. Then 50 *Aedes aegypti* L1 obtained from laboratory culture and a female *M. aspericornis* were added at the same time. The same procedure was done for *M. ogunnus*. The control group did not receive *Mesocyclops*. The experiment was replicated six times and was observed every day for five consecutive days, with daily replacements of new L1. The daily number of L1 destroyed or consumed by *M. aspericornis* and *M. ogunnus* and those that died in the control group were determined. Copepods' feeding behaviour was observed.

**Field trials**

Permission to conduct the study in Estero de Tanque located at P Nieto Street, Barangay 674, Zone 73, Paco, Manila, was secured from the community health officials and Barangay chairperson. Members of the households were informed regarding the procedures to be undertaken and the possible benefit they could derive from it. Consent from the caretakers of the sample households was also secured.

A preliminary study was conducted for 15 days. Twenty houses were chosen as study sites. Two drums per household or a total of 40 drums were emptied and filled almost to the brim with tap water, pH 7.2. A litre of water as sample from each drum container was collected in sterile bottles and brought to the laboratory to exclude the presence of fungi, bacteria and indigenous copepods. Mosquito eggs that hatched into larvae in drums were monitored and collected daily for
recording and identification. Water pH, water temperature and ambient temperature were taken daily. Drums were checked daily for the presence of mosquito eggs and larvae with the aid of a magnifying glass. Drums with mosquito larvae were marked and female copepods were introduced in experimental drums using the ratio one Mesocyclops aspericornis per 50 Aedes aegypti first instar larvae (L1). No copepods were introduced in the 20 control drums. Observation was made daily for 15 consecutive days. Surviving larvae were collected from all drums and brought to the laboratory for recording and species identification.

**Results and discussion**

**Laboratory trials**

The results of the predatory capacity of M. aspericornis and M. ogunnus as evaluated under laboratory conditions and analysis of variance are presented in Tables 1 and 2. The mean L1 consumed by M. aspericornis was 23.96 while that of M. ogunnus was 15.00. Control means were 0.63 L1 and 0.60 L1, respectively. The findings showed that there was a highly significant difference between the mean number of Ae. aegypti L1 consumed by M. aspericornis and that of M. ogunnus and the control group.

**Table 1.** Predatory capacity of female Mesocyclops aspericornis vs Aedes aegypti first instar larvae in 500 ml filtered water under laboratory conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>F value</th>
<th>Tabular f</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group</td>
<td>23.96</td>
<td>338.83</td>
<td>7.12</td>
<td>Highly significant</td>
</tr>
<tr>
<td>Control group</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Predatory capacity of female Mesocyclops ogunnus vs Aedes aegypti first instar larvae in 500 ml filtered water under laboratory conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>F value</th>
<th>Tabular f</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group</td>
<td>15.00</td>
<td>319.18</td>
<td>7.12</td>
<td>Highly significant</td>
</tr>
<tr>
<td>Control group</td>
<td>0.60</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To determine if there was a difference between the mean number of L1 consumed by M. aspericornis and M. ogunnus, the analysis of variance was carried out (Table 3). The findings showed that there was a highly significant difference between the mean number of L1 consumed by M. aspericornis and by M. ogunnus. The study showed that M. aspericornis was a more efficient predator of Aedes aegypti larvae.
Table 3. Comparison of the predatory capacity of M. aspericornis and M. ogunnus under laboratory conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean</th>
<th>F value</th>
<th>Tabular f</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. aspericornis</td>
<td>23.96</td>
<td>36.11</td>
<td>7.12</td>
<td>Highly significant</td>
</tr>
<tr>
<td>M. ogunnus</td>
<td>15.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Predatory capacity of Mesocyclops aspericornis vs Aedes larvae in small-scale field trials using drum containers

<table>
<thead>
<tr>
<th>Treatments</th>
<th># Replicates</th>
<th>Mean ± SD</th>
<th>P value</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group</td>
<td>20</td>
<td>63.10 ± 59.43</td>
<td>0.0002</td>
<td>Significant</td>
</tr>
<tr>
<td>Control group</td>
<td>20</td>
<td>202.95 ± 140.43</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Field trials
The results of the field trials are presented in Table 4.

The mean L1 in experimental drums was 63.10 and 202.95 L1 in control drums. To determine the difference between the mean number of surviving larvae in the drums with M. aspericornis and in the drums without M. aspericornis, a Student t-test was used. The findings indicated that there was a significant difference between the mean number of surviving larvae in the drums with and without Mesocyclops. This significant difference suggests that M. aspericornis is a good biological control agent, for it consumed about two-thirds of the wild, dengue mosquito larvae population.

Conclusion
The results of the study showed that Mesocyclops aspericornis was an efficient predator of Aedes aegypti larvae both in laboratory and field conditions. These copepods could be effectively used for the control of Aedes breeding in non-removable containers, viz. drums and used tyres.

Acknowledgements
The World Health Organization and the Yaman Lahi Foundation, Inc. – Emilio Aguinaldo College provided financial support for this project. We acknowledge the kind assistance of Dr Kevin Palmer of the WHO Western Pacific Region; Dr Jose Paulo E. Campos of Yaman Lahi Foundation, Inc. – Emilio Aguinaldo College (YLFI-EAC), Philippines; Dr Remigio M. Olveda of the Department of Health – Research Institute for Tropical Medicine (DOH-RITM), Philippines; Dr Maria Holynska of Museum and Institute of Zoology – Polish Academy of Science (MIZ-PAS), Poland; Dr Vu Sinh Nam of the National Institute of Hygiene and Epidemiology (NIHE), Viet Nam; and Dr Brian H. Kay of the Queensland Institute of Medical Research (QIMR), Australia.
References


Susceptibility of Aedes aegypti to Insecticides in Viet Nam

Vu Duc Huong*, Nguyen Thi Bach Ngoc, Do Thi Hien and Nguyen Thi Bich Lien

Department of Entomology, National Institute of Malariology, Parasitology and Entomology, B.C. 10 200 Tu Liem, Hanoi, Viet Nam

Abstract
During 2000 -2002, studies on the susceptibility of Aedes aegypti to insecticides were conducted at 22 places in 11 provinces and cities in four different regions of Viet Nam. Aedes aegypti was found susceptible to malathion, but resistant to DDT in almost all the study sites. It continues to be susceptible to the pyrethroid group of insecticides (permethrin, lambda-cyhalothrin, deltamethrin and alpha-cypermethrin) in many places in the North and Centre regions, but is resistant to these insecticides in many places in the South and Central Highlands in Viet Nam. However, the species was found highly and widely resistant to etofenprox.

Keywords: Aedes aegypti, pyrethroids, insecticides, Viet Nam.

Introduction
Insecticidal measures, especially in the outbreak-risk areas, are the most important for the control of Aedes aegypti, the main vector of DHF. Many insecticides of the group organochlorine (DDT), organophosphorous (fenthion, malathion and temephos) and pyrethroid (permethrin, lambda-cyhalothrin, deltamethrin, etc.) have been used for the malaria control programme and for Aedes aegypti. Aedes aegypti has been resistant to DDT since the early 1960s and cross-resistant to many insecticides of the pyrethroid and temephos groups in many countries, but is not yet resistant to malathion[1-3]. When this species is resistant to the insecticides of the pyrethroid group, the organophosphorous ones could take their place[4].

Both malaria and DHF were endemic in many mountainous, forested and coastal plain areas of Viet Nam where house spray and bednet treatment were applied for years. DDT was widely used before 1990 and later lambda-cyhalothrin, permethrin and deltamethrin[5] were introduced. In 1999, Aedes aegypti was found resistant to DDT and some insecticides of the pyrethroid group in many places in Nam Bo (the South), Central Highlands, but not yet to malathion[6]. This study provides more data on the susceptibility of Aedes aegypti to insecticides in different regions of Viet Nam.

Materials and methods
Time and study regions
During 2000-2002, studies were conducted at 22 places (located in 11 provinces and
cities) - six places in the North, six in the Centre, six in the South and four in the Central Highlands.

Methods

The WHO standard bioassay tests (1998) were followed and the papers treated with DDT 4%, the control paper with OC (organochlorine control), malathion 5% and the control paper with OP (organophosphate control) and 5 insecticides of the pyrethroid group (permethrin 0.75%, lambda-cyhalothrin 0.05%, deltamethrin 0.05%, alpha-cypermethrin 30mg/m² and etofenprox 0.5%) with the same control paper PY (pyrethroid control).

The tests were done at a temperature of 25 °C ± 2 °C and humidity of 75-85%. The unfed, F1 mosquitoes, one or two days old - at least 150 mosquitoes for each insecticide, 100 for the test and 50 for the control, were used. Twenty-five mosquitoes were put in each test tube and the per cent mortality count was done 24 hours after the exposure. The mosquitoes in the resting tubes were then fed with glucose 10% in soaked cotton.

The susceptibility to the insecticides was evaluated on the following criteria:
- Mortality 98-100%: Susceptible to insecticide.
- Mortality 80-97%: Possibility of resistance to insecticide.
- Mortality < 80%: Resistant to insecticide.

Results

North

Of the six study places, Aedes aegypti was found resistant to DDT at five places and possibly resistant to DDT at one place; it was susceptible to malathion at five places and possibly resistant to this insecticide at one place. It was susceptible to all the tested insecticides of the pyrethroid group such as permethrin, lambda-cyhalothrin, deltamethrin and alpha-cypermethrin in at five places and the possibility of resistance to these four insecticides existed at one place. Aedes aegypti was resistant to etofenprox at four places and possibly resistant to etofenprox at two places (Table 1).

Table 1. Results of susceptibility tests on Aedes aegypti to insecticides in the North and Centre, Viet Nam

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Places</th>
<th>Permethrin 0.75%</th>
<th>Lambda-cyhalothrin 0.05%</th>
<th>Deltamethrin 0.05%</th>
<th>Alpha-cypermethrin 30mg/m²</th>
<th>Etofenprox 0.05%</th>
<th>DDT 4%</th>
<th>Malathion 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Thi Cau (Co) Bac Ninh (T) Bac Ninh (P)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>68</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Phu Lang (Co) Que Vo (D) Bac Ninh (P)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>92</td>
<td>94</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>Cat Ba (S) Cat Hai (D) Hai Phong (C)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>42</td>
<td>14</td>
<td>100</td>
</tr>
</tbody>
</table>
### Susceptibility of Aedes aegypti to Insecticides in Viet Nam

#### % Mortality

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Places</th>
<th>Permethrin 0.75%</th>
<th>Lambda-cyhalothrin 0.05%</th>
<th>Deltamethrin 0.05%</th>
<th>Alpha-cypermethrin 30mg/m²</th>
<th>Etofenprox 0.05%</th>
<th>DDT 4%</th>
<th>Malathion 5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.</td>
<td>Niem Nghia (Co)</td>
<td>91</td>
<td>92</td>
<td>94</td>
<td>95</td>
<td>92</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Le Chan (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hai Phong (C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Ly Thai To (Co)</td>
<td>99</td>
<td>96 (200)</td>
<td>100</td>
<td>100</td>
<td>18</td>
<td>21</td>
<td>97.33 (150)</td>
</tr>
<tr>
<td></td>
<td>Hoan Kiem (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ha Noi (C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Thinh Liet (Co)</td>
<td>100</td>
<td>99</td>
<td>98</td>
<td>98</td>
<td>37</td>
<td>60</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Thanh Tri (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ha Noi (C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Thach Phu (Co)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>63</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>HaiTinh (T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ha Tinh (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Duc Tho (S)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>93</td>
<td>37.39 (115)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Duc Tho (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hai Tinh (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Song Cau (S)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>98</td>
<td>51</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Song Cau (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phu Yen (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>No. 6 (Co)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>91</td>
<td>76</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Tuy Hoa (T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phu Yen (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Dong Luong (Co)</td>
<td>94</td>
<td>96</td>
<td>97</td>
<td>95</td>
<td>16</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Dong Ha (T)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quang Tri (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Trieu Do (Co)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>86</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Trieu Phong (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quang Tri (P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

No. 1 - 6 in the North
No. 7 - 12 in the Centre
Co: Commune; S: Small town; D: District; T: Town; P: Province; C: City
Figures in parenthesis indicate the number of mosquitoes tested

### Central

Aedes aegypti was found resistant to DDT but was susceptible to malathion in all six places. It was susceptible to four insecticides of the pyrethroid group such as permethrin, lambda-cyhalothrin, deltamethrin and alpha-cypermethrin in five places and possibly resistant to them in one place, and resistant to etofenprox in three places, and possibly resistant and susceptible to etofenprox at two and one places, respectively (Table 1).

### South

Aedes aegypti was resistant to DDT at all six places; but susceptible to malathion at four places and possibility of resistance to malathion at two places; it was resistant to
Susceptibility of Aedes aegypti to Insecticides in Viet Nam

permethrin and lambda-cyhalothrin at four places and the possibility of resistance to permethrin and lambda-cyhalothrin at two places. It also showed resistance to deltamethrin at one place and possibility of resistance to deltamethrin at five places; resistance to alpha-cypermethrin at two places and possibility of resistance to alpha-cypermethrin at four places as well as resistance to etofenprox at all six places (Table 2).

**Table 2.** Results of susceptibility tests on Aedes aegypti to insecticides in the South and Central Highlands, Viet Nam

<table>
<thead>
<tr>
<th>No.</th>
<th>Places</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Permethrin 0.75%</td>
</tr>
<tr>
<td>1</td>
<td>No. 6 (Co) Ben Tre (T) Ben Tre (D)</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>Binh Thuan (Co) Binh Dai (D) Ben Tre (P)</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td>Binh Khanh (Co) Can Gio (D) Ho Chi Minh (C)</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>Binh Trung Tay (Co) No. 2 (D) Ho Chi Minh (C)</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>An Loc (S) Binh Long (D) Binh Phuoc (P)</td>
<td>79</td>
</tr>
<tr>
<td>6</td>
<td>Tan Xuan (Co) Dong Xoai (T) Binh Phuoc (P)</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>Plei Can (S) Ngoc Hoi (D) Kon Tum (P)</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>Quyet Thang (Co) Kon Tum (T) Kon Tum (P)</td>
<td>57</td>
</tr>
<tr>
<td>9</td>
<td>Buon Trap (Co) Krong Ana (D) Dak Lak (P)</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>Thang Loi (Co) Buon Me Thuot (C) Dak Lak (P)</td>
<td>24</td>
</tr>
</tbody>
</table>

No. 1 - 6 in the South
No. 7 - 10 in the Central highlands
Co: Commune; S: Small town; D: District; T: Town; P: Province; C: City
Figures in parenthesis indicate the number of mosquitoes tested
Aedes aegypti was resistant to DDT, permethrin, lambda-cyhalothrin, deltamethrin, alpha-cypermethrin and etofenprox at all four places in this region and susceptible to malathion at three places and showed possibility of resistance to malathion at one place (Table 2).

**Discussion**

Aedes aegypti was susceptible to malathion and resistant to DDT at almost all study places in Viet Nam. It was still susceptible to the four insecticides of the pyrethroid group (permethrin, lambda-cyhalothrin, deltamethrin and alpha-cypermethrin) in many places in North and Centre Viet Nam, but resistant to these insecticides in many places in the South and Central Highlands. These results were not completely comparable with the observations made by Reiter and Gubler (1997)\[3\], and Vu Duc Huong and Nguyen Thi Bach Ngoc (1999)\[6\]. Aedes aegypti was possibly resistant to malathion in some places. This discrepancy in different regions was possibly due to the longer and more extended use of the insecticides of the pyrethroid group in malaria and dengue haemorrhagic fever control programmes and in agriculture in the Southern and Central Highlands. It is therefore suggested that the susceptibility tests should be conducted on all insecticides before use. Moreover, the cross-resistance of Aedes aegypti to insecticides belonging to the pyrethroid group should also be checked. Aedes aegypti was highly and widely resistant to etofenprox and further studies should be conducted in this context.

**References**


Ovipositioning Behaviour of Aedes aegypti in Different Concentrations of Latex of Calotropis procera: Studies on Refractory Behaviour and its Sustenance across Gonotrophic Cycles

Manju Singhi, Vinod Joshi*, R.C. Sharma and Keerti Sharma

Desert Medicine Research Centre (Indian Council of Medical Research), New Pali Road, Jodhpur 342 005, India

Abstract

Dengue fever associated with dengue haemorrhagic fever is gaining endemicity in India. Due to lack of any chemotherapy against this arboviral infection, the control of the disease depends largely on preventive measures against Aedes mosquito vectors. A wild shrub, Calotropis procera, commonly growing in the desert areas of Rajasthan has shown a remarkable effect as a larvicide against Aedes aegypti. However, different water concentrations of this biocide have also brought forward very important observations on the ovipositioning behaviour of Aedes aegypti. At 0.7% concentration of latex, the oviposition was avoided by the gravid female mosquitoes and this behaviour continued till three gonotrophic cycles. However, at lower concentrations (0.2% and 0.1%) of the larvicidal latex, the refractory behaviour of ovipositioning could not be retained up to the third gonotrophic cycle. The concentration of latex such as 0.7% and 0.2% were observed as ovicidal also and this effect continued across all the gonotrophic cycles. The behavioural observations reported in the present study may serve as significant information on choosing bio-larvicides for vector control against dengue.

Keywords: Dengue, Aedes aegypti, Calotropis procera, ovipositioning, refractory behaviour, gonotrophic cycle.

Introduction

Dengue fever and dengue haemorrhagic fever (DF/DHF) is gaining endemicity in many states in India[1]. In the absence of chemotherapy and vaccines, vector control, largely based on larval control, is the only option available. In Rajasthan, a number of epidemics of dengue associated with DHF have been reported[2-4]. It has also been established that dengue virus undergoes transovarial transmission across generations of Aedes aegypti under natural as well as experimental conditions[5,6]. Latex of Calotropis procera, a milky weed plant growing all across the desert areas in the state has shown promising larvicidal properties in a series of laboratory
Materials and methods

Six experimental sets were designed for the study. Cages of 30 cm$^3$ size, with wooden frame and iron mesh with muslin cloth on one side, were used as units of present experiments. In each cage, 16 gravid females of *Aedes aegypti* were released. The concentration of latex in water, which showed the highest (0.7%), moderate (0.2%) and no mortality (0.1%) effect in the larvicidal efficacy experiments, were prepared and put in beakers in the cages A, B, C, D and E while cage F was kept without latex solution and contained only water. A beaker containing plain water was also placed in each of the six cages to serve as control. While in cage D all the experimental concentrations were placed along with the control, in cage E all the concentrations were placed without choice of a control. In the sixth cage (cage F) two beakers with plain water were placed without any experimental lethal concentration. The eggs laid by female *Aedes aegypti* were counted after 48 hours in each of the experimental cages. Second and third blood meals were provided to facilitate $G_2$ and $G_3$ (gonotrophic cycles) to mosquitoes.

Results and discussion

Table 1 shows relative observations on different concentrations of latex of *Calotropis procera* on the ovipositioning behaviour of *Aedes aegypti*. In experimental cage A, while in the control set, 65 eggs were laid, in the corresponding lethal concentration of 0.7% of the same cage, no eggs were laid. In this cage all the mosquitoes showed persistent refractiveness of ovipositioning across all gonotrophic cycles from $G_1$-$G_3$. In cage B where 0.2% concentration was offered along with control, refractive behaviour up to two cycles only was observed while in the third cycle ($G_3$), 127 eggs were laid. Similar observations were made in cage C. It was interesting to note that in cage D where all the experimental concentrations were offered to mosquitoes along with choice of control, even in $G_1$ cycle no refractiveness was shown. In experiment E where choice of control (plain water) was not offered, maximum preference for ovipositioning was shown in lowest concentration (0.1%). The different preference among different concentrations continued across all the gonotrophic cycle (Table 1).
Table 1. Ovipositing preference of Aedes aegypti in different larvicidal concentrations of latex of Calotropis procera

<table>
<thead>
<tr>
<th>Cage</th>
<th>Latex concentration (%) in experimental cages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Eggs laid within 48 hrs</td>
<td>C</td>
</tr>
<tr>
<td>G1</td>
<td>65</td>
</tr>
<tr>
<td>G2</td>
<td>93</td>
</tr>
<tr>
<td>G3</td>
<td>275</td>
</tr>
</tbody>
</table>

C: Control
G1-G3: Gonotropic cycles

Table 2. Effect of latex on the percentage viability of eggs of Aedes aegypti

<table>
<thead>
<tr>
<th>Cage</th>
<th>Experimental cages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Latex (%)</td>
<td>C</td>
</tr>
<tr>
<td>Eggs immersed</td>
<td>65</td>
</tr>
<tr>
<td>Eggs hatched</td>
<td>55</td>
</tr>
<tr>
<td>% eggs hatched</td>
<td>84.6</td>
</tr>
</tbody>
</table>

C: Control

Dengue fever associated with DHF has become a problem of public health importance. Available evidence shows that the virus undergoes vertical transmission across generations of mosquitoes\(^9\). Larval control, therefore, is the most effective approach to restrict the vector and virus sustenance in nature. The wildly grown plant, Calotropis procera, has shown very encouraging results in its different lethal concentrations. However, if this plant...
species is to be used as a material of choice, its other aspects such as the ovipositioning behaviour of gravid females towards the larvicide can also become known.

The significance of the reported observations is that the refractiveness developed by the species is sustained across two gonotrophic cycles when one larvicidal concentration was offered with a corresponding control (Table 1). However, when all the concentrations viz. 0.1%, 0.2% and 0.7% were offered with one control, such avoidance was not shown. Similarly, in the experimental set E where all concentrations of latex were made available without any control, the maximum egg laying was preferred in 0.1% of latex. The data suggest fine chemo-sensation in Aedes aegypti where the ovipositioning female could distinguish to choose the least larvicidal concentration for its egg laying.

The observation showed that if in the various types of domestic containers where water is stored a non-lethal concentration of latex (0.1%) is used, the refractiveness of ovipositioning will not be there and the domestic mosquito fauna in such premises will lay the eggs but they will lose their viability to hatch into larvae (Table 2).

The role of Calotropis procera as a larvicide has been reported by other workers from India[7]. However, contrary results have been reported by some workers[8] where insect growth regulators actually enhanced ovipositioning.

The observations reported by us not only present the results of the ovipositioning behaviour induced by a bio-larvicide, but also add the information on relative preference in ovipositioning and its sustenance across gonotrophic cycles when different larvicidal concentrations were offered.

Acknowledgements
The authors gratefully acknowledge the guidance and inspiration received from Mr N.L. Kalra, Member, Scientific Advisory Committee of the Desert Medicine Research Centre, Jodhpur, India.

References


Community-based Assessment of Dengue-related Knowledge among Caregivers

Khynn Than Win*#, Sian Za Nang** and Aye Min***

*Health Systems Research Division, Department of Medical Research (Lower Myanmar), Myanmar
**Health Education Bureau, Department of Health Planning, Myanmar
***Vector Borne Disease Control Programme, Department of Health, Myanmar

Abstract

The study was conducted in Thaketa township in Myanmar involving 405 respondents aged 18 years and above. It was aimed: (i) to explore the extent of dengue-related knowledge among caregivers; (ii) to identify the exposure of community members to the existing IEC materials; and (iii) to find out the factors related to high knowledge scores. The findings were triangulated by results from personal interviews, focus group discussions and observational checklist. The difference of mean scores among males and females was not statistically significant. Knowledge scores of the caregivers were not statistically different whether there was a primary DHF case at home or not. Almost 60% of the interviewees had received information on DHF by watching television and they observed that television was the most effective medium. Females with more than six years of schooling, persons who had access to pamphlets/posters, television, newspapers and journals got higher scores than the unexposed group. Less than 15% were not exposed to any of the IEC materials. Aedes aegypti larvae were found in 67% of water storage tanks and 15.9% of flower vases when using observational checklist. Focus group discussions were held for drafting IEC materials. Community members were more interested in the mode of DHF transmission to children rather than in the elimination of the Aedes mosquitoes. A low practice score was observed in those with high knowledge level, which means that high knowledge does not necessarily lead to high practice. Less than half of the respondents had seen posters and pamphlets. IEC materials need to be improved so that they present the message most effectively and they should be extensively distributed in the community.

Keywords: Dengue, caregivers, community, IEC material, knowledge score, Myanmar.

Introduction

Dengue haemorrhagic fever (DHF) is endemo-epidemic in 12 out of 14 states and divisions in Myanmar and is transmitted by Aedes aegypti. Most of the reported cases are under 15 years of age[1]. The Myanmar National Health Plan (NHP) (1996-2001) termed DHF as one of the diseases under national surveillance. DHF is also listed as the 17th priority disease in Myanmar. One of the strategies devised in NHP for the prevention and control of DHF is production of guidelines for basic health staff (BHS) as part of the information, education and communication (IEC) programme.

# E-mail: thaint@mail4u.com.mm
IEC initiatives are based on the concepts of prevention and primary health care. They create awareness, increase knowledge, change attitudes and motivate people to adopt new ideas[2].

Communication participation appears to be one of the most promising innovative means to prevent and control DHF. Simple elimination of vector-breeding water collections or “source reduction” is the possible answer to the problem. Community activities are identified mainly as reduction of non-essential water containers, protection of water containers from larvae breeding, larviciding and release of larvivorous fish. Community participation needs to be sustained by dissemination of health messages through various channels[3].

Existing IEC materials in Myanmar included health talks routinely carried out in schools and in the community. Health messages were distributed through radio, television, newspapers and journals before and during the epidemic season. Pamphlets were developed locally in states and divisions. However, it is necessary to find out the most appropriate IEC materials and means that would be relevant for various communities.

This study attempted to improve the existing IEC materials on DHF control based on the knowledge and practice of child-care providers in the Thaketa township of the Yangon division in Myanmar. DHF control will be more effective in the future by strengthening community participation on case information and source reduction.

This research aimed to: (i) explore the extent of dengue-related knowledge among caregivers; (ii) identify the exposure of community members to the existing IEC materials; and (iii) to find out the factors responsible for high knowledge scores.

Methods and materials

This community-based cross-sectional study was based on multistage sampling to identify 405 caregivers in Thaketa township, Yangon. This area was one of the dengue endemic regions in the Yangon division and the case-fatality rate (CFR) was 1.65% in 2002. Both quantitative and qualitative data collection methods, including observation checklist, were used in the study. The study sample included household members aged 18 years and above. The households with children were selected randomly. In these households, we chose one subject from each household, regardless of sex. The respondent was a relative of the child (mother/father/grandfather/grandmother/brother/sister/uncle/aunt). Mothers were the key persons to be interviewed after taking their consent. The questionnaire was pilot-tested for clarity and validity; all questions were reviewed by epidemiologists, public health experts, health educators and by investigators experienced in conducting community-based surveys in DHF. In order to ensure the accuracy and completeness of data, our surveyors were trained before and after pre-testing.

Ten sessions of Focus Group Discussions (FGDs) were performed among basic health staff (BHS), general practitioners, Maternal and Child Welfare Association (MCWA) members, other volunteers including Ward Law and Order Restoration Council members, voluntary fire brigade members, etc., for recommendation of existing IEC activities (including television, radio, newspapers, local journals and pamphlets). In every FGD, the moderator
explained thoroughly the purpose of conducting the discussions. The Township Health Centre, Ward Law and Order Restoration Council offices and homes were chosen for holding FGDs.

Caregivers were those who took care of children at home, or supervised at the health centre or clinic, or advised parents on home care of a child with fever.

Data checking, cleaning and validation were performed using Epi-info 6.0, and data analysis was conducted using SPSS 10.0. $P<0.05$ was used as the definition of statistical significance. The study period lasted one year starting in June 2002.

Results

About respondents

The sex ratio of the respondents was 7:1 for females and males. The mean age was $35.9\pm10.3$. The majority of the respondents were aged 18-35 years, literate and dependants. About 8.6% of them had received $\geq 12$ years' school education. Nearly 40% lived in their own wooden houses. Most of the households (77.8%) had 1 to 2 children under 15 years of age. Only 34 (8.4%) of the households that participated in the interview had a child with history of DHF.

The difference of mean score among males and females was not statistically significant ($P=0.271$).

Dengue-related knowledge responses

The responses of caregivers are included in Table 1.

<table>
<thead>
<tr>
<th>Responses</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHF is common in children 3-8 years of age</td>
<td>254</td>
<td>44.4</td>
</tr>
<tr>
<td>DHF is transmitted by the mosquito</td>
<td>331</td>
<td>81.7</td>
</tr>
<tr>
<td>Mosquito species of DHF vector is Aedes</td>
<td>204</td>
<td>61.6</td>
</tr>
<tr>
<td>Biting time of mosquitoes is at daytime</td>
<td>266</td>
<td>80.4</td>
</tr>
<tr>
<td>Aedes breed in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• clean water</td>
<td>135</td>
<td>33.3</td>
</tr>
<tr>
<td>• polluted water</td>
<td>166</td>
<td>41.0</td>
</tr>
<tr>
<td>The Aedes mosquito breeds inside the house</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• flower vases</td>
<td>253</td>
<td>62.5</td>
</tr>
<tr>
<td>• ant traps</td>
<td>144</td>
<td>35.6</td>
</tr>
<tr>
<td>• blocked gutters</td>
<td>96</td>
<td>23.7</td>
</tr>
<tr>
<td>The Aedes mosquito breeds outside the house</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• water containers</td>
<td>292</td>
<td>72.1</td>
</tr>
<tr>
<td>• old tyres, broken pots, and coconut shells</td>
<td>178</td>
<td>61.0</td>
</tr>
<tr>
<td>• blocked gutters</td>
<td>75</td>
<td>25.7</td>
</tr>
<tr>
<td>• ant traps</td>
<td>9</td>
<td>2.2</td>
</tr>
<tr>
<td>DHF transmission is highest in the rainy season</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>85.9</td>
</tr>
<tr>
<td>DHF may be fatal</td>
<td>382</td>
<td>94.3</td>
</tr>
<tr>
<td>There is a vaccine for prevention of DHF</td>
<td>243</td>
<td>60.0</td>
</tr>
<tr>
<td>A previously infected child may get DHF again</td>
<td>293</td>
<td>72.3</td>
</tr>
<tr>
<td>If the child is febrile, DHF should be observed</td>
<td>262</td>
<td>64.7</td>
</tr>
</tbody>
</table>

The total knowledge scores were categorized as low (0-19) and high (20-39). The percentage of the high score group was more than that of the low score group.
(68.6% vs 31.4%). Signs and symptoms of DHF were fever (57.3%), vomiting (51.6%), purpura (36.3%), drowsiness (28.1%), cold extremities (17%), etc.

Knowledge score of respondents with and without history of DHF case in their homes

Knowledge scores with and without past history of primary DHF cases at home are given in the Figure.

![Figure. Past history of primary DHF cases at home and knowledge scores](image)

The figure illustrates that the knowledge scores of the caregivers were not statistically different according to the presence of primary DHF case at home ($P=0.137$).

Prevention of mosquito bites

To prevent and protect children from mosquito bites, the following measures were taken: use of mosquito nets (47.9%), use of repellants (47.2%), wearing of long sleeves (12.6%), others (8.6%), and none (5.2%).

Existing exposure to IEC material in the community

Table 2 contains the responses of the communities to IEC materials.

**Table 2. Exposure to IEC materials in the community**

<table>
<thead>
<tr>
<th>Community members exposed to IEC materials</th>
<th>(n)</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>• had seen pamphlets</td>
<td>197</td>
<td>48.6</td>
</tr>
<tr>
<td>• listened to radio</td>
<td>137</td>
<td>33.8</td>
</tr>
<tr>
<td>• watched on television</td>
<td>246</td>
<td>60.7</td>
</tr>
<tr>
<td>• read in newspapers/journals</td>
<td>133</td>
<td>32.8</td>
</tr>
<tr>
<td>• Percent exposed to any type of IEC</td>
<td>352</td>
<td>87.0</td>
</tr>
<tr>
<td>• Percent not exposed to any IEC</td>
<td>53</td>
<td>13.0</td>
</tr>
<tr>
<td>• Percent exposed to all types of IEC</td>
<td>87</td>
<td>21.5</td>
</tr>
</tbody>
</table>

Are the facts easily recognized?

- Yes                                           317   78.3
- No                                            23    5.7

The percentage of people watching television was the highest as compared to other types of exposure. Nearly half of the respondents had seen the pamphlets about DHF (48.6%). Exposure to radio talks and information in newspapers and journals was very low. The extent of respondents exposed to any type of IEC was 87%. The facts in those IEC materials were concise and easily recognized (78.3%) (Table 2).

Almost 60% of the interviewees felt that television was the most effective medium for dissemination of knowledge on DHF in the community.

The logistic regression model of knowledge score by the respondents’ characteristics and exposure to health education media is given in Table 3.
Table 3. Logistic regression model of knowledge scores, by respondents’ characteristics and exposure to health education materials

<table>
<thead>
<tr>
<th>Variables</th>
<th>(n)</th>
<th>Percent</th>
<th>Odds ratio (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Respondent’s characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Male</td>
<td>54</td>
<td>13.3</td>
<td>1</td>
</tr>
<tr>
<td>• Female</td>
<td>351</td>
<td>86.7</td>
<td>0.379 (0.198-0.727)**</td>
</tr>
<tr>
<td>Years of formal schooling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• 0 to 5 (r)</td>
<td>133</td>
<td>32.8</td>
<td>1</td>
</tr>
<tr>
<td>• 6 to 20</td>
<td>272</td>
<td>67.2</td>
<td>0.588 (0.364-0.950)*</td>
</tr>
<tr>
<td>Mean years of schooling</td>
<td>7.6±3.6</td>
<td>67.2</td>
<td></td>
</tr>
<tr>
<td><strong>Exposure to health education media</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pamphlets/posters</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Not seen</td>
<td>197</td>
<td>48.6</td>
<td>1</td>
</tr>
<tr>
<td>• Seen</td>
<td>208</td>
<td>51.4</td>
<td>0.478 (0.291-0.784)**</td>
</tr>
<tr>
<td>Television</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Not watched (r)</td>
<td>159</td>
<td>39.3</td>
<td>1</td>
</tr>
<tr>
<td>• Watched</td>
<td>246</td>
<td>60.7</td>
<td>0.353 (0.218-0.571)**</td>
</tr>
<tr>
<td>Newspapers/journals</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Not read (r)</td>
<td>272</td>
<td>67.2</td>
<td>1</td>
</tr>
<tr>
<td>• Read</td>
<td>133</td>
<td>32.8</td>
<td>0.443 (0.245-0.803)**</td>
</tr>
</tbody>
</table>

An analysis of the findings suggested that females with over six years of schooling were significantly related to total knowledge scores. Survey respondents with 6-20 years of schooling were more likely to obtain high scores than those with 0-5 years of schooling. Respondents who were exposed to health education media such as pamphlets/posters, television, newspapers and journals obtained higher scores than the unexposed group. The respondents who watched television were more likely to score higher than those who did not (Table 3).

Cross-check by observation checklist

The researchers used an observation checklist to support the findings. Nearly 55% of the households had two to three water containers. Although 46.5% of the water containers had lids, only 19% were covered tightly. Larvae were found in 67% of the water storage tanks and 15.9% of the flower vases at the time of interviews. Gutter blockage was observed in 3.2% of cases. Old tyres, coconut shells and tins were found in the compounds and larvae existed in half of these solid wastes.
Approaches considered for improvement of IEC activities

Many recommendations were extrac ted from FGDs for the improvement of existing IEC materials. It was suggested that messages should be short and clear. Pamphlets should be widely distributed among the community, especially in schools. The same messages could be published in newspapers and journals once a week before and during the rainy season. Health magazines and magazines on astrology were the most preferred media. They also pointed out the most suitable times for telecast and broadcast of health messages. Regular clearing of gutters should be mentioned in the health message as well as in the book, “Facts for Life”. The fact that “DHF may attack again a previously-infected child” was important to remind mothers of the danger. Cartoons and art competitions and exhibitions on DHF would be the most effective media for schoolchildren and mothers.

Discussion

The mean scores of male and female respondents were not significantly different in the community. Community members knew a lot (more than 80%) about transmission of DHF by mosquito bite, the biting habit of Aedes and the resultant fatality. Nevertheless, only 61.6% correctly identified the main vector of DHF. Nearly 20% answered that Aedes usually bit at nighttime. Some people were still confused that polluted water was also a breeding place. Most of the interviewees responded that the common breeding sites of Aedes were water containers and flower vases. Not more than 30% identified ant traps, broken pots, tins, old tyres and coconut shells as the vector breeding places. The subjects knew little of blocked gutters as possible breeding sites. Nearly 80% thought that DHF could be prevented by immunization. A majority of the interviewees used measures to prevent children from being bitten by mosquitoes. A very small percentage did not use any measure. Some points that require emphasis for community awareness include:

- Aedes is the main vector for DHF.
- Aedes only breeds in clear water, not in polluted water.
- The biting time of Aedes is daytime.
- Blocked gutters should be mentioned as possible breeding sites for Aedes in existing IEC materials.
- There is no vaccine available for the prevention of DHF.

The chance to get high scores was better in females with high education. Caregivers with low educational levels should be targeted for health education. The level of knowledge scores was not related to the history of a previously infected child at home. Knowledge scores may be changed through exposure to various media. Also, sufficient numbers of IEC materials should be distributed in the community. Only a small percentage of the interviewees were not exposed to any type of existing IEC. Ways and means should be found to improve people’s exposure by further discussions with community members and health workers using participatory approaches.

Pamphlets/posters, television, newspapers and journals are still popular media for the public. Television is the most effective medium and various types of programmes, such as songs, comedies, short movies with famous actors, actresses, etc., apart from
discussions and health talks can be telecast. However, radio is still a valuable tool in the health education process because of its easy availability ad popular use in semi-urban and rural areas. The effectiveness of various existing IEC materials should be reviewed for further improvement.

Many water containers were not covered tightly to prevent larval breeding. Social mobilization for sustainability of larvae control activities should be implemented. Coordination and cooperation with volunteers and local NGOs should also be strengthened.

Acknowledgement

We express our gratitude to the Township Medical Officer and basic health staff in Thaketa township for their support. We are grateful to volunteers, NGO members and the study area residents for their active participation in the study.

References


Students’ Perceptions about Mosquito Larval Control in a Dengue-Endemic Philippine City

Jeffrey L. Lennon*

Foundation University, School of Education, Dumaguete City, Negros Oriental, The Philippines

Abstract

A study was carried out among university students to find out their perceptions about mosquito larval control in a dengue-endemic Philippine city. This formative research was conducted to obtain information for formulating future school-related dengue control strategies. The study was carried out in-class through a semi-structured, open-ended question format. The study yielded information on students’ perceptions about the most important measures for mosquito larval control and perceived reasons why people did not implement them. The study also explored the opinions of the students by gender. The study yielded students’ knowledge on the types of mosquito larval habitats. Little was expressed about specific indoor mosquito larval control nor about the frequency of conducting source-reduction activities. Perceived barriers to constructing mosquito larval control centred on themes such as apathy, laziness and lack of time. Further studies are necessary to follow-up on these themes in depth.

Keywords: Dengue, formative research, mosquito larval control, students, open-ended questionnaire, Philippines.

Introduction

The control of Aedes aegypti mosquito larvae is essential for the control of dengue fever (DF) and dengue haemorrhagic fever (DHF)[1]. The need to know the perceptions of key informants is necessary in order to better address the dengue-related control issues in a specific area or community[2-5]. Schools are potential mosquito breeding sites[6-8]. Also, primary, secondary and tertiary school-age students are principal targets of the Aedes mosquitoes[9-11].

Dengue has become a steadily increasing health problem in the Philippines[9]. Consequently, it has become endemic in Dumaguete city, Philippines[12].

Aedes control is largely based on source reduction. Therefore, knowledge of the types of mosquito breeding sites is a prerequisite for health personnel, schoolteachers and children and the community at large for the control of dengue. Various types of containers have been identified as potential mosquito breeding sites. These include plastic and metal containers, animal-feeding dishes, tyres, flower vases, coconut shells and water storage drums[3,4,13,14].

* E-mail: jeffchona2@yahoo.com

---

196 Dengue Bulletin - Vol 28, 2004
The knowledge about the types of breeding containers alone is not enough to achieve mosquito control. Attitudes and beliefs impact a person's knowledge about mosquito control. For example, the belief that dengue is not a fatal or serious problem impairs a person from carrying out adequate mosquito control practices. Some people believe that mosquitoes within the home and outside are different. So it is believed that mosquitoes inside the house do not carry disease. Gender-related responsibilities for control of certain mosquito breeding containers might also exist. There may be different responsibilities for one gender inside the residence compared to the outside surroundings. Or, there may be a distribution of clean-up activities depending upon ownership of specific items such as tyres.

One study in Mexico indicates that informants devalued the use of screens in dengue prevention and believed that the screens were not effective in keeping out mosquitoes.

Since dengue is already endemic in this study city in the Philippines, it was presupposed that there would already be a high level of awareness about dengue in general. So, this study sought to explore the more specific topic related to dengue control, through source reduction. Therefore, this objective of the study was to explore the opinions of Philippine university education students about the mosquito larvae control in a dengue endemic city.

**Materials and methods**

**Subjects**

The subjects consisted of 43 university major students at Foundation University, College of Education, Dumaguete city, Philippines. There were 36 female and 7 male subjects. All subjects were Filipinos. The participants were students in a college-based “personal and community hygiene” course. This study took place prior to any course coverage or discussions about dengue fever or any of the mosquito-borne diseases. The participation was voluntary and no student in the class declined it. The results and data were confidentially held. All results were tabulated and reconciled so that the responses would be anonymous.

**Procedure**

Two open-ended semi-structured questions were administered to the subjects on January 16, 2003. The students' instruction was the facilitation of the questionnaire.

The two questions were as follows:

(i) What is the best way to control mosquito larvae?

(ii) Why don't some people use the measure you suggest for question No. 1?

The questions were given verbally. The students were also told that they were allowed to give more than one response per question. The students gave written responses to the questions. The responses were classified by gender. There were no predetermined categories. Categories of responses were created from post-survey results. As categories emerged, sub-categories were created according to their relationship to the broader response categories. The time allotted for the whole process of issuing instructions to the students and completion of response-writing was approximately 15 minutes.
Results

On the responses to Question 1, the principal categories that emerged concerning mosquito larvae control activities were as follows: outside activities, inside activities, activities not specified as inside or outside, activities related to specific types of containers, use of insecticide, unrelated activities, and not familiar with mosquito larvae (Table 1).

Table 1. Responses to Question 1

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Outside activities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. General outside activities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.1. Clean surroundings or yard</td>
<td>5</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>A.2. Clean surroundings daily</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>B. Specific water-containing items outside</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.1. Turn over coconuts</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>B.2. Dispose of water in coconut shells</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B.3. Put tyres in a safe place</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B.4. Dispose of tyres</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>B.5. Dispose of stagnant water in drums</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. Other environmental activities</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C.1. Clean canals</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>C.2. Burn grass</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C.3. Put trash cans in a safe place (to avoid rain-water entry)</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C.4. Throw garbage properly</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td><strong>D. Activities involving other participants</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D.1. Clean-up drive participation</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>II. Inside activities</strong></td>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>A. Clean house (No specific activities mentioned)</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><strong>III. Activities not specified as inside or outside</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Non-specific cleaning</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A.1. Clean things with stagnant water</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>A.2. Clean or eliminate stagnant water</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>A.3. Empty all stagnant water</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A.4. Avoid or prevent stagnant water</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>A.5. Dispose of anything where mosquito eggs are laid</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A.6. Kill mosquito larvae in stagnant water (not specified)</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A.7. Clean things where mosquito larvae are laid (non-specific item)</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
### Activities related to non-specific types of containers

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.1. Prevent water from entering open containers</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B.2. Cover any water container</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>B.3. Clean water containers</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B.4. Dry water containers</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B.5. Empty containers</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. Clothes-hanging arrangement</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

### Activities related to specific types of containers

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Avoid open cans</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>B. Cover water containers such as gallon containers</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. Cover water barrels and tanks</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>D. Put open cans in a safe place</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>E. Dispose of cans</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>F. Cover jars</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G. Cover basins</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>H. Clean bottles</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>I. Replace flower vase water (no time mentioned)</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>J. Replace flower vase water daily</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>K. Replace flower vase water weekly</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>L. Cover garbage</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>M. Put garbage in a can (No mention of placing over)</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

### Other control-related activities

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Spraying insecticide</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

### Unrelated activity

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Remove an injured person</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

### Not familiar with mosquito larvae

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Spraying insecticide</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Totals</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
<td>94</td>
<td>108</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>36</td>
<td>43</td>
</tr>
<tr>
<td>No. of students with multiple responses</td>
<td>5</td>
<td>29</td>
<td>34</td>
</tr>
</tbody>
</table>
Table 2. Responses to Question 2

<table>
<thead>
<tr>
<th>Category</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>I. Knowledge-related categories</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Lack of education</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>B. Lack of knowledge</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>C. Lack of consciousness of surroundings or environment</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>D. Lack of consciousness of health</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>E. Specific responses related to knowledge</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E.1. Do not know that the suggested method of larval control is effective</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>E.2. Do not know the cause and effect of the mosquito to dengue</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E.3. They think that they know everything about mosquito control already</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>E.4. Not aware of the consequences</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>E.5. Do not know that the mosquito is dangerous</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>II. Attitude-related categories</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Don’t care – apathy</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>B. Don’t care because they have enough money to pay hospital bill</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C. Lazy</td>
<td>3</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>D. Busy</td>
<td>2</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>E. Since people are busy, they only use mosquito sprays to kill adult mosquitoes instead of mosquito larvae</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>F.1. No time</td>
<td>0</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>F.2. Insufficient time or little time</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G. No money especially to pay for insecticide spray</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>H. Tired</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>III. Practice-related categories</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Lack of cooperation with others</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>B. They just use insecticide spray instead of source restriction environment control</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>IV. Invalid or unrelated responses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Misunderstood the question (Answered in the affirmative)</td>
<td>1</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>B. Either answered Question 1 incorrectly or unfamiliar with the term “mosquito larvae”</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>V. No response</strong></td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>63</td>
<td>76</td>
</tr>
<tr>
<td>n</td>
<td>7</td>
<td>36</td>
<td>43</td>
</tr>
<tr>
<td>Students with multiple responses</td>
<td>4</td>
<td>20</td>
<td>24</td>
</tr>
</tbody>
</table>
For the responses to Question 2, the principal categories that emerged concerning reasons why people do not undertake mosquito larval control were as follows: knowledge-related categories, attitude-related categories, practice-related categories, invalid or unrelated responses and no response (Table 2).

**Discussion**

In response to Question 1, both female and male students rated “clean surroundings” (Table 1, Category I A.2) with the largest number of responses. This reflects the need for emphasis on specific mosquito control efforts while developing training and health education strategies\[5,14\].

The higher rating of outside activities compared to inside activities reflects the perceived importance of outside clean-up, and conversely, the relative lack of importance of inside clean-up and control of inside mosquito breeding sites. This may follow the perception as recorded in literature that “house mosquitoes” do not transmit dengue\[2,5\]. Perhaps students believe that the Aedes mosquitoes do not live inside houses or other living quarters.

No male subject gave any response related to indoor mosquito control measures (Table 1, Category II). Besides the perception that the indoors lack importance in mosquito control, the males may have deemed the indoors as the domain of females. The role of women as homemakers needs to be considered in vector control programmes. This was reviewed in a number of international settings\[15\].

Female subjects responded to all but one response, “activities not specified as inside or outside” (Table 1, Category III). Perhaps, the male respondents were not carrying out these activities. The male response emphasis on general response items Table 1 A, “General outside activities” may suggest their lack of specificity in the details necessary to conduct proper dengue-related mosquito control. The largest response in Table 1, Category III, was on the section “cover any water container” (Category III B.2). This practice is consistent with recommendations throughout the literature\[4,6,7,13,14\]. However, these responses in Table 1, Category III B.2, are less than one-third the number of the responses given in Table 1, Category I A.1.

The training of students in environmental surveys and in the environmental action plan known as “4 o’clock habit”, has previously been successful\[6\]. It is suggested that this be projected on a larger scale to include other members of the community.

The responses in “activities related to specific types of containers” Table 1, Category IV, were scattered across the item sub-categories by the respondents. Many of these response items could be used as inside containers. Not only is it necessary to cover, empty, and clean containers, but also to indicate the frequency of the activity in order to conduct adequate mosquito control. Only the responses in Table 1, Category IV J, and Category IV K, both dealing with flower-water replacement, and Category I A.2 “clean surroundings daily” mentioned the frequency and timing of activities. This amounted to four responses out of a total of 108 responses in Table 1. Perhaps,
programmes need to stress health education on the mosquito life-cycle and include emphasis on the time interval to conduct successive mosquito control clean-ups. The lack of responses on the frequency of environmental clean-up is in agreement with previous literature on the incomplete understanding about the mosquito life-cycle, and thus, the need to include this topic in future health education programmes[2,5].

Only four female respondents and no male respondent in Table 1, Category V A, indicated that insecticide spraying was the most important mosquito control measure. The overwhelming majority of the respondents discussed general or specific environmental clean-up activities. Source reduction of mosquito breeding sites without the use of adult insecticides has been stressed in dengue-related health education programmes in Honduras[3], Mexico[4], and the Philippines[6,13,16]. Since larvicides are not currently being used or promoted in this Philippine study site, the respondents most likely were referring to the use of adult insecticides. In contrast, dengue control programmes in Puerto Rico utilized both source reduction of mosquito breeding sites and insecticide use[14]. However, for developing countries, regular adult aerosol insecticides were deemed to be too expensive for regular home use, and thus not recommended for routine control[13].

Only one respondent as seen in Table 1, Category VI, gave a response to an activity unrelated to mosquito control. Also, there was only one respondent as seen in Table 1, Category VII, who responded to the category, “Not familiar with mosquito larvae”. These responses validated that the respondents in general had awareness about mosquito larvae. However, only a minority gave specific details either in types of control measures or in the timing of control activities.

For Question 2, the greatest number of responses among the knowledge-related category was the general category, “Lack of knowledge”, Table 2, Category I B. There was no concentration of specific knowledge-related responses. There were nearly twice as many attitude-related responses as knowledge-related responses. Attitude responses were greater than knowledge responses for both male and female subjects (Table 2). Since dengue is endemic in Dumaguete City[12], and various health education programmes have continued for years, it is reasonable to agree that there is a high general awareness about dengue. However, knowledge alone is generally not sufficient to change attitudes and behaviour[17]. Health education to increase only knowledge without addressing health behaviours has also been ineffective in the dengue control experience[9].

The greatest numbers of responses were in the attitude-related categories of “Don’t care – apathy”, Table 2, Category II A, and “Lazy”, Table 2, Category II C. The responses of apathy or laziness could have been a result of a weak belief in the effectiveness of the proposed measures from Table 1 to control mosquitoes and dengue. The health belief model may help to explain these responses. Two key components of the health belief model are “perceived benefits” and “perceived barriers.” A lower perception of benefits coupled with elevated barriers may result in a lower possibility for change[18]. Likewise, people having low self-efficacy (a construct of the
Social Cognitive Theory and also the Health Belief Model), or, in other words, confidence in doing something\[18,19\] could have resulted in their lack of interest to carry out the suggested mosquito control tasks.

The large number of responses found in Table 2, Categories II D, II E, II F1 and II F2, related to the factors of insufficient time to perform clean-up tasks is also suggested in the literature\[15\]. While people may know about various individual mosquito breeding sites source reduction tasks, they may lack the self-confidence necessary to perform a regular, comprehensive environmental clean-up task. Thus, skill development on how to conduct the steps of an environmental action plan for dengue control should be emphasized in order to increase self-efficacy and mosquito control behaviours. This promotion of skill development may, in turn, increase personal efficiency to perform source reduction tasks and thus, decrease the perception of time as a limitation to perform mosquito control activities.

There were only two responses to Table 2, Category III A, “Lack of cooperation with others”. This low number may have been due to a high value placed on the important relational imperative or supportive Filipino norm of “bayanihan” or cooperation\[20\]. Yet, there were still responses to perceived lack of cooperation as a cause for lower rate of mosquito larval control activities. Addressing this norm through various communications and other health promotion means may help in increasing mosquito larvae control activity.

Increasing the self-efficacy of individuals in the community may help in increasing the collective efficacy for the desired behavioural change activities. High levels of perceived collective efficacy may increase the likelihood of a group carrying out desired behavioural change activities\[21\]. Therefore, promoting strategies to increase collective efficacy may enhance the likelihood that community mosquito control activities will be carried out and sustained.

Asking the questions orally, rather than in a written form, had some limitations. This may have contributed to the nine respondents who misunderstood Question 2. They answered Question 2 by explaining their reason of choice for the best mosquito larval control, rather than explain why people were not using the best mosquito larval control method. See these responses in Table 2, Category IV A.

This form of questioning was also limited in the lack of in-depth follow-up. The procedure in this study did not allow for follow-up of such responses as the reason for “apathy” as a response to Question 2. Unlike other studies that used interviews\[2-5,14\] or focus groups\[4,5\], the facilitator of the questions did not interact with the respondents, nor probe for follow-up responses.

The method and procedure also had its strengths. In spite of utilizing open-ended questions, the procedure was very efficient in time and in organization. Unlike a previous study where interviews lasted for an hour per respondent, and at home\[9\], respondents in this study were able to complete the questions in a matter of minutes, and in one sitting.

All questions were completed at the same time, reducing potential biases that
could result from interaction with others in the outside environment. Also, having the students complete the questions individually, without interaction with fellow students and reduced biased or blended responses.

The procedure also demonstrated the strength of producing a large variety of response categories, multiple responses and overall total responses in a very short period of time. This was evident for both sexes and in response to both questions.

Summary and recommendations

• The study is suggestive that there was an understanding by most students of the term “larvae”, and its general relationship to mosquito and dengue control. This was exemplified by the low number of incorrect responses to content unrelated to mosquito larval control as indicated in Table 1, Categories VI and VII, and Table 2, Categories III B, IV B and V.

• There were no male responses for indoor control measures to Question 1. Also, there were almost no specific mosquito control measures mentioned by the male respondents. Further studies should explore the possible gender relationship to mosquito control practices.

• The majority of male and female respondents did not mention indoor mosquito larval control. Therefore, future mosquito control programmes should stress the importance of indoor mosquito larval control measures.

• Efforts are needed to create awareness regarding frequency of mosquito activities.

• There should be an in-depth exploration of the reasons behind the perceived causes of non-participation in mosquito larval control. Especially, the reasons behind “apathy” and “laziness” as perceived causes for non-participation in mosquito larval control should be further explored.

• Activities such as an environmental control action plan and creation of a mosquito control “checklist” and implementation for houses and schools as a means to increase self-efficacy should be promoted.

• The determinants of collective efficacy in mosquito larval control and dengue control should be explored.

Teachers play an important role in facilitating of health promotion in dengue endemic areas. Students and teachers should be properly oriented to carry out personal, school and community mosquito and dengue control measures. Antecedent to this is an understanding of students' perceptions about mosquito-related dengue control. The in-class semi-structured question method is one tool to carry out this type of formative research.

Acknowledgement

The author is associated with the International Technical Assistance Group (ITAG), Seattle, WA, USA. ITAG’s support is gratefully acknowledged. Also, thanks to Chona F. Lennon and Fernando N. Florendo for their assistance.
References


In India the first major outbreak of dengue fever (DF) accompanied with dengue haemorrhagic fever (DHF) was reported in Kolkata (Calcutta) in 1963\textsuperscript{1}. More than 60 outbreaks have been reported since 1956 to date\textsuperscript{2}. Of these two major outbreaks of DF/DHF occurred in 1996 and 2003 in Delhi and its adjoining states. Surveillance is the most cost-effective approach for prevention and control of dengue. A strong surveillance system will help in detecting early warning signals of an outbreak, instituting timely and appropriate control measures, assessing the impact of intervention measures and early containment of the outbreak. Considering the above facts, the arbovirus laboratory at the National Institute of Communicable Diseases, Delhi, has started sero-surveillance and monitoring of dengue fever in Delhi since 1996 as an ongoing activity. It was intended to develop an early warning signal for timely detection of an impending outbreak and institution of preventive and control measures in high-risk areas.

Sera samples of clinically suspected cases of DF and/or DHF are received from various hospitals of Delhi round the year. These samples were tested for dengue by haemagglutination inhibition (HI) test\textsuperscript{3} or IgM Capture ELISA Test\textsuperscript{4}. A titre of $\geq 1:1280$ in HI test in acute phase serum is considered a presumptive diagnosis of a current dengue infection\textsuperscript{5}. Samples positive for IgM antibodies against dengue virus indicate recent infection with dengue virus.

The results of the sentinel sero-surveillance from 1996 to 2003 are summarized in the Table. The analysis of data over the period of eight years shows that dengue strikes Delhi every year. The positivity ranges from approximately 13%-33%, except in 1996 and 2003 when dengue fever occurred in epidemic proportions along with DHF. The positivity in these two years was 53.4% and 57.8% respectively.

The month-wise distribution of samples tested from 1996-2003 shows that the positivity for dengue starts appearing in the month of August and reaches a peak in October and continues till mid-November and then a decline starts and the last cases are reported up to 2\textsuperscript{nd} week of December. The data for 2003 also shows a similar trend (Figure).
### Table: Dengue serology during the years 1996 - 2003 in Delhi

<table>
<thead>
<tr>
<th>Year/Month</th>
<th>1996</th>
<th>1997</th>
<th>1998</th>
<th>1999</th>
<th>2000</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tested</td>
<td>+ve</td>
<td>Tested</td>
<td>+ve</td>
<td>Tested</td>
<td>+ve</td>
<td>Tested</td>
<td>+ve</td>
</tr>
<tr>
<td>JAN</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>21</td>
<td>1</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>FEB</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MAR</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>24</td>
<td>0</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>APR</td>
<td>0</td>
<td>0</td>
<td>39</td>
<td>1</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>MAY</td>
<td>3</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>67</td>
<td>0</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td>JUN</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>JUL</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>0</td>
<td>31</td>
<td>1</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>AUG</td>
<td>3</td>
<td>0</td>
<td>20</td>
<td>3</td>
<td>48</td>
<td>0</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>SEP</td>
<td>159</td>
<td>73</td>
<td>169</td>
<td>65</td>
<td>70</td>
<td>5</td>
<td>5</td>
<td>71</td>
</tr>
<tr>
<td>OCT</td>
<td>643</td>
<td>343</td>
<td>369</td>
<td>159</td>
<td>201</td>
<td>38</td>
<td>38</td>
<td>145</td>
</tr>
<tr>
<td>NOV</td>
<td>104</td>
<td>74</td>
<td>81</td>
<td>26</td>
<td>293</td>
<td>140</td>
<td>130</td>
<td>35</td>
</tr>
<tr>
<td>DEC</td>
<td>11</td>
<td>3</td>
<td>15</td>
<td>1</td>
<td>47</td>
<td>21</td>
<td>40</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>931</td>
<td>493</td>
<td>764</td>
<td>255</td>
<td>868</td>
<td>206</td>
<td>482</td>
<td>93</td>
</tr>
</tbody>
</table>

Note: Percentages are calculated based on the total number of tests for each year.

- 1999: +ve 450 / 1438 (31.3%)
- 2000: +ve 450 / 2356 (19.2%)
- 2001: +ve 877 / 4072 (21.6%)
- 2002: +ve 94 / 42 (22.1%)
- 2003: +ve 0 / 701 (0.0%)
The studies for the estimation of the House Index (HI) of mosquitoes also shows that the house index for Aedes aegypti, the vector of dengue fever, starts building-up during the rainy season, i.e. from July and reaches a peak in August-September\(^6\).

A regular monitoring of suspected dengue cases by detection of IgM antibodies to dengue virus can act as an early warning signal for an impending outbreak. The above observations show that serological surveillance throughout the year, especially during the outbreak-prone period, can play an important role in the detection of early cases.

References

Detection of Dengue Virus in Wild Caught Aedes albopictus (Skuse) around Kozhikode Airport, Malappuram District, Kerala, India

B.P. Das*, L. Kabilan**, S.N. Sharma*, S. Lal*, K. Regu*** and V.K. Saxena*

*National Institute of Communicable Diseases, 22 Sham Nath Marg, Delhi – 110 054, India
**Centre for Research in Medical Entomology, 4 Sarojini Street, Chinna Chokkikulam, Madurai – 625 002, India
***National Institute of Communicable Diseases, Kozhikode Branch, Kerala, India

Introduction

In India, outbreaks of dengue fever (DF)/dengue haemorrhagic fever (DHF) have been reported in various parts of the country during the past four decades[1]. Aedes aegypti is the only vector that has so far been implicated in dengue transmission[1,2], even though Aedes albopictus is known to be present in some of the peri-urban and rural areas[2]. Recently, a survey was carried out in the Kozhikode (earlier known as Calicut) airport area of Malappuram district, Kerala. During 2002 and 2003 (up to July), 75 and 150 clinical dengue fever cases, respectively, were reported from the district[3]. Earlier, reports of Aedes survey in Kerala had shown the presence of Aedes albopictus in rubber plantation areas[4] and in plastic cups[5].

This communication presents the results of the detection of dengue virus from the wild and dry preserved, adult females of Aedes albopictus and their breeding indices in and around the airport area. The survey was carried out in May 2004.

Materials and methods

The Kozhikode airport is situated at 11°.15' N latitude and 75°.49' E longitude, in a hilly area of Malappuram district, Kerala. It became functional as an international airport in 1988. A larval survey was carried out in various types of water-holding containers to detect the breeding of Aedes (Stegomyia) mosquitoes, both inside the airport premises and its periphery up to about 600 metres. The larvae were identified as per the method described earlier[6,7]. Adults of Aedes (Stegomyia) mosquitoes were collected while landing on human baits by aspirator tube in a forested residential area about 600 metres away from the airport.

The wild Aedes albopictus females caught from outside the Kozhikode airport, the adults reared from larval collections...
from inside the Kozhikode airport and the city, and the adults of Aedes aegypti reared from larval collection from the Thiruvananthapuram international airport were separated sex-wise, pooled by species (about 15 adults per pool) and transported to the Centre for Research in Medical Entomology (CRME), Madurai, Tamil Nadu, in a dry state, for detection of dengue virus. The methodology followed was similar to that used for the detection of JE virus and based on the protocol developed and standardized by CRME[8]. However, the antibody (D3-5C9-1) was diluted at 1:5000 as being followed by CRME.

Results and discussion

Aedes survey

The survey for larval infestation in 52 houses/premises around the airport area revealed 16 premises as positive for Aedes albopictus breeding (house index 30.7%). A total of 272 wet containers searched for Aedes breeding revealed 41 containers as positive for Aedes albopictus and two for Aedes vittatus (container index 15.1% and 0.7% respectively). The most preferred containers for Aedes albopictus breeding were discarded tyres, coconut shells and plastic containers. The average landing rate of Aedes albopictus on humans was 20 females/human bait/hour.

Dengue virus detection in Aedes albopictus

Of the three pools of Aedes albopictus tested for dengue virus infection following antigen-capture enzyme immunoassay (EIA), one pool was found positive for dengue virus (OD-0.32), thereby indicating dengue viral activity in this mosquito species. The mosquitoes in the positive mosquito pool were collected as landing collection around the Kozhikode airport on 28 May 2004, and transported as dry specimens to the CRME laboratory and processed on 8 June 2004 (Table). Earlier dengue virus was also isolated from Aedes albopictus collected in a village in Vellore district of Tamil Nadu[9].

Table. Aedes mosquito pools tested for dengue virus infection by ELISA

<table>
<thead>
<tr>
<th>Mosquito species</th>
<th>Locality</th>
<th>Collected on (Processed on)</th>
<th>No. of pools tested/No. of adults/No. of pools positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild caught adults (landing)</td>
<td>Reared adults (immature)</td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>Kozhikode airport</td>
<td>27/05/04 (08/06/04)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Residential area around the airport</td>
<td>28/05/04 (08/06/04)</td>
<td>1/10/1*</td>
</tr>
<tr>
<td></td>
<td>City area (2 kms from airport)</td>
<td>28/05/04 (08/06/04)</td>
<td>–</td>
</tr>
</tbody>
</table>

* Positive pool
Detection of Dengue Virus in Wild Caught Ae. albopictus (Skuse) around Kozhikode Airport

The present study confirms that antigen-capture enzyme immunoassay is a useful surveillance tool for monitoring dengue virus infection in mosquitoes.

Acknowledgements

The provision of laboratory facilities and the permission for the study by the Director, Centre for Research in Medical Entomology, Madurai, Tamil Nadu, is gratefully acknowledged. The authors are thankful to Mrs V. Thenmozhi and Mr S. Venkatesan, CRME, for providing laboratory assistance.

References


Entomological Investigations for DF/DHF in Alwar District, Rajasthan, India

Kalpana Baruah* , Avdhesh Kumar and V.R. Meena

National Institute of Communicable Diseases, 22 Shamnath Marg, Delhi-110 054

Introduction
During 2001, small outbreaks of DF/DHF were reported in many districts in Rajasthan, including the capital city of Jaipur and the industrial city of Alwar. There was a total of 1,820 laboratory-confirmed cases (based on serology using kits for IgG and IgM antibodies) with 30 deaths (CFR: 1.65%)[1]. The present communication deals with the entomological investigations carried out by the National Institute of Communicable Diseases, Delhi, during the outbreak period in a few urban and rural areas of Alwar district that were affected.

Study area
Alwar district is situated in the north-eastern part of Rajasthan between 27°4′ and 28°4′ north latitude and 76°7′ and 77°13′ longitude. The central part of the district is occupied by the Aravali hills. The population of the district is 29,90,862 (2001 census), of which 85% is predominantly rural. The monsoon season is usually of a short duration (July-August), the average rainfall being 61.16 cm. The highest temperature during June goes up to 47 °C whereas the lowest may go down to freezing point.

Alwar town has a number of industrial units. Migration of labour thus poses an increased threat for malaria and other vector-borne diseases, including DF/DHF. The city has irregular piped water supply resulting in water storage practices for household purposes. In rural areas no such piped water supply system exists; therefore, water from wells, bore-wells and natural streams is used for household purposes with minimal storage practices.

Larval survey
An Aedes survey, as per WHO guidelines[2], was carried out in four localities out of 10 in urban areas and in five localities under four primary health centres (PHCs) in rural areas, all reporting fever cases. The results of the Aedes survey are given in Table 1.

* E-mail: kalpana_baruah@yahoo.co.in
Table 1. Aedes aegypti larval indices in the urban and rural areas, Alwar, Rajasthan

<table>
<thead>
<tr>
<th>Name of the locality (or villages)</th>
<th>Total houses searched</th>
<th>Houses found positive</th>
<th>House Index</th>
<th>Containers searched</th>
<th>Containers positive</th>
<th>Container Index</th>
<th>Breteau Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urban areas</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonwa</td>
<td>30</td>
<td>10</td>
<td>33.3</td>
<td>62</td>
<td>17</td>
<td>27.4</td>
<td>56.7</td>
</tr>
<tr>
<td>Karaulikund</td>
<td>30</td>
<td>9</td>
<td>30.0</td>
<td>57</td>
<td>13</td>
<td>22.8</td>
<td>43.3</td>
</tr>
<tr>
<td>Arya Nagar</td>
<td>20</td>
<td>4</td>
<td>20.0</td>
<td>47</td>
<td>4</td>
<td>8.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Kalakuan</td>
<td>25</td>
<td>9</td>
<td>36.0</td>
<td>111</td>
<td>15</td>
<td>13.5</td>
<td>60.0</td>
</tr>
<tr>
<td><strong>Rural areas</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indok</td>
<td>25</td>
<td>0</td>
<td>-</td>
<td>47</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Madhogarh</td>
<td>20</td>
<td>0</td>
<td>-</td>
<td>32</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malakhera (Kalachara)</td>
<td>25</td>
<td>0</td>
<td>-</td>
<td>34</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bhartahari Tiraha</td>
<td>25*</td>
<td>5</td>
<td>20.0</td>
<td>20</td>
<td>5</td>
<td>25.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Kushalgarh</td>
<td>10*</td>
<td>1</td>
<td>10.0</td>
<td>15</td>
<td>1</td>
<td>6.7</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Shops and nearby houses just outside the villages

**Results and discussion**

**Larval surveys**

In urban areas, the House, Container and Breteau indices ranged from 20.0% to 36.0%, 8.5% to 27.4% and 20.0 to 60.0, respectively. Mixed breeding of Aedes aegypti and Anopheles stephensi was also detected in cement tanks in some areas.

In comparison, the House Index was nil in rural residential areas as no mosquito breeding could be detected; however, shops in the marketplaces near the villages and their adjacent houses were found to be positive. The House/Premise Index in these localities ranged from 10.0% to 20.0%, Container Index 6.7% to 25.0% and Breteau Index from 10.0 to 20.0 only. The shops used earthen pots for storing drinking water wherein co-breeding of Aedes aegypti and Aedes albopictus was detected. The dwelling houses along the shops were also found positive, as Aedes breeding was detected in cement containers used for providing drinking water to cattle.

The area-wise infestation by containers is given in Table 2. In the urban areas, out of the total of 49 positive containers, 67.35% were domestic water-storing containers (like cement tanks, clay pots and overhead tanks), followed by 30.61% evaporation coolers and the remaining 2.04% trash.
Table 2. Area-wise infestation of Aedes aegypti in the urban areas, Alwar district, Rajasthan

<table>
<thead>
<tr>
<th>Area</th>
<th>Evaporation coolers</th>
<th>Cement tanks</th>
<th>Clay pots</th>
<th>Overhead tanks</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>% +ve</td>
<td>S</td>
<td>% +ve</td>
<td>S</td>
</tr>
<tr>
<td>Sonwa</td>
<td>27</td>
<td>18.5</td>
<td>16</td>
<td>56.3</td>
<td>12</td>
</tr>
<tr>
<td>Karaulikund</td>
<td>19</td>
<td>21.1</td>
<td>21</td>
<td>38.1</td>
<td>17</td>
</tr>
<tr>
<td>Arya Nagar</td>
<td>24</td>
<td>8.3</td>
<td>9</td>
<td>11.1</td>
<td>14</td>
</tr>
<tr>
<td>Kalakuan</td>
<td>37</td>
<td>10.8</td>
<td>27</td>
<td>25.9</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>14.0</td>
<td>73</td>
<td>34.2</td>
<td>84</td>
</tr>
</tbody>
</table>

S= Searched

Adult surveys

Landing collections were also undertaken in the same urban and rural areas where larval surveys were carried out. In urban areas the adult density of Aedes aegypti ranged from 2.0 to 7.0 per man-hour. The houses with poor ventilation and light yielded higher numbers.

In rural areas both Aedes aegypti and Aedes albopictus were detected in shopping areas only. The cases reported from these rural areas could be attributed to population movement from rural to urban areas (city) during daytime for earning their livelihood or shopping purposes. A similar observation was made earlier by Kalra et al. in Ajmer (Rajasthan), where people from the periphery picked up the infection during their day visit to the city area where Aedes aegypti breeding indices were very high.

Acknowledgments

The authors are grateful to the Director, National Institute of Communicable Diseases, Delhi, for providing the opportunity and necessary facilities to undertake this investigation. The cooperation extended by the staff of the Chief Medical Officer’s office, Alwar district, and the technical support of the staff of the NICD, Alwar branch, is gratefully acknowledged.

References


Delhi, the Capital of India, reported the first-ever outbreak of dengue fever in 1967[1]. Since then the city has experienced cyclic epidemics every 2-3 years. During 1996, a large epidemic swept the city when 10,252 cases were hospitalized and 423 deaths were recorded[2]. The disease has now become endemic and the yearly incidence has varied between 160 and 300 cases of DF/DHF, with a couple of deaths[3]. Aedes aegypti has been invariably found to be associated with these outbreaks in Delhi. Overhead tanks (OHTs) and ground level tanks (GLTs) as key containers, and evaporation coolers have repeatedly been reported as seasonal amplification sites[4,5]. The key containers maintain mosquito breeding throughout the year, whereas coolers amplify the Aedes population from May to November, and thereafter they go dry[6].

During 2003, DF/DHF resurged in some localities in Delhi and a total of 2,604 DF/DHF cases and 33 deaths were registered up to 9 November 2003 by the city Municipal Corporation of Delhi (MCD). The MCD organized a campaign aimed at source reduction, which was backed by vehicle-mounted thermal fogging for the control of the outbreak. However, the reduction in the DF incidence was not found to be commensurate with the inputs made into the campaign. At the request of the MCD, a team of experts from the Malaria Research Centre undertook a comprehensive survey in five affected localities, viz. Dayanand Colony (Lajpat Nagar Phase IV), Lajpat Nagar (Phases I and II), Kotla Mubarakpur, Dayalpur Extension and Harsh Vihar - Tulsi Niketan, to identify both the key containers (OHTs and GLTs) and the amplification containers (evaporation coolers, earthen pots, flower vases, household ornamental fountains, etc.) during November 2003 as per WHO techniques. The results of the study are included in this communication.

The results of the larval survey of Aedes aegypti in five dengue-affected localities in Delhi as mentioned above are given in the
Table. The results revealed that among the key containers, out of 243 OHTs and 20 GLTs, 64 OHTs (26.34%) and 8 GLTs (40%) were found positive for breeding of Aedes aegypti. On the other hand, out of the 533 evaporation coolers, which are identified as the major amplification-breeding site, checked, none was found positive for breeding of Aedes aegypti. Ornamental fountains inside the drawing rooms and mud-pots containing water for birds were supporting the heavy breeding (>1,000 larvae in each site) of Aedes aegypti. The maximum positivity of OHTs was found in Kotla Mubarakpur (39.39%), followed by Phases I and II of Lajpat Nagar (30%) and Dayanand Colony (20.69%). Two OHTs located on the roofs of the market area were found supporting the breeding of Aedes albopictus.

IEC Activities of MCD

The IEC activities of the MCD generally covered the following aspects:

(1) Creating awareness through media and spreading vocal messages through the use of loudspeakers in DF-affected localities, distribution of pamphlets, etc.;

(2) MCD workers who visit houses for physical verification of breeding sites also interact with householders and impress upon them to remove small water collections indoors/outdoors, specially water evaporation coolers.

During the survey it was found that fogging generally lacked a ‘pre-fogging public information campaign’ requiring the public to keep the house doors/windows open to permit the entry of fog. Health workers, while interacting with householders, invariably talked about the removal of breeding from coolers and tanks but did not point out other sites of breeding. The crosschecking teams of the MCD also laid emphasis on inspection of evaporation coolers as focal points and did not verify OHTs/GLTs and trash materials, as they found it a time-consuming affair. In urban areas, for security reasons or houses being locked, access by health workers into the houses was also a constraint.

In view of the aforesaid, it can be concluded that: (i) source reduction should cover both the key containers as well as the amplification breeding sites, viz. evaporation coolers and other trash articles breeding the vector species; (ii) the fogging operation should be preceded by a pre-fogging information campaign in order to seek full cooperation of communities to derive optimal benefits from fogging; (iii) the IEC campaign based on KAP studies should be prepared with particular emphasis on community participation; (iv) special efforts should be focused on behavioural change in accordance with the guidelines laid down by WHO; and (v) health staff of the MCD should be trained in entomological surveys/techniques related to Aedes breeding, prevention and its control.

Dengue Bulletin - Vol 28, 2004
Table. Breeding of *Aedes aegypti* in key and amplifier containers in dengue-affected localities of Delhi during November 2003

<table>
<thead>
<tr>
<th>Locality</th>
<th>Key containers</th>
<th>Amplification containers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overhead tanks (OHTs)</td>
<td>Ground level tanks (GLTs)</td>
</tr>
<tr>
<td></td>
<td>Examined</td>
<td>+ve</td>
</tr>
<tr>
<td>Dayanand Colony (Lajpat Nagar Phase IV)</td>
<td>58</td>
<td>12</td>
</tr>
<tr>
<td>Lajpat Nagar (Phases I and II)</td>
<td>80</td>
<td>24</td>
</tr>
<tr>
<td>Kotla Mubarakpur</td>
<td>66</td>
<td>26</td>
</tr>
<tr>
<td>Dayalpur Extension</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Harsh Vihar – Tulsi Niketan</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>243</td>
<td>64</td>
</tr>
</tbody>
</table>

* Ornamental fountain and trash
–: NIL
Acknowledgement

The authors are thankful to Dr K.N. Tiwari, Municipal Health Officer-cum-Director, Health Services, Municipal Corporation of Delhi, for providing assistance in the conduct of the study, and to Mr N.L. Kalra, former Deputy Director, National Vector-Borne Diseases Control Programme, for his valuable suggestions and guidance. We acknowledge the efforts of the field staff for assisting in carrying out the survey. Thanks are also due to Mr Sanjeev Gupta for his help in various ways.

References


Breeding of Dengue Vector *Aedes aegypti* (Linnaeus) in Rural Thar Desert, North-western Rajasthan, India

B.K. Tyagi and J. Hiriyan

Centre for Research in Medical Entomology, Indian Council of Medical Research, 4 Sarojini Street, Chinna Chokkikulam, Madurai 625 002, India

*Aedes aegypti*, the vector of dengue fever, is widely present in India\cite{1,2}, including the Thar desert in north-western Rajasthan. Jalore town in the Thar desert experienced the first-ever epidemic of dengue fever in 1985. Entomological studies carried out in Jalore during 1990 and subsequently in 1996 observed extensive breeding of *Aedes aegypti*\cite{3,4}. Recently, dengue fever again struck the Thar, this time in Jodhpur district, warranting a serious review of the vector ecology in the region, particularly in view of the prodigious breeding of *Aedes aegypti* in discarded household and community-based underground water reservoirs called tankas, of which thousands are formed in different forms in the Thar\cite{3}. These tankas, which originally attracted only the malaria vector *Anopheles stephensi* as long as the water remained potable, started breeding *Aedes aegypti* only after being discarded by local populations in the wake of the recent availability of conduit-based water supply under Indira Gandhi Nahar Pariyojana (IGNP canal project). It is, therefore, considered worthwhile to highlight the association of *Aedes aegypti* and tankas in sustaining the vector population under extreme xeric conditions.

A total of 33 villages in the three districts of Jodhpur, Jaisalmer and Sri Ganganagar (now incorporated partly in the newly created Hanumangarh district) were surveyed for the presence of tankas (Table 1). Compared to those of Jodhpur and Jaisalmer, most villages in Sri Ganganagar are highly irrigated and adequately supplied with the conduit-water system. Four major types of water storing facilities were identified which supported the breeding of *Aedes aegypti*. About 13.6% of the tankas, which constituted 77.1% of the four water bodies, were found to be breeding *Aedes aegypti*. It is noteworthy that the typical century-old traditional earthen-type tanka was almost invariably present in most villages of Jodhpur (88.8%) and Jaisalmer (85.7%) but less so in Sri Ganganagar (17%) where canal-based water storage sources

---

\*E-mail: bk_tyagi@sify.com*
had greatly reduced the existence of the tankas. As many as 11 (24.4%) out of a total of 45 community tankas existing peripherally in Kanasar village were abandoned for want of proper water storage facility and rendered uncared for by the villagers. All such tankas, wherein the water turned turbid in course of time including vegetation growth, Aedes aegypti was invariably found to breed, replacing in the process its earlier and original occupant, the malaria vector Anopheles stephensi. The first instar larvae abounded the most (52.3%), while the fourth instar larvae (7.8%) was present the least, followed by pupae (2.5%). This situation clearly indicated a sustained breeding of Aedes aegypti in the tankas. Cement tanks, invariably constructed in close vicinity of bore-wells, for cattle drinking bred Aedes aegypti as soon as the water there turned turbid due to prolonged use by cattle, sometimes with Anopheles subpictus. None of the beris, another type of earthen reservoir in the desert environment, supported the breeding of Aedes aegypti.

Table 1. Distribution of different kinds of tankas and beris in various villages in three districts currently under varying degrees of irrigation and/or conduit water supply from canals

<table>
<thead>
<tr>
<th>District</th>
<th>No. of villages</th>
<th>Typical intra-domestic earthen tankas</th>
<th>Metallic mobile tankas</th>
<th>Central community tankas</th>
<th>Peripheral village tankas assembly</th>
<th>Beris</th>
<th>Cement tanks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Sri Ganganagar</td>
<td>17</td>
<td>9</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>Jodhpur</td>
<td>9</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Jaisalmer</td>
<td>7</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>11</td>
<td>16</td>
<td>6</td>
<td>4</td>
<td>28</td>
<td>1</td>
</tr>
</tbody>
</table>

- = Absence of breeding habitat
+ = Presence of breeding habitat, with potable water breeding Anopheles stephensi
++ = Presence of abandoned breeding habitat positive for Aedes aegypti breeding

Conclusion

The dengue vector, Aedes aegypti, has so far been collected from the Thar desert only from townships and/or desert fringe areas in the vicinity of urban environment, breeding mostly in household pitchers and cement tanks. The breeding of Aedes aegypti in the tankas in the rural areas of the Thar desert is considered to be a rather recent phenomenon, possibly due to the easy accessibility of newly provided conduit-water supply to villages, which has led to the abandoning of the traditional water reservoirs.
References


This review was developed in response to a recommendation of the WHO Informal Consultation on Strengthening Implementation of the Global Strategy for Dengue Fever/Dengue Haemorrhagic Fever Prevention and Control, held in October of 1999, urging “the refinement of existing entomological indicators and/or the development of new indicators that better reflect transmission potential.” The Consultation “recommended that such indicators should provide clear, meaningful information for communities as well as for programme managers and policy-makers.” Whereas the traditional Stegomyia indices (the House, Container, and Breteau indices, and various related derivations) are of some operational value for measuring the entomological impact of larval control interventions against the mosquito vectors of dengue virus, they are not proxies for adult vector abundance. 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 seroconversion rates in the human population.

The document reviews and critiques current methods, focusing especially on sampling methods that provide information on (1) the risk of transmission as a function of vector abundance, and (2) the relative or absolute importance of the various types of containers in the environment. This second aspect is essential when considering a suppression strategy designed to minimize costs or to improve sustainability by targeting only a subset of the breeding containers for control or elimination – specifically those container types that are responsible for the majority of adult production. In reviewing current and generally-used sampling methods, each is discussed with respect to transmission risk assessment and evaluated in terms of being useful for either “research or special studies” or as a practical operational tool providing useful information for planning and management of vector control programmes.
Epidemic dengue fever (DF) and dengue haemorrhagic fever (DHF) have emerged as a global public health problem in recent decades. In fact, the problem has become hyperendemic in many urban, periurban and rural areas, with frequent epidemics. The South-East Asia Region is one of the regions at highest risk of DF/DHF, accounting for 52% of the global risk. Dengue outbreaks now occur in India, as in other high-burden countries in the Region, such as Indonesia, Myanmar and Thailand.

Strengthening epidemiological and laboratory surveillance of dengue and dengue haemorrhagic fever including, the implementation of DengueNet, is one of the priorities of the global and regional strategies for dengue prevention and control. DengueNet is WHO’s global data management system created on the Internet to collect and analyse standardized epidemiological and laboratory surveillance data with the objective to improve capacity for effective national and international planning for the prevention and control of epidemic dengue and DHF.

Following the pilot use of DengueNet in the Americas, a joint WHO HQ/SEARO/WPRO meeting on DengueNet implementation in South-East Asia and the Western Pacific was held in Kuala Lumpur on 11-13 December 2003. The objective of the meeting was to expand the pilot project to these two regions, building upon the lessons learned from the pilot project in the Americas. Based on the recommendations of this meeting, two country workshops were organized in India in March 2004, supported by the WHO/CSR and USAID project to strengthen surveillance in India. The first took place in New Delhi on 11-12 March 2004 with the northern states and the second in Bangalore on 16-17 March 2004, with the southern states. WHO collaborating centres attended both meetings. The proceedings and recommendations from the New Delhi meeting were discussed at the Bangalore meeting to ensure that the consensus recommendations addressed national issues, needs and priorities. Experts from health service departments of all states and the Delhi City Corporation, the National Institute for Communicable Diseases (NICD), the National Institute of Virology (NIV) in Pune, the WHO Department of Communicable Disease Surveillance and Response, WHO Representative for India and SEARO participated.

1 See No. 36, 2002, pp. 300-304.

2 See No. 6, 2004, pp. 57-62.
The main objective of the workshops was to strengthen disease surveillance and response to vector-borne diseases using DengueNet as an entry point. Work focused specifically on assessing current surveillance practices (including the use of case definitions, reporting formats and mechanisms for flow of information), laboratory facilities and tests for DHF, on identifying and strengthening regional collaborative laboratories and on establishing a framework for participation in DengueNet.

Experiences both from India and from the region on surveillance and control were discussed. The need for an integrated approach to surveillance of vector-borne diseases and application of lessons and experiences from malaria and other vector-borne diseases was identified. The consensus was to implement DengueNet in accordance with the Integrated Disease Surveillance Programme (IDSP) that is starting in India. This would require capacity building for disease surveillance and response at national, state and district levels, through training of health workers and programme managers and strengthening of laboratory services.

Given the vastness of the country, the heterogeneity of the disease burden and the organizational network, the group recommended piloting the participation of selected states in global DengueNet through focal points at state and national level. States in which implementation would be piloted included Delhi, Karnataka, Maharashtra, Tamil Nadu and Uttaranchal. Maharashtra and Tamil Nadu have a well developed disease surveillance system and a network of public health laboratories at both district and state level, with strong linkages to national-level laboratories. Delhi, following the recent dengue outbreak, has significantly improved its surveillance system, including strengthening of laboratory services. Uttaranchal, although lacking a good network of laboratories, was included because of its close proximity to Delhi.

It was also decided to designate two WHO laboratory collaborating centres (WHO CC) for northern and southern states to ensure practical use of these facilities. Accordingly, NIV-Pune will continue as WHO CC for the south, and it is recommended that WHO designate NICD to serve as WHO CC for the northern states (following a request from NICD). The two centres would be responsible for training, quality control, use of standard procedures and networking at national and international level.

Based on the recommendations of the workshops, a follow-up meeting has been planned to develop an activity plan for 2004 focusing on laboratory strengthening, training, disease and vector surveillance, networking, information sharing and reporting to DengueNet. NICD will take the lead role in this follow-up activity, which is scheduled for the end of May 2004, working closely with all major stakeholders.

1 Major stakeholders include the Indian Council for Medical Research, IDSP Cell, Delhi City Corporation, NICD - WHO CC for Training and Epidemiology, WHO CC for Rabies Epidemiology, WHO Regional Reference Laboratory for Polio Surveillance, National Reference Laboratory for SARS and Avian Influenza, state nodal officers of the pilot states and states that were not represented in the earlier meetings, national-level institutes such as the Central Bureau of Health Intelligence, Vector Control and Research Centre - WHO CC for Filarialis and Vector Control, National Vector Borne Disease Control Programme and NIV - WHO CC for Arboviral Disease Diagnosis and Research and National Influenza Surveillance Centre.
Dengue/DHF - Global public health problem

Epidemic dengue fever and dengue haemorrhagic fever (DHF) have emerged as a global public health problem in recent decades, with the development of hyperendemicity in urban and peri-urban centres of many tropical and subtropical countries. Asia-Pacific countries have more than 70% of the disease burden; in several of them, DHF has become a leading cause of hospitalization and death among children. Latin America and the Caribbean appear to be following the same DHF epidemic trend, with the disease affecting all ages and case-fatality rates as high as 10-15% in areas with limited health service infrastructure. The African and Eastern Mediterranean regions are much less affected. Air travel is also facilitating the rapid global movement of dengue viruses and increasing the risk of DHF epidemics through the introduction of new serotypes. Globally, 2.5 billion people live in areas where dengue viruses can be transmitted: an estimated 50 million dengue infections occur each year, with 500,000 cases of DHF and at least 22,000 deaths, mainly among children. Although dengue is a notifiable disease in many endemic countries, only a small proportion of cases are reported to WHO.

Rationale for DengueNet

DengueNet, WHO’s global surveillance system for dengue fever and DHF, has been created as a web-based central data management system to collect and analyse standardized epidemiological and virological data in a timely manner and to present epidemiological trends as soon as new data are entered. Strengthening epidemiological and virological surveillance of dengue and DHF, including implementation of DengueNet, for early detection, planning and response is one of the four main priorities of WHO’s global prevention and control strategy, adopted in resolution WHA55.17 in May 2002. DengueNet, when fully implemented, will facilitate WHO’s global outbreak and response activities and support the GOARN.

Epidemiological and laboratory-based surveillance is required to monitor and guide dengue/DHF prevention and control programmes whether these are based on vector control or possible future vaccination with a safe, effective and affordable vaccine. Recent and encouraging research developments have made it likely that a dengue vaccine will become available. As a consequence, the public health community

---

1 See No. 36, 2002, pp. 300-304.
2 Global Outbreak Alert and Response Network.
needs to define the burden of dengue for society, so that adequate cost-benefit analyses can be presented to government leaders before they decide to use the vaccine. Standardized global dengue surveillance data, one of the principal results expected from the establishment of DengueNet, have become critical.

Phased implementation of DengueNet

First meeting in San Juan, Puerto Rico, July 2002

The first DengueNet meeting was held jointly with WHO/PAHO and the WHO collaborating centre for dengue/DHF at the Centers for Disease Control and Prevention (CDC Dengue branch; San Juan, Puerto Rico). Its objective was to describe and demonstrate DengueNet to prospective users and to launch a pilot, building on the existing reporting systems and network of dengue laboratories in the Americas. Epidemiologists and virologists from eight countries in the Americas, three countries in Asia and five WHO collaborating centres provided recommendations for the administrative and technical procedures involved in making DengueNet operational.

Second meeting in Kuala Lumpur, Malaysia, December 2003

After pilot use of DengueNet by four Member States and one network representing 20 island countries in the Americas, and after changes to the supporting computer hardware, software, and routines, a second meeting was convened jointly with WHO/WPRO/SEARO and the WHO collaborating centre for dengue/DHF at the University of Malaya in Kuala Lumpur, 11-13 December 2003. The objective was to expand the pilot to South-East Asia and the Western Pacific, building on the lessons learned from the pilot conducted in the Americas. About 70 participants included national epidemiologists, laboratory specialists, and clinicians from 19 Asia-Pacific countries, three countries in the Americas, six WHO collaborating centres, and WHO HQ, regional and country staff.

The plenary presentations and discussions focused on: (1) the challenges and need for standardized global epidemiological and laboratory surveillance of dengue and DHF; (2) the activities of the Pediatric Dengue Vaccine Initiative (PDVI); (3) the national surveillance and reporting systems in the participating countries in South-East Asia and the Western Pacific; (4) the activities of the participating WHO collaborating centres; (5) the WHO global

---

3 This meeting was organized by the WHO Department of Communicable Disease Surveillance and Response, Global Alert and Response, jointly with the WHO Regional Offices for South-East Asia and the Western Pacific, and the WHO Collaborating Centre for dengue/DHF at the University of Malaya in Kuala Lumpur, Malaysia, with technical and financial support from the US Centers for Disease Prevention and Control.

4 Participants included:

- **South-East Asian and Western Pacific regions**: national programmes from Bangladesh, Cambodia, China, Fiji, French Polynesia, India, Indonesia, Lao People’s Democratic Republic, Maldives, Malaysia, Myanmar, Nepal, Philippines, Sri Lanka, Singapore, Thailand, Viet Nam; the bi-regional Mekong Basin Disease Surveillance Network; WHO collaborating centres and research institutes in Australia, India, Japan, Malaysia, Thailand.

- **Americas**: DengueNet pilot country Brazil; WHO collaborating centres in Cuba and USA; interim director of the Dengue Pediatric Vaccine Initiative (PDVI).

- **WHO**: HQ; regional offices (PAHO, SEARO, WPRO); country offices (India, Malaysia).
strategy and regional programmes; (6) WHO’s global outbreak and response activities and GOARN; (7) the DengueNet pilot and lessons learned; (8) presentation of DengueNet and a “hands on” session with the “new” prototype web site in Global Atlas.

Two working groups were convened. The first reviewed and defined the epidemiological data and reporting requirements for DengueNet, modifications needed to the present format, identification of countries for expanding the DengueNet pilot to Asia-Pacific regions, and roles and responsibilities of national and international partners. A subgroup also reviewed and defined the objectives of DengueNet. The second working group reviewed the existing laboratory capacity in South-East Asian and Western Pacific countries in relation to DengueNet, identifying the current needs (and gaps) for laboratory standards, quality control, and dengue serological diagnosis and virus isolation, as well as for reporting and information exchange. The group made recommendations that focused on strengthening regional dengue laboratory diagnosis capacity, so that laboratories participating in DengueNet are able to report data of the highest quality possible within their working environment.

Meeting outcomes

Objectives of DengueNet

The participants agreed that the overall objective of DengueNet is to improve capacity for effective national and international planning for the prevention and control of dengue and that the specific objectives for implementing this global surveillance system are:

- to provide early warning of potential outbreaks of dengue disease or of the introduction of dengue viruses into epidemiologically silent areas, for the purpose of implementing timely control measures and notifying decision-makers in institutions whose occupations or livelihood may be affected;
- to strengthen and standardize epidemiological surveillance of DF and DHF;
- to promote the use of standardized clinical case definitions and reporting criteria for dengue illnesses, permitting comparisons between countries and over time;
- to strengthen the network of collaborating centres and national laboratories for serotype determination and strain characterization;
- to promote improvement in the quality of laboratory data reported at national and international levels;
- to provide a standardized database for epidemiological research and analysis;
- to provide data useful for estimating the burden of disease (including the social and economic burden) on a national, regional, or global scale;
- to support the improvement in national and international alert and response capacity for dengue/DHF outbreaks; and
- to promote the free and timely exchange of epidemiological information between affected countries, their neighbours, and other stakeholders in order to facilitate and promote dengue control activities within the region.
Recommendations of the laboratory working group

To strengthen the regional dengue laboratory diagnosis capacity, the participants of this group made the following recommendations for national laboratories, WHO collaborating centres, WHO, and government health authorities.

Quality control

- Quality control/proficiency testing should be undertaken by the national laboratory/WHO collaborating centre for other laboratories in the country concerned.

- A reference centre should be established at the WHO Collaborating Centre for Tropical Viral Diseases, Nagasaki, Japan, to undertake coordination of quality assurance/control for other WHO collaborating centers and designated national laboratories in the two Regions.

- The Nagasaki reference centre should coordinate, organize, and distribute a WHO panel of reference sera for validation of tests/kits/rapid tests by WHO, national laboratories, and WHO collaborating centres.

Reference services

- Countries that do not have facilities for virus isolation should send appropriate samples to a WHO collaborating centre of their choice after consultation with that centre.

- WHO should recommend capacity-building for virus isolation to the ministries of health of countries that lack facilities.

- In collaboration with WHO country and regional offices, WHO collaborating centres should provide reference reagents to national laboratories - standard inactivated antigens, monoclonal antibodies, standard sera, cell lines for virus isolation, and prototype dengue virus strains.

Laboratory training

- WHO should organize regional training courses on laboratory diagnosis of dengue and other flaviviruses.

- WHO should develop a laboratory manual for dengue diagnosis.

- WHO HQ should establish a global technical advisory group, including representatives from collaborating centres, to meet annually to advance laboratory training, capacity building, reagents, quality issues, and DengueNet.

Reporting and information exchange

With regard to collection of laboratory data and information transfer, the group identified a strong need for government health authorities to develop a reporting system to collect, centralize, and disseminate these data, identify key laboratories to participate in this system, and designate a focal point for DengueNet.

The group recommended that WHO support national health ministries to assess current laboratory status in Asia-Pacific countries and to plan mechanisms to strengthen laboratories for DengueNet. A draft DengueNet laboratory assessment tool is available for review and use.

The group expressed appreciation of the efforts made to develop DengueNet and
recommended that WHO work with partners to develop strategies for raising crucial resources.

**Recommendations of the epidemiology working group**

The group reviewed currently available data and reporting practices in the Asia-Pacific countries in relation to DengueNet. The discussion was organized around the principal epidemiological variables of time, place and personal characteristics, plus information about the virus. The group made recommendations on the modifications to be made to the present format of the DengueNet prototype in Global Atlas, on strengthening epidemiological surveillance, and on a framework for implementation of the DengueNet in Asia-Pacific regions with emphasis on the quality of available data and the active participation of national programmes.

**Data collection**

**Epidemiological data**

- To accommodate currently available case classification reporting practices, countries should provide three categories – DF cases, DHF/DSS (dengue shock syndrome) cases, total dengue cases (DF/DHF/DSS). These data should be provided monthly, at state/department level by large countries and at island level by island nations.
- Countries should provide data, monthly when available, on “dengue deaths” (probable or confirmed).
- Countries should provide annual epidemiological data by sex and age groups.

**Rate calculations**

- Both incidence and mortality rates should be expressed per 100 000 mid-year population; countries should provide updates to DengueNet in the event of significant change.
- The system should not show incidence and mortality rates for countries that report data only from sentinel sites.

**Virus serotype data – all available**

- Data should be entered (when provided by the laboratory) as the cumulative number of isolations of each serotype in the country from 1 January.

**General recommendations**

**For countries**

Countries should promote implementation of the WHO recommended surveillance standards for dengue. (Participants were provided with copies of these standards.)

**For DengueNet**

A country information page should be provided on the DengueNet web site for all country-specific information, definitions, and methods used (e.g. sentinel site information, reporting by time of onset or notification, case classification other than according to the WHO definition, etc.).
WHO-recommended surveillance standards should be made available on the DengueNet web site.

Roles and responsibilities of the partners in this network

Countries will collect, validate, and provide epidemiological and laboratory data. They will designate the participating centres and focal points, and WHO country offices will facilitate the process. WHO collaborating centres will provide laboratory support, proficiency panels, and training to national laboratories. WHO regional offices will implement the country support activities, and WHO HQ will maintain and moderate the DengueNet web site. WHO regional offices and HQ will seek financial support for dengue surveillance activities.

Country participation

A major outcome of the meeting was that representatives of all participating countries showed interest in collaborating with DengueNet and agreed to present the meeting’s recommendations to their health ministries. Country participation will require a letter of request from WHO to the ministry of health; ministry authorization for participation and designation of a national DengueNet focal point; and, for some countries, an external budget.

The DengueNet pilot will be expanded to countries in American, South-East Asian, and Western Pacific regions in 2004 after modifications to the system have been made in Global Atlas. The lessons learned from the pilot will be used to develop a consensus framework for DengueNet implementation for standardized global surveillance of dengue and DHF.
Instructions for Contributors

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

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

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


Figures and tables (Arabic numerals), with appropriate captions and titles, should be included on separate pages, numbered consecutively, and included at the end of the text with instructions as to where they belong. Abbreviations should be avoided or explained. Graphs or figures should be clearly drawn and properly labeled, preferably using MS Excel, and all data clearly identified.
Articles should include a self-explanatory abstract at the beginning of the paper of not more than 300 words explaining the need/gap in knowledge and stating very briefly the area and period of study. The outcome of the research should be complete, concise and focused conveying the conclusions in totality. Appropriate keywords and a running title should also be provided.

Articles submitted for publication should be accompanied by a statement that they have not already been published, and, if accepted for publication in the Bulletin, will not be submitted for publication elsewhere without the agreement of WHO, and that the right of republication in any form is reserved by the WHO Regional Offices for South-East Asia (SEARO) and the Western Pacific (WPRO).

One hard copy, original and clear figures/tables and a computer diskette indicating the name of the software, of the manuscript should be submitted to:

The Editor  
Dengue Bulletin  
WHO Regional Office for South-East Asia  
Mahatma Gandhi Road  
New Delhi 110002  
India  
Telephone: 91-11-23370804  
Fax: 91-11-23379507, 23370972  
E-mail: dengue@whosea.org

Manuscripts received for publication are subjected to in-house review by a professional expert and are peer-reviewed by experts in the respective disciplines. Papers are accepted on the understanding that they are subject to editorial revision, including, where necessary, condensation of the text and omission of tabular and illustrative material.

Original copies of articles will not be returned. The principal author will receive 10 reprints of his article published in the Bulletin. A PDF file can be supplied on request.