The WHO Regional Office for South-East Asia, in collaboration with the Western Pacific Region, has been jointly publishing the annual Dengue Bulletin.

The objective of the Bulletin is to disseminate updated information on the current status of DF/DHF infection, changing epidemiological patterns, new attempted control strategies, clinical management, information about circulating DENV strains and all other related aspects. The Bulletin also accepts review articles, short notes, book reviews and letters to the editor on DF/DHF-related subjects. Proceedings of national/international meetings for information of research workers and programme managers are also published.

All manuscripts received for publication are subjected to in-house review by professional experts and are peer-reviewed by international experts in the respective disciplines.
From the Editor’s Desk

The WHO regions of South-East Asia and the Western Pacific have become hyperendemic by reporting progressively larger and more frequent cyclical dengue epidemics.

During 2008, the South-East Asia Region showed about 18% increase in the number of reported cases and about 15% increase in the number of reported deaths. Indonesia, Myanmar and Thailand reported substantial increases in the number of cases. The case-fatality rate (CFR) was below 0.2% in Thailand, and in Indonesia and Myanmar it was around 1%.

Similarly, in the Western Pacific Region, out of 37 countries, 27 countries reported 213,190 dengue cases and 671 deaths. Viet Nam, Malaysia and Philippines were hit the hardest.

The WHO African Region has, till now, reported epidemics of classical dengue only. The populations there have not suffered from DHF because of their innate resistance. However, in 2008, with the entry of DENV-3, subtype III, in a large outbreak in Pemba, Mozambique, two deaths were reported due to DHF (for details, refer to the Book Review section of this issue).

Member States of the WHO South-East Asia and Western Pacific regions have developed a biregional Asia-Pacific Strategic Plan (2008-2015) that focuses on reversing the increasing trend of DF/DHF. In this plan, community ownership and multisectoral interventions are the key strategies to be pursued for the prevention and control of dengue.

The current volume of the Dengue Bulletin (No. 32, 2008) contains contributions received from South-East Asia (15); the Western Pacific (8); the Eastern Mediterranean (1); the Americas (3); and Europe (1).

We now invite contributions for Volume 33 (2009). The deadline for receipt of contributions is 31 October 2009. Contributors are requested to please follow the instructions given at the end of the Bulletin while preparing their manuscripts. Contributions, accompanied by CD-ROMs using MS Word for Windows, should be sent to the Editor, Dengue Bulletin, WHO Regional Office for South-East Asia, Mahatma Gandhi Road, I.P. Estate, Ring Road, New Delhi-110002, India, or by e-mail as a file attachment to the Editor at dengue@searo.who.int. Readers desirous of obtaining copies of the Dengue Bulletin may write to the WHO Regional Offices in New Delhi or in Manila or the WHO Country Representative in their country of residence.

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The quality and scientific stature of the Dengue Bulletin is largely due to the conscientious efforts of the experts and also due to the positive response of contributors to comments and suggestions.
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WHO's efforts for the development of a dengue vaccine

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Abstract

**Background**: Dengue fever and dengue haemorrhagic fever (DF/DHF) are caused by dengue (DENV) viruses. There are four antigenically related, but distinct, DENV serotypes (DENV-1 through DENV-4). Humans are the amplifying vertebrate hosts and Aedes mosquitoes are the primary mosquito vectors as well as the reservoir of infection.

DENV infections cause a spectrum of diseases, ranging from asymptomatic infections to infections complicated by haemorrhage, shock and death. Infection with DENV of one serotype results in apparent life-long monotypic immunity against that serotype but not against any other serotype. Thus, separate infections with all four DENV serotypes are theoretically possible in a single host. It should be noted that in Thailand, all the four DENV serotypes co-circulate, thereby resulting in multiple exposures and the potential for re-infection with different serotypes.

**Initial vaccine development**: In 1980, Mahidol University committed to develop a live-attenuated tetravalent DENV vaccine. The DENV vaccine development project was supported by a grant from the WHO Regional Office for South-East Asia (ICP RPD 002/DHF). DENV-1 and -2 obtained from DHF patients and DENV-4 obtained from a DF patient were serially passaged in primary dog kidney (PDK) cells certified to be free from human and canine infectious agents. DENV-3 obtained from DHF patients was first passaged in primary green monkey kidney (PGMK) cells and then in certified Fetal Rhesus Lung (FRhL) cells. The degree of attenuation was empirically based on certain biological markers.

Bulk seed productions were eventually prepared in pilot production facilities at Mahidol University’s Centre for Vaccine Development at the Institute of Molecular Biosciences. They were subjected to general safety tests and monkey neurovirulence tests in accordance with the US FDA requirements. These pre-clinical tested candidate DENV viruses were approved for proceeding to the clinical evaluation phase by a WHO-appointed Scientific Steering Committee and by the Ethical Review Committee of the Thai Ministry of Public Health.

The monovalent live-attenuated viruses – DENV-1 PDK-13, DENV-2 PDK-53, DENV-3 PGMK-30/FRhL-3 and DENV-4 PDK-48 – were first tested in flavivirus non-immune adult subjects, followed by bivalent, trivalent and tetravalent vaccine clinical trials. All vaccine recipients developed either a mild or no adverse reaction to the vaccine. The immunogenicity data were discussed.

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Due to viral interference of each DENV components in the combinations, 12 DENV formulations were evaluated for confirmation of safety and immunogenicity profiles in 155 Thai children aged 3–15 years. Preliminary data were analysed and processed for further development.

**Collaboration with Sanofi Pasteur:** In order to make productive use of this research, Mahidol University entered into a collaborative licensing agreement in DENV vaccine production in 1993 with France-based Sanofi Pasteur, the vaccine division of Sanofi-Aventis Group and the largest company in the world devoted entirely to human vaccines. DENV vaccine based on this approach was prepared for production on an industrial scale in France using specific-pathogen-free (SPF) dog colony and FRhL cells. The vaccine was presented in a lyophilized (freeze-dried) form and reconstituted with water for injection in order to deliver a 0.5 ml specified dose. Multiple dose presentations were planned for a target population of children and adults living in or travelling to DENV-endemic areas.

The current strategy of creating tetravalent DENV vaccine formulations can lead to an unbalanced immune response. This is attributed to viral interference that apparently comes into play when three monovalent vaccine viruses DENV-1, DENV-2 and DENV-4 are mixed with DENV-3 to create a tetravalent formulation.

More research is needed on a priority basis to work out the viral interference factor in order to make the production of a tetravalent vaccine out of our attenuated DENV-3 candidate vaccine strain a success.

**Keywords:** Live attenuated tetravalent dengue vaccine; WHO/SEARO; PDK cells.

**Historical development**

Dengue haemorrhagic fever (DHF) was first recognized as a new disease in Manila in 1954[1]. The disease affected mainly children and was characterized by the acute onset of high fever, petechial haemorrhage and shock. The second large outbreak occurred in Manila again in 1956 which resulted in more than 1200 cases, with 10 to 15 per cent case-fatality rate[2]. In 1958, an outbreak of DHF occurred in Bangkok and its nearby areas. Almost 2500 cases with 10 per cent case-fatality rate were recorded[3]. Since then, DHF has become a serious public health problem, causing large-scale morbidity and mortality among children in the South-East Asia and the Western Pacific regions of WHO. Well-established epidemics have also been reported from Myanmar, China, Cambodia, Indonesia, Laos, Malaysia, Philippines, Thailand and Viet Nam.

In the WHO South-East Asia Region, DHF is a major public health problem in Indonesia, Myanmar and Thailand[4].

The first meeting of the South-East Asia Regional Advisory Committee on Medical Research (SEA/ACMR), held in New Delhi, 5–9 January 1976, recommended that research on DHF should be given a high priority. During the second session of the SEA/ACMR held in New Delhi, 23–27 August 1976, a review was made of the history of the spread of this disease through several countries of the Region, with an evaluation of the current state of knowledge on its epidemiology, virology, pathogenesis and the related problems of clinical management.

A meeting of the Research Study Group on DHF was held in New Delhi on 24-25 February 1977. Several measures with potential for the prevention and control of this disease were considered. After detailed discussions, the group made its recommendations, of which the two important ones were: (i) vaccine research; and (ii) control of Aedes aegypti.

The first plan of vaccine research was developed, which, *inter alia*, proposed that
virologists from the South-East Asia and the Western Pacific regions be trained in research and development of the vaccine at the School of Tropical Medicine and Medical Microbiology, University of Hawaii. On the completion of the training (1980), the participants on return to their respective countries were impressed upon to get directly involved in the national DENV vaccine development programme. The time frame needed for the development of the DENV vaccine programme was proposed to be 3–5 years.

It was understood that most countries with DHF problem would like to participate in the field trials of DENV vaccine at a later stage when the vaccines would be ready.

In 1978, a research steering committee recommended to WHO to take positive steps towards DENV vaccine development by designating the then Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, now known as the Centre for Vaccine Development, Mahidol University at Salaya, Thailand, to undertake the research for the development of the vaccine.

Funding of this project began in April 1980. Three laboratories were equipped for DENV vaccine research and development. A virologist was recruited and sent to the University of Hawaii for the initial phase of research as well as for advanced training while equipping of the laboratory continued. The laboratory was ready for operation in early November 1980. Detailed and comprehensive standard operating procedures (SOPs) for vaccine development were prepared. Protocols were available. Tests were signed by operators and were checked and signed by the supervisor.

In 1987, the site for DENV vaccine development moved to the Centre for Vaccine Development of Mahidol University at Salaya, Nakhonpathom, Thailand. Equipments for the centre were donated by the Italian Government. Another four additional buildings were constructed between 1988–1990, which included an experimental animal house and vaccine pilot plant buildings. The entire vaccine compound was designated for DENV vaccine development. Airlocks and a hepafiltered air supply were generated to control potential cross-contamination.

**Rationale for dengue vaccine development**

The scientific hypothesis behind the development of a tetravalent DENV vaccine against DHF can be summarized as follows:

1. Adults developed a higher rate of seroconversion of antibody response against DENV viruses and appeared to be less susceptible to DHF. The naturally-acquired immunity appeared to protect the individuals against the infection. The immunization of target populations could result in the development of a protective antibody response in individuals and could help in protection against the disease.

2. It had also been shown that a mono or bitypic antibody response could be a risk factor for DHF if sequential infection by other serotypes of DENV viruses occurred. It was imperative that the DENV vaccine should be able to confer the protective immunity against all four serotypes of DENV infection and provide life-long immunity. This called for the development of a live-attenuated tetravalent DENV vaccine.

3. The target population for immunization against DHF should be toddlers 1–3 years old.
Technical consideration on dengue vaccine development at Mahidol University

The objectives of this programme were to select strains of DENV-1, -2, -3 and -4 which showed promise of being attenuated for human use and produced in cell substrates. All the four DENV virus serotypes being developed in Thailand were passaged serially in cell culture without specific selection.

Dengue virus strains selected for attenuation attempts

**DENV-1 (16007-TC-10 2/14/74)**

Isolated from a DHF patient in Thailand in 1964 had been passaged in tissue culture before inoculation into *Toxorhynchites amboinensis*. The first intrathoracic passage was No. 167164 and the second was No. 167376 (received from Dr. Robert E. Tesh on 17 June 1980).

**DENV-2 (16681 LLC-1 1/22/73)**

Isolated from a DHF case in 1964 from Thailand had been passaged in tissue culture before inoculation into *Toxorhynchites amboinensis*. The first intrathoracic passage was No. 167165 and the second passage was No. 167377 and were received on 17 June 1980. The parent culture strain was virulent for man, having produced typical DF in a laboratory worker who was exposed accidentally (unpublished observations, Dr S.B. Halstead).

**DENV-3 (16562 TC-7 1/31/72)**

Virus was isolated in 1964 from a DHF case from Indonesia using *Aedes aegypti* and kindly furnished by Dr Duane G. Gubler. The fourth passage was used to initiate the vaccine studies.

**DENV-4 (1036)**

Virus was isolated from a DF case from Indonesia in 1976 using *Aedes aegypti* and kindly furnished by Dr Duane G. Gubler. The fourth passage was used to initiate the vaccine studies.

Mosquito inoculation

At the University of Hawaii (Pacific Research Unit), five adult laboratory-reared *Toxorhynchites amboinensis* were inoculated intrathoracically with strains of DENV-1 to -4. The inoculum was approximately 0.0003 ml. Mosquitoes were maintained on 10% sucrose solution at 28 °C for 12 days. At the end of the incubation period, each group of insects was killed by freezing, their heads removed and triturated in 5.0 ml phosphate buffer saline, containing 0.5% gelatin, 30% heat-inactivated calf serum and penicillin and streptomycin. After centrifugation at 5 °C for 30 minutes, each supernatant fluid was inoculated into another group of five *Toxorhynchites amboinensis*. These insects were also held at 28 °C for 13 days. At the end of the incubation period, they were killed by freezing.

Preparation of mosquito suspensions

The head was removed from the infected mosquitoes with a sterile surgical blade and placed in a mortar. Body parts were kept in a
sterile vial and frozen at –70 °C. The virus diluent, with 30% heat-inactivated calf serum in phosphate-buffered saline, pH 7.5, penicillin/streptomycin, was added to ground mosquito heads, 2.5 ml/5 mosquitoes. After centrifugation at 10 000 rpm for 30 minutes at 5 °C, the supernatant fluids were filtered through at 0.45 micron millipore filter. Filtrates were used to inoculate PDK and PGMK cells.

**Preparation of primary dog kidney cells**

The work was done in the laboratories of the Department of Tropical Medicine and Medical Microbiology, University of Hawaii, and was supported, in part, by a grant from the Rockefeller Foundation to Dr S.B. Halstead.

Each lot and sub-lot of dog kidney cells were subjected to safety tests to assure that the cells and supernatant fluid were free of infectious agents. Tests included for exclusion of bacterial, fungi, mycoplasma and cytopathic and haemadsorbing agents.

**Development of attenuated strains of DENV 1-4 viruses**

Mosquito suspensions of the DENV-1 (16007), DENV-2 (16681) and DENV-4 (1036) were attenuated by serial passages in PDK cell culture at 32°C without cloning or deliberate selection. The procedure relied on the spontaneous appearance of variants and selection for attenuated variants by the biological pressure of the abnormal host cell. This general method had been successful with several other live virus vaccines, e.g. rubella and mumps.

The DENV-3 (16562) virus was attenuated by serial passages in PGMK cell; however, attempts to adapt it to PDK cells had failed.

DENV viruses were serially passaged in PDK cells as illustrated in Figure 1.

**Figure 1: Schema for attempted attenuation of dengue viruses**

![Figure 1: Schema for attempted attenuation of dengue viruses](image)

At every fifth passage level, a moderate-sized virus seed was prepared. This virus was studied for plaque size morphology in LLC-MK2 cells, temperature sensitivity to replication shut-off, suckling mouse neurovirulence and growth in human monocytes, viraemia and antibody response in primates.

When the passaged virus presented a reduction in plaque size, temperature sensitivity for replication and absence of viraemia and reduced antibody response in monkeys, “Masters seed”, “Production seed” and “Candidate vaccine” were prepared. Safety tests on the Production seed and Candidate vaccine included inoculation of neutralized virus into adult and suckling mice, guinea pigs, rabbits and several tissue culture systems. Furthermore, it was also assured that the candidate vaccine produced no neurovirulence following intracerebral inoculation in monkeys. The attenuated strains thus developed could help towards worldwide stock of candidate dengue vaccines[6].

What constitutes a satisfactory level of attenuation remains uncertain. Hypothetically, we would like to have a vaccine which was
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avirulent, i.e. viruses which did not have the capability to cause direct cell injury, but the protective antigenic epitopes of these avirulent viruses should still be preserved and effectively presented to both the B and T lymphocytes of the vaccines to confer both humoral and cellular immunities. It was very difficult to define the attenuation of the dengue viruses by a specific series of the biological markers. These observations remained unsubstantiated due to the fact that there was no known animal model for DHF. Man represented the only alternative testing model of vaccine efficacy.

Markers of attenuation

To define the level of attenuation of the viruses at the present, it could at best be empirical. The assessment was based on the findings of a combination of markers.

Evidence for attenuation was based on a comparison of the high passage viruses with the parent virus in several in vivo and in vitro tests: plaque size, temperature sensitivity, replication in human monocytes, and monkey viraemia. These characteristics had been shown to be related to human virulence with other experimental DENV vaccines.

The DENV-1 PDK 43, DENV-2 PDK 53, DENV-3 PGMK 33 and DENV-4 PDK 48 candidate viruses produced a uniform small plaque size when assayed in LLC-MK2 cells. They revealed temperature sensitivity by the plaquing efficiency test. High PDK or PGMK passages had significantly reduced virulence for suckling mice by the intracerebral route. All the DENV candidate viruses produced low or no ability to replicate in human monocytes in vitro. All of them showed low or no viraemia after inoculation with 10^4–10^5 plaque forming unit (pfu) of each candidate viruses with moderate specific neutralizing antibody responses. Reduced neurovirulence for mice was observed with DENV-1, DENV-2 and DENV-3 candidate viruses. However, the DENV-4 PDK 48 candidate viruses still revealed modulate neurovirulence in suckling mice.

Safety test

Safety tests of the cell substrate, the candidate viruses and the candidate vaccines were designed according to the United States FDA regulations as applied to live attenuated vaccines produced in the United States. Tests included microbial sterility; and search for adventitious agents in PGMK cells, adult and infant mice, guinea pigs, and rabbits. Haemadsorption agents were sought in cell-culture experiments. A second tier of tests required for additional safety were performed at the virology laboratory of the Department of Tropical Medicine and Medical Microbiology, University of Hawaii.

The team could establish the capability to perform monkey neurovirulence test in Thailand and slides of monkey tissue were independently reviewed by an experienced neuropathologist.

Peer review of the vaccine development project

Candidate DENV vaccines considered to be sufficiently attenuated were submitted to an international panel of experts in DENV for review. This panel had met annually once a year in Bangkok for twelve times from 1983 to 1994. The function of the panel of experts was to review the scientific work, including visit to the site of vaccine development, in order to pursue and examine the facilities, and to audit the raw data. The record books were reviewed by two of the peer reviewers in detail for completeness and for the accuracy of summary
WHO's efforts for the development of a dengue vaccine

Data presented. The peer group gave recommendations to the Ministry of Public Health of Thailand and to WHO-SEARO based on their assessment whether the candidate vaccines were suitable for vaccine trials in human beings or not. This process was unique for WHO programmes\(^\text{7-18}\) (Table).

The DENV-1 (16007) PDK 43, DENV-2 (16681) PDK 53, DENV-3 (16562) PGMK 30 FRhL 3 and DENV-4 (1036) PDK 48 met the US FDA requirement for microbial safety and monkey neurovirulence test for live attenuated viral vaccine conducted by laboratory at Mahidol University as well as by an independent laboratory at the Walter Reed Army Institute of Research, USA. They were approved by an international peer review group based on the examination of the result of safety test and by an on-site examination of the facilities, laboratory record and log books. Confirmatory histopathological examination was done at the ethical review conducted by a committee for human experimentation of the Mahidol University and by a similar committee of the Ministry of Public Health, which was satisfactory and these candidate vaccines were approved for clinical trials.

Clinical trials of monovalent dengue vaccines\(^\text{19}\)

The site for the small-scale experimental clinical trial was Lampoon and Loei provinces, an area where there was low prevalence of \textit{Ae. aegypti} mosquitoes. The trials were conducted during the cold season so as to minimize the risk of other arbovirus infections and possible transmission of vaccine viruses. The population chosen to conduct the trials consisted of flavivirus non-immune young male adults. The initial trial was conducted in two phases using first two and then eight volunteers to increase the safety factor. The protocol called for close observation during the first 21 days.

DENV-1 (16007) candidate vaccines

The candidate DENV-1 (16007) PDK 43 vaccine was passed 43 times in PDK cell. The evolution of the biological markers tested was as follows: plaques in LLC-MK2 cells became of small size (= 1 mm) after passages 10–15. Temperature sensitivity at 39 °C was achieved at passage 30. Ability to grow in human peripheral blood lymphocytes (PBL) was lost at passage 20. Suckling mouse neurovirulence was reduced to minimal level at passage 15. After 43 passages, all monkeys that received the DENV-3 PDK 43 showed no or low viraemia. Based on these results, passage 43 was selected for phase 1 trial.

Six flavivirus non-immune subjects were inoculated with a dose of 2.1 to 3.5 \(\times\) 10\(^4\) pfu. Clinical symptoms were mild in all volunteers and only one of them showed very minimal nose bleeding, without other haemorrhagic

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**Table: Dengue Virus Development Programme WHO Peer Review Meeting, Bangkok, Thailand**

Organized by WHO/SEARO, WHO Project: ICP RPD/002

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manifestations. In no case was absolute leukopenia observed. Clinical chemistry was normal in all volunteers. Immune response, as measured by plaque reduction neutralization test (PRNT), was detected in only 1 out of 6 flavivirus non-immune volunteers. Two of the seronegative subjects were challenged again with the same dose of DENV-1 PDK 43 at 3 months. Again, they failed to develop any neutralizing antibody. The conclusion was that DENV-1 PDK 43 had been over-attenuated and that it was necessary to try lower passage levels. A candidate vaccine was then prepared from DENV-1 PDK 30, and after the usual safety tests, was infected into five adult male volunteers who were seronegative for both Japanese encephalitis (JE) and DENV viruses. Two of the five seroconverted, but the responses were low. The exercise was thus repeated using DENV-1 PDK 20 but again the antibody responses were low and only three of the five seroconverted. The conclusion was thus reached that DENV-1, PDK 20, PDK 30 and PDK 43 were over-attenuated to be useful as candidate vaccines for DENV-1 in people that were seronegative for previous exposure to flaviviruses. The DENV-1 PDK 13 virus that was used in the next trial showed evidence of lesser attenuation than PDK 20, PDK 30 or PDK 43 in that it still replicated in human monocytes. When seven DENV and JE antibody negative male volunteers were injected with DENV-1 PDK 13, five seroconverted to DENV-1 within 30 days. There was some evidence of rhinitis but this may have been coincidental and the significance of the observation could not be assessed. There was also a slight fall in leukocyte counts on day 10, but the virus was not isolated from blood at any stage.

DENV-2 (16681) candidate vaccine

The trial of the DENV-2 candidate vaccine, DENV-2 (16681) PDK 53, was carried out as phase 1a and 1b trial.

The initial phase 1a of DENV-2 PDK 53 candidate vaccine in ten 18–30-years-old male human subjects showed encouraging results. None of the 10 persons vaccinated were febrile or incapacitated; side-reactions possibly attributable to the candidate vaccine were limited to slight leukopenia, occasionally abnormally large platelets and a few transient complaints such as mild aches and pains.

All vaccinated persons developed DENV-2 neutralizing antibody. Those subjects with preexisting antibody to JE virus responded serologically more rapidly than those subjects without preexisting flavivirus antibody before vaccination.

Serological tests carried out one year after the vaccination showed that neutralizing antibodies were present in 100 per cent of the volunteers.

The phase 1b trial of DENV-2 PDK 53 candidate vaccine involved sixteen 15–30-years-old male volunteers, 15 of whom were flavivirus non-immune. Four doses of varying virus dilutions were given to groups of four volunteers each, and every person developed DENV-2 neutralizing antibodies, regardless of vaccine virus dilutions. Abnormal lymphocytes and a slight decrease in lymphocyte numbers were consistently observed between days 6 and 10. As in the phase 1a trial, no adverse reactions to the vaccine were observed.

Viraemia was detected in one volunteer and virus isolated in C6/36 cells from day 6 serum. The virus had growth characteristics similar to those of the candidate DENV vaccine virus. On the basis of 1a and 1b studies it was revealed that viraemia occurred between days 6 and 10. It was unlikely to occur after day 14 because of the onset of neutralizing antibodies. It is possible that viraemia may precede the time of the lowest white cell counts, which frequently occurred on day 6.
A dose response study in adults, based on 5-fold dilutions of the vaccine, showed an estimated 50 per cent infectious dose of 5–7 pfu.

**DENV-3 (16562) candidate vaccines**\(^{19}\)

The DENV-3 (16562) parent virus did not grow in PDK cells and was passaged in PGMK cells. At passage PGMK 30, the virus was still able to replicate in PBL and produced plaques of varying sizes. After passage 34, plaques were uniformly small. Two PGMK passages were selected for adaptation to FRhL cells: 30 and 35. In FRhL cells, DENV-3 attained titres one log higher than in PGMK cells. With both passages (PGMK 30/F2) and PGMK 35/F2), biological markers were considered to be satisfactory: plaques were of small size, no CPE in LLC-MK2 cells, temperature sensitive at 38.3 °C, no growth in human PBL, and reduced neurovirulence for suckling mice.

No adventitious agents were found in PGMK cells analysed by electron microscopy. Safety tests of PGMK cells were being completed at the National Institute for Biological Standards and Control (London) and the National Biological Standards Laboratory (Canberra). The cells had been found to be free of mycoplasma, mycobacteria and other adventitious agents. Tests to detect simian retroviruses, SV5 and SV40, were negative.

**Biological characteristics of DENV-3 candidate vaccine viruses**

The DENV-3 (16562) PGMK 30 passage virus had mixed plaque morphology (medium and small), a restrictive temperature of 40 °C caused CPE in LLC-MK2 cells and grew in human PBL. DENV-3 PGMK 30, FRhL-3 virus had small and pinpoint plaque morphology, restricted growth at 38 °C, and did not cause CPE in LLC-MK2 cells. Considerable change, presumably selection, had occurred with FRhL passage. The virus recovered from a volunteer who received PGMK 30, FRhL-3 vaccine had biological markers (medium) plaque size, CPE in LLC-MK2 similar to earlier passage vaccine was either genetically prone to reversion or contained an undetected subpopulation of more virulent virus.

Three passage levels of DENV-3 (16562) were given to volunteers; PGMK 33, PGMK 30-FRhL-2, and PGMK 30 FRhL-3. The FRhL-passaged viruses differed from the PGMK 33 in being more temperature-sensitive, less able to produce CPE in LLC-MK2 cells, and having uniform small plaque morphology.

Four volunteers received the PGMK 30 FRhL-3 virus at doses of 1 × 10⁴ to 6.5 × 10⁴ pfu. One of two volunteers seroconverted at the lower dose. The volunteer who failed to convert at the lower dose was revaccinated at the higher dose and seroconverted. Two volunteers seroconverted at a higher dose. Only minor symptoms and no fever were observed. Satisfactory primary immune responses were observed in three volunteers; the fourth, who was JE immune, had a secondary-type serological response. The virus isolated from the serum of one volunteer exhibited medium-sized plaque morphology and its characteristics of earlier passage virus.

In other trials, two volunteers received PGMK 33 vaccine and four volunteers received PGMK 30 FRhL-2 vaccine. Both of those vaccines contained both medium and small plaque sizes and were less temperature-sensitive than the PGMK 30 FRhL-3 vaccine. Both vaccines immunized satisfactorily at doses of 10⁴; however, brief febrile responses and mild symptoms were observed.

The PGMK-30, FRhL-3 vaccine appeared to be less reactogenic than the other two DENV-3 candidate vaccines and was immunogenic at a dose of 5 × 10⁴ pfu.
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DENV-4 (1036) PDK 48 candidate vaccine

DENV-4 (1036) virus was passaged in PDK cells to passage 48. Biological markers of PDK 48 included small plaque and no cytopathic effect in LLC-MK2 cells, temperature replicative shut-off at 39 °C, and average survival of 12 days in suckling mouse. PDK 48 virus replicated in peripheral blood mononuclear cells. Rhesus monkeys inoculated with PDK 48 virus did not develop viraemia but seroconverted. On evaluation of the monkey neurovirulence tests, the panel of experts concluded that there was no significant difference between the parental DENV-4 virus and DENV-4 PDK 48 candidate vaccine, and it was thus acceptable to proceed with phase 1a clinical trial. An additional four rhesus monkeys had been tested with PDK 48 virus; enhanced neurovirulence was not found. The monkey neurovirulence test result was considered satisfactory, and it appeared feasible to proceed with PDK 48 as a candidate vaccine.

The phase 1a clinical trial was the inoculation of five flavivirus seronegative volunteers with 1-2 × 10^4 pfu of DENV-4 PDK 48. All volunteers developed specific neutralizing antibodies which first appeared from days 13–16, and peaked in titre at day 30 post-inoculation. Clinical signs were unremarkable in all volunteers, and no volunteer developed fever. Clinical symptoms were generally absent, although two volunteers reported eye pain and headache. In one of these volunteers the headache re-occurred for a period of about 2 weeks. All volunteers showed normal blood chemistry profiles. Haematological studies revealed a transient increase in atypical lymphocytes in three volunteers. All volunteers showed a temporary depression in total white blood cell counts; however, there was no absolute leukopenia. Virus was recovered only from plasma, and the viraemia appeared to be low in titre, since no virus could be detected by direct plaque assay.

The recovered virus strains shared all biological markers with the vaccine candidate, except that one strain showed an extended mean day to death in suckling mice of 20 days.

The phase 1b trial was designed to determine the minimum infective dose of the DENV-4 vaccine candidate. The 1b trial was temporarily divided into two phases with groups of seven and five volunteers. The vaccine was diluted from 1:5 (3700 pfu) to 1:1000 (12-15 pfu) and each dilution was inoculated into 1–3 volunteers. None of the volunteers showed fever or rash, and clinical signs and symptoms were mild, although eight of the twelve volunteers reported transient headache and eye pain. Blood chemistries were normal, and haematological findings were similar to those seen in the phase 1a trial.

All groups of volunteers inoculated with a dilution of 7 × 10^2 pfu or greater developed specific neutralizing antibody. Two of the two volunteers at 7 × 10^2 pfu seroconverted, one of the two at 1.5 × 10^2 pfu seroconverted, and none of the three volunteers at 63–77 pfu seroconverted. In total, combining the phase 1a and phase 1b results, 10 of the 10 volunteers inoculated with vaccine doses of 7 × 10^2 pfu or greater seroconverted.

Polyvalent vaccine clinical trials[20]

Bivalent vaccine DENV-2 (16681) PDK 53 and DENV-4 (1036) PDK 48 clinical trial

The aim of the DENV vaccine development programme was to develop and administer a vaccine containing a mixture of multiple DENV serotypes. The rationale was based on the provision of providing protection to all serotypes that would minimize any chances of future DENV infection enhancement and adverse host reaction. This trial was designed to conduct in
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Humans in support of the hypothetical concept that multiple simultaneous infections with candidate vaccines were possible, safe, and effective. Eleven male flavivirus non-immune subjects aged 16 to 31 years received bivalent DENV-2 and DENV-4 candidate vaccines.

The neutralizing antibody responses to DENV-2 at 6 months ranged from 1:52 to 1:440 and that of DENV-4 ranged from 1:44 to 1:310. A low titre of neutralizing antibodies to DENV-1, DENV-3 and JE viruses were detected early, but this disappeared by 6 months.

The bivalent DENV-2–DENV-4 candidate vaccine was both immunogenic and without unacceptable reactions. Moreover, the dose of DENV-2 and DENV-4 viruses was acceptable and formed to be the basis for future trials[20].

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-4 (1036) PDK 48 clinical trial[20]

The bivalent DENV-1 and DENV-4 vaccine’s human clinical trial was conducted in Loei province, Thailand. Seven male flavivirus non-immune subjects, aged 16 to 30 years, received bivalent DENV-1 and DENV-4 candidate vaccine.

All seven subjects seroconverted to both DENV-1 and DENV-4 since the presence of neutralization antibodies were detected by PRNT to both DENV-1 and DENV-4. There was no difference in response between those receiving candidate vaccines in separate arms and among those receiving mixed vaccine in another arm.

The bivalent DENV-1 and DENV-4 candidate vaccine induced specific response to both DENV-1 and DENV-4 but low titres of heterologous neutralizing antibody were found and the vaccine was without any adverse reactions among the recipients[25].

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-2 (16681) PDK 53 clinical trial

Seven male subjects aged between 17 and 32 years received bivalent DENV-1 and DENV-2 candidate vaccine.

All seven subjects seroconverted to both DENV-1 and DENV-4 since the presence of neutralization antibodies were detected by PRNT to both DENV-1 and DENV-4. There was no difference in response between those receiving candidate vaccines in separate arms and among those receiving mixed vaccine in another arm.

The bivalent DENV-1 and DENV-4 candidate vaccine induced specific response to both DENV-1 and DENV-4 but low titres of heterologous neutralizing antibody were found and the vaccine was without any adverse reactions among the recipients[25].

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-2 (16681) PDK 53 clinical trial

Seven male subjects aged between 17 and 32 years received bivalent DENV-1 and DENV-2 candidate vaccine.

All subjects seroconverted to DENV-1 and DENV-2 by 30 days. Titres of DENV-1 neutralizing antibody ranged between 1:27 and 1:70 in the six subjects who were non-immune before vaccination. Titres of DENV-2 ranged from 1:26 to 1:120 and at 30 days no cross-reaction with other flaviviruses was observed.

The bivalent DENV-1 and DENV-2 candidate vaccine was both immunogenic and without any adverse reactions[25].
WHO’s efforts for the development of a dengue vaccine

**Trivalent vaccine DENV-1 (16007)**
PDK 13, DENV-2 (16681) PDK 53 and DENV-4 (1036) PDK 48 clinical trial[21]

The human clinical trial comprised of a mixture of three monovalent DENV vaccines (DENV-1, -2 and -4) which was inoculated into each of 12 male adults. The study was performed in a subdistrict of Loei province in north-east Thailand, where the prevalence of *Ae. aegypti* or *Ae. albopictus* mosquitoes was low. The objective of this study was to determine the safety and feasibility of simultaneous administration of three DENV candidate vaccines.

Of the 12 recipients, nine were flavivirus non-immune; they all developed serum neutralizing antibodies to all the three DENV viruses.

The results of this study showed that it was possible to infect humans safely with three attenuated DENV viruses. The median dose of DENV-1 virus was close to optimum whereas the dose of DENV-4 was too low. The successful administration of a trivalent DENV vaccine indicated that Mahidol University had achieved another important milestone on the road to the development of a tetravalent dengue vaccine[21].

**Trials of tetravalent vaccine in children aged 5–12 years[22]**

The tetravalent DENV vaccine candidate appeared to be safe when administered to children aged 5–12 years. Children became just febrile, and this usually did not last for more than a day. One volunteer had a rash that persisted for three days.

Two trends of serological response to the tetravalent vaccine were observed among the volunteers. First, the infectious dose that was calculated for adults was not equivalent for the children in the age groups studied. A trend of an increasing rate of seroconversion among children was noted with a decreasing vaccine dosage; however, an optimum dose for children 5–12-years-old still had to be determined.

Second, children with preexisting antibodies to either DENV or JEV appeared to respond better to the tetravalent vaccine than did children who were completely non-immune. Even so, not all volunteers with preexisting flavivirus antibodies responded to all four DENV serotypes.

**Collaboration with manufacturer[23]**

The Mahidol vaccine was licensed to Pasteur Mérieux (now Sanofi Pasteur) in France for large-scale production under Good Manufacturing Practice (GMP) conditions.

The master seed, the production seed and the candidate DENV vaccines were sent to Pasteur Merieux in February 1993, shortly after the agreement was signed in January 1993. Three technical meetings at Pasteur Merieux were held in Lyon, France, between 1994–1996. Industrial production of the four monovalent vaccines was achieved by 1995. All biological studies, including monkey neurovirulence studies were repeated.

The first clinical trial carried out using the Mahidol/PMsv tetravalent vaccine in US volunteers suggested that the combination of four attenuated strains appeared to result in increased reactogenicity and diminished tolerability. Antibody responses were predominantly directed against DENV-3 with low or undetectable titres against the remaining three serotypes[23]. This outcome appeared to be the result of preferential replication of
DENV-3 in the tetravalent vaccine. The mechanism of such viral interference was not known. But it had been suggested that the ratio of the four attenuated viruses in the tetravalent formulation may be an important factor. A subsequent clinical study in Thailand\cite{24} showed that varying and reducing the concentrations of the DENV-3 strain resulted in an improved clinical safety profile of the tetravalent vaccine. About 71% seroconversion (against all 4 serotypes) was observed after a two-dose vaccination schedule in this study. Several different reformulations of the tetravalent vaccine were being evaluated in order to provide a more balanced immune response to each serotype\cite{23}.

Second generation recombinant vaccines

A Cooperative Research and Development Agreement (CRADA) was entered into with the Division of Vector-Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado, USA, to develop the second-generation recombinant vaccines using complementary DNA (cDNA) technology. As per the memorandum of understanding (MoU), the first shipment of candidate DENV vaccines (DENV-1 and DENV-2) was sent to CDC in August 1994. The DENV-3 and DENV-4 vaccines were sent shortly thereafter. The agreement called for Mahidol University to provide support for one locally-trained technician and for CDC to provide support to one Thai investigator engaged in research and capacity-building activities at Fort Collins. Vaccine development studies were realized at CDC while biological marker testing was partially done in Thailand.

Conclusion on present status of PDK-based live-attenuated dengue vaccine

The importance of DENV vaccine development was imperative in order to improve public health throughout the world and was highly desirable for WHO to provide financial support for this programme. The peer group summarized the progress as follows:

(1) Monovalent candidate vaccines
   (a) DENV-1: A usable candidate vaccine
   (b) DENV-2: A near-perfect candidate vaccine
   (c) DENV-3: The most recently developed candidate vaccine, somewhat more reactogenic than the other candidate vaccines. A search for a better vaccine should proceed.
   (d) DENV-4: A very good product.

(2) Bivalent and trivalent combinations using DENV-1 PDK 13, DENV-2 PDK 53 and DENV-4 PDK 48 had undergone phase 1 trials in adults with satisfactory results.

(3) Tetravalent vaccine was acceptably safe. Interference was noticed after mixing of the DENV-3 PGMK-30/F3 in the combination.

Lessons learned

There was a general consensus that vaccination can be one of the most cost-effective ways to prevent DF and DHF. The aim of this project was to develop a safe and immunogenic
vaccine against the four DENV serotypes. Each of the four monovalent vaccines as well as the bivalent and the trivalent vaccines were developed and tested step by step in the laboratory and in human volunteers. By 1992, the attenuated, tetravalent vaccine was being tested for immunogenicity and safety in human volunteers. Formal phase 1 and phase 2 clinical trials had proven the vaccines to be both safe and immunogenic in humans. Human trials of the tetravalent vaccine were successfully concluded.

In November 1992, WHO headquarters and WHO/SEARO announced the attainment of the objective of the dengue vaccine development project at Mahidol as follows: “Vaccine for Dengue Haemorrhagic Fever”.

From this study, it was proved that PDK cells could be used successfully for attenuation attempts. The DENV-2 PDK 53, which was one of the important outcomes of this study, has been further used as a backbone to construct live molecular DENV vaccines in the USA.

Research as well as relevant capability-building activities at Mahidol University were established with the advice of the international peer group which met annually. However, the initial expectation in 1985 that DENV vaccine development would be completed within three years proved too optimistic.

Considerable research capacity building took place as part of the research project support during that decade. The various technologies required for vaccine development and laboratory-scale production were transferred. They included continuous tissue culture, development of PDK and other cell lines, monkey tests for neurovirulene, etc. The annual meetings of the peer group itself provided valuable scientific advice to the project. In addition, Mahidol University scientists were supported for visits and contacts with various scientists and institutions in other countries.

Meanwhile, Mahidol University expanded the physical and other infrastructure required for vaccine development and pilot scale up. A vaccine development centre building and a laboratory animal centre were completed at the new Salaya campus. Equipment for upscaling was received as donation from the Italian Government.

The DENV vaccine development project was acknowledged to be a worthy scientific achievement in the area of health. Such achievements could occur due to the long-term commitment of scientists in Thailand, the continuous support of the Government of Thailand, and the initial impetus and sustained commitment and support provided by WHO/SEARO. The Government of Thailand and Mahidol University provided the major resources. WHO provided about US$ 2.5 million during a period of 15 years. Other donors contributed substantial amounts at various stages of the project for specific components of the programme. Success was due to scientific correctness of the research, the outstanding leadership of late Prof. N. Bhamarapravati of Mahidol University, sound research management by several parties, and the sustained commitment and technical support through the years of the WHO Regional Office for South-East Asia.

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References


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Socioeconomic determinants of dengue incidence in Singapore

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Abstract
Community participation is critical in sustaining vector population control activities in order to prevent dengue transmission. However, disease exposure in a community is often not uniform across the entire population and the identification of “at-risk” groups would enable the disease prevention effort to be focused and thus cost-effective. We performed ecological data analyses to study the association between socioeconomic variables and dengue incidence in Singapore from 1998 to 2002. Our results indicated that the DF/DHF incidence was ecologically associated with some socioeconomic/demographic characteristics of the population. Areas with a high proportion of socioeconomically disadvantaged residents had also a significantly higher DF/DHF incidence. The *Aedes* population density of larvae was not related to this difference in the DF/DHF incidence, indicating that additional risk factors were present in these population sub-groups, and that dengue control in Singapore could benefit from a more focused effort in outreach to the population of relatively lower socioeconomic levels.

Keywords: Dengue; Socioeconomic; Geographical; Singapore.

Introduction
Dengue fever/dengue haemorrhagic fever (DF/DHF) remains a major health problem in many areas of the world, especially in south-east Asia[1–3]. Much effort has been focused on the prevention and control of dengue infection. The only effective strategy to control a DF/DHF outbreak, in the absence of a vaccine, is to eliminate *Aedes* mosquitoes and its larval breeding habitats[4].

In Singapore, DHF was first recognized as a public health problem during the early 1960s and a nationwide *Aedes* control programme, which incorporated source reduction, public education and law enforcement, was implemented in 1969. The *Aedes* house index (HI) (% of premises positive for *Aedes* breeding) was markedly reduced from more than 25% before 1970 to 1–2% for the entire country since 1982. The significant decline of the DF/DHF incidence was ecologically associated with some socioeconomic/demographic characteristics of the population. Areas with a high proportion of socioeconomically disadvantaged residents had also a significantly higher DF/DHF incidence. The *Aedes* population density of larvae was not related to this difference in the DF/DHF incidence, indicating that additional risk factors were present in these population sub-groups, and that dengue control in Singapore could benefit from a more focused effort in outreach to the population of relatively lower socioeconomic levels.

Keywords: Dengue; Socioeconomic; Geographical; Singapore.
DHF incidence (about 60 per 100 000 population in 1973 to below 10 per 100 000 in 1982) was the result of an effective Aedes surveillance and control programme\[5\]. Although the Aedes mosquito population density has been reduced and maintained to a relatively low level, as indicated by the overall house index, there was a progressive resurgence of DF (but proportionately less DHF) with a periodicity of about 5 to 6 years from 1992 onwards\[6-8\].

In the last two decades, several studies have investigated the risk factors for DF/DHF in affected communities, including those with poor living conditions, social inequalities and illiteracy\[3\]. Identified DF/DHF risk factors vary greatly depending on the location, population density, previous exposure to specific serotypes and availability of oviposition sites. Seasonal distribution has also been reported with the Aedes aegypti population density and DF/DHF incidence being associated with elevated temperature and rainfall in certain regions\[9\]. However, not much is known about how socioeconomic or demographic variables could influence the occurrence of DF/DHF in urban centres, such as Singapore. This may have particular importance since DF/DHF outbreaks are likely to initiate from urban centres\[10,11\]. Geographical correlation analysis may help to answer this question so as to shed some light on providing various perspectives for public health policy-makers when designing control measures for DF/DHF.

The aim of this study was to examine whether or not there was any correlation between socioeconomic/demographic variables and DF/DHF incidence by geographical areas using the Development Guide Plan (DGP) zones in Singapore. Our findings have direct and immediate implications for dengue prevention.

**Materials and methods**

**Units of analysis**

The analyses were based on the geographical units, namely, Development Guide Plan areas. The DGP is a detailed urban plan used for each of the 55 planning areas in Singapore designated by the Urban Redevelopment Authority of Singapore, the nation’s planning and conservation authority. Each DGP covers a planning area with a population of around 150 000 served by a town centre. In order to obtain stability and reduce sampling variability of disease incidence, 4 DGP areas with less than 10 000 persons (ranging from 1085 to 9403 persons) have been merged with the adjacent zones in our analysis. We also excluded 23 DGP zones from our analyses that are composed of rural areas with very small population size in which no census enumeration was conducted. In addition, only a few cases of dengue (ranging from 0 to 11 cases per year) were notified from these 23 excluded DGP zones during the study period. Hence, only 28 of the DGP groups with enumerated population denominators were included in the final analyses.

**Population census**

The socioeconomic/demographic (SED) variables by DGP were extracted from the Singapore Population Census 2000 reports\[12\] and are described in Table 1. The proportions with individual SED characteristics computed for each DGP group were used as SED variables for the analysis.

**Data analyses**

The residential address of each notified case of DF/DHF was first geo-coded into 28 DGP groups
Table 1: Spearman’s rank correlation coefficient (r) between dengue incidence and proportions with individual socioeconomic/demographic characteristics (SED1-18), and average densities of *Aedes aegypti* and *Aedes albopictus* (%) (AED1-2)

<table>
<thead>
<tr>
<th>Socioeconomic/demographic variable (denominator)</th>
<th>r</th>
<th>(p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SED1: Landed properties* and others (resident population)</td>
<td>0.645</td>
<td>(0.001)</td>
</tr>
<tr>
<td>SED2: Services production industries (working residents aged 15 years and over)</td>
<td>0.592</td>
<td>(0.002)</td>
</tr>
<tr>
<td>SED3: ‘Other’ ethnic group (resident population)</td>
<td>0.560</td>
<td>(0.004)</td>
</tr>
<tr>
<td>SED4: Aged 65 years and above (resident population)</td>
<td>0.559</td>
<td>(0.004)</td>
</tr>
<tr>
<td>SED5: Non-owner (resident private households)</td>
<td>0.533</td>
<td>(0.007)</td>
</tr>
<tr>
<td>SED6: Financial &amp; business services industries (working residents aged 15 years and over)</td>
<td>0.500</td>
<td>(0.010)</td>
</tr>
<tr>
<td>SED7: Widowed female (resident population aged 15 years and over)</td>
<td>0.475</td>
<td>(0.014)</td>
</tr>
<tr>
<td>SED8: Economically inactive (resident population aged 15 years and over)</td>
<td>0.464</td>
<td>(0.016)</td>
</tr>
<tr>
<td>SED9: Attending upper secondary education (resident students aged 5 years and over)</td>
<td>0.453</td>
<td>(0.019)</td>
</tr>
<tr>
<td>SED10: Monthly gross income from work below SGD1000 (resident private households)</td>
<td>0.430</td>
<td>(0.026)</td>
</tr>
<tr>
<td>SED11: Female Chinese (resident population)</td>
<td>0.429</td>
<td>(0.026)</td>
</tr>
<tr>
<td>SED12: No family nucleus** (resident private households)</td>
<td>0.427</td>
<td>(0.027)</td>
</tr>
<tr>
<td>SED13: Female living alone (resident population aged 15 years and over)</td>
<td>0.403</td>
<td>(0.037)</td>
</tr>
<tr>
<td>AED1: Average density of Ae. aegypti (%)</td>
<td>0.395</td>
<td>(0.040)</td>
</tr>
<tr>
<td>SED14: Household size: 8 or above (resident private households)</td>
<td>0.394</td>
<td>(0.041)</td>
</tr>
<tr>
<td>SED15: Household size: 1 (resident private households)</td>
<td>0.394</td>
<td>(0.041)</td>
</tr>
<tr>
<td>SED16: Attending university education (resident students aged 5 years and over)</td>
<td>0.365</td>
<td>(0.058)</td>
</tr>
<tr>
<td>AED2: Average density of Ae. albopictus (%)</td>
<td>0.273</td>
<td>(0.156)</td>
</tr>
<tr>
<td>SED17: Indian (resident population)</td>
<td>0.260</td>
<td>(0.177)</td>
</tr>
<tr>
<td>SED18: Workers: agriculture &amp; fishery, craftsmen, etc.</td>
<td>-0.450</td>
<td>(0.019)</td>
</tr>
</tbody>
</table>

* Refers to residents who are living at residential unit with individual ground contact which do not include multi-level apartment buildings. Types of landed properties include bungalow/detached house, linked house, semi-detached, terrace house, town house, and cluster housing, etc. For the latter housing type, it is a hybrid between conventional landed housing and condominium housing and all these units have ground contact but with shared facilities similar to those found in condominiums. Most of these landed property buildings usually are less than 4 floors per building.

** Refers to a household formed by a person living alone or living with others but which does not constitute any family nucleus. Thus it can refer to individuals, but not necessarily to people living alone.
using ArcView software version 9.1 (ESRI, Redlands, CA). Crude DGP-specific DF/DHF annual incidence was calculated based on the total number of cases reported from 1998 to 2002 inclusive, with the person-years denominator being the sum of the annual population estimates for each of the years. The population estimates were obtained by interpolating or extrapolating linear trend of each DGP zone from the Population Census 1990 to the Population Census 2000 to account for population changes over the study period.

Three levels of data analysis, namely, correlation analysis, factor analysis and linear regression analysis, were conducted in this study.

Firstly, the possible geographical correlation between the crude DF/DHF incidence (on log scale) and each SED variable was assessed by using the Spearman’s rank correlation coefficient.

Secondly, the potential SED variables identified by the correlation analysis were then summarized into factor scores obtained by using the exploratory factor approach, which examine how underlying constructs influence the response on a number of measured variables\(^{13}\). In the factor analysis, the maximum likelihood estimation was used to determine the number of factors to retain, followed by orthogonal (varimax) rotation to assist in the interpretation of the factors and to ensure that the factors were uncorrelated. SED variables with rotated factor loadings (equivalent to Pearson’s correlation coefficients between each variable and each factor) having absolute values of 0.6 or greater were used in interpreting the factors and considered “dominant” as the defining SED variables for the identification of specific factors\(^{13}\). Scores were computed for rotated factors as the sum of products of observed variables multiplied by their factor loading.

Thirdly, six weighted linear regression models were then used to study the associations between each of the respective factor score-based variables and the DGP group-specific DF/DHF incidence, with or without adjustment for mosquito indices, as a sensitivity analysis (namely, models 1–6 in Figure 1). This analysis allowed for the investigation of the association between the socioeconomic influence and DF/DHF incidence because factor score-based variables minimized the multi-collinearity problem present in conventional regression analyses. It, therefore, allowed us to obtain a stable estimation. Log-transformation of the DGP group-specific DF/DHF incidence was taken as a dependent variable. The regression was weighted by the DGP group’s population size.

The average densities of the two Aedes mosquitoes were defined as the total number of Ae. aegypti and Ae. albopictus larvae observed, divided by the number of premises where larval breeding was found, respectively. These two mosquito variables were also subsequently included together with 2-factors score-based variables in the weighted linear regression analyses.

All analyses were performed with S-Plus software version 6.0 (Insightful Corporation, Seattle, Washington) and Stata software version 8.0 (Stata Corporation, College Station, TX, USA).

Results

Geographical variations in DF/DHF incidence

During the study period (1998–2002), there were 16.4 million person-years of observation with 11 888 reported cases of DF/DHF (after excluding 210 cases that occurred in the non-
sampled DGP zones). Figure 2 shows the incidence of DF/DHF in each of the 28 DGP groups studied in Singapore. The overall incidence of DF/DHF was 72.7 per 100 000 person-years. The incidence of DF/DHF ranged from 18.8 to 271.2 per 100 000 and all the incidence rates were significantly different (p<0.01) from the DGP average, except for two DGP groups (DGP groups 16 and 17) (Figure 2).

**Association with socioeconomic/demographic factors**

From the pair-wise ecological correlation analyses (Table 1), the following variables were most significantly associated (p<0.01) with the incidence of DF/DHF: landed properties and others (r=0.65); services production industries (0.59); and ‘other’ ethnic group (0.56); aged 65+ (0.56); and non-owner tenancy (0.53).
In the factor analysis, two factors were extracted based on 16 SED variables that could explain 80% of the total variations (Table 2). The first factor with the first six greatest in absolute value of factor loadings were considered “dominant”: low gross monthly income from work (<Singapore dollars, SGD1000), no family nucleus, aged 65+, living alone, female widowed and economically inactive. This is consistent with “retired elderly”. Likewise, for the second factor identified had: workers in the business industries, ‘other’ ethnic group (minority living in Singapore), household size 8 or above, landed property residents, and attending upper secondary school as dominant variables (Table 2). This appears to be consistent with “middle-class adults”.

These 2-factors score-based variables were then included in the weighted linear regression analysis. The two indices for the average density of \textit{Aedes} mosquitoes were also included in the regression analysis for further adjustment. Four variables together could explain 32% of the variations ($R^2=0.32$) in the regression model (model 6 in Table 3). However, only scores in the first factor consistent with “retired elderly” remained significantly and positively associated ($p=0.022$) with DF/DHF incidence.

**Discussion**

Singapore is a modern and highly urbanized tropical island city state with one of the highest urban population densities in the world (6004 residents per square kilometre). In this study, substantial geographical variations in the incidence of DF/DHF were observed, and these variations have been shown to be associated with differences in the socioeconomic/demographic characteristics of the population.
Exploratory factor analysis is a data-reduction statistical method that has been widely used to identify and summarize many inter-relationships that exist among individual variables. In a factor analysis used in this study (schematic diagram shown in Figure 3), inter-correlated variables (variables SED1-16) are grouped into smaller numbers of new variables (2 factors). Such an approach allows for the simplification of the data set analysed in order to gain practically relevant insights into the underlying risk factors and true exposures that are linked to adverse health effects.

Our study had several methodological limitations. Firstly, the actual extent of dengue infection is probably an underestimate since it is based on the notified cases. However, the

### Table 2: Results of factor analysis estimated using maximum likelihood estimation: rotated factors and factor loadings

<table>
<thead>
<tr>
<th>Socioeconomic/demographic variable (denominator)</th>
<th>Factor 1 (Retired elderly)</th>
<th>Factor 2 (Middle-class adults)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SED1: Landed properties and others (resident population)</td>
<td>0.826</td>
<td></td>
</tr>
<tr>
<td>SED2: Services production industries (working residents aged 15 years and over)</td>
<td>0.724</td>
<td></td>
</tr>
<tr>
<td>SED3: 'Other' ethnic group (resident population)</td>
<td>0.938</td>
<td></td>
</tr>
<tr>
<td>SED4: Aged 65 years and above (resident population)</td>
<td>0.962</td>
<td></td>
</tr>
<tr>
<td>SED5: Non-owner (resident private households)</td>
<td>0.906</td>
<td></td>
</tr>
<tr>
<td>SED6: Financial &amp; business services industries (working residents aged 15 years and over)</td>
<td>0.956</td>
<td></td>
</tr>
<tr>
<td>SED7: Widowed female (resident population aged 15 years and over)</td>
<td>0.945</td>
<td></td>
</tr>
<tr>
<td>SED8: Economically inactive (resident population aged 15 years and over)</td>
<td>0.904</td>
<td></td>
</tr>
<tr>
<td>SED9: Attending upper secondary education (resident students aged 5 years and over)</td>
<td>0.747</td>
<td></td>
</tr>
<tr>
<td>SED10: Monthly gross income from work below SGD1000 (resident private households)</td>
<td>0.995</td>
<td></td>
</tr>
<tr>
<td>SED11: Female Chinese (resident population)</td>
<td>0.622</td>
<td></td>
</tr>
<tr>
<td>SED12: No family nucleus (resident private households)</td>
<td>0.963</td>
<td></td>
</tr>
<tr>
<td>SED13: Female living alone (resident population aged 15 years and over)</td>
<td>0.784</td>
<td></td>
</tr>
<tr>
<td>SED14: Household size: 8 or above (resident private households)</td>
<td>0.877</td>
<td></td>
</tr>
<tr>
<td>SED15: Household size: 1 (resident private households)</td>
<td>0.938</td>
<td></td>
</tr>
<tr>
<td>SED16: Attending university education (resident students aged 5 years and over)</td>
<td>0.709</td>
<td></td>
</tr>
<tr>
<td>Percentage total variance</td>
<td>46.3%</td>
<td>34.1%</td>
</tr>
<tr>
<td>Percentage cumulative variance</td>
<td>46.3%</td>
<td>80.4%</td>
</tr>
</tbody>
</table>

Data are factor loadings, the correlation between the individual variable and each factor. Only variables with loadings ≥ ±0.60 are shown. SED17 and SED18 are not shown because their loadings < ±0.60.
Table 3: Summary of multiple linear regression analysis weighted by the DGP group’s population size

<table>
<thead>
<tr>
<th>Model</th>
<th>Variable(s) included in the regression analysis</th>
<th>R²‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Factor 1* (Retired elderly)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Factor 2* (Middle-class adults)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average density of Ae. aegypti (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average density of Ae. albopictus (%)</td>
<td></td>
</tr>
<tr>
<td>Model 1: DF/DHF incidence = Scores of Factor 1</td>
<td>0.391 (0.015)</td>
<td>–</td>
</tr>
<tr>
<td>Model 2: DF/DHF incidence = Scores of Factor 2</td>
<td>–</td>
<td>0.313 (0.145)</td>
</tr>
<tr>
<td>Model 3: DF/DHF incidence = Av. density of Ae. aegypti %</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Model 4: DF/DHF incidence = Av. density of Ae. albopictus %</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Model 5: DF/DHF incidence = Scores of Factor 1 + Scores of Factor 2</td>
<td>0.383 (0.015)</td>
<td>0.293 (0.132)</td>
</tr>
<tr>
<td>Model 6: DF/DHF incidence = Scores of Factor 1 + Scores of Factor 2 + Av. density of Ae. aegypti % + Av. density of Ae. albopictus %</td>
<td>0.399 (0.022)</td>
<td>0.092 (0.724)</td>
</tr>
</tbody>
</table>

* Scores of Factor 1 and Factor 2 obtained from factor analysis and socioeconomic variables contributed to each factor referred to in Table 2.
† Regression coefficient estimated from weighted linear regression.
‡ R-squared interpreted as percentage of variation explained by respective model.

A local study showed that these meteorological conditions preceded the DF/DHF incidence by 8–20 weeks[18]. While there may be differences in such environmental factors in different parts of Singapore, these may not be significant given the very small geographical size of the island. Furthermore, as the DF/DHF incidence was aggregated over a 5-year period in our analysis, the short- and medium-term fluctuations of meteorological conditions are less likely to have an impact on our analysis.

Thirdly, the association between socioeconomic/demographic factors and DF/DHF incidence was assessed based on ecological data. Therefore, as with any ecological analysis, interpretation of these findings must be done with caution. Individual-
Based studies are needed to validate the hypothesis generated from these findings[19].

In a cancer study conducted in Thailand, the socio-demographic characteristics that influenced the decision-making of the patient’s caretaker to receive alternative therapy included the level of education, occupation, residential areas and lay symptom assessment[20]. For the economic factors, the capability to reimburse the cost of treatment, the family income and the financial resources were also important[20]. In our study, the DF/DHF incidence was ecologically associated with some socioeconomic/demographic characteristics of the population, such as those with low income (economically inactive; Spearman’s r=0.46 or monthly gross income from work below SGD1000; 0.43), living alone (household size 1; 0.39), gender (female living alone; 0.40 or widowed female; 0.48), and less attention paid to environmental hygiene (aged 65+; 0.56, no family nucleus; 0.43, household size 1; 0.39, and widowed female; 0.48), a group we have collectively referred to as “retired elderly”. In such a population group, the Aedes larval breeding sites in the domestic and peri-domestic environment could increase due to poor hygiene[21] and failure to check for breeding and reluctance to have their homes fogged with
insecticide\textsuperscript{22}. In addition, non-owner tenancy householders (r=0.53) could be less responsible in cleaning up their premises. Living in landed properties was also associated with a higher DF/DHF incidence (r=0.65) as it has been consistently observed that there are more breeding habitats in these premises\textsuperscript{5}.

We found that areas with a high proportion of socioeconomically disadvantaged residents have significantly higher incidence rates of DF/DHF. \textit{Ae. aegypti} and \textit{Ae. albopictus} population densities, taken individually without the inclusion of other factors, are insufficient to account for the observed difference in the DF/DHF incidence rates (Models 3 and 4 in Table 3). It is interesting to note that in the same multivariable regression analysis, the regression coefficient of the average density of \textit{Ae. albopictus} on DF/DHF incidence was significantly reduced (regression coefficients: 0.035 in Model 4 vs –0.007 in Model 6), but the \textit{Ae. aegypti} variable had less reduction although both variables did not reach statistical significance (regression coefficients: 0.041 in Model 3 vs 0.036 in Model 6, Table 3). This suggests that \textit{Ae. aegypti} remains the principal vector for dengue virus transmission, despite the greater abundance of \textit{Ae. albopictus}.

Because this finding is based on ecological data, we cannot conclude that persons from poor families have a higher risk of DF/DHF without further prospective studies. However, it is consistent with the hypothesis that susceptibility to infection is associated with low socioeconomic status\textsuperscript{23}. The higher DF/DHF incidence in the socioeconomically disadvantaged residents could also likely be due to socio-behavioral barriers in seeking health care\textsuperscript{24,25} or some other behavioural or environmental processes operating at household or individual levels that supported breeding of \textit{Aedes} mosquitoes (e.g., monthly gross income from work below SGD1000 was correlated with average densities of \textit{Ae. aegypti}, r=0.703, data not shown) and transmission of dengue viruses\textsuperscript{26}.

Until a safe and effective vaccine is available, controlling \textit{Aedes} mosquitoes, active laboratory-based case as well as entomological surveillance\textsuperscript{27,28}, and improved case management are the principal options available for reducing the burden of DF/DHF in Singapore. More cost-effective integrated control measures such as public health educational campaign targeting ‘hot-spot’ areas, in which both DF/DHF incidence and factor scores are high, could be a logical approach to minimize the impact of the disease\textsuperscript{26-30}. (The ‘high-high’ areas defined as the first one-third of the DGP groups with high DF/DHF incidence accounted for 28.3% of the total annual cases but only 10.4% of the total population size, and the first one-third of the factor scores (data not shown)).

In conclusion, the results of this study suggest that dengue control in Singapore could benefit with a more focused effort in outreach to the population in relatively lower socioeconomic regions. Further efforts should be directed at addressing the barriers to behavioural change, correcting misconception on the spread of dengue by social and close contact, and educating them and the illiterate on measures to prevent dengue\textsuperscript{22}.

Acknowledgements

We thank Ms J.K.Y. Wong, Ms E. Loh, Mr C.T. Heng and Ms P.Y. Soh of the National Environmental Agency for their assistance in geo-coding of data to the DGP zones. We also thank Ms L. Kurupatham and Dr P.L. Ooi of the Communicable Diseases Division and Dr Y.B. Cheung for their helpful comments on an early version of the manuscript.
References


Forecasting dengue incidence in Dhaka, Bangladesh: A time series analysis

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c Department of Agricultural Statistics, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
d Southamton Statistical Sciences Research Institute, University of Southampton, UK

Abstract

This article attempts to model the monthly number of dengue fever (DF) cases in Dhaka, Bangladesh, and forecast the dengue incidence using time series analysis. Seasonal Autoregressive Integrated Moving Average (SARIMA) models have been developed on the monthly data collected from January 2000 to October 2007 and validated using the data from September 2006 to October 2007. The results showed that the predicted values were consistent with the upturns and downturns of the observed series. The SARIMA (1,0,0)(1,1,1)_{12} model has been found as the most suitable model with least Normalized Bayesian Information Criteria (BIC) of 11.918 and Mean Absolute Percent Error (MAPE) of 595.346. The model was further validated by Ljung-Box test (Q_{18}=15.266 and p>.10) with no significant autocorrelation between residuals at different lag times. Finally, a forecast for the period November 2007 to December 2008 was made, which showed a pick in the incidence of DF during July 2008, with estimated cases as 689.

Keywords: Dengue; Time series analysis; SARIMA; Disease prediction; Dhaka, Bangladesh.

Introduction

Dengue is one of the most important emerging viral diseases of major public health concern in Bangladesh. The disease is transmitted through the bite of the Aedes aegypti and Ae. albopictus mosquitoes[1]. It causes a broad spectrum of clinical manifestations in humans ranging from the acute febrile illness, dengue fever (DF), to the life-threatening dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS)[2].

Dengue was first reported as “Dacca fever” in Bangladesh in 1964 by Aziz and his colleagues[3]. Subsequent reports suggested that
dengue fever may have been occurring sporadically in Bangladesh from 1964 to 1999\cite{3-9}. The first epidemic of dengue was reported in the capital city, Dhaka in the year 2000\cite{10,11}. Since then the disease has shown an annual occurrence in all major cities of the country. During January 2000–December 2007, Bangladesh recorded a total of 22 245 cases and 233 deaths (1.04%). Of these, Dhaka accounted for 20 115 cases and 181 deaths (0.9%).

In the absence of a vaccine and specific treatment available for dengue, vector control remains the only option. Early warning about the disease based on forecasting, therefore, becomes crucial for the prevention and control of dengue in Bangladesh. The time series analyses methodology has been increasingly used in the field of epidemiological research on infectious diseases, particularly in the assessment of health services\cite{12-16}. In health science research, Autoregressive Integrated Moving Average (ARIMA) models\cite{12-18} as well as Seasonal Autoregressive Integrated Moving Average (SARIMA)\cite{19-20} models are useful tools for analysing time series data containing ordinary or seasonal trends to develop a predictive forecasting model. There have been efforts in forecasting dengue incidence in different parts of the world using both ARIMA\cite{21,22} and SARIMA\cite{19} modelling. This study is aimed at developing univariate time series models to forecast the monthly dengue incidence in Dhaka based on reported monthly cases available from 2000–2007. This forecasting offers the potential for improved and consistent planning of public health interventions.

**Materials and methods**

**Study area**

Dhaka is the capital and principal city of Bangladesh located at 23° 42’ 0” N, 90° 22’ 30” E, covering an area of 815.85 km² (315 sq miles). According to the World Gazetteer (2006), the population in the Dhaka region was 11 million and the density was 14 608/km² (37 834.5/sq mile) making it the largest city in Bangladesh and the eleventh most populous city in the world\cite{23}. The Dhaka region was chosen as the study area because of its relatively high incidence of DF between 2000 and 2007 (average annual incidence: 2515.75 cases).

**Data collection**

We obtained computerized data sets of notifications of monthly DF cases in the Dhaka region for the period 1 January 2000 through 31 October 2007 from the Directorate-General of Health Services, Mohakhali, Dhaka-1212\cite{24}. It may be noted that the Directorate-General of Health Services collects information on dengue cases separately as DF, DHF and DSS but clubs this data as data for DF only.

**Data analysis**

A SARIMA \((p,d,q)(P,D,Q)_s\) model\cite{25} was fitted, where \(p\) is the order of autoregression, \(d\) is the order of integration, \(q\) is the order of moving average, \(P\) is the order of seasonal autoregression, \(D\) is the order of seasonal integration, \(Q\) is the order of seasonal moving average and \(s\) is the length of seasonal period. The analyses were performed using SPSS 17 software. The stationarity of the series was made by means of seasonal and non-seasonal differencing\cite{25}. Then the order of autoregression and moving average were identified using autocorrelation function (ACF) and partial autocorrelation function (PACF) of the differenced series. A model was fitted with a training set of data from January 2000 to October 2007 and the fitted model was used to predict values for a validation period (from...
September 2006 to October 2007) to evaluate the time series model. Most suitable models were selected on the basis of their ability of reliable prediction. Two measures, namely, Normalised Bayesian Information Criteria (BIC)\textsuperscript{[26]} and Mean Absolute Percent Error (MAPE)\textsuperscript{[25]}, were used. Lower values of Normalised BIC and MAPE were preferable. Furthermore, Ljung-Box test (portmanteau test) was performed to test if the residual ACF at different lag times was significantly different from zero, where not being different from zero was expected\textsuperscript{[27]}. After the best model was identified, forecast for future values from November 2007 to December 2008 was made.

**Results and discussion**

The observed series of DF (January 2000 to October 2007) shows that the series is non-stationary and there are seasonal fluctuations in the dataset (Figure 1). ACF and PACF of one seasonal differenced series (Figure 2a, Figure 2b) as well as of one seasonal with one non-seasonal differenced series (Figure 2c, Figure 2d) instigated to explore a set of models based on the training set of data (January 2000 to October 2007), which are listed in Table 1. Among these models, SARIMA(1,0,0)(1,1,1)\textsubscript{12} has both lowest normalised BIC (11.918) and MAPE (595.346) values and appeared to be the best model. Moreover, the Ljung-Box test suggested that the ACF of residuals for the model at different lag times was not significantly different from zero ($Q_{18}=15.266$ and $p>.10$). All the coefficients of SARIMA (1,0,0)(1,1,1)\textsubscript{12} model were significant (Table 2). The model has been used to predict values from September 2006 to October 2007 (Figures 3 and 4) for validation. It appeared that the predicted values could follow the upturn and downturn of the observed series reasonably well. Finally, Figure 5 represents the forecast values for the period from November 2007 to December 2008, which indicates a seasonal pick in July 2008, with the estimated number of patients as 689 and a sharp decrease from September 2008. The predicted values as well as the forecast values show some negative values, which is a common case with a series with too many zeros as observed values in the series.

**Figure 1:** Observed dengue fever from January 2000 to October 2007

![Graph showing observed dengue fever from January 2000 to October 2007]
Forecasting dengue incidence in Dhaka, Bangladesh: A time series analysis

Table 1: MAPE and normalized BIC of Time Series Models

<table>
<thead>
<tr>
<th>Models</th>
<th>MAPE</th>
<th>Normalized BIC</th>
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<tr>
<td>SARIMA(2,1,1)(1,1,0)_{12}</td>
<td>1026.050</td>
<td>12.072</td>
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<tr>
<td>SARIMA(2,1,0)(1,1,0)_{12}</td>
<td>766.310</td>
<td>12.215</td>
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<td>SARIMA(1,1,1)(1,1,0)_{12}</td>
<td>945.640</td>
<td>12.052</td>
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<td>SARIMA(0,1,0)(1,1,0)_{12}</td>
<td>805.376</td>
<td>12.236</td>
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<td>791.408</td>
<td>12.269</td>
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<td>SARIMA(1,1,1)(1,1,1)_{12}</td>
<td>947.663</td>
<td>12.059</td>
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<td>SARIMA(1,0,1)(1,1,1)_{12}</td>
<td>600.730</td>
<td>11.952</td>
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<td>SARIMA(1,0,0)(1,1,1)_{12}</td>
<td>595.346</td>
<td>11.918</td>
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</table>

Table 2: Model parameters of SARIMA (1,0,0)(1,1,1)_{12}

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$</th>
<th>SE</th>
<th>$p$-value</th>
</tr>
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<tbody>
<tr>
<td>AR (Lag 1)</td>
<td>0.385</td>
<td>0.106</td>
<td>0.000</td>
</tr>
<tr>
<td>AR, Seasonal (Lag 1)</td>
<td>-0.587</td>
<td>0.109</td>
<td>0.000</td>
</tr>
<tr>
<td>Seasonal Difference</td>
<td>1.000</td>
<td></td>
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<td>MA, Seasonal (Lag 1)</td>
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<td>0.009</td>
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Figure 2a: ACF of transformed series with seasonal difference (1, period 12)
**Figure 2b:** PACF of transformed series with seasonal difference (1, period 12)

![Partial Autocorrelation Function (PACF)](image)

**Figure 2c:** ACF of transformed series with non-seasonal difference (1) and seasonal difference (1, period 12)

![Autocorrelation Function (ACF)](image)
Forecasting dengue incidence in Dhaka, Bangladesh: A time series analysis

**Figure 2d:** PACF of transformed series with non-seasonal difference (1) and seasonal difference (1, period 12)

![Partial Autocorrelation Function](image)

**Figure 3:** Dengue incidence: Observed values and predicted values of SARIMA (1,0,0)(1,1,1)_{12}

![Graph showing observed and predicted dengue incidence](image)
Figure 4: Dengue incidence – Observed values and predicted values of SARIMA $(1,0,0)(1,1,1)_{12}$ for the period September 2006 to October 2007

Figure 5: Forecast of dengue incidence from November 2007 to December 2008 by SARIMA $(1,0,0)(1,1,1)_{12}$
### Conclusion

The incidence of dengue fever every year in Bangladesh, especially in Dhaka city, is a constant threat to the population and a recurring problem for the health authorities. Furthermore, all environmental conditions that can trigger an outbreak are more or less present in the country. Forecasting a dengue outbreak can help the authorities to take effective measures to handle any unexpected situation. Such an effort is cost-effective considering the financial constraints of the health sector. The present study is the first of its kind in Bangladesh. The SARIMA results revealed that the number of dengue patients in 2008 will have a seasonal pick with the highest value as 689 in July, which is concordant with our previous experience. SARIMA models are well-practised tools in epidemiological research which may offer further accuracy in prediction if some relevant variables\[20,21\], for example, temperature, humidity, rainfall, are considered during the modelling process. Efforts should be made in the future to use such additional information, which was not possible in the current study due to lack of coordination between different sources as well as dissimilarity of area of coverage by different authorities. Separate modelling approaches for DF, DHF and DSS would provide better information to policy-makers and planners. Relevant data should be made available in a timely manner, possibly from one service point, with proper coordination between different data sources.

### Acknowledgements

We thank the Disease Control Directorate, Directorate-General of Health Services, Dhaka, Bangladesh, for providing the data of notified dengue cases between 2000 to 2007. We also thank Professor Dr Md Alimul Islam for his suggestions and inspiration. Finally, we would like to thank the three anonymous reviewers for their valuable comments on the previous version of the manuscript.

### References


Dengue vector surveillance in Hong Kong – 2007

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Abstract

A dengue vector surveillance programme was implemented in the city and port areas in Hong Kong. As a result of the surveillance, only *Aedes albopictus* was detected to be present in various areas in summer. *Aedes aegypti* was, however, not detected in any area under surveillance. Although a rather high index of 70.9% was recorded in July, the activity of *Ae. albopictus* was immediately brought down through concerted efforts of various agencies and the public. The swift response of concerned agencies were facilitated by the use of Geographic Information System (GIS) in the dissemination of surveillance results. Users were able to access the system at any time for the latest results of the surveillance for taking immediate remedial measures. The public was also informed of the results regularly through the Internet and press releases to arouse awareness to prevent and control the local dengue vector.

Keywords: Dengue vector surveillance; Hong Kong SAR, *Aedes albopictus*; Community efforts; Vector control.

Introduction

Hong Kong is located on China’s south coast (22°20′N and 114°11′E); it is surrounded by the South China Sea on the east, south and west, and borders the city of Shenzhen in Guangdong Province to the north over the Sham Chun river. The territory’s 1104 sq. km (426 sq. miles) land area consists primarily of Hong Kong Island, Lantau Island, Kowloon Peninsula and the New Territories as well as some 260 other islands. Hong Kong has a hilly terrain with steep slopes. Most of the urban development exists on the Kowloon peninsula, along the northern edge of Hong Kong Island and in scattered settlements throughout the New Territories. Hong Kong exhibits a monsoonal climate, in which the south-west monsoon occurs from May to September, characterizing Hong Kong’s hot, wet summers; while the north-west monsoon occurs from November to March, bringing to Hong Kong cold, dry winters. Because of the climatic influence, most of the annual rainfall occurs in summer and the mean air temperature ranges between 25–28 °C. Even during winter, the temperature ranges between 15–21 °C.

Hong Kong is one of the world’s leading financial centres. It is an important centre for international finance and trade with the largest concentration of corporate headquarters in the Asia-Pacific region, and is known as one of the four “Asian Tigers” for its high growth rates and rapid industrialization between the 1960s and 1990s. The territory’s population also increased sharply throughout the 1990s, reaching 6.99 million in 2006.
Dengue fever has been made statutorily notifiable in Hong Kong since 1994[1]. All the infections reported to the Department of Health of Hong Kong Special Administrative Region, China, are investigated to establish their source. The Department of Health works jointly with the Department of Food and Environmental Hygiene of Hong Kong Special Administrative Region, China, which plays the leading role in the control of the disease vector. Between 1994 and 2001, the annual number of notifications ranged from 3 to 17 cases; all these cases acquired the infection from outside of Hong Kong (i.e. imported cases), mostly from South-East Asian countries. In 2002, there were 36 confirmed cases recorded, of which 20 cases were locally infected. There was another local case recorded in 2003 but none since 2004. The number of imported cases remained at 31 from 2004 to 2006 while these increased to 58 in 2007 (Table).

In Hong Kong, a total of 13 Aedes species have been recorded that include Ae. albopictus and Ae. aegypti[2,3]. Ae. albopictus is one of the most commonly found mosquitoes in Hong Kong. It has wide distribution both in urban and rural areas. Ae. aegypti, on the other hand, probably has not been an indigenous species in Hong Kong. It was once discovered on board a vessel from another country in mid-1950s. In 2000, a dengue vector surveillance programme, using ovitraps at selected sites, to monitor and evaluate the effectiveness of dengue vector control work carried out by various agencies, and for making timely adjustments to dengue vector control strategies and measures, was put in place by the Food and Environmental Hygiene Department. The programme was expanded in 2003 with an increase in the areas covered and the frequency of surveillance. The surveillance programme was further extended in 2004 to cover all major port areas, including all seaports. A dengue vector surveillance programme by using ovitraps had already been in place for the Hong Kong International Airport since 1998.

**Table**: Number of imported and indigenous dengue fever cases from 1994 to 2007[2]

<table>
<thead>
<tr>
<th>Year</th>
<th>Imported cases</th>
<th>Indigenous cases</th>
<th>Total</th>
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<tbody>
<tr>
<td>1994</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1995</td>
<td>6</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>1996</td>
<td>5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>1997</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>1998</td>
<td>15</td>
<td>0</td>
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</tr>
<tr>
<td>1999</td>
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</tr>
<tr>
<td>2000</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>2001</td>
<td>17</td>
<td>0</td>
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</tr>
<tr>
<td>2002</td>
<td>24</td>
<td>20</td>
<td>44</td>
</tr>
<tr>
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<td>1</td>
<td>49</td>
</tr>
<tr>
<td>2004</td>
<td>31</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>2005</td>
<td>31</td>
<td>0</td>
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</tr>
<tr>
<td>2006</td>
<td>31</td>
<td>0</td>
<td>31</td>
</tr>
<tr>
<td>2007</td>
<td>58</td>
<td>0</td>
<td>58</td>
</tr>
</tbody>
</table>

**Methods and materials**

The oviposition trap (ovitrap) was used in this surveillance programme as a tool to detect the prevalence and distribution of aedine mosquitoes. The device was locally manufactured as per specification. It comprised of a simple plastic container of approximately 200 ml capacity, painted black inside with a straight and slightly tapered sides. The opening measured 6.5 cm in diameter, the base diameter was 5.0 cm, and the container was 10.0 cm in height. The ovitrap was covered by a black cap with four openings and a grey-colour umbrella-shape raised cover to protect the content inside the ovitrap from contamination by unwanted materials. A brownish wooden tongue depressor was placed diagonally inside the container as an oviposition paddle.
Thirty-eight areas with high human concentration were selected such as housing estates, schools and hospitals. All the 38 areas were surveyed every month to closely monitor the situation of each location and to obtain a territory-wide picture of the vectorial situation. On an average 55 ovitraps were placed at each selected site. The ovitraps were set at a distance of about 100 m from each other for one week and collected back to the laboratory. The percentage of positive ovitraps was recorded as the ‘Ovitrap Index’. To serve as a quick reference for taking prompt follow-up mosquito control actions, each of the ovitraps collected was examined immediately for the presence of mosquito larvae. The larvae found were identified under compound microscope to species level and the Provisional Ovitrap Index (POI) was worked out. The ovitraps were then incubated at room temperature for one week for the eggs in the ovitraps, if any, to hatch out. The number of ovitraps found with *Ae. albopictus* or *Ae. aegypti* in the first and second examination were pooled together for the calculation of the Area Ovitrap Index (AOI). Another index, Monthly Ovitrap Index (MOI), was then calculated by pooling the results of all the ovitraps retrieved in the same month from the 38 areas which reflected the overall vector situation of the month.

A total of 33 land ports, which are categorized into seven groups according to the nature of the ports, were also surveyed. Twenty ovitraps were used at land ports and 650 ovitraps were used in the airport.

**Results**

**Community surveillance**

The MOIs of 2007 followed a similar trend as previous years but were generally lower (Figure 1). The MOIs in the first quarter were maintained at a rather low level of 0.2% to 1.4%. However, the indices rose gradually in the second quarter from 7.6% in April to 20.7% in August.

**Figure 1:** Comparison of Monthly Ovitrap Index of 2007 with the average of previous years (from 2000 to 2006)
in June and reached a peak of 23.1% in July. Although the MOI recorded in July 2007 (23.1%) was the highest recorded in July since 2003 and higher than the average MOI of July from 2000 to 2006 (19.4%), it was lower than the average MOI of June from 2000 to 2006 (23.8%). A marked drop from 23.1% to 11.3% was observed in August and the MOIs declined gradually thereafter and reached the lowest in December (0.2%).

In respect of individual survey areas, only one AOI exceeded 20% in April. The number of AOIs greater than 20% increased sharply to 12 in May and further to 18 in June. Three locations were found to have AOIs greater than 40% in June and increased further to 6 in July. A record high AOI of 70.9% was also recorded in July. After reaching the peak in July, the indices came down rapidly in August. All AOIs recorded in August were lower than 40%. The number of AOIs reaching 20% also decreased from 16 in July to 8 in August and 3 in September. The indices remained at a lower level in the last quarter. Activity of Aedine mosquitoes was not detected in most of the survey areas after November.

**Port surveillance**

In 2007, the Port Monthly Ovitrap Index (PMOI) ranged from 0.0% in January through February to 3.2% in June. The variation in PMOIs showed a similar trend as in previous years (Figure 2). The ovitrap indices of all port groups were below 20.0%. The highest index of 13.8% was recorded in the port group of Cross Boundary Check Points on Land in June. The ovitrap index at the Hong Kong International Airport was also the highest in June (2.6%). In the months of June, July and August, all port groups had records of positive indices.

**Discussion**

The results of the urban and port areas surveillance indicated that *Ae. albopictus* existed in various areas in summer. The breeding places of the vector include a variety of small water bodies such as discarded buckets, empty lunch boxes, sand pits, surface drainage channels, keyholes of manhole covers, bamboo stumps, and saucers underneath plant

![Figure 2: Comparison of Port Area Ovitrap Index: 2004-2006 and 2007](image)
pots. High ovitrap indices were recorded repeatedly in some of the areas covered by the surveillance programme, indicating the presence of persistent breeding grounds that needed particular attention. *Ae. aegypti*, the important vector for the transmission of dengue fever and yellow fever, was however not detected in all the areas covered by the urban and port surveillance programmes.

It was well recognized that community participation was the key to success in controlling mosquitoes, particularly dengue vectors, and an annual territory-wide anti-mosquito campaign was organized to promote community participation and forge close partnership of government departments and nongovernmental organizations in controlling the mosquitoes. The dengue vector surveillance programme served as a tool not only to monitor the local dengue vector distribution but also to provide objective information for taking appropriate actions by the community against dengue vectors. The Area Ovitrap Index and Monthly Ovitrap Index numbers were released to the public through press releases and the Internet to arouse awareness in preventing mosquitoes. Government departments were able to access detailed information of the surveillance, including location of positive ovitraps through a Geographical Information System which is accessible by registered users through the government intranet. They are able to target mosquito control action at venues that fall within the 100 m radius of all positive ovitraps under their purviews. The people were also advised to pay particular attention to any water accumulation in and near their residences. A detailed and comprehensive advice on mosquito prevention and control was issued together with the press release. The public was also able to access the information through the Internet.

For operational purposes, the ovitrap indices were classified into four different categories – Level One: for indices less than 5%; Level Two: for indices between 5% and less than 20%; Level Three: for indices between 20% to less than 40% and Level Four: for indices at 40% or above. Different actions were taken based on the levels reached. At lower levels (levels 1–3), control measures mainly relied on source reduction, e.g. proper disposal of disused articles, lunch boxes, containers, etc. Potential breeding sites such as saucers underneath plant pots, surface drainage channels, roadside gully traps or keyholes of manhole covers were inspected weekly and accumulation of water was removed promptly. Larvicides were applied whenever immediate elimination of breeding sources was not feasible. When the Ovitrap Index reached Level Four, space spraying of insecticides was carried out at the resting places of the adult mosquito to contain the mosquito problem.

On health education, health talks were organized for schoolchildren, managements of estates, construction sites as well as local organizations such as area committees to disseminate the message of mosquito prevention and control. Training was also organized for pest control personnel in the government. Operatives of pest control contractors providing mosquito control services funded by the government were also required to receive proper training on general pest control, including mosquito control and dengue fever.

**Conclusions**

According to the results of the dengue vector surveillance in 2007, *Ae. aegypti* was not detected and the activity of *Ae. albopictus* was, in general, under control. The Monthly Ovitrap Indices were mostly lower than the averages of the past few years except in July where a surge in the ovitrap indices was observed.
However, with concerted efforts made and swift actions taken by relevant agencies and the public, the indices were brought down quickly in the following month and maintained at a lower level till the end of the year. This indicates that the vector problem had been put under control in 2007.

Active participation of the government, local organizations and the public were the key to success in controlling dengue vector. The results of dengue vector surveillance were released to the public and other parties concerned through different channels to facilitate prompt remedial actions. Timely target-specific control efforts were achieved through the coordination of district-based anti-mosquito task force led by the government.

References


Re-emergence of dengue in Argentina: 
Historical development and future challenges

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Abstract

After 82 years of the absence of dengue in Argentina, a dengue outbreak occurred in the northern provinces of the country in 1998. Aedes aegypti, the vector mosquito, was eradicated in the 1960s, mainly due to the use of residual insecticides at an enormous cost of resources and through a vertical health programme. Since then, the country has gradually become reinfested due to the deterioration of the surveillance system and vector control programmes. At present, DENV-1 to 3 have been found in circulation and 3162 cases of dengue fever (DF) have been reported in the country. However, as autochthonous cases have been recorded during this epidemic only, the disease is still not considered endemic in the country, although there is a regular occurrence of outbreaks in neighbouring countries.

The control strategies currently being used are the same ones as used in the past century although socioeconomic and demographic conditions have greatly changed. Consequently, alternative methods are proposed as potential tools to establish new ways of controlling the vector, which is the only way of preventing new outbreaks in the region.

Keywords: Dengue; Argentina; Control strategies; Aedes aegypti.

Introduction

Argentina is the southern-most country in Latin America. With a surface area of 3 761 274 km², it has a wide diversity of geographical areas such as the cold and dry steppes of Patagonia, the Pampa grasslands, the humid and dry Chaco region and the jungle highlands or “yungas” in the north[1]. The great climatic and topographic diversity of this vast extension of land determines different forms of fauna and flora, as well as different types of human settlements that develop different lifestyles and socioeconomic activities that are directly related to their environment.

The growth of urban centres, viz. the city of Buenos Aires, where nearly 40% of the country’s population is concentrated[2], in conjunction with movement of people from and to the neighbouring countries, supported by congenial environmental conditions in the north and centre, render this country prone to explosive epidemic outbreaks. The prevailing
socioeconomic aspects of Latin America in general and Argentina in particular, especially the extreme polarization of resources, are extremely relevant in the re-emergence of dengue.

During the mid-20th century, the health authorities of American countries, together with the Pan American Health Organization (PAHO), carried out important *Ae. aegypti* eradication campaigns, which were developed in Argentina in 1965[3]. However, by the end of the 1980s, the country was re-infested by the mosquito, a situation that currently prevails[4].

The present article describes some of the variables that contributed to the re-emergence of dengue in Argentina, placing particular emphasis on mosquito vector control, and discusses possible contributions to the current vector control strategies.

**History of dengue fever in Argentina**

The first outbreak of dengue in Argentina was recorded by Nicolás Gaudino[5] in 1916. The virus entered the country via Paraguay and affected the provinces of Corrientes and Entre Ríos. Although no cases were reported in the city of Buenos Aires, it affected 50% of the mesopotamian population.

Since then, in Argentina, the disease was not recorded for 82 years, in spite of the occurrence of severe outbreaks in the Caribbean and Central America in the 1960s, and the later appearance of dengue haemorrhagic fever (DHF) in the Cuban epidemic of 1981 which spread to all the other American countries except Canada and Uruguay[6]. During those eight decades, dengue was considered a problem affecting south-east Asia and other far-off regions. However, it has slowly re-entered our continent via Central America. Today, almost all the American countries from Mexico to the southern tip of the continent are affected by this disease[7].

In 1998, there was an epidemic caused by DENV-2 restricted to the Chaco-Salta region of Argentina, with its epicentre in the city of Tartagal. The epidemic reached its peak in May[8], which caused several hundreds of cases of dengue fever (DF) (incidence rate: 45/10 000 inhabitants). All indications suggest that the virus was introduced from Bolivia[9]. However, this was just the beginning. Since then, a series of outbreaks have occurred in Argentina – in 1998, 2000, 2002, 2003, 2004, 2006, 2007 and 2008 (Figure 1). Five provinces, namely Salta, Jujuy, Corrientes, Formosa and Misiones reported autochthonous cases. More than 70% of the cases were reported in the province of Salta[10]. Only imported cases were reported in 2005, among people having travelled to Bolivia, Paraguay, Brazil, Puerto Rico and Nicaragua. Figure 2 shows the provinces affected by the outbreaks, active serotypes and relationship with outbreaks in neighbouring countries. At present, the outbreaks of dengue in Argentina have always

**Figure 1: Dengue fever cases in Argentina since the re-emergence of the disease**

![Graph showing dengue fever cases in Argentina from 1997 to 2008](image_url)
Re-emergence of dengue in Argentina

Figure 2: Outbreak localization and its relationships with outbreaks in border countries by year, province and circulating serotype in Argentina, 1998–2007

had a direct relationship with neighbouring countries, with the entry of viraemic subjects to initiate transmission. As such, Argentina is still considered a non-endemic country\textsuperscript{[11]}. Three serotypes have been detected in Argentina since the first emergency situation, and have only appeared simultaneously in 2003 in the province of Salta.

In addition to DENV-2, serotypes DENV-3 and DENV-4 started circulating in the north-eastern frontier with limited epidemic potential until 2004, when there was an extended outbreak with thousands of DENV-3 cases in several cities of the Chaco-Salta region. Despite the circulation of several serotypes in successive years and sequential infections, no clinical cases of DHF had been detected\textsuperscript{[12]}.

In 2006, the situation in the north-eastern frontier was aggravated by floods. Dengue outbreaks were recorded in the area of Embarcación in Salta and Puerto Iguazú in Misiones due to DENV-1. Sixty-nine cases were

\textbf{Source:} Sistema Nacional de Vigilancia de la Salud (National System of Surveillance of Health), National Ministry of Health of Argentina
detected in Salta and 112 in Misiones, all of which were confirmed by a laboratory or epidemiological nexus\(^{13}\). This was followed by yet another outbreak in north-eastern Argentina and Iguazú (province of Misiones): where 55 and 90 cases were reported in the Chaco-Salteño area and in Iguazú respectively. The latter cases were mostly imported through the significant flow of people in the “triple frontier” area around the falls\(^{14}\).

Towards the end of 2006, the authorities of Paraguay reported cases of dengue in the city of Asunción, which rapidly developed into a great epidemic. Like in the beginning of 2006, DENV-3 probably entered from Brazil via the state of Mato Grosso. With the entry of new DENV-3 serotype, the population of Asunción, which was previously exposed to DENV-1 in 1999-2000, presented DHF cases as expected due to sequential infections. This event marked a turning point in the history of dengue in the region as it was the first time that this severe clinical form was recognized in Paraguay\(^{15}\). Although by mid-February 2007 there were under 20 cases of DHF, serious cases of classical dengue were detected without plasma extravasation, and the physiopathological and clinical event defining DHF. Such DENV-3 cases had been previously observed in Brazil. The affected individuals presented acute attacks in one or several parenchyma: myocarditis, brain haemorrhage, or hepatocellular deficiency. Acute symptoms appeared 48–72 hours after the onset of dengue, sometimes in the absence of any apparent bleeding and without the haematocrit modifications as normally observed in DHF. The term “visceral dengue” has recently been coined to name this clinical variant, which must be carefully considered in the event of circulation of DENV-3. Due to the dengue epidemic situation in Paraguay, Argentine provinces are now considered high-risk areas\(^{16}\).

### Historical evolution of Ae. aegypti in Argentina

At the beginning of the 20\(^{th}\) century, Ae. aegypti was present in every American country except Canada, from the southern states of United States to Buenos Aires, Argentina. In Argentina, it was widely distributed, covering 14 provinces in the northern and central regions of the country\(^{17}\). In 1947, a continental programme coordinated by PAHO was launched to eradicate yellow fever and its vector, Ae. aegypti\(^{18}\). It started out as a highly successful campaign and by 1954 and 1962 achieved its goal in 18 continental countries, including Argentina. Since 1962, only three additional countries have managed to eradicate this vector. During the 1970s, the support for mosquito surveillance and control programme got slackened, with the result that Ae. aegypti re-infested. By 1995, Ae. aegypti had a distribution similar to that in the 1940s before the eradication effort was initiated. Only Bermuda and Chile remained free of this infestation\(^{19}\).

### Presence of Ae. albopictus

In August 1998, the presence of Ae. albopictus was reported in the locality of San Antonio, province of Misiones; in February 2004 it was also found in Eldorado, another locality in Misiones. These are the first reports of this species in our country\(^{20,21,22}\). In the surveillance studies performed during February and March of 2007 in the open spaces and suburbs of the city of Puerto Iguazú, 24 foci of Ae. albopictus were detected, 18 of which were shared with Ae. aegypti\(^{23}\). The presence of Ae. albopictus conveys a potential risk in the epidemiological context of the region regarding the circulation and transmission of dengue, yellow fever and other related arboviruses\(^{24}\).
Current situation

The reinfestation of this region with *Ae. aegypti* forced the authorities to re-launch monitoring and control activities based on the new criteria of health service decentralization established by PAHO. According to these norms, the National Government transferred the responsibility of monitoring and control activities to the local municipalities, contributing to them with supplies and staff training. This new modality made it necessary to modify old criteria used by the centralized system, generating local difficulties in the provincial facilities regarding their resources and staff training. Furthermore, it is still difficult to combine criteria regarding the monitoring method, rational purchase of supplies (equipment, insecticides, security equipment, etc.) and development of control activities[25].

The high-risk situation of viral transmission still prevails in several localities in north Argentina despite the intervention of national, provincial and local governments, as well as of NGOs, that have been working on vector control for several years. The *Ae. aegypti* indices are still high enough to produce autochthonous outbreaks. In most municipalities (Figure 3), the House Index (HI) (*Ae. aegypti* breeding sites/houses inspected) remains over 10%[26]. Therefore, the entire northern region of the country must be considered a high-risk area. The current floods in Santa Cruz de la Sierra, Bolivia, put the provinces of Salta and Jujuy in an outbreak-prone area with the additional risk of yellow fever transmission as the flooded rural areas being evacuated lie in the jungle yellow fever-endemic zone.

In 2008, 2 996 183 tourists arrived in Argentina from dengue-endemic neighbouring countries, 285 073 of which entered from Paraguay. Approximately 46% of these tourists arrived by plane. In 2008 >2 400 000 Argentines left the country via Buenos Aires to travel to dengue-endemic countries. The level of migration in border areas, especially in the tropical regions of northern Argentina, is under-reported[27]. The number of imported dengue cases in Buenos Aires and other cities of Argentina detected during the current period is substantially higher than the number detected in previous years.

During 2008, the National Ministry of Health reported only 28 cases of dengue in the country, 9 of which were imported. National government workers together with the local provincial staff of Salta are currently carrying out intense house-by-house control activities against mosquito breeding sites, with the collaboration of the community and using insecticide space spraying. These activities have

Figure 3: *Ae. aegypti* infestation in Argentina by province. Cumulative values of 2008. Numbers indicate municipalities with the presence of the vector

Source: Sistema Nacional de Vigilancia de la Salud (National System of Surveillance of Health), National Ministry of Health of Argentina
extended to the border town of Yacuiba in coordination with the health workers of this Bolivian district. No dengue deaths have been reported as yet in Argentina\cite{28}.

**Current control strategies**

Ever since the outbreak in Tartagal in 1998, all the routine and emergency activities recommended for the control of dengue were implemented in the country by the National Ministry of Health. The monitoring, control and evaluation methods implemented were the classical methods used for many years in similar situations\cite{29,30}. The necessary supplies and equipment were purchased and field workers were trained on their correct usage. An emergency control strategy included the application of ultra low volume (ULV) thermal fog spray treatments, portable mist blowers, and house-by-house focal treatment. Simultaneously, diffusion activities were carried out to alert the population of the current situation. Adulticide treatments were only performed during epidemics and not as a means of prevention.

The active substances used were Temephos\textsuperscript{\textregistered} sand granules as a larvicide, and the organophosphate Sumithion\textsuperscript{\textregistered} and the pyrethroid Deltamethrin\textsuperscript{\textregistered} as adulticides in spatial sprays in an oily base using gas oil as solvent. These are obviously not the best tools for implementing control activities in urban areas where the inhabitants suffer a high degree of exposure to the insecticides used.

**Innovation in control strategies**

Although certain epidemic outbreaks were controlled in some areas of northern Argentina, the inadequacy of implementing actions extrapolated from similar situations in other countries or regions with different socioeconomic conditions was soon obvious. We needed to modernize, improve, change and/or adapt future vector control strategies to meet our national and local requirements.

Some social events have triggered these changes. For example, focal treatments in Argentina were possible due to the implementation of social plans during the 2001 recession for the unemployed, who were obliged to contribute four working hours for vector control activities. However, since the economic recovery of the country, these plans were de-activated and now it is impossible to carry out these activities. Major constraints included security risk, refusals, locked houses, etc. These obstacles and inconveniences required the development of alternative strategies.

The CIPEIN, Pest and Insecticide Research Centre, in Buenos Aires, Argentina, is a World Health Organization Collaborating Centre for the evaluation of Chagas disease and dengue vector resistance. Among other tasks, it carries out basic and operational research studies with the object of optimizing control activities for insect vectors of human disease. Among the Centre’s many contributions to mosquito control, we can mention the development of new active substances (permethrin cis-isomer, permethrin trans-isomer)\cite{31,32,33}, isolation of natural products with insecticide properties\cite{34,35} and new insecticide formulations as fumigants in cans or tablets\cite{36,37}, Insect Growth Regulators (IGR) formulations in sand\cite{38}, and ULV formulations for spatial treatments\cite{39}.

Another proposal is the use of adulticides in complete cycles throughout the city, in addition to the control of immature forms, as a control strategy in case of an imminent outbreak of dengue. In countries like Argentina, this type of methodology would be particularly important due to the short periodicity of risk of transmission, which is generally from January
to April, coinciding with the period of higher temperatures and greater rainfall. Furthermore, the outbreaks of dengue in Argentina are closely associated with the epidemiological situation in neighbouring countries, evidenced by the coincidence in time and circulating serotypes in each affected area. Therefore, it has been suggested that the control of adult mosquitoes in border areas with epidemiological risk is a strategy that might avoid autochthonous outbreaks and, at the same time, is cost-beneficial at an incidence of more than 29 cases for every 1000 inhabitants[40].

However, developing such a tool is only part of the vector control challenge. To supplement focal house-to-house treatment, and in the frame of an integral mosquito vector control, a combination of treatments has been proposed that involves spraying a larvicidal-adulticidal mixed formulation[41] using units set up on vehicles in addition to intra-domiciliary actions performed by the dwellers themselves. This proposal is currently under evaluation and could constitute an efficient alternative for controlling this disease.

In spite of the lack of an extended success of campaigns based only on the use of insecticide tools, other strategies of vector control without chemical treatment involving the community have not been organized either. A good review of the achievements of the community-based dengue control programmes was done by Heintze et al.[42].

The PLICOV (Latin American Programme for Innovation in Vector Control) initiative was conceived due to the need of regional countries to develop novel strategies, which can be adapted to the particular situation of each country[43]. A group of six countries, comprising of Argentina, Bolivia, Peru, Panama, Cuba and Colombia, are jointly developing evaluation and control activities of new tools to verify their potential use for vector control in our continent.

This objective has been supported not only by field studies, but also by laboratory research carried out in Latin American countries.

### Resistance to insecticides

As recommended by the World Health Organization[44], the main preventive activities include monitoring of *Ae. aegypti* oviposition and larviciding sites. Since 1998, extensive chemical control operations were performed in the northern part of Argentina. A massive control programme began in 2002 in Clorinda (Formosa)[45] and in 2003 in Iguazú (Misiones), carried out by the Mundo Sano Foundation in collaboration with the National Ministry of Health, the local municipal government, and CIPEIN. The insecticides generally used in the event of an outbreak were temephos for larvicidal treatment in water containers (focal treatment) and cis-permethrin as an adulticidal ULV formulation (spatial treatment). For the control strategies to succeed it is important to know the level of susceptibility to the insecticides used, because the development of resistance could lead to control failures[46]. Therefore, our Centre implemented the first monitoring programme in Argentina in the cities of Clorinda and Iguazú, based on a protocol established during a meeting of the Latin American Network for Vector Control held in Iguazú (Misiones) in December 2004[47], and compared the susceptibility data obtained to the mosquito reference strain at CIPEIN. The results indicated an incipient resistance to temephos in these mosquito populations, posing an alert for this region. The Brazilian Ministry of Health considers that Resistance Ratio (RR) values of 3 are a reason to alternate temephos with another insecticide such as *Bacillus thuringiensis* var. *israelensis* or methoprene[48]. No control failures have been observed yet, but if these values rise to 10, the current control strategies would need to be completely revised[49].
Conclusions

The vertical plans of the mid-20th century based on the mobilization of huge resources and DDT, and centred on the eradication of *Ae. aegypti*, the vector, provided extraordinary results. However, their application in the current situation is highly impracticable and, as demonstrated by Brazil, not only a budgetary issue.

Judging by the progression of the disease in our continent, and in the world in general, the problem is far from being solved. The complex situation that Argentina and the rest of the South American countries face not only depends on the development of new active substances or more efficient formulations, but also on adopting an integral approach to the problem that includes active participation of all parties, reasonable allocation of resources, cost-benefit analyses, insecticide-resistance monitoring, establishing adequate entomological and epidemiological surveillance and, most importantly, the political will.

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References


Re-emergence of dengue in Argentina


Duration of short-lived cross-protective immunity against a clinical attack of dengue: A preliminary estimate

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Abstract

It is believed that primary infection with a single serotype of dengue virus elicits short-lived cross-protective immunity against other heterologous serotypes; however, the duration of cross-protection has not been explicitly estimated using epidemiological data. To offer an empirical estimate of the duration, the present study re-analysed historical cohort data of multiple clinical attacks of dengue among American soldiers in the Philippines from 1922–24. In the original study, the historical cohort of 299 cases with a first clinical attack of dengue were closely surveyed; 99 (33.1%) experienced a second attack, while the remaining 200 returned to the United States without further attacks. The time intervals from first to second attack among the 99 cases, and from first attack to departure to the United States among the 200 soldiers, were used for estimating the duration of cross-protective immunity based on a simple mathematical model. Employing an exponential distribution or Kronecker’s delta function as the loss function of cross-protection against a second clinical attack, the mean duration of cross-protective immunity since the first clinical attack was estimated as 6.90 (4.87, 11.83) days and 7.52 (4.88, 16.38) days, respectively. The force of infection, which was jointly estimated with the duration of cross-protection, reasonably explained the other observed epidemiological information in the data, supporting the finding of a short cross-protection period. Even though the estimates suggested that the first clinical attack most likely elicited cross-protective immunity, the length of cross-protection lasted only 1–2 weeks, far shorter than previously believed.

Keywords: Dengue; Epidemiology; Immunity; Serotype; Statistical model.

Introduction

Dengue fever (DF) is a vector-borne disease caused by four closely related dengue viruses (DENV-1-4)[1-2]. It is distributed in most tropical and subtropical areas where Aedes aegypti and/or Aedes albopictus are abundant[3]. Infection with DENV can also cause dengue haemorrhagic fever (DHF), which is a clinical syndrome characterized by increased vascular permeability, plasma leakage, hypovolemia and shock[4,5]. Although the pathogenesis of DHF has yet to be fully clarified, several risks have been reported; these include secondary infection with heterologous serotypes[6,7],

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primary infection in infants born to dengue-immune mothers, differing virulence of a strain and differing human susceptibility according to race or genetic factors.

Although the epidemiological risks of DHF have been explored for more than 30 years, many aspects of the transmission dynamics of dengue remain to be clarified. The transmission dynamics, especially of the interactions between two or more serotypes, have been explored using mathematical models. Despite a recent increase in the number of relevant studies, only those based on epidemiological observations in the field have provided detailed insights into the pathogenesis of DHF or interactions between dengue transmission and disease. There is a general lack of field data complete with serotype, time, age and space measurements that would allow scientists to investigate and model dengue at a population level. Questions that could be clarified with modeling exercises include: (i) more specific information on the mechanisms of innate dengue viral virulence (if any); (ii) the result of infections with any two specific heterologous serotypes; and (iii) the mechanisms and duration of protective immunity.

Among these unknowns, the present study focuses on cross-protective immunity among those who have experienced primary infection against further infection caused by a heterologous serotype. The duration of acquired cross-protective immunity has never been explicitly estimated and various epidemiological models have employed a number of different and unsupported assumptions. For instance, Ferguson et al. assumed the absence of cross-protection, although a historical study conducted by Sabin suggests that the presence of cross-protective immunity for a short time-period is plausible following primary infection. The presence of transient cross-protective immunity was once supported by explicit data analyses by Adams et al., but the data were from DHF cases in Thailand in a time-series (with serotype-specificity) that required a number of other epidemiological assumptions. Other studies have assumed differing mechanisms of cross-protective immunity following an exposure to a heterologous serotype shortly after primary infection (e.g. exposure to the heterologous serotype results either in infection or sero-conversion and/or permits developing immunity against the heterologous serotype). Even though the presence of cross-protective immunity seems likely, and although the majority of previous studies have acknowledged the critical importance of short-lived cross-protective immunity in describing the oscillatory transmission dynamics of dengue, the actual duration remains unknown. An implicit suggestion has been that the duration is 2–9 months.

Accordingly, it would be important to offer an empirical estimate of the duration using existing informative data. The present study aims to estimate the duration of cross-protective immunity against a second clinical attack of dengue as a function of the time since the first attack. For the estimation, historical cohort data from American soldiers in the Philippines from 1922–24 are re-analysed.

**Methods**

The historical data of DENV infection originated from a well-known and rigorous study by Joseph Franklin Siler, Milton Weston Hall and Arthur Parker Hitchens that took place in 1924–25. The study was originally published in the Philippine Journal of Science and was reprinted with appendices by the Bureau of Printing, Manila. Further details of the publication were revisited by Nishiura and Halstead in a recent study. The experimental transmission of DENV-4 in human volunteers
recruited from US Army personnel is widely known[26,27]. Siler et al. also conducted an epidemiological study of the natural infection of dengue among American soldiers in the Thirty-first Infantry from 1922–24[24,25]. This investigation revisits that study.

As well as the results from the transmission experiment in human volunteers, Siler et al.[24,25] hoped that the study would achieve further insights into the frequency of infection, acquired immunity and recurrence of dengue in the average American soldier in Manila under natural conditions. Accordingly, an epidemiological survey of the clinical attack of dengue was attempted among American soldiers; multiple clinical episodes of dengue in each individual were recorded. After obtaining the preliminary results of the epidemiological observations, the authors noted serious technical problems in interpreting the data and precisely estimating the frequency of infection at a population level. The issues included: (i) varying time-intervals between the first attack of dengue and the end of military duty (i.e. some cases experienced the first attack at a time close to the end of duty, and thus were unlikely to experience a second attack); (ii) the duration of military service was variable and some soldiers left for home during the period of observation while others remained in the Philippines; and (iii) mild attacks were less likely to be recorded compared with severe cases. To resolve these technical problems, Siler et al. conducted further epidemiological observations in the Thirty-first Infantry; subjects were limited to those who had their initial attack of dengue between 1 July 1922 and 30 June 1923. All of the enrolled soldiers started their duty on or after 1 January 1922 and left the Philippines no earlier than 31 December 1923. The usual length of duty in the Philippine Islands was 2 years. The period of observation ended 31 December 1924, a time by which the soldiers had been closely monitored for any possible signs or symptoms of dengue. Individuals with irregular military assignments or transfer were excluded because of the above-mentioned epidemiological problems. Except for a few individuals, all of the included subjects were men from the United States who could be assumed to be fully susceptible at the beginning of their tour of duty in the Philippines, or at least were stated as “could not have been exposed to dengue for more than six months before.” Unfortunately, the severity of the cases was not well detailed, and it is unknown if there was any indication of DHF among those with clinical attacks.

The Thirty-first Infantry numbered 1086 personnel, among which there were 562 potential episodes of dengue. Because of missing observations of 28 potential cases, the authors proportionally decreased the total sample (n = 1032). The first attack of dengue was clinically assessed and detailed clinical records were obtained for 421 cases. Furthermore, strictly applying the exclusion criteria to satisfy the authors’ concern regarding epidemiological problems (especially, to meet the condition of the time of assignment being on or after 1 January 1922), only 299 cases (71.0% of those with clinical records) were selected for further analyses. Again, proportionally decreasing the total sample size (n = 733), the authors concluded that a clinical attack of dengue was observed at least once among 40.8% of the soldiers. Of the 299 cases that had a first attack, 99 (33.1%) experienced a second attack while the remaining 200 left the Philippines without any further clinical attacks. For all of the included subjects, the time interval between their arrival in the Philippines and the first attack was recorded. Moreover, the time from the first attack to departure to the United States was recorded among the 200 cases without a second attack. The time interval from the first to the second attack as well as the time interval from the second attack to departure back to the United States was recorded among the 99 cases with
a second clinical attack. In the original publication, the data for those with only a first clinical attack were reported as a group (i.e. given as just summary tables) by discrete time intervals and there was no individual information (such as time from arrival-to-attack and attack-to-departure), but the data for those with a second attack (n = 99) were recorded for each individual. Although third and fourth clinical attacks were observed among 14 (14.1%) and 1 (1.0%) cases among the 99 experiencing a second attack, the information was discarded in the present study for simplicity.

Using the historical cohort data of those who experienced at least a first clinical attack of dengue, the present study estimates the duration of cross-protective immunity against a second clinical attack as a function of time since the first attack. Cases, both with and without a second clinical attack (n = 99 and 200), are analysed. First, the descriptive statistics of the time intervals were examined. The time intervals between arrival and the first attack and between the first attack and departure to the United States were compared between those who did and those who did not experience a second clinical attack. For these comparisons, a t-test and the Welch analysis of variance (ANOVA) were employed following the use of a F-test. Subsequently, a mathematical model was developed and applied to estimate the duration of cross-protective immunity against a second clinical attack of dengue.

Figure 1 shows a schematic diagram of a mathematical model that describes the cohort episode of first and second clinical attacks of dengue. Let t denote the time since the first clinical attack. Moreover, \( l_1(t) \), \( S(t) \) and \( l_2(t) \) denote, respectively, the fractions of those who experienced a first attack and are still immune to another clinical attack, who are susceptible to another clinical attack caused by a heterologous serotype, and who experienced a second clinical attack, at time t since the first attack of dengue. Supposing that the rate to loose cross-protective immunity and the force of infection (i.e. the rate at which susceptible individuals experience infection) are \( \delta \) and \( \lambda \) (per day), respectively, the model for the observed intervals is given by

\[
\begin{align*}
\frac{dl_1(t)}{dt} &= -\delta l_1(t) \\
\frac{dS(t)}{dt} &= \delta l_1(t) - \lambda S(t) \\
\frac{dl_2(t)}{dt} &= \lambda S(t)
\end{align*}
\]

\(\text{(1)}\)

**Figure 1:** Compartmental model to describe the time interval between first and second clinical attacks of dengue in the Thirty-first Infantry in the Philippines from 1922-24

[Although infected soldiers are assumed as transiently immune against other serotypes immediately after the first clinical attack, they loose the cross-protective immunity at rate \( \delta \) and become susceptible to other heterologous serotypes. The susceptible individuals experience infection at rate \( \lambda \) and experience a second clinical attack.]
It should be noted that a constant force of infection $\lambda$ assumes an endemic equilibrium in the Philippines (see discussion on seasonality). Since $I_1(0) = 1$ and $S(0) = I_2(0) = 0$, the probability density and the cumulative distribution of the second clinical attack at time $t$ since the first attack, $f(t)$ and $F(t)$, respectively, are

$$
 f(t) = \frac{dI_2(t)}{dt} = \frac{\delta \lambda}{\lambda - \delta} \left[ \exp(-\delta t) - \exp(-\lambda t) \right]
$$

$$
 F(t) = I_2(t) = \frac{\delta \lambda}{\lambda - \delta} \left[ \frac{1 - \exp(-\delta t)}{\delta} - \frac{1 - \exp(-\lambda t)}{\lambda} \right]
$$

(2)

Let the time interval from the first to the second attack of case $i$ be $t_i$ (where $i$ belongs to the 99 cases with a second attack), and let the time interval from the first attack to the return to the United States of case $j$ be $t_j$ (where $j$ belongs to the 200 cases without a second attack). The observed $t_j$ among the 200 without a second clinical attack are dealt with as censored data. That is, the likelihood of not observing a second clinical attack for $t_j$ days is given by $1 - F(t_j)$. Accordingly, the total likelihood is given by

$$
 L(\delta, \lambda) = \prod_i f(t_i) \prod_j (1 - F(t_j))
$$

(3)

The parameters, $\delta$ and $\lambda$, were estimated by minimizing the negative logarithm of equation (3). Profile likelihood confidence intervals were computed. In addition to the exponentially distributed immune-loss function in model (1), Kronecker’s delta function was also employed as the loss function of cross-protective immunity. Delta function assumes that the duration of cross-protection does not differ between individuals (i.e. is constant), and yields an estimate of the duration that can be regarded as maximum. Under the alternative assumption, $f(t)$ and $F(t)$ are replaced by

$$
 f(t) = \lambda \exp(-\lambda(t-\delta))
$$

$$
 F(t) = 1-\exp(-\lambda(t-\delta))
$$

(4)

which was also applied to the data using the likelihood function (3).

It should be noted that the following assumptions were made for inference: (i) all included subjects were fully susceptible to dengue at the beginning of their military duty in the Philippines; (ii) the first clinical attack elicited life-long immunity against the causative homologous serotype; (iii) the force of infection was independent of time, and seasonality was ignored because of the absence of adequate data; (iv) multiple serotypes were co-circulating during the period of observation in the Philippines with identical transmission potential (though the exact number of co-circulating serotypes does not have to be known); and (v) the second clinical attack does not have to reflect secondary infection, and the assumed loss of immunity reflected the waning of cross-protection against a second ‘clinical’ attack. Although the results in the present study are deemed preliminary because of these simplistic assumptions, it is critically important to validate the realism of these assumptions (especially, iii, iv and v) to appropriately interpret the results. Thus, these points are later discussed in more detail (see Discussion).

**Results**

Figure 2A shows the distribution of time from arrival in the Philippines to the first attack for 299 cases. The mean (and standard deviation (SD)) and median (and lower-upper quartiles) were 153.9 (115.1) and 144 (48-213) days, respectively. The mean (SD) intervals from arrival to the first attack among those who did or did not experience a second clinical attack were 124.7 (103.6) and 168.4 (118.0) days; significantly different by a t-test ($t$ ratio = -3.27, $p < 0.01$). Figure 2B shows the distribution of time from the first to the second attack of dengue among 99 cases. The mean (SD) and median (lower-upper quartiles) were 142.4 (129.6) and 142 (72-279) days, respectively.
Duration of cross-protection against second attack of dengue

**Figure 2:** Frequency distributions of the time from arrival to the first attack and the time from the first to the second attack in the Thirty-first Infantry in the Philippines from 1922-24

[A. The time since arrival in the Philippines to the first clinical attack of dengue (n = 299). B. The time interval between the first and second attacks among 99 American soldiers. The other 200 soldiers did not experience a second clinical attack.]

**Figure 3:** Comparison of the time from first attack to departure between those with and those without a second attack of dengue in the Thirty-first Infantry in the Philippines from 1922-24

[Departure denotes the end of military service in the Philippines (soldiers then returned to the United States)].
Figure 3 compares the time from the first attack to departure to the United States between those with and those without a second clinical attack. The mean (SD) and median (lower-upper quartiles) lengths for the entire samples (n = 299) were 574.2 (141.0) and 575 (475-675) days, respectively. The mean (SD) intervals from the first attack to departure for those who did and those who did not experience a second attack were 600.8 (107.7) and 561.0 (153.4) days, respectively. Since the F-test revealed a significant difference in variance (F-ratio = 2.03, p < 0.01), a Welch ANOVA was subsequently employed for the comparison. This showed that the time from the first attack to departure among those with a second attack was significantly longer than those without (F-ratio = -6.75, p = 0.01). All of the above-mentioned time intervals among those with a second attack were given as individual data, permitting an estimation of the total length of stay in the Philippines. The mean (SD) length of stay was 725.5 (100.2) days, roughly corresponding to 2 years as described in the original study[24,25].

Assuming an exponentially distributed immune-loss function, the maximum likelihood estimates (and the corresponding lower and upper 95% CI) of δ and λ were 0.14 (0.08, 0.21) and 7.52×10⁻⁴ (6.13×10⁻⁴, 9.12×10⁻⁴) per day, respectively. The mean length of cross-protective immunity against a second clinical attack is given by 1/δ, i.e., 6.90 (4.87, 11.83)

**Figure 4:** Estimated duration of cross-protective immunity against a second clinical attack of dengue

[The estimated fraction of those still protected against a second clinical attack, from a heterologous serotype, is shown as a function of time since the first attack. Two different models, exponential distribution (thick line with dotted-and-dashed 95% confidence intervals) and Kronecker’s delta function (thin line with dotted 95% confidence intervals), were assumed as the survival function of cross-protective immunity. The 95% confidence intervals were derived from profile likelihood.]
Discussion

Despite the critical importance of cross-protective immunity for understanding the epidemiological dynamics of dengue, there has been no previous determination of the duration of cross-protection against a heterologous serotype; thus, the present study re-analysed historical case cohort data among US Army personnel in the Philippines from 1922-24. As it was observed from the experimental transmission of dengue in human volunteers\[26,27\], another original data set of Siler et al.\[25\] also yielded critically important information on the time intervals of exposure and transmission events, permitting empirical assessment of the length of acquired cross-protective immunity against a second clinical attack. The most important conclusion drawn from the simple exercise undertaken in the present study is that the duration of transient cross-protective immunity was estimated as short as 1 or 2 weeks, which is far shorter than has been implicitly suggested (i.e. 2-9 months)\[23\]. The finding of short-lived cross-protective immunity can explain Sabin’s note in which mild systemic inflammation was observed by inoculating subjects who had been thought to be cross-protected\[23\].

Although it was not possible to quantify the degree (or strength) of cross-protection, to the best of the author’s knowledge, the present study is the first to explicitly estimate the duration based on empirical epidemiological data. The role of cross-protective immunity has been recognized as critical for describing the transmission dynamics of dengue, and especially, oscillatory epidemiological patterns\[15,20\]. Despite the successful quantification of the short length of cross-protection, it should be noted that the estimated duration reflected cross-protection against a second clinical attack. Considering that DENV infection involves a substantial fraction of sub-clinical infections\[28,29\], cases with a second attack could have been infected before their second clinical attacks were observed. Considering that experimental infection of animals with silent sero-conversion has been observed\[30\], the sub-clinical secondary infection is indeed plausible. Nevertheless, the possible presence of sub-clinical secondary infection indicates that the duration of cross-protective immunity against infection (rather than clinical attack) is shorter than the estimated duration in the present study. This supports the main conclusion of the present study, i.e. that the duration of cross-protection is extremely short. Considering the fact that infection with a second heterologous serotype tends to enhance the severity of a secondary infection\[6,7\], the duration of cross-protective immunity against infection with a second heterologous serotype may be reasonably close to the estimate in the present study, and may be slightly shorter than the estimated duration against a second clinical attack.
Since the present study was intended to present preliminary results of estimates based on a simple model structure, the mathematical model employed a number of unrealistic assumptions, among which (v), the interpretation of a second ‘clinical’ attack, was discussed above. The remaining two important issues, (iii) the constant force of infection, and (iv) equal frequency of co-circulation among heterologous serotypes, are discussed here. Although the force of infection could vary as a function of time in reality (e.g. reflecting seasonal ecological dynamics of the vector population), during the study period from July 1922 to December 1924, there was no month without a clinical attack of dengue. Moreover, the largest difference in incidence (i.e. highest minus lowest incidence) was as small as 1560 per 1000 per annum among the entire population of American soldiers[25], indicating that the seasonal forcing was not critical quantitatively. That is, even though assumption (iii) could have influenced the precision of the estimate, the main conclusion of an extremely short duration of cross-protection is still deemed valid.

The contention of short-lasting cross-protection (and validity of the model) is also supported by two other calculations, one of which is also relevant to the interpretation of the above-mentioned point (iv). First, using the estimated force of infection $\lambda = 7.5 \times 10^{-4}$ per day, the fraction of those who experienced a clinical attack of dengue during 2 years of military service would be given by $1-\exp(-2 \times 365 \times \lambda) = 0.422$, which gives an estimate very close to the observed cumulative incidence of first clinical attack by the end of the study period (i.e. 40.8%). Accounting for the possible presence of sub-clinical infection, $\lambda$ could have been greater than the estimate, but, in fact, a greater $\lambda$ supports the finding of a short duration of cross-protection (i.e. if $\lambda$ is greater than $7.5 \times 10^{-4}$ per day, the estimated mean duration of cross-protection, $1/\delta$, will be shorter than that in the present study). Second, if there were 2, 3 or 4 co-circulating serotypes, the assumption of identical transmissibility would yield the force of infections for all 2, 3 or 4 circulating serotypes as $15.0, 11.3$ and $10.0 \times 10^{-4}$, respectively, per day. The median time from arrival to first attack is then given by $-\ln(0.5)/\lambda = 462, 616$ and 693 days. Although a direct comparison between these estimates and the observed data cannot be made because of the absence of detailed data about those who avoided a clinical attack of dengue, the estimated median times from arrival to first attack are shorter than the observed median among those experiencing a first attack (144 days in Figure 2A), indicating that the force of infection could have been larger than that estimated, which again supports the finding of short-lived cross-protective immunity. Also, if the force of infection of a specific serotype was much greater than those of other serotypes, this could reasonably explain the discrepancy between the expected median time from arrival to first attack and the median estimate in Figure 2A. Assuming that the average life expectancy of the host was $L = 50$ years and exponentially distributed, and adopting an approximate estimator of the basic reproduction number (i.e. the average number of secondary cases generated by a single primary case in a fully susceptible population), $R_0 = \pi L$ (where $\pi$ is the serotype-specific force of infection), equal frequency of transmissibility among co-circulating 2, 3 or 4 serotypes yields $R_0$ of $9.13, 6.84$ and $6.08^{[31,32]}$. Obtaining a more precise estimate utilizing an extended modelling approach is a subject for future study.

In summary, the present study re-analysed the distribution of time intervals between first and second clinical attacks of dengue, jointly estimating the duration of cross-protective immunity against a second clinical attack and the force of infection among US Army personnel who experienced at least a first attack in the Philippines. Although transient cross-protective immunity most likely exists, the
length is estimated to be just 1-2 weeks, far shorter than previously suggested. Considering that DENV infection involves a substantial number of sub-clinical infections, and because the present study reported preliminary results based on simplistic model assumptions, future improvements that address the presence of sub-clinical infection, adjust seasonal characteristics of infection, and obtain more precise estimate of the duration of cross-protection, are considered crucial.

Acknowledgment

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References


Duration of cross-protection against second attack of dengue


Discrimination between primary and secondary dengue virus infection by using an immunoglobulin G avidity test

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Abstract

Discrimination between primary and secondary dengue infections is important, as the possibility of DHF is more in secondary infection. Therefore, there is need to develop a test that can distinguish between primary and secondary serological responses. The traditionally-used haemagglutination inhibition (HI) test, which is recommended by the World Health Organization, is complicated to perform. We standardized an enzyme-linked immunosorbent assay kit with some modifications to discriminate between primary and secondary dengue infections. Sera from 72 patients with acute dengue infection were tested. Seventy-one of the 72 patients were correctly classified (18 of 18 patients with primary dengue and 53 of 54 patients with secondary dengue). We conclude that this rapid and simple test is an excellent alternative to the HI test for discriminating between primary and secondary dengue virus infections during the acute phase of dengue.

Keywords: Dengue; Discrimination; Primary and secondary infections; Immunoglobulin G avidity test.

Introduction

Dengue infection (DI) is among the most important arboviral diseases in India in terms of both morbidity and mortality[1]. Dengue virus is a member of the flaviviridae family, with four serologically related but antigenically distinctive serotypes (DENV-1, DENV-2, DENV-3 and DENV-4). Acute infection due to dengue virus is generally asymptomatic and may present with classical dengue fever (DF), a mild illness, or its severe form, dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS)[1]. DHF, which is life-threatening, has been postulated to result from immune enhancement after a second (heterotypic) infection by a different serotype. The hypothesis on antibody-dependent enhancement can be used for the establishment of an early diagnostic test to distinguish the primary from the secondary infection and to know the immunological status of the patients infected with dengue virus. Keeping in view the increased possibility of DHF in secondary infections, it is important to discriminate between primary and secondary infections[2,3].
Traditionally, the haemagglutination inhibition (HI) test has been used to detect and differentiate between primary and secondary dengue virus infections\(^4\). Patients are classified as having secondary dengue virus infections when the HI test titre in their sera is greater than or equal to 1:2560, and are classified as having primary dengue virus infection if the HI test titre is less than 1:2560\(^4\). However, when the interval between the acute- and the convalescent-phase samples is less than 7 days, or the convalescent phase specimens are not available, haemagglutination inhibition test is difficult to interpret\(^4,5\). Moreover, the requirements of serum pre-treatment with acetone or kaolin to remove non-specific inhibitors makes HI test a tedious one. Furthermore, this test cannot give an early diagnosis\(^4,5\).

Innis et al.\(^6\) first proposed the classification of primary and secondary dengue infections by determining the ratio of dengue virus IgM antibodies to the dengue virus IgG antibodies. The acute-phase sera of patients with primary dengue virus infections show higher IgM/IgG ratios, as compared to the patients with secondary infections who show lower IgM/IgG ratios. The ratio of IgM/IgG higher than 1.78 was considered as a marker of primary infection and less than that was considered as a marker of secondary infection\(^6\). The IgG antibody avidity test is a very useful tool for differentiating between primary and secondary immune responses\(^7\). The avidity assay is based on the fact that the first antibodies synthesized after an antigenic challenge or primary infections have a lower affinity for the antigen than those produced later on. In the secondary infection, the rapid antibody response is characterized by the production of high-avidity antibodies\(^2\). The avidity levels are reported as the avidity index, expressing the percentage of IgG bound to the antigen following treatment with denaturing agents. Recently, a few studies have standardized the avidity test and discriminated between primary and secondary dengue infections. A study carried out by de Souza et al.\(^2\) reported for the first time that by using a commercial kit for serological dengue diagnosis, it was possible to discriminate between a primary dengue infection and a case of secondary dengue infection by detecting avid IgG\(^2\). Matheus et al.\(^3\) developed an in-house ELISA to standardize avidity test and concluded that the avidity test was more useful than the HI test for the discrimination of primary from secondary dengue virus infection, whatever the type of dengue antigen used\(^3\). Since this aspect has not been worked in our settings, there is a dearth of data from India. Thus, the aim of the present study was to standardize an IgG avidity test using a commercially available kit for differentiating between primary and secondary dengue infections in our settings.

### Materials and methods

The present study was performed in the Department of Microbiology, Maulana Azad Medical College and Associated Lok Nayak Hospitals, New Delhi, from September 2005 to December 2006. The study group included 150 patients clinically suspected of having dengue infection, attending the outpatient department and admitted in medical wards of Lok Nayak Hospital, New Delhi. The WHO criteria were followed for inclusion or exclusion of a case of dengue infection\(^4\). Acute-phase blood samples were collected within 4–8 days of infection, and a convalescent-phase sample was obtained after 8–15 days of onset of fever. Confirmation of acute dengue was obtained by the detection of IgM antibodies and demonstration of a $\geq$ 4-fold change in reciprocal IgM antibody titres in paired serum samples. IgG and IgM antibodies were detected by using PanBio IgG and IgM capture ELISA kit.
Primary and secondary dengue infections were defined by using the following diagnostic criteria. Primary dengue virus infection was characterized by the presence of IgM antibodies during the acute-phase and seroconversion (appearance of the IgG antibodies along with IgM antibodies) in the convalescent phase, or the acute phase serum sample was positive for both IgM and IgG and the ratio of IgM/IgG was greater than 1.78. A secondary dengue infection was characterized by the presence of both IgM and IgG antibodies in the acute-phase serum sample and also the IgM/IgG ratio was lesser then 1.78.

Following the above criteria, 18 patients were classified as primary dengue infection cases and 54 patients as secondary infection cases. Samples whose absorbances were above the limit of the ELISA reader were retested using higher dilution, i.e. 1:1000. The IgG avidity test was standardized by using dengue indirect IgG ELISA kit (PanBio), the procedure of which was modified by introducing an urea incubation step. The test was performed in the same manner as reported in a previous study on standardization of dengue IgG avidity test, excepting that the commercial kit used by us was PanBio dengue IgG indirect ELISA kit. Serum samples were diluted 1:100. The samples were then dispensed in duplicate into dengue antigen-coated wells. The samples were then incubated for half-an-hour at room temperature. After the incubation period first differential washing with PBS was done. After first washing half of the wells were washed with phosphate-buffered saline (pH 7.2) which contained urea, and the other half were rinsed with phosphate-buffered saline without urea. After five washing cycles, the test was performed as per the manufacturer’s instructions. The avidity index (AI), expressed as a percentage, was calculated as the ratio of the optical density with urea to the optical density without urea multiplied by 100.

The test was performed several times using different concentrations of urea, 6M urea for 10 min, 7M urea for 10 min, and 8M urea for 5 min. Since variable results have been obtained by several investigators when ELISA was used to test avidity using different commercial plates, different sources of antigen and different urea concentrations, we tested different formats such as concentration of urea and time for urea incubation step to standardize the procedure and followed the same method of de Souza et al.

**Statistical analysis**

We used SPSS version 12 statistical software for the statistical analysis. Mann Whitney’s test was used to check whether the avidity level was significantly different between primary and secondary dengue infections. A receiver operating characteristic (ROC) curve analysis was employed using Analyze-it software, to evaluate the accuracy of the test.

**Results**

The study used various incubation schedules of 6M for 10 min, 7M for 10 min and 8M for 5 min. The mean avidity index for primary infection was 38.24, 22 and 15.11 for urea at 6M for 10 min, 7M for 10 min and 8M for 5 min respectively, while the mean avidity indices for secondary infection were 78.94, 72 and 43.7 for urea at 6M for 10 min, 7M for 10 min and for urea at 8M for 5 min, respectively (Figure). The use of 6M urea for 10 min could differentiate only 13 out of 18 primary dengue infections and 44 out of 54 secondary dengue infections. Whereas 8M urea for 5 min could differentiate 15 out of 18 primary dengue infections and 49 out of 54 secondary dengue infections. The use of 7M urea for 10 min differentiated best between the primary and secondary infections.
Immunoglobulin G avidity test to discriminate between primary and secondary dengue virus infections

the secondary infections. This washing schedule could correctly classify all 18 primary and 53 secondary dengue infections, and was chosen to evaluate IgG AI. Avidity indices ranged from 14–32 for primary infections and 27–106 for secondary infections with 7M for 10 min. The mean AI for primary infection was $22 \pm 5.4$ and for secondary infection was $72 \pm 12.2$. The mean AI of the 18 primary dengue infections was significantly lower than the 54 secondary dengue infections ($P < 0.001$) (Table). The cut-off point of $\geq 27.4\%$ IgG AI was chosen for the

**Figure:** IgG antibody avidity indices in sera from patients with primary and secondary dengue infections with washing schedules of 7M urea for 10 min and 8M urea for 5 min and 6M for 10 min

<table>
<thead>
<tr>
<th>Avidity Index (AI) range</th>
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<th>Secondary infection</th>
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<tr>
<td></td>
<td>No. (%) of patients</td>
<td>Mean AI (SD)</td>
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<td>Total</td>
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<td>22 (5.4)</td>
</tr>
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</table>

**Table:** Performance of IgG avidity test
Immunoglobulin G avidity test to discriminate between primary and secondary dengue virus infections


classification of primary and secondary infections. At this cutoff point, the IgG avidity test provided correct classifications of 71 of 72 patients [18 of 18 patients with primary dengue (100%) and 53 of 54 patients with secondary dengue (98.14%)].

The avidity test showed 98.67% sensitivity, 100% specificity.

Discussion

Dengue hemorrhagic fever and dengue shock syndrome have been observed to occur frequently with secondary dengue infection. Therefore, it is important to discriminate between primary and secondary infections and to assess the immunological status of patients to know the progression of the disease[4]. The haemagglutination inhibition test is conventionally used as a standard test to differentiate between primary and secondary dengue virus infections. The main disadvantages of the HI test are the requirement of paired samples, serum pre-treatment with acetone or kaolin and goose red blood cells[5,10]. Keeping in mind the disadvantages of HI, alternative assays are needed for differentiating between primary and secondary dengue infections. The utility of the assay in diagnosing a primary infection has been reported for a variety of parasites and viruses like leishmania[11], respiratory syncytial virus (RSV)[12], and rubella[13]. Recently, a few studies have standardized the avidity test and discriminated between primary and secondary dengue infection. To the best of our knowledge, no study is reported from India which has used avidity test for differentiating between primary and secondary dengue infections. The studies carried out by de Souza et al.[2] have shown for the first time that, by using a commercial kit for the diagnosis of dengue, it was possible to discriminate between a case of primary dengue infection and a case of secondary dengue infection by detecting avid IgG antibodies. Our results, obtained by using a commercial IgG indirect ELISA kit, confirmed the results obtained by de Souza et al.[2] We observed a mean AI of 22% during a primary infection and 72% during a secondary infection. The sensitivity and specificity of the test were 98.67% and 100%, respectively, with a single sample. These findings were in tune with the previous studies, which showed that the avidity test was an excellent alternative to HI assay for differentiating between primary and secondary dengue infections. Thus, the avidity test standardized by this study is a simple test which can differentiate between primary and secondary dengue infections.

References


DENV-3 genotype III circulating in São Paulo, Brazil, from 2003 to 2008 is not associated with dengue haemorrhagic fever/dengue shock syndrome

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Abstract

Dengue viruses (DENV) are the most important arboviruses of public health significance, and comprise of four distinct antigenic serotypes (DENV-1 to 4) that show substantial genetic diversity. These viruses usually cause dengue fever (DF) but some patients progress to a more severe form of the illness, i.e. dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS). The first reports of DENV-3 cases in Brazil occurred in the year 2000 with co-circulation of DENV-1 and 2. Thereafter, DENV-3 spread throughout the country. DENV-3 phylogenetic analysis has revealed the existence of four to five DENV-3 genotypes. Genotype III of DENV-3 has been the main genotype circulating in Brazil, but recent studies have indicated that DENV-3 genotype I and genotype V are also circulating in some states of Brazil. In order to evaluate DENV-3 genotypes circulating in São Paulo state from 2003 through 2008 we analyzed the NS1 region of DENV-3 isolated from patients residing in Ribeirão Preto and presenting with different clinical manifestations of dengue disease. Nucleotide sequences from 31 viruses were obtained and compared to 105 DENV-3 corresponding sequences retrieved from GenBank. Phylogenetic analysis showed that São Paulo DENV-3 sequences belong to genotype III and that Puerto Rico strains are closely related to South American strains. There was no association between DENV-3 genotype and DHF/DSS.

Keywords: Dengue genotyping; Phylogenetic analysis; São Paulo; Brazil.

Introduction

Dengue is an acute febrile disease caused by a flavivirus with four antigenically distinct serotypes (DENV-1, -2, -3, and -4) and is mainly transmitted to humans by Aedes aegypti mosquitoes. Dengue virus (DENV) infections are currently the most important human arboviral disease in terms of morbidity and mortality in several countries of America, Asia and Africa[1]. DENV contains a single-stranded
positive-sense RNA genome of about 10.7 kb that encodes three structural proteins (envelope glycoprotein, E; membrane, M; and capsid, C) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) [2]. The clinical manifestations of dengue range from inapparent or mild disease (dengue fever) to severe forms known as dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS). It currently affects around 100 million people every year worldwide, and around 500 000 people have DHF/DSS with 2.5% deaths [3]. The mortality rates vary according to the affected region, being close to 11% in Brazil which has accounted for nearly 65% of the reported cases of dengue fever in the American regions in the last 10 years [4].

The sequential introduction of different dengue serotypes in Brazil has contributed to the high incidence of the disease. The first epidemic, which occurred in the north-west Amazon region (Roraima state) in 1982, was associated with DENV-1 and DENV-4 [5]. After a 4-year interval without any confirmed dengue cases, an epidemic due to DENV-1 occurred in Rio de Janeiro state and was followed by several epidemics in highly populated cities in the south-east and north-east regions of Brazil [6]. In 1990–1991 an outbreak of DHF was recorded in Rio de Janeiro, and it was associated with DENV-2 [7]. The first reports on DENV-3 cases in Brazil occurred in 2000 [8] and a period of co-circulation of DENV-1, -2, and -3 was observed. Serotyping analysis of dengue virus strains isolated after 2002 showed that DENV-3 has spread to new areas of the country and replaced the other dengue virus serotypes [9], confirming a high infection capacity of this virus in both humans and vectors. In 2008, DENV-2 was the predominant serotype isolated in Rio de Janeiro and Ceará states, which increased the disease severity after almost seven years of DENV-3 circulation. However, DENV-3 is still the predominant serotype detected in Brazil, and in São Paulo state. The city of Ribeirão Preto (estimated population 558 137), located in the north-eastern region of São Paulo state, is among the cities with highest incidence of dengue in the state. Since 1990, yearly epidemics have occurred in the city of Ribeirão Preto, and, in 2006, the dengue incidence reached its peak with 1153 cases/100 000 inhabitants, but, fortunately, this incidence rate has been reduced in recent years [10].

Molecular analyses showed the existence of different variants among DENV serotypes which led to the recognition of different genotypes within each serotype. Genetic diversity of DENV-1 and DENV-2 identified five viral genotypes [11,12]. DENV-4 viruses were first separated into two distinct genotypes [13], but more recently, a third genotype has been identified [14]. DENV-3 was initially classified into four geographically distinct genotypes [15]; however, recent studies have suggested the existence of an additional group within genotype I that was named genotype V [16]. DENV-3 strains circulating in Brazil since 2000 belong to genotype III [17,18], and are closely related to strains from Sri Lanka and India, which are associated with DHF/DSS cases in those countries [15]. However, circulation of DENV-3, genotype I, has been reported in Minas Gerais state, a state located in the south-eastern region of Brazil, in 2003–2004 [19], and the co-circulation of genotypes III and V was reported in patients living in the northern region of Brazil during the 2002–2004 epidemics [20].

Molecular characterization of DENV with the identification of circulating genotypes is an important task for laboratories performing virological surveillance, as it has been demonstrated that intratypic variations among different genotypes could be associated with the disease severity [21,22,23]. In order to evaluate DENV-3 circulating genotypes in São Paulo state from 2003 to 2008, we analysed the NS1 region of DENV-3 isolated in the state from patients with different clinical presentations of dengue disease.
Materials and methods

Clinical samples

This study was conducted between 2003 and 2008 at the School of Medicine of Ribeirão Preto – University of São Paulo (FMRP-USP), and the samples were collected at the Clinical Hospital of FMRP-USP and at the Centro de Saúde Escola (CSE), a community health centre supervised by the School of Medicine of Ribeirão Preto – University of São Paulo. Ethical clearance was granted by the Research Ethical Committee of the Clinical Hospital of FMRP-USP and by the

<table>
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DENV-3 genotype III circulating in São Paulo, Brazil

Academic Board of Teaching and Research at CSE. Patients were recruited if they gave informed written consent and if the responsible physician suspected of dengue virus infection on clinical grounds based on the World Health Organization (WHO) guidelines[24]. Thirty-one isolates obtained from acute-phase serum samples from patients with DF or DHF (previously identified by RT-PCR as DENV-3) were selected for this study (Table 1). All serum samples were from patients living in the city of Ribeirão Preto or neighbouring cities.

RNA extraction and RT-PCR

Viral RNA was isolated from 140 µL of each serum sample using QIAamp® Viral RNA Mini Kit (QIAGEN, USA) according to the manufacturer’s directions. The RT-PCR was carried out using QIAGEN® OneStep RT-PCR kit in a 25 µL final volume containing 5 µL 5X One-Step RT-PCR buffer, 5 µL of dNTPs (10 mM), 0.5 pmol/µL of each primer, 1 µL of enzyme mix (Omniscript and Sensiscript) and 5 µL of RNA. The reaction was performed using serotype-specific primers described previously by Lanciotti[25] and also using primers described by Henchal[26]. The amplifications were performed under the following parameters: 50 °C for 30 min and 95 °C for 15 min for reverse transcription, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and final cycle of 72 °C for 10 min. The amplicons were detected on a 2% agarose gel electrophoresis stained with 1 µg/mL of ethidium bromide, and then visualized with the Kodak Electrophoresis Documentation and Analysis System 120 (Kodak, USA).

Sequencing, multiple sequence alignment and phylogenetic analysis

In order to obtain the nucleotide sequences of NS1 region from 31 virus samples, PCR amplicons were purified with Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and directly sequenced twice in both orientations using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, UK).

Phylogenetic analysis of the NS1 partial coding sequence (cds) also included 105 sequences of DENV-3 retrieved from GenBank. The new sequences obtained in this work were submitted to the GenBank (Table). Prior to phylogenetic analysis, DENV-3 sequences were aligned by using the multiple sequence alignment program CLUSTAL W vs.1.8[27], and edited using the BioEdit software v7.0.0[28] and MEGA 4.1[29]. Phylogenetic trees were constructed according to the best-fit model of nucleotide substitution implemented in ModelTest (TrN+G)[30] and were implemented with model Maximum Composite Likelihood (ML). The phylogenetic relationships among strains were reconstructed by the neighbour-joining (NJ) and maximum parsimony (MP) methods using Mega 4.1. Branch topology was verified by generating 1000 bootstraps for NJ and 10 for MP and a representative sequence of DENV-4 was used to root the trees. Phylogenetic approaches yielded identical or nearly identical topologies, but only NJ tree is shown.

Results and discussion

In order to characterize the DENV-3 strains circulating in Ribeirão Preto, São Paulo state, and to determine their relationships with other DENV-3 strains, clinical samples from different epidemics were analysed. This study is important due to the fact that despite having one of the highest incidence rates of dengue in the country, and having experienced epidemics caused by DENV-1 and DENV-2, the city of Ribeirão Preto has a very low mortality rate associated with dengue illness. Thirty-one samples collected from 2003 to 2008 in
DENV-3 genotype III circulating in São Paulo, Brazil

Ribeirão Preto were evaluated in this study. The majority of the patients had clinical symptoms of DF and only two patients presented with symptoms of DHF. The samples belonged mainly to adults ranging from 19 to 50 years and the male-to-female ratio was 1:1. The nucleotide sequences were obtained from viral RNA extracted directly from the patients’ sera, and then submitted to GenBank (Table). Sequence analysis revealed that all viruses sequenced in this study belonged to DENV-3 genotype III (Figure 1).

NS1 was chosen for DENV-3 genotyping because it has been shown to be important in disease outcome[31], for having a number of B and T cell epitopes[32,33], and being increasingly important in the diagnosis of dengue acute disease[26,34,35]. Thus, even though NS1 has not been well evaluated in genotyping studies, in our view, it represented a good target for genotyping DENV-3 isolates from Brazil[36]. All the NS1 DENV-3 sequences segregated into four distinct genotypes as established on the basis of C, prM and E genes in earlier studies[15,37]. Genotype IV is not shown due to unavailability of sequences. Genotype I comprised viruses isolated in Taiwan (China), Indonesia, Philippines, Timor-Leste and French Polynesia from 1978 to 2005. Genotype II consisted of viruses from Thailand, Taiwan (China), Bangladesh and Viet Nam isolated from 1987 to 2007. Genotype III was further divided in three clades: the Indian clade consisting of Indian isolates of 2006; the American clade consisting of isolates from Martinique, Argentina, Venezuela, Puerto Rico and Brazil isolated from 1999 to 2008; and the Asian clade comprising of isolates from Singapore, Sri Lanka and Taiwan (China) isolates. The existence of intragenotypic groups have also been observed by other authors, e.g. Messer[37] analysing the C, prM and a portion of E genes showed that genotype III viruses includes four groups: Latin America, East Africa and groups A and B from Sri Lanka. Kochel et al.[38] have also observed four main groups within genotype III, a South American group, a Central American group and also groups A and B from Sri Lanka. Our analyses showed a similar distribution of genotype III viruses in the three analysed trees; however, by analysing the available sequences for the NS1 region we have found that genotype III forms three main clades as already described above.

Within the American clade two main clusters within genotype III were observed (Figure 1). One cluster groups Puerto Rico DENV-3 strains isolated in 1998 to 2007 and Venezuelan strains isolated in 2001. The second cluster contains four groups of isolates. One group is composed by isolates from Puerto Rico and one Venezuelan strain of 2005. There is another minor group with Argentinean strains isolated in 2007, a big group with two Argentinean isolates, the Martinique isolate and some Brazilian isolates from São Paulo, Rio de Janeiro and Acre states, and another group with two isolates from Puerto Rico from 2003–2004 and the Brazilian isolates from Acre and São Paulo states.

Several studies have applied phylogenetic methods to analyse the epidemiology of dengue viruses and to understand the genetic relationships between them[37-40]. These studies have shown that dengue viruses could travel short distances between neighbouring countries[38,40] as well as long distances between continents[39]. In this study, it was possible to determine that Brazilian DENV-3 isolates grouped into separate clusters: the majority of samples isolated in Ribeirão Preto were grouped altogether and were related to another Brazilian strain from Acre state, whereas another isolates grouped with two Puerto Rico strains from 2003 and 2004, and the rest of sequences from Brazilian isolates were grouped with Argentinean and Martinique viruses (Figure 1). These different groups show that the viruses are constantly moving within the country, as described by Aquino et al.[18]. This tree also suggests that some
DENV-3 genotype III circulating in São Paulo, Brazil

Figure 1: Neighbour-joining phylogenetic tree of DENV-3 using a 419 bp fragment of the NS1 gene

[This phylogenetic tree is showing the presence of genotype III and V in Brazil. The Tamura-Nei nucleotide substitution model was used to estimate distance matrix. Sequences obtained in the present study are marked with ♦. It is possible to observe two main clusters within the American clade in genotype III. Cluster A is composed of strains from different Latin American countries and cluster B is composed only with strains from Puerto Rico and Venezuela. Bootstrap values greater than 80% were maintained in the tree. Horizontal branch lengths are drawn to scale.]
Brazilian strains are similar to the Puerto Rico strains. In fact, Puerto Rico strains are related to South American strains included in this analysis, which could indicate a strong relationship between these strains, reinforcing the theory of a single introduction of DENV-3 genotype III in Latin America\(^\text{18,41}\). Even though it is not clear whether the American genotype III lineage came from Africa or Asia, a migration analysis study done by Araujo et al.\(^\text{41}\) has suggested that the genotype III was first introduced into the Americas through Mexico, and from there these viruses spread to other countries in the region using independent migration routes to reach Central America, the Caribbean and South American countries.

Another interesting point to make is that these closely related strains are responsible for very distinct disease manifestations in each country, inducing severe disease in Puerto Rico and mild disease in Ribeirão Preto city.

Figure 2: Distribution of DENV-3 genotypes in Brazil

[A red ▲ represents the presence of genotype I; a green ■ represents the presence of genotype V; and a yellow ● represents the circulation of genotype III. The co-circulation of two genotypes is registered in two states of Brazil (Minas Gerais and Rondonia).]
We have shown that DENV-3 genotype III is the circulating genotype in São Paulo state and is still the most prevalent genotype in Brazil and the Americas. However, the recent reports of DENV-3 genotype I and V co-circulation in Brazil associated with cases of DF and DHF\textsuperscript{[19,20]} may change this situation. These genotypes were isolated in different states of Brazil (Figure 2), and given that Brazil is a tropical country, under optimal conditions, this DENV-3 genotype may spread to other areas of the country and cause a more severe disease.

Studies such as this one are important surveillance strategies that should be taken to follow the DENV path across the country, and to investigate the association of a specific genotype with distinct disease manifestations. Also, the emergence of DENV-3 genotype I and V in the Americas supports future research to follow up the movement of these genotypes in Brazil, and also to identify the possible introduction and emergence of these genotypes into other countries in South America. Moreover, given the limited options available for dengue control, active surveillance programmes with continuous monitoring of dengue infection in communities is still the best strategy available to detect the introduction of new serotypes/genotypes, and, consequently, to prevent the occurrence of epidemics. Genetic studies investigating substitutions across different genes within the DENV-3 viruses are also necessary to know, with more certainty, the evolutionary directions of DENV-3 in South America.

**Acknowledgments**

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**References**


DENV-3 genotype III circulating in São Paulo, Brazil


DENV-3 genotype III circulating in São Paulo, Brazil


Application of monoclonal antibody DSSC7 for detecting dengue infection in *Aedes aegypti* based on immunocytochemical streptavidin-biotin peroxidase complex assay (ISBPC)

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Abstract

*Aedes aegypti* mosquito is the important vector of dengue fever and chikungunya fever. Therefore, for virus detection in the mosquito, the possibility of cross-reactivity with chikungunya virus must be considered. The laboratory studies were aimed at characterizing the monoclonal antibody DSSC7, and its application for detecting dengue (DENV) antigen on the various organs of orally-infected *Ae. aegypti* in the paraffin-embedded tissue sections, viz. head squash, abdomen squash, based on immunocytochemical streptavidin-biotin-peroxidase complex (ISBPC) assay. Determination of the antibody class and subclass was based on antigen-mediated ELISA (enzyme-linked immunosorbent assay). The specificity of monoclonal antibody DSSC7 was determined by Western blotting method, using DENV-1, DENV-2, DENV-3, DENV-4, and chikungunya antigen. The presence of DENV antigen in the various organs of the orally-infected *Ae. aegypti* were microscopically optimized in the paraffin-embedded tissue section using ISBPC assay and monoclonal antibody DSSC7 (diluted 1:10, 1:50, 1:100 in phosphate buffer saline) as a primary antibody. The specificity of the immunocytochemical procedure is validated by negative controls and by positive controls that show that the antibody is binding to an appropriate structure. The monoclonal antibody DSSC7 recognize DENV complex specific and does not recognize chikungunya antigen. The monoclonal antibody belongs to IgG class, IgG1 subclass, and it is used as primary antibody to detect DENV infection in *Ae. aegypti* on tissue section in experimental infection based on ISBPC method. The infection rates of abdomen squash and head squash after incubation period of five days were 75% and 33.33% respectively.

Keywords: *Aedes aegypti*; Antigen; DENV; Chikungunya; Monoclonal antibody DSSC7.

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Introduction

The incidence of dengue fever (DF) occurs every year in Indonesia, but the number of cases were unusually high in at least 12 of the 32 provinces of the country during 2004. From 1 January to 4 April 2004 a total of 52,013 cases, mainly hospitalized cases of DENV and 603 deaths, have been registered with the Indonesian Ministry of Health. It was double the figure when compared with the same period in the previous year. Provinces in Java, including West Java, Central Java and East Java, were particularly severely affected, with more than 35% of the cases reported from DKI-Jakarta\[1\].

*Aedes aegypti* is the important vector of dengue haemorrhagic fever (DHF) as well as chikungunya fever\[2\]; therefore, detection of dengue virus in the mosquito is a matter of great concern in view of its cross-reactivity with chikungunya virus.

There are several methods of virus detection in the mosquito, such as the direct fluorescent-antibody (DFA) test on mosquito tissues, usually brain or salivary glands or head squashes, and reverse transcriptase polymerase chain reaction (RT-PCR). However, the DFA method is labour-intensive and requires fluorescent microscope and cryofreezer. RT-PCR provides a rapid serotype-specific diagnosis for RNA viruses. The method is rapid, sensitive, simple, and reproducible if properly controlled and can be used to detect viral RNA in human clinical samples, autopsy tissues, or mosquitoes\[3,4,5\]. Although immunofluorescence tests were used in the past, newer methods involving enzyme conjugates such as peroxidase and phosphatase in conjunction with either polyclonal or monoclonal antibodies have greatly improved\[6\].

Monoclonal antibodies (mAb) are used extensively in basic biomedical research, in the diagnosis of disease, and in the treatment of illnesses, such as infections and cancer. Antibodies are important tools used and recent research works have led to many medical advances. Producing mAb requires immunizing an animal, usually a mouse; obtaining immune cells from its spleen; and fusing the cells with a cancer cell (such as cells from a myeloma) to make them immortal, which means that they will grow and divide indefinitely. A tumor of the fused cells is called a hybridoma, and these cells secrete mAb. A major advantage of using mAb rather than polyclonal antisera is the potential availability of almost infinite quantities of a specific monoclonal antibody directed toward a single epitope (the part of an antigen molecule that is responsible for specific antigen-antibody interaction). To produce the desired mAb, the cells must be grown in either of two ways: by injection into the peritoneal cavity of a suitably prepared mouse (the in vivo, or mouse ascites, method) or by in vitro tissue culture. The mouse ascites method is generally familiar, well understood, and widely available in many laboratories; but the mice require careful handling to minimize pain or distress induced by excessive accumulation of fluid in the abdomen or by invasion of the viscera. When injected into a mouse, the hybridoma cells multiply and produce fluid (ascites) in its abdomen; this fluid contains a high concentration of antibody\[7,8,9\].

Monoclonal antibody against DENV-3 was produced by the Dengue Team of Gadjah Mada University through three-time fusion from 1993–1995. The first fusion generated 4 hybridomas (clones) producer, whereas the second fusion generated 13 clones producer; meanwhile, the third fusion generated 22 clones producer. Hybridoma cell lines producing the DENV antibodies were stored in the liquid nitrogen tank in the Laboratorium Hayati, Gadjah Mada University\[10\].
Among the hybridoma cell lines generated from the third fusion, DSSC7 and DSSF1 clones were still growing very well in the tissue culture, after storing it in the liquid nitrogen for several years\[11]. Once a cellular source of monoclonal antibody has been established in culture, it is usual to obtain a small quantity of ascitic fluid for further characterization before preparing a larger stock of antibody. A number of tests need to be carried out in order to relate the outcome to the final application and required specificity of the antibody such as isotyping, cross-reactivity and epitope analysis\[8,12].

The objective of this study was aimed at producing, characterizing, optimizing and applying the monoclonal antibody DSSC7 for the detection of DENV antigen on the various organs of the orally-infected *Ae. aegypti* in the paraffin-embedded tissue sections, viz. head squash, abdomen squash, based on immunocytochemical streptavidin-biotin-peroxidase complex (ISBPC) assay.

**Characterization of monoclonal antibody**

**Determination of isotype and cross-reactivity**

Determination of the antibody class and subclass was carried out based on antigen-mediated ELISA (enzyme linked immunosorbent assay). The specificity of monoclonal antibody DSSC7 was determined by Western blotting method, using DENV-1, DENV-2, and DENV-3, DENV-4, and chikungunya antigen\[13]. In a previous study, analysis of the DENV novel anti-dengue monoclonal antibodies (DSSC7 and DSSF1) with different binding specificities for DENV-1, DENV-2, DENV-3, DENV-4 and other flavivirus (Japanese B encephalitis virus) and chikungunya antigens were carried out based on indirect and inhibition ELISA. The result showed that the mAb DSSC7 showed high immunoreactivity toward DENV-1, DENV-2, DENV-3, DENV-4 and no cross-reactivity toward Japanese encephalitis and chikungunya antigen based on indirect ELISA\[10]. The viruses were obtained from Naval Medical Research Unit 2 (NAMRU-2), Jakarta. Antigens were prepared as follows: monolayers of C6/36 cells were grown to 90% confluence in 75 cm² flasks, then inoculated with dengue virus, and incubated for 1 hour at 28 °C in an atmosphere of 5% CO₂. Flasks were supplemented with 15 mL of maintenance medium (minimal essential medium, 2% fetal bovine serum [FBS], 1x non-essential amino acids, 100 U/mL of penicillin, and 100 µg/mL of streptomycin) and maintained at 28 °C in an atmosphere of 5% CO₂. Infection was monitored daily by an inverted microscope and cell supernatants were harvested at seven or eight days post-infection. Maintenance medium was changed after 2 to 4 days (depending on the virus) and the culture supernatants and infected cells were harvested when cytopathic effect was apparent throughout the monolayer. The culture supernatants were clarified by centrifugation for 10 minutes at 1000 rpm at 4 °C, and stored in
 aliquots at –80 °C until use. The infected monolayers were washed with PBS and lysed in 2 ml of a hypotonic buffer containing 1% TX-100. Intact nuclei were removed by brief centrifugation at 14 000 rpm in a micro centrifuge and the lysate supernatants (referred to as “lysates”) were aliquoted and stored at –80 °C until use. Virus stocks were stored as individual 1 mL aliquots in 20% FBS at –70 °C.

Immunoblotting

DENV-1, DENV-2, DENV-3, DENV-4 and chikungunya antigens were prepared from lysate supernatants in eppendorf tubes by mixing 25 µl antigen with gliserol 3 µl, SDS (10%) 3 µl, (bromophenol blue) and xylene cyanol 0.1% 2 µl and boiling it for a minute. Antigen were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were probed with DSSC7 antibodies from ascites at a 1:100 dilution. Bound antibodies were detected with an alkaline phosphatase-conjugated goat anti-mouse IgG antibody, followed by the addition of substrate containing 0.15 M sodium barbital 9 ml, BCIP 1mg in DMF 200 µl, NBT 1mg and 40 µl M MgCl₂. Protein loading and transfer efficiency were monitored by Coomassie blue and silver staining and the use of pre-stained molecular weight markers, respectively.

Detection of DENV infection of Ae. aegypti based on ISBPC assay

Positive control tissue specimens

Five-day-old male Ae. aegypti mosquitoes were injected intrathoracally with DENV-3 strain H87, and female Ae. aegypti were orally infected with the blood sample of patient. DENV-3 strain H87 virus was obtained from NAMRU-2 Jakarta. Blood samples from patients clinically diagnosed as DHF were also used as source of infection because they were positive dengue IgM and IgG based on the immunochromatography test. Head squash preparation made at incubation period of 8 weeks was used as positive controls. The slides were air-dried, wrapped in aluminum foil, and stored at –80 °C. Before use, the head squash preparation was fixed in acetone for 20 min at 4 °C.

Negative control tissue specimens

Negative controls comprised (1) uninfected Ae. aegypti mosquitoes from non-endemic area of DHF and Anopheles mosquitoes from Bantul district, Yogyakarta province. The Anopheles mosquitoes were used as negative control tissue because they are not the vector of dengue virus.

The infected mosquitoes were held in small cylindrical cages covered with mosquito netting, and they were incubated at 27±1 ºC and a relative humidity of 88±6%. The presence of dengue antigen on head squash of intrathoracally-infected male Ae. aegypti were detected based on ISBPC assay using mAb 2D3B10 and commercially mAb as positive controls. The mAb 2D3B10 is specific for DENV-3 and it reacts to viral E protein at the molecular weight of 57.9 kDa, whereas the commercially mAb reacts to DENV-1, DENV-2, DENV-3, and DENV-4. Negative control tissue specimens without primary antibody were used as negative controls. Positive result was detected as discrete brownish granular deposits throughout most fields having brain tissue. Negative result were detected as blue colour throughout most field having brain tissue and no brownish colour other than the chitinous mosquito tissues and non-specific background distinctly different from specific positive result⁶.

Infection of mosquitoes

Four- to-five-day-old Ae. aegypti females were orally infected with DENV-3 strain H87 virus. The infected mosquitoes were held in small cages
covered with mosquito netting and incubated at 27±1 °C and a relative humidity of 88±6%. One day after inoculation, the presence of dengue antigen on the various organs of orally-infected Aedes aegypti mosquitoes were optimized in the paraffin-embedded tissue section based on ISBPC assay using monoclonal antibody DSSC7 (diluted 1:10, 1:50, 1:100 in phosphate buffer saline) as a primary antibody. The presence of dengue antigen on the various organs of orally-infected Aedes aegypti mosquitoes were also observed 2 days, 3 days, 4 days and 5 days after inoculation in the paraffin-embedded tissue sections, head squashes and abdomen squashes preparations based on ISBPC assay using monoclonal antibody DSSC7 (1:50 in phosphate buffer saline) as a primary antibody.

**Result**

**Production of monoclonal antibody**

It was possible to produce 50 cc of antibody against DENV secreted by DSSC7 hybridoma in ascites of two BALB/c mice. Monoclonal antibodies in ascitic fluid were also secreted by DSSF1. Typical antibody concentrations in ascites of hybridoma-bearing mice ranged from 2–20 mg/l, and they represent a significant fraction of all protein present. In contrast, the antibody levels in culture supernatants of hybridomas were of the order of 5–50 µg/ml. It is, therefore, obvious that purification of antibodies from serum or ascites will be much easier than from culture supernatants. If antibodies must be purified from culture supernatants, affinity chromatography is usually the method of choice[7]. In this study, monoclonal antibody secreted by DSSC7 clone were not purified. According to Sutaryo et al.[10], monoclonal antibodies secreted by 3E9E12, 2D3B10 and 1D10C5 clones were purified by affinity chromatography on protein A, and the mAb concentrations were 2.955 mg/ml, 2.645 mg/ml and 2.485 mg/ml respectively.

**Characterization of monoclonal antibody**

**Determination of isotype**

A commercially available testing kit isotypic-specific reagent (Sigma ISO-2) based on antigen-mediated ELISA was used to perform isotype analysis during this study. In this study, monoclonal antibody secreted by DSSC7 clone belonged to IgG class, IgG1 subclass.

**Protein dengue**

Dengue virus is a single-stranded, positive-sense RNA virus with 11 kb unfragmented genome surrounded by a lipid bilayer envelope. RNA codes for three structural proteins and seven non-structural proteins. Three structural proteins are C (nucleocapsid), M (membrane-associated protein) and E (envelope protein), and the seven non-structural proteins have been named as NS1, NS2, NS3, NS4, NS5, NS6 and NS7[14]. The antigen used in this study comprised NS-3 protein (68.9 kDa), E protein (57.9 kDa), and NS-1 protein (48.0 kDa) (Figure 1).

**Figure 1:** DENV-3 viral protein (Yogyakarta isolate) gel (SDS-PAGE) that has been stained with silver dye showing strong bands at molecular weight of 68.9 kDa (NS-3 protein), 57.9 kDa (E protein), and a weak band at 48.0 kDa (NS-1 protein)
Monoclonal antibody secreted by a single hybridoma (DSSC7) was generated from the third fusion-recognized dengue complex-specific epitope (DENV-1, DENV-2, DENV-3, DENV-4) at molecular weight of about 48,000 Da (48 kDa) based on Western blotting analysis (Figure 2).

**Figure 2:** Monoclonal antibody DSSC7 (1:100) recognized dengue antigen (DENV-1, DENV-2, DENV-3, DENV-4) epitope at molecular weight of 48 kDa showing no cross-reactivity toward chikungunya antigen based on Western blotting method.

According to Henchal and Putnak\[14\], nonstructural (NS-1) DENV protein has molecular weight of 48,000 Dalton. The NS-1 protein could be found in the cell, at plasma membrane, or it is secreted out of the cell during the infection. The figure also showed that there was no cross-reactivity between the mAb DSSC7 toward chikungunya antigen. This finding supported the previous study that the mAb DSSC7 showed high immunoreactivity toward DENV-1, DENV-2, DENV-3, DENV-4 and no cross-reactivity toward Japanese encephalitis and chikungunya antigen based on indirect ELISA\[10\]. Meanwhile, the previous study showed that mAb secreted by a single hybridoma (2D3B10), which generated from the second fusion, recognized a DENV-3 virus type-specific determinant (epitope), based on immune dot blot assay by using DENV-1, DENV-2, DENV-3, and DENV-4 antigen (Yogyakarta isolate)\[15\].

**Detection of DENV infection of *Ae. aegypti* based on ISBPC assay**

**Positive control**

Dengue antigen was detected as brownish colour in the cytoplasm of infected cell throughout most fields having brain tissue of infected *Ae. aegypti* mosquito with DENV-3 strain H87 at incubation period of 11 days under light microscope based on ISBPC assay using mAb 2D3B10 (1:50) as primary antibody. The previous study showed that the mAb 2D3B10 recognized DENV-3 viral E protein. Antigen was also detected as discrete brownish granular deposits throughout most fields having brain tissue of orally-infected *Ae. aegypti* mosquito with DENV-3 strain H87 at incubation period of 8 days under light microscope based on ISBPC assay using commercial mAb as primary antibody. According to the manufacturer (Chemicon Laboratory), the mAb reacts to DENV-1, DENV-2, DENV-3, and DENV-4. Both brownish colour in the cytoplasm of infected cells and discrete brownish granular deposits throughout most fields having brain tissue of orally-infected *Ae. aegypti* mosquito with DENV-3 strain H87 at incubation period of 11 days were shown under light microscope based on ISBPC assay using mAb DSSC7 (1:50) as primary antibody. The negative result was shown on head squashes of uninfected *Ae. aegypti* from non-endemic area of DHF and head squashes of *Anopheles* mosquitoes as blue colour throughout most fields having brain tissue and no brownish colour other than the chitinous mosquito tissues and non-specific background distinctly different from specific positive result (Figure 3).
Application of monoclonal antibody DSSC7 for detecting dengue infection in Aedes aegypti

Figure 3: Head squashes immunocytochemical preparation of orally-infected *Ae. aegypti* with DENV-3 strain H87 at incubation period of 11 days showing positive reaction as brownish coloration in the cytoplasm of infected cells with mAb 2D3B10 (A), and showing positive reaction as discrete brownish granular deposits throughout most fields having brain tissue with commercial mAb as positive controls (B). Positive reaction was also shown as brownish coloration in the cytoplasm and discrete brownish granular deposits with mAb DSSC7 as primary antibody (C) and negative reaction was shown on head squashes of uninfected *Ae. aegypti* preparation as blue coloration (D).

The result indicated that DENV antigen was detected on head squash preparation of *Ae. aegypti* infected with DENV-3 at incubation period of 11 days based on ISBPC assay using mAb DSSC7 at concentration of (1:50) as primary antibody.

The result indicated that DENV antigen was also detected on paraffin-embedded section of *Ae. aegypti* orally infected with DENV-3 at incubation period of 1 day based on ISBPC using mAb DSSC7 at concentration of (1:10) and (1:50) and mAb 2D3B10 (1:50) as primary antibodies (Table).
The table indicates that there was no significant difference between the positive rate of mosquito preparation using mAb DSSC7 1:10, DSSC7 1:50, and mAb 2D3B10 1:50 as primary antibody based on Fisher’s Exact Test \((P=1; >0.05)\). The result also revealed that there was no significant difference between the negative control and mosquito preparation using mAb DSSC7 (1:100) as primary antibody \((P=0.62; >0.05)\).

The monoclonal antibody DSSC7 (diluted 1:50 in PBS) was optimum to be used as primary antibody to detect DENV antigen in various organs of orally-infected *Ae. aegypti* in the tissue section preparation based on ISBPC assay under light microscope (Figures 4–7). The DENV viral antigen was also detected on head squash and abdomen squash preparation (Figure 8).

In the head squash preparation, positive result was detected as discrete brownish granular deposits throughout most fields having brain tissue. DENV viral antigen were immunolocalized to the cytoplasm of >100 cells per field at 100x magnification in high infection, 10–100 cells per 100x field in moderate infection. Low infection cannot be seen at 100x magnification, and the infection can be seen at 400x magnification. Meanwhile, negative result was detected as blue colour throughout most fields having brain tissue and no brownish color other than the chitinous mosquito tissues and non-specific background distinctly different from specific positive result. The result also exhibited that DENV viral antigens were detected in oocytes at incubation period of 4 days and 5 days and it also indicated that the infection rate of abdomen squash and head squash at incubation period of five days were 75% and 33.33% respectively.

**Table:** Result of microscopic examination of dengue antigen on paraffin-embedded section of *Ae. aegypti* orally infected with DENV-3 at incubation period of 1 day based on ISBPC assay using mAb DSSC7 1:10, DSSC7 1:50, DSSC7 1:100 from ascitic fluid and purified mAb 2D3B10 1:50

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**Figure 4:** Tissue section preparation of orally-infected *Ae. aegypti* with DENV-3 at difference incubation period showing dengue viral antigen (brownish colour) in various organs of orally-infected *Ae. aegypti* in the mid gut in the cytoplasm cells at incubation period of 1 day (B), 3 day (C), haemocytes at incubation period of 1 day (E), and fat at incubation period of 1 day (H) 2 day (I) based on ISBPC assay using monoclonal antibody DSSC7 (1:50). Blue colour is shown in the cytoplasm cells of mid gut (A), hemocyte (B), and fat (G) as negative control without primary antibody.
Application of monoclonal antibody DSSC7 for detecting dengue infection in Aedes aegypti

**Figure 5:** Tissue section preparation of orally-infected Ae. aegypti with DENV-3 at different incubation periods showing dengue viral antigen in the brain – deutocerebrum (A), protocerebrum, tritocerebrum ang facet eye (C) based on ISBPC assay using monoclonal antibody DSSC7 (1:50)
Figure 6: Tissue section preparation of orally-infected Ae. aegypti with DENV-3 at incubation period of 2 days showing dengue viral antigen in the cytoplasm of salivary gland based on ISBPC assay using monoclonal antibody DSSC7 (1:50)

Figure 7: Tissue section preparation of orally-infected Ae. aegypti with DENV-3 showing dengue viral antigen in the ovary at incubation period of 1 day (B) and at incubation period of 2 days (C, D) based on ISBPC assay using monoclonal antibody DSSC7 (1:50)
Figure 8: Head squash and abdomen squash of orally-infected Ae. aegypti with DENV-3 at incubation period of 5 days showing dengue viral antigen in the brain (A), and eggs in the ovary (D,F) based on ISBPC assay using monoclonal antibody DSSC7 (1:50)

Discussion

Hybrid cells (DSSC7) producing antibodies were grown in flask containing complete RPMI medium. The hybrid cells were inoculated intraperitoneal into pristane-treated BALB/c mice. After 2–3 weeks, the ascitic fluid produced by each mouse was collected with a syringe or by puncturing the abdomen, and then stored for further steps, while the hybrids were stored in liquid nitrogen. It has produced 50 cc of antibody against DENV secreted by DSSC7 hybridoma in ascites of two BALB/c mice. Monoclonal antibody secreted by DSSC7 clone belongs to IgG class, IgG1 subclass. An important early characterization test of any panel of antibodies is the analysis of whether they react with the same, close or totally different epitopes. Monoclonal antibody secreted by a single hybridoma (DSSC7) which generated from the third fusion recognized as DENV complex specific epitope and showing no cross-reactivity toward CHIK antigen based on Western blotting analyses. The mAb DSSC7 reacts to non-structural protein (NS1).

The viral NS1 protein circulates in the sera of infected patients throughout the clinical phase of the disease. Novel diagnostic tests based on NS1 detection have been recently developed and marketed. During in vitro infection, the flavivirus NS1 protein is expressed as an intracellular membrane-associated form essential for viral replication[16,17]. In solution, secreted NS1 protein behaves as a hexamer; it circulates and accumulates in the sera of dengue virus-infected patients throughout the clinical phase of the
A recent study demonstrated that soluble NS1 protein binds to endothelial cells and, following recognition by anti-NS1 antibodies, could contribute to plasma leakage during severe dengue virus infection\(^{21}\). The detection of secreted NS1 protein represents a new approach to the diagnosis of acute dengue infection. A recently developed, commercially available diagnostic test based on dengue NS1 antigen-capture ELISA (Platelia Dengue NS1 Ag test, Bio-Rad Laboratories, Marnes la Coquette, France), was investigated in two studies (one in South America and the other in South-East Asia); the test had an overall sensitivity of 88.7% and 93.4% in the two studies, with 100% specificity\(^{22,23}\).

The monoclonal antibody DSSC7 was able to be used as primer antibody to detect DENV infection in \textit{Ae. aegypti} on tissue section, head squash, abdomen squash preparation of orally-infected \textit{Ae. aegypti} with DENV-3 at different incubation periods based on ISBPC assay. Therefore, it will be suitable for detecting the DENV infection in \textit{Aedes}.

Immunocytochemistry is a powerful method for the identification of proteins or antigen in cells and tissues. However, this method is dependent on the specificity of the antibody binding to the epitope of the protein used as an immunogen. The specificity of the result depends on two independent criteria: the specificity of the antibody and of the method used. The antibody specificity is best determined by immunoblot and/or immunoprecipitation. The specificity of the method is best determined by both a negative control, replacing the primary antibody with non-immune serum, and a positive control, using the antibody with cell, known to contain the protein or antigen\(^{24}\).

This study followed the principle of ISBPC techniques to demonstrate antigen in cells or tissue as follows (Figure 9).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image.png}
\caption{The principle of immunohistochemistry (immunoperoxidase SBC) techniques to demonstrate antigen in cells or tissue}
\end{figure}

1. The endogenous peroxidase activity may be destroyed by treating a specimen with hydrogen peroxide solution.
2. The non-specific background is eliminated by incubating the specimen with non-immune serum.
3. The primary antibody to specific antigen is incubated to target antigens. This is followed by addition of biotinilated second antibody which serves as the linker between the primary antibody and peroxidase-streptavidin conjugate.
4. Streptavidin-peroxidase is then added to bind to the biotin residues on the linking antibody.

The presence of enzyme can be revealed by addition of a mixture of substrate-chromogen solution. The enzyme peroxidase will catalyse the substrate, hydrogen peroxide, and convert the chromogen to a brown coloured deposit demonstrating the location of the antigen.
This finding indicated that the transovarial infection of DENV in *Ae. aegypti* could be observed both in the tissue section and abdomen squash preparation of the mosquito based on immunocytochemical assay using mAb DSSC7 as primary antibody. Other study (Umniyati, unpublished data) indicated that natural transovarial infection of DENV virus in *Ae. aegypti* were identified in Gondokusuman sub-district, Yogyakarta Municipality, post outbreak of DHF in 2004, based on the identification of DENV antigen in the brain on head squash of mosquito reared from larvae and pupae collected from domestic household wells and water containers for bathing (*bak mandi*) without blood feeding, using mAb DSSC7. Based on these reports, natural transovarial transmission in the domestic household wells and *bak mandi* have great epidemiological significance and may play an important role in the maintenance of virus in nature, and may act as reservoirs of these viruses. Therefore, vector surveillance and control activities in domestic household wells as part of an active community DENV control strategy should be performed.

Several studies suggest the existence of transovarial DENV transmission in *Aedes* infected female mosquitoes, allowing propagation of virus to their progeny. Such a process would allow it to act as a reservoir for virus maintenance during interepidemic periods. The transovarial transmission rate of DENV was found to be seven times higher in the high susceptible isofemale lines than in the low susceptible lines. The rate of transovarial transmission initially increased in the initial two generations (F1-F2), but in further generations it was steady. It was also reported that a higher transovarial transmission rate in the progeny was obtained from the longer desiccated egg[25].

**Conclusion**

Monoclonal antibody DSSC7 recognized DENV complex specific epitope at molecular weight of 48 kDa (NS1) protein and showed no cross-reactivity toward chikungunya antigen. The monoclonal antibody belongs to IgG class, IgG1 subclass, and it is able to be used as primary antibody to detect DENV infection in *Ae. aegypti* on tissue section, head squash and abdomen squash preparation in experimental infection based on ISBPC method.

**Suggestion**

Monoclonal antibody DSSC7 could be applied as primary antibody to investigate the natural transovarial infection of DENV in *Ae. aegypti*, for developing vector surveillance and early warning system to anticipate a DHF epidemic.

**Acknowledgement**

The authors gratefully thank the Dean of Faculty of Medicine, and the Head of Department of Parasitology, the Head of Department of Pathology – Anatomy, Gadjah Mada University, for their support in the conduct of these studies. Thanks are also due to Suprihatin, Purwono, T.M. Joko, Agustin and Yunadir for their valuable assistance in the laboratory.

**References**


Application of monoclonal antibody DSSC7 for detecting dengue infection in Aedes aegypti


Application of monoclonal antibody DSSC7 for detecting dengue infection in Aedes aegypti


Enhancement of MHC class I binding and immunogenic properties of the CTL epitope peptides derived from dengue virus NS3 protein by anchor residue replacement

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dDepartment of Infectious Biology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki, Japan

Abstract

The immunogenecity of the defined H-2Kd-restricted, murine cytotoxic T lymphocyte (CTL) epitopes of dengue viruses were examined for CTL induction in epitope peptide / H-2Kd tetramer assays. The peptides used in the study included those corresponding to amino acid (a.a.) residues 298-306 (GYISTRVEM) of NS3 of dengue virus types 2 and 4 (named DENV-2/4), and to a.a. residues 299-307 (GYISTRVGM) of NS3 of dengue virus types 1 and 3 (named DENV-1/3), and their respective modified epitope peptides, DENV-2/4-9L (GYISTRVEL) and DENV-1/3-9L (GYISTRVG), in which the C-terminal residue M of the original epitope peptide was replaced by L, in order to provide the complete H-2Kd-binding motif. Immunization of BALB/c mice with the original epitope peptide, DENV-2/4 or DENV-1/3, did not induce specific CTLs, while that with the modified epitope peptide, DENV-2/4-9L or DENV-1/3-9L, induced epitope peptide/H-2Kd tetramer-binding CD8+ cells indicating specific CTLs. Competition-based binding assay with biotinylated epitope-related reference peptides (DENV-2/4-9L-Biotin and DENV-1/3-9L-Biotin) demonstrated that the modified epitope peptide, DENV-2/4-9L and DENV-1/3-9L, had higher avidity to H-2Kd than the respective original epitope peptides. These results indicate that modification of dengue virus-derived CTL epitope peptide by replacing a.a. residue at the position of anchor residue increases the binding avidity to MHC class I, resulting in the induction of specific CTLs. The strategy to enhance the immunogenecity of CTL epitope peptide may contribute to investigation of CTL biology in dengue virus infection.

Keywords: Dengue virus; CTL epitope; Binding motif; Anchor residue; MHC class I; Affinity; Immunogenicity.

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Introduction

MHC class I – restricted, CD8+ cytotoxic T lymphocytes (CTLs) play an important role in the elimination of virus-infected and tumor cells by antigen-specific lysis\(^1,2,3\). They recognize specific structures on the surface of target cells as their antigens, which are composed of self MHC class I molecules and the peptides of 8 to 11 a.a. in length. The peptides are derived from the endogenous protein, fitting to the groove of the MHC class I molecule\(^4,5,6\). Recently, there has been a great deal of interest in the CTL epitope-based immunomodulation therapy, including peptide vaccines, mainly for the treatment of malignancies\(^7,8,9,10,11,12\).

Dengue viruses, of which there are four serotypes, cause dengue fever and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS), the severe manifestation of infection, which is often fatal\(^13\). They are of a great global health importance, particularly in the tropical regions, causing up to 100 million infections including a couple of thousands of deaths each year\(^14\). Protective immunity against the same serotype virus is life-long, while re-infection with a different serotype can occur. The secondary infections are often complicated with DHF/DSS, suggesting that pre-existing immunity to a different serotype virus may contribute to the pathogenesis of DHF/DSS\(^15\). One of the strategies for the prevention of dengue virus infection is vaccination. However, a vaccine has not been developed yet. Immunization with dengue vaccine may have the potential risk of inducing DHF/DSS manifestation. In this context, peptide vaccine based on CTL epitopes that binds to MHC class I molecule is thought to be a candidate, because it is anticipated that this vaccine induces the least cross-reaction due to its minimal component. Furthermore, immune response elicited by immunization with a single epitope peptide is thought to be much simpler than those elicited by virus infection, which evokes multiple immune responses against various epitopes on viruses. Establishment of a strategy by immunization with a single dengue virus-derived epitope peptide, thus, is anticipated to facilitate dissection of immunobiology of dengue virus infection. This strategy is expected to contribute to investigation of the immunopathogenesis (DHF/DSS may be involved).

Rothman et al.\(^{16}\) elucidated CTL responses to an immunodominant epitope on the dengue virus NS3 protein in BALB/c mice after primary infection. They mapped the minimal CTL epitopes consisting of nine amino acids. By using CTL clones, they defined the H-2K\(^d\)-restricted CTL epitopes, which corresponded to the amino acid (a.a.) residues 298-306 (GYISTRVEM) of NS3 of dengue virus types 2 and 4, or a.a. residues 299-307 (GYISTRVGM) of NS3 of dengue virus types 1 and 3\(^{16,17}\). Immunodominant epitopes for human CD8+ CTLs have been also defined on dengue virus NS3 protein\(^{18,19,20,21}\) suggesting that NS3 is the main target for the CTL response in humans as well.

Previously, by using cytotoxicity assay, we have demonstrated that immunization with the modified epitope peptide, DENV-1/3-9L (GYISTRVGL) or DENV-2/4-9L (GYISTRVEL), in which the original epitope peptide C-terminal residue M was replaced by residue L to provide a complete H-2K\(^d\)-binding motif\(^{22,23}\), induced specific CTLs with little affection to antigen specificity, while immunization with original one, DENV-1/3 or DENV-2/4, which corresponded to the defined CTL epitope spanning a.a. residues 299-307 (GYISTRVGM) of NS3 of dengue virus types 1 and 3 or which corresponded to that spanning a.a. residues 298-306 (GYISTRVEM) of NS3 of dengue virus types 2 and 4, respectively, did not\(^{24}\).
In the present study, we analysed immunogenic properties of the modified epitope peptides for CTL induction more in detail. We first examined whether immunization with the modified epitope peptide induces epitope peptide/H-2Kd tetramer-binding CD8+ cells, the specific CTLs more significantly than that with the original peptide, as was observed in cytotoxicity assays. We also analysed the avidity of the original epitope peptides and modified peptides to H-2Kd molecule by competition-based binding assay, using the biotinylated epitope peptide-related reference peptides. We demonstrated that the modification of the original epitope peptides by substitution of the C-terminal a.a. residue increased the binding avidity to H-2Kd, and that this modification enhanced the immunogenicity of the epitope peptides in CTL induction.

**Materials and methods**

**Mice:** Female BALB/cAJcl mice were purchased from Clea Japan (Tokyo, Japan), and maintained in the Animal Facility of Kinki University School of Medicine under the conventional condition. Mice were used at the ages of 6 to 12 weeks.

**Cells:** Murine mastcytoma line P815 (H-2d), fibroblast cell line L929 (H-2b), and cell line L-Kd-172 (kindly provided by Dr Jack R. Bennink, NIAID, NIH), which is H-2Kd gene transfectant cell line derived from L929, were maintained in RPMI 1640 medium (Sigma, St. Louis, MO) with 5x10^{-5} M 2-mercaptoethanol (2-ME), 100 U penicillin, 100 mg/ml streptomycin, 10 mM HEPES, and 10% heat-inactivated fetal calf serum at 37 °C in 5% CO2.

**Peptides:** The sequences and derivation of peptides DENV-2/4 (GYISTRVEM), DENV-2/4-9L (GYISTRVEL), DENV-2/4-9L-Biotin (GYISTRVELGEAC-Biotin), DENV-1/3 (GYISTRVGM), DENV-1/3-9L (GYISTRVGL), and DENV-1/3-9L-Biotin (GYISTRVGLGEAC-Biotin) are shown in the Table. They were synthesized with 9-fluorenylmethoxycarbonyl chemistry by Sigma Genosis Japan (Ishikari, Hokkaido, Japan). Peptides were purified by reverse phase HPLC in the conditions of 5% to 80% gradient elution with acetonitrile in 0.1%

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Virus derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-2/4</td>
<td>GYISTRVEM</td>
<td>Dengue virus types2/4</td>
</tr>
<tr>
<td>DENV-2/4-9L</td>
<td>GYISTRVEL</td>
<td>NS3 298-306</td>
</tr>
<tr>
<td>DENV-2/4-9L-Biotin</td>
<td>GYISTRVELGEAC-Biotin</td>
<td>*1</td>
</tr>
<tr>
<td>DENV-1/3</td>
<td>GYISTRVGM</td>
<td>Dengue virus types1/3</td>
</tr>
<tr>
<td>DENV-1/3-9L</td>
<td>GYISTRVGL</td>
<td>NS3 299-307</td>
</tr>
<tr>
<td>DENV-1/3-9L-Biotin</td>
<td>GYISTRVGLGEAC-Biotin</td>
<td>*3</td>
</tr>
</tbody>
</table>

Peptide sequence is expressed by Dayhoff’s one-letter code of amino acid except “-Biotin”.

Note that the difference between DENV-2/4 and DENV-1/3 is only the residue at position 8, that is “E” in DENV-2/4 or “G” in DENV-1/3.

*1 The sequence corresponds to the residues NS3 298-306 of dengue virus types 2 and 4 except the residue of C-terminus substituted for “L”, and to the residues NS3 299-307 of kunjin virus.

*2 The sequence corresponds to the residues NS3 298-310 of dengue virus type 4 except the substituted residue “L” and the biotinylated residue “C”.

*3 The sequence corresponds to the residues NS3 299-307 of dengue virus types 1 and 3 except the residue of C-terminus substituted for “L”.

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trifluoroacetic acid using TSKgel ODS-80Ts QA column (Toso, Tokyo, Japan). The purity of peptides was determined to be more than 95.0%, and mass-spectrometry (Applied Biosystems Voyager System 1162) analysis proved every peptide molecular weight to be the anticipated one.

**Immunization and CTL induction:** Immunization and CTL induction were carried out as described before. Briefly, mice were immunized by subcutaneous injection with peptide DENV-2/4-9L or peptide DENV-1/3-9L with complete Freund adjuvant (CFA). Then, more than three weeks later, lymph node cells prepared from the draining lymph nodes were co-cultured with irradiated syngeneic spleen cells pulsed with the same peptide in EHAA medium (Sigma), supplemented with 100 µg/ml nucleic acid precursors, 2mM L-glutamine, 5x10^{-5}M 2-ME, 100U penicillin, 100 µg/ml streptomycin, 10mM HEPES, and 10% fetal calf serum (FCS) at 37 °C in 5% CO₂. On day 4, half volume of the medium was replaced with a fresh one, and 10 U recombinant mouse IL-2 was added. On day 7, viable cells were harvested and used as CTL effector cells.

**Detection of MHC tetramer-binding cells:** The phycoerythrin (PE)-labelled MHC tetramer composed of four monomeric complexes which consisted of the modified H-2K^{d} heavy chain, β₂-microglobulin, and peptide DENV-2/4-9L (named DENV-2/4-9L-tetramer) or peptide DENV-1/3-9L (named DENV-1/3-9L-tetramer) were prepared by MBL Co., Ltd. (Nagoya, Aichi, Japan). The CTL effector cells (1x10⁶) suspended in 100 µl of phosphate buffered saline (PBS) containing 0.02% NaN₃ (PBS/Na₃) were incubated with 5 µl of FITC-conjugated anti-mouse CD8 antibody (Immunotech, Marseille, France) and 5 µl of DENV-2/4-9L-tetramer or DENV-1/3-9L-tetramer at room temperature for 30 minutes. Cells were washed three times with PBS/Na₃ at 4 °C, then fixed with 1 ml of PBS containing 1% paraformaldehyde, and analysed by a FACS Calibur (Becton Dickinson, San Jose, CA) and CELL Quest™ version 3.3 software.

**Analysis of the avidity between MHC molecule and peptide:** Peptide binding competition assay was carried out to determine the relative avidity of the original epitope peptides (DENV-2/4 and DENV-1/3) and that of the modified epitope peptides (DENV-2/4-9L and DENV-1/3-9L) to H-2K^{d} molecule, using H-2K^{d}-gene transfectant L-K^{d}-172 cells and the biotinylated, epitope-related reference peptide. Briefly, 1x10⁶ of L-K^{d}-172 cells suspended in complete medium were incubated with various concentration of non-biotinylated, original or modified epitope peptide in the presence of 1 µM of biotinylated, epitope-related reference peptide (DENV-2/4-9L-Biotin or DENV-1/3-9L-Biotin, respectively) at 37 °C for two hours. Cells were washed three times with PBS/Na₃ at 4 °C, and then incubated with 0.5 µg of streptoavidin-conjugated Cy-Chrome™ (SA-CyC) (BD PharMingen) at 4 °C for 30 minutes. Cells were washed three times, fixed with 1 ml of PBS containing 1% paraformaldehyde, and subjected to FACS analysis.

Geometric mean fluorescence intensity (MFI) was measured, and we defined delta MFI (ΔMFI) by subtracting the background MFI (i.e. MFI in the case stained with SA-CyC only). Per cent fluorescence intensities were calculated by the formula: % fluorescence intensity = 100 x (ΔMFI with the non-biotinylated epitope peptide/ΔMFI without the non-biotinylated epitope peptide), and plotted against non-biotinylated epitope peptide concentrations in logarithmic scale. Concentration of the non-biotinylated epitope peptide that correspond to the per cent fluorescence intensity fifty (IC₅₀; 50% inhibitory concentration) was obtained by the chart, and used as an index of the relative avidity of each peptide to H-2 K^{d} molecule.
**Results and discussion**

Because of incomplete set of anchor residues (only one anchor residue Y at position 2) in the original epitope peptide for preparation of peptide/H-2K\(^d\) tetramers, and because of less affection to the specificity recognized by CTLs with replacement of C-terminal residue M by L\(^{24}\), we prepared phycoerythrin-labelled DENV-1/3-9L/H-2K\(^d\) and DENV-2/4-9L/H-2K\(^d\) tetramers. We then examined whether the modified epitope peptides, DENV-2/4-9L and DENV-1/3-9L, induce DENV-2/4-9L/H-2K\(^d\) tetramer-binding cells and DENV-1/3-9L/H-2K\(^d\) tetramer-binding cells, respectively, more efficiently than the original peptides, DENV-2/4 and DENV-1/3, as was observed in cytotoxicity assays.

Immunization with the modified peptide DENV-2/4-9L followed by in vitro stimulation with same peptide-pulsed APC induced 5.59% of CD8-positive DENV-2/4-9L/H-2K\(^d\) tetramer-binding cells, while that with the original peptide DENV-2/4 did 1.25% (Fig. 1A). Similarly, immunization with the modified peptide DENV-1/3-9L followed by in vitro stimulation with same peptide-pulsed APC induced 5.81% of CD8-positive DENV-1/3-9L/H-2K\(^d\) tetramer-binding cells, while that with the original peptide DENV-1/3 did 0.34% (Fig. 1B). In addition, immunization with PBS emulsified with CFA followed by in vitro stimulation with non-pulsed spleen cells (i.e., pulsed with PBS only) induced 0.15% of CD8-positive DENV-1/3-9L/H-2K\(^d\) tetramer-binding cells (data not shown). The results indicate that immunization and in vitro stimulation with the modified epitope peptides, which possessed a complete binding motif to H-2K\(^d\) (i.e., Y at position 2 and hydrophobic L or I at C-terminus of 9-mer peptide), efficiently induced CD8-positive, epitope peptide/H-2K\(^d\) tetramer-binding cells implying CTLs, as was observed in cytotoxicity assays.

It has been reported that the affinity of a peptide for MHC binding is an important parameter determining the immunogenicity of an MHC-presented epitope peptide\(^{25,26}\). Indeed, only peptides derived from tumour-associated antigens, hepatitis B virus or influenza A virus with a high binding affinity for MHC class I molecules, have been demonstrated to be immunogenic enough for inducing CTL response\(^{27-30}\). However, so far, there had been no report regarding the correlation of binding affinity to MHC class I molecules and immunogenicity of dengue virus-derived CTL epitope peptides for CTL induction. Thus, we assessed the avidity of the modified epitope peptides, DENV-2/4-9L and DENV-1/3-9L, to H-2K\(^d\) molecules in comparison with the original epitope peptides, DENV-2/4 and DENV-1/3, respectively. Rothman et al.\(^{16}\) demonstrated that elongation of the epitope peptide at the C-terminus did not affect the specific lysis of the target cells\(^{17}\). We, thus, prepared the biotinylated epitope peptides of 13-mer elongated at the C-terminal side with 4 a.a. residues spanning NS3 307-310 of dengue virus types 2 and 4, except for C-terminal residue A replaced with C to conjugate biotin, DENV-2/4-9L-Biotin (GYISTRVELGEAC-Biotin) and DENV-1/3-9L-Biotin (GYISTRVGLGEAC-Biotin) (Table), and examined whether they bound to H-2K\(^d\) molecules. L-Kd-172 cells, which are H-2K\(^d\)-gene transfectant cells derived from cell line L929, gained increased fluorescence intensity in dose-dependent manner after incubation with various concentration of the biotinylated-modified epitope peptide (Fig. 2). In contrast, no specific staining with SA-CyC was observed in L929 cells incubated with the biotinylated epitope peptide. The results indicate that the biotinylated epitope peptides, DENV-2/4-9L-Biotin (GYISTRVELGEAC-Biotin) and DENV-1/3-9L-Biotin (GYISTRVGLGEAC-Biotin) (Table), bound to H-2K\(^d\) molecule specifically, suggesting that they could be used as epitope-related reference peptides for binding competition.
**Figure 1:** Induction of the tetramer-binding cells by immunization with the modified epitope peptides

(Source of the CTLs was pooled lymph node cells (5 mice each).

(A) CD8-positive, DENV-2/4-9L/H-2Kd-tetramer-binding cells (right upper quadrant) accounted for 5.59% of the analysed cells after immunization and in vitro stimulation with modified epitope peptide DENV-2/4-9L, while 1.25% after those with the original epitope peptide DENV-2/4.

(B) CD8-positive, DENV-1/3-9L/H-2Kd-tetramer-binding cells (right upper quadrant) accounted for 5.81% of the analysed cells after immunization and in vitro stimulation with the modified peptide DENV-1/3-9L, while 0.34% after those with the original epitope peptide DENV-1/3.)
Based on the findings mentioned above, we carried out competitive binding inhibition assays with non-biotinylated peptide (i.e. the original epitope peptide or the modified epitope peptide), and compared the relative avidity to H-2K\textsuperscript{d} molecule. L-K\textsuperscript{d}-172 cells were incubated with various concentrations of the non-biotinylated peptides in the presence of 1 mM biotinylated reference peptide, and stained with SA-CyC. The geometric mean fluorescence intensity was measured by FACS analysis. Per cent fluorescence intensities was plotted against non-biotinylated peptide concentrations, and 50% inhibitory concentrations (IC\textsubscript{50}) were estimated to evaluate the avidities, meaning that the lower the IC\textsubscript{50} value is, the higher the avidity of the peptide to H-2K\textsuperscript{d} is. Per cent fluorescence intensity decreased in a dose-dependent manner. No specific staining was observed on L929 cells with the biotinylated peptide.

**Figure 2:** Specific binding of the biotinylated peptide to H-2K\textsuperscript{d} molecule

[L929 cells (H-2\textsuperscript{k}) and L-K\textsuperscript{d}-172 cells (H-2\textsuperscript{k} + H-2K\textsuperscript{d}) were incubated with the biotinylated peptide at concentrations of 10 \mu M, 1 \mu M, 0.1 \mu M and 0.01 \mu M. After incubation, cells were stained with SA-CyC, and analysed by flowcytometry. The cells without incubation with biotinylated peptide, but stained with SA-CyC were used as controls. Fluorescence intensity increased with DENV-2/4-9L-Biotin (A) and DENV-1/3-9L-Biotin (B) on L-K\textsuperscript{d}-172 cells in a dose-dependent manner. No specific staining was observed on L929 cells with the biotinylated peptide.]
Avidity and immunogenecity of dengue virus CTL epitope

manner as concentration of the non-biotinylated peptide increased (Fig. 3). The IC_{50} of DENV-2/4-9L (4.5 µM) was 10.7 times lower than that of DENV-2/4 (48.0 µM). Similarly, the IC_{50} of DENV-1/3-9L (7.3 µM) was 2.4 times lower than that of DENV-1/3 (17.8 µM). These results indicate that the modified epitope peptides (DENV-2/4-9L and DENV-1/3-9L) demonstrated higher avidity to H-2K\(^d\) molecule than the original epitope peptides (DENV-2/4 and DENV-1/3), respectively. These findings, taken together, indicate that modification of dengue virus-derived CTL epitope peptide by replacing a.a. residue at the position of anchor residue to provide a complete binding motif for MHC class I increases the binding avidity to MHC class I, resulting in immunogenicity augmentation for CTL induction by its immunization as has been reported about the CTL epitope peptides derived from tumour-associated antigens or other viruses, and that this strategy may be applicable for induction of dengue virus-specific CTLs by immunization with other CTL epitope peptides of relatively poor immunogenicity.

**Figure 3:** Increase in the avidity of the peptides to H-2K\(^d\) molecule by substitution of amino acid residue to provide a complete H-2K\(^d\)-binding motif

[L-K\(^d\)-172 cells were incubated with various concentrations of non-biotinylated peptides in the presence of 1 µM of the biotinylated peptide. Percent fluorescence intensity (See Materials and methods.) was plotted against logarithmic scale of non-biotinylated peptide concentration, and lines were drawn by using Microsoft Excel 2003® software (DENV-2/4-9L : y = – 44.237x + 78.87, DENV-2/4 : y = – 94.446x + 208.76, DENV-1/3-9L : y = – 57.164x + 99.239, DENV-1/3 : y = – 47.947x + 109.9). The similar experiments were repeated more than three times, and the representative data are shown. The fluorescence intensity of the biotinylated peptide decreased in a dose-dependent manner as concentration of the non-biotinylated peptides increased.

(A) *A : The 50 % inhibitory concentration (IC_{50}) of peptide DENV-2/4-9L was 4.5 µM.

*B : The IC_{50} of peptide DENV-2/4 was 48.0 µM.

(B) *C : The IC_{50} of peptide DENV-1/3-9L was 7.3 µM.

*D : The IC_{50} of peptide DENV-1/3 was 17.8 µM.

Note that X-axis (peptide concentration) is expressed as logarithmic scale. The modified epitope peptides (DENV-2/4-9L and DENV-1/3-9L) with the substitution of C-terminal residue L for M, that possessed a complete H-2K\(^d\)-binding motif, demonstrated higher avidity to H-2K\(^d\) molecule than the original epitope peptides (DENV-2/4 and DENV-1/3).]
There was no significant difference in the avidity to H-2K^d between DENV-2/4-9L (IC_{50}: 4.5 µM) and DENV-1/3-9L (IC_{50}: 7.3 µM). However, there was an apparent difference between DENV-2/4 (IC_{50}: 48.0 µM) and DENV-1/3 (IC_{50}: 17.8 µM). It was reported that the immunogenicity of MHC class I-restricted peptide is determined not only by binding affinity to MHC molecule but also by T cell repertoire[31]. Because immunization with the modified epitope peptide, DENV-1/3-9L, induced the CTLs that lysed the target cells pulsed with the original epitope peptide, DENV-1/3, and that these CTLs are cross-reactive to the other original epitope peptide, DENV-2/4 (data not shown), it is not plausible that low immunogenicity of DENV-1/3 is attributed to poor T cell repertoire. We, thus, think of other two possibilities. One possibility is that the avidity to H-2K^d needs to be between 7.3 µM and 17.8 µM of IC_{50} to induce CTLs. The other possibility is that not only anchor residues but also the non-anchor residues at secondary position contribute to increased MHC class I avidity and peptide-MHC complex stability[32,33]. In the present study, we only evaluated the binding avidity. Thus, a detailed study including analysis of the stability of H-2K^d/DENV-1/3 complex and H-2K^d/DENV-2/4 complex will be a future subject.

In this paper, we present the first report that modification of dengue virus-derived CTL epitope peptide increasing the binding avidity to MHC class I augmented immunogenicity for CTL induction. The strategy to augment the immunogenicity of dengue virus-derived CTL epitope peptide, established here, is expected to contribute to the immunobiology analysis of dengue virus infection, such as investigation of the immunopathogenesis, protection against infection, and vaccine development.

**Acknowledgments**

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**References**


Avidity and immunogenecity of dengue virus CTL epitope


Liver function tests in patients with dengue viral infection

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Abstract

To assess the frequency and degree of hepatic dysfunction in patients with dengue infection, records of 214 serologically confirmed cases of dengue infection with available biochemical liver tests, admitted to our tertiary-care institute, were analysed. Patients were classified as classical dengue fever (DF) – 81.3%, dengue haemorrhagic fever (DHF) – 13.6% and dengue shock syndrome (DSS) – 5.1%. The mean age was 31.6 years (male:female = 3.3:1). Deranged total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin and prothrombin time index (PTI) [international normalized ratio (INR)] was present in 19.5% (29/143), 97.7% (209/214), 93.9% (199/214), 32.6% (47/144), 29.1% (44/151) and 15.5% (22/156) patients respectively. The mean (± SE) total bilirubin, AST, ALT, ALP, albumin and INR values were 0.93 ± 0.09 mg/dl, 353.7 ± 49.6 U/L, 218.6 ± 27.2 U/L, 135.2 ± 6.5 U/L, 3.2 ± 0.04 g/dl and 1.2 ± 0.03 respectively. The mean value of AST was significantly higher than ALT. The degree of rise of AST and ALP was significantly more in DHF and DSS, as compared to DF; but the frequency of rise was similar in all groups. Mean serum bilirubin, ALT and ALP values were significantly higher in patients with haemorrhage as compared to those without haemorrhage, in patients with secondary dengue infection as compared to primary infection, and in non-survivors. Hepatic dysfunction was very common in all forms of dengue infection, with AST rising significantly more than ALT. Serum bilirubin, ALT and ALP were significantly higher in patients with DSS, haemorrhage, sequential infection and non-survivors. While preferentially high AST may serve as an early indicator of dengue infection, high bilirubin, ALT and ALP may act as poor prognostic markers.

Keywords: Dengue infection; Hepatic dysfunction; Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Alkaline phosphatase (ALP).

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Liver function tests in patients with dengue viral infection

Introduction

Dengue infection, an arthropod-borne viral haemorrhagic fever, continues to be a major challenge to public health, especially in South-East Asia[1]. It has a wide geographical distribution and can present with a diverse clinical spectrum[2]. Although dengue virus is a non-hepatotropic virus, liver injury due to dengue infection is not uncommon and has been described since the 1960s[3]. The degree of liver dysfunction in dengue infection varies from mild injury with elevation of aminotransferases alone to severe injury with jaundice and even fulminant hepatic failure[2,4]. The liver dysfunction could be a direct viral effect or an adverse consequence of dysregulated host immune response against the virus[5]. Several outbreaks of dengue infection have been reported from India[5,6,7,8]. However, large clinical studies documenting hepatic involvement in dengue infection, especially in adults, are scarce.

The aim of this study was to assess the frequency and degree of hepatic dysfunction in patients with dengue infection presenting to a tertiary-care medical facility in Punjab. Punjab is a state located in north India with an area of 50 362 sq km, extending from latitudes 29° 30’ to 32° 32’ North and longitudes 73° 55’ to 76° 50’ East.

Materials and methods

An outbreak of dengue infection was noted in the state of Punjab during the last quarter of 2006. During this period, 2205 patients presented to Dayanand Medical College and Hospital, Ludhiana, Punjab, India, with acute febrile illness. The provisional clinical diagnosis of dengue infection was made on the basis of a history of fever of short duration (<15 days) and constitutional symptoms, with or without haemorrhagic manifestations. These patients were screened for dengue-specific IgM and IgG antibodies by IgM and IgG capture ELISA respectively (Panbio Diagnostics, Brisbane, Australia). IgM and IgG antibody positivity was found in 366 and 76 patients respectively. The mortality rate was 1.9% (7/366). Medical records of all adult patients with available liver function tests (LFT) (n=214) were analysed for their clinical presentation, degree of hepatic involvement, hospital course and outcome. Hepatitis markers (HBsAg, IgM antibody to HAV and HEV) were done in the clinically suspected cases (53 patients).

Patients were divided into three categories: (a) classical dengue fever (DF); (b) dengue haemorrhagic fever (DHF); and (c) dengue shock syndrome (DSS), according to the WHO criteria[9]. Non-survival was taken as poor outcome and survival as good outcome in our study.

The statistical analysis was done using the Fischer’s exact test and Student’s unpaired t-test for significance of difference in proportions and means between two groups respectively.

Results

Of the 214 patients reactive for dengue virus-specific IgM antibody, dengue virus-specific IgG antibody was also positive in 43 (20.1%) patients. One hundred and seventy four (81.3%) patients were classified as dengue fever, 29 (13.6%) as dengue haemorrhagic fever, and 11 (5.1%) as dengue shock syndrome. Further, 17.8% (31/174) patients with DF, 13.8% (4/29) patients with DHF, and 72.7% (8/11) patients with DSS had positive IgG antibody, indicating sequential infection. Markers for hepatitis A, B, C and E viruses were done in 53 patients and found to be negative in all.

The mean age of patients in our study was 31.6 years with a range of 15 to 80 years. The maximum number of patients (n=71; 33.2%) belonged to the age group of 21–30 years. There were 164 (76.6%) males and 50 (23.4%) females (male:female ratio = 3.3:1).
The main presenting symptoms were fever (100%, 214/214), myalgias (43%, 92/214), haemorrhagic manifestations (40.6%, 87/214), vomiting (37.4%, 80/214) and abdominal pain (20%, 43/214). Encephalopathy was observed in 3 (1.4%) patients; one each belonged to DF, DHF and DSS groups. The gastrointestinal tract was the most common site of haemorrhage (n=42/214, 19.6%), followed by skin rash/petechia (n=22/214, 10.3%). The clinical examination revealed hepatomegaly in 26 (12.1%) patients, splenomegaly in 4 (1.9%) and ascites in 4 (1.9%) patients. The mean (±SE) haemoglobin, haematocrit, total leukocyte count and platelet count at admission were 13.8 ± 0.17 gm/dl, 40.6 ± 0.5%, 6123 ± 339 cells/mm³ and 48.5 ± 2.6 x 1000/mm³ respectively. Hepatic dysfunction, in the form of deranged total bilirubin, AST, ALT, ALP, albumin and PTI (INR) was present in 19.5% (29/143), 97.7% (209/214), 93.9% (199/214), 32.6% (47/144), 29.1% (44/151) and 15.5% (22/156) patients respectively. The mean (±SE) total bilirubin, AST, ALT, ALP, albumin and INR values were 0.93 ± 0.09 mg/dl, 353.7 ± 49.6 U/L, 218.6 ± 27.2 U/L, 135.2 ± 6.5 U/L, 3.2 ± 0.04 g/dl and 1.2 ± 0.03 respectively. The mean value of AST was significantly higher than the mean ALT value (p=0.017). A comparison between the degree of rise of ALT and AST is shown in Fig. 1. Significantly more percentage of patients had AST values >10 times elevated as compared with ALT (p < 0.0001).

**Figure 1:** Comparison between ALT and AST elevation in patients with dengue infection (n=214)

<table>
<thead>
<tr>
<th>Percentage of patients</th>
<th>AST</th>
<th>ALT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of times increased</td>
<td>≤1x</td>
<td>&gt;1-3x</td>
</tr>
<tr>
<td>DF</td>
<td>7.0%</td>
<td>19.2%</td>
</tr>
<tr>
<td>DHF</td>
<td>5.1%</td>
<td>11.1%</td>
</tr>
<tr>
<td>DSS</td>
<td>3.3%</td>
<td>14.4%</td>
</tr>
</tbody>
</table>

DF= Classical dengue fever, DHF = Dengue hemorrhagic fever, DSS = Dengue shock syndrome, ULN = Upper limit of normal
**Liver function tests in patients with dengue viral infection**

**Table 1**: Comparison of biochemical liver test derangements in patients with DF, DHF and DSS

<table>
<thead>
<tr>
<th>Liver biochemical test</th>
<th>DF (n=174)</th>
<th>DHF (n=29)</th>
<th>p value*</th>
<th>DSS (n=11)</th>
<th>p value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. bilirubin (mg/dl)</td>
<td>0.79 ± 0.08</td>
<td>0.89 ± 0.13</td>
<td>0.623</td>
<td>2.7 ± 0.97</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>No (%) of patients with &gt; ULN</td>
<td>16/118 (13.5%)</td>
<td>4/14 (28.6%)</td>
<td>2.26</td>
<td>9/11 (81.8%)</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>277.18 ± 20.5</td>
<td>478.4 ± 163.5</td>
<td>0.016</td>
<td>1234.7 ± 787.6</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>No (%) of patients with &gt; ULN</td>
<td>169/174 (97.1%)</td>
<td>29 (100%)</td>
<td>1.00</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>174.6 ± 11.05</td>
<td>254.5 ± 80</td>
<td>0.059</td>
<td>819 ± 431.5</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>No (%) of patients with &gt; ULN</td>
<td>161/174 (92.5%)</td>
<td>27/29 (93.1%)</td>
<td>1.00</td>
<td>11 (100%)</td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>122.44 ± 4.7</td>
<td>166.7 ± 28.9</td>
<td>0.007</td>
<td>241.5 ± 60</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>No (%) of patients with &gt; ULN</td>
<td>36/119 (30.3%)</td>
<td>6/15 (40%)</td>
<td>0.056</td>
<td>5/10 (50%)</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.3 ± 0.04</td>
<td>3.0 ± 0.17</td>
<td>0.013</td>
<td>2.7 ± 0.18</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>No (%) of patients with &lt; LLN</td>
<td>32/121 (26.4%)</td>
<td>8/20 (40%)</td>
<td>0.283</td>
<td>4/10 (40%)</td>
</tr>
<tr>
<td>PTI (n=158)</td>
<td>1.1 ± 0.02</td>
<td>1.2 ± 0.06</td>
<td>0.069</td>
<td>1.3 ± 0.11</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>No (%) of patients with &gt; ULN</td>
<td>12/127 (9.4%)</td>
<td>4/20 (29%)</td>
<td>0.236</td>
<td>6/11 (54.5%)</td>
</tr>
</tbody>
</table>

All data expressed as mean ± standard error (SE).

† = Number of patients in which the particular test was available.

*p value = p value between DF and DHF groups.

**p value = p value between DF and DSS groups.

DF = Classical dengue fever, DHF = Dengue hemorrhagic fever, DSS = Dengue shock syndrome, ALP = Alkaline phosphatase, ALT = Alanine transaminase, AST = Aspartate transaminase, PTI = Prothrombin time index, LLN = Lower limit of normal, ULN = Upper limit of normal.

A comparison between the mean values of various liver biochemical tests in different groups of dengue infection, and the number of patients with abnormal tests is shown in Table 1 and Figures 2(a), 2(b) and 2(c). All the liver biochemical tests were significantly more deranged in the DSS group as compared to the DF group. Also, the percentage of patients...
**Table 2: Comparison of biochemical liver test derangements between various patient groups**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Bilirubin (mg/dl)</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>ALP (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (164)</td>
<td>0.99 ± 0.1</td>
<td>380 ± 63.4</td>
<td>241 ± 34.9</td>
<td>129 ± 6.7</td>
</tr>
<tr>
<td>Female (50)</td>
<td>0.69 ± 0.08</td>
<td>266.4 ± 40.3</td>
<td>144.3 ± 14.9</td>
<td>159 ± 17.4</td>
</tr>
<tr>
<td>p value</td>
<td>0.109</td>
<td>0.333</td>
<td>0.131</td>
<td>0.053</td>
</tr>
<tr>
<td><strong>2. Haemorrhage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With haemorrhage (n=87)</td>
<td>1.13 ± 0.18</td>
<td>464.5 ± 116.3</td>
<td>290.4 ± 64.0</td>
<td>154.6 ± 13.6</td>
</tr>
<tr>
<td>Without haemorrhage (n=127)</td>
<td>0.79 ± 0.08</td>
<td>277.8 ± 24.4</td>
<td>169.3 ± 11.8</td>
<td>121.8 ± 5.5</td>
</tr>
<tr>
<td>p value</td>
<td>0.05</td>
<td>0.064</td>
<td>0.028</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>3. Gastrointestinal haemorrhage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GI haemorrhage (n=42)</td>
<td>1.4 ± 0.32</td>
<td>591.4 ± 212.9</td>
<td>326.9 ± 107.7</td>
<td>158.9 ± 22.3</td>
</tr>
<tr>
<td>Without haemorrhage (n=127)</td>
<td>0.79 ± 0.08</td>
<td>277.8 ± 24.4</td>
<td>169.3 ± 11.8</td>
<td>121.8 ± 5.5</td>
</tr>
<tr>
<td>p value</td>
<td>0.008</td>
<td>0.016</td>
<td>0.016</td>
<td>0.021</td>
</tr>
<tr>
<td><strong>4. Primary/secondary dengue infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM reactive (n=171)</td>
<td>0.68 ± 0.05</td>
<td>312 ± 33.1</td>
<td>190 ± 16.7</td>
<td>123.5 ± 5.8</td>
</tr>
<tr>
<td>IgM and IgG reactive (n=43)</td>
<td>1.8 ± 0.36</td>
<td>519 ± 56.8</td>
<td>332 ± 117.2</td>
<td>174.5 ± 20.9</td>
</tr>
<tr>
<td>p value</td>
<td>0.0001</td>
<td>0.096</td>
<td>0.036</td>
<td>0.0012</td>
</tr>
<tr>
<td><strong>5. Survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivors (n=207)</td>
<td>0.84 ± 0.07</td>
<td>349.9 ± 50.6</td>
<td>207.5 ± 25.1</td>
<td>130.1 ± 5.9</td>
</tr>
<tr>
<td>Non-survivors (n=7)</td>
<td>3.15 ± 1.6</td>
<td>463.3 ± 250.5</td>
<td>544.7 ± 372.1</td>
<td>241.7 ± 72.4</td>
</tr>
<tr>
<td>p value</td>
<td>0.0001</td>
<td>0.684</td>
<td>0.026</td>
<td>0.001</td>
</tr>
</tbody>
</table>

All data expressed in mean ± standard error.

ALP = Alkaline phosphatase, ALT = Alanine transaminase, AST = Aspartate transaminase
having hyperbilirubinemia and deranged PTI was significantly more in the DSS group as compared to the DF group. On a comparison of the DF and DHF groups, it was observed that the mean values of AST, ALP and albumin were significantly different in the two groups.

A comparison of the biochemical liver tests in various patient subgroups (Table 2) showed that serum bilirubin, ALT and ALP values were significantly higher in patients with haemorrhage as compared to those without haemorrhage, and were even higher in the patients with GI haemorrhage. It was also noted that bilirubin, ALT and ALP were significantly higher in patients with secondary infection as compared to primary infection and in non-survivors as compared to those who survived. The AST value was significantly more deranged only in patients with GI haemorrhage as compared to those without haemorrhage. There was no significant difference in the LFTs between male and female patients and in the patients with or without encephalopathy.

Discussion

The clinical and biochemical impact of dengue virus on liver function was studied on 214 serologically confirmed cases of dengue infection during an outbreak in north India in 2006. In this study, DHF and DSS were present in 13.6% (29/214) and 5.1% (11/214) patients respectively. This is in accordance with the results of a recent study from Delhi[10] (DHF and DSS in 9.3% and 2.2% respectively). However a few other studies had reported a higher percentage of DHF[5,6,7].

Impaired consciousness was seen in only three patients in our study. LFT abnormalities in these patients were not significantly different from those without encephalopathy, indicating that liver failure was not the cause of altered sensorium in these patients. Other possible reasons for the neurological symptoms in dengue infection are metabolic acidosis, severe disseminated intravascular coagulation, gross haemorrhage or edema in the brain, or hyponatremia due to excessive fluid administration.

Hepatomegaly was observed in 12.1% patients in this study, compared to 17.6%–20.4% in other Indian studies[5,6]. The relative higher incidence of hepatomegaly reported by Sharma et al.[5] could be attributed to the fact that all their patients belonged to the DHF group. Although liver size does not correlate with disease severity, an enlarged liver is observed more frequently in shock than in non-shock cases[9]. In our study, too, hepatomegaly was more frequent in the DSS group as compared to DF group (45.5%; 5/11 v/s 10.9%; 19/174; p < 0.05).

Biochemical liver dysfunction, in the form of increased transaminases, was found in most of the patients in our study (93.9%–97.7%), similar to the results of other studies[5,6,7,10]. However, in a study by Souza et al.[11], AST and ALT were deranged only in 63.4% and 45% patients respectively. In our study, increased levels of ALP and serum bilirubin were noted in a smaller proportion of patients, in accordance with the results of Itha et al.[7].

The aspartate aminotransferase (AST) levels in dengue infection tend to be greater than alanine aminotransferase (ALT) levels[12,13]. This differs from the pattern in viral hepatitis but is similar to that seen in alcoholic hepatitis. The exact cause of this is uncertain, but it has been suggested that it may be due to excess release of AST from damaged monocytes during dengue infection[10]. We also noted a preferential elevation of liver enzymes, with AST being significantly higher than ALT. This abnormality may act as an early indicator of dengue infection.
Liver function tests in patients with dengue viral infection

Comparing the three subgroups of dengue infection (DF, DHF and DSS), we observed that the frequency of liver dysfunction (raised AST, ALT and ALP) was equally common in all the groups (Table 1, Figures 2(a), 2(b) and 2(c)). Similar results were noted in another Indian study[7]. However, Wahid et al.[14] found liver dysfunction to be more common in DHF than in DF patients.

The severity of hepatic dysfunction in dengue infection has been associated with disease severity. Indeed, liver injury has been proposed to be a good positive predictive factor for the development of DHF[13]. We noted a greater degree of hepatic injury in the DHF group (significantly higher values of AST and ALP) and DSS group (significantly higher values of all biochemical liver tests) as compared to the DF group, suggesting that the degree of liver injury may be related to the severity of dengue infection. Similar data have been suggested by Seneviratne et al.[2] and Souza et al.[11]. However, in two other studies, the degree of elevation of liver enzymes in the DF and DHF groups was not significantly different[7,14].

In our study, the mean bilirubin, ALT and ALP values were significantly higher in patients with haemorrhage as compared to those without haemorrhage, and were even higher in those with GI haemorrhage. Wahid et al.[14] also observed that the ALT and ALP levels were significantly higher in DHF patients with spontaneous bleeding than those without bleeding (p < 0.05), while Nguyen et al.[15] noted significantly higher elevation of AST and ALT in DHF patients with gastrointestinal haemorrhage. A possible reason for this could be an ischaemic injury to the liver due to acute blood loss.

In the present study, the mean bilirubin, ALT and ALP values were significantly higher in patients with secondary dengue infection as compared to those with primary dengue infection, while the mean AST value in the two groups was similar. Nguyen et al.[15] observed that the results of transaminases did not differ significantly between the two groups, while Souza et al.[11] noted that transaminases were significantly higher in cases with secondary infection.

Jaundice in dengue infection has been associated with fulminant liver failure and by itself is a poor prognostic factor[15]. We found hyperbilirubinemia to be significantly more common in patients with DSS, in patients with haemorrhage and in non-survivors. Thus, our observations support the fact that high bilirubin may act as a bad prognostic marker in patients with dengue infection.

The percentage of patients with deranged PTI was significantly more in the DSS group as compared to the DF group. There was no significant difference of biochemical liver tests between males and females in our study. However, in another study[11], transaminases were significantly higher among females.

Liver dysfunction was found to be significantly more severe in non-survivors as compared with survivors (Table 2). The causes of mortality (n = 7) were adult respiratory distress syndrome in three patients, underlying cardiac dysfunction causing arrhythmias in one patient, underlying decompensated cirrhosis in one patient, sepsis in one patient and refractory shock in one patient.

We thus report the liver function test abnormalities and their clinical implications in a large group of patients with dengue infection. Non-availability of the baseline LFT values in the same patient group is a possible limiting factor. Future studies with assessment of viral titers, and their correlation with LFTs, may help to define the cause of hepatic injury in dengue infection.
In summary, liver involvement is very common in all forms of dengue infection, with AST rising significantly more than ALT. Serum bilirubin, ALT and ALP are significantly higher in patients with DSS, haemorrhage and sequential infection and in non-survivors. Therefore, while preferentially high AST may serve as an early indicator of dengue infection, high bilirubin, ALT and ALP may act as poor prognostic markers.

References


Changing clinical manifestations of dengue infection in north India

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Abstract

Dengue infection is endemic in many parts of India, including the state of Uttar Pradesh. This study describes the changing clinical picture of dengue viral infections observed by us in children admitted to a teaching hospital in Lucknow, India.

A total of 139 children with suspected dengue were admitted during this period, of which 124 could be tested by dengue IgM capture ELISA and 102 were positive. However, only 80 of these 102 patients could be followed up. Average age was 5.9 (±3.1) years and 87.5% of them were from rural areas. The male:female ratio was 1.6:1. Seizures were observed in 45% cases, altered sensorium in 53.7%, vomiting in 41.2%, haemorrhage in 38.8%, skin rash in 37.5%, abdominal pain in 25%, headache in 18.8% and jaundice in 2% cases. Gastrointestinal tract was the commonest site of bleeding. On examination, edema was present in 47.5% cases, hepatomegaly in 62.5%, splenomegaly in 60.0% and hypotension in 10.0% cases. The investigations revealed a low platelet count of less than 100,000/mm³ in 60.3% cases. Mean liver enzyme levels were mildly raised. Definitions of WHO criteria for DHF were present in only 18 (22.5%) cases. Mean total duration of fever in survivors was 14.9±7.3 days. The overall fatality rate in hospital was 5.0%.

The results indicated a significant proportion of children presented with little-described features of encephalopathy, edema, splenomegaly and prolonged fever rather than the typical dengue presentation. These features were not noted during the past epidemics and in previous years.

Keywords: Dengue viral infection; Dengue fever; Dengue encephalopathy; Dengue haemorrhagic fever.

Introduction

Dengue infection is the most important arbovirus infection of humans and the most important tropical infectious disease after malaria. Although dengue fever is a very old disease, more complicated forms of the infection – dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) have been recognized in the last century[11]. In India, the

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first virologically confirmed epidemic occurred in Calcutta (now known as Kolkata) and the eastern coast of India in 1963–1964[2]. All four serotypes of the virus are circulating now[3]. A major widespread epidemic of DHF occurred in 1996 involving areas around Delhi, and, since then, there has been a remarkable resurgence of the infection in north Indian plains that include the state of Uttar Pradesh. Once considered an urban problem, it has now penetrated into rural areas also due to high population density and other factors[4].

As observed in this part of the country, dengue infection is showing an increasing trend. The illness occurs throughout the year with a peak during monsoon and post-monsoon season due to high vector density. Major outbreaks have occurred in this region in 2003 and 2006. Besides the increasing frequency of the infection, even the manifestations observed have been varied. In 2008, we observed manifestations of dengue which were not commonly observed in the previous years. We, therefore, undertook to prospectively study and describe the varied manifestations of dengue viral infection as seen in hospitalized children in northern India.

Materials and methods

This study was conducted in the Department of Paediatrics of Chhatrapati Shahaji Maharaj Medical University (CSMMU) Hospital, Lucknow – a tertiary-care teaching hospital.

Over a period of five months from August to December 2008, we carefully screened admissions for suspected diagnosis of dengue, as made by the admitting physician, usually on the basis of febrile illness with rash, or bleeding with or without alteration of consciousness. A detailed clinical history was taken, physical examination was performed and baseline investigations were noted using a structured proforma. Laboratory investigations and treatment of the patients were decided by the treating physician. Tests that were usually done included haemoglobin (Hb), total and differential leukocyte count (TLC and DLC), platelet count (PLT count), haematocrit (HCT), and liver function tests (LFT) including prothrombin time (PT), serum proteins and albumin. Lumbar puncture and cerebrospinal fluid (CSF) examination was usually done in patients who presented with a history of altered sensorium and/or seizures. Serology for dengue infection was also done as part of routine clinical work. Blood samples were collected and transported to the Department of Microbiology, CSMMU. They were tested for dengue IgM by antibody capture ELISA (Mac ELISA) test using commercial kits marketed by IVD Research Inc., USA. Thus, the study was purely observational.

Diagnosis of dengue infection, DF and DHF was made according to WHO criteria[5]. If altered sensorium was present, the child was classified as dengue encephalopathy (DE) with or without DHF.

Statistical analysis

Data were entered into a Microsoft Excel sheet. Frequencies, mean and standard deviation was calculated by using Epi-info software for statistical analysis.

Results

During the period of the study, a total of 139 suspected dengue patients were admitted to the hospital, of which 124 could be tested for dengue IgM, and 102 were positive. Of these 102 patients, 80 could be followed up and documented. The clinical features of these 80 patients are given in Table 1.
In the 80 serologically-confirmed cases, 18 (22.5%) satisfied WHO criteria for DHF, while 43 (53.7%) had encephalopathy. Seven patients with DHF had encephalopathy also. One case with clinical presentation of Guillian Barré syndrome was also dengue IgM-positive. Mean duration of fever at presentation was 10.7±6.2 days. After follow-up, mean total duration of fever in survivors was found to be 14.9±7.3 days. Other main complaints besides fever were: swelling over body, rash, altered sensorium, seizures, vomiting, bleeding, abdominal pain and headache.

On examination, a discrete maculopapular erythematous rash was present in 37.5% cases. Edema was present in 47.5% children. It was generalized in 36.3%, over extremities in 6.2% and facial in 5% children. Haemorrhage was found in 31 (38.8%) children. Gastrointestinal tract was the most common site for bleeding (23.7%) followed by the skin (16.2%).

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Clinical features</th>
<th>Dengue IgM +ve cases (n=80) No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mean age in years ± SD</td>
<td>5.9±3.1</td>
</tr>
<tr>
<td>2.</td>
<td>Male:Female ratio</td>
<td>1.6:1</td>
</tr>
<tr>
<td>3.</td>
<td>Residence in rural area</td>
<td>70 (87.5)</td>
</tr>
<tr>
<td>4.</td>
<td>Fever</td>
<td>80 (100)</td>
</tr>
<tr>
<td>5.</td>
<td>Average duration of fever at admission in days ± SD</td>
<td>10.7±6.2</td>
</tr>
<tr>
<td>6.</td>
<td>Altered sensorium</td>
<td>43 (53.7)</td>
</tr>
<tr>
<td>7.</td>
<td>Seizures</td>
<td>36 (45)</td>
</tr>
<tr>
<td>8.</td>
<td>Abdominal pain</td>
<td>20 (25)</td>
</tr>
<tr>
<td>9.</td>
<td>Haemorrhage</td>
<td>31 (38.8)</td>
</tr>
<tr>
<td>10.</td>
<td>Diarrhoea</td>
<td>5 (6.2)</td>
</tr>
<tr>
<td>11.</td>
<td>Vomiting</td>
<td>33 (41.2)</td>
</tr>
<tr>
<td>12.</td>
<td>Headache</td>
<td>15 (18.8)</td>
</tr>
<tr>
<td>13.</td>
<td>Rash</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td>14.</td>
<td>Edema</td>
<td>38 (47.5)</td>
</tr>
<tr>
<td>15.</td>
<td>Hepatomegaly</td>
<td>50 (62.5)</td>
</tr>
<tr>
<td>16.</td>
<td>Splenomegaly</td>
<td>48 (60.0)</td>
</tr>
<tr>
<td>17.</td>
<td>Hypotension</td>
<td>8 (10.0)</td>
</tr>
<tr>
<td>18.</td>
<td>Meningeal signs</td>
<td>7 (8.7)</td>
</tr>
<tr>
<td>19.</td>
<td>Raised intracranial tension (ICT)</td>
<td>5 (6.2)</td>
</tr>
<tr>
<td>20.</td>
<td>Jaundice</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td>21.</td>
<td>Total duration of fever in days ± SD</td>
<td>14.9±7.3</td>
</tr>
<tr>
<td>22.</td>
<td>DHF</td>
<td>18 (22.5)</td>
</tr>
<tr>
<td></td>
<td>DE</td>
<td>43 (53.7)</td>
</tr>
<tr>
<td></td>
<td>DE+DHF</td>
<td>7 (8.7)</td>
</tr>
<tr>
<td>23.</td>
<td>Duration of hospital stay in days ± SD</td>
<td>7.6±4.7</td>
</tr>
<tr>
<td>24.</td>
<td>Mortality in hospital</td>
<td>4 (5.0)</td>
</tr>
</tbody>
</table>

Table 1: Clinical features of dengue IgM-positive cases
Conjunctival haemorrhage and epistaxis were noted in 2 patients (4.1%) and 1 patient (2.0%) respectively. Intracranial haemorrhage was suspected in one child, but cranial imaging could not be done in this case. One child developed haemorrhagic pleural effusion and another had pulmonary haemorrhage. Gum bleeding was not present in any child. Hepatomegaly and splenomegaly were present in 62.5% and 60% cases respectively. Mean liver size was 4.1±1.1 cm below costal margin and mean spleen size was 2.8±1.4 cm below costal margin at the time of admission.

Seizures occurred in 36 (45%) of cases. Altered sensorium was present on admission in 41 (51.2%). In children with altered sensorium, rash was seen in 18 (43.9%), bleeding manifestations were seen in 15 (38.5%) and swelling in 13 (31.7%). Alteration of sensorium developed later in another 2 patients. The average duration of altered consciousness on admission was 2.8±5.2 days and for seizures 2.8±5.3 days. Generalised hypertonia was found in 21 (48.8%) subjects with encephalopathy, meningeal signs in 7 (16.8%) and focal neurological deficit in 2

Table 2: Laboratory investigations in dengue IgM-positive cases

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Investigation</th>
<th>Dengue IgM +ve cases (n=80) No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Mean Hb (gm%) ± SD</td>
<td>9.8±2.0</td>
</tr>
<tr>
<td>2.</td>
<td>Mean total leukocyte count (per mm3) ± SD</td>
<td>98±3908</td>
</tr>
<tr>
<td>3.</td>
<td>Mean % polymorphs in blood</td>
<td>61.5±11.9</td>
</tr>
<tr>
<td>4.</td>
<td>Platelet count (per mm3) in blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;20 000</td>
<td>11 (13.7)</td>
</tr>
<tr>
<td></td>
<td>21 000–40 000</td>
<td>12 (16.2)</td>
</tr>
<tr>
<td></td>
<td>41 000–60 000</td>
<td>11 (13.7)</td>
</tr>
<tr>
<td></td>
<td>61 000–80 000</td>
<td>9 (11.2)</td>
</tr>
<tr>
<td></td>
<td>81 000–100 000</td>
<td>5 (6.2)</td>
</tr>
<tr>
<td></td>
<td>&gt;100 000</td>
<td>32 (40.0)</td>
</tr>
<tr>
<td>5.</td>
<td>Mean packed cell volume (PCV) (%) ± SD</td>
<td>26.8±5.7</td>
</tr>
<tr>
<td>6.</td>
<td>Mean serum bilirubin (in mg%) ± SD</td>
<td>1.0±0.7</td>
</tr>
<tr>
<td>7.</td>
<td>Mean sGOT (IU) ± SD sGOT(IU) &gt; 40 IU</td>
<td>98.4±69.820/26 (76.9)</td>
</tr>
<tr>
<td>8.</td>
<td>Mean SGPT (IU) ± SD sgPT(IU)&gt; 40 IU</td>
<td>78.1±66.422/33 (66.7)</td>
</tr>
<tr>
<td>9.</td>
<td>Mean International Normalized Ratio (INR) ± SD</td>
<td>1.8±2.0</td>
</tr>
<tr>
<td>10.</td>
<td>Mean serum sodium (in mEq/l) ± SD</td>
<td>132.5±5.3</td>
</tr>
<tr>
<td>11.</td>
<td>Mean urea (in mg%) ± SD</td>
<td>35.0±18.2</td>
</tr>
<tr>
<td>12.</td>
<td>Mean serum protein (in gm%) ± SD &lt;6.1 gm%</td>
<td>5.9±0.814/26 (53.8)</td>
</tr>
<tr>
<td>13.</td>
<td>Mean serum albumin (in gm%) ± SD</td>
<td>3.1±0.45</td>
</tr>
<tr>
<td>14.</td>
<td>CSF findings (38 patients)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CSF pleocytosis (&gt;10 cells/mm³)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean cell count ± SD (per mm³)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean polymorphs% ± SD Mean CSF protein (in mg%) ± SD</td>
<td>17/38 (44.7) 30.4±80.6</td>
</tr>
<tr>
<td></td>
<td>CSF sugar – normal (&gt;=2/3rd of blood sugar)</td>
<td>11.9±24.2</td>
</tr>
<tr>
<td></td>
<td>CSF sugar – decreased (&lt;2/3rd of blood sugar)</td>
<td>84.4±61.1</td>
</tr>
<tr>
<td></td>
<td>CSF sugar – decreased (&lt;2/3rd of blood sugar)</td>
<td>29 (80.6)</td>
</tr>
<tr>
<td></td>
<td>CSF sugar – decreased (&lt;2/3rd of blood sugar)</td>
<td>7 (19.4)</td>
</tr>
</tbody>
</table>
(4.6%) children. Five (6.2%) patients developed features of raised intracranial tension (ICT) such as hypertension, bradycardia and hyperventilation.

The laboratory findings are given in Table 2. The platelet count was below 100,000/mm³ in 48 (60%) cases. In 13.7% cases the platelet count was below 20,000 mm³. Liver enzymes sGOT and sGPT were raised above the normal limit in 76.9% and 66.7% cases respectively. Packed cell volume was greater than 36 in 2 patients only.

Examination of the cerebrospinal fluid was done in 38 patients, of which 17 (44.7%) showed pleocytosis with mean cell count of 30.4 ± 80.6/mm³. Mean CSF protein was 84.4 ± 61.1 mg%.

Discussion

Dengue is a major public health problem in Lucknow and surrounding districts in the state of Uttar Pradesh in north India. Over the last 7–8 years we have been observing varied clinical manifestations of dengue, which are rather different from the past reports from this region as well as from other parts of the country. An ‘encephalopathic’ presentation was noted by us from 2003 itself, which led us to test consecutive children hospitalized with acute febrile encephalopathy (AFE) for dengue IgM and genome in CSF and serum. Of a total of 265 patients of AFE tested, 39 (14.7%) were conclusively proven to have dengue viral infection[6]. We also observed dengue viral infection presenting as acute hepatic failure. A total of 27 children admitted with acute hepatic failure were tested for dengue IgM of which 13 were unequivocally positive, and 7 of these were tested for dengue genome by RT-PCR, of which 4 were positive[7]. In 298 patients of acute undifferentiated febrile illness, dengue IgM was positive in 56 (18.8%) and dengue genome was detected in 15 of 44 IgM-positive cases[8]. It was observed that in addition to the well-known WHO criteria for case definition of DF, altered liver function with moderate elevation of transaminases is a differentiating feature of dengue. Over the last two seasons we have observed a further shift in the clinical manifestations, which we think is worthy of dissemination through this communication.

Our patients did not include all dengue IgM-positive cases over the study period. However, they were unselected cases and therefore unlikely to represent a biased group. Most of the dengue cases belonged to rural areas. This may only reflect the predominantly rural population admitted to this hospital. In two studies conducted between 2003 and 2006 we found no significant difference in the incidence between rural and urban areas[4].

The major difference from the previous reports is the frequent occurrence of encephalopathy, swelling, splenomegaly and prolonged fever. Encephalopathy, an important manifestation of dengue infection seen here, has been reported by us previously. It was observed in 53.7% patients in this series, but was not reported by earlier workers from Lucknow[9,10] and was seen in only 4% cases in the Delhi epidemic of 1996[11]. Rash, swelling and/or bleeding manifestations in these patients are suggestive of dengue and prompts testing for dengue IgM. Encephalopathy has been reported in several studies from Thailand[12-16]. Encephalopathy in dengue was believed to be due to cerebral edema, hyponatremia, hypoperfusion or intracranial bleed, but, more recently, the actual dengue viral invasion of the brain is recognized[17,18].

Another manifestation observed by us frequently over the last few years is the presence of swelling which was found in almost
Changing clinical manifestations of dengue infection in north India

half of our patients (Figure). This is a peculiar generalized non-pitting edema which may be explained by plasma leak in DHF. However, no earlier workers from India or abroad have mentioned this finding. Only 15 of the patients with swelling had received intravenous fluids prior to presentation here. Swelling was not seen in patients with other diagnoses seen here even if fluids had been administered outside.

Although hepatomegaly is among the WHO clinical criteria for DF, splenomegaly is not generally held to be a feature of dengue infection. Earlier reports from Lucknow[9,10] and other parts of India[11,19-21] do not describe a high frequency of splenomegaly. In our earlier studies we too did not usually find splenomegaly in DF, DHF or DE[6-8]. However, in this season (2008), it was observed commonly in almost 3/5th of the cases. Peripheral smears for malaria were negative in all cases. A recent study from Delhi has reported a somewhat high percentage (32.4%) of splenomegaly in children with dengue[22].

Dengue fever is generally described as a short febrile illness. The WHO criteria mention an illness of 2–7 days’ duration[5]. Over the

last two years we have observed a longer duration of fever than in previous years. The mean duration of fever in survivors in this study was almost 15 days.

On an analysis of the laboratory findings, it was observed that platelets were below 100 000/mm³ in a majority of the cases, with roughly 1/6th having counts below 20 000/mm³. Liver transaminases showed a mild-to-moderate elevation in around 3/4th patients. Alterations in liver functions are well-known in dengue infection[23-26], but are not listed in the WHO criteria for case definition[5]. Packed cell volumes (PCV) were almost always low in our patients, presumably due to high prevalence of anaemia. The diagnosis of DHF rests heavily on finding a high PCV, but in our patients, we have to rely on other evidences of capillary leak like low serum proteins. Even this may be misleading because serum proteins may be low due to malnutrition also. Demonstration of pleural fluid or ascites is difficult because these findings may not be found in all stages of the illness and involves transportation of a sick child. Therefore, only about a fourth of our hospitalized patients had definite WHO features for case definition of DHF. We strongly feel that these case definitions need revision as these cannot be applied in settings such as ours where dengue regularly occurs.

Our study patients had a severe presentation and rather high mortality. This is because all were hospitalized patients. If all dengue cases occurring in the community were to be described then we expect the severe manifestations and mortality to be certainly lower. However, such severe manifestations and encephalopathy are not described from other parts of the country even in hospitalized patients, which makes us think that the differences are at least in part due to greater virulence and neurotropism of the serotypes circulating in this region.

Figure: Characteristic swelling on the face of a child with dengue encephalopathy
Changing clinical manifestations of dengue infection in north India

Only serological diagnosis by IgM ELISA was possible in our patients. IgM, however, has its limitations in the diagnosis of dengue infection. Studies have shown 80% positivity in the first 5 days of illness, 93% positivity between 6 and 10 days of illness onset, and 99% positivity after the 10th day\(^\text{[1]}\). A negative IgM in a patient sampled early in the illness therefore does not totally rule out dengue infection. On the other hand, a positive IgM does not always mean that the current illness is dengue, but that dengue infection has occurred in the recent past, i.e. 60–90 days. IgM positivity therefore may merely mean that dengue transmission is going on. Further, although IgM-type antibodies are held to be specific between flaviviruses, some cross-reactivity was still possible, especially with Japanese encephalitis, which is endemic here. Due to these limitations, WHO has put the diagnosis of dengue infection on the basis of positive acute phase IgM ELISA test as ‘probable’ only. However, all our patients were clinically suspected as dengue, and, therefore, are very likely to have had dengue viral infection.

In conclusion, clinical manifestations of dengue as seen by us in Lucknow over the last few years appear to be different from those seen in other parts of the country, or even in the same region in earlier epidemics. The manifestations also seem to be changing over this period. DF and DHF/DSS are not the only clinical presentations. Encephalopathy is an important presentation in hospitalized children. The spectrum of findings may be explained by the presence of different circulating serotypes in this region. It would be interesting to correlate serotype with clinical features in this infection. Our report, however, is based on only a small study and should be corroborated by a larger, detailed study.

References


Dengue virus serotype 3 (genotype III) from Colombia: 
A perspective of its pathogenic potential

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Flor Angela Torres Pimiento⁴, Daniel Rafael Miranda-Esquivel⁷⁺, 
Raquel Elvira Ocazionez Jimenez*#

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³Laboratorio de Sistemática & Biogeografía, Escuela de Biología, 
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Abstract

The introduction of DENV-3 genotype III in Latin American countries has been associated with dengue outbreaks, and the role of the virus with respect to the occurrence of dengue haemorrhagic fever (DHF) has been different depending on the country. We have conducted research on the relative abundance of DENV-3 in relation to the incidence of DHF in a Colombian endemic area. Additionally, it was explored using phylogenetic analyses whether or not viruses are genetically distinct in relation to the severity of dengue. Viral isolation was made from serum samples collected during the period from January 2007 to October 2008. Sequences from the envelope gene of viruses from Colombia and Latin American countries isolated from DF and DHF patients and submitted to GenBank were compared. We found that in 2007–2008 the predominance of DENV-3 declined as compared to 2002–2004 (28.3% versus 87.8%), whereas the DENV-1 and DENV-2 predominance increased (54.7% versus 2.7% and 16.9% versus 5.4%, respectively). This relative abundance of serotypes coincided with an increase of DHF compared with the period of the highest DENV-3 dominance (25.9% versus 4.6%). Phylogenetic analyses showed that: (i) there is no relationship between DENV-3 clades and the severity of the disease; and (ii) Colombian viruses clustered apart from those coming from countries where DENV-3 has caused severe dengue. The results suggest that DENV-3 could not play any important role in the occurrence of DHF in Colombia, and that local viruses are genetically distinct from Latin American viruses associated with epidemics of DHF.

Keywords: Dengue serotypes; Dengue haemorrhagic fever; DENV-3 genotype III; Colombia.

Introduction

Dengue virus exists as four antigenically distinct viruses designated as serotypes (DENV-1, -2, -3, and -4), belonging to genus Flavivirus of family Flaviviridae[1]. Infection with any one of these serotypes generally leads to a mild, self-limiting febrile illness called dengue fever (DF).
Nonetheless, in a few cases, the viral infection leads to severe, sometimes fatal, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS)\[2\]. Epidemiological studies have identified sequential infection with different serotypes as a risk factor for DHF/DSS\[3,4\]. Despite the much higher frequency of secondary infections in areas where two or more DENV serotypes are present, only a small percentage of patients develop DHF\[5,6\]. The infecting viral strain is hypothesized to influence the severity of dengue. It has been demonstrated that dengue virus serotypes and strains within a serotype may vary in their ability to cause DHF\[7-9\].

DENV-3 viruses are phylogenetically grouped into four genotypes by Lanciotti et al.\[10\] or five genotypes by Wittke et al.\[11\]. In Latin America, this serotype was present between 1963 and 1977, and reappeared in Nicaragua and Panama in 1994\[12\]. It then dispersed to Central and Caribbean American countries\[13-15\]. In South America, DENV-3 appeared first in Brazil\[16\] and Venezuela\[17\] in 2000, and then dispersed to neighbouring countries in the following years\[18-21\]. Viruses isolated before 1994 were DENV-3 genotype IV, and those isolated after 1994 were DENV-3 genotype III\[10,17,18-20\]. Recently, Brazilian isolates in 2002 and 2004 were grouped into genotype I\[22\], but the precise classification has been controversial, considering that this genotype was classified as genotype V by Nogueira et al\[23\].

In Colombia, the presence of DENV-3 genotype III was detected for the first time in 2001 in the Departamento de Santander, the region where the present study was conducted. The reappearance of the virus coincided with an extended epidemic but increase in the number of DHF cases was not observed\[21,24\]. The same was seen in Mexico\[13\], Puerto Rico\[14\], Venezuela\[17\] and Peru\[20\], where a great number of dengue cases occurred after the re-introduction of the virus, but DHF cases were rare. In contrast, in Brazil\[25\], Paraguay\[26\] and Cuba\[15\], DHF/DSS in DENV-3-infected patients was frequent and some of them died. Likewise, the predominance of this dengue serotype in India and Sri Lanka has been associated with an increased incidence of DHF/DSS\[27,28\].

It is difficult to determine the causes for different clinical outcomes in dengue patients infected with DENV-3 of genotype III. This is due to the limitations of our knowledge about the role of host factors and the virus-specific determinants of virulence. In this study we investigated the predominance of the virus circulating during 2007 and 2008, five years after its occurrence in the Departamento de Santander, with relation to the occurrence of DHF. We have also studied the genetic relationships of Colombian DENV-3 isolates to determine if viruses in DF patients diverge or have distinct geographical origin from those in DHF patients.

**Methods**

**Study area**

Santander is one of the 32 states (departments) of Colombia with a total number of 87 municipalities. Located in the north-central part of the country close to Venezuelan border, it covers an area of 30 537 km². Its Capital is Bucaramanga, which together with three nearby municipalities, constitutes the seventh largest metropolitan area of Colombia with one million inhabitants (2005). It has a population density of 1012/km², and an annual average temperature of 22 °C. At least 94% of dengue cases occurring in Santander originated in Bucaramanga and the metropolitan area. The numbers of DHF cases out of total dengue cases in Santander during the period 1998–2008, as reported by the state Secretariat of Health\[29,30\], are shown in Table 1.
Dengue virus serotype 3 (genotype III) in Colombia

A total of 680 serum samples from clinically suspected dengue patients, reported to the dengue surveillance programme set up by the state Health Secretariat, were included in the study. Between 18 January 2007 and 11 October 2008, acute serum samples for virus isolation were selected every week from total samples sent to the Public Health Laboratory in Bucaramanga. Only sera from patients with fever of unknown origin (not from respiratory, diarrhoea or other apparent causes) were included, and a reporting form was completed with clinical and laboratory data collected from each patient.

### Serosurveillance

<table>
<thead>
<tr>
<th>Year</th>
<th>Dengue cases</th>
<th>DHF Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>23 826</td>
<td>881</td>
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</tr>
<tr>
<td>1999</td>
<td>4956</td>
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<td>130</td>
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<td>2001</td>
<td>10 530</td>
<td>779</td>
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</tr>
<tr>
<td>2002</td>
<td>10 356</td>
<td>523</td>
<td>5.0</td>
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<tr>
<td>2003</td>
<td>6638</td>
<td>288</td>
<td>4.3</td>
</tr>
<tr>
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<td>1669</td>
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<td>2.9</td>
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<tr>
<td>2005</td>
<td>1586</td>
<td>419</td>
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<tr>
<td>2006</td>
<td>2341</td>
<td>496</td>
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</tr>
<tr>
<td>2007</td>
<td>5167</td>
<td>1 331</td>
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</tr>
<tr>
<td>2008*</td>
<td>2579</td>
<td>676</td>
<td>26.2</td>
</tr>
</tbody>
</table>

Source: [29,30]; *From January to October, information supplied by Luis Gualdrón, División de Epidemiología, Secretaría de Salud, Santander, Colombia.

### Virus isolation

The isolation of viruses from the acute phase samples was attempted in C6/36 cells as previously described[21]. Briefly, 100 µl of serum was added onto cell monolayers and, after centrifugation, 1 ml of culture medium was added. Cells were incubated at 32 °C, and analysed for the presence of virus on the 12th post-infection day by using a polyclonal anti-dengue antibody (Instituto Evandro Chagas, Brazil) in a direct immunofluorescence assay.

### Typing of viruses

Serotype identification of the virus isolates was carried out by a seminested reverse transcription-PCR (RT-PCR) protocol on the basis of that described by Lanciotti et al[31]. Briefly, viral RNA was extracted from 140 µl of cell-infected culture supernatant by using Trizol® (GIBCO BRL, Grand Island, NY), followed by reverse transcription with forward primer D2. cDNA was subjected to PCR amplification with D1 and D2 primers for 42 cycles, and a second round of amplification was conducted with a mixture of type-specific reverse primers (TS1-TS4). PCR reaction product was electrophoresed through a 2.5% agarose gel, stained with ethidium bromide, and photographed.

### Anti-dengue IgG antibodies

IgG antibodies were screened in the sera collected 0–4 days after the onset of symptoms from dengue virus isolation-positive cases by using the PanBio IgG ELISA kit (PanBio Inc., Brisbane, Australia). Primary or secondary infection status was determined by the absence or presence of IgG antibody in an acute-phase sample.

### Sequence analysis

The envelope gene sequences of Colombian DENV-3 genotype III isolates were used along with some representative global isolates. Sequences of the remainder genotypes were included as outgroup. All sequences used in the
Table 2: Isolates of DENV-3 analysed

<table>
<thead>
<tr>
<th>Location*</th>
<th>Strain</th>
<th>Year</th>
<th>Clinical status</th>
<th>Accession no.</th>
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<tr>
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study were deposited in GenBank (Table 2). Colombian viruses were isolated in our laboratory from patients suffering from either DF or DHF in previous studies\(^\text{[21,24]}\). The sequences were aligned in the Muscle software v. 3.7\(^{[32]}\) using the default parameters and the model of nucleotide substitution that best fits the data set was determined using a hierarchical likelihood ratio test\(^{[33]}\) using the Modeltest software\(^{[34]}\). A maximum likelihood phylogenetic tree was reconstructed in the phyML software v. 3.0\(^{[35]}\) where the starting tree was found using the neighbour-joining method. A Bootstrap analysis with 10 000 pseudo-replicates was conducted to place confidence values on grouping within the tree.

### Results

#### Dengue serotypes and infection pattern

A total of 53 dengue viruses were isolated from 426 and 254 serum samples collected from febrile cases enrolled in 2007 and 2008.
respectively. The serotypes detected were DENV-1 (n=29), DENV-2 (n=9) and DENV-3 (n=15), and no isolates of DENV-4 was obtained. Primary infection was more frequent in DENV-1 (86.9%) and DENV-3 (73.3%) than DENV-2 (55.6%)-infected patients.

**DENV-3 predominance and DHF**

In 2007, DENV-3 (42.2%) was the most prevalent serotype followed by DENV-1 (36.3%) and DENV-2 (21.2%). In 2008, in contrast, DENV-1 (85%) became the dominant serotype followed by DENV-2 (10%), while DENV-3 (5%) was detected to a much lesser extent. This temporal relative abundance of dengue serotypes coincided with an increase in the frequency of DHF cases with respect to the period of the highest DENV-3 dominance. This is, from 4.6% (861/18 663; DENV-3=87.8%) between 2002 and 2004 to 25.9% (2922/11 673; DENV-3=28.3%) between 2007 and 2008 (Tables 1 and 3).

**DENV-3 phylogenetic diversity**

The aligned final data set comprised 60 sequences. The ingroup included 42 sequences of the entire E gene (1479 bp in length) from patients suffering from either DF or DHF/DSS (Figure). The Tamura and Nei plus Gamma (TrN + Γ) model was the best fit to the data with an α value (shape parameter) of 0.24. The single phylogenetic tree obtained revealed five different groups of DENV-3 viruses that could be assigned to genotypes. The analysis clearly distinguished the two different genotypes (III and IV) detected in Latin America. All the 2001–2004 Colombian DENV-3 isolates grouped into genotype III, along with viruses from Latin American countries that were isolated after 1994. Viruses isolated from DHF patients did not cluster apart with isolates from DF patients. Consequently, there were no phylogenetically distinct groups related with disease severity when using the envelope gene. Nonetheless, Latin American viruses could be grouped in two clades. One clade grouped strains from Mexico, Venezuela, Colombia and Nicaragua, where the disease outcome has been benign in the majority of DENV-3-infected patients[13,17,24,36]. The second clade grouped the isolates from Cuba, Brazil and Paraguay, where infections resulted in either fatalities or serious visceral and nervous system involvement[15,25,26].

<table>
<thead>
<tr>
<th>Table 3: Annual temporal predominance of dengue virus serotype, Departamento de Santander, Colombia</th>
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<td>2008</td>
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</tbody>
</table>

*: Ocazionez et al.[21]. §: this study.
Dengue virus serotype 3 (genotype III) in Colombia

**Figure:** Maximum likelihood phylogenetic tree of 60 DENV-3 envelope gene sequences. Viruses are listed by abbreviation for country, year and strain (Table 2). Bootstrap values above 50 are shown above the branches.
Discussion

Our results show that at least three dengue serotypes have been simultaneously present in the Departamento de Santander between 2007 and 2008, and that the relative abundance of serotypes had a distinct pattern each year. To our knowledge, dengue virological surveillance in 2005 and 2006 was not carried out in Santander. Under these circumstances, we used data from previous studies in the same region of the country for the period 1998–2006 to identify the relationship between the occurrence of DHF and the abundance of DENV-3.

DENV-3 was the predominant serotype in Santander in the period 2002–2004, while DENV-1, DENV-2 and DENV-4 were found in considerably lower frequency. This temporal serotypes distribution coincided with a decrease in the frequency of DHF with respect to the previous year\cite{21,24}. Between 2007 and 2008, in contrast, DENV-3 declined and DENV-1 and DENV-2 increased, and the frequency of DHF was six-fold higher with respect to 2002–2004 (Tables 1 and 3). Although the severity of dengue in accordance with WHO parameters\cite{37} in patients enrolled in the present study was not determined, however, in previous studies\cite{21,24} conducted in Santander, DHF was less frequent in DENV-3-infected patients compared with DENV-2-infected patients (10.9% versus 27.5%), and that there was no DSS or fatal case caused by DENV-3. Moreover, the period of the highest predominance of DENV-3 coincided with a decrease of DHF, compared with the period of DENV-2 dominance.

Phylogenetic analyses of isolates of DENV from severe cases or DHF epidemics suggest that viral factors can have an influence on the outcome of viral infection\cite{8}. In this study, segregation of DF- versus DHF-associated viruses on the basis of E gene sequences was not observed. This finding is in agreement with a study of DENV-3 viruses from Venezuela\cite{17}.

Likewise, Miagostovich et al.\cite{38} did not find any differences among the untranslated region (UTR) sequences of viruses isolated from fatal or DF patients in Brazil. Additional studies investigating other genes within the DENV-3 viruses are necessary to infer with more certainty the genetic basis of virulence.

It seems that the strains of DENV-3 genotype III circulating in the Americas exhibit different pathogenic potential. During the 1998 epidemic in Nicaragua, 11.8% out of DENV-3-infected patients developed DHF and fatalities were not registered\cite{36}. In Venezuela, even though the virus caused the largest epidemic seen after 1989, during the period of its highest predominance only 8% of dengue cases were severe and death in DENV-3-infected patients was not reported (Dirección de Epidemiología y Análisis Estratégico, 2001). Although, in Mexico, the severity of dengue increased in the mid-1990s after the introduction of DENV-3, the continuous presence of Asian genotype of DENV-2 seems to have played a more important role in DHF outbreaks\cite{13}. On the contrary, the introduction of DENV-3 into Brazil, Paraguay and Cuba was associated with severe disease. The virus caused an epidemic in the state of Rio de Janeiro with 1831 DHF cases, and a total of 91 deaths in DENV-3-infected patients\cite{16}. In Paraguay, the predominance of the virus resulted in 28 129 dengue cases, out of which 55 were haemorrhagic and 14 ended fatally. The fatality rate was 25.4%\cite{26}. In Cuba, 12 886 dengue cases occurred during the 2001 DENV-3 epidemic, of which 70 suffered from DHF and 3 of them ended fatally\cite{15}.

Our phylogenetic analyses clearly revealed that isolates of DENV-3 from Colombia belong to the same genotype as isolates from Brazil, Paraguay and Cuba. Why did the predominance of the virus result in epidemics with a distinct severity of dengue? One explanation involves genetic diversity within the isolates of the virus. It has been suggested that DENV-3 viruses
Dengue virus serotype 3 (genotype III) in Colombia

might have gone through a period of in situ evolution within Latin American countries after its introduction, diversifying into distinct phylogenetic groups\(^\text{[17]}\). A genetic shift in DENV-3 of genotype III has been suggested as the cause for the emergence of an invasive strain responsible for the increased frequency of DHF in Sri Lanka\(^\text{[9,28]}\). We found that isolates coming from countries where DENV-3 has not been associated with severe disease (Colombia, Venezuela and Mexico) grouped apart from isolates coming from countries where the virus has caused deaths (Brazil, Paraguay and Cuba)\(^\text{[19,39,40]}\). Additional studies are necessary to evaluate whether or not a genetic shift in viruses from Latin America is occurring.

Other aspects might influence the severity of dengue during the DENV-3 outbreaks. Virus-specified determinants of virulence at the level of susceptibility to cross-neutralization, more prone to enhancement by dengue antibodies, and variation in the ability to infect and be transmitted by their mosquito vector, have been proposed\(^\text{[27]}\). On the other hand, human genetic resistance to infection caused by viral strains more virulent, and herd immunity of the respective populations, should also be considered.

An observation that caught our attention in the present study was the increased incidence of DHF during the period 2005–2008 (25%) compared with the period 1998–2004 (4.5%). We did not investigate the temporal distribution of dengue serotypes in Santander in 2005–2006. Nonetheless, we conducted a study in the municipality of Ocaña, located 299 km from Bucaramanga in the north-eastern part of Santander (data not published). In Ocaña, in 2005, DENV-2 (77.5%) was the dominant serotype followed by DENV-3 (12.5%), and DENV-1 (5%) was isolated to a much lesser extent. In 2006, DENV-1 (57.5%) became dominant, followed by DENV-3 (27.5%) and DENV-2 (22.5%). We can speculate that in Bucaramanga the predominance of DENV-2 and DENV-1 could have also increased in 2005 and 2006, respectively, despite DENV-3 continuing as the prevalent serotype; and, in this context, the frequency of DHF cases could have increased. One explanation could be that the co-dominance of DENV-2 and DENV-3 in 2001 coincided with increased DHF cases in Bucaramanga during the period 1998–2001 coincided with increased DHF cases in Bucaramanga during the period 1998–2001 coincided with increased DHF cases in Bucaramanga during the period 1998–2001 coincided with increased DHF cases in Bucaramanga during the period 1998–2004 (Tables 1 and 3), and that DENV-1 and DENV-2-infections were more associated with DHF than DENV-3-infections\(^\text{[21]}\). In Nicaragua (1999–2003), the predominance of DENV-2 was associated with infections with shock to a greater extent, and the predominance of DENV-1 with an increased number of infections with severe manifestations\(^\text{[41]}\). In addition, the marked increase of DHF in Latin America has been largely attributed to the increased frequency of DENV-2 infections\(^\text{[4,42]}\).

We cannot exclude that the increase of DHF cases in Santander between 2005 and 2008 could be due to the failure of physicians in clinics to collect sufficient data to fulfil the requirements for the WHO case definition. In the case of some patients, serial haematocrit tests required to estimate the degree of haemoconcentration were not available; and as such, patients with platelet counts below 100 000/mm\(^3\) and/or with haemorrhages might have been classified as a DHF case.

Taken together, our results in this study and previous studies suggest that the presence of DENV-3 in the north-central part of Colombia since 2001 has had a minor role in the occurrence of DHF. More studies need to be conducted to clarify the pathogenic potential of the virus in Colombia. The phylogenetic analysis suggests that DENV-3 Colombian viruses could be genetically distinct from the viruses of the same serotype coming from neighbour countries with potential to cause DHF. Thus, continuous virological surveillance should be a priority in the Colombian dengue endemic areas.
Acknowledgments

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References


Dengue virus serotype 3 (genotype III) in Colombia


Dr. Wiwanitkit houses and his work. The first group relates to descriptive study focusing mainly on the natural history of dengue. The good examples are studies on dengue prevalence and clinical cases summarization. The second group pertains to analytical study. This kind of work focuses mainly on the cause and result to derive odd ratio and risk estimation. The third group of studies focuses on the development of sensitive and dengue-specific diagnostic tests. The last group of work is the experimental group. This mainly focuses on clinical studies for dengue as previously mentioned. This kind of work is considered useful for establishing of real-time clinical practices. However, in addition to these classical approaches, use of database and information technology is important. In the era of information technology, scientists can successfully scan the heap of information available on dengue for usage in diagnosis, treatment and control of the disease. This

Abstract

Dengue is an important tropical infectious disease. This infection has been widely studied and there are many reports on its pre-clinical and clinical aspects. In the era of information technology, scientists can successfully manipulate the large amount of information available on dengue for use in the diagnosis, treatment and control of this disease. In this article, the author briefly summarizes and comments on applied informatics manipulation for the prevention and control of dengue.

Keywords: Dengue; Informatics; Manipulation.

Introduction

Dengue is an important tropical infectious disease that has become a focused disease and its effective control for sustainability requires all-round efforts. There are many research studies on different aspects of this effort: epidemiology, clinical, diagnostics, treatment, and prevention and control of dengue. Various types of dengue researches can be summarized by their characteristics into pre-clinical and clinical studies. For pre-clinical study, scientists usually study the pathophysiology of dengue by standard methods of scientific research. For clinical study, physicians are concerned with diagnosis and therapeutic research on dengue.

Basically, these dengue research areas can be divided into four main groups. The first group relates to descriptive study focusing mainly on

#E-mail: wviroj@yahoo.com
article tries to briefly summarize the application of informatics technology for the development of new technologies/strategies in all aspects of control and management of DF/DHF.

Database searching for natural history study of dengue

As previously mentioned, case summarization is a basic descriptive study for dengue research. In the past, the summarization could be undertaken when there was enough data for analysis. The analysis can be done only if the sample size of the data is sufficient for statistical analysis. However, with advances in database technology, there are numerous databases in medicine, which can serve as primary data source. Of several databases, there is an interesting specific database, DengueInfo, which can be a good gateway to dengue information resources[1]. Many useful data can be freely accessed using this database.

There are many new research studies where scientists have made use of database searching and utilized the same in metaanalysis technique to derive the natural history of dengue. The best example is the series of Cochrane Reviews. The paper by Panpanich et al. is within this group[2]. Panpanich et al. carried out metaanalysis on the use of corticosteroids for treating dengue shock syndrome (DSS)[2]. In this review, Panpanich et al. searched the Cochrane Infectious Disease Group Specialized Register (January 2006), CENTRAL (The Cochrane Library 2005, Issue 4), MEDLINE (1966 to January 2006), EMBASE (1974 to January 2006), LILACS (1982 to January 2006), and concluded that there was insufficient evidence to confirm the efficacy of the use of corticosteroids in managing dengue shock syndrome[2]. Wiwanitkit recently reported on liver dysfunction due to dengue infection by an analysis of the previously published Thai cases[3]. In this work, Wiwanitkit used Pubmed and Thai Index Medicus database search for deriving primary data to conclude his research results[3]. According to this work, Wiwanitkit noted the importance of detection of abnormal high transaminase enzyme among patients with dengue infection, which consequently developed into hepatic encephalopathy[3]. Wiwanitkit also used a similar technique to summarize the magnitude and pattern of neurological pathology in fatal dengue haemorrhagic fever and found that neurological pathology was also common[4].

Prediction for pathobiology mechanism in dengue

With the advent of computational biology, some new research studies have been carried out on predictive pathobiology of dengue by means of “omics” science. Indeed, there are many newly launched bioinformatics tools that can be applied for dengue research. The basic tools on genomics and proteomics can provide many new data to the scientific community. For example, Wiwanitkit recently explained the pathobiology of DSS by means of predictive bioinformatics. Firstly, Wiwanitkit used phylogenomics analysis to predict the phylogenetic interrelationship and reported that platelet CD61 might have an important role in causing haemorrhagic complication in dengue infection[5]. However, Wiwanitkit further clarified by means of gene ontology that there was no existence of functional similarity between DNS1 and CD61[6]. A study on functional similarity between dengue non-structural protein 1 and platelet integrin/adhesin protein, CD61, showed no confirmative result[6]. Secondly, Wiwanitkit also focused the work on the immune complex generation in dengue. Wiwanitkit successfully demonstrated weak binding affinity of immunoglobin G, which could be an explanation for the immune
mimicking theory in pathophysiological findings in the recovery phase of dengue\cite{7}. The molecular docking technique was mainly used for this predictive-specific study\cite{7}. Wiwanitkit also used the docking study to estimate the size of immune complex and reported its possible relationship to renal pathology in dengue\cite{8}. The author concluded that because entrapment of the immune complex is believed to occur when a previous glomerular lesion causes narrowing of the glomerulus’s diameter, the immune complex should not have a significant role in the pathogenesis of renal failure in dengue infection\cite{8}. Hibberd et al. used a genomics approach to understand the host response during dengue infection and found many new host pathways involved in viral replication in vitro, and also host immune responses that were influenced by viral sequence\cite{9}. For the nature of outbreak, Halide and Ridd recently used a predictive model for describing dengue haemorrhagic fever epidemics\cite{10}. Halide and Ridd also found that the most important determinant in the predictive model was the present number of cases followed by the relative humidity three to four months previously\cite{11}.

## In silico mapping of dengue virus epitopes

Epitope finding is the basic principle in applied immunology. This activity is useful for understanding the immunopathology of infectious diseases as well as to help search for vaccine candidate. In silico mapping of dengue virus epitopes is the current useful application of bioinformatics technology in dengue research. Kutubuddin et al. used basic bioinformatics to describe recognition of CD4(+) T cell epitopes in envelope (E) glycoprotein of Japanese encephalitis, West Nile and dengue viruses\cite{12}. In this work, analysing the occurrence of amphipathic segments, Rothbard-Taylor tetra/pentamer motifs and presence of alpha helix-prefering amino acids were used for epitopes prediction\cite{12}. Wen et al. recently used computational prediction to identify dengue virus-specific CD4(+) T-cell epitopes\cite{13}. According to this work, As a result, C(45-57) (KLVMFIFLFRL), E(396-408) (SSIGKMFETARG), NS3(23-35) (YRLQRGLGRSO), and NS3(141-155) (NREGKIVGLGNVGV) were the identified epitopes\cite{13}. A similar work was also recently published by Leclerc et al.\cite{14}.

## Vaccine search by means of immunomics

Immunomics is the new specific “omics” science for the study of epitope for production of new vaccines. Immunomics is presently focused on vaccine research. For dengue, the disease without an effective vaccine, immunomics can be useful. In 2007, Khan et al. introduced a systematic bioinformatics approach for the selection of an epitope-based vaccine, targeted to assess its efficacy against dengue\cite{15}. In this study the number of unique protein sequences required to represent complete antigenic diversity of short peptides in dengue virus is significantly smaller than that required to represent complete protein sequence diversity\cite{15}. Recently, in 2008, Khan et al. also published another work on the identification of conservation and variability of dengue virus proteins by mean of bioinformatics\cite{16}. In 2007, Mazumder et al. reported on computational analysis and identification of amino acid sites in dengue E proteins relevant to development of diagnostics and vaccines\cite{17}. They found that six singular sites [N(37), Q(211), D(215), P(217), H(244), K(246)] in dengue E protein that were conserved, were part of the predicted consensus T(h)-cell epitopes and were exposed in the dimer or trimer\cite{17}. They also proposed
these sites and corresponding epitopic regions as potential candidates for prioritization by experimental biologists for development of dengue vaccines[17].

**Computer-aided drug design**

Computer-aided drug design is another useful bioinformatics application in dengue research. Similar to vaccine search, it is possible to search for a new antiviral compound for dengue based on bioinformatics technology. For example, Zhou et al. recently used virtual screening of small-molecule libraries against dengue virus E protein to identify new antiviral compound for dengue[18]. According to the study of Zhou et al., P02, a new compound with a change for the development of an effective treatment against dengue virus and related flaviviruses could be identified[18]. Luzhkov et al. used a similar technique, virtual screening and bioassay study, to find new inhibitors for dengue virus mRNA cap (nucleoside-2’O)-methyltransferase[19]. According to this study, a novel inhibitor, with a previously unknown scaffold that has an IC(50) value of 60 microM, could be identified[19]. Yang et al. also used combinatorial computational approaches to identify tetracycline derivatives as flavivirus inhibitors[20]. Yang et al. described that rolitetracycline and doxycycline were the two compounds that have their inhibitory effect on dengue virus propagation with IC(50)s estimated to be 67.1 microM and 55.6 microM, respectively[20].

**Plan for dengue control by GIS**

Finally, it should also be noted that not only the information in textual format but also in figure format can be manipulated. The Geographic Information System (GIS) is a good example of figure format data manipulation. There are some recent interesting reports on GIS and dengue. Wiwanitkit reported an observation on the correlation between rainfall and the prevalence of clinical cases of dengue in Thailand[21]. In this work, the collected primary data in general report was used for further manipulation and a predictive map was also generated. A similar work was also reported by Bonet et al. in the Latin America-Caribbean region[22]. In addition, GIS can also be applied for the actual field study data. This is usually used for the vector or mosquito survey. A good example is the paper by Chansand and Kittayapong[23]. According to this work, the immature survey data and the GPS coordinates of house location were combined into GIS maps showing the distribution of immature density and clustering of immature stages and positive containers in the study area[23]. The authors concluded that this approach could be used to improve the efficiency and accuracy of dengue vector surveillance for targeting vector control[23]. Vanwambeke et al. concluded that the great variation of determinants for recent dengue infection in space and time should be taken into account when designing local dengue control programmes with the help of GIS[24].

**Diagnosis and prediction of disease outbreaks**

As mentioned before, the bioinformatics technology is useful for the assessment of pathobiology of dengue. The diagnosis and prediction of disease outbreaks can successfully be performed by the bioinformatics technique. Shaw et al. recently used dimension reduction to improve outbreak predictability of multistrain diseases including dengue[25]. Shaw et al. showed that this technique allowed the centre to use manifold equations, which are mainly used for prediction and are applicable even to
noisy systems\cite{25}. Jackson et al. also proposed a new technique, mass cataloguing, based on mass spectrometry and genotyping, to identify outbreaks of flaviviruses including dengue\cite{26}. For dengue, the method can help distinguish major subgroupings within each serotype\cite{26}.

**Informatics analysis of dengue vector genomes**

Not only the applications for the dengue virus but also for the dengue vector can be performed based on advanced informatics technology. Informatics analysis of dengue vector genomes is presently performed in entomology. For example, Lobo et al. performed analysis of 14 BAC sequences from the *Aedes aegypti* genome\cite{27}. The data from this study is useful for genome annotation and assembly\cite{27}. Takahashi et al. used mathematical models for the *Ae. aegypti* dispersal dynamics\cite{28}. Travelling waves by wing and wind could be identified in this study\cite{28}.

**Conclusion**

This paper has tried to review the application of informatics for the diagnosis, treatment and control of dengue virus. As the field of dengue virus research has seen an increased application of informatics over the years, presentation of a review summarizing informatics applications, their impacts and future potentials seems to be in order.

**References**


Applied informatics manipulation for fight against dengue

Research articles:


Community participation and social engagement in the prevention and control of dengue fever in rural Cambodia

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Abstract

The prevention and control strategies for dengue fever require community involvement to succeed. Drawing on data collected in 2003–2004 as part of an ethnographic study in eastern Cambodia, we explore the role of community participation and the factors that influence its success in the prevention and control of dengue fever in Cambodia. Community participation has the potential for effective and efficient control of the disease, but this is subject to how communities are engaged in specific activities. Historical, political, social and economic factors have undermined the social institutions and conventions in the study villages that could facilitate community involvement. In particular, poverty and differences in local interests influence the capacity for people to be involved. Villagers regarded the maintenance of the domestic environment as a personal responsibility and were reluctant to extend their action to a wider domain. Comprehensive programmes, which draw on local institutions and understandings of community and enable community members to participate in the planning and management of prevention and control activities, are essential to ensure programme sustainability and effectiveness.

Keywords: Cambodia; Community participation; Dengue; Social engagement.

Introduction

Community participation is a process of engaging various stakeholders and members of communities, however defined, to participate in the development and management of particular programmes or projects. It is conventionally represented as the lynchpin for the success of targeted health interventions and sustainable programmes. Various pilot projects have been conducted, well described in the literature in relation to the strategies and processes of participation\textsuperscript{[1-5]}. However, little research has been conducted on how community participation, once under way, is perceived by community members, health workers or other stakeholders. Similarly, the translation of community participation from...
Community participation in the prevention of dengue in Cambodia

Policy to practice in health, development and disease control programmes has received little attention, and the effectiveness of community participation both as a process and an outcome in disease prevention and control, including for dengue fever, remains unclear[2,3,6,7].

Cambodia adopted community participation as one of the principles of its primary health care policy and health system in 1999. Yet, dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) remain critical public health problems. Because of the domestic habitat and behaviour of Aedes mosquitoes, community participation and effective health education are central to sustainable dengue prevention and control. Most interventions in which community members are urged to be involved are relatively straightforward – maintaining safe water storage to prevent breeding, ensuring that there are no pools of stagnant water, carefully disposing of hard waste and using local biocontrol agents (e.g. copepods). Yet, there has been uneven success in the sustained commitment of communities in DF/DHF-endemic areas to environmental management, in part because of the lack of attention to social factors influencing dengue transmission, and in part because of the superficial nature of community involvement.

In this article, we explore how Cambodian villagers perceive community participation in the prevention and control of dengue fever. We focus on women, as the central players in dengue-related interventions, for it is they who are assumed to be responsible for permitting control programme staff to distribute temephos (as 1% sand granules with a dosage of 1 ppm per litre of water) and to fog and spray outside houses, and for maintaining the domestic environment, disposing of waste, ensuring larva-free water supplies, protecting children from bites, and taking appropriate action if and when their children are sick[8,9]. Women are also more likely than men to face challenges in seeking health care for themselves and their children, due to multiple demands on their time and their lesser role in determining how household cash resources are used[10]. This study tries to highlight how the social institutions within the study villages, that have supported community cooperation and reciprocity in the past, have been eroded in recent decades for political and economic reasons.

Materials and methods

Data were collected from March 2003 to February 2004 in the province of Kampong Cham (KPC), eastern Cambodia, where dengue has especially high prevalence compared to other regions of the country. During 2002–2003, there were 3713 cases of DF, DHF and DSS and 49 deaths in KPC. In the first eight months of 2007, there were 5105 cases of DF/DHF/DSS and 65 deaths in KPC[11]. Dengue continues to be endemic despite the National Dengue Control Programme (NDCP) that conducts activities in the area.

Study area

An ethnographic study was conducted in two villages with the highest reported incidence of the disease in the province. The villages, Khun and Nekry (pseudonyms), are located approximately 30 kilometres in opposite directions from the provincial town centre (also known as Kampong Cham) and around 100 kilometres from the Capital, Phnom Penh. Most villagers are poor farmers, growing rice for subsistence and sale, supplementing this by selling other produce and re-selling non-food goods in small quantities. Environmental conditions in the villages are conducive to dengue transmission. Broken coconut shells,
plastic bags, used packages and other disposable items are indiscriminately scattered in house yards and lanes; these provide ideal conditions for the breeding of the vector, *Aedes* mosquito. Wooden houses are built on stilts using bamboo and thatch. Water jars, stored under houses, are rarely covered and breed larvae all the year round.

**Data collection**

Data collection methods included key informant interviews, focus group discussions, in-depth interviews and ongoing participant observation, as well as structured observations and entomological surveys[12]. Key informant interviews were conducted with all village health volunteers about villagers’ awareness and perceptions about DF, and their participation in prevention, control and development activities. Four focus group discussions were conducted with mothers or other family caretakers of children who had been infected with dengue, to gain insight into their understanding of DF and their involvement in prevention and control measures. In-depth interviews were then conducted with 29 women whose children had been infected in the past year or during the research period, including about their participation in dengue prevention and control activities and their views about control programme activities. Women were also asked about changes in their village, and so could speak of issues about which they may have been reticent publicly. These data were supplemented by questionnaires with 38 other women, representing 15% of households where children had no history of dengue, on their participation in prevention and control activities. Sixteen interviews were conducted with health staff at health centres, provincial and national levels on community participation. All data were entered into computer, codes were developed, and the data were analysed thematically.

Ethics approval to conduct this research study was granted by the Human Research Ethics Committee of The University of Melbourne (Australia), the Ministry of Health (Cambodia), and WHO/TDR. All potential participants were provided with plain language Participation Information Sheets in Khmer (Cambodia’s national language), and the project was explained to them verbally. Consent to participate was verbal, since the collection of signatures held negative connotations for most people.

**Results**

**Social engagement in villages in Cambodia**

The idea of community, if taken to refer not to co-location but also to a spirit of common purpose, shared identity and trust, has declined in Cambodia as a result of its recent bitter history of autocracy, violence, genocide, and poverty. Interviews with participants aged 60 and older, born in both villages, illustrated the extent to which villages were believed to have changed since the 1940s. Prior to political turbulence and civil war in Cambodia in 1970, development activities in the villages, primarily road maintenance and building wells, was conducted under the leadership of Buddhist abbots and monks, senior citizens and local leaders. Under Pol Pot’s regime, in contrast, the population was forced to provide labour for developmental purposes in the villages.

Today, villagers passively participate in village development. Most roads and water wells are constructed with substantial financial and logistic support and management from the government, international organizations and national NGOs. This partly reflects changes in ideas of the role of government, and in some cases, the implementation style of development agencies and NGOs. Villagers
have contributed some labour, such as digging water drains along the road in front of their own houses. While this involvement is sometimes relatively spontaneous, it often follows the explicit requests of outside organizations, which undertake development activities on the provision that householders contribute in cash or kind. The rationale is that by contributing, people will have greater ownership of the projects, and so will participate in their maintenance.

But various factors discourage community engagement and involvement. Villagers believe that the level of trust among them decreased significantly during the three decades of domestic political unrest since 1970, and this has continued. During the period of Khmer Rouge rule under Pol Pot (1975–1979), people were divided into “locals” (nak mul tharn) and “evacuees” (nak chum leas). Most evacuees were urban dwellers, sent to rural areas by force to contribute to economic development, in which context “locals” exerted extreme dominance and authority. Those who were forcibly resettled were subject to random terror, murder, forced labour, starvation and absence of basic services. This significantly undermined trust and ties among villagers. As one woman explained, “From the time of the Khmer Rouge, there were more and more unreliable people and there were fewer good people, most of them were cheats and they even cheated the government” (In-depth interview, 11).

Elderly interviewees believed that the level of cooperation among villagers – referred to in Khmer as provass, has gone down. They claimed that in the past, fellow villagers were enthusiastic about helping each other, and were involved on a voluntary basis in a range of activities, from digging water wells to helping to build entire houses; now almost all work involves payment. Political party representatives donate gifts in cash or kind to villagers to gain popularity, but there is little evidence of long-term political commitment to development. Others make public donations to those who are especially disadvantaged or vulnerable, or donate to temples or schools when high profile politicians visit. But the motivation of these activities is questionable, and is seen by villagers themselves as having less to do with aspirations for community development and more with power and popularity.

The standard of living of villagers has also declined. Most villagers reported that, over time, they have had poorer crop yields as a result of lack of irrigation and because of drought and floods in dry and rainy seasons. People reported that they can no longer make a profit from farming because of the expenses of petrol and hiring water pumps and oxen. To meet various recurrent and emergency cash needs, including for medical care, villagers sell or pawn their land and other resources such as cows or pigs and take out loans with exorbitant interest rates. Most villagers generate too little cash to repay loans or retrieve property, and so lose their property and continue to pay interest, so spiralling into extreme poverty[13].

Villagers have to meet direct and indirect costs for medical care to treat sick children. Delays occur because of the difficulty in locating resources, and the poorest households at times have no choice but to rely on home care only[13]. Villagers felt that the level of care and support from others, and from social welfare and public health services, had declined. One elderly woman reported that her neighbours used to accompany her at the Kampong Cham Provincial Hospital whenever she had a medical problem, because she had no relatives. The surgery was free of charge if a person was too poor to pay the fee. Now, this is rare:

“I had a surgery in 1962 or 1963. The surgery was very clean and I was discharged from the hospital after staying only one week. At first I was afraid that I had no money and
Community participation in the prevention of dengue in Cambodia

People do not necessarily share the same health-related problems and socioeconomic or political status that could result in collective action[3,14]. This is true for dengue and for the prevention and control activities in the study villages. Key informants in Khun village argued that women whose children had been affected by dengue were far more interested in discussions and health education messages than those whose children had never had dengue. Similarly, mothers of small children were more influenced than mothers of older children because of the pervasive belief that dengue was unlikely to affect older children. Other participants had other health priorities. But, in addition, participants mentioned lack of water for their rice fields and lack of money to buy petrol for water pumps to irrigate drought-affected rice fields, buy piglets or calves to raise them for sale, or buy oxen for farm work. Others reported difficulties in finding employment to earn money to meet their basic expenses or to repay interest and loans to fellow villagers. Dengue prevention was a minor concern against these major problems.

Political divisions at the national level strongly influence village politics. The three main parties in the country have their own party activists and supporters in villages, and everywhere, billboards promote their particular interests. In 2001, Commune Councils were established, to be responsible for the management and development of communes. Although their members were chosen through general election, candidates were elected under the name of their political party. As a result, each elected council member was strongly allied to his or her political party, using village issues for political advantage and working within the community to build networks for their own party and gather support for their platforms. The politicization of village government and of social relations within villages also appears to hamper the spirit of togetherness in village development among people with different political ideologies.

Village cooperation and participation

Development committees were established in 1998 in Nekry and Khun villages, as in most villages nationwide; these included a village development committee (VDC), school committee, temple committee, canal committee, water-well committee, women’s association, and village health volunteers (VHV) and others belonging to the village health supporting group (VHSG)[15]. The tasks of VHVs and VDC included providing health education, assisting with health outreach activities, giving first aid to villagers, and referring patients to health centres. Few of the committees received technical and logistic support however, so they quickly dissolved. In the study villages, the VDC and VHSG are still prominent as entities but are not functional; many other committees no longer exist even in name. As one member of a VHSG explained, “I had no time. I had nothing for my children to eat. It wasn’t just me, everyone quitted” (Key informant interview, 38).

Even so, the cooperation of villagers is critical for village development. Most cooperation occurs through social and cultural activities, but also through the exchange of
Community participation in the prevention of dengue in Cambodia

labour or provass. Provass is a Khmer term, connotative of reciprocity: according to Nekry and Khun villagers, the term means "working together to share outcomes". Three types of provass occur in the villages. At the time of the study, the first and dominant type of provass was shared animal husbandry. Many households raised young pigs or cows which belonged to another household, and would share with the owners any piglets or calves according to prior agreement. A second type of provass was the exchange of labour, when householders farmed for a short period for another householder when extra labour was needed. The latter householder would later work for the former to return the labour. Provass also referred to borrowing oxen for farming, with the loan repaid with rice or labour. Provass provides mutual benefits and only occurs between villagers of relatively equal socioeconomic status. In Khun and Nekry, this meant that poor villagers were generally excluded. But this reciprocal assistance has also diluted as a cash economy has taken hold in the villages and other types of help no longer exist as families lack the means to reimburse in cash or kind: “Nobody helped. The word 'help' doesn’t exist. I had no oxen to farm so I had to use a hoe to dig the soil. If I wanted oxen, I would have to hire and pay for them. Once I'd paid for the oxen, I wouldn’t have any rice left” (In-depth interview, 5).

Participation in prevention and control of dengue

Aedes mosquito breeding sites are ubiquitous\[12,16\], explaining the continued endemicity of DF. Women who participated in interviews were asked what villagers should do to prevent dengue. Many suggested that villagers should clean up their own house yards, collecting or burning rubbish such as tyres and coconut shells, to get rid of mosquito breeding sites. The majority believed that people knew of the danger of such breeding sites, and that they should work together to get rid of them to prevent disease. Many said that they would clean up their own yards if someone requested them to do so but they were reluctant to ask others. “I was afraid that they’d be angry. They would say they didn’t need to be told to clean their house. But if someone told me to do so, I’d be happy to follow their advice” (In-depth interview, 14). However, most women complained that while they cleaned up their own houses and yards, others did not, resulting in indiscriminate garbage throughout in the villages; they claimed too that neighbours ignored them when they asked them to clean their yards. Many villagers also felt that all villagers should use temephos to prevent dengue but this did not happen.

Mothers of children not infected with dengue reported individual and collective activities to prevent and control the disease (Table). All but five of the women knew about the disease and appropriate prevention and control activities. Most perceived dengue control to be a personal responsibility, with about half (19/33) reporting that they regularly cleaned their water jars and a third (10/33) stating that they kept their houses clean to discourage mosquitoes. Almost one of four women (9/33) claimed that they used temephos in water jars. A few women also used other (ineffective) activities to prevent and control dengue, such as using mosquito nets at night, removing sewage, clearing bushes and using mosquito coils. The majority overlooked discarded containers, the most common source of larval breeding in the rainy season\[12\], and made no effort to get rid of them. Only three women reported telling their neighbours to turn coconut shells upside down, or to remove discarded cans and plastic packing bags. Hence despite claims of high knowledge, few women undertook all necessary tasks on a regular basis.
Health workers at the village health centres and at the NDCP faced significant challenges in encouraging villagers’ participation in disease prevention. They complained that villagers prioritized income-generation activities over vector source reduction because of the dictates of their economic status, their reliance on the NDCP to undertake such activities, and the low effectiveness of the dengue health education campaign[12,16]:

“In my opinion, people do not have enough time to clean up their yard because their standard of living is so low; they even do not have enough food to eat... from season to season. When they return home from work in the late afternoon, they cook for their children, then find another job... they have no time to clean up the house. They are too busy to clean the jars even once a month or wash their clothes. Some people never think about their house or hygiene, they only think about food to eat; that was why our health education was not successful.” (Health worker, FGD, 30).

**Discussion**

Community participation as an approach seems to have been most successful in countries with strong political authority, as in Cuba[1,17]. In contrast, in fragmented societies where community members have different interests and problems, and lack trust and confidence
in political leadership\cite{7,10}, community participation has faced significant challenges. Local social, political and economic factors and associated structural barriers and inequalities compound to affect the ability of members of communities to sustain the activities required of them for disease control\cite{18}. These various factors have influenced the introduction and sustainability of community participation in Cambodia.

The literal translation of community in Khmer is sahakum, indicating a group of villages or regions whose residents share the same jobs\cite{19}. While sahakum is used in reference to community participation in official contexts, Cambodians prefer to speak of “villagers” (nak phum), “residents” (nak strok), “provincial dwellers” (nak khet) and “city-dwellers” (nak krong) to indicate geographical identity, invoking the administrative structures of village, commune, district, province and city. The term sahakum only became popular when health and development-related programmes were introduced in the 1990s. In the study on which we report, villagers rarely understood questions when the word sahakum was used in relation to participation in preventing or controlling dengue. Women stated bluntly that they did not understand the term and could not explain what it meant. However, all villagers clearly understood the terminology and ideas associated with provass, with tveu kar cheamuy knea (working together) and nak phum tveukar cheamuy knea (villagers working together) to prevent or control dengue.

A number of scholars have argued that community-based programmes are more sustainable than vertical ones\cite{4,20,21}. Studies show that top-down dengue prevention and control activities have a temporary effect but do not lead to the behavioural changes needed to reduce larval indices from the local domestic environment to ensure prevention and control\cite{22}. However, a recent review of community-based dengue control studies\cite{7} indicated that the implementation of community-based interventions has been variable, and noted the lack of involvement by villagers, and specifically village committees, in planning and implementation, so threatening sustainability. The study recommended intersectoral cooperation and stressed the importance of involving local health services, civil authorities and key community members to encourage individuals to take part in and sustain dengue prevention and control strategies.

Village participation in development in Cambodia, as has occurred with road construction, and the tradition of reciprocal labour exchanges, points to the potential for participatory development. However, the low level of village cooperation, the lack of a spirit of collaboration, and economic pressures combine to create significant challenges. Cooperation occurs in villages only if there is material or financial involvement\cite{13,23}. As noted above, willingness and ability to work together is relatively low even when the rewards are tangible (as occurs with labour exchange). The idea of working for the public good is far less familiar. Villagers work individually, including undertaking dengue control activities on request, but they are reluctant to encourage each other to do so. Effective dengue health education is needed to encourage people to undertake such activities on a continuing basis\cite{16}. There is an urgent need to restore trust, confidence and cooperation between villagers and in the society as a whole. This requires the political commitment of the government.

Whiteford\cite{7,10} has suggested that people’s vision of their future plays a major role in their participation in government programmes. She has argued that Cubans consider health to be a collective achievement, and this, coupled
with confidence in the government and feelings of hope for the future, have supported government-community partnerships for disease control, including for dengue. In contrast, in the Dominican Republic, unfulfilled political promises, lack of political will and the lack of belief in community-based social action have resulted in the failure of community participation despite good community understandings of dengue control\(^\text{10}\). The situation in Cambodian villages echoes that of the Dominican Republic. Suspicion, distrust, increasing poverty, food insecurity, unemployment, landlessness and indebtedness, against the backdrop of violent history, contribute to a lack of confidence in government capacity, its long-term commitment to village development or to improved health, and consequently, people show little interest in community participation.

**Conclusions**

Community-based programmes involving local responsibility and for the participation in the elimination of breeding sites are the only cost-effective and sustainable ways to ensure control in any dengue-affected country, in particular, in poorly resourced countries. However, in Cambodia, community participation has been implemented primarily by international organizations, NGOs, government departments and vertical disease control programmes. Local knowledge and local institutions, including those that would serve to achieve the same goals, have largely been overlooked. In this study, community members claimed that community-based dengue control occurred, but, in practice, people had limited opportunity to participate in planning and managing dengue prevention and control in their own villages, and so had little interest in or awareness of the need to ensure that basic control activities were sustained.

In poor communities and poor countries such as Cambodia, disease control programmes need to take into account factors affecting community welfare, engagement and participation. In Cambodia, despite differences in wealth, different health problems and often, different political views and affiliations, everyday life is dominated by the struggle to survive. Given this scenario, health programmes such as DF programmes, using community participation, need to deal not only with source reduction to control the larvae and the mosquitoes but to address larger questions of poverty and income. Until such fundamental issues are addressed, people’s engagement in programme planning and management and in the work of disease prevention will remain partial and episodic.

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References


Dengue in the National Capital Territory (NCT) of Delhi (India): Epidemiological and entomological profile for the period 2003 to 2008


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Abstract

Dengue is endemic in the National Capital Territory (NCT) of Delhi. During the period 2003 to 2008, 9737 confirmed cases of dengue fever (DF)/dengue haemorrhagic fever (DHF) and 115 deaths were recorded compared with 1341 cases and 6 deaths that happened during 1997 to 2002, representing an increase of 626%. During this period two outbreak peaks were also recorded. In addition, the satellite town of Gurgaon (Haryana) bordering Delhi also suffered a severe outbreak of DF/DHF during 2008.

Aedes aegypti, the responsible vector, is fully entrenched in both urban and rural areas. DF/DHF transmission in years of extended winter rains occurs both during the summer and rainy seasons. Evaporation coolers during summer maintain low temperature and high humidity to ensure dengue transmission in some highly congested localities.

Keywords: National Capital Territory (NCT); Delhi; Endemic; Room water coolers; DF/DHF transmission.

Introduction

The National Capital Territory (NCT) of Delhi is endemic for dengue and has experienced several outbreaks since 1967[1]. In the recent past, Delhi recorded an outbreak in 1988, which resulted in 33% mortality among children admitted in hospitals[2]. This was followed by yet another severe outbreak in 1996 throughout the NCT region of Delhi when a total of 10 252 cases and 423 deaths were recorded[3]. All the serotypes (DENV 1-4) have been detected circulating in the NCT Region[1].

In view of the severity of the outbreak in 1996, the Directorate of the National Vector-Borne Disease Control Programme (NVBDCP) initiated several measures. These included regular epidemiological and entomological surveillance for timely prediction of an
impending outbreak. The present communication incorporates the epidemiological and entomological databases for the years 2003 to 2008.

Study area

The National Capital Territory (NCT) of Delhi, with an area of 1485 sq km, is located between $28^\circ 75\text{'}$ north latitude and $76^\circ 22\text{'}$ east longitude\cite{4}. The population of NCT increased from 9.43 million in 1991 to 13.7 million in 2001, recording a decadal growth rate of 51.3\% (Census report, Government of India, 2001). Since 2001, the population has further increased to 15.46 million with a growth rate of 12.2\% (communication from Municipal Corporation of Delhi).

Local civic bodies: The NCT of Delhi has multiple agencies responsible for the control of vector-borne diseases. These include: two civic bodies, the cantonment authority and the Railways. The Municipal Corporation of Delhi (MCD) has twelve zones and 272 wards covering about 1399 sq km area. The New Delhi Municipal Council (NDMC) has one zone and eight wards covering 42.74 sq km area. The cantonment covers 42.89 sq km area\cite{4} (Figure 1). The railways look after its residential colonies and railway stations.

Figure 1: Map showing the zones of Municipal Corporation of Delhi, New Delhi Municipal Council and Cantonment
Materials and methods

Epidemiological surveillance: The National Vector-Borne Disease Control Programme (NVBDCP) is a nodal agency for the monitoring of DF and DHF throughout the country. Hospitals follow the under-mentioned case definitions for the purpose of reporting.

- Patients with clinical symptoms like sudden onset of high fever, severe body pain and headache, myalgia, nausea, vomiting and rash, with positive dengue-specific IgM in a single serum specimen, to be considered as a dengue case.
- Clinical symptoms with low thrombocytopenia and leucopenia are also taken as cases of dengue fever. The presence of both these two criteria with haemorrhagic manifestation and death are taken as death due to dengue fever.

Entomological monitoring: Monitoring of Aedes aegypti throughout the year has been carried out by the Central Cross Checking Organization (CCCO) under NVBDCP to work out the House Index (HI), Container Index (CI) and Breteau Index (BI) as per WHO guidelines\(^5\).

Results

Epidemiological profile of dengue fever and dengue deaths in NCT Delhi: The DF incidence in NCT Delhi has been showing a rising trend over the last six years (2003 to 2008), when 9737 confirmed DF cases and 115 deaths were recorded compared with 1341 DF cases and 6 deaths that were reported during 1997–2002 (Source: NVBDCP). This represented an increase of 626% in the incidence, with an average mortality rate of 1.2%. The DF attack rate recorded was 20.9, 4.2, 6.9, 22.3, 3.5 and 7.9 per 100 000 population respectively during 2003 to 2008. During this period, two outbreak peaks were recorded, first in 2003 and then second in 2006 (Figure 2). The satellite town of Gurgaon (Haryana) bordering Delhi recorded a severe outbreak of DF/DHF during 2008 (Source: NVBDCP).

![Figure 2: Status of dengue fever and deaths in NCT Delhi (2003–2008)](image)

Seasonal distribution: Although DF cases were reported throughout the year but a majority, i.e. 96%, were recorded during September to November, with a peak in the month of October (Figure 3). Dengue deaths also coincided with this period.

Spatial distribution of dengue cases in National Capital Territory of Delhi: The dengue incidence pertaining to NCT areas and other agencies is given in Table 1. The

![Figure 3: Seasonal incidence of dengue fever and deaths (2003–2008)](image)
Municipal Corporation of Delhi comprises 12 zones (Figure 1). Six zones border the states of Haryana and Uttar Pradesh and the remaining six are urban zones. The six bordering zones contributed 57% of the cases while the urban zones recorded 38% cases. The NDMC, Railways and Cantonment areas contributed 3.7%, 0.9% and 0.4% cases respectively.

**Table 1: Disease incidence pertaining to NCT areas and other agencies (2003–2008)**

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<td>(a) Zones bordering Haryana and Uttar Pradesh (6 zones)</td>
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<td></td>
<td>(b) Urban zones (6 zones)</td>
<td>38.0</td>
</tr>
<tr>
<td>2</td>
<td>NDMC area</td>
<td>3.7</td>
</tr>
<tr>
<td>3</td>
<td>Railway area</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>Cantonment area</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Vector surveillance:** The *Ae. aegypti* surveillance was carried out throughout the year, with special vigilance being maintained in the zones reporting active cases. The search for the breeding habitats of *Ae. aegypti* to monitor the entomological indices, viz. House Index (HI), Container Index (CI) and Breteau Index (BI), was carried out (Table 2).

From Table 2 it is evident that the *Ae. aegypti* breeding in NCT Delhi started rising from April, reaching the peak in August (BI=7.3), and then the decline in September (BI=5.8).

**Breeding potential of *Ae. aegypti* (indoors and outdoors):** The break-up of the preferred breeding sites of *Ae. aegypti*, both indoors and outdoors is given in Figure 4.

(1) **Indoor breeding:** Indoor water coolers (cooling by water evaporation) were the most preferred sites, followed by cement water tanks, flowers vases and overhead water tanks (OHT). High breeding in water coolers is facilitated by the community’s lax behaviour towards weekly cleaning which is essential to prevent breeding. Underground cement tanks remained covered, but whenever broken, they do not find replacement. Overhead water tanks, mostly with plastic bodies, get heated up in summer and do not support breeding.

**Figure 4: Breeding potential of *Ae. aegypti* in NCT Delhi**

![Breeding potential of *Ae. aegypti* in NCT Delhi](image)
(2) Outdoor breeding: During the rainy season, trash comprising of packaged consumer items, broken pots, iron scrap and used tyres are the most common items holding rain-water, which supports breeding and multiply the vector population.

DF transmission season: NCT Delhi has four distinct seasons – (i) hot and dry (April–June); (ii) rainy season (July–September); (iii) autumn (October–November); and (iv) winter (December–March). The transmission season starts in the rainy season and extends into the autumn because of congenial temperature and humidity combination. However, possibilities of DF transmission during the hot and dry season cannot be ruled out. Room water coolers, which is the cheapest product available locally for the cooling of human dwellings, is affordable even by the people belonging to lower socioeconomic groups living in slums. Room coolers not only cool the houses effectively but also provide high humidity. *Ae. aegypti* breeds prolifically in water troughs and rests indoors in humid dark places. Extended periods of winter rains and the combination of low temperature and high humidity in summer months permit completion of the extrinsic incubation cycle of the virus when active transmission can take place.

Discussion

Entomological surveillance: The first-ever comprehensive survey of *Ae. aegypti* population was carried out in 1964 to assess the potential threat to Delhi after the Kolkata episode in 1963. The study revealed that *Ae. aegypti* was confined to the central part of city and the peripheral areas were free of the infestation. In the walled (old) city, the breeding index was reported to vary from 50 to 100, thus indicating very high receptivity of the vector species[6].

(1) High endemicity of *Ae. aegypti*: The longitudinal studies by the National Anti-Malaria Programme (NAMP), now called NVBDCP, indicated that the vector species had now fully established itself in all zones of MCD, NDMC, Cantonment and Railways. The monthly cumulative indices fully synchronized with the disease incidence[7] (Figure 5).

(2) Receptivity of schools/hospitals: A spatial study carried out in 1998 showed high receptivity of schools and hospitals in view of the high vulnerability in a randomly selected 12 schools/hospitals. The Container Index in schools varied from 2.5 to 28.3, while in hospitals it varied from 2 to 45.1[8]; these are the most common places of DF transmission.

(3) Epidemiological impact: A graphic integration of the epidemiological and entomological data is presented in Figure 5. It is evident that August to October were the most crucial months epidemiologically. Subsequently, with the onset of winter, the transmission ceased by December.

Conclusion

From the forgoing, it is evident that the NCT Delhi region carries a high receptivity and

**Figure 5:** Seasonal incidence of dengue fever and dynamics of *Ae. aegypti* (2003–2008)
vulnerability to *Ae. aegypti* because of high international traffic as well as from the bordering satellite towns of Gurgaon (Haryana) and Noida (Uttar Pradesh), which are highly endemic for DF/DHF. The transmission of dengue here, unlike in other parts of the country where it occurs only during the rainy season, has an extended transmission period covering both the hot and dry and rainy seasons. The transmission may start in summer (hot and dry season) in the years of extended winter rainfall up to April, followed by the rainy season. This requires round-the-year vector control measures to ensure effective control of the vector. To meet these challenges, strengthening of the health infrastructure of all agencies involved in the prevention and control of dengue is essential. These activities require a well-organized and coordinated effort to control the incidence of dengue in the NCT region.

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**References**


Increased utilization of treatment centre facilities during a dengue fever outbreak in Kolkata, India

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Abstract
An outbreak of febrile illness occurred in Kolkata (formerly Calcutta), India, which led to an increased utilization of treatment centre facilities during August – September 2005. The etiological agent was confirmed to be dengue by analysing 308 acute-phase clinical specimens for virus-specific IgM antibodies.

Keywords: Dengue fever; Outbreak, Treatment centres.

Background
In dengue-hyperendemic countries such as Indonesia, Thailand, the Philippines and Viet Nam, the circulation of multiple virus serotypes is well-established, regular dengue outbreaks occur, and the severe form of the disease is a common problem readily recognized by experienced clinicians. In contrast, in South Asian countries such as India, Bangladesh, and Sri Lanka, dengue is considered as an emerging disease and epidemics have been more recently recognized[1]. Clinicians have less experience with dengue, and laboratory confirmation of the etiology of the viral disease is urgently needed to highlight this problem. Recently, an increasing number of outbreaks have been reported from various parts of India[2-9]. We report a laboratory-confirmed outbreak of dengue fever in an urban slum community of Kolkata during the course of a community-based fever surveillance study.

Methods
Kolkata is the third largest city in India and is one of the world’s most densely populated cities; 13 million residents live within an area of 1450 sq. kms. Kolkata has three seasons,
the cool dry months from November to February, the hot dry period from March to May, and the monsoon season from June to October. As part of a typhoid fever vaccine trial, we conducted a community-based passive surveillance for febrile illness in a well-defined urban slum population of about 60,000 individuals living in Wards 29 and 30. Blood samples were collected from patients residing in the study area who presented to treatment facilities in the study area with fever of 3 days or longer \textsuperscript{[10]}. The blood samples were used to inoculate Bactec Plus Aerobic bottles (Becton Dickinson, New Jersey, USA) for bacterial culture, to make thick and thin blood films for malaria diagnosis, and preserved as sera for serological testing. A detailed description of the methods is presented elsewhere \textsuperscript{[10]}. The study was approved by the Institutional Ethics Committee of the National Institute of Cholera and Enteric Diseases, the Ministry of Health Screening Committee of the Government of India, and the Institutional Review Board of the International Vaccine Institute.

An alarming increase in febrile episodes was noted in 2005 (Figure). The number of fever episodes evaluated in August and September 2005 was 1637, nearly double the 935 cases evaluated in August and September 2004. The possibility of a dengue outbreak was investigated using a Commercial Pathozyme Dengue IgM kit (Omega Diagnostics Limited, Omega House, Carsebridge Court, Whins Road, Scotland, UK) \textsuperscript{[11]}. This is an in vitro diagnostic test based on an indirect enzyme immunoassay for screening dengue IgM antibody in infections caused by all four serotypes. Briefly, diluted sera (after absorption of IgG antibody) were added to the wells coated with dengue-specific antigen. After a thorough wash, peroxidase conjugated

\textbf{Figure:} The number of fever episodes evaluated and confirmed malaria, typhoid and paratyphoid fever from January 2004 to December 2005, Ward 29 and 30, Kolkata, India
A dengue fever outbreak in Kolkata, India

Antihuman IgM followed by specific substrate were added. A colour development indicated the presence of human anti-dengue antibody. The reaction was stopped by the addition of diluted sulphuric acid and absorbance was measured. Positive and negative controls supplied with the kit were used during each run. We compared the characteristics of patients with and without dengue IgM. We used the chi-square test for comparison of categorical variables and the Wilcoxon rank sum test for comparison of medians. Statistical significance was designated as a p-value less than 0.05 (2-tailed).

Results

From 1 August to 30 September 2005, a total of 1637 residents in the study area presented to a treatment centre with fever of 3 days or more. Of these 1637 fever cases, 471 (29%) presented with fever of 5 days or longer, and 308 (65%) were tested for dengue. 87/308 (28%) were positive for dengue IgM antibodies, suggestive of primary dengue infection. The ages ranged from 3 years to 60 years for those who tested positive and 1 year to 77 years for those who were negative. The characteristics of the patients with a positive and negative test for dengue IgM were compared (Table). The patients who had a positive dengue IgM test had a slightly lower median age and were more likely to have vomiting. There were no significant differences in the other characteristics between the two groups.

Discussion

Our surveillance detected an outbreak of dengue fever during the rainy season in a densely-populated area of India, where dengue has not traditionally been considered a local cause of fever. The principal vector for dengue fever is the female Aedes aegypti which breeds around human dwellings, in water containers, vases, cans, old tyres and other discarded objects\(^1\), which are common in the study site. The presence of this vector in Kolkata has been documented. Ae. aegypti prevalence coincides with the rainy season which sets in Kolkata from July to September\(^2\), the same months when this dengue outbreak occurred.

We confirmed acute dengue fever in our study area with no evidence of severe manifestations (i.e. plasma leakage, haemorrhage, shock). There are four dengue

| Table: Characteristics of febrile patients presenting for treatment with a positive and negative test for dengue IgM, Ward 29 and 30, Kolkata, India |
|-------------------------------------------------|-------------------------------------------------|-----------------|-----------------|
| Females (%) | Positive for dengue IgM (n = 87) | Negative for dengue IgM (n = 221) | p value |
| Median age in years | 17 | 20 | 0.05 |
| Median number of days fever | 5 | 6 | N.S. |
| With continuous fever (%) | 24 (28%) | 56 (25%) | N.S. |
| With nausea (%) | 27 (31%) | 56 (25%) | N.S. |
| With vomiting (%) | 17 (20%) | 18 (8%) | 0.01 |
| With abdominal pain (%) | 15 (17%) | 21 (10%) | N.S. |

N.S.: Not significant
serotypes. Infection with one provides life-long immunity against the same serotype, but not against the other serotypes. The risk of severe disease is increased about 15-fold during repeat infection due to a serotype different from a previous dengue infection[14]. Thus, populations previously infected by one or more dengue serotypes are at an increased risk for more severe manifestation during subsequent dengue episodes due to other serotypes.

The observations made in Kolkata in 2005 are consistent with a population with little to no pre-existing, anti-dengue immunity and where dengue is an emerging disease. Although the illnesses in the current outbreak were self-limiting, the sudden increase in consultations was an unexpected burden to the existing treatment facilities. Furthermore, there is the potential for more severe disease manifestations in future outbreaks. The proportion of febrile episodes caused by dengue during the coming years warrants further investigation. It would also be important to follow any changes in signs and symptoms of the disease, particularly the occurrence of severe manifestations.

We did not perform virological confirmation of the disease and therefore could not evaluate the circulating dengue serotype(s). We were not able to check for other etiologies, especially other flaviviruses with potential diagnostic cross-reaction. Our serological diagnosis of dengue infection relied on the presence of IgM antibody. By day five of illness, 80% of dengue cases had detectable IgM antibody, and by day six to ten, 93 to 99% of cases have detectable IgM that may persist for over 90 days[15]. We were unable to check for dengue IgG antibodies in the acute sera, nor did we collect convalescent sera to assess a rise in IgG antibody titre. However, we believe that the test for IgM antibodies among those with fever of 5 days or longer is appropriate to confirm an outbreak, particularly in this relatively dengue-naïve community.

The realization that dengue fever is an increasing cause of febrile disease in South Asia suggests the urgent need for preventive interventions. A safe, highly protective, long-lasting vaccine at an affordable price for large populations at risk in the tropical regions of Asia and the Americas would be the ideal control strategy. Meanwhile, preventive activities have to focus on vigorous vector control.

Acknowledgement

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References


A dengue fever outbreak in Kolkata, India


Entomological survey of dengue vectors as basis for developing vector control measures in Barangay Poblacion, Muntinlupa City, Philippines, 2008

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Abstract

An entomological survey of dengue vectors was carried out in Barangay Poblacion Muntinlupa City in Philippines. The survey revealed the presence of only one Aedes species, i.e. Aedes aegypti. The House Index (HI), Container Index (CI) and Bretaeu Index (BI) were estimated as 23%, 23.4% and 63 respectively. Drums, used tyres and soft-drink cases were the major breeding habitats. Although communities kept the water storage containers covered, but this failed to prevent the breeding.

Keywords: Aedes aegypti; Drums; Used tyres; Aedes indices; Philippines.

Introduction

Dengue fever (DF) and dengue haemorrhagic fever (DHF) was first recognized in the Philippines in 1953[1]. Dengue virus is transmitted by Aedes aegypti, which abounds in all cities and towns in the country.

In recent years, a major outbreak was recorded in 1998, with 35 100 cases and 500 deaths for all regions (Department of Health, 2001). Seventy per cent of those admitted were children less than 15 years of age.

Dengue control in Philippines is a community-based programme. Hence, the participation of all members of the community as well as all sectors of society is a must for the success of the programme.

The present entomological study was undertaken in Barangay Poblacion Muntinlupa city during 2008 to detect the prevalence of Aedes species and their breeding habitats for developing a control strategy for the same.
Study area

The entomological survey was conducted in Barangay Poblacion, Muntinlupa City. Barangay Poblacion has a population of 33,096 [City Health Office (CHO), 2008]. According to the records of the CHO Surveillance Unit, Barangay Poblacion ranks number one in 2007 with 87 dengue cases and three deaths in the nine barangays of the city. Clustering of the dengue cases was recorded in Magdaong, hence it was chosen to be the study site.

Magdaong is located in the National Bilibid Prison (NBP) Reservation area in Barangay Poblacion, Muntinlupa City. It has a total population of 3,312 with 552 households. Most of the houses are constructed with light materials while others are made of mixed wood and hollow blocks. There is no piped water supply in the area. The community gets water for washing/bathing purposes from the NBP delivery truck and free water delivery project of Mayor Aldrin San Pedro. People buy water for drinking purposes. There is no artesian well in the area.

Methodology

A total of 100 households were chosen for *Aedes* larval survey. The sampling unit is the household defined as one unit of accommodation sharing a common pot, irrespective of the number of persons residing therein.

Every fifth house was included in the survey. The larval survey and computation of entomological indices, viz. House Index (HI), Container Index (CI) and Breteau Index (BI), were carried out as per WHO guidelines [2].

Results

Out of the 100 houses searched, 23 houses were found infested with immatures for *Aedes* mosquitoes. There were 269 water-holding containers, out of which 63 containers were breeding *Aedine* mosquitoes (Table 1). Identification of both the larval and pupal stages revealed the presence of only one *Aedes* species, i.e. *Ae. aegypti*, in the study area. Computed larval indices are presented in Table 1.

From the table it is apparent that the threshold levels of House Index (HI), Container Index (CI) and Breteau Index (BI) were high. The BI of 63 containers, which establishes direct relationship between positive containers and houses, indicate a high transmission potential for dengue.

Key breeding sites of *Ae. aegypti*

The distribution of breeding habitats is given in Table 2.

From the table it is apparent that metal drums, used tyres and soft-drink cases were the most preferred breeding sites, while pails, jars and jugs were the least attractive.

<table>
<thead>
<tr>
<th>Study area</th>
<th>Total houses searched</th>
<th>House Index (HI) +ve</th>
<th>Index %</th>
<th>Container Index (CI) Wet containers searched</th>
<th>Containers +ve</th>
<th>Index %</th>
<th>Breteau Index (BI) per 100 houses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magdaong</td>
<td>100</td>
<td>23</td>
<td>23</td>
<td>269</td>
<td>63</td>
<td>23.4</td>
<td>63</td>
</tr>
</tbody>
</table>

Table 1: Computed larval indices of *Ae. aegypti* as observed in Magdaong Poblacion, Muntinlupa City.
Entomological survey of dengue vectors in the Philippines

Table 2: Number of positive containers by habitats inspected in Magdoang Poblacion, Muntinlupa City

<table>
<thead>
<tr>
<th>Type of container</th>
<th>Total number of containers inspected</th>
<th>Number of containers positive</th>
<th>Percentage positive containers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drums</td>
<td>107</td>
<td>40</td>
<td>37.3%</td>
</tr>
<tr>
<td>Used tyres</td>
<td>43</td>
<td>17</td>
<td>40%</td>
</tr>
<tr>
<td>Soft-drink cases</td>
<td>2</td>
<td>2</td>
<td>100%</td>
</tr>
<tr>
<td>Pails</td>
<td>69</td>
<td>2</td>
<td>2.9%</td>
</tr>
<tr>
<td>Jars</td>
<td>36</td>
<td>1</td>
<td>2.8%</td>
</tr>
<tr>
<td>Jugs</td>
<td>12</td>
<td>1</td>
<td>8.3%</td>
</tr>
<tr>
<td>Total</td>
<td>269</td>
<td>63</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Number of containers with cover and without cover positive for Ae. aegypti larvae at Magdoang Poblacion, Muntinlupa City

<table>
<thead>
<tr>
<th>Type of container</th>
<th>With cover</th>
<th>Without cover</th>
<th>Total</th>
<th>Percentage +ve with cover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drums</td>
<td>37</td>
<td>3</td>
<td>40</td>
<td>92.5</td>
</tr>
<tr>
<td>Used tyres</td>
<td>0</td>
<td>17</td>
<td>17</td>
<td>0.0</td>
</tr>
<tr>
<td>Soft-drink cases</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0.0</td>
</tr>
<tr>
<td>Pails</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Jars</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Jugs</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>22</td>
<td>63</td>
<td>65</td>
</tr>
</tbody>
</table>

Classification by coverage of breeding containers

To know the impact of ‘covering’ the breeding containers as practised by the local communities, 63 positive containers were classified into (i) covered; and (ii) without cover (Table 3).

From the table it is clear that a majority of the drums and, to some extent, pails, jars and jugs, were kept covered. However, a majority of them failed to prevent breeding. Metal drums, which were the main storage containers and were kept covered, were found breeding to the extent of 92.5 per cent. Hence, there is a need to render the covers more airtight to prevent the entry of mosquitoes.

The results showed that the study area carries a high potential for dengue outbreaks. Since dengue control in Philippines is community-based, there is an urgent need (i) to strengthen the health infrastructure, (ii) for advocacy to sensitize local populations; and (iii) for intersectoral coordination to improve dependable water supply and professional management of solid waste disposal.

Acknowledgement

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References


Aedes survey of selected public hospitals admitting dengue patients in Metro Manila, Philippines

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Abstract
Entomological investigations were carried out in five selected public hospitals admitting dengue patients in Metro Manila, Philippines. The results revealed the presence of only one species, i.e. Aedes aegypti, mostly breeding in fresh water plant vases, drums, basins, plastic cups, tin cans, and empty paint cans. The water plant vase/bowl was found to be the most preferred container for Ae. aegypti breeding. The percentage positive rates of fresh water plant vases of the five public hospitals were: RMC (40.69%), TMC (76.62%), SLH (80.60%), NCH (64.06%), and EAMC (40.40%). An analysis of data revealed that the Premise, Container, and Breteau indices varied from 0.0 to 4.0, 0.0 to 41.1 and 0.0 to 11.0 respectively, indicating thereby the high receptivity of the area to DF/DHF transmission. The egg density ranged from 0.0 to 48.5 which showed the presence of Ae. aegypti vector in the five public hospitals. The presence of productive breeding sites indoors and outdoors in the study area revealed that outbreaks could possibly occur in the future if no vector control plan is adopted and implemented.

Keywords: Aedes aegypti; Hospitals; Receptivity; Ovitrap index; Philippines.

Introduction
The Philippines is one of the dengue endemic countries in Asia. Since the first outbreak of dengue fever (DF) and dengue haemorrhagic fever (DHF) in 1953[1], Philippines has been experiencing an increasing incidence of dengue cases. Based on the data of the National Epidemiology Centre of the Department of Health[2], there were 12 900 cases in 1997, 35 600 in 1998, 9221 in 1999, 8761 in 2000 and 25 050 cases in 2001.

Entomological surveys of communities have been conducted in the Philippines since the development of the Dengue Prevention and Control Programme (DPCP) of the Department of Health was initiated. The increase and peak in the number of dengue cases were observed to coincide with the rainy months of August to November, and the Aedes aegypti density was the highest during this period, while the months of February to April are low-density months. In 1993, Schultz[3], in his study on the seasonal abundance of dengue, confirmed that Ae.
Aedes aegypti was the major container breeder in residential areas while Ae. albopictus predominated in cemeteries in Manila.

In view of the high vulnerability of major hospitals, which admit dengue patients, no study has been carried out to assess the receptivity for Ae. aegypti.

The present study was conducted in five major hospitals during September 2001 to April 2002 and the results are incorporated in the present communication.

**Study area**

An entomological survey of dengue vectors was conducted in five selected public hospitals in Metro Manila, which had the highest number of dengue admissions for five years (1995–2000) based on the Department of Health (DOH) – National Capital Region (NCR) Surveillance data 1995–2000[2]. These were: San Lazaro Hospital (SLH), Rizal Medical Centre (RMC), Tondo Medical Centre (TMC), East Avenue Medical Centre (EAMC) and National Children’s Hospital (NCH).

**Methodology**

**Aedes larval/pupal survey**

A total of 500 rooms were surveyed monthly for Ae. aegypti in the five selected hospitals. Larval/pupal survey was done monthly for six months. The survey for Ae. aegypti mosquito breeding was carried out using the single larva techniques. The larvae/pupae were collected from all infested containers and identified in the laboratory for species identification. Subsequently, water was removed to destroy the foci of breeding.

The level of infestation of Ae. aegypti mosquitoes, i.e. Premise (House) Index (PI), Container Index (CI) and Breteau Index (BI), was estimated as per WHO guidelines[4].

**Ovitrap survey**

The ovitrap was made from a tin can 76.2 mm in diameter, 101.6 mm in height, and painted black inside and outside. Water was added to a level of 76.2 mm. A wooden paddle (25.4 mm x 152.4 mm) was placed diagonally inside the tin can with the rough surface on top. The paddle served as oviposition substrate. The number of ovitraps used was calculated through sequential sampling (Lee, 1987)[5]. Thirty ovitraps were set up indoors of hospitals and 30 ovitraps were placed outdoors. “Indoors” refers to the interior of the room while “outdoors” refers to the outside of the hospital building but within the hospital compound. The ovitraps were set up at different strategic places indoors – under the sink, in the corner of rooms, under the cabinet, under the bed, inside the comfort room, in the hospital chapel, and the nurse station; while outdoors, these were placed in corners of the hospital building, in the parking area, near vegetation, near piles of wood/hollow blocks, and plants/trees in the garden. Lost or damaged ovitraps were replaced regularly.

The ovitraps were collected after seven days with the water and paddle placed separately in plastic bags. The eggs were counted in all positive ovitraps. Plastic bags of positive ovitraps were submerged into a pan for larval emergence. The larvae were counted and identified for species confirmation. Similarly, the collected paddles were submerged into a pan with water for larval emergence. Fish flakes were used as larval food. The third and fourth instar larvae were identified for confirmation of species.
Analysis of data

The PI, CI and BI were computed using the WHO guidelines. At the same time, the Ovitrap Index and Ovitrap Density Index were computed.

Results and discussion

The monthly averages of Premise Index (PI), Container Index (CI) and Breteau Index (BI) of San Lazaro Hospital (SLH) varied from 0 to 2, 5 to 25 and 2.0 to 8 respectively; in the Tondo Medical Centre (TMC) these were 1.0 to 3, 2.7 to 30.55 and 1 to 11; in the National Children’s Hospital (NCH) 1 to 3, 7.14 to 27.27 and 1 to 4; in the East Avenue Medical Centre (EAMC) these were 0 to 3; 0 to 13.04 and 0-4; and in the Rizal Medical Centre (RMC) the averages were 0 to 2, 5.12 to 25 and 2 to 8 respectively, thereby indicating the low receptivity of the five public hospitals to DF/DHF transmission (Table 1). The results revealed that the breeding of *Ae. aegypti* occurred throughout the six months in the five public hospitals in Metro Manila. The breeding index was very low during the cool months of January and February and the hot months of March and April in all the hospitals surveyed. The proportion of breeding containers of *Ae. aegypti* as presented in Table 2 showed that fresh water plant vases were the major indoor containers inspected in the five public hospitals.

The results revealed that *Ae. aegypti* bred in both indoor and outdoor breeding sites. The indoor containers searched and found infested with larvae were fresh water plant vases, drums

| Table 1: Monthly *Ae. aegypti* indices of five public hospitals in Metro Manila during September 2001 to April 2002

San Lazaro Hospital (SLH)

<table>
<thead>
<tr>
<th>Month</th>
<th>PI</th>
<th>ICI</th>
<th>OCI</th>
<th>CI</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>October</td>
<td>4.0</td>
<td>0</td>
<td>36</td>
<td>4.0</td>
<td>4</td>
</tr>
<tr>
<td>November</td>
<td>2.0</td>
<td>31.0</td>
<td>75</td>
<td>41.1</td>
<td>7</td>
</tr>
<tr>
<td>December</td>
<td>4.0</td>
<td>8.3</td>
<td>100</td>
<td>15.38</td>
<td>4</td>
</tr>
<tr>
<td>January</td>
<td>1.0</td>
<td>4.76</td>
<td>33.3</td>
<td>8.3</td>
<td>2</td>
</tr>
<tr>
<td>February</td>
<td>2.0</td>
<td>4.50</td>
<td>33.3</td>
<td>8.0</td>
<td>2</td>
</tr>
<tr>
<td>March</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Tondo Medical Centre (TMC)

<table>
<thead>
<tr>
<th>Month</th>
<th>PI</th>
<th>ICI</th>
<th>OCI</th>
<th>CI</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>October</td>
<td>3</td>
<td>18.75</td>
<td>40.0</td>
<td>30.55</td>
<td>11</td>
</tr>
<tr>
<td>November</td>
<td>2</td>
<td>28.57</td>
<td>27.77</td>
<td>28.0</td>
<td>7</td>
</tr>
<tr>
<td>December</td>
<td>1</td>
<td>5.55</td>
<td>25.0</td>
<td>14.70</td>
<td>5</td>
</tr>
<tr>
<td>January</td>
<td>1</td>
<td>18.75</td>
<td>40.0</td>
<td>30.55</td>
<td>11</td>
</tr>
<tr>
<td>February</td>
<td>1</td>
<td>11.11</td>
<td>0</td>
<td>2.70</td>
<td>1</td>
</tr>
<tr>
<td>March</td>
<td>1</td>
<td>11.11</td>
<td>0</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>
and basins, while the outdoor containers positive for *Aedes* larvae were fresh water plant vases, empty paint cans, discarded plastic cups, drums, used automobile tyres, empty bottles and abandoned toilet bowls. These receptacles provided breeding in both dry and wet seasons (Table 3).

### Ovitrap survey

The Ovitrap Index indoors was also observed to build up from October to January (Table 4). The findings were contrary to reports that the breeding was only confined to the wet season. The Mean Ovitrap Index varied from 0.0 to 48.5
Table 2: Percentage of indoor containers inspected in five public hospitals

<table>
<thead>
<tr>
<th>Type of containers</th>
<th>RMC</th>
<th>TMC</th>
<th>NCH</th>
<th>EAMC</th>
<th>SLH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh water plant vase</td>
<td>59 (40.69%)</td>
<td>59 (76.62%)</td>
<td>41 (64.06%)</td>
<td>90 (46.40%)</td>
<td>166 (80.60%)</td>
</tr>
<tr>
<td>Basin</td>
<td>1 (0.69%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pails</td>
<td>43 (29.65%)</td>
<td>13 (16.88%)</td>
<td>9 (14.06%)</td>
<td>98 (50.51%)</td>
<td>9 (4.36%)</td>
</tr>
<tr>
<td>Plastic drums (orocan)</td>
<td>42 (28.97%)</td>
<td>3 (3.90%)</td>
<td>14 (21.88%)</td>
<td>6 (3.09%)</td>
<td>29 (14.07%)</td>
</tr>
<tr>
<td>Plant saucer</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (0.97%)</td>
</tr>
<tr>
<td>Total</td>
<td>145 (100%)</td>
<td>77 (100%)</td>
<td>64 (100%)</td>
<td>194 (100%)</td>
<td>206 (100%)</td>
</tr>
</tbody>
</table>

Table 3: Number of indoor and outdoor containers positive for Ae. aegypti in five hospitals in Metro Manila, during September 2001 to April 2002

<table>
<thead>
<tr>
<th>Type of containers</th>
<th>Indoors</th>
<th>Outdoors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. searched</td>
<td>Positive</td>
</tr>
<tr>
<td>Freshwater plant vase</td>
<td>356</td>
<td>50 (14)</td>
</tr>
<tr>
<td>Drums</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Basins</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Empty coconut shells</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Plastic cups</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tin cans</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Used tyres</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Empty paint cans</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abandoned toilet bowls</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Decorative jars</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figures in brackets indicate percentage

and showed the presence of Ae. aegypti vector in the five public hospitals. The Mean Ovitrap Density Index ranged from 24.98 to 32.58 per positive ovitrap (Table 5). There were 8464 eggs counted and 10% were reared to Aedes larvae, 90% were Ae. aegypti and 10% were Ae. albopictus. This showed that Ae. aegypti bred in the hospitals predominantly over Ae. albopictus. Our study showed that the ovitrap technique could be a reliable tool in vector surveillance. The ovitrap detected density of Aedes mosquito in hospitals even during hot months.

Conclusion

Our study established the high receptivity of five hospitals in Metro Manila (breeding potential) for dengue vectors. The presence
### Table 4: Ovitrap Index

#### San Lazaro Hospital (SLH)

<table>
<thead>
<tr>
<th>Month</th>
<th>Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oli</td>
</tr>
<tr>
<td>October</td>
<td>23.3</td>
</tr>
<tr>
<td>November</td>
<td>3.3</td>
</tr>
<tr>
<td>December</td>
<td>6.6</td>
</tr>
<tr>
<td>January</td>
<td>20.0</td>
</tr>
<tr>
<td>February</td>
<td>37.0</td>
</tr>
<tr>
<td>March</td>
<td>0.0</td>
</tr>
</tbody>
</table>

#### East Avenue Medical Centre (EAMC)

<table>
<thead>
<tr>
<th>Month</th>
<th>Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oli</td>
</tr>
<tr>
<td>November</td>
<td>26.6</td>
</tr>
<tr>
<td>December</td>
<td>16.6</td>
</tr>
<tr>
<td>January</td>
<td>6.7</td>
</tr>
<tr>
<td>February</td>
<td>17.0</td>
</tr>
<tr>
<td>March</td>
<td>13.3</td>
</tr>
<tr>
<td>April</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Oli = Ovitrap Index indoor (%), OIo = Ovitrap Index outdoor (%), Olm = Mean Ovitrap Index

#### Tondo Medical Centre (TMC)

<table>
<thead>
<tr>
<th>Month</th>
<th>Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oli</td>
</tr>
<tr>
<td>October</td>
<td>3.3</td>
</tr>
<tr>
<td>November</td>
<td>10.0</td>
</tr>
<tr>
<td>December</td>
<td>13.3</td>
</tr>
<tr>
<td>January</td>
<td>20.0</td>
</tr>
<tr>
<td>February</td>
<td>3.4</td>
</tr>
<tr>
<td>March</td>
<td>0.0</td>
</tr>
</tbody>
</table>

#### Rizal Medical Centre (RMC)

<table>
<thead>
<tr>
<th>Month</th>
<th>Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td>September</td>
<td>17.0</td>
</tr>
<tr>
<td>October</td>
<td>37.0</td>
</tr>
<tr>
<td>November</td>
<td>7.0</td>
</tr>
<tr>
<td>December</td>
<td>20.0</td>
</tr>
<tr>
<td>January</td>
<td>17.0</td>
</tr>
<tr>
<td>February</td>
<td>3.3</td>
</tr>
</tbody>
</table>

#### National Children's Hospital (NCH)

<table>
<thead>
<tr>
<th>Month</th>
<th>Indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oli</td>
</tr>
<tr>
<td>October</td>
<td>27.0</td>
</tr>
<tr>
<td>November</td>
<td>3.33</td>
</tr>
<tr>
<td>December</td>
<td>3.33</td>
</tr>
<tr>
<td>January</td>
<td>7.0</td>
</tr>
<tr>
<td>February</td>
<td>10.0</td>
</tr>
<tr>
<td>March</td>
<td>13.3</td>
</tr>
</tbody>
</table>

### Table 5: Ovitrap Density Index

<table>
<thead>
<tr>
<th>Name of hospital</th>
<th>No. of eggs (indoor)</th>
<th>No. of eggs (outdoor)</th>
<th>Total</th>
<th>No. of (+) ovitrap</th>
<th>Ovitrap Density Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAMC</td>
<td>898</td>
<td>1741</td>
<td>2639</td>
<td>81</td>
<td>32.58</td>
</tr>
<tr>
<td>SLH</td>
<td>891</td>
<td>690</td>
<td>1581</td>
<td>49</td>
<td>32.26</td>
</tr>
<tr>
<td>RMC</td>
<td>613</td>
<td>811</td>
<td>1424</td>
<td>57</td>
<td>24.98</td>
</tr>
<tr>
<td>NCH</td>
<td>296</td>
<td>792</td>
<td>1088</td>
<td>35</td>
<td>31.08</td>
</tr>
<tr>
<td>TMC</td>
<td>523</td>
<td>1209</td>
<td>1732</td>
<td>56</td>
<td>30.92</td>
</tr>
</tbody>
</table>
of key breeding receptacles indoors and outdoors confirmed the presence of dengue vectors, *Ae. aegypti* and *Ae. albopictus*, in all public hospitals surveyed. Hospitals are a priority area for surveillance and control of DF/DHF as they are highly vulnerable, i.e. source of virus through patients. As a result of the study, guidelines for vector control in hospitals were prepared to prevent mosquito breeding in all potential breeding sites of dengue vectors in hospital premises.

**Acknowledgements**

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**References**


Epidemiological and entomological aspects of an outbreak of chikungunya in Lakshadweep Islands, India, during 2007

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Abstract

Since 2006, the Indian state of Kerala has reported outbreaks of chikungunya (CHIK). During July-August 2007, an unusual increase in the incidence of fever was noticed in Kadmat, Amini and Kavaratti Islands in the Union Territory of Lakshwadeep, a group of Indian islands adjacent to the Kerala coast in the Arabian Sea.

The populations affected as per the primary health centre (PHC) records of three islands, viz. Kadmat, Amini and Kavaratti, was 85%, 1.4% and 0.15% respectively. Entomological surveys revealed very high larval indices of *Aedes albopictus* only in the three surveyed islands. *Aedes aegypti*, the classical vector of dengue, was not detected. The maximum breeding of *Ae. albopictus* was found in coconut shells (57%), tyres (9%), metal containers (9%) and plastic containers (8%). The breeding was also detected in tree holes and rat-bitten coconuts on top of the trees. The House Index for *Ae. albopictus* ranged between 95.4% in Kavaratti to 79% in Amini. Kadmat island which was the worst affected, recording the maximum Container Index of 90%, compared with 40% in Amini island. The CHIK outbreak seemed to have been caused by importation of the virus from Kerala, because of heavy movement of the islanders to the mainland.

Keywords: Chikungunya; *Aedes albopictus*; Lakshadweep Islands.

Introduction

In India, a chikungunya (CHIK) outbreak was reported for the first time in Calcutta (now Kolkatta) in 1963[1]. Subsequently, epidemics of chikungunya fever were reported in Pondicherry (now Puducherry), Chennai, Rajahmundry, Vishakapatnam and Kakinada in 1965. Chikungunya reappeared in India after quiescence of four decades in 2005[2]. A large number of cases were reported from the states of Andhra Pradesh, Kerala, Karnataka, Tamil Nadu, Gujarat and Maharashtr[2,3]. As per the National Vector-Borne Diseases Control

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![Image](image-url)
Programme (NVBDCP) records, a total of 1828 CHIK virus-specific IgM confirmed cases were recorded from 210 districts of 12 states after its re-emergence in 2007. In India, both dengue and chikungunya have been reported to be transmitted by *Aedes aegypti* [4].

During July–August 2007, an outbreak of chikungunya was recorded in Kadmat, Amini and Kavaratti islands of the Union Territory of Lakshadweep in the Arabian Sea. The present study was carried out from 2 August to 7 September 2007 to investigate the epidemiological and entomological aspects of the CHIK outbreak. The results of this study are incorporated in the present communication.

**Study area**

Lakshadweep comprises 36 islands, 11 of which are inhabited. The islands are scattered in the Arabian Sea between longitude 71°–74° east and latitude 8°–12°. The total area of Lakshadweep is 32 sq. kms and the population as per the 2001 census is 60 650 (31 131 males and 29 519 females). The population density is 2255 per sq. km. The people have regular contact with Ernakulam in the state of Kerala on the mainland. Ferry services are frequent from Kochi (earlier Cochin) (Kerala) to different islands of Lakshadweep (Figure 1).

The climate is tropical, humid and warm. The average annual rainfall is more than 1500 mm, mainly during the monsoon season (May to September). The relative humidity is always above 70%–75%. The average maximum temperature varies from 29.5 °C to 33.2 °C and the minimum from 23.6 ° to 27 °C.

Kadmat is one of the Lakshadweep group of islands, with an area of 3.2 sq. km. with a population of 5319. Kavaratti island has 10 113 population with an area of 4.22 sq. km. Amini is a small island with 2.60 sq. km. area and 7340 population. Coconut cultivation is the main occupation of the people here.

**Materials and methods**

**Epidemiological data**

House-to-house surveys to find out the attack rate of fever with joint pains were conducted in the affected islands. These surveys were combined with entomological surveys. Epidemiological information was also collected from the primary health care (PHC) centres in the affected islands.
Entomological data

The entomological studies were carried out in Kadmat, Amini and Kavaratti islands as per WHO guidelines\textsuperscript{[5]}. Larval collections were reared into adults to determine the \textit{Aedes} species. After the identification of the species, the emerging adults were destroyed. The searches were carried out in domestic and peri-domestic habitats, viz. coconut shells, grinding stones and metal containers, tyres, plastic containers, cement tanks, glass bottles, mud pots, plastic sheets, etc. The House Index (% houses positive for \textit{Aedes} breeding), Container Index (% containers positive for \textit{Aedes} breeding) and Breteau index (positive containers per 100 houses) were calculated to make an assessment of the vector population in the affected islands.

Results

Epidemiological

Signs and symptoms: In Kadmat island the fever cases started reporting on 2 July 2007 and the daily trend was increasing. The fever cases reported to the PHC centres were mainly with high fever, malaise, headache and arthralgia, particularly pain in small gouts, and vomiting. Skin rashes were also seen in some of the patients. Twenty-three blood samples were collected from eligible patients from PHC and 10 were found positive for CHIK-specific IgM antibody.

Disease incidence: The island-wise CHIK-affected population as per PHC centres recorded from 2 July to 7 September 2007 is included in Table 1.

From Table 1 it is evident that the outbreak was centred around Kadmat island where 85\% of the population was affected. Similarly, in Amini and Karavatti islands, 1.4\% and 0.15\% respectively of the population was affected.

Trend of outbreak build-up: The daily fever cases reported at Kadmat PHC centre from 2 July to 7 September 2007 are shown in Figure 2.

From Figure 2, it may be seen that during 2 July to 30 July 2007, the number of cases reported ranged between 10 and 50, but on 31 July, it climbed to 200 cases. Thereafter, it showed a declining trend, and, by 7 September, the figure had come down to below 100 cases.

House-to-house survey: In a house-to-house survey to assess the attack rate of CHIK, the numbers of sick persons who had fever with joint pains in each house on the three affected islands were recorded. The results are presented in Table 2.

From Table 2, it is evident that the attack rate in Kadmat island of 31.3\% showed a declining trend, while in Amini and Kavaratti, each showing 1.9\% attack rate, showed a rising trend.

<table>
<thead>
<tr>
<th>Affected PHC centres</th>
<th>Area in sq. km.</th>
<th>Population</th>
<th>No. affected with CHIK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kadmat</td>
<td>3.20</td>
<td>5319</td>
<td>4565 (85%)</td>
</tr>
<tr>
<td>Amini</td>
<td>2.60</td>
<td>7340</td>
<td>1025 (1.4%)</td>
</tr>
<tr>
<td>Kavaratti</td>
<td>4.22</td>
<td>10113</td>
<td>16 (0.15%)</td>
</tr>
</tbody>
</table>

Figures in brackets indicate percentage of people affected.
Epidemiological and entomological aspects of an outbreak of chikungunya in Lakshadweep, 2007

Figure 2: Daily reported CHIK fever cases at PHC, Kadmat island (2 July to 7 September 2007)

Table 2: Attack rate of CHIK as determined during house-to-house survey on Kadmat, Amini and Kavaratti islands

<table>
<thead>
<tr>
<th>Date of survey</th>
<th>No. of houses visited</th>
<th>Total no. of persons in the houses visited</th>
<th>Total no. of persons affected at the time of visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kadmat 27 August 2007</td>
<td>100</td>
<td>647</td>
<td>203 (31.3%)</td>
</tr>
<tr>
<td>Amini 29-30 August 2007</td>
<td>200</td>
<td>1278</td>
<td>25 (1.9)</td>
</tr>
<tr>
<td>Kavaratti 31 August - 1 September 2007</td>
<td>200</td>
<td>1292</td>
<td>25 (1.9)</td>
</tr>
</tbody>
</table>

Figures in brackets indicate percentage of people affected clinically

Entomological

Aedes species prevalence: The entomological investigations on all the three islands revealed the presence of only one species, Ae. albopictus. Ae. aegypti, the classical vector of dengue, could not be detected.

Larval indices of Ae. albopictus: The larval indices as worked out on the three affected islands are included in Table 3.

From Table 3, it may be seen that the threshold levels of HI, CI and BI on all the three islands were very high. However, the
maximum BI (380) which establishes a direct relationship between positive containers and houses was recorded on Kadmat island, indicating a high transmission potential as evidenced by the population affected (85%).

**Key breeding habitats:** The maximum breeding of *Ae. albopictus* was recorded in tender coconut shells (57%), grinding stones (3%) and metal containers (9%), tyres (9%), plastic containers (8%), cement tanks (2%), glass bottles (2%), mud pots (2%) and plastic sheets (2%) (Figure 3). The indoor larval habitats were: coconut shells, plastic containers, metal containers, grinding stones, cement tanks, mud pots and plastic sheets. Outdoor breeding places were: tree holes, tyres, broken glass bottles, clogged roof gutters, etc., on all the three islands (Figure 4).

**Figure 3:** Key breeding habitats of *Ae. albopictus* observed in Kadmat, Amini and Kavaratti islands

### Table 3: Larval indices for *Aedes albopictus* in different islands

<table>
<thead>
<tr>
<th>Date of survey</th>
<th>No. of houses surveyed</th>
<th>Vector indices</th>
<th>House Index (%)</th>
<th>Container Index (%)</th>
<th>Breteau Index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kadmat</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 August 2007</td>
<td>100</td>
<td></td>
<td>88</td>
<td>90</td>
<td>380</td>
</tr>
<tr>
<td><strong>Amini</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>29–30 August 2007</td>
<td>200</td>
<td></td>
<td>79</td>
<td>40</td>
<td>146</td>
</tr>
<tr>
<td><strong>Kavaratti</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31 August – 1 September 2007</td>
<td>200</td>
<td></td>
<td>95.4</td>
<td>67.1</td>
<td>222.7</td>
</tr>
</tbody>
</table>
Discussion

During 2007, an outbreak of chikungunya was reported from Lakshadweep island. The outbreak was serologically confirmed (CHIK-specific IgM) to be due to chikungunya virus and this was the first recorded outbreak in the history of Lakshadweep. All the vector indices in all islands were very high and conducive for the transmission of the disease (Table 2).

Out of the three islands surveyed as per PHC centre records, Kadmat island was worst affected, where 85% of the population had suffered from the infection. In Amini and Karavatti islands, only 1.4% and 0.15%
respectively of the population were affected, despite the fact that all three islands had very high vector indices. These variable results could be explained by high vulnerability (introduction of virus) at Kadmat island as compared to the two other islands. The high build-up of the CHIK epidemic can be explained by the shorter extrinsic incubation period (3–5 days) as compared to dengue virus (8–14 days). Consequently, even if the longevity of the vector is adversely affected by climatic factors, the transmission still continues unabated\[6\]. The CHIK transmission continues till all the population develops immunity, which is long-lasting. During the inter-epidemic periods the virus enters into wild animals. Lakshadweep has high rodent and bird populations which possibly act as a reservoir of infection during the inter-epidemic period.

Data collected during the house-to-house survey explains the declining trend in Kadmat, as 85% of the population had already developed immunity due to infection, whereas on the other two islands, the population affected was low and the susceptible population was still high, as reflected by higher indices (1.9%).

In India, Ae. aegypti has been incriminated as the principal vector of CHIK virus in all urban and rural areas\[4\]. However, in Lakshaweep, Ae. albopictus is the CHIK vector in the absence of Ae. aegypti. In La Réunion island in the Indian Ocean, Ae. albopictus was also incriminated as the CHIK vector\[4\]. In nature, Ae. albopictus is assumed to have a low vectorial capacity (i.e. efficacy as a vector) because blood meals taken from non-susceptible hosts do not contribute to the transmission\[7\]. However, in Lakshadweep, Ae. albopictus, in the absence of monkeys and other domestic animals, maintains a high degree of contact with humans. However, this aspect needs to be further assessed.

Kerala on the Indian mainland was one of the endemic states for chikungunya during 2006. In all probability the virus was introduced in these islands through frequent movement of the islanders to the mainland. A similar phenomenon was observed in Italy in 2007 when the virus was again introduced from Kerala\[8,9\].

The control of Ae. albopictus is most difficult as the species occupies both natural habitats, viz. leaf axils and tree holes as well as man-made domestic/peridomestic receptacles which can hold rainwater. The control strategy would require source reduction/larvicidal application in domestic/peridomestic habitats. Professional management of solid waste material outdoors is essential. Coconut shells should be removed or stored under sheds to prevent breeding. All these activities require active participation of the community. Advocacy programmes need to be developed for providing information to the community as well as inter-sectoral cooperation.

**Acknowledgement**

The authors thank Mr N.L. Kalra, WHO Consultant, for his valuable suggestions and help in the preparation of the manuscript. The help and cooperation extended by the Lakshadweep Administration is gratefully acknowledged.
References


Effect of pyriproxyfen in Aedes aegypti populations with different levels of susceptibility to the organophosphate temephos

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Abstract

Vector control with larvicides is an important component in dengue control programmes. In Brazil, the extensive use of temephos has led to the evolution of resistance in Aedes aegypti populations in many parts of the country. One of the strategies proposed for managing temephos resistance is the use of the insect-growth regulator – pyriproxyfen. This study evaluated the lethal concentration for this product in mosquito populations with different profiles of susceptibility to temephos and semi-field residual response to a commercial product. The results suggest the possibility of cross-resistance between temephos and pyriproxyfen.

Keywords: Aedes aegypti; Susceptibility to insecticides; Cross-resistance; Insect-growth regulators.

Introduction

It is estimated that about 975 million people around the world live in areas with dengue transmission risk[1]. In Brazil, all states are infested by Aedes aegypti[2], the main dengue vector in the tropical region[3].

The organophosphate temephos has been used as larvicide since the beginning of the 1980s for controlling Aedes aegypti in Brazil, and its use has been indicated by the National Program for Dengue Control (PNCD)[4]. The prolonged use of this larvicide has selected resistant populations in many parts of the world[5,6]. In Brazil, there are many reports on Ae. aegypti resistance to temephos in several states[7-15].

The first strategy for managing temephos resistance adopted by the Brazilian government was the substitution of larvicides by Bacillus thuringiensis var. israelensis (Bti)[16]. Under field conditions the use of biolarvicide presents the disadvantage of a shorter residual effect than the chemical one. Bti residual effect evaluated under field conditions varied 30–36 days[17] to 7–12 weeks[18,19]. In Brazil, a residual effect of

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larvicide lasted nearly 60 days for the control of *Ae. aegypti*, and this coincides with the frequency of visits of vector control teams in the context of PNCD[4].

In order to manage the temephos resistance in *Ae. aegypti*, besides Bti-based larvicides, products that are classified as insect growth regulators (IGRs) have been pointed out as an alternative by the Ministry of Health in Brazil[20]. The World Health Organization (WHO) recommends pyriproxyfen (IGR) technical grade ingredient for the control of *Ae. aegypti* population. Many studies indicated that pyriproxyfen will not adversely affect a non-target species when applied at rates usually <50 ppb in mosquito control programmes[21]. Among IGRs, pyriproxyfen, which is a mimic of juvenile hormone, is a potent inhibitor of embryogenesis, metamorphosis and adult formation and shows a long residual effect[22,23]. Although resistance to pyriproxyfen has not been reported in *Ae. aegypti* populations, it is important to evaluate its effect on insects that are resistant to organophosphates as they would be the target for the management of resistance to temephos.

The objectives of this study were to estimate lethal concentrations of pyriproxifen and evaluate the residual effect of one commercial formulation of this product on *Ae. aegypti* populations with different susceptibility levels to the organophosphate temephos.

**Methods**

**Origin of *Ae. aegypti* populations**

Field populations were collected through ovitraps according to the sampling methodology used in the Brazilian network for the evaluation of resistance of *Ae. aegypti* to insecticides[16]. *Ae. aegypti* populations were collected from two different regions of Brazil. From the south-
Effect of pyriproxyfen in Aedes aegypti populations with different levels of temephos resistance

east, four populations were collected from São Paulo state, cities of Araçatuba (AT), Bauru (BR), Marília (MA) and Santos (SA) and one from Parana state, Maringa (MG). The second region, the north-east, was represented by three states: Salvador (SS) and Barreiras (BA) from Bahia state, Recife (RE) from Pernambuco state and Fortaleza (FO) from Ceará state. The geographical distribution of these cities is illustrated in the Figure. The eggs collected were used to rear laboratory colonies. The Rockefeller susceptible strain, provided by the Centers of Disease Control, Puerto Rico, was used for comparison.

Laboratory assays

Pyriproxyfen technical grade 98.5%, batch 2006018 [4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propylether] was evaluated for field tests. The stock solution (250 mg/L) was prepared in deionized water and stored at 4 °C. The work solution (2.5 mg/L) was prepared immediately before each test.

The effect of the insect growth regulator was evaluated by the estimation of concentrations that caused inhibition of adult emergence, according to the World Health Organization methodology[24]. This estimation was done through dose-response bioassays. Four Ae. aegypti populations were evaluated: Rockefeller, an insecticide-susceptible reference strain, and three field populations, on the second generation reared in laboratory (F2). The field populations used had been classified according to their temephos-resistance ratio (R.R.) which were calculated at the lethal concentration 95% (LC95). The population Salvador (SS), highly resistant[25,26] (R.R. 11), the population Barreiras (BA), moderately resistant (R.R. 6.9) and the population Bauru (BR) with low level of resistance (R.R. 3.8) were compared to the susceptible Rockefeller strain. For each population, three bioassays were performed, each one with 720 third instar larvae exposed to eight pyriproxyfen concentrations. Eighty larvae were used per dose and for the control group, in four replicates with 20 larvae each. All larvae were fed every other day during observation period with 0.5 ml of solution made of 10% of fish food (Tetra Marine Granules®). Evaluations were done 48 hours after exposure and after each remaining 24 hours, through quantification of live and dead larvae, pupae and adult. The exposure lasted till the last individual died or emerged as adult. Bioassay data were pooled by doses and the percentage of inhibition of adult emergence at each dose was calculated dividing the percentage of adult emergence by the percentage of adult emergence at the control replicates[24]. After that, the estimation of doses that caused 50% and 95% of emergence inhibition (EI) was obtained by the software Polo-PC[27]. The EI doses obtained with Rockefeller strain were used for the calculation of resistance ratio of pyriproxyfen for field populations.

The comparison of populations was performed by analysing the overlapping of EI doses confident intervals.

Simulated field trial

The persistence of two products, Sumilarl® 0.5G (pyriproxyfen, SUMITOMO batch 5099X31) and Temefós Fersol 1G (Fersol Indústria e Comercio Ltda., Product batch 287SP0214000), was evaluated in two kinds of containers, i.e. glass vases and tyres, which are important breeding sites in Sao Paulo state. Glass vases are commonly used for maintaining

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* Technical material was provided by Sumitomo Chemical Co., Ltd. First, Tokyo, Japan
plants (cut or live plants) and contribute to around 30% of *Ae. aegypti* foci in many cities of Sao Paulo state[28].

The Rockefeller strain and six *Ae. aegypti* field populations with different susceptibility to temephos were used in the test. Among the field populations one was considered susceptible MA (R.R. 1.8), two with low level of resistance; MG, and AT (R.R. 2.7 and 2.6 respectively) and three moderately-resistant populations: SA, FO and RE (R.R. 4.6, 8.4 and 9.0 respectively).

For each population five breeding sites of each type were used, one for control and two for each product. Glass vases were filled with 4 l of water and tyres with 800 ml.

Pyriproxyfen at 0.05 ppm and 1 ppm temephos were applied as per the manufacturers’ instructions. All containers, vases and tyres, were kept in a shaded area in an open garage.

Insecticide application was performed at day “zero” and 30 third instar larvae were exposed one day later. New larvae were added at fifteen-day intervals in a period of two months. Mortality was recorded every 24 hours after exposure and daily until the emergence of adults. Pupae were transferred and observed daily to register adult emergence or death. The temperature and pH of treated containers were recorded once a week along with the test.

One-third of the water of each vase was replaced at 15-days interval, before a new exposition of larvae, in order to simulate the domestic situation. There was no water replacement in tyres. In this case the water volume was just filled to the original level before each new larvae exposure.

The control group was used for Abbott correction when the observed mortality was between 5% and 20%.

As pyriproxyfen – the candidate IGR act on different stages of mosquito development, the results were expressed as inhibition of adult emergence following the scheme presented by Pinzon et al.[29] where $EI = 1 - \frac{Ad}{Lexp}$, where $Lexp = \text{larvae exposed}$ and $Ad = \text{adults}$. The result was multiplied by 100 to express it as percentage of inhibition.

The effect of candidate IGR was evaluated through their capacity for providing at least 95% mortality along the time after treatment. As in Brazil, the cycle of visits for vector control proposed by the National Programme (PNCD)[4] is of 60 days, the expected effect of a larvicide should suit this period of time.

The results obtained in *Ae. aegypti* populations were compared after transformation of the mortality data into arcsin values. Data were pooled by group according to resistance status of populations being MG, MA and AT pooled at one group for presenting low level of resistance (R.R. below 4) and populations SA, RE and FO pooled in a second group for presenting moderate resistance to temephos (R.R. between 6 and below 10). Comparisons were made between the two groups and between each group and Rockefeller strain with Student t-test.

**Biochemical assays**

The activity of metabolic enzymes alpha and beta esterases, mixed function oxidases (MFO) and glutathione-S-transferase (GST) were evaluated in larvae according to the Centers of Disease Control protocol[30,31]. The enzymatic activity obtained by each larvae was corrected by the respective protein values. The results were analysed as proposed by Montella et al.[15], which is the standard method for analysis at the Brazilian Network for the evaluation of resistance of *Ae. aegypti* to insecticides[32]. The
enzyme activity of field populations was compared with the susceptible Rockefeller strain. The percentage of individuals with enzyme activity higher than the Rockefeller percentage 99 classifies that activity as “normal” when it is below 15%, “altered” when it is between 15% and 49%, and “highly altered” when it is more than 50%.

Results

Estimation of adult emergence inhibition concentrations

Pyriproxyfen exhibited a major effect on adult emergence with high mortality at pupal stage (97.2%) and very low mortality of larvae (2.8%). The estimated EI 50 and EI 95 of pyriproxyfen for the susceptible Rockefeller strain and the three field Ae. aegypti populations are presented in Table 1 where data of temephos lethal concentrations and resistance ratios of both products are compared.

By the analysis of confidence interval of EI concentrations, all the populations differed from the Rockefeller strain and also among each other. SS population presented the higher R.R. for both temephos and pyriproxyfen. Population BA, moderately resistant to temephos, had a lower R.R. for pyriproxyfen than population BR which had the smallest R.R. for temephos.

Enzyme activity

The enzyme activity was evaluated for all populations except the field RE and MFO enzyme for MA population. The number of

Table 1: Estimated adult emergence inhibition concentrations of pyriproxyfen and lethal concentrations of temephos (confidence interval). Resistance ratios based on Rockefeller concentrations.

<table>
<thead>
<tr>
<th>Pyriproxyfen</th>
<th>Population</th>
<th>EI 50(ppb)</th>
<th>EI95(ppb)</th>
<th>R.R. 50</th>
<th>R.R. 95</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>3.37 (3.10 – 3.60)</td>
<td>6.44 (5.90 – 7.20)</td>
<td>6.5</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>0.74 (0.64 – 0.84)</td>
<td>2.70 (2.31 – 3.33)</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>BR</td>
<td>1.86 (1.70 – 2.00)</td>
<td>4.13 (3.75 – 4.63)</td>
<td>3.6</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Rockefeller</td>
<td>0.52 (0.48 – 0.55)</td>
<td>1.17 (1.06 – 1.32)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temephos</th>
<th>Population</th>
<th>LC 50 (ppm)</th>
<th>LC 95 (ppm)</th>
<th>R.R. 50</th>
<th>R.R. 95</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>0.028 (0.027 – 0.029)</td>
<td>0.053 (0.05 – 0.056)</td>
<td>11.0</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>0.013 (0.012 – 0.013)</td>
<td>0.029 (0.026 – 0.033)</td>
<td>5.2</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>BR</td>
<td>0.0051 (0.0048 – 0.0053)</td>
<td>0.0110 (0.014 – 0.016)</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Rockefeller</td>
<td>0.0025 (0.0024 – 0.0026)</td>
<td>0.0042 (0.004 – 0.0045)</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

ppb: parts per billion; ppm: parts per million.
### Table 2: Quantification of enzyme activity in *Ae. aegypti* populations

<table>
<thead>
<tr>
<th>Populations</th>
<th>Alpha esterase (nmoles/mg ptn/min)</th>
<th>Beta esterase (nmoles/mg ptn/min)</th>
<th>Glutathion-S-transferase (mmoles/mg ptn/min)</th>
<th>MFO (nmoles/mg ptn/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Med</td>
<td>sdev</td>
<td>p99</td>
</tr>
<tr>
<td>Rockefeller</td>
<td>150</td>
<td>11.1</td>
<td>2.6</td>
<td>21.7</td>
</tr>
<tr>
<td>Field populations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marília (MA)</td>
<td>120</td>
<td>11.5</td>
<td>1.8</td>
<td>12.6</td>
</tr>
<tr>
<td>Bauru (BR)</td>
<td>117</td>
<td>16.1</td>
<td>2.7</td>
<td>6.0</td>
</tr>
<tr>
<td>Maringá (MG)</td>
<td>119</td>
<td>17.9</td>
<td>2.8</td>
<td>0</td>
</tr>
<tr>
<td>Barreiras (BA)</td>
<td>146</td>
<td>19.4</td>
<td>4.4</td>
<td>30.1</td>
</tr>
<tr>
<td>Araçatuba (AT)</td>
<td>150</td>
<td>19.5</td>
<td>3.7</td>
<td>26.7</td>
</tr>
<tr>
<td>Santos (SA)</td>
<td>147</td>
<td>24.7</td>
<td>16.5</td>
<td>67.3</td>
</tr>
<tr>
<td>Fortaleza (FO)</td>
<td>150</td>
<td>21.0</td>
<td>3.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Salvador (SS)</td>
<td>150</td>
<td>27.1</td>
<td>3.3</td>
<td>93.3</td>
</tr>
</tbody>
</table>

n: number of larvae assayed; med: median; sdev: standard deviation; p99: percentage 99
p% > p99: percentage of individuals with enzyme activity higher than Rockefeller percentage 99 activity
-: Not determined

- Normal enzyme activity
- Altered enzyme activity
- Very altered enzyme activity

Effect of pyriproxyfen in *Aedes aegypti* populations with different levels of temephos resistance
Effect of pyriproxyfen in Aedes aegypti populations with different levels of temephos resistance

larvae assayed, median activity of enzymes, standard deviation and percentage of individuals with activity higher than the percentage 99 of Rockefeller strain are given in Table 2. A higher activity of all four metabolic enzymes was observed on population SS, followed by SA and FO, which were characterized as resistant to temephos. The enzyme GST was altered in all field populations but at a higher level of alteration in SS and SA. Populations BA and MG, with respectively moderate and low level of resistance to temephos, presented normal activity of MFO and alteration on beta esterase and GST. Populations with the lowest R.R. to temephos, MA and BR, presented normal activity for all enzymes, except for GST with the smallest percentage of individuals with alteration on that enzyme activity (15.7% and 18.0% respectively).

Residual effect of commercial products

The adult emergence of all non-treated containers was higher than 90%. Temephos induced mortality at larval stage while pyriproxyfen treatment affected mainly the pupal stage. Larval mortality with pyriproxyfen was 2.3% on average. Exceptions were observed only in MA and AT populations, with larval mortality higher than 10.8%.

The residual effect of both products varied according to the containers. Both treatments presented a shorter residual effect in tyres.

Treatment of glass vases with temephos (Table 3) resulted in 100% inhibition of adult emergence during the whole test period, except for the FO population where inhibition ended in 92%. In tyres the effect of temephos decreased over time, especially for populations FO (13 days), RE and SA (29 days), which had the higher RRs for temephos. The populations MA and Rockefeller were the only ones to arrest 100% inhibition of adult emergence for 44 days.

On the vases treated with pyriproxyfen (Table 4), 100% of inhibition of adult emergence was observed at the 58-days period.

Table 3: Adult emergence inhibition observed in vases and tyres treated with temephos (Temefós Fersol 1G)

<table>
<thead>
<tr>
<th>Days*</th>
<th>Rockefeller</th>
<th>MA</th>
<th>AT</th>
<th>MG</th>
<th>SA</th>
<th>FO</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vases</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
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<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>13</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>29</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>44</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>58</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>Tyres</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>100</td>
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<td>84</td>
<td>9</td>
<td>0</td>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* Days after treatment
Table 4: Percentage of adult emergence inhibition observed in vases and tyres treated with pyriproxyfen (Sumilarv® 0.5 G)

<table>
<thead>
<tr>
<th>Days*</th>
<th>Rockefeller</th>
<th>MA</th>
<th>AT</th>
<th>MG</th>
<th>SA</th>
<th>FO</th>
<th>RE</th>
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<td>100</td>
<td>100</td>
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<td>82</td>
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<td>Tyres</td>
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<td>100</td>
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<td>97</td>
<td>61</td>
<td>43</td>
<td>80</td>
<td>39</td>
<td>20</td>
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</table>

* Days after treatment

only for Rockefeller and the field population MA. The shorter effect was observed in the SA population (29 days), and in all the other populations inhibition of adult emergence was observed to be higher than 95% for 44 days. In tyres, only Rockefeller showed inhibition during the whole test period. While AT presented a shift in inhibition (86% to 91%) during the observation period, MG presented the longer effect among field populations (44 days) followed by MA and RE (29 days). Again, the populations FO and SA presented the shorter residual effect, i.e. 1 and 13 days respectively.

The mortality data were converted into arcsin values and analysed by Student-t test to compare the response of groups of populations. The analysis of data from treatment in vases showed that while temephos treatment did not cause significant difference between the two groups of populations, treatment with pyriproxyfen presented a significantly lower effect on the moderately resistant population groups when compared to the Rockefeller strain (p=0.01), and a significantly shorter effect when the results of all populations were compared with the treatment results with temephos (p=0.002).

The treatment with temephos in tyres showed a significantly shorter residual effect only on the group of moderately resistant populations (p=0.02), while treatment with pyriproxyfen presented significant difference between the two groups of populations (p=0.02) and between each group and Rockefeller strain (p<0.05).

Discussion

The pyriproxyfen doses that caused adult emergence inhibition estimated in this study were higher than the lethal concentrations observed by Hatakoshi et al.[33] (L.C. 50 of 0.023 ppb); Itoh et al.[34] (L.C. 50 of 0.056 ppb) and Henrick[35] (L.C. 50 of 0.0039 ppb). Our estimations are closer to the lethal concentrations described by Estrada and Mulla[36] (L.C. 50 of 0.33 ppb and L.C. 95 of 2.6 ppb).
Population SS, which presents the highest R.R. to temephos, also presented the highest R.R. to pyriproxyfen. No field population evaluated in this study had been previously exposed to pyriproxyfen, so they had not been under selection for resistance to this IGR.

Although there is no recorded evidence of pyriproxyfen resistance to *Ae. aegypti*, the possibility of cross-resistance between conventional insecticides and IGRs has been reported for other insects like *Tribolium castaneum* [37] and houseflies [38]. Oxidase activity seems to be involved on resistance to juvenile hormone in houseflies [39-42]. In the present study, differences on pyriproxyfen IE concentrations among susceptible and resistant organophosphate populations were also observed, suggesting the possibility of cross-resistance between the IGR and temephos.

Braga et al. [43] discuss the possibility of cross-resistance between temephos and methoprene, another juvenile hormone analogue, in *Ae. aegypti* populations that presented high esterase and monooxygenase activity. The metabolism of endogenous juvenile hormones is associated to both classes of enzymes in other insects [40-43].

The role of the studied metabolic enzymes on the observed resistance to temephos is not easy to define since all four enzymes were highly altered on resistant populations (SS, FO and SA). It is possible that the multiple metabolic alterations are responsible for temephos resistance and also for the higher pyriproxyfen observed on population SS. Nevertheless, the alteration on beta esterases observed for populations BA and MG, less susceptible than BR and MA, which had normal activity, also indicate a possible role of this class of enzymes. The enzyme GST might also play an important role on temephos resistance as it is highly altered on resistant populations, especially on population SS. Although esterases have been previously related to temephos resistance in *Ae. aegypti* [13,44-46] this class of enzyme is not the sole enzyme to be more active in resistant populations. GST was also characterized in Cuba [44]. Braga et al. [43] relates alterations not only in esterases activity but also in MFO and GST enzymes on temephos-resistant Brazilian *Ae. aegypti* populations, making it difficult to ascribe temephos resistance to only one class of enzymes. The same could be said for pyriproxyfen, as strain SS presented the higher R.R. and a high activity of all enzymes, although MFO presented the higher alteration on that population (75.6% of individuals) and the role of this enzyme on IGR resistance is well-documented in literature [47-50].

Data from the simulated field trial test in vases indicate that the commercial product pyriproxyfen showed a significant shorter effect on adult emergence when compared with temephos (p=0.02). Temephos-treated vases exhibited 100% inhibition compatible with the 60-day treatment cycle proposed by the PNCD [4] while treatment with pyriproxyfen was effective for this period only for Rockefeller and MA (temephos-susceptible population).

On tyres, pyriproxyfen treatment promoted a 100% inhibition of adult emergence on temephos-susceptible population Rockefeller. For the three resistant populations, temephos lasted more for two of them (SA and FO) and less for RE. The duration of effect of inhibition of adult emergence above 95% in both products for tyres is not compatible with the cycle of treatments as they lasted half of the expected period in many populations.

The lack of temephos effectiveness for 60 days against the susceptible Rockefeller strain contrasts with previous studies [51] and raises suspicion on the quality of commercial products.
Melo-Santos et al.\[52\] tested pyriproxyfen in water-storage cement boxes and plastic buckets at the same concentration 0.05 ppm with water replaced three times a week, and found a complete inhibition of adult emergence of 160 days at shaded area and 46 days at sunlight exposure. Resende\[53\], with pyriproxyfen trials found total inhibition of adult emergence of 90 days at 0.05 ppm dose and 45 days at 0.01 ppm dose for the Rockefeller strain in cement boxes and glass vases; but in plastic buckets it was 30 days. Vythilingam\[54\] testing an *Ae. aegypti* population from Malaysia, resistant to temephos, observed pyriproxyfen complete inhibition of adult emergence of 160 days at 0.02 ppm even with water reposition every fifteen days in earthen jars and plastic tubs and 100% EI was obtained for 10 weeks in earthen jars where water was replaced daily.

The variation found in literature for the time of complete inhibition of adult emergence of products might be explained by the difference in the surface of containers used in tests and also by the variation in climate conditions. Also, bigger volumes of water tend to promote a more stable situation for larvicide action. This might also play a role in persistence effect.

The choice of containers tested in this study was based on their distinct surface of absorption, aiming to reach the best and worst availability of larvicides, respectively, for glass vases and tyres. We do believe that the treatment of those kinds of recipients should not be encouraged. More sustainable actions should be encouraged. In this respect, community participation has shown very satisfactory results in controlling *Ae. aegypti* foci in plant-related containers\[28\].

**Conclusions**

The development of resistance to temephos in many *Ae. aegypti* populations makes the change for alternative larvicides for dengue control programmes most desirable.

Commercial products based on the IGR pyriproxyfen are one of the alternatives listed by the Brazilian Health Ministry for substituting temephos.

A temephos-resistant *Ae. aegypti* population showed higher pyriproxyfen adult inhibition concentrations than susceptible ones. Besides, in semi-field assays, pyriproxyfen exhibited a lower residual effect against populations characterized as temephos-resistant, suggesting interference of temephos resistance with pyriproxyfen action. The mechanism by which this interference acts is not clear since MFOs, cited on literature as involved, are not the only class of enzymes altered in temephos-resistant populations.

Data presented here should be considered to make further evaluations and studies about this IGR action as well as on the choice of this product for use in management strategies.

**References**

Effect of pyriproxyfen in *Aedes aegypti* populations with different levels of temephos resistance


Effect of pyriproxyfen in Aedes aegypti populations with different levels of temephos resistance

[38] Cerf DC, Georghiou GP. Evidence of cross-resistance to a juvenile hormone analogue in some insecticide-resistant houseflies. Nature. 1972; 329: 401-2.


Effectiveness of pyriproxyfen-controlled release block against larvae of Aedes (Stegomyia) aegypti in Kuala Lumpur, Malaysia

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Abstract

This study was conducted to evaluate the effect of a commercially available pyriproxyfen, an insect growth regulator (IGR) on the larvae of a dengue vector, Ae. aegypti. The study site was the surrounding area of the Medical Entomology Unit, Institute for Medical Research (IMR), Kuala Lumpur (N03°10.167', E101°41.919'). Pyriproxyfen-controlled release blocks with dosages of 10% w/w and 20% w/w were used to treat a set of earthen jars placed outdoors. Untreated jars were also set up as controls. Fifty laboratory-bred 2nd instar larvae were introduced into each earthen jar and observed daily. The number of adults that emerged was recorded and the larval mortality was calculated. The indicators of effectiveness of IGR for these studies were: (i) duration of effectiveness, and (ii) percentage of emergence inhibition (EI). There was a significant difference in the number of emerged adults obtained from the untreated and treated earthen jars up to 25 weeks (p<0.05). The duration of effectiveness of pyriproxyfen caused 80% emergence inhibition in earthen jars treated with 10% w/w and 20% w/w pyriproxyfen up to 22 and 25 weeks, respectively. Pyriproxyfen-controlled release block is an effective method of controlling mosquito larvae for several months. The method of application of the block is simple and straightforward and can therefore be used easily.

Keywords: Aedes aegypti; Pyriproxyfen; Controlled release block; Duration of effectiveness; Emergence inhibition.

Introduction

Aedes aegypti is a principle vector of dengue in many parts of the world. It is one of the major domestic groups of mosquitoes that are pests of man as well as a vector of disease. In many areas of the world, this species commands considerable attention in term of its management and control[1]. The number of options available for mosquito control at the present time are also limited. The control of this mosquito is still dependent on the use of chemical insecticides.

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Although insecticides are invaluable in preventing and controlling damage to agricultural products and to the health of man and animals, they are not without side-effects on the environment and its biota\cite{2}. There is a critical need to find and develop new agents and products for the control of this and other important species of mosquitoes. Insect growth regulators (IGRs) are now increasingly used to control Aedes and other mosquito larvae. These compounds have unique modes of action, and are often selective and do not persist in the environment. Such attributes are desirable when dealing with the problem of pest resurgence, secondary pest outbreaks and insecticides resistance\cite{3}.

Pyriproxyfen, 2-[1-methyl-2-(4-phenoxyphenoxy) ethoxyl] pyridine, is a new generation of IGR. It is a juvenile hormone analogue and a relatively stable aromatic compound. It functions as an insecticide by overloading the hormonal system of the target insect, ultimately affecting its egg production, brood care and other social interactions, and inhibiting its growth\cite{4}. Pyriproxyfen works well against public health insects like houseflies and mosquitoes\cite{5}. Pyriproxyfen is reported to exhibit 95% inhibition of the emergence of mosquito larvae and its effects on mosquito larvae having lasted for two months after application\cite{6}. Although the treated mosquito larvae continue to pupate, however, their emergence is inhibited by the action of pyriproxyfen\cite{7}.

The controlled release block used in this study was impregnated with 10% w/w and 20% w/w a.i. (active ingredient) of pyriproxyfen granules. Pyriproxyfen-controlled release block is claimed to be an easy method applicable in areas such as drains, ponds, lakes, etc., where mosquitoes breed.

This study was conducted to evaluate the commercially available pyriproxyfen-controlled release block used for the control of Ae. aegypti larvae in earthen jars.

**Materials and methods**

**Test site**

The study was conducted in the area surrounding the Medical Entomology Unit, Institute for Medical Research (IMR), Jalan Pahang, Kuala Lumpur (N03°10.167', E101°41.919').

**Insect growth regulator**

A formulation of insect growth regulator, pyriproxyfen-controlled release block, was used in this study. Two concentrations of controlled release block were provided, each containing pyriproxyfen at 10% w/w a.i. and 20% w/w a.i. The formulation was provided by Zero-Moz Japan Sdn. Bhd.

**Test containers**

Earthen jars were used as mosquito breeding containers in this study. Earthen jars each with an opening of 52 cm in diameter, base diameter of 35 cm and 47 cm in height were prepared and placed outdoors. Three replicates of each were used in each research arm of the study (Table 1). Each earthen jar held 60 L tap water. Before initiating the study, all containers were washed with tap water and tested for the presence of contaminant, such as insecticides, by introduction of 50 Ae. aegypti 2nd instar larvae. The larvae were observed until complete emergence as adult.
Effectiveness of pyriproxyfen-controlled release block against larvae of Aedes aegypti

Table 1: Set-up of earthen jars for testing

<table>
<thead>
<tr>
<th>Earthen jar</th>
<th>Chemical (active ingredient)</th>
<th>Number of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Treated (with controlled release block)</td>
<td>Granular pyriproxyfen 10% w/w</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Granular pyriproxyfen 20% w/w</td>
<td>3</td>
</tr>
</tbody>
</table>

**Trial procedures**

Each pyriproxyfen-controlled release block was placed into earthen jar (3 replicates) and labelled. Three earthen jars without pyriproxyfen-controlled release block served as untreated control. In each test, 50 laboratory-bred 2nd instar larvae were introduced into each earthen jar and observed daily. Pupae were collected, recorded and transferred into paper cups covered with net. The total number of adults emerged was recorded and the larvae mortality rates were calculated. A total of 50% of water (30 L) was removed and added into the earthen jars every alternate day. The same procedure was repeated by adding fresh batch of larvae (50 larvae) into each earthen jar weekly.

**Data analysis**

The indicators of effectiveness of pyriproxyfen-controlled release block for these studies were:

1. duration of effectiveness of each dosage, and
2. percentage of emergence inhibition (EI) =

\[
\text{Number of larvae introduced} - \text{Number of adult emerged}\times 100%
\]

Number of larvae introduced

A cut-off point of EI ≥ 80% was considered to be effective.

If percentage of untreated EI was > 5%, the percentage of treated EI was corrected by Abbott’s formula:

\[
\frac{\% \text{ treated EI} - \% \text{ untreated EI}}{100 - \% \text{ untreated EI}} \times 100%
\]

**Results and discussion**

Table 2 shows the number of pupae, adult emergence and emergence inhibition obtained from earthen jars treated with 10% w/w and 20% w/w pyriproxyfen-controlled release block that were impregnated with 10% w/w and 20% w/w pyriproxyfen granules. The result showed a significant difference on the number of pupae collected from all treated (10% w/w and 20% w/w) and untreated earthen jars (p<0.05). However, there was no significant difference on the number of pupae collected from the 6th week onwards (p>0.05), indicating that pyriproxyfen exhibited low larvicidal activity against Ae. aegypti. This is similarly reported by Lee et al. in which studies were carried out on the bio-efficacy and duration of the effectiveness of pyriproxyfen (Sumilarv 0.5%) as direct applications for the control of larvae of Ae. aegypti and Ae. albopictus. At 79.5 mg/L and 159.0 mg/L, pyriproxyfen showed low larvicidal activity but provided very effective control of adult emergence against larvae of Ae. aegypti and Ae. albopictus.

A significant difference in the number of adult emergence was observed in both the
Table 2: Number of pupae, emergence of adult and emergence inhibition obtained from earthen jars treated with 10% w/w and 20% w/w pyriproxyfen-controlled release block

<table>
<thead>
<tr>
<th>Week</th>
<th>Test period</th>
<th>Mean ± SE of collected pupae</th>
<th>Mean ± SE adult emerged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Un treated</td>
<td>Treated 10% w/w</td>
</tr>
<tr>
<td>1</td>
<td>5 Feb – 11 Feb</td>
<td>48.00 ± 1.00</td>
<td>15.67 ± 10.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12 Feb – 18 Feb</td>
<td>43.18 ± 3.18</td>
<td>21.67 ± 11.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>19 Feb – 25 Feb</td>
<td>40.67 ± 2.03</td>
<td>2.00 ± 0.58</td>
</tr>
<tr>
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</tr>
<tr>
<td>4</td>
<td>26 Feb – 4 Mar</td>
<td>39.00 ± 2.65</td>
<td>10.33 ± 5.90</td>
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<tr>
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<td></td>
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<tr>
<td>5</td>
<td>5 Mar – 11 Mar</td>
<td>20.50 ± 7.50</td>
<td>0.33 ± 0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>12 Mar – 18 Mar</td>
<td>45.33 ± 1.76</td>
<td>39.33 ± 1.33</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>7</td>
<td>19 Mar – 25 Mar</td>
<td>41.47 ± 0.88</td>
<td>35.33 ± 2.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>8</td>
<td>26 Mar – 1 Apr</td>
<td>30.33 ± 14.40</td>
<td>0.67 ± 0.33</td>
</tr>
<tr>
<td></td>
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<td></td>
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<tr>
<td>9</td>
<td>2 Apr – 8 Apr</td>
<td>49.67 ± 0.33</td>
<td>47.00 ± 3.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9 Apr – 15 Apr</td>
<td>49.33 ± 0.67</td>
<td>42.33 ± 3.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>16 Apr – 22 Apr</td>
<td>47.33 ± 2.19</td>
<td>46.33 ± 2.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>23 Apr – 29 Apr</td>
<td>44.00 ± 2.08</td>
<td>36.33 ± 2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>30 Apr – 6 May</td>
<td>42.00 ± 2.08</td>
<td>38.67 ± 2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week</td>
<td>Test period</td>
<td>Mean ± SE of collected pupae</td>
<td>Mean ± SE adult emerged</td>
</tr>
<tr>
<td>------</td>
<td>------------------</td>
<td>------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Untreated 10% w/w 20% w/w</td>
<td>One way ANOVA Untreated 10% w/w 20% w/w</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Treated</td>
</tr>
<tr>
<td>14</td>
<td>7 May – 13 May</td>
<td>43.33 ± 2.73 38.00 ± 3.21 43.00 ± 4.73</td>
<td>F = 1.64 p = 0.271 41.67 ± 2.40 0.00 ± 0.00 0.00 ± 0.00</td>
</tr>
<tr>
<td>15</td>
<td>14 May – 20 May</td>
<td>41.67 ± 2.91 39.67 ± 2.96 35.33 ± 3.76</td>
<td>F = 1.00 p = 0.420 38.33 ± 1.67 0.00 ± 0.00 0.00 ± 0.00</td>
</tr>
<tr>
<td>16</td>
<td>21 May – 27 May</td>
<td>44.33 ± 3.48 37.67 ± 2.33 33.67 ± 6.33</td>
<td>F = 1.51 p = 0.294 43.67 ± 3.76 0.00 ± 0.00 0.00 ± 0.00</td>
</tr>
<tr>
<td>17</td>
<td>28 May – 3 June</td>
<td>46.00 ± 2.65 35.33 ± 4.91 33.33 ± 5.04</td>
<td>F = 2.46 p = 0.166 41.67 ± 1.67 0.00 ± 0.00 0.00 ± 0.00</td>
</tr>
<tr>
<td>18</td>
<td>4 June – 10 June</td>
<td>41.33 ± 1.86 34.67 ± 3.84 33.00 ± 3.21</td>
<td>F = 2.04 p = 0.210 39.67 ± 2.19 0.00 ± 0.00 0.00 ± 0.00</td>
</tr>
<tr>
<td>19</td>
<td>11 June – 17 June</td>
<td>37.67 ± 2.19 36.00 ± 1.53 36.00 ± 3.61</td>
<td>F = 0.14 p = 0.874 36.67 ± 1.76 0.00 ± 0.00 0.00 ± 0.00</td>
</tr>
<tr>
<td>20</td>
<td>18 June – 24 June</td>
<td>36.67 ± 5.78 42.00 ± 2.08 40.67 ± 2.60</td>
<td>F = 0.52 p = 0.620 30.00 ± 8.33 1.67 ± 0.08 0.00 ± 0.00</td>
</tr>
<tr>
<td>21</td>
<td>25 June – 1 July</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>2 July – 8 July</td>
<td>44.00 ± 1.15 37.67 ± 1.45 26.33 ± 10.68</td>
<td>F = 2.05 p = 0.210 44.00 ± 1.15 3.00 ± 0.58 0.00 ± 0.00</td>
</tr>
<tr>
<td>23</td>
<td>9 July – 15 July</td>
<td>46.50 ± 1.50 27.00 ± 5.51 16.33 ± 1.86</td>
<td>F = 13.21 p = 0.010 45.50 ± 2.50 12.33 ± 3.53 0.33 ± 0.33</td>
</tr>
<tr>
<td>24</td>
<td>16 July – 23 July</td>
<td>Not available</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>24 July – 30 July</td>
<td>48.00 ± 1.00 35.00 ± 14.50 41.67 ± 4.91</td>
<td>F = 0.35 p = 0.719 47.67 ± 0.33 31.67 ± 12.86 9.33 ± 6.98</td>
</tr>
<tr>
<td>26</td>
<td>31 July – 6 Aug</td>
<td>36.67 ± 1.20 37.67 ± 2.85 34.33 ± 5.17</td>
<td>F = 0.24 p = 0.792 33.00 ± 3.06 33.67 ± 2.40 30.67 ± 5.36</td>
</tr>
<tr>
<td>27</td>
<td>7 Aug – 13 Aug</td>
<td>34.33 ± 12.20 46.00 ± 1.53 43.67 ± 2.33</td>
<td>F = 0.73 p = 0.520 31.33 ± 11.17 42.00 ± 0.58 19.33 ± 9.33</td>
</tr>
</tbody>
</table>

SE = standard error; p>0.05 = no significant difference; p<0.05 = significant difference; p<0.01 = highly significant difference
Effectiveness of pyriproxyfen-controlled release block against larvae of Aedes aegypti

treated and untreated earthen jars up to 25 weeks (p<0.05). The result indicated that in
the untreated jar, not all the pupae successfully emerged as adults throughout the trial period. In
the earthen jars treated with 10% w/w and 20% w/w pyriproxyfen granules, although some
larvae pupated successfully, none of these pupae could emerge as adults up to 19 and 22
weeks, respectively. This finding was similar to that reported by Sihuincha et al.[7], where
larvae continued to pupate but failed to emerge. After this, both earthen jars (treated
with 10% and 20% pyriproxyfen granules) exhibited 80% emergence inhibition for
another 3 weeks, indicating that both 10% and 20% pyriproxyfen were able to inhibit the
emergence of adult Aedes aegypti for 22 weeks (5 months) and 25 weeks (6 months),
respectively (Figure).

In Cambodia, the inhibition of adult emergence of Aedes aegypti in simulated domestic
water storage containers by using controlled-release formulation of pyriproxyfen showed
that at target dosages of 18, 27 and 36 µg/L of a.i., inhibition of adult emergence remained
above 95% for at least two months. After three months at 18 µg/L a.i., the residual efficacy
was significantly lower than for the higher dosages (p<0.05)[9]. At the higher dosages,
inhibition of adult emergence was > or = 87% for six months[9].

The persistence and efficacy of pyriproxyfen were evaluated in two final
concentrations of 0.01 and 0.05 mg/L against Aedes aegypti larvae in laboratory conditions using
three types of containers, i.e. cement box, glass bottle and plastic bucket, in the University of

**Figure:** Percentage emergence inhibition of Aedes aegypti exposed to 10% w/w and 20% w/w
pyriproxyfen-controlled release block in earthen jars

![Percentage emergence inhibition of Aedes aegypti](image_url)

Cut-off point of adult emergence inhibition = 80%
Federal de Minas Gerais. The study indicated that a persistency of 45 and 90 days by using 0.01 and 0.05 mg/L final concentrations of pyriproxyfen respectively was observed\[10\].

In another study, pyriproxyfen was tested against Ae. aegypti at 0.01 and 0.02 mg of active ingredient (a.i.) per litre of water in 60 litre earthen jars. Both concentrations provided 100% control for four months. Moreover, in field trial condition, pyriproxyfen at a dosage of 0.02 mg a.i. per litre provided 100% control for 10 weeks against Ae. albopictus\[11\].

In Japan, blood-fed female Ae. aegypti were exposed to a surface treated with pyriproxyfen at 1.0 g/m\(^2\). The results showed that transmission of pyriproxyfen from females to the water was revealed\[12\]. Pyriproxyfen affected the egg maturation of females treated before blood meals, as the number of eggs deposited decreased concurrently with the number of days before the blood meal\[12\].

The use of pyriproxyfen-treated oviposition containers to achieve horizontal transfer of pyriproxyfen to mosquito oviposition sites also can be a field management technique based on mosquito biology and behaviour. A study conducted in the North Carolina State University showed that horizontal transfer of pyriproxyfen by Ae. albopictus from a container with a treated ovistrip (0.3 or 0.4 mg/cm\(^2\)) to an untreated microcosm resulted in 14% – 38% inhibition\[13\].

Besides controlling Aedes, many researchers have also reported that pyriproxyfen was able to control Culex mosquitoes in Israel\[14\], Egypt\[15\], Florida\[16\] and Bangladesh\[17\]; and Anopheles mosquitoes in Sri Lanka\[18\] and Solomon Island\[19\].

The current study showed that pyriproxyfen-controlled release block is an effective method of controlling mosquito larvae for several months. The method of application of the block is simple and straightforward, and can therefore be used easily. This method can be applied in areas such as drains, ponds and lakes where mosquitoes breed and in which a long-term control is desired.

**Acknowledgements**

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**References**


Effectiveness of pyriproxyfen-controlled release block against larvae of Aedes aegypti


Laboratory evaluation of *Mesocyclops aspericornis* as a biocontrol agent of *Aedes aegypti*

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**Abstract**

*Mesocyclops aspericornis* abounds in village ponds. Hence, the predatory capacity of *M. aspericornis* was considered for use as a biological control agent for *Aedes aegypti* mosquitoes. In laboratory experiments, *M. aspericornis* consumed 33–50 mosquito larvae within 24-hours time period. *M. aspericornis* preyed upon only the first instar larvae of *Ae. aegypti* within a few seconds after their introduction. It started feeding on the tail portion first and ended with the head capsule. The mean value (triplicate) showed that the predatory capacity was 45.76 against the control 1.2. *M. aspericornis* prefers only the first instar mosquito larvae and feeds on them voraciously. When the *Aedes* larvae attained the second instar stage, *M. aspericornis* attacked and killed them.

**Keywords:** Biological control; *Aedes aegypti*; *Mesocyclops apericornis*; Predatory capacity.

**Introduction**

In India, particularly in the state of Tamil Nadu, dengue and chikungunya have been reported from many places. The National Vector-Borne Disease Control Programme (NVBDCP) recommended the Integrated Vector Management (IVM) approach. This includes biocontrol agents. It has been proved that larvicidal measures sustain mosquito population for a short period and require repeated applications of chemicals and eventually develop resistance against that chemical[11]. Therefore, search for an effective biocontrol agent to control mosquito population has become top priority among researchers. Predatory fishes and zooplankton have been widely used as a biocontrol method to control vector population[2,3,4]. Integration of these methods can be a low-cost and environmentally-friendly approach in controlling mosquito vectors[5,6]. Cyclopoid copepods (planktonic microcrustaceans) have been extensively used as biocontrol agents in the South-East Asian countries for container-breeding mosquito species like *Ae. aegypti*[7]. The copepod, *Mesocyclops aspericornis*, is an effective predator of *Ae. aegypti*. It is known

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Laboratory evaluation of Mesocyclops aspericornis as a biocontrol agent of Aedes aegypti

for its wide distribution and predatory efficiency against several species of mosquito larvae. The present study was conducted as a brief laboratory experiment designed to understand the mode of destruction of mosquito larvae by *M. aspericornis*.

**Methodology**

Out of a few preliminary surveys carried out in the nearby environs of Chennai, Capital of Tamil Nadu, two ponds were identified for the collection of cyclopoid copepods. The plankton mesh size used for the collection was 100 µm. Mesocyclops were isolated from the sample and identified up to species level with the help of standard keys[^8][^9]. *M. aspericornis*, once its species identity was confirmed, was selected for experimental studies and reared in the laboratory. Females with egg sacs collected from the stock were placed on a petridish and were examined under the dissection microscope. These were transferred into 600 ml beaker where 50 newly-hatched *Ae. aegypti* larvae were introduced. We sacrificed the second-generation *Mesocyclops* collected from our stock for experimental purposes. Fully-fed *Ae. aegypti* females were collected from the house and kept in a small cloth cage for egg-laying. The mosquitoes were provided a small dish, half filled with water, and a paper strip for egg-laying. The eggs were hatched in a Petri dish and used for experiment.

Fifty newly-hatched first instar larvae were introduced into a 600 ml beaker containing 500 ml dechlorinated water where a single *M. aspericornis* was introduced. The experiment lasted for 24 hours. The number of larvae that survived at the end of 24 hours was recorded. Triplicates were maintained simultaneously at 26±1 °C under photoperiod 12L:12D along with the control without the introduction of *M. aspericornis*.

**Results**

*M. aspericornis* preyed upon the first instar of *Ae. aegypti* larvae within 24 hours, which was recorded. In general, they attacked the tail region of the mosquito larvae and consumed them. On a few occasions they left out the head capsule of the mosquito larvae, and, at times they killed the mosquito larvae without consuming them. Ten experiments were conducted for 10 days in the laboratory on relay basis. *M. aspericornis* consumed about 33 to 50 first instar larvae of *Ae. aegypti* within 24 hours time period. The number of mosquito larvae left inside the experimental beakers ranged from nil to 17 nos. On the whole, the mean predatory capacity of a single *M. aspericornis* was calculated at 45.75 (see Table).

**Discussion and conclusions**

According to Nam et al.\(^{10}\), the daily consumption/killing average of a single *M. aspericornis* ranged between 16 to 41 larvae. Through continuous observations, *M. aspericornis* attacked the first instar larvae within a few seconds. They mainly consumed the central portion, leaving the head capsule. Occasionally, they just killed the larvae without consuming it. Using their strong mandible they pierced and crammed the larvae into pieces. According to Lardeux et al.\(^{11}\), *M. aspericornis* served as a good biocontrol agent against *Ae. aegypti* within a three-weeks time period. In the present study, 10 experiments were conducted simultaneously to know the predatory capacity of *M. aspericornis* under laboratory conditions. The maximum predatory capacity of *M. aspericornis* was found to be 49.3 (mean value) and the minimum 39.3 (Table). Our results demonstrate that *M. aspericornis* is an efficient predator of *Ae. aegypti* under laboratory conditions.
Laboratory evaluation of Mesocyclops aspericornis as a biocontrol agent of Aedes aegypti

Table: The mean value of Ae. aegypti larvae consumed by M. aspericornis

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<tr>
<th>S. no.</th>
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Total mean: 45.75

Acknowledgements

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References


Laboratory evaluation of Mesocyclops aspericornis as a biocontrol agent of Aedes aegypti


Management dilemmas in the treatment of dengue fever*

Kolitha H Sellahewa#

Abstract

This paper is aimed at highlighting some of the dilemmas faced by clinicians in the management of adult patients with dengue and my views in resolving these issues.

Even though early diagnosis and prompt fluid therapy are central to reduce morbidity and mortality in dengue, achieving these goals are contentious issues and are often hampered by the limited access to expensive laboratory data in most developing countries which would enable a rapid and accurate diagnosis. My viewpoint on overcoming these dilemmas is to make an early diagnosis on the clinical features, and apply clinical predictors of disease severity in selecting patients for interventions. In this regard, diffuse blanching erythema in a patient with features of a viral fever during dengue epidemics would suffice to diagnose and treat the patient as a dengue case. Laboratory confirmatory data are expensive, not readily available and could delay treatment. Fluid therapy and intervention modalities for thrombocytopenia should be judged clinically on an individual basis rather than the blind, strict adherence to theoretical fluid regimens with the potential risk of fluid overloading. Capillary refill time, pulse pressure, cervical lymphadenopathy, and changes in the sensorium are useful clinical parameters for selection of patients for intervention as well as subtle adjustments and termination of fluid therapy. A practically feasible step-wise approach to dengue management is described in this paper.

Keywords: Dengue; management; disease severity predictors.

Introduction

The management dilemmas faced by busy clinicians in developing countries where dengue has reached epidemic proportions are centred on:

- Early diagnosis of dengue;
- Prediction of disease severity;
- Selection of patients for aggressive interventions;
- Implementation of the concept of judicious fluid therapy on an individual basis to prevent fluid overloading and its attendant complications;

* The conclusions and recommendations in this paper are based on the personal experiences of the author while treating adult patients with dengue. The views expressed here are entirely of the author and are subject to technical analysis or confirmation by other experts. These do no necessarily reflect the opinions or decisions of WHO. – Editor

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Concerns on thrombocytopenia and decisions on when to intervene, and how to intervene.

It has been widely recognized that early diagnosis and prompt appropriate treatment of dengue prevents both morbidity and mortality.[1] The vast majority of adult patients with dengue recover completely without specific aggressive interventions (personal experience). It is thus necessary to select the minority of patients early in the disease for close monitoring and appropriately timed fluid therapy to prevent progression to dengue shock syndrome (DSS). Achieving this objective, which is a fundamental requirement for proper management, requires dengue to be diagnosed early and then be able to predict disease severity. Dengue-specific IgM is positive in only 55% of patients in 4th to 7th days while 94% positivity is evident after the 7th day. No IgM is detected in 1 to 3 days after infection.[2] PCR amplification for dengue RNA provides a fast diagnosis but is expensive and false positive reports are seen. In numerous acute dengue fever patients an early diagnosis will be obtained only by combining IgM antibody detection with detection of virus or virus RNA using RT-PCR.[2,3]

These facilities are also not available to most clinicians at the point of delivery of care. Consequently, they need to rely on clinical skills to arrive at an early diagnosis. The diagnostic dilemma, however, is that the classic clinical features of dengue such as saddle-back fever and break-bone pain are not seen in all the patients, and varying degrees of headache, myalgia, arthralgia and vomiting are common to most non-specific viral fevers often encountered in the community and among inpatients in a medical unit. However, what is encountered very often in dengue patients is diffuse blanching erythema (personal experience).[4,5,6] Any patient with features of a viral fever having diffuse blanching erythema should be treated as dengue fever during epidemics.

The management dilemma is compounded by the current WHO classification of grading dengue haemorrhagic fever (DHF) from I to IV, as there is an overlap of DHF grades III and IV with dengue shock syndrome (DSS). Also, the case definition of DHF requires there to be evidence of plasma leakage.[7] However, there are many patients with haemorrhage, particularly skin haemorrhages, who do not have evidence of plasma leakage and therefore cannot be classified as DHF despite the presence of haemorrhages. This can confuse the inexperienced clinician and lead to unnecessary and inappropriate overzealous fluid therapy.

A more practical classification based on the clinical findings would be more appropriate and simpler to determine. Dengue patients without any haemorrhages or shock are classified as dengue fever. Patients who have diffuse blanching erythema or blanching papular erythema would also fall into this category. Blanching papular erythema should not be mistaken for petechiae. Petechiae will not blanch on pressure and implies skin haemorrhages. Dengue patients with haemorrhages, irrespective of its magnitude or site, are classified as DHF. Dengue patients in shock are classified as DSS. Intervention decisions at all levels of care could then be based on accurate and easily determined clinical parameters such as capillary refill time and pulse pressure.

Accurate prediction of disease severity requires the analysis of specific data related to pathogenesis and plasma leakage such as viral serotype, serum levels of non-structural protein 1 (NS1), immunoglobulin G (IgG) subclass, dengue-specific and total immunoglobulin E (IgE), serum concentration of antiplatelet antibodies, and levels of cytokines such as TNF α, IFN α and IL-10.[8,9,10,11,12] Non-availability of such information to clinicians at the point of delivery of care leaves no option but to rely on clinical parameters to predict disease severity.
Thrombocytopenia is a common problem in dengue, which causes a lot of concern not only to the patient but also to the relations as well as the attending physician. No clear guidelines exist for its management. The natural tendency is to transfuse platelets. Thrombocytopenia in dengue is primarily immune-mediated. It can therefore be surmised that platelet transfusions by presenting a strong antigenic stimulus can aggravate the thrombocytopenia by an exalted immune response. Besides, the short life span of transfused platelets would result only in a transient non-sustained elevation of the platelet count. Additionally, platelet transfusions can evoke hypersensitivity reactions and fluid overloading with attendant complications of pleural effusions, such as ascites, and pulmonary oedema.

Clearly, prophylactic platelet transfusions for dengue are baseless and appear to be an irrational and inappropriate intervention. However, transfer of patients from peripheral hospitals to tertiary care hospitals primarily for platelet transfusions reflect the dilemmas confronting clinicians in managing thrombocytopenia in patients with dengue.

**Management**

From a practical point of view, reduction in morbidity and mortality of dengue rests firstly in the early diagnosis of dengue and then on the ability to identify the minority of patients with a propensity to develop severe disease early in the disease course. Individually-tailored parameters designed to detect potential complications should be monitored in such patients. Critically-timed appropriate interventions based on changes in the monitoring parameters could thereby thwart disease progression and an adverse outcome.

Diffuse blanching erythema is a very useful sign in the early clinical diagnosis of dengue in adults during epidemics. This is recognized as a generalized flushed appearance in the skin, particularly in the posterior aspect of the chest when observed in a good light (Figure 1). The erythematous areas have no clear boundaries and blanch on light pressure applied with the fingers. Some patients have blanching papular erythema, particularly in the limbs (Figure 2). These appear as minute erythematous papules which blanch when light pressure is applied with the finger tip. It should not be mistaken for petechiae which do not blanch on pressure. Serological confirmation is not required for management and should be requested only if necessary.
there is a doubt in the diagnosis, specially to differentiate from other febrile illnesses with myalgia and thrombocytopaenia like leptospirosis.

Patients with a normal sensorium, good peripheral circulation with warm extremities, bounding pulse, capillary refilling time <2 seconds, posterior cervical lymphadenopathy and platelet counts over 50,000/μL are bound to make an uneventful recovery. Patients with a pulse pressure of <20 mmHg and poor capillary refilling will require aggressive fluid therapy to prevent progression to DSS. Interventions in such patients should be early, decisive and aggressive. The quantum and quality of the fluid infused should be dictated by clinical judgement and changes in the monitoring parameters, particularly pulse pressure, haematocrit and platelet count.

Peripheral pulse, capillary refill time, and pulse pressure are the most consistent and important parameters to base decisions on interventions at all levels of care. However, in difficult and ambiguous situations, seeking additional information on predictors of disease severity and capillary leakage facilitates decision-making. These include cervical lymphadenopathy, acute right hypochondrial pain and tenderness, retro-orbital pain, altered sensorium, pleural effusions, ascites, oedematous gall bladder on ultrasonography, positive tourniquet test, platelet count, and aspartate amino transferase (AST) levels. It should be noted that in this context the presence of cervical lymphadenopathy[4] and normal levels of AST[13] are strong negative predictors of dengue fever progressing to DHF or DSS. Such patients very often will not require aggressive fluid therapy. On the contrary, the presence of any one or more of the other predictors of disease severity mentioned above should alert the clinician to the potential probability of an adverse outcome and the need for close monitoring of pulse pressure, capillary refill time and haematocrit and to be more liberal on fluid therapy. Less than optimal care, both with regard to monitoring and fluid therapy early in the disease course in such patients, could result in DSS[14].

Clinicians are cautioned against aggressive and overzealous fluid therapy in the face of stable haemodynamics even in patients with extensive blotchy erythema and the above-mentioned predictors of disease severity, which imply incipient increase in vascular permeability, and increased vulnerability to fluid overloading and attendant mortality. A dynamic approach to management and subtle adjustments in fluid therapy, based on astute clinical judgement, requires one to strike the correct balance between augmentation of fluid therapy to offset a drop in the pulse pressure and capillary filling on the one hand and decrementation of fluids without compromising the haemodynamics on the other, when increased capillary permeability has shifted the balance towards aggravation of pleural effusions and pulmonary oedema. The entire quantum of intravenous fluid as calculated per guidelines need not necessarily be given to patients with pleural effusions provided haemodynamic parameters are satisfactorily maintained, usually with 1 to 1.5 litres of isotonic saline infused over 24 hours. Any deterioration in the haemodynamics should prompt the immediate administration of an intravenous saline bolus (10 ml/kg). Satisfactory circulation is reflected by a pulse pressure of over 20 mmHg, capillary refill time <2 seconds, and hourly urine output of >0.5 ml/kg body weight. These are the parameters to be monitored and utilized to optimize fluid therapy.

Platelet transfusions are hardly ever required even with counts as low as 10,000/μL because the circulating platelets are haematologically active and sufficient to prevent bleeding by thrombocytopaenia per se.
Besides, the survival of transfused platelets is very short in cases with DSS\textsuperscript{[15]}. In general, platelet transfusions are given only when there are serious haemorrhagic manifestations. Transfusion requirements correlate with the occurrence of bleeding in the gastrointestinal tract but not with the platelet count\textsuperscript{[16]}. There is no place for prophylactic platelet transfusions\textsuperscript{[17]}.

Fresh frozen plasma is an useful and safer therapeutic option than platelet transfusions for patients with severe thrombocytopaenia. Its use, however, should be reserved only for highly selected patients with severe thrombocytopaenia early in the disease\textsuperscript{[18]}.

In summary, the management dilemmas can be resolved by applying the following steps when treating a febrile patient with suspected dengue in an adult medical ward.

**Step I: Early diagnosis of dengue**

It should be a clinical diagnosis based on the presence of diffuse blanching erythema in a patient with features of a viral fever.

**Step II: Classify the clinical type as DF, DHF or DSS**

**Step III: Risk stratification and selection of patients for specific interventions**

1. **Minor disease:** Patients with a normal sensorium who do not look too ill, have a good appetite, warm extremities, pulse pressure of 20 mmHg, bounding peripheral pulse, capillary refill time <2 seconds and enlarged posterior cervical lymphnodes. A majority of patients fall into this category (personal experience). Intravenous fluids are not mandatory and can be managed effectively with oral fluids\textsuperscript{[7,17]}. Intravenous fluids will be required if there are excessive fluid losses due to undue vomiting.

2. **Severe disease:** Patients with altered sensorium, sustained right hypochondrial pain, absence of cervical lymphadenopathy, cold extremities, pulse pressure <20 mmHg, capillary refill time >2 seconds or any other predictors of severe disease should be selected for monitoring and judicious fluid therapy.

**Step IV: Monitoring and critical care**

Patients with clinical markers of severe disease or predicted to develop severe disease should be shifted to a high dependency area in the ward where close observation and monitoring is feasible with the prioritized utilization of limited facilities in a resource-poor setting. Monitoring charts in these selected patients should be simple and practical. The monitoring parameters should be individualized and should have predictive utility to base interventional decisions. These include the pulse rate, pulse pressure, capillary refill time, respiratory rate, and hourly urine output. Serial estimation of haematocrit and platelet count in the first 24 to 48 hours where facilities are available will provide additional and useful information to base decisions on interventions.
the minimal quantum of intravenous fluid to maintain a pulse pressure of 20 mmHg or more and an hourly urine output of 0.5 ml/kg body weight. This usually amounts to approximately 1000 to 1500 ml of isotonic saline over 24 hours. Necessarily the fluid intake has to be adjusted appropriately for those patients with excessive fluid losses due to undue vomiting. Plasma leakage continues for about 24 to 48 hours and good pulse volume, wide pulse pressure diuresis and stable haemotocrit are indicators to stop fluid therapy\(^{17}\). Over-treatment with fluids at this stage will have an adverse outcome and will lead to pleural effusions and pulmonary oedema. Progressive increase in the respiratory rate in the monitoring chart should alert the clinician to the possibility of this complication, prompting a careful evaluation of the lungs clinically, radiologically and measurements of oxygen saturation as well as the need to withhold intravenous fluids provided minimal haemodynamic stability is maintained.

**Step V: Aggressive fluid therapy**

Urgent and aggressive fluid therapy is required for those with a pulse pressure <20 mmHg, cold clammy skin, rapid weak pulse and restlessness. Aggressive intervention entails an intravenous bolus of isotonic saline or Hartman’s solution at a dose of 10 ml/kg body weight. If the pulse pressure remains less than 20 mmHg the same dose is repeated twice. If there are still no signs of improvement, up to two doses of colloid (plasma or dextran) at a dose of 10 ml/kg body weight should be given\(^{17}\).

**Step VI: Consider intervention for thrombocytopaenia**

Platelet transfusions are hardly ever indicated even with counts as low as 10 000. It should be considered only if there is significant bleeding attributable to thrombocytopaenia\(^{17}\).

Consider fresh frozen plasma as a therapeutic option for thrombocytopaenia in highly selected patients early in the disease course\(^{18}\).

**Conclusion**

The management of dengue patients, particularly during epidemics, should be based on arriving at a clinical diagnosis early in the disease course. Recognition of clinical predictors of disease severity at the time of presentation, avoiding unnecessary platelet transfusions, and judicious fluid therapy dictated by clinical judgement early in the disease course are of crucial importance. Proper utilization of simple parameters for monitoring such as pulse pressure, capillary refill time and haematocrit play a central role in the selection of patients for interventions as well as determination of end points and optimization of fluid therapy to reduce morbidity and mortality due to dengue.

**Recommendations**

1. Utilize a simple and practical six-step approach to manage adult dengue patients in a ward setting. This approach is particularly applicable to busy overcrowded hospitals with limited resources and little access to intensive care units, as is the case with most rural and district hospitals in developing countries where dengue is common.

2. Develop clear consensus guidelines on the management of thrombocytopaenia in dengue.
(3) Current classification of the grading of dengue needs to be reviewed.

(4) Aggressive educational programmes targeting care-providers at all levels to avoid unnecessary transfers, irrational platelet transfusions and fluid overloading.

References


Management dilemmas in the treatment of dengue fever


An outbreak of dengue in Moreh: A small rural town in Manipur near Indo-Myanmar border

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Introduction

Dengue is endemic in most countries of south-east Asia. As of December 2007, out of the 11 countries in the WHO South-East Asia Region, 10 countries (Bangladesh, Bhutan, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, Thailand and Timor-Leste) have been reporting the incidence of dengue every year. Thailand, Indonesia and Myanmar continue to be hyperendemic countries in the Region (Source: WHO-SEARO, New Delhi, 2007)[1].

In India, out of the 35 states/Union Territories in the country, 23 have reported cases and deaths due to dengue. All the four serotypes, i.e. DENV-1 to -4 have been isolated in the country (Source. www.nvbdcp.gov.in)[2]. *Aedes aegypti* is the most efficient vector of dengue in India[2]. Ecology is a great determinant of the occurrence of dengue. Due to the man-made ecological and lifestyle changes, DF/DHF has now spread to rural areas as well[3,4,5,6].

In November 2007, Moreh town in Chandel district of Manipur state reported the death of an 11-year-old girl due to dengue haemorrhagic fever. The girl was admitted with high fever with haemorrhagic manifestations and was serologically confirmed for dengue-specific IgM. Following this reported dengue death, a sero-surveillance study was carried out in the area. This note incorporates the results of the investigations.

Study area

Moreh is a small border town adjacent to Myanmar having a municipal council of nine wards and is a border trade centre. The population of the town is about 20 000, of which 4–5000 people comprise the floating population. The area is subtropical, hot and humid, with variable temperature ranging from a minimum of 10 °C to a maximum of 35 °C. The annual rainfall varies from 1000 mm to 1200 mm.
Results

Sero-surveillance

Blood samples were collected from 281 clinically-suspected dengue cases with duration of ≥5 days for serological confirmation of DENV. Out of the 281 samples tested, 51 were found positive for DENV by IgM Mac ELISA. For analyses of the cases, all the 281 cases were divided into three age groups 0 to 5 years, 5.1 to 14 years, and above 14 years. The results are given in the Table. There was no significant difference in the prevalence of the disease among the three age groups (p=0.07797 at 5%), indicating fresh introduction of the virus infecting all age groups in the absence of immunity. Furthermore, when the cases were analysed for disease prevalence among children 0 to 14 years against those aged 14.1 years and above, the findings revealed significant differences in the prevalence among both the age groups (p=0.02645 at 5%). The prevalence was significantly higher in the age group above 14 years. Thus, the probability of extradomiciliary transmission of dengue at the work site (border areas, trade centres) in the affected areas cannot be ruled out. Both sexes were equally affected. There was no significant difference in the prevalence of the disease among males and females (p=0.2415 at 5%). Among the 51 serologically-positive cases, haemorrhagic manifestation was observed in 2% of the cases.

This is the first report of DF/DHF outbreak from Manipur, a state in the north-eastern part of India, with 51 serologically-confirmed dengue cases and 9.6% DHF cases. However, dengue is rampant in the bordering country of Myanmar[1]. Since the area is endemic for both Ae. aegypti and Ae. albopictus, it is desirable to undertake comprehensive entomological studies to establish the breeding habitats of both the species indoors and outdoors and to identify the areas of overlap and/or co-breeding. This information is essential for developing any vector control strategies in the state.

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References


Imported dengue fever cases in Gunma prefecture, Japan

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Dengue virus (DENV), which is classified into four serotypes designated as DENV-1 to DENV-4, is endemic in more than 100 countries in both tropical and subtropical regions, with south-east Asia and the western Pacific being the most seriously affected[1]. According to the World Health Organization (WHO), the global prevalence of dengue fever (DF) and dengue haemorrhagic fever (DHF) has dramatically increased in recent decades (http://www.who.int/mediacentre/factsheets/fs117/en/). Concern is therefore growing over DF/DHF as one of the most important mosquito-borne human infectious diseases[2]. In Japan, relatively large epidemics of DF occurred between 1942 and 1944 in Nagasaki, Kobe and Osaka, originating from persons repatriating from the tropics during World War II; these epidemics were eliminated in 1946[3]. In recent years, between 10 to 70 cases of DF/DHF have been reported annually in Japan, all of which were imported as a traveller’s disease[3-6]. The number of the reported DF/DHF cases has shown an increasing trend since the late 1990s (http://idsc.nih.go.jp/idwr/kansen/k04/k04_50/k04_50.html [in Japanese]). In 2007, a total of 89 cases of DF/DHF were reported, the highest number recorded in Japan thus far.

Gunma prefecture, located in the north-west corner of the Kanto region on Honshu island, has a population of approximately 2 million, and 10% of its residents travel overseas. In 2007, we experienced 2 cases of DF: one patient (Case 1) diagnosed with DENV-3 was plausibly infected in Ho Chinh City, Viet Nam[7], and the other patient (Case 2) diagnosed with DENV-2 was infected in Kingston, Jamaica. In general, when hospital doctors in Japan suspect a case of DF, specimens are sent to a local public health institute for confirmation of DENV infection. In the present two cases, specimens were sent to our institute (Gunma Prefectural Institute of Public Health and Environmental Sciences) and virological analysis was performed for each case. In Case 1, from a blood sample collected on hospital day 3, we detected the DENV gene by reverse transcription-
polymerase chain reaction (RT-PCR) using the DENV-3-specific primers covering E-NS1 gene (GenBank accession number: AB362210)\cite{8} (Figure 1), although we could not isolate the virus using various cell lines (Vero, RD, HEP-2, and HEL cells). In Case 2, from serial blood samples collected on hospital days 3 and 4, DENV was not detected by RT-PCR, but specific IgG and IgM antibodies against DENV were significantly raised. In addition, DENV was propagated and isolated from Vero cell cultures. From the isolate, we confirmed DENV-2 by RT-PCR and phylogenetic analysis for the E gene (AB470342) of the virus (Figure 2).

**Figure 1:** Phylogenetic tree based on the E-NS1 sequences of DENV-3

[Phylogenetic distance was calculated using Kimura’s two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Inscriptions indicate the country where the dengue virus gene was detected, GenBank accession numbers, and collection year.]
As mentioned above, DF is an important infectious disease not only in tropical and subtropical regions but even in countries in temperate zones such as Japan. There is currently no vaccine available for human use, and as in the present cases, there is the potential for relatively severe clinical manifestations such as continuous high fever, hepatic disorder, leukocytopenia and thrombocytopenia to develop\(^2\). There is no evidence of domestic DENV transmission in Japan; however, *Aedes albopictus*, one of the main vectors, is widely distributed across the country, with the exception of Hokkaido\(^9\), and an outbreak of DF/DHF is possible in Japan once the virus enters the country\(^1\). This is especially important to consider in the light of the large numbers of overseas travellers who are at risk of DENV infection. Thus, attention to trends in DENVs and the incidence of DF/DHF in Japan is required.

*Figure 2: Phylogenetic tree based on the E sequences of DENV-2*

[Phylogenetic distance was calculated using Kimura’s two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Inscriptions indicate the country where the dengue virus gene was detected, GenBank accession numbers, and collection year.]
References


Concurrent dengue fever and bacterial septicemia during the 2008 dengue outbreak in Delhi

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There have been reports of unusual clinical presentations associated with dengue virus infection with sporadic reports of cardiac involvement[1], altered consciousness with electroencephalographic changes[2] and liver pathology[3]. During 2008, there was a sudden spurt in dengue cases in the Indian Capital, Delhi, and its adjoining areas[4]. We found concurrent dengue virus infection and bacterial septicemia in three hospitalized cases at Sant Parmanand Hospital, a 140-bedded tertiary-care hospital catering to the population of Delhi and adjoining townships.

During the period September to November 2008, 125 suspected cases of dengue were hospitalized. Of these, 114 were confirmed cases of dengue; three of them were also blood culture positive: two Staphylococcus aureus and one Salmonella typhi/paratyphi A, B group. S. aureus was isolated from two females, aged 28 and 30 years. The female patient, aged 30 years, platelet count 0.98x10^3/µl, total leukocyte count 9600/mm^3, positive for anti-dengue virus IgG and IgM, was not on hand for any antibiotic treatment. The other female, with platelet count 0.22x10^3/µl, leukocyte count 2000/mm^3, positive for anti-dengue virus IgM, was prescribed parenteral amoxicillin-clavulanic acid during hospitalization and ofloxacin during her convalescence.

The S. typhi/paratyphi group A, B was isolated from a 30-year-old male, platelet count 0.38x10^3/µl, total leukocyte count 2500/mm^3, and positive for anti-dengue virus IgG and IgM. Isolate was susceptible to amoxicillin-clavulanic acid, amikacin, piperacillin, ceftriaxone, aztreonam, chloramphenicol and tetracycline, but resistant to ampicillin-sulbactam, ceftazidime, cefaclor, cefotaxime, ceftriaxone, cefuroxime and trimethoprim-sulphamethoxazole. The patient responded to parenteral amoxicillin-clavulanic acid and amikacin.

Reports on concurrent bacterial infections have been meagre, with not many dengue patients with concurrent typhoid fever[5]. Among 5000 cases with symptomatic dengue infection during an outbreak in Taiwan, China, there were only seven cases with becteremia at Chang Gung Memorial Hospital, Kaohsiung, in southern Taiwan[6]. Furthermore, during the 1990s, only one of the 19 serologically confirmed infants at Chon Buri Regional Hospital, Thailand, had Staphylococcus aureus sepsis, who recovered with appropriate management and treatment[7].

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Dengue patients, upon a significant disease resolution, would by and large be asymptomatic with normal platelet count and haematocrit values during a short hospital stay. The involvement of several organs[1-3] would imply a prolonged stay. There are not many reports on concurrent dengue infection and septicemias, which is really not all that odd. Initial symptoms of fever would camouflage any septicemia. Dengue outbreaks are known to overwhelm the limited outpatient and inpatient facilities available in hospitals in countries like India. Furthermore, during such episodes, the medical personnel there are overworked and completely exhausted[8].

The recommended essential laboratory investigations during a dengue outbreak include prothrombin time, partial thromboplastin time, thrombin time, electrolytes and liver function tests[8]. Addition of blood culture would be desirable for detection and treatment of any concurrent septicemia.

References


Fibre-glass drums as the key containers of Aedes aegypti breeding in apartments occupied by expatriates in Jeddah, Saudi Arabia

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bVector Control Research Centre, Medical Complex, Indira Nagar, Puducherry 605006, India

Dengue fever (DF) and its severe forms dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are endemic in many parts of the tropical world. The disease is caused by four antigenically-related dengue viruses (DENV-1 to 4) belonging to the genus Flavivirus and transmitted by Aedes (Stegomyia) aegypti.

In recent times DF made its first appearance in 1994 with 289 cases in Jeddah[1] (21°29'31"N 39°11'24"E), a cosmopolitan city which is situated on the coast of the Red Sea and divided into 14 sub-municipalities covering a total of 95 districts. The Ministry of Health (MoH) initiated dengue surveillance as per WHO guidelines[2] without effective vector control measures. However, noticing the upward trend of DF cases during 2004-2005[3], the Government of Saudi Arabia established the dengue crisis management and mosquito control programme under the Department of Pest Control and Public Health of Jeddah municipality. The main focus of the programme was on vector control activities equipped with men and materials. Control of dengue vectors was started by the municipality through reputed “company contractors” to cover the outside building areas which included open areas, building under construction, blocks and bricks factories, gardens, shopping complexes, etc. The contractors applied various insecticides in the form of larvicides and adulticides and space-spray applications with ULV thermal fogging. Mosquito surveillance activities were carried out using light traps and ovitraps within the operational area. This operation was assisted by the Geographical Information System (GIS) unit, in preparing baseline maps and updating relevant data.

For indoor activities student volunteers (SVs) studying in various colleges, schools, universities and other educational institutions were involved in house-to-house campaign in different operational areas of sub-municipalities to carry out source reduction measures. Under each operational area, 22 to 25 SVs were engaged on a day-to-day control operation between 4.30 PM and 10.00 PM everyday except Friday. Two SVs formed a team and minimum nine such teams were involved in the campaign under the supervision of a team leader of the respective operational areas. One SV involved recorded all potential Aedes

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breeding sources with the help of hand-held torch lights while the other SV treated all breeding sources with required larvicides or Insect Growth Regulator (IGR) granules. One of the authors (T. Mariappan) who was recruited as a consultant from the Vector Control Research Centre (VCRC), Puducherry, India, provided all SVs a practical hand in training in the field with regard to identification of Aedes mosquito breeding habitats\(^4\), collection of data, sampling methods of larvae from different water storage containers, and utility of both larvicides and adulticides and handling of spray application equipment, viz. compression sprayers, including hand-held thermal fogging machines.

**Table:** Number of houses surveyed and Aedes breeding status

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Street name</th>
<th>No. of houses surveyed (+ve)</th>
<th>Drums &gt;100 litres</th>
<th>Drums &lt;100 litres</th>
<th>No. of drums with lids and locked (+ve)</th>
<th>No. of drums without lock (+ve)</th>
<th>+ No. of drums open (+ve)</th>
<th>Total no (+ve)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1.</td>
<td>Shawas Lane</td>
<td>2/16 (12.5)</td>
<td>0</td>
<td>1/22 (4.6)</td>
<td>1/18 (5.6)</td>
<td>2/40 (5)</td>
<td>0/86</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Shaman Lane</td>
<td>3/9 (33.3)</td>
<td>0</td>
<td>3/14 (21.4)</td>
<td>0/15 (0)</td>
<td>3/29 (10.3)</td>
<td>0/51</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Al-Zahab Street (3000)</td>
<td>1/5 (20)</td>
<td>0</td>
<td>1/11 (9.1)</td>
<td>0/8 (0)</td>
<td>1/19 (5.3)</td>
<td>0/45</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>SurorBaa’selle (4)</td>
<td>4/12 (33.3)</td>
<td>0</td>
<td>3/17 (17.7)</td>
<td>1/29 (3.5)</td>
<td>4/46 (8.7)</td>
<td>0/59</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Amnai Lane (21)</td>
<td>4/15 (26.7)</td>
<td>0</td>
<td>4/19 (21.1)</td>
<td>0/24 (0)</td>
<td>4/43 (9.3)</td>
<td>0/85</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Marash Street (2)</td>
<td>0/18 (0)</td>
<td>0</td>
<td>0/8 (0)</td>
<td>0/6 (0)</td>
<td>0/14 (0)</td>
<td>0/142</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Muhaymed Lane (6)</td>
<td>4/10 (40)</td>
<td>0</td>
<td>3/12 (25)</td>
<td>1/28 (3.6)</td>
<td>4/40 (10)</td>
<td>0/77</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Al-Shurakari Street (109)</td>
<td>1/8 (12.5)</td>
<td>3/14 (21.43)</td>
<td>0/6 (0)</td>
<td>0/7 (0)</td>
<td>3/27 (11.1)</td>
<td>0/28</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td>Al-Badia Lane (59)</td>
<td>0/14 (0)</td>
<td>0</td>
<td>0/21 (0)</td>
<td>0/8 (0)</td>
<td>0/29 (0)</td>
<td>0/238</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>19/107 (17.76)</td>
<td>3/14 (21.43)</td>
<td>15/130 (11.5)</td>
<td>3/143 (2.1)</td>
<td>21/287 (7.32)</td>
<td>0/811</td>
<td></td>
</tr>
</tbody>
</table>

Figures in brackets in columns 1 2, 3, 4 and 5 indicate percentage.
The present communication incorporates the field collection data collected on 15 July 2007 (Table) at Al-Balad district from apartments in nine lanes occupied by non-Saudi expatriates. Larvae and pupae collected from the field were reared in the laboratory for confirmation of species.

A total of 107 houses searched for Aedes breeding and 19 were found positive. Computation of House Index (HI) was estimated at 17.76. A total of 1098 drums/containers were grouped into two categories (i.e. drums/containers with >100 litres (columns 2, 3, 4) and <100 litres (column 6)). The proportion of drums/containers of >100 litres (i.e. 287 numbers) versus <100 litres (i.e. 811 numbers) were 1: 2.83. The drums/containers with >100 litres further classified into (i) L-Ds {i.e. drum covered with lid along with independent lock (column 2)} (ii) drum covered with lid without lock (column 3) and (iii) drum without any lid (open). The percentages of contribution of L-Ds, drum with lid without lock and drum without any lid (open) were 4.9%, 45.3% and 49.8% respectively. The drums/containers with <100 litres were unclassified ones due to absence of Ae. aegypti breeding. Other water storage containers included ornamental plant jars/bottles, flower pots, draining water from air-conditioners and their contribution of Ae. aegypti breeding was nil. It is apparent that only drum with >100 litres only contributed Aedes breeding. Though an overall 7.32% drums supported Aedes breeding, however the percentage of contribution by L-Ds, drums covered without lock and open ones were 21.43% and 11.5% and 2.1%. The study has indicated that drums with lids have contributed Ae. aegypti significantly higher than open ones. This can be explained by negative phototropism of Ae. aegypti for sunlight. The overall calculated Container Index (CI) was 7.32.

The absence of breeding in other containers as outlines above may largely be due to scarcity of rains. Locked drums had a cover where the design of cover was not mosquito proof, hence allowed breeding.

**Conclusion**

There is a need for more extensive field surveys and statistically significant data on sampling basis covering both Saudi and non-Saudi apartments to unravel the varied types of storage containers used by the civil society to assess the Aedes breeding potential to further strengthen the vector control programme and to raise material for Information, Education and Communication (IEC) material.

**Acknowledgements**

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References


The Asia-Pacific Dengue Strategic Plan (2008-2015) has been prepared in response to the increasing threat from dengue, which is spreading to new geographical areas and causing high mortality during the early phase of the outbreaks. Among an estimated 2.5 billion people at risk globally, about 1.8 billion (more than 70%) reside in the Asia-Pacific region. The development of this Strategic Plan is also important to meet the requirements of the International Health Regulations (IHR) 2005. The goal is to reverse the rising trend of dengue in countries of the Asia-Pacific region.

Countries of the region vary in terms of their preparedness, their capacity to respond and in the allocation of financial resources in the prevention and control of dengue. The Strategic Plan provides generic recommendations to allow local adaptation of strategies.

Dengue does not respect international boundaries. Effective dengue control is not possible if control efforts are limited to one country or a few. It requires the adoption of a regional approach through collaboration between countries and sustained partnerships to enable countries to implement evidence-based interventions and the use of best practices.

The Asia-Pacific Strategic Plan would assist countries to enhance their preparedness; enable them to promptly detect, characterize and contain outbreaks; and limit epidemics of dengue for effective prevention and control. This plan should be implemented in harmony with the Strategic Framework for Asia-Pacific Partnership for Dengue Prevention and Control (APDP).

The Strategic Plan should be used for elaborating national operational plans; to develop capacity and strengthen the health system; establish networking; harmonize itself with the APDP Strategic Framework for mobilization of resources and sustain ongoing information exchange; and be able to advocate for prevention and control of dengue. It would also assist in increasing access to innovations such as with tools for the diagnosis, prevention and treatment of dengue.
Dengue prevention and control

Resolution of the WHO Regional Committee for South-East Asia (SEA/RC61/R5)

The Regional Committee,

Concerned with the emergence and re-emergence of dengue as a serious public health threat in countries of the Region,

Understanding that global climate change has resulted in the emergence and reemergence of dengue in the Region with an increase in outbreaks,

Recognizing that dengue has far-reaching cross-border and international implications,

Noting that most Member States in the Region need to strengthen dengue surveillance, prevention and control systems,

Appreciating efforts made by the South-East Asia and Western Pacific regions for developing the Asia-Pacific Dengue Strategic Plan, 2008-2015 focusing on reversing the increasing trend of dengue,

Recognizing the importance of community ownership and multisectoral interventions as key strategies in prevention and control of dengue, and

Having considered the report and recommendations by the Meeting of the Advisory Committee held in the Regional Office, New Delhi from 30 June – 3 July 2008,

1. URGES Member States:

(1) to implement the bioregional Asia-Pacific Dengue Strategic Plan, 2008-2015;

(2) to implement the primary health care approach to promote community ownership, intersectoral collaboration and coordination among relevant ministries for effective implementation of dengue prevention and control, as well as intercountry activities,

(3) to strengthen national and cross-border surveillance to assess the burden of dengue;

(4) to implement an integrated vector management strategy as a major preventive strategy, and

(5) to strengthen clinical competency in diagnosis and management of cases, and

2. REQUESTS the Regional Director:

(1) to advocate for and mobilize additional financial resources for strengthening the dengue prevention and control programmes in Member States;

(2) to provide technical support to Member States in implementing the bioregional Asia-Pacific Dengue Strategic Plan (2008-2015);
Dengue prevention and control

(3) to facilitate the acceleration of dengue vaccine research and development;

(4) to support Member States in assessing and monitoring the impact of climate change on dengue, and

(5) to provide technical support to Member States in prioritizing operations research in order to support evidence-based policy decisions and effective prevention interventions.
Six countries test dengue interventions

TDRnews, March 2009 (No.82)

Eco-bio-social research

A multi-country research effort in Asia designed to study dengue transmission and then test social and ecosystem-based interventions is launching its Phase II. Following the completion of the Phase I situation analysis, all six sites will initiate tests of various community-based management approaches in April. These are designed to address locally identified factors in disease transmission.

The initiative, funded by the EcoHealth Programme of the Canadian International Development Research Centre (IDRC), involves multi-disciplinary research teams at universities and research centres in India, Indonesia, Myanmar, the Philippines, Sri Lanka and Thailand.

The initiative has been designed to improve dengue prevention through better understanding of its ecological, biological and social (“eco-bio-social”) determinants.

Eco-bio-social research is a trans-disciplinary research concept that integrates research on environmental, vector-epidemiological (entomological) and social factors that make communities vulnerable to vector borne diseases such as dengue. The aim of such research is to develop inter-sectoral approaches to disease control, addressing issues that extend beyond traditional boundaries of health-sector activities.

In the current study, research teams are examining both effectiveness and community acceptance of locally developed vector control measures. For instance, in Yangon, Myanmar, teams will examine how use of natural predators and biological larvicides such as dragonfly nymphs and Bacillus thuringiensis serovar israelensis (Bti), as well as water covers, window curtains and waste control measures, may reduce vector densities. Stakeholder alliances, community partner groups and volunteers will be involved in these activities.

In Muntinlupa City, Philippines, part of the metropolitan Manila region, the Phase II work also aims to evaluate relative acceptance by communities and local governments of community-based vector control efforts involving solid waste management, lid covers for key containers, larvicides and health education measures. The social research element will include stakeholder meetings, key informant interviews and focus group discussions.

Similar projects are taking place in Gampaha District, Sri Lanka; Chennai, India; Yogyakarta, Indonesia; and Chachoengsao Province, Thailand. The project in Thailand aims to measure the efficiency of insecticide impregnated window curtains and water container covers on the reduction of vector density measured by standard entomological indices. EcoHealth volunteers and students will be engaged in applying the vector control methodologies at the community level.
Six countries test dengue interventions

“The aim is to show that dengue transmission can be reduced with an appropriate management of ecosystems, in different ecological environments.” said Dr Olaf Horstick, a TDR technical officer involved in TDR’s eco-bio-social efforts. “If this concept is successful the studies should underline the importance of inter- and intrasectoral approaches to the control of vector-borne, but also other communicable diseases.”
Dengue in Africa

Dengue fever is an emerging pandemic that has spread globally during the past 30 years as a result of changes in human ecology. Around 2.5 billion people live in the >100 countries in tropical and subtropical areas where the dengue virus is transmitted. More than 70% of the disease burden occurs in Asia and the Pacific, followed by the Americas, the Middle East and Africa. Dengue is caused by 4 serologically distinct, but closely related, viruses: dengue virus (DENV) 1, 2, 3 and 4 of the Flaviviridae family. This family of viruses also includes yellow fever virus and West Nile virus. There is good evidence that sequential infection with the different serotypes of dengue virus increases the risk of more severe disease that can result in shock syndrome and death. The increase in incidence and the geographical expansion of dengue have been facilitated by the rapid movement of all 4 viruses and mosquito vectors through international air travel and trade; population increases; global urbanization; and the abundance of disposable, non-degradable containers that serve as breeding sites in the peridomestic environment for the principal vector, Aedes aegypti, which maintains the urban dengue transmission cycle among humans[1]. Additionally, a sylvatic transmission cycle has been documented in west Africa, where DENV-2 has been found circulating among Erythrocebus patas monkeys and various sylvatic Aedes species, including Ae. taylori, Ae. fuscifer, and Ae. luteocephalus[2].

Map 1 shows instances of dengue occurring in Africa during 1948–2008; the data are based on published reports of outbreaks and serosurveys, and reports of dengue diagnosis in suspected cases of travellers returning from Africa[3,4]. These reports indicate there was a substantial increase in epidemic dengue activity in Africa during the 1980s. However, because epidemic dengue activity in Africa has mostly been classical dengue fever caused by DENV-1 and DENV-2 without associated mortality, dengue has not been seen as a disease that should be given priority when compared with malaria, HIV/AIDS and other diseases that cause high morbidity and mortality in Africa. Because of this, dengue surveillance data from Africa are sparse, and cases and outbreaks are not reported to WHO. During 1984–1985, the first major outbreak of DENV-3 in Africa was documented in Pemba, Mozambique. During this outbreak, most patients experienced secondary infections, and 2 deaths were attributed to dengue haemorrhagic fever and shock. DENV-3 was documented again in a mixed outbreak caused by DENV-2 and DENV-3 in Somalia in 1993. During the 2000s, DENV-3 caused major outbreaks with high incidence and severity in many countries in the Americas, Asia and the Middle East[5].
Although many arboviruses – such as dengue, chikungunya, Crimean-Congo haemorrhagic fever, Rift Valley fever, yellow fever and West Nile virus – affect human health in west Africa, surveillance programmes and reference diagnostics are not consistently available except for yellow fever, for which there is an effective vaccine and a programme for implementing mass vaccination following an outbreak alert.

DENV-3 alert, Côte d’Ivoire, 2008

In April 2008, an international alert was issued when 3 cases of yellow fever in urban areas were confirmed in Abidjan through the active surveillance system for yellow fever in west Africa. In response to this alert, WHO, the Global Outbreak Alert and Response Network and the Ministry of Health conducted a yellow fever vaccination campaign, finalized a seroprevalence survey and completed an environmental survey to investigate yellow fever in the mosquito vector and the monkey reservoir[6].

A second alert was issued following the diagnosis of an acute infection caused by DENV-3 in 1 Japanese tourist and 1 French expatriate, both of whom were hospitalized (in Tokyo and Marseille, respectively) upon their return from Abidjan; their visits occurred between May and July 2008. The virus isolate was similar to the DENV-3 genotype that had been circulating in Saudi Arabia in 2004. Additionally, the Centre National de Référence des Arbovirus in Paris also identified positive anti-dengue immunoglobulin M (IgM) in 7 of 14 specimens collected from suspected dengue cases occurring in travellers returning from Abidjan between 1 May and 31 August 2008. Further analysis by reverse-transcriptase polymerase chain reaction (RT-PCR) identified
additional cases who were positive for DENV-3. One case, returning to France after a 2-month stay in Yamoussoukro, was RT-PCR positive for West Nile virus. In 2008, reports from the Institut de Veille Sanitaire in Paris also identified a sharp increase in imported cases of dengue in travellers returning from Côte d’Ivoire; of 12 cases reported between 1 January 2006 and 31 August 2008, 8 were reported in 2008, 3 in 2007 and 1 in 2006.

In 2008, Côte d’Ivoire was faced with the co-circulation of yellow fever virus and DENV-3 in Abidjan city and the surrounding suburban areas. This raises questions of whether there was an urban transmission cycle for dengue in Abidjan and whether it was caused by an emerging serotype since DENV-3 has never been reported in west Africa. To better assess the extent of the epidemic and the risk of dengue in Abidjan, a team from WHO and the Ministry of Health followed up with an investigation in Abidjan during 2–16 September 2008. The yellow fever surveillance system helped the followup team to obtain specimens from humans, mosquitoes and monkeys. The investigation team also collected mosquito and serum specimens from around the residences of confirmed dengue cases (where this information was available) and established an active case-finding system in health-care centres in districts reporting confirmed cases of yellow fever.

The surveillance systems established in health-care centres by the Ministry of Health identified many cases of suspected classical dengue but did not detect any severe cases or deaths. However, specimens were not collected and laboratory diagnosis was not available. DENV-3 was confirmed by RT-PCR in 2 specimens collected as part of routine surveillance during week 19 (1 from Cocody Bingerville and 1 from Adzopé). The vector and larval indices were high and above the threshold of 5% in these areas.

Transfer of technology for laboratory diagnosis

In response to this concurrent epidemic and the need for differential laboratory diagnosis of dengue and yellow fever, the Institut Pasteur in Dakar and in Paris, in coordination with WHO, deployed a task force to transfer this technology to the Institut Pasteur in Côte d’Ivoire during 2 successive missions in late September and October 2008. Several diagnostic platforms were put in place. Serological tools for detecting IgG and IgM antibodies to dengue and yellow fever were introduced. Confirmatory platforms, such as the plaquereduction neutralization test and dengue serotype-specific enzyme-linked immunosorbent assays (ELISAs), were also introduced. Direct detection platforms (such real-time RT-PCR) for detecting yellow fever and dengue, and for dengue serotype determination, were implemented for human diagnosis and entomological investigations. Cell cultures for virus isolation from human and mosquito specimens were established. Laboratory protocols and assays were validated on site by testing specimens from the 2008 epidemic. Analyses are in progress. ELISA and RT-PCR platforms were provided by the Institut Pasteur in Paris. Recombinant antigens, monoclonal antibodies, cell lines and other diagnostic and reference reagents have been provided by the Institut Pasteur in Paris and in Dakar. An external quality control schedule has been organized for all methods. The continuous exchange between the 3 institutes in Côte d’Ivoire, Dakar and Paris will likely sustain the diagnostic capability for rapid diagnosis and confirmation of dengue virus in Côte d’Ivoire in 2009. However, there is a need to plan to assess the risk and prepare for an appropriate response to dengue in Africa should an outbreak occur.
References


Instructions for contributors

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

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

References to published works should be listed on a separate page at the end of the paper. References to periodicals should include the following elements: name and initials of author(s); title of paper or book in its original language; complete name of the journal, publishing house or institution concerned; and volume and issue number, relevant pages and date of publication, and place of publication (city and country). References should appear in the text in the same numerical order (Arabic numbers in 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 at the first mention. Graphs or figures should be clearly drawn and properly labelled, 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 of the manuscript with original and clear figures/tables and a computer diskette/CD-ROM indicating the name of the software should be submitted to:

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The WHO Regional Office for South-East Asia, in collaboration with the Western Pacific Region, has been jointly publishing the annual Dengue Bulletin.

The objective of the Bulletin is to disseminate updated information on the current status of DF/DHF infection, changing epidemiological patterns, new attempted control strategies, clinical management, information about circulating DENV strains and all other related aspects. The Bulletin also accepts review articles, short notes, book reviews and letters to the editor on DF/DHF-related subjects. Proceedings of national/international meetings for information of research workers and programme managers are also published.

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