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Detection of Dengue Virus Serotype 2 in *Aedes aegypti* in Quintana Roo, Mexico, 2011

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Abstract. In October 2011, the State Health Department announced that several laboratory-confirmed cases of dengue had occurred among residents in two neighborhoods of Benito Juarez, Quintana Roo State, Mexico. To identify the dengue virus serotype(s) temporally and spatially associated with the cases, entomologic-based virus surveillance was initiated in October 2011 in both neighborhoods. Adult mosquitoes were collected from 88 houses by CDC-backpack aspirator, and all female *Aedes aegypti* L. (n = 419) were individually homogenized and assayed in pools of as many as 10 by reverse transcription-polymerase chain reaction (RT-PCR) using dengue virus-specific primers. Five (12%) of 41 pools were positive for dengue virus RNA. The individual mosquitoes that comprised the pools were analyzed separately by RT-PCR using dengue virus serotype-specific primers. Six mosquitoes were positive for dengue virus serotype-2 (DENV-2) RNA, three of which were collected in the same house. The mean number of female *Ae. aegypti* collected in each house was 4.76 ± 6.19 . The overall dengue virus-infection rate in female *Ae. aegypti* was 1.4%. Interestingly, most (60%) of mosquito females were collected only from 15 (17%) houses. In summary, we provide evidence of recent DENV-2 transmission in Quintana Roo State.

Introduction

Dengue virus (family *Flaviviridae*, genus *Flavivirus*) is the most important arthropod-borne virus in terms of human morbidity and mortality (Simmons et al. 2012). An estimated 2.5 billion people in more than 100 countries are at risk of dengue viral infection, almost 1 billion of whom live in urban areas in tropical and

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subtropical countries in the Americas, Pacific, and Southeast Asia. Dengue virus can cause a spectrum of diseases, ranging from a flu-like illness known as dengue fever to severe and sometimes fatal manifestations known as dengue hemorrhagic fever and dengue shock syndrome. An estimated 50 to 100 million cases of dengue fever occur each year, including 500,000 hospitalizations for dengue hemorrhagic fever and 22,000 deaths. Dengue virus predominantly affects children younger than 15 and in some countries is the leading cause of hospitalization and death of individuals in this age group. There has been an alarming increase in the frequency and severity of dengue virus outbreaks in the last few decades, as illustrated by a 500-fold rise in dengue hemorrhagic fever cases during the last 50 years.

Dengue virus exists as four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. Infection with one serotype results in life-long protection from homologous challenge, but it does not protect against heterologous infection (Vasilakis and Weaver 2008, Vasilakis et al. 2009). Secondary dengue viral infection is the single greatest risk factor for the development of dengue hemorrhagic fever and dengue shock syndrome. Before 1970, secondary dengue viral infections were relatively uncommon because no more than one dengue virus serotype was found in most regions of the world, the exceptions being Central America and Africa where DENV-1 and DENV-2 were present and Southeast Asia where all serotypes were found (Guzman et al. 2010). However, all four serotypes currently co-circulate in most tropical and subtropical regions of the world and therefore the risk of acquiring a secondary dengue viral infection is much greater. Other factors contributing to the dramatic rise in dengue hemorrhagic fever/dengue shock syndrome cases include unprecedented population growth, uncontrolled urbanization, increased movement of humans and vectors, and discontinuation of vector control programs (Guzman et al. 2010).

Dengue virus is maintained in a primate-mosquito transmission cycle. In urban areas, the virus is maintained in an endemic/epidemic cycle between humans and peridomestic *Aedes aegypti* L., although certain other *Aedes* spp. mosquitoes can also serve as vectors. Sylvatic dengue virus is transmitted in an enzootic cycle between non-human primates and various species of arboreal *Aedes* mosquitoes, including *Ae. aegypti* (Vasilakis and Weaver 2008, Vasilakis et al. 2009). In urban settings, *Ae. aegypti* larvae can be found in a wide variety of containers, both indoors (i.e., vases and flower pots) and outdoors (i.e., bottles, cans, buckets, tires, drums, and water storage tanks) (Focks and Alexander 2006, Halstead 2008). Destruction of the oviposition sites can dramatically reduce transmission of dengue virus as well as decrease the likelihood and severity of dengue virus outbreaks. Insecticidal fogging of adult mosquitoes is another strategy often used to control vector populations.

The detection and serotyping of dengue viruses in mosquitoes in endemic and epidemic areas is of critical importance. During epidemic periods, these studies can identify areas where transmission of dengue virus is greatest, thereby allowing vector control authorities to prioritize their efforts and focus on areas where individuals are at greatest risk of disease. During endemic periods, mosquito-based dengue virus surveillance provides an early warning system for predicting future dengue outbreaks, therefore allowing for the timely implementation of prevention and control measures (i.e., insecticidal fogging and elimination of oviposition sites). The detection of dengue virus-infected mosquitoes as early as 6 weeks before the first human cases highlights the important need for mosquito-based virus surveillance during endemic periods (Mendez et al. 2006).

Several studies have provided information on the molecular epidemiology of dengue virus in Mexico (Gardella-Garcia et al. 2008, García-Rejón et al. 2011). However, there is limited information on the molecular epidemiology of the dengue virus serotype(s) in Quintana Roo State. Therefore, the overall goal of this study was to collect *Ae. aegypti* in two neighborhoods of Benito Juárez, Quintana Roo State where laboratory-confirmed cases of dengue fever and dengue hemorrhagic fever had recently occurred and assay the mosquitoes by reverse transcription-polymerase chain reaction (RT-PCR) using consensus dengue virus primers. Mosquitoes positive for dengue virus RNA were further tested by RT-PCR using serotype-specific primers. The resulting data were used to calculate the overall mosquito infection rate and determine the identity of the dengue virus serotype present in the study area. Additionally, distribution of mosquitoes within sampled households was also analyzed.

Material and Methods

In October 2010, the State Health Department reported that a total of 330 cases, of which 115 were dengue fever and 215 dengue hemorrhagic fever, had occurred among residents of several mid-to low-socioeconomic neighborhoods (designated regions 102 and 103) of Benito Juárez, Quintana Roo State, Mexico (Fig. 1) (CENAVECE 2012). Benito Juárez has undergone rapid population increase in the last decade, and its current population is 676,000 (INEGI 2010). Tourism is the main economic activity, and the ongoing construction of new resorts and hotels has resulted in a steady influx of construction workers and tourism personnel from throughout the state as well as from nearby states, particularly Chiapas and Oaxaca (Villafuerte et al. 2008). Benito Juárez has an average annual temperature of 25.5°C and rainfall of 12,000 mm. Rain usually occurs every month, but most precipitation occurs in the summer (INAFED 2009).

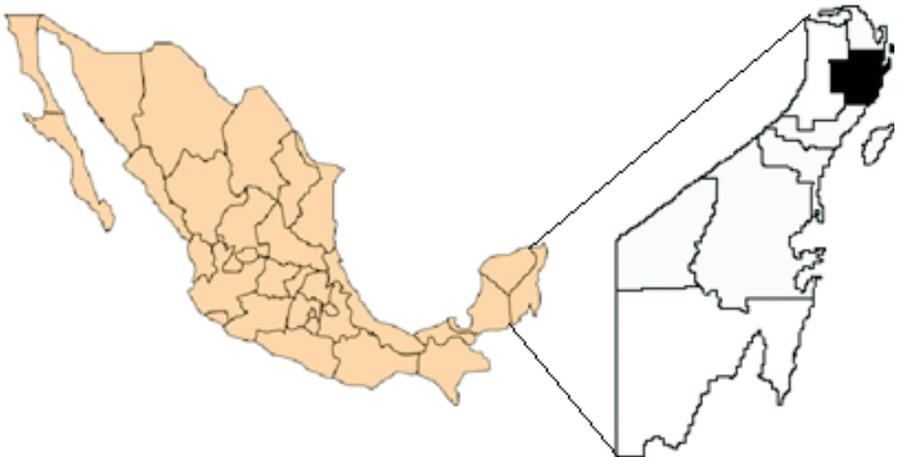


Fig. 1. Benito Juárez geographical location in the Yucatan Peninsula, Southern Mexico.

In total, 88 households (45 from Region 102 and 43 from Region 103) were inspected for resting mosquitoes. Every room as well as the backyard of each residence was searched. Mosquitoes were collected by using a CDC-style backpack aspirator (Clark et al. 1994). Mosquitoes were collected in October 2011. Indoor collections included aspirations from walls, furniture, hanging clothes, flags, curtains, blinds, and any dark and humid places where mosquitoes might rest. Outdoor collections included aspirations from vegetation, fences, equipment, and pet houses. The average amount of time spent collecting mosquitoes at each site was 25 minutes per visit. Aspirated mosquitoes were placed into screw-capped vials placed on dry ice and transported to the Laboratorio de Arbovirologia at the Universidad Autonoma de Yucatan in Merida, Mexico. Morphological characteristics were used to identify mosquitoes on chill tables to species and sex (Darsie and Ward 1981). All female *Ae. aegypti* were stored at -70°C until processed.

Mosquitoes were placed into individual Eppendorf tubes with 0.2 ml of cold L-15 medium (Invitrogen, Carlsberg, CA) supplemented with 2% fetal bovine serum (HyClone, Logan, UT), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), and homogenized for 30 seconds using a blue pestle rod attached to a hand-held, cordless motor (Daigger, Vernon Hills, IL). One hundred microliters of each homogenate was mixed with 0.5 ml of Trizol (Invitrogen, Carlsberg, CA) (Chomezynski and Sacchi 1987), and total RNA was extracted following instructions of the manufacturer.

Complementary DNAs were generated using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), and PCRs were done using *Taq* polymerase (Invitrogen, Carlsbad, CA) and primers specific to a 470-bp region of the NS3 gene of all four dengue virus serotypes (Seah et al. 1995a,b). RT-PCR products were resolved on 2.0% LE agarose gels (Promega Corp., Madison, WI) stained with ethidium bromide. Mosquito homogenates were tested in pools of as many as 10. If a pool tested positive, the individual mosquitoes that comprised the pool were tested separately by semi-nested PCR using the forward consensus primer and four dengue virus serotype-specific reverse primers.

Mean numbers of mosquitoes per house and between sampled neighborhoods were statistically compared. Raw data were transformed ($\ln x+1$) and analyzed using SPSS™ 19.0 (Chicago, IL). Dengue virus infection rates of field-collected *Ae. aegypti* populations were also calculated.

Results

Adult mosquitoes were collected by battery-powered backpack aspirator in two neighborhoods of Benito Juarez. *Ae. aegypti* were found in 66 of 88 (75%) households sampled (Table 1). A similar percentage of houses in each region yielded *Ae. aegypti*; this mosquito species was obtained from 36 of 45 (80%) houses in Region 102, and 30 of 43 (70%) houses in Region 103. The total number of *Ae. aegypti* collected was 965. Of these, 419 (43%) were identified as female. One hundred sixty females were collected in Region 102, and 259 females were collected in Region 103.

The mean number of female *Ae. aegypti* collected in each house was 4.76 ± 6.19 . Student-*t* analysis revealed that the mean number of females collected per house in Region 103 (6.02 ± 7.30) was significantly greater compared to Region 102 (3.56 ± 4.66) ($t = 14.221$, $df = 44$, $P < 0.000$). Interestingly, most (60%) of the

female *Ae. aegypti* were collected from 15 (17%) houses. The numbers of female *Ae. aegypti* collected in these houses ranged from 10 to 24 (Fig. 2).

Table 1. Summary of Numbers of Adult *Aedes aegypti* Collected in Benito Juarez, Quintana Roo, Mexico, October 2011

Neighborhood	Households sampled	Households with <i>Ae. aegypti</i>	<i>Ae. aegypti</i> collected	Female <i>Ae. aegypti</i> collected	Mean \pm SD females/house
Region 102	45	36 (80%)	434	160	3.56 \pm 4.66
Region 103	43	30 (70%)	531	259	6.02 \pm 7.30
Total	88	66 (75%)	965	419	4.76 \pm 6.19

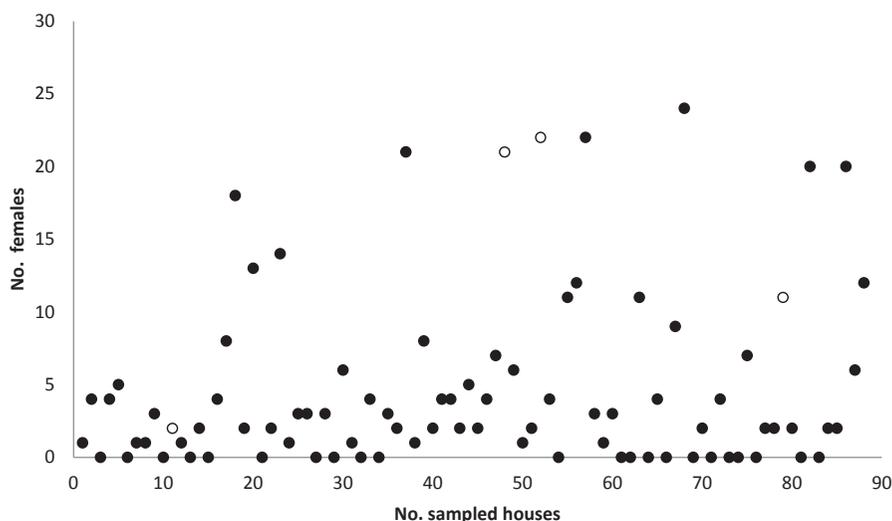


Fig. 2. Density of houses infested with adult *Aedes aegypti* using CDC-style backpack aspirator in two neighborhoods of Benito Juarez, Mexico, October 2011.

Dengue virus RNA was detected by RT-PCR in 5 (12%) of the 41 mosquito pools tested (Table 2, Fig. 3). A greater number of positive pools, four (16.6%), was found for Region 103 as compared to two (11.7%) ($t = 7.22$, $df = 87$, $P < 0.000$) for Region 102. Further analysis by semi-nest RT-PCR of the individual homogenates that comprised these pools revealed the presence of DENV-2 RNA in six mosquitoes. Five infected mosquitoes were collected from three houses in Region 103 (three mosquitoes were from the same house), while the other was collected in Region 102. The three houses in Region 103 that yielded infected mosquitoes were located within a radius of less than 100 m (Fig. 4). Overall, 1.4% of the female *Ae. aegypti* collected in this study were positive for DENV-2 RNA. None of the mosquitoes was positive for DENV-1, DENV-3, or DENV-4 RNA.

Table 2. Summary of Numbers (%) of *Ae. aegypti* Positive for Dengue Virus RNA in Benito Juarez, Quintana Roo, Mexico, October 2011

Region	Total mosquito pools	Pools positive for DENV RNA	Individual mosquitoes positive for DENV RNA	Houses with DENV-infected mosquitoes
102	17	1 (6%)	1 (0.6%)	1 (2.2%)
103	24	4 (17%)	5 (1.9%)	3 (7.0%)
Total	41	5 (12%)	6 (1.4%)	4 (4.5%)

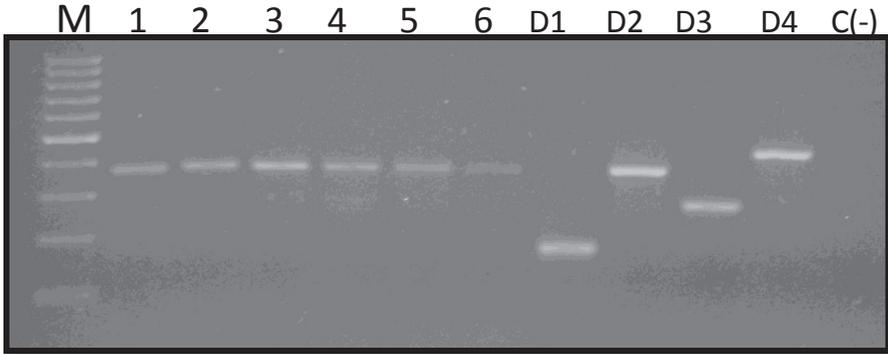


Fig. 3. Agarose gel 1.5% electrophoresis of RT-PCR products. M: molecular weight. 1-6: individual field-collected *Ae. aegypti* females. D1: DENV1, D2: DENV2, D3: DENV3, D4: DENV4, C (-): negative control.



Fig. 4. Spatial distribution of houses where DENV-2 *Ae. aegypti* were collected in two neighborhoods of Benito Juarez, México. Above: Region 102, below: Region 103 (Google Earth free software™).

Most of the dengue virus-infected mosquitoes were collected in houses where *Ae. aegypti* were most abundant. For example, a total of 21 female *Ae. aegypti* was collected in the house that yielded three dengue virus-infected mosquitoes. Another dengue virus-infected mosquito was from a house where 11 female *Ae. aegypti* were collected. Only one infected mosquito was collected in a house where less than five female *Ae. aegypti* were found. The spatial locations of houses with dengue virus-infected mosquitoes were mapped using Google Earth Software™ (Fig. 4).

Discussion

Serotype-specific RT-PCR revealed the presence of DENV-2 RNA in six of 419 (1.4%) female *Ae. aegypti* collected from households in two neighborhoods of Benito Juarez. During October, when mosquitoes were collected, the State Health Department announced that several residents from several neighborhoods had 215 confirmed cases of dengue hemorrhagic fever and 115 had dengue fever (CENAVECE 2012). The patients were considered to have acute dengue virus infections because they had presented symptoms described by WHO guidelines for dengue fever and dengue hemorrhagic fever (severe manifestations) classifications. In addition, IgM antibody was detected in their acute-phase serum by antibody-capture enzyme-linked immunosorbent assay. However, the identity of the dengue virus serotype(s) responsible for the infections was not routinely determined by the laboratory analysis. Data obtained in this entomologic investigation clearly can be used to associate which dengue virus serotype(s) was responsible for the human infection; we consider DENV-2 the etiological agent because this serotype was identified by RT-PCR molecular technique and temporally and spatially associated with the cases. In this regard, Garcia-Rejon et al. (2008) detected three serotypes (DENV-1, DENV-2, and DENV-3) in *Ae. aegypti* collected from households of dengue patients in Merida, Yucatan State, Mexico, and observed that the serotype matched with the serotype of the patients when serotype-specific information was available.

As already noted, dengue virus RNA was detected in 1.4% of the female *Ae. aegypti* collected in this study. Similar infection rates have been documented for female *Ae. aegypti* from other dengue epidemic/endemic regions (Garcia-Rejón et al. 2008, Yoon et al. 2012). For example, Garcia-Rejon et al. (2008) reported a minimal infection rate of 1.8% in female *Ae. aegypti* collected from March 2007 to February 2008 inside houses in Merida, Yucatan State, Mexico. The minimal infection rate for mosquitoes collected in October was 1.3%. However, several differences exist between the two studies; most notably, all of the houses sampled in Merida belonged to dengue patients, and the mosquitoes were processed in pools of one to 30. Guedes et al. (2010) reported a much lower infection rate (10.2%) in female *Ae. aegypti* collected during 18 months in households of dengue patients in Brazil.

Adult *Ae. aegypti* were collected in most (75%) households sampled in this study. A moderate to high proportion (24 to 80%) of houses within other dengue endemic/epidemic neighborhoods also yielded adult *Ae. aegypti* (Mendez et al. 2006, Garcia-Rejon et al. 2008). The mean number of female *Ae. aegypti* collected from each house in our study was 4.76. However, mosquitoes were particularly abundant in some households, with at least 20 female *Ae. aegypti* collected from seven of the 88 (8.0%) residences sampled during the 1-month collection period

(Fig. 2). Other studies also reported numerous female *Ae. aegypti* in certain houses within dengue endemic/epidemic regions (Guedes et al. 2010, Yoon et al. 2012). These findings suggest adult mosquito control practices should be implemented for indoor *Ae. aegypti* to quickly stop dengue transmission.

In summary, serotype-specific RT-PCR revealed the presence of DENV-2 RNA in adult *Ae. aegypti* collected from households in Benito Juárez, Quintana Roo State. Residents from this area were diagnosed with clinical dengue virus infections immediately before and during the course of our entomologic investigations, but a serotype-specific diagnosis was not made for any patient. In the absence of a serotype-specific diagnosis, we suggest entomological-based dengue and serotype surveillance could be implemented to detect silent disease activity caused by asymptomatic manifestations.

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