MONITORING OF DENGUE VIRUSES IN FIELD-CAUGHT Aedes aegypti and Aedes albopictus Mosquitoes by a Type-Specific Polymerase Chain Reaction and Cycle Sequencing


Department of Microbiology, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore; Vector Control and Research Department, Ministry of the Environment, Singapore

Abstract. Virologic surveillance for dengue through the detection of the prevalent serotype(s) circulating in the human population during inter- and intra-epidemic periods constitutes a reliable sentinel system for dengue outbreaks. We have applied a rapid and sensitive, semi-nested, reverse transcription–polymerase chain reaction (RT-PCR) assay using nonstructural protein 3 gene primers for the type-specific-detection of dengue viruses in artificially infected and in field-caught adult Aedes mosquitoes. In laboratory experiments, the assay was sensitive enough to detect one virus-infected mosquito head in pools of up to 59 uninfected heads. In a prospective field study conducted from April 1995 to July 1996, female adult Ae. aegypti and Ae. albopictus mosquitoes were caught from selected dengue-sensitive areas in Singapore and assayed by RT-PCR. Approximately 20% of 309 mosquito pools were positive for dengue viruses. Of the 23 RT-PCR-positive Ae. aegypti pools (containing 1–17 mosquitoes each), 18 pools (78.3%) were positive for dengue 1 virus. The predominant virus type responsible for the current dengue epidemic since 1995 was also dengue 1. The geographic locations of the virus-infected mosquitoes correlated with the residences or workplaces of patients within dengue outbreak areas. A total of 43.5% of the positive Ae. aegypti pools and 25.0% of the positive Ae. albopictus pools contained only a single mosquito. Both Aedes species showed similar overall minimum infection rates of 57.6 and 50 per 1,000 mosquitoes. Infected Ae. aegypti were detected as early as six weeks before the start of the dengue outbreaks in 1995 and 1996. However, infected Ae. albopictus appeared later, when the number of cases was increasing. Virologic surveillance by RT-PCR for detecting dengue virus-infected Aedes mosquitoes in the field may serve as an early warning monitoring system for dengue outbreaks.

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are caused by the four serotypes of dengue virus, types 1 to 4, belonging to the Flavivirus family. In many tropical and subtropical areas of the world, these viruses are endemic and cause periodic or annual outbreaks of disease. Dengue viruses are transmitted principally by Aedes aegypti that breeds in stagnant water in all forms of receptacles in urban areas, especially following intermittent rainfall in these tropical regions. Dengue infection has expanded to other geographic areas such as the Americas due to changes in human behavior and travel. Dengue mosquitoes are widely distributed in tropical areas, plays an important but secondary role (Committee on Epidemiic Diseases, Ministries of the Environment and Health, Singapore, unpublished data). Dengue hemorrhagic fever was first reported in Singapore in 1960, and in recent years, increasingly larger outbreaks have occurred. There were 1,239, 2,008, and 3,128 cases of DF/DHF reported in 1994, 1995, and 1996, respectively. The high incidence of DF/DHF persisted in spite of a relatively low Aedes mosquito population as indicated by an overall Aedes house index of 1–2% for the entire country. The low Aedes population density was the result of an effective Aedes surveillance and control program implemented in the early 1970s. Dengue outbreaks were partly attributed to localized increases of Aedes mosquitoes in outbreak areas.

The prevention and control of dengue outbreaks depend upon the surveillance of cases and mosquito vectors. Vector surveillance allows timely implementation of emergency mosquito control measures such as insecticidal fogging of adults and destruction of breeding places to limit an impending outbreak from spreading. In Singapore, where the Aedes population has been reduced to a very low level, the monitoring of population densities of mosquito adults and larvae has not been a sensitive indicator to serve as an early warning surveillance system for an impending dengue outbreak because it has failed to detect pockets of high Aedes populations.

Virologic surveillance, which involves the monitoring of dengue virus infection in humans, has been used as an early warning system to predict outbreaks. Such surveillance is based on isolation of dengue virus from human serum by cell culture or mosquito inoculation and type-specific identification by immunofluorescence.

We have developed a rapid, sensitive, and specific semi-nested, reverse transcription–polymerase chain reaction (RT-PCR) assay for the amplification and typing of dengue virus serotypes in patient serum. The RT-PCR assay has been previously shown to detect virus types in serum specimens from which dengue viruses had been isolated by the gold standard test of virus isolation in C6/36 cells followed by identification with monoclonal antibodies.

This RT-PCR method was applied in the detection of type-specific dengue viral RNA in artificially infected and field-
caught *Aedes* mosquitoes. In this report, we describe the results of these experiments, as well as a prospective field study of dengue virus infections in *Ae. aegypti* and *Ae. albopictus* mosquitoes from April 1995 to July 1996.

**MATERIALS AND METHODS**

**Semi-nested RT-PCR.** This assay uses six oligonucleotide primers within the nonstructural protein 3 (NS3) genes of dengue viruses. Two consensus primers common to several flaviviruses including dengue viruses were used in the RT step and for the first round of the PCR. In the second round of the PCR, the upstream consensus primer DV1 was used together with four internal type-specific primers (DSP1 to DSP4). Semi-nested RT-PCR products of diagnostic sizes of 169, 362, 265, and 426 basepairs (bp) were thus generated for the four dengue virus types 1, 2, 3, and 4, respectively. The specific dengue type(s) could be readily identified according to the size of the RT-PCR product(s) determined by agarose gel electrophoresis and staining with ethidium bromide.

**Field-collected *Aedes* mosquito larvae.** *Aedes* larvae were collected from 16 sites of known dengue transmission. A total of 53 pools (in water) each containing from one to 10 larvae of the third or fourth instar were obtained. RNAs were extracted from whole larvae by a modified silica-based method and assayed by semi-nested RT-PCR.

**Experimental infection of adult *Aedes* mosquitoes.** Three to four-day-old male *Ae. aegypti* mosquitoes were individually infected by intrathoracic inoculation with dengue virus. Using the silica-based method, total RNAs were extracted from excised mosquito heads and assayed for dengue viral RNA by semi-nested RT-PCR.

**Prospective field study of adult *Aedes* mosquitoes.** From April 1995 to July 1996, adult *Aedes* mosquitoes were caught at weekly intervals from five indoor *Ae. aegypti* and six outdoor *Ae. albopictus* monitoring stations. These stations were located in dengue-sensitive areas that were selected on the basis of a history of high disease incidence, *Aedes* density, and human population density, as well as building activities. Additional indoor *Aedes* mosquitoes were caught during the 1995 and 1996 DF/DHF outbreaks from areas where cases occurred.

For each indoor station, *Aedes* mosquitoes were caught from 10 premises at each visit between 9:00 AM and 11:00 AM by one vector control officer by suction using a battery-operated, back-packed aspirator. It took 10–15 min to catch the mosquitoes in each premise. For each outdoor station, *Aedes* mosquitoes were caught by one officer using himself as bait between 9:00 AM and 10:00 AM.

Dead mosquitoes were discarded, and live ones were identified, pooled according to species, stored at -20°C until tested. The excised heads of each mosquito pool were homogenized in a sterile glass homogenizer and RNAs were extracted by the silica-based technique. A single-step RT-PCR was performed using consensus primers, and the products were electrophoresed in alternate lanes in agarose gels. Gel slices corresponding to the 470-bp target size were excised and DNAs were eluted by freezing, thawing, and centrifugation in Costar (Cambridge, MA) tubes. The DNA eluates were subjected to the semi-nested PCR with upstream consensus primer and the four dengue type-specific primers. Semi-nested PCR product bands of sizes corresponding to one or more of the type-specific target fragments were excised and the DNA was eluted. The eluted DNAs were subjected to a further PCR with four separate primer pairs, i.e., consensus primer DV1, with each of the four type-specific primers to confirm unequivocally the dengue virus type. Every batch of assays of mosquitoes was accompanied by a no DNA negative control sample, and stringent precautions were taken to avoid carryover contamination. The first 20 dengue 1-positive and five dengue 2-positive PCR products were subjected to cycle sequencing using 32P-labeled primers and autoradiographed.

**Minimum infection rate (MIR).** The MIR was used to compare virus infection rates in *Ae. aegypti* and *Ae. albopictus* at the monitoring stations. The MIR was calculated as (number of viruses detected in mosquitoes by species ÷ total number of that species tested) × 1,000.

**RESULTS**

**Experimental *Aedes* mosquito larvae.** To establish the sensitivity of the RT-PCR technique when applied to mosquito larvae, we spiked RNA extracted from whole *Ae. aegypti* larvae with varying amounts of purified dengue 3 viral RNA. The RT-PCR detected at least 0.01 ng of viral RNA. No positive RT-PCR bands were observed for uninfected larvae. These findings indicate that the RT-PCR assay could successfully detect dengue virus against a background of *Aedes* larval RNA.

**Field-collected *Aedes* larvae.** We initially attempted to detect dengue virus in larvae by RT-PCR because it was easier to collect larvae from the field compared with adult mosquitoes. None of the 53 larval pools was positive for dengue virus by RT-PCR, and only nonspecific amplified products were observed, implying that field mosquito larvae are less likely to harbor the virus through parental (vertical) transmission. A minimum field infection rate of *Ae. aegypti* larvae of 1:2,067 has been reported, suggesting the relatively infrequent occurrence of transovarial transmission of dengue virus in nature.

**Type-specific identification of dengue viruses in artificially infected adult *Ae. aegypti* mosquitoes.** Excised heads of artificially infected adult *Ae. aegypti* mosquitoes were mixed with heads of un inoculated mosquitoes, pooled, and assayed by RT-PCR. The RT-PCR products of diagnostic sizes were amplified from mosquitoes infected individually with each of the four dengue virus types, but not from an un inoculated mosquito control, or from C6/36 cells infected with Japanese encephalitis, Kunjin, or yellow fever viruses. This technique could detect a single virus-infected mosquito head in pools containing from nine, 19, 29, 39, and 49–59 uninfected mosquito heads. Furthermore, dengue virus was detectable in infected mosquitoes from days four to 42 postinoculation. These data underscore the ability of this technique to detect specific dengue virus types against a background of *Aedes* mosquito DNA, even though the experimentally-inoculated mosquitoes do not exactly mimic natural infections in the field.

**Prospective study of dengue virus infections in field-caught *Aedes* mosquitoes by RT-PCR.** During the 16-
TABLE 1

Reverse transcription–polymerase chain reaction–positive Aedes mosquito pools and dengue (DEN) virus serotypes detected

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of pools assayed</th>
<th>Total no. of mosquitoes</th>
<th>No. (%) of positive pools</th>
<th>DEN1</th>
<th>DEN2</th>
<th>DEN3</th>
<th>DEN4</th>
<th>Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ae. aegypti</td>
<td>110</td>
<td>409</td>
<td>23 (20.9%)</td>
<td>18</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2 (DEN1 + DEN2)</td>
</tr>
<tr>
<td>Ae. albopictus</td>
<td>199</td>
<td>784</td>
<td>40 (20.1%)</td>
<td>31</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>3 (DEN1 + DEN2; DEN1 + DEN4; DEN1 + DEN4)</td>
</tr>
</tbody>
</table>

TABLE 2

Number of mosquitoes in positive pools and dengue virus serotypes detected

<table>
<thead>
<tr>
<th>No. of mosquitoes in pool</th>
<th>No. of positive pools and dengue virus serotypes detected</th>
<th>Total no. (%) of positive pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ae. aegypti</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9 1 0 0 0</td>
<td>10 (43.5%)</td>
</tr>
<tr>
<td>2</td>
<td>2 0 0 0 0</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>1 1 0 0 0</td>
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<tr>
<td>4</td>
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</tr>
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<td>8</td>
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<td>1</td>
</tr>
<tr>
<td>17</td>
<td>0 0 0 0 1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>18 2 1 0 2</td>
<td>23</td>
</tr>
<tr>
<td>Ae. albopictus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8 1 0 1 0</td>
<td>10 (25.0%)</td>
</tr>
<tr>
<td>2</td>
<td>4 2 0 0 1</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
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<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>31 5 0 1 3</td>
<td>40</td>
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monitoring stations and dengue outbreak areas were expressed as MIRs and compared (Table 3). Aedes aegypti caught at the five indoor stations had MIRs ranging from 27.5 to 222.2 per 1,000 mosquitoes, with an MIR of 57.6 for the combined stations. The MIR for mosquitoes caught in outbreak areas was 50.6. The MIRs for Aedes albopictus showed a range from 17.3 to 114.3 for mosquitoes caught in the six stations, and an MIR of 50.0 for the combined stations.

**Aedes mosquito population density and infection in dengue outbreaks.** The prospective study began in week 14 of 1995 and ended in week 27 of 1996. In Figure 2, mosquito population densities of Ae. aegypti and Ae. albopictus are shown by the overall house index (HI) for the whole country and by the total number of female adults caught weekly from three stations for each species. These stations were selected based on their close proximity to the dengue outbreak areas. Except for weeks 40–47 in 1995 and weeks 6–8 in 1996, weekly catches of the two Aedes species were assayed by RT-PCR for dengue virus infection. The MIRs were used for comparing infection rates for the two Aedes species caught each week. The study period spanned two DF/DHF outbreaks, i.e., the 1995 outbreak that began in week 20, and the beginning of the 1996 outbreak from week 24 (Figure 2).

The disease incidence of the 1995 outbreak correlated significantly with the HIs for both Ae. aegypti and Ae. albopictus. The weekly Ae. aegypti HIs fluctuated below 1.0% throughout 1995, increasing from 0.31% in week 15 and reaching a peak of 0.49% in week 19 before the outbreak began. A second HI peak of 0.71% was observed in week 34, before the peak in dengue cases in week 39. The numbers of female Ae. aegypti caught each week in the monitoring stations were small, and fluctuated between 0 and 11 mosquitoes with multiple peaks throughout 1995.

Dengue virus–infected Ae. aegypti mosquitoes were already detected by RT-PCR at the start of the study in week 14 of 1995, which was six weeks before the increased number of cases was apparent, and again in weeks 20, 24, and 25 after the outbreak began. No infected Ae. aegypti were detected in subsequent weeks 26–39 when the 1995 outbreak was reaching its peak. No results were available from weeks 40–47. Infected Ae. aegypti mosquitoes were again detected at the end of the 1995 outbreak in week 51, and in week 4 of 1996.

The 1996 outbreak was marked by a sharp increase in the Ae. aegypti HI from 0.58% in week 14 to a peak of 0.98% in week 19, five weeks before the outbreak began in week 24. There was a concomitant increase in the number of female mosquitoes that fluctuated widely from one to 24 in the weekly catches. Infected mosquitoes were detected before the outbreak began, in weeks 18, 20, and 21, and again in week 26.

In the case of Ae. albopictus, the HI and number of female mosquitoes were already increasing when the study began, reaching 0.94% and 10, respectively, in week 18 before the 1995 outbreak. The fluctuations of the HIs and the numbers of female mosquitoes followed closely those of Ae. aegypti. During the 1995 outbreak, the Ae. albopictus HIs fluctuated from 0.56% to 1.06% and the numbers caught weekly fluctuated from 0 to 13 mosquitoes. Infected Ae. albopictus female mosquitoes were detected in weeks 16 and 18 before the 1995 outbreak began, and again in weeks 21, 24, 28, 36, 37, and 50 of 1995, and weeks one and 10 of 1996. From weeks 11 to 23 before the 1996 outbreak began in week 24, no infected Ae. albopictus mosquitoes were detected. Infected mosquitoes appeared again in weeks 26 and 27 after the 1996 outbreak began.

The geographic locations of the dengue virus–infected mosquitoes coincided with the residential or workplace addresses of 46 local patients with dengue confirmed by virus isolation during the same overlapping period. This geographic correlation was concentrated chiefly within the major dengue outbreak areas, i.e., the eastern and southeastern sectors of Singapore’s main island (Figure 3). From the 46 patients, dengue 1 virus was isolated from 20 patients (43.5%), dengue 2 virus from six patients (13.0%), dengue 3 from 17 patients (37.0%), and dengue 4 from three patients (6.5%). The predominant dengue virus type identified in both mosquitoes and patients was dengue 1.

**Nucleotide sequencing of dengue viruses in field-caught adult Aedes mosquitoes.** Cycle sequencing of 20 dengue 1 type-specific PCR products each yielded 123 readable NS3 base ladders from nucleotides 4920 to 5042 encoding 123 amino acid residues from positions 1614 to 1654. Within this nucleotide stretch, one specimen shared identity with the prototype dengue 1 virus (Hawaii strain). Compared with the latter, phenotypically silent mutations were observed at nucleotide 5012 (A to C) in another specimen, at nucleotide 4997 (A to C) in five specimens, and at nucleotides 4997 (A to C) as well as 5000 (T to C) in 13 specimens (Figure 4a).

Cycle sequencing of five dengue 2 virus type-specific PCR products each generated 315 readable base ladders from nucleotides 4939 to 5253 encoding 105 amino acids from residues 1615 to 1719. In comparison with the nucleotide sequence of the prototype New Guinea C strain of
dengue 2 virus, all five specimens demonstrated mutations at nucleotides 4965, 4974, 4983, 4986, 5013, 5034, 5067, 5133, 5154 (T to C); 5055, 5167, 5199, and 5223 (C to T); 4999, 5078, 5136, 5151, 5163, 5172, and 5202 (G to A); 4939, 5081, and 5205 (A to G); 5139 (C to A); and 5226 (A to T). The mutations at nucleotides 4939, 4999, 5078, and 5081 were predicted to lead to substitutions of amino acid residues, i.e., isoleucine to valine (1615), alanine to threonine (1635), arginine to lysine (1661), and lysine to arginine (1662), respectively. In addition to the above mutations, one specimen exhibited transitions at nucleotides 5029 (A to G) and 5138 (C to T), culminating in lysine to glutamic acid and alanine to valine substitutions at residues 1645 and 1681, respectively. Another harbored additional transitions at nucleotides 4992 (G to A), 5100 (C to T), and 5069 (T to C), with the latter resulting in a nonconservative phenylalanine to serine replacement at codon 1658 (Figure 4b).

These sequence data verified the authenticity of the dengue RT-PCR products, illustrated the heterogeneity, albeit limited, of the NS3 nucleotide sequences, and excluded the possibility of carryover contamination. In addition, five and three of the sequences of dengue 1 and dengue 2 PCR products were identical with those of dengue 1 virus strain D1S8393 and dengue 2 virus strain D2SINGR8 isolated from Singapore patients in April 1995 and in 1993–1994, respectively, thereby reaffirming the correlation between dengue virus strains in mosquito vectors and in human hosts.

DISCUSSION

In the absence of a safe and effective vaccine for mass immunization, the prevention and control of DF/DHF outbreaks are dependent upon the control of the vector mosquitoes *Ae. aegypti* and *Ae. albopictus*. In Singapore, a comprehensive mosquito control program incorporates source reduction, public health education, community participation, and law enforcement against mosquito breeding. The *Aedes* control strategy has focused mainly on surveillance for and elimination of *Aedes* larval breeding habitats and emergency control of adults during outbreaks. Although this strategy has successfully reduced the *Aedes* mosquito population to a relatively low level, as indicated by the overall HI, it has not prevented the emergence of progressively larger outbreaks in recent years.

Dengue fever/dengue hemorrhagic fever is highly endemic in Singapore, with more than one dengue virus serotype cocirculating since 1960. Outbreaks occur when a new dengue virus serotype is introduced into the human population and there are localized increases in the *Aedes* mosquito population. Virologic surveillance based on the isolation and identification of dengue viruses infecting the human popu-
Figure 4. Cycle sequencing of representative dengue virus nonstructural protein 3 polymerase chain reaction products. a, sequencing gel of a sample showing nucleotides 4992-5009 of dengue virus type 1, with the asterisks denoting silent mutations in comparison with the published sequence. b, sequence ladders corresponding to nucleotides 5065-5082 of dengue virus type 2 from another sample, illustrating an A to C mutation (*) resulting in a phenylalanine to serine substitution at codon 1658.

lation provides an important means of early detection of any change in the prevalence of dengue virus serotype(s). The reappearance of serotype(s) that have not been widely circulating in human cases in the preceding years may signal an impending outbreak triggered by new emergent dengue serotype(s). The monitoring of the dengue virus type(s) infecting Aedes mosquitoes during inter- and intra-epidemic periods will complement the current virologic surveillance for dengue outbreaks.

In the prospective field study, dengue virus–infected female Ae. aegypti and Ae. albopictus were detected in all the monitoring stations, reflecting the wide distribution of dengue viruses. There was also a wide variation in mosquito infection rates for both Aedes species in the different stations. However, overall MIRs for Ae. aegypti and Ae. albopictus calculated from the combined stations for the respective species were similar (Table 3). Overall MIRs for Ae. aegypti and Ae. albopictus were also very close to the MIR for Ae. aegypti caught from outbreak areas. These findings suggest that both Aedes species were involved in virus transmission in dengue outbreaks.

Compared with 30 years ago, dengue virus infection rates for Ae. aegypti and Ae. albopictus have increased almost 100-fold. In 1966, the mean MIRs for Ae. aegypti and Ae. albopictus were 0.51 and 0.59, respectively. During a dengue outbreak in 1960, infection rates of 18.6 and 0.8 were reported for Ae. aegypti and Ae. albopictus, respectively. Many factors could have contributed to the disparities in MIRs, e.g., the use of virus isolation in infant mice (known to be an insensitive method for the recovery of dengue virus) in the 1960s, and the sizes of the mosquito pools used in the different studies. In the 1966 study, pools of 25–45 mosquitoes were used for virus isolation, whereas the present study used smaller pools of mosquitoes for virus detection by the more sensitive RT-PCR method. More than 90% of the RT-PCR-positive pools of each Aedes species contained less than 10 mosquitoes each, and about 40% of the Ae. aegypti and 25% of the Ae. albopictus pools contained only a single mosquito (Table 2). Since the sizes of the mosquito pools used were comparable, the MIRs for Ae. aegypti and Ae. albopictus probably represent the true infection rates for both species. The higher infection rates for the two Aedes species in the present study are to be expected since dengue viruses are now highly endemic in the human population.

In the Singapore outbreaks of 1990 and 1991, it was observed that the increase in Ae. aegypti density as measured by the HI preceded the increase in dengue cases by 2–6 weeks (Committee on Epidemic Diseases, Ministries of the Environment and Health, Singapore, unpublished data). The numbers of infected Ae. aegypti and Ae. albopictus are expected to increase with the increase in mosquito population. In the 1995 dengue outbreak, infected Ae. aegypti mosqui-
toes were already detected by RT-PCR when the study began in week 14, i.e., six weeks before the outbreak began. In the 1996 outbreak, infected *Ae. aegypti* mosquitoes were first detected in week 18, i.e., also six weeks prior to the outbreak, but not earlier (from weeks 9–17). These findings are consistent with the HI data. Infection in *Ae. albopictus* was detected later in week 16, i.e., four weeks before the outbreak. In the 1996 outbreak, infected *Ae. albopictus* mosquitoes were not detected throughout the 14 weeks prior to the outbreak, but appeared only after the outbreak. Furthermore, MIRs for *Ae. aegypti* in the weeks prior to and at the beginning of the outbreak were generally higher (ranging from 333.3 to 1,000) than those for *Ae. albopictus* (ranging from 125 to 200), suggesting that more *Ae. aegypti* than *Ae. albopictus* were infected during this period.

Infection of *Ae. albopictus* appeared to persist for a longer period than infection of *Ae. aegypti* in the early phase of the outbreak. Following the initiation of the 1995 outbreak, infected *Ae. aegypti* could not be detected in the subsequent 14 weeks when the number of dengue cases was increasing and the *Ae. aegypti* population (as reflected by HI) was increasing. In contrast, *Ae. albopictus* infection continued for an additional 17 weeks until week 37, i.e., two weeks before the peak of the outbreak was reached. From these preliminary observations, it is tempting to speculate that *Ae. aegypti* played a major role in the initiation of the outbreak, while *Ae. albopictus* was largely responsible for the increase in the number of cases after the outbreak had started.

In the 1995 dengue outbreak, dengue type 1 was identified as the predominant serotype infecting both *Ae. aegypti* and *Ae. albopictus* mosquitoes (Table 1). Virus isolations from 46 DF/DHF patients during the study period showed that dengue 1 was the prevalent serotype, followed by cocirculating dengue 3. In 1994, the two most prevalent circulating serotypes were dengue 3 (44.4 %) and dengue 2 (35.6 %) based on the virus serotypes isolated from patients. Lam\(^8\) has observed that in Malaysia, the predominant virus serotype usually persisted for at least two years before it was replaced by another serotype. In Singapore, dengue 3 first appeared as the most prevalent serotype in 1994, and remained as one of the most prevalent serotypes in 1995, until it was replaced by dengue 1 in 1995 and 1996, as shown by virus isolations from patients and RT-PCR assays of *Ae. aegypti* and *Ae. albopictus* mosquitoes.

Other researchers have used molecular techniques for dengue virus detection in artificially infected or field-caught *Aedes* mosquitoes.\(^{14,30–32}\) In the present study, we have demonstrated that the NS3 gene-based RT-PCR assay coupled with cycle sequencing could successfully type and sequence dengue viruses in field-caught adult *Aedes* mosquitoes. These techniques constitute practical molecular diagnostic and epidemiologic tools for the virologic surveillance of dengue virus–infected *Aedes* mosquitoes to serve as an early warning system for dengue outbreaks.

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Authors’ addresses: Vincent T. K. Chow, Y. C. Chan, and Rita Yong, Department of Microbiology, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore 119260. K. M. Lee, L. K. Lim, Y. K. Chung, S. G. Lam-Phua, and B. T. Tan, Vector Control and Research Department, Ministry of the Environment, Singapore 228321.

REFERENCES


20. Irie K, Mohan PM, Sasaguri Y, Putnak R, Padmanabhan R,


