COMMON OCCURRENCE OF CONCURRENT INFECTIONS BY MULTIPLE DENGUE VIRUS SEROTYPES

M. A. LOROÑO-PINO, C. B. CROPP, J. A. FARFÁN, A. V. VORNDAM, E. M. RODRÍGUEZ-ANGULO,
E. P. ROSADO-PAREDES, L. F. FLORES-FLORES, B. J. BEATY, AND D. J. GUBLER

Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Mérida, Yucatán, México; Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado; Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Colorado State University, Fort Collins, Colorado; San Juan Laboratories, Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, San Juan, Puerto Rico

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. 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. 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. 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). 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. 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. In New Caledonia, DEN-1 and DEN-3 viruses were isolated from 6 patients with DF in 1989. In Thailand, DEN-1 and DEN-2 were identified by RT-PCR in the serum samples of 2 patients with DHF in 1990. 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. 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.

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.

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. 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. 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. At that time, the serotype of the viruses was identified by the complement fixation test using viral antigens obtained from inoculated mosquitoes. 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 µg/ml of gentamicin sulfate, and 50 µg/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. 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.
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 twice in PBS, pH 7.2, and then incubated with fluorescein-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 rpm for 1 min. The column was washed twice with 500 µl of wash buffer (AW) and centrifuged at 6,000 x g for 1 min followed by centrifugation at 20,000 x 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 x 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 dithiothreitol. 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’-TCAATATGCTGAACAACGGCGGAGAACCG-3’, was common for the 4 dengue virus serotypes. The reverse primers, which were specific for dengue 1 to 4, respectively, were TS1, 5’- CGTCCTCACTGATCCGGGGG-3’, TS2, 5’-CGGCCACAAGGGCCATGAACAG-3’, TS3, 5’-TAAACTCATCATGAGACAGAGC-3’, and TS4, 5’-CTCTGTGTGCTTTAAAACGAGA-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.
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.

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
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 have no explanation for the apparent increased sensitivity of the C6/36-IFA system in detecting concurrent infection with 2 or more dengue serotypes, but are confident of the results presented 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.19 *Aedes aegypti* mosquitoes experimentally infected with dengue viruses spend a longer time probing to acquire a blood meal compared with uninfected mosquitoes.20 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.12 Dengue patients have the viruses circulating in their blood for an average of 5 days after onset of illness, but it may be longer.14,25-27 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.28,29

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.30,31 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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Authors’ addresses: M. A. Loroño-Pino, J. A. Farfán, E. M. Rodríguez-Angulo, E. P. Rosado-Paredes, and L. F. Flores-Flores, Centro de Investigaciones Regionales Dr. Hideo Noguchi, Universidad Autónoma de Yucatán, Mérida, Yucatán, México 97000. C. B. Crop and D. J. Gubler, Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, PO Box 2087, Fort Collins, CO 80522. A.V. Vorn- dam, San Juan Laboratories, Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, San Juan, PR 00921. B. J. Beaty, Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Colorado State University, Fort Collins, CO 80523.

Reprint requests: D. J. Gubler, Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, PO Box 2087, Fort Collins, CO 80522.

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