Impact of dengue virus infection and its control

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Abstract

Dengue virus infection has been counted among emerging and re-emerging diseases because of (1) the increasing number of patients, (2) the expansion of epidemic areas, and (3) the appearance of severe clinical manifestation of dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), which is often fatal if not properly treated. In the meantime, there are no effective dengue control measures: a dengue vaccine is still under development and vector control does not provide a long-lasting effect. In order to obtain direct evidence for the virulent virus theory concerning the pathogenesis of DHF/DSS, type 2 dengue virus strains isolated from patients with different clinical severities in the same epidemic area in northeast Thailand, during the same season, were comparatively sequenced. The result revealed a DF strain specific amino acid substitution from I to R in the PrM, and a DSS strain specific amino acid substitution from D to G in the NS1 gene regions, which could significantly alter the nature of these proteins. Moreover, DF strain specific nucleotide substitutions in the 3’ noncoding region were predicted to alter its secondary structure. These amino acid and nucleotide substitutions in other strains isolated in different epidemic areas during other seasons, together with their biological significance, remain to be confirmed. In order to innovate dengue vector control, field tests were carried out in dengue epidemic areas in Vietnam to examine the efficacy of Olyset Net screen, which is a wide-mesh net made of polyethylene thread impregnated with permethrin. The results show that Olyset Net (1) reduced the number of principal dengue vector species, *Aedes aegypti*, (2) interrupted the silent transmission of dengue viruses and (3) was highly appreciated by the local people as a convenient and comfortable vector control method. This encouraging evaluation of the Olyset Net screen should be confirmed further by other tests under different settings.

Keywords: Dengue virus; Disease severity; Molecular difference; Vector control

1. Introduction

Dengue viruses with four different serotypes (D1, D2, D3, D4) belong to the family *Flaviviridae*, genus *Flavivirus*, which is represented by yellow fever virus [1,2]. From their mode of transmission, dengue viruses are typical mosquito-borne arboviruses [3]. *Aedes aegypti*, which breeds in various man-made containers in and around human dwellings, has been documented as the principal dengue vector species, while humans are the most susceptible vertebrate hosts of dengue viruses.

During the 25 year period from 1955 to 1980, approximately a million cases of dengue hemorrhagic fever (DHF) were reported to WHO. The number of reported DHF cases was almost similar during the subsequent 5 year period from 1981 to 1985 corresponding to a 5-fold increase in number per year, and again showed a nearly 20% increase in the following 5 year period from 1985 to 1990. These figures represent the increased magnitude of dengue virus transmission.

Fig. 1 shows the geographical distribution of...
dengue virus infection. In the black areas on the map, both severe DHF and mild DF were reported, in contrast to the gray areas where only mild DF cases were reported. This map should be modified continuously, because the gray areas have been expanding and some of them will soon be black.

The expansion of dengue is closely related to economic growth in tropical countries, and to the mode of dengue virus transmission. Economic growth is interrelated with population growth, and increasing numbers of people have been migrating into large cities in order to find jobs, resulting in urbanization. In many large cities in tropical, developing countries with several million inhabitants, crowded people in areas with poor sanitation co-exist with a large number of *Ae. aegypti* breeding sites: an ideal situation for dengue virus transmission. At the same time accelerated travel, particularly by aeroplane, increases the chance of introducing the dengue virus by migration of a patient, during the incubation period, from an endemic area to dengue receptive areas where *Ae. aegypti* and a susceptible population exist. Such an introduction of dengue virus has often resulted in explosive outbreaks to expand epidemic areas of dengue virus infection.

2. Pathophysiology, clinical manifestations and case management of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS)

There are two major abnormalities in the pathophysiology of DHF/DSS leading to a fatal outcome: (1) increased vascular permeability, plasma leakage, hemoconcentration, and hypovolemic shock and (2) coagulopathy including thrombocytopenia leading to disseminated intravascular coagulation and massive hemorrhage [4]. Therefore, proper case management of DHF/DSS should be directed to correct these two abnormalities, by (a) intravenous infusion to correct hemoconcentration/hypovolema and (b) blood transfusion to compensate for the massive hemorrhage [5]. Thanks to improved case management, the case fatality rate of DHF/DSS, which was almost 40–50% at the beginning of its appearance, was remarkably reduced to below a few percent. However, these achievements have not solved the problem of dengue virus infection, because the number of cases has been increasing. During epidemic seasons, the pediatric wards in epidemic areas are occupied by a large number of dengue patients, and cannot function for other emergency cases.
It was estimated that the direct medical cost to treat a single DHF/DSS patient was US$50.00 [6]. This may be trivial for the developed countries, but is a substantial amount for a country whose per capita GNP is around several hundred US dollars. For example in Vietnam in 1987, more than 350,000 DHF cases were reported. Simple calculation can figure out that more than US$17 million would be required as the direct medical cost of treatment, which is equivalent to almost 10% of GDP of this country.

3. Pathogenesis of DHF/DSS and comparative sequence analysis of dengue type 2 virus strains isolated from patients with different clinical manifestations in northeastern Thailand

3.1. Background

There have been serious discussions on the pathogenesis of DHF between two hypotheses: the virulent virus theory, which was strongly postulated by Rosen [7], and the secondary infection theory, divided into the hypersensitivity theory of Russell [8] and the immune enhancement theory of Halstead [9]. The virulent virus theory is simple and easy to understand but direct evidence for it has not been obtained until recently. At the same time, in spite of supporting evidence for the secondary infection theory, it is difficult to explain the iceberg phenomenon. That is, DHF/DSS cases constitute only a minor fraction of a huge population of dengue virus infected people.

During 1992 and 1993, the author carried out an investigation on dengue virus infection in northeastern Thailand supported by the Ministry of Education, Science and Culture of Japan, as well as by the Japan International Cooperation Agency, in collaboration with several investigators in Thailand. During this study, dengue virus strains were isolated, both from severe DHF/DSS cases and from mild DF cases in the same epidemic area within 4 weeks. We have been trying to find direct evidence for the virulent virus theory by comparative sequence analysis of type 2 dengue virus strains isolated from patients with different clinical manifestations.

3.2. Materials and methods

3.2.1. Virus strains

All strains used for comparative sequence analysis were isolated from patient serum specimens by inoculation into Ae. albopictus clone C6/36 cell cultures [10], and their serotypes were determined as type 2 by reverse transcription polymerase chain reaction (RT-PCR) [11]. Table 1 shows the record of the patients from whom each strain was isolated. ThNH7/93 was isolated from a male DF case in the Outpatient Department. ThNH28/93 and ThNH28/93 were isolated from hospitalized male patients with DHF grade I and grade II, respectively, while ThNH7/93 was isolated from a hospitalized female patient showing DHF grade III or DSS. From IgG-ELISA, DHF and DSS cases were categorized as secondary infections, while the DF patient was a primary infection [12]. The age of the patients ranged from 7 to 14 years.

3.2.2. Nucleotide sequencing of viral genomes

The E/NS1 junction of these viral genomes has been sequenced showing that all strains belonged to the same genotype II of dengue type 2 virus [13,14]. Before sequencing, all strains were amplified once by inoculation to C6/36 cells, the infected culture fluid was harvested after 7 days incubation at 28°C, and stored in aliquots at −80°C. For the analysis of the structural protein genes (C, PrM/M, E) and nonco-

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Clinical severity of the patient</th>
<th>Age of the patient (years)</th>
<th>Sex of the patient</th>
<th>Serological response</th>
</tr>
</thead>
<tbody>
<tr>
<td>ThNH7/93</td>
<td>DSS</td>
<td>12</td>
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<td>Secondary</td>
</tr>
<tr>
<td>ThNH28/93</td>
<td>DHF grade II</td>
<td>10</td>
<td>Male</td>
<td>Secondary</td>
</tr>
<tr>
<td>ThNH52/93</td>
<td>DHF grade I</td>
<td>7</td>
<td>Male</td>
<td>Secondary</td>
</tr>
<tr>
<td>ThNHp11/93</td>
<td>DF</td>
<td>14</td>
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<td>Primary</td>
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ing regions, the target sequences were first amplified by rapid RT-PCR with infected C6/36 culture fluid [11]. The amplified gene products were purified from the visualized cDNA bands in ethidium bromide stained 2% agarose gels with a Gene Clean II kit (Bio-101, La Jolla, CA, USA). The cDNA fragment was phosphorylated with the T4 Kinase kit (Takara, Kyoto, Japan), followed by blunting with T4 DNA polymerase (Takara). The prepared cDNA fragment was cloned into the pUC19 vector plasmid, which had been digested with Smal restriction enzyme and dephosphorylated by CIAP (calf intestinal alkaline phosphatase), with a DNA Ligation kit (Takara). The resulting recombinant plasmid was transformed into competent Escherichia coli cells of strain JM-109, transformant colonies were screened by the boiling method, and insertion was confirmed by PCR. Confirmed recombinant colonies were cultured overnight and plasmid DNA was extracted with a Wizard Minipreps DNA purification system (Promega, USA). The nucleotide sequence of the inserted cDNA was determined by the primer extension deoxy chain termination method using the Taq Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems). Viral genome sequences covering the whole structural protein genes were determined by sequencing both strands of the cDNA clones corresponding to six overlapping regions, with more than two independent clones for each region to avoid possible artifactual errors.

In the case of the NS1 gene, two overlapping regions were amplified by RT-PCR, and the products were purified as above, followed by an optional precipitation step with 20% polyethylene glycol and 2.5 M NaCl. The purified PCR products were directly sequenced as described above. At least two amplified products from independent PCR runs were sequenced for each gene region.

For sequencing the 3' noncoding region, each strain was first amplified by inoculation into suspension culture C6/36 cells [15], the virion was concentrated from the infected culture fluid by polyethylene glycol precipitation and ultracentrifugation. Phenol extracted RNA from the pellet was used as the template of the RT-PCR to prepare cDNA before cloning as described above.

The sequence data were analyzed by the DNASIS-MAC Version 2.4 NEW CD3 Software System (Hitachi, Tokyo, Japan).

3.3. Results and discussion

The nucleotide sequences of four newly analyzed dengue type 2 strains from northeastern Thailand were compared with those of the reference strains. The results showed that all four strains possessed the highest homology to the New Guinea C strain (95.09–95.29%), followed by the Jamaica 1409 strain (93.13–93.37%), and the lowest homology to the S1 strain (90.44–90.65%). Nucleotide differences were scattered throughout the length of the sequenced region. Most of the changes were transitions and occurred at the third position of the codons, resulting in silent mutations. Among sequenced regions, PrM showed the highest divergence (6.59–7.32%) compared with the New Guinea C strain, while the C and M regions were most highly conserved.

By amino acid sequence homology analysis, the four newly sequenced strains again showed the highest homology to New Guinea C (97.24–97.78%), fol-

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Strain specific amino acid replacements among four dengue type 2 strains in their structural protein genes and a major nonstructural protein NS1 gene</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain code</th>
<th>Clinical severity</th>
<th>Genome region, amino acid number and substitutions</th>
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<tr>
<td></td>
<td>PrM</td>
<td>E</td>
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<tr>
<td></td>
<td>130</td>
<td>163</td>
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<td>ThNH7/93</td>
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<td>I*</td>
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<td>I</td>
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<td>ThNH5/293</td>
<td>DHF grade I</td>
<td>I</td>
</tr>
<tr>
<td>ThNHp11/93</td>
<td>DF</td>
<td>R</td>
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*Single letter amino acid code.
Fig. 2. Secondary structure predicted for the 3' noncoding regions of dengue type 2 virus strains isolated from different clinical manifestations in northeast Thailand in 1993.

followed by the Jamaica strain (96.80–97.24%), and the least homology to the S1 strain (96–96.53%). Amino acid differences were also scattered throughout the sequenced region. In comparison with the New Guinea C strain, the PrM protein showed the highest divergence (3.29–5.49%), while the M protein was completely conserved. Among the strain-specific amino acid replacements shown in Table 2, the change
from I to R at amino acid number 130 in PrM was specific to the DF strain and could significantly alter the nature of the PrM protein. On the other hand, the change from D to G at amino acid number 1053 in NS1 was specific to the DSS strain and could also significantly alter the nature of the NS1 protein.

We have also analyzed the 5' and 3' ends of these dengue type 2 strains. In contrast to the complete conservation of the 5' end sequence, there were DF strain specific nucleotide changes in the 3' end region. The secondary structure of the 3' end predicted for the DF strain was completely different from the structure predicted for DHF and DSS strains as shown in Fig. 2.

In summary, certain molecular differences were found among dengue type 2 strains isolated from mild and severe cases in the same epidemic area in the same season. These results should be confirmed by the analysis of additional strains from different epidemic areas and different seasons. Comparative sequence analysis on dengue type 2 strains isolated in central Thailand [16,17], Malaysia [18-23], and northeast Thailand [24] as reported by other groups and our group could not detect disease severity related molecular differences among strains isolated from patients with different clinical manifestations. However, when a cluster of these strains isolated in the same epidemic area in the same season were compared to strains isolated from the most severe case as the standard, it was concluded that as the sequences diverge, the clinical severity of the disease is lowered [14].

At this moment, we do not know the biological significance of these amino acid replacements and different secondary structures predicted for the 3' end region, which await further investigations. In the case of poliovirus, the nucleotide sequence difference in the 5' noncoding region is related to the temperature sensitivity [25] or attenuation [26] of the virus strains. Complete conservation of the 5' noncoding region in our cluster of dengue type 2 virus strains indicated that this region was not related to the disease severity of dengue virus infection. On the other hand, six nucleotide sequence differences in the 3' noncoding region were reported between virulent 1668 strain and its attenuated vaccine candidate strain PDK53 of dengue type 2 virus [27].

4. Field test on Olyset Net screen to control dengue vector mosquitoes in Vietnam

4.1. Background and study design

Since a dengue vaccine is still under development, vector control is the only practical measure to control a dengue epidemic. Unfortunately, dengue vector control did not provide the expected results although it has been routinely carried out in epidemic areas. Since 1985, the author has been to Vietnam as a short-term consultant of WHO-WPRO and postulated a field trial of dengue vector control, which, however, could not be implemented until recently.

In 1994, we carried out a field study on dengue vector control with the Olyset Net screen as a collaborative study between the National Institute of Hygiene and Epidemiology (NIHE), Vietnam, Sumitomo Chemical Co. Ltd., and the Institute of Tropical Medicine, Nagasaki University.

Olyset Net is a wide mesh net which is woven from polyethylene thread impregnated with permethrin, an insecticide of low toxicity [28]. The slow release of the permethrin to the surface of the net leads to a long-lasting effect. But its efficacy for the control of dengue vector mosquitoes had not been demonstrated until our field trial [29].

The idea came from a publication in Dengue Newsletter on the efficacy of permethrin-impregnated bamboo curtains to control dengue vector mosquitoes, in WHO-WPRO [30]. Bamboo curtains, however, have two disadvantages: short efficacy and poor ventilation/illumination. By consultation and discussion in NIHE, we reached an agreement to carry out a field trial of Olyset Net in Hai Hung Province, northern Vietnam in 1994.

4.2. Materials and methods

4.2.1. Olyset Net

Olyset Net screen was the product of Sumika Like-Tech. Co. Ltd., Osaka Japan, related to Sumitomo Chemical Co. Ltd., Osaka, Japan. A sufficient number of rolls were shipped to NIHE at the beginning of 1994, and its anti-Ae. aegypti effect was examined in the Insecticide Resistance Testing Laboratory, Department of Epidemiology, NIHE, by the
contact test. The practical applicability of Olyset Net to common households was examined by a small-scale test in Hanoi, followed by the preliminary application in three households in the study area in early April 1996.

Each household in this study area, Cam Dien village in Cam Binh District, possessed a huge outdoor cement container to collect rain water for use during the dry season from November to April, and this container was a major breeding site of vector mosquitoes. In late April 1994, before the epidemic season, Olyset Net was set up in 500 households in the study area: at the entrance, windows, and ventilators, thus covering all openings of the house to prevent entry of *Ae. aegypti* to the house, in addition to the routine anti-*Aedes* health education and control measures.

Another village, Kim Gian, of similar size and setting as the study area in the same district, about 10 km from the study area, was chosen as the control area, where routine anti-*Aedes* health education and control measures were carried out.

4.2.2. Vector surveillance and monitoring of dengue virus transmission

Vector surveillance was carried out by eight persons divided into four groups collecting adult and larval mosquitoes fortnightly in 30 houses each in the study and control areas, from 7:00 to 11:30 a.m.

Monitoring dengue virus transmission was carried out as follows. First, all possible dengue cases were reported to the Virology Department, NIHE, from April to December 1994. Secondly, blood specimens were collected from the cohort group of 78 healthy schoolchildren each in the study and control areas in April (before the epidemic season) and November (after the epidemic season), and their serum anti-dengue IgM antibodies were measured by ELISA [12,31].

Opinions of the local people on the use of Olyset Net screen were collected by written questionnaires in the study area 8 months after the placing of the net.

4.3. Results and discussion

The laboratory studies on the effect of Olyset Net using a local strain of *Ae. aegypti* showed that all adult mosquitoes dropped down after 9–12 min exposure to the nets which were hung inside houses or unused Olyset Net. This effect remained 100% after 8 months. When the Olyset Net was hung outdoors, its effect was reduced after 2 months, and the mortality of *Ae. aegypti* after 24 h exposure was decreased to 40% in 5 months, and 20% in 8 months. In the control test, the mortality of *Ae. aegypti* without exposure to Olyset Net was only 2.5–5%.

Fig. 3 shows the monthly changes in the *Ae. ae-
gypti adult density index (DI) in the study area and in the control area. In the study area, the Ae. aegypti DI was significantly reduced after setting up the net and remained at an undetectable level. In the control area, however, the Ae. aegypti DI gradually increased from 0.68 to 2.0 during the epidemic season from May to October. Similar results were observed for the larval index of Ae. aegypti (data not shown).

These results indicate that Olyset Net gave a positive control effect on the principal dengue vector mosquito, Ae. aegypti. In addition, the Olyset Net also showed a control effect on other genera of mosquitoes, such as Culex, Anopheles and Mansonia, as well as flies (Musca domestica) and cockroaches.

In the year 1994, no DF/DHF cases were reported from either the study area or the control area. The results of serological tests with IgM-ELISA in healthy schoolchildren are shown in Table 3. Before the epidemic season in April, the anti-dengue IgM positive rates in the study and control areas were 1/78 and 4/78, respectively, the difference not being statistically significant \((P \geq 0.1\) by \(\chi^2\) test). These positive cases probably represent a carry-over of dengue antibodies from the previous epidemic season in 1993, thus indicating that the magnitude of dengue transmission was comparable in both study and control areas. In contrast, the IgM-ELISA on sera collected in November 1994 after the epidemic season showed a definite increase in the number of positives in the control area (26/78 or 33%), and dengue virus was isolated from four out of seven blood specimens collected during the epidemic season (data not shown). In the study area the positive antibody rate remained at a low level (5/78 or 6.4%) even in November after the epidemic season. This difference in anti-dengue IgM positive rates between the study and control areas after the epidemic season was statistically significant \((P < 0.005\) by \(\chi^2\) test).

These results indicate that Olyset Net screen effectively prevented dengue virus transmission and also that silent dengue virus infection was present even in the absence of apparent DF/DHF cases in the control area.

Written questionnaires were collected from 467 of 500 households \((93.4\%)\) in which Olyset Net screen was set up, and the results are summarized below:

1. DHF patients were reported from 89% of the households in the year 1991.
2. Agreement to set up the Olyset Net was obtained from 100% of the householders.
3. Freedom from mosquito bites after setting up the Olyset Net was observed in 100% of the households.
4. Color preference for the Olyset Net: 100% of the householders preferred blue or white.
5. Likelihood to purchase a new Olyset Net when the present one deteriorates was obtained in 85% of the householders.
6. Obvious effect of the Olyset Net on the control of the dengue vector was observed in 100% of the households.
7. Evaluation of the Olyset Net screen: 100% of the householders agreed that Olyset Net is a simple, convenient and comfortable method of vector control. Its large mesh size allows better ventilation and illumination than other nettings.

Since community participation is essential for successful dengue vector control [32], the good evaluation of the Olyset Net by the local people, and its apparent control effect on the dengue vector to prevent dengue virus transmission indicated that it is a possible control measure against DF/DHF. The result in 1994 prompted us to carry out additional confirmatory tests in three areas in Vietnam with different environmental and social settings in 1995. The test areas were (1) a suburban area of Hai Phuong city, (2) an urban area of Nha Trang city

<table>
<thead>
<tr>
<th>Area</th>
<th>Number of specimens tested</th>
<th>Number of positive specimens (%)</th>
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<tr>
<td></td>
<td>April, before epidemic season</td>
<td>November, after epidemic season</td>
</tr>
<tr>
<td>Study area</td>
<td>78</td>
<td>1 (1.3)</td>
</tr>
<tr>
<td>Control area</td>
<td>78</td>
<td>4 (5.1)</td>
</tr>
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and (3) a rural area of Ho Chi Minh city. These studies showed that the *Ae. aegypti* density index was reduced in all test areas after setting up the Olyset Net compared with the control areas, at least for several months. However, prevention of dengue virus by the Olyset Net was not positively demonstrated because anti-dengue IgM antibodies did not show a significant seroconversion rate in the control areas. The possibility of using the Olyset Net as a control measure against DF/DHF should be confirmed by further tests.

References


in the 5'-noncoding region of the viral RNA. Virology 155, 498–507.


