COMMON OCCURRENCE OF CONCURRENT INFECTIONS BY MULTIPLE DENGUE VIRUS SEROTYPES

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Abstract. The co-circulation of all 4 dengue virus serotypes in the same community, common since the 1950s in Southeast Asia, has now become a frequent occurrence in many Caribbean Islands, Mexico, and Central and South America in the past 20 years. As a consequence, the frequency of concurrent infections would be expected to increase in these areas. To assess this, using state of the art technology, we screened viremic serum samples and mosquitoes inoculated with serum samples collected during epidemics involving multiple dengue virus serotypes in Indonesia, Mexico, and Puerto Rico for virus isolation. Of 292 samples tested, 16 (5.5%) were found to contain 2 or more dengue viruses by an indirect immunofluorescence test and/or the reverse transcriptase-polymerase chain reaction.

The global epidemiology of dengue/dengue hemorrhagic fever (DF/DHF) has changed dramatically in the past 50 years, first in Southeast Asia during and following World War II, and in the past 20 years in other tropical regions of the world, especially in the Americas.¹² The factors responsible for this change have involved demographic and societal changes that have resulted in the expanding geographic distribution of both the mosquito vectors and the 4 dengue virus serotypes, resulting in the co-circulation of multiple serotypes in a city or country (hyperendemicity), the single most common factor associated with the emergence of severe and fatal dengue in a country.¹ This change has been followed most closely in the American region where prior to the 1980s, countries had only one (hypoendemic) or no (nonendemic) viruses present at any given time. During the 1980s and 1990s, many countries in the Americas became hyperendemic (Table 1), followed in most, by the emergence of DHF.¹² During 1996, 11 countries in the region reported the circulation of multiple virus serotypes: 2 countries reported the presence of 4 serotypes, 3 reported the presence of 3 serotypes and 6 reported the presence of 2 serotypes (Table 1).³ It should be noted, however, that not all countries have an adequate laboratory-based virologic surveillance system. Therefore, Table 1 is incomplete and likely represents an underestimation of hyperendemicity in the region.

During epidemics in which multiple dengue virus serotypes are being transmitted, patients with concurrent serotype infections undoubtedly occur; however, such reports are few. The use of serotype-specific monoclonal antibodies and the reverse transcriptase–polymerase chain reaction (RT-PCR) for virus identification during routine virologic surveillance allowed detection of concurrent infections for the first time.⁴⁻⁶ The first case of a dual infection with 2 dengue virus serotypes was reported in Puerto Rico in 1982; dengue 1 (DEN-1) and DEN-4 circulated in the population during that time, and both virus serotypes were isolated from a single patient with dengue fever.⁷ In New Caledonia, DEN-1 and DEN-3 viruses were isolated from 6 patients with DF in 1989.⁸ In Thailand, DEN-1 and DEN-2 were identified by RT-PCR in the serum samples of 2 patients with DHF in 1990.⁹ Eight cases with dual infections of DEN-2 and DEN-4 viruses were identified by RT-PCR in the serum samples of DF patients during epidemics in China from 1991 to 1995.¹⁰ In serum obtained from patients in Somalia during 1993, DEN-2 and DEN-3 were isolated from one of 13 cases that were virologically confirmed.¹¹

In areas where multiple dengue serotypes are transmitted concurrently, clinical cases caused by more than 1 serotype of dengue virus may be more common than previously thought. The high attack rates of cases that occur during epidemics would likely result in many infections with multiple virus serotypes in humans (both clinical and sub-clinical), and also provide opportunities for mosquitoes to become infected with two or more serotypes.⁷¹²

It has been suggested that concurrent infection by multiple dengue serotypes might influence clinical expression, and this was initially considered as 1 explanation for the emergence of DHF.¹¹ The changing global epidemiology of dengue has resulted in increased frequency of epidemic dengue and the emergence of DHF in the American and Pacific tropical areas coincidentally with the occurrence of hyperendemicity in these regions.¹² Modern diagnostic technology provides more powerful tools to diagnose concurrent infections of dengue viruses, and it is now possible for the first time to determine the frequency of multiple infection with two or more dengue serotypes, and whether concurrent infection with 2 or more serotypes is associated with more severe disease. The purpose of this study was to determine the frequency of concurrent infection in epidemics where the co-circulation of multiple virus serotypes has been documented.

MATERIALS AND METHODS

Specimens. A total of 292 specimens were analyzed in this study. Ninety-nine specimens were human serum samples or viruses isolated in mosquitoes inoculated with serum from patients collected during epidemics in Indonesia from 1975 throughout 1978.¹⁴,¹⁵ At that time, the serotype of the viruses was identified by the complement fixation test using viral antigens obtained from inoculated mosquitoes.¹⁴–¹⁶ One hundred nineteen isolates were from serum samples obtained during epidemics that occurred in Mexico between 1983 to
with 100 U/ml of penicillin, 100 mg/ml of fungizone, and 50 µg/ml of gentamicin sulfate. Suspensions were centrifuged for 1 min at 8,000 rpm, filtered in 0.45-µm pore filters, and stored at −70°C until use.

Dengue viruses from Mexico in 1995 and 1996 were isolated using the C6/36 cells at CDC in Fort Collins. Confluent monolayers of the C6/36 cells were grown in a 25-cm² flask at 28°C without CO₂ using Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated FBS and supplemented with 0.01 mM of non-essential amino acids, 0.01 mM of sodium pyruvate, 0.9 mg of sodium bicarbonate, 100 U of penicillin, 100 µg of streptomycin, and 1 µg of fungizone per milliliter as the growth medium.

To infect the C6/36 cells, the growth medium was replaced with 1 ml of maintenance medium (DMEM containing 2% FBS instead of 10%). Thirty microliters of each specimen, serum, or mosquito triturate were added to the 25-cm² flask and incubated at 28°C with gentle rotation every 15 min. After an adsorption period of 1 hr, 7 ml of maintenance medium were added to each flask. The flasks were incubated at 28°C for 15 days. At day 7 postinfection, the medium was replaced with fresh medium. The infected cells were harvested on days 10 and 15 postinfection, pelleted by centrifugation, and then resuspended in phosphate-buffered saline (PBS), pH 7.2. Twelve-well spot slides were prepared using 15 µl of cell suspension per spot. The slides were air-dried, fixed in cold acetone (−20°C) for 15 min, and stained immediately or stored at −70°C until use.

**Table I**

Reports, by country, of multiple dengue serotypes circulating the same year in the American region

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of countries</th>
<th>Country (serotypes)*</th>
<th>Dengue serotypes in region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td>5</td>
<td>Colombia (1, 2), Jamaica (2, 4), Puerto Rico (1, 4)</td>
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<td>1982</td>
<td>9</td>
<td>Barbados (1, 4), Brazil (1, 4), Colombia (1, 2, 4), Jamaica (2, 4), Mexico (1, 2), Puerto Rico (1, 4), Suriname (1, 4)</td>
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<tr>
<td>1983</td>
<td>4</td>
<td>Colombia (1, 2, 4), Jamaica (2, 4), Mexico (1, 2, 4), Trinidad and Tobago (1, 2, 4), US Virgin Islands (1, 4)</td>
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<tr>
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<td>8</td>
<td>Colombia (1, 4), Dominican Republic (1, 4), Haiti (1, 2), Honduras (1, 2), Mexico (1, 2, 4), Puerto Rico (1, 2), Trinidad and Tobago (1, 4), Venezuela (1, 2)</td>
<td>1, 2, 4</td>
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<tr>
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<td>7</td>
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<td>1, 2, 4</td>
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<tr>
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<td>6</td>
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<td>1, 2, 4</td>
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<tr>
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<td>8</td>
<td>Colombia (1, 2, 4), Dominican Republic (1, 2, 4), El Salvador (1, 2, 4), Honduras (1, 4), Mexico (1, 4), Puerto Rico (1, 2, 4, 5, 7, 9)</td>
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<td>6</td>
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<td>9</td>
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<td>1, 2, 4</td>
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<tr>
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<td>11</td>
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<td>1992</td>
<td>12</td>
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<td>12</td>
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<td>14</td>
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<td>11</td>
<td>Brazil (1, 2), Costa Rica (1, 3, 4), Ecuador (1, 2, 4), El Salvador (1, 2, 3, 4), Guatemala (1, 2, 3, 4), Honduras (1, 2, 3, 4), Mexico (1, 2, 3, 4), Nicaragua (1, 2, 3), Peru (1, 2), Puerto Rico (1, 2, 4), Venezuela (1, 2, 4)</td>
<td>1, 2, 3, 4</td>
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<tr>
<td>1996</td>
<td>11</td>
<td>Brazil (1, 2), Colombia (1, 2, 4), Costa Rica (1, 3, 4), Ecuador (1, 2, 4), French Guiana (1, 2), Guatemala (1, 2, 3, 4), Mexico (1, 2, 3, 4), Peru (1, 2), Puerto Rico (1, 2, 4), Trinidad and Tobago (1, 2), Venezuela (1, 2, 4)</td>
<td>1, 2, 3, 4</td>
</tr>
</tbody>
</table>

* Data from Pan American Health Organization. 15,16

1996 (Loroño-Pino MA, unpublished data). Seventy-four isolations from serum samples from Puerto Rico were collected during an epidemic occurring in 1994 (Centers for Disease Control and Prevention [CDC], unpublished data).

**Virus isolation and identification.** The methods for dengue virus isolation and identification in Indonesia and Puerto Rico have been previously described. 5,14,15 Briefly, serum from suspected DHF patients in Indonesia was inoculated into Aedes aegypti mosquitoes that were incubated for 14 days at 30°C. 15 Ten to 20 individual mosquitoes inoculated with a specific serum sample were tested for dengue virus infection by direct fluorescent antibody assay (DFA) on mosquito head squashes. 15 The viruses were identified by complement fixation test using antigen prepared from infected mosquitoes. 15,16

The serum samples from Puerto Rico and the 1983 Mexico isolate were inoculated into both mosquitoes and into monolayer cultures of the C6/36 clone of Ae. albopictus cells grown in 16 × 125 mm screw cap tubes. 5,10 The virus isolates from Indonesia, Puerto Rico, and the 1983 Mexico isolate were then reanalyzed at CDC in Fort Collins, Colorado.

Mosquitoes infected by intrathoracic inoculation with viruses from Indonesia had been stored since the 1970s at −70°C. 15,17 One or two infected mosquitoes of each sample were triturated in 1.0 ml of medium M-199 containing 5% of heat-inactivated fetal bovine serum (FBS) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin, 1 µg/ml of fungizone, and 50 µg/ml of gentamicin sulfate.
Immunofluorescence (IFA) tests. Cells inoculated with serum were screened for infection by DFA using a conjugate prepared from high-titered, pooled human serum samples. The IFA test using serotype-specific monoclonal antibodies was used to identify the virus serotype. Slides were incubated in a humidified chamber at 37°C for 30 min. The monoclonal antibodies used in the present study were D2-1F1-3 (anti-dengue 1), 3H5-1-21 (anti-dengue 2), D6-8A1-12 (anti-dengue 3), 1H10-6-7 (anti-dengue 4), and 4G2 (group specific for flaviviruses detection). Slides were washed twice in PBS, pH 7.2, and then incubated with fluorescent-labeled goat anti-mouse IgG (heavy + light chain) at 37°C for 30 min. Slides were rinsed twice in PBS and mounted in glycerol-saline-DABCO (1.4 Diazobicyclo (2,2,2) Octane, # D-2522; Sigma, St. Louis, MO).

Extraction of RNA. Total cellular and viral RNA were extracted from the infected cells by using the QIAamp viral RNA kit (QIAGEN, Santa Clara, CA) following the instructions of the manufacturer. Briefly, 280 μl of cell culture suspension was lysed with 1,120 μl of lysis buffer (AVL) by incubation at room temperature for 10 min. The suspension was mixed with 1,120 μl of ethanol (96–100%), placed in a QIAamp spin column, and centrifuged at 6,000 × g for 1 min. The column was washed twice with 500 μl of wash buffer (AW) and centrifuged at 6,000 × g for 1 min followed by a centrifugation at 20,000 × g for 3 min. The RNA was eluted from the column with 50 μl of preheated RNAse-free water (80°C) by centrifugation at 6,000 × g for 1 min.

Synthesis of cDNA. The cDNA was reverse transcribed with the use of a SuperScript II RNase H Reverse Transcriptase kit (Life Technologies [Gibco], Grand Island, NY). Briefly, for each reaction, RNA and 50–100 pM of the dengue serotype-specific reverse primers were denatured at 70°C for 10 min and placed on ice before addition of 500 μM of each deoxynucleoside triphosphate (dNTP) and 200 units of SuperScript II in 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiotreitol. The final reaction volume was 20 μl. The samples were incubated for 50 min at 42°C before heating the reaction for 15 min at 70°C to inactivate the enzyme.

Primers for the RT-PCR. The primers and the nucleotide sequences used in this study were those published by Lanciotti and others: the forward primer D1, 5′-TCAATATGCTGAAGAGTGGAGAAACCG-3′, was common for the 4 dengue virus serotypes. The reverse primers, which were specific for dengue 1 to 4, respectively, were TS1, 5′-GACAGAGC-3′; TS2, 5′-TCTGCTGAAACGCGCGAGAAACCG-3′; TS3, 5′-CCGACACAAGGGCCCATGAACAGC-3′; TS4, 5′-CTCTGGTTGTCTTTAACAAGAGA-3′.

Polymerase chain reaction. For the DNA amplification, 2 μl of first-strand cDNA reaction was amplified in a total volume of 50 μl containing 50 pM of each forward and reverse primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 μM each of the 4 dNTPs, 2.5 units of the Taq polymerase (Perkin-Elmer Corp., Norwalk, CT), and 4.0 units of RNase Inhibitor (RNasin, Promega, Madison, WI). The PCR was carried out in a thermocycler set to incubate the samples at 94°C for 1 min for denaturation of the cDNA, at 55°C for 2 min for primer annealing, and at 72°C for 3 min for primer extension for 35 cycles, followed by incubation at 72°C for 7 min. The amplified products were visualized by electrophoresis on 3% NuSieve agarose gels (FMC Bioproducts, Rockland, ME) with ethidium bromide using TAE (40 mM Tris-acetate, 1 mM EDTA) as buffer for the electrophoresis. The bands were visualized under UV light.

RESULTS

Two hundred ninety-two dengue virus isolates were studied from Indonesia, Mexico, and Puerto Rico. Sixteen concurrent infections with multiple serotypes were detected by IFA and/or RT-PCR (Table 2). Ten samples were corroborated by both methods, 4 only by IFA, and 2 only by RT-PCR.
FIGURE 1. Detection of dual dengue (DEN) virus infections. The RNAs extracted from infected cells were used for the reverse transcriptase–polymerase chain reaction (RT-PCR). The RNA extracted was reverse transcribed with the specific primer. Two microliters of the cDNA was used in a PCR that included primer DS1 and the specific primer for the amplification of a 482–basepair (bp) PCR product for DEN-1, a 119-bp PCR product for DEN-2, a 290-bp PCR product for DEN-3, and a 392-bp PCR product for DEN-4. Ten microliters of the PCR product was subjected to electrophoresis in a 3% agarose gel. Lane 1 contains TS1 primer; lane 2 contains TS2 primer; lane 3 contains TS3 primer; lane 4 contains TS4 primer. Positive controls are the dengue prototypes. Lane MM contains a 1-kb DNA basepair ladder. Values on the right are in basepairs.

PCR. An agarose gel with representative dual dengue virus infections identified by RT-PCR is shown in Figure 1.

The overall prevalence of concurrent dengue infections, defined as the simultaneous presence of 2 or more dengue virus serotypes in an individual, was 5.5% (16 of 292). The frequency of concurrent infections varied from epidemic to epidemic. In the samples from Indonesia, 11 (11.1%) of 99 had multiple virus serotypes; in the samples from Mexico, 5 (5.2%) of 119 were multiple infections; and no concurrent infections were found in samples from Puerto Rico (0 of 74). Of the 11 concurrent infections identified in the samples from Indonesia, 3 contained DEN-3 and DEN-4, 2 contained DEN-2 and DEN-3, 2 contained DEN-1 AND DEN-3, 1 each had DEN-1 and DEN-2 and DEN-2 and DEN-4, and 2 had 3 virus serotypes, DEN-1, DEN-3, and DEN-4. In 1 specimen, only DEN-1 was identified by IFA, but RT-PCR analysis revealed the presence of DEN-1 and DEN-2. Interestingly, DEN-2 was originally identified in this specimen by the complement fixation test. In the 5 multiple infections from Mexico, 2 specimens contained DEN-1 and DEN-4, 1 contained DEN-1 and DEN-2, 1 contained DEN-1 and DEN-3, and 1 contained DEN-1, DEN-3, and DEN-4.

Three of the concurrent infections were in patients diagnosed as DHF, 8 in patients with DF, and 5 in dengue case-patients with clinical data that were inadequate to determine whether they had mild or severe dengue illness.

DISCUSSION

Concurrent infections with more than 1 serotype of dengue virus might be expected to occur when multiple serotypes of dengue virus co-circulate in the same population. Epidemics caused by multiple serotypes have become more frequent on a global basis in the past 18 years. Our demonstration that 5.5% of patients in 3 epidemics had multiple infections with multiple serotypes (Table 2) indicates that large numbers of concurrent infections are likely occurring worldwide. In areas where dengue is hyperendemic, such as many countries in Southeast Asia and tropical America, humans would have greater risk for multiple infections. Indeed, multiple infections were detected in 11% of the patients specimens collected during Indonesian epidemics (Table 2).

The Indonesia viruses were recovered from whole mosquitoes that had been inoculated for long-term storage with
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zero passage virus suspensions. The possibility must be considered that the mosquitoes inoculated with the zero passage virus suspension were already infected with another serotype of dengue virus, thus giving a false-positive result for dual infection. While this might be a very remote possibility, it is highly unlikely for the following reasons. First, the mosquitoes used in these assays were from an uninfected colony of Ae. aegypti that was started in 1975 from larvae collected in Jakarta, Indonesia. Second, mosquitoes from the colony were used for many experimental purposes in addition to virus isolation. Over a 3-year period, hundreds of mosquitoes from this colony were used as negative controls in the above work and none were found to be naturally infected. Lastly, Ae. aegypti is not known to be an efficient host for vertical transmission of dengue viruses. During the period of 1976–1977, approximately 10,000 larvae collected in the epidemic area of Jakarta were tested for dengue virus infection. All were negative (Gubler DJ, 1978, unpublished data).

We thus are confident that the concurrent infections detected in these mosquitoes represent the viruses isolated from patients' serum.

The lack of concurrent dengue infections in Puerto Rico in 1994 during the largest epidemic in that island's history is puzzling. Although DEN-2 was the predominant virus serotype isolated, both DEN-1 and DEN-4 were also present on the island. One explanation might be that DEN-1 and DEN-4 viruses were more localized on the island, where it is not uncommon for a particular serotype to be found in only a small numbers of towns, quite separated from the other serotypes. The Puerto Rican samples tested in this study were selected from towns with high attack rates where at least two serotypes were documented. However, DEN-1 and DEN-4 were isolated at a low frequency compared with DEN-2. At this point we do not have a good explanation for this difference, although it is likely associated with lack of hyperendemicity in most towns of Puerto Rico. The reason for the lack of detectable dual infections in Puerto Rico remains to be determined.

There were 9 discrepancies in the results between IFA using monoclonal antibodies and RT-PCR (Table 2). Most of these (7) were the failure of RT-PCR to detect a single serotype that was identified by IFA while 2 were the failure of the IFA to detect a serotype identified by RT-PCR. It has been shown that virus isolation in C6/36 cells and identification by monoclonal antibodies has a sensitivity similar to the RT-PCR using nested primers. We have no explanation for the apparent increased sensitivity of the C6/36-IFA system in detecting concurrent infection with 2 or more dengue virus serotypes, but are confident of the results presented because all controls in the tests were correct and repeat experiments gave the same results. It should be noted that the sensitivity of the C6/36-IFA system was increased by reducing the multiplicity of infection, extending the incubation period and carefully reading the IFA using serotype-specific monoclonal antibodies before passage of the virus.

We observed that the detection of multiple dengue virus serotypes in cell culture can be increased by infecting the C6/36 cells with a smaller amount of the original specimen and allowing the cultures to incubate for a longer period of time. In routine isolations, between 100 and 200 μl of specimen were inoculated into 1–3 × 10⁶ cells. Detection of multiple infections was increased by using only 30–50 μl of sample to inoculate 3 x 10⁶ cells, which were then harvested at day 15 postinfection. The proportion of cells infected with different dengue serotypes varied during the course of the infection. When the first passage was analyzed by IFA, single infections were more likely to occur if 80% or more of the cells were infected. Two or more dengue serotypes were more likely to be detected when 50% or less of the cells were infected. After the second or third passage, 1 serotype typically predominated, and finally only 1 serotype prevailed. In our experience, if multiple infections are not detected in the initial isolation culture, many will be lost on the first passage. Thus, blind passage of the isolate, as done in many laboratories, is not conducive to identifying multiple infections.

In addition to the co-circulation of multiple virus serotypes in a community, opportunities for dual infections of humans are also increased by the feeding behavior of Ae. aegypti. This mosquito frequently feeds multiple times during a single gonotrophic cycle. Aedes aegypti mosquitoes experimentally infected with dengue viruses spend a longer time probing to acquire a blood meal compared with uninfected mosquitoes. Longer feeding periods increase the chance of host defensive behavior against blood-seeking mosquitoes, and increase the possibility that mosquitoes will feed on more hosts to complete their blood meals. This type of Ae. aegypti feeding behavior may thus increase the chances that they will become dually infected and subsequently transmit multiple viruses to a single host.

In dengue-endemic regions, the density of vectors is usually high, allowing intense transmission of dengue viruses between humans. In 1 study, it was reported that up to 87% of dengue virus infections in children are asymptomatic or minimally symptomatic. Dengue patients have the viruses circulating in their blood for an average of 5 days after onset of illness, but it may be longer. Even though there is no information available regarding the viremia in asymptomatic cases, such infected persons are potential sources of viruses for the mosquitoes. However, it has been shown that some persons with mild dengue illness have low or undetectable viremia that may not be enough to infect mosquitoes.

In our study, no statistically significant association was found between the proportion of cases with concurrent dengue virus infection and clinical outcome. However, the numbers are small and this aspect requires further study before a definitive conclusion can be drawn. Also, the virologic and epidemiologic significance of dual infections remains to be determined.

Of interest are recent reports of recombination among dengue viruses based on evidence from published sequence data. Recombination would be expected to occur more frequently in hyperendemic areas where multiple virus serotypes co-circulate and where concurrent infections would be expected to occur. The role such genetic changes play in the virulence or epidemic potential of dengue viruses is unknown.

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