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Detection of *Aedes aegypti* Mosquitoes Infected with Dengue Virus as a Complementary Method for Increasing the Sensitivity of Surveillance: Identification of Serotypes 1, 2, and 4 by RT-PCR in Quintana Roo, Mexico

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Abstract. Sensitivity of monitoring *Aedes aegypti* (L.) populations was determined to identify the distribution of dengue virus (DENV) during epidemics in Quintana Roo. From September to November 2012, we used a motorized aspirator to collect 2,144 female *Ae. aegypti* from 569 homes. These were grouped into 220 to use semi-nested RT-PCR for DENV, and positive groups were analyzed individually. Five groups (2.27%) were positive for DENV. Individual analysis yielded eight groups that tested positive, six with DENV-2, one DENV-1, and one DENV-4. The latter was not reported by the surveillance system that year. The mean number of female mosquitoes per household was 3.77 ± 5.71, and the rate of viral infection of *Ae. aegypti* was 0.4%. Most infected mosquitoes (49%) were concentrated in 10% of the houses. Monitoring *Ae. aegypti* infected with DENV has the potential to complement the current system of clinical and entomological surveillance.

Resumen. Se determinó la sensibilidad del monitoreo de poblaciones *Ae. aegypti* para identificar la circulación de DENV durante epidemias en Quintana Roo. De Septiembre a Noviembre del 2012, en 569 viviendas se colectaron 2,144 hembras *Ae. aegypti* con un aspirador motorizado. Se agruparon en 220 lotes para realizar la RT-PCR semi-anidada para DENV y los lotes positivos se analizaron individualmente. Cinco lotes (2.27%) fueron positivos para DENV. El análisis individual de los lotes arrojó ocho mosquitos positivos: seis DENV-2, uno DENV-1, y uno DENV-4. Este último no fue reportado por el sistema de vigilancia epidemiológica en ese año. El promedio de hembras colectadas por casa fue 3.77 ± 5.71 y la tasa de infección viral de *Ae. aegypti* 0.4%. La mayoría (49%) se concentró en el 10% de las casas. Monitorear *Ae. aegypti* infectados a DENV tiene

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un potencial para complementar el actual sistema de vigilancia clínica y entomológica.

Introduction

Dengue is the fastest spreading mosquito-borne viral disease in the world (WHO 2009). Each year, an estimated 50 million infections occur, and about 2,500 million people live in dengue-endemic countries. In Mexico, the situation is worse because of recurrent epidemics, as in 2012 when 50,368 cases were reported and in 2009 when 55,961 cases occurred (CENAVECE 2013). Dengue virus (DENV 1-4) has a positive-strand of RNA of the genus *Flavivirus*, family Flaviviridae (WHO 2009). It can cause symptoms ranging from mild febrile illness, classified as dengue without warning signs, to severe manifestations such as mucosal bleeding, abdominal pain, lethargy, hepatomegaly (<2 cm), and a reduced hematocrit and platelet count, classified as dengue with warning signs. The most dangerous condition is severe dengue, which presents with fluid extravasation, severe bleeding, and shock, on occasions being fatal (WHO 2009).

One of the main problems in epidemiological surveillance of dengue is underestimation of the number of cases. Dengue has been described as an iceberg with the tip representing less than 10% of symptomatic dengue cases reported (Kyle and Harris 2008). Dengue with warning signs and severe dengue are at the top of a pyramid, because they are more obviously diagnosed in clinical surveillance and confirmed by serology. However, surveillance and statistics do not reflect a substantial 50-90% of all asymptomatic infections that undoubtedly play a role as amplifying hosts of DENV transmission in the man-vector cycle (Halstead et al. 1970, Graham et al. 1990). This demonstrates the alarming magnitude of silent virus transmission and the weakness of the current surveillance system, which are responsible for permanent epidemics in most endemic areas.

Studies have shown that detection of dengue virus and its serotypes in *Ae. aegypti* in endemic and epidemic areas is critical for surveillance systems based only on clinical and laboratory diagnosis. During epidemics, the studies may identify areas where greater transmission of dengue virus occurs, thus allowing vector-control authorities to prioritize efforts and focus on areas where people are most at risk for disease (Sánchez-Casas et al. 2013). During endemics, surveillance of circulating dengue virus in mosquitoes provides early warning for predicting future outbreaks of dengue; this allows timely implementation of prevention and control measures (insecticide fogging and elimination of oviposition sites) (Halstead 2008). Dengue virus in infected mosquitoes can be detected as long as 6 weeks before the first human case occurs, emphasizing the importance of virological surveillance in mosquitoes during endemic periods (Mendez et al. 2006). An increase in the sensitivity of the current dengue surveillance system is urgently needed, even to calculate future distribution of vaccine in the human population. A comparative example of another arbovirus with more complete epidemiological surveillance is West Nile Virus. Besides surveillance in humans to issue epidemiological alerts, it includes frequent monitoring of mosquito vectors, serological and virological sampling in horses, and a surveillance and alert program in resident and migratory birds (CDC 2013). Because of the nature of circulation of dengue virus, incorporation of infected female *Ae. aegypti* into the current alert system based on clinical cases has the potential of increasing the sensitivity of the surveillance system. The aim of this study was to use reverse transcription

polymerase chain reaction (RT-PCR) to evaluate effectiveness of detection of DENV and its serotypes in indoor female *Ae. aegypti* (L.) with silent transmission of dengue virus in the state of Quintana Roo.

Materials and Methods

From September to November 2012, the Secretary of Health of Quintana Roo reported an outbreak of dengue. In total, 375 cases were reported, of which 177 were described as dengue fever and 198 as dengue hemorrhagic fever (Fig. 1) (CENAVECE 2013).

The municipalities of Benito Juarez and Othon P. Blanco were studied. The cities have 661,000 and 244,000 inhabitants, respectively, and both have experienced rapid growth in the last decade (INEGI 2010). Increasing tourism demands creation of infrastructure including hotels and residential areas. This activity attracts migrant workers from neighboring states, especially Chiapas and Oaxaca (Villafuerte-Solís et al. 2008). Quintana Roo has an average annual temperature of 25.5°C and rainfall of 12,000 mm (INAFED 2012).

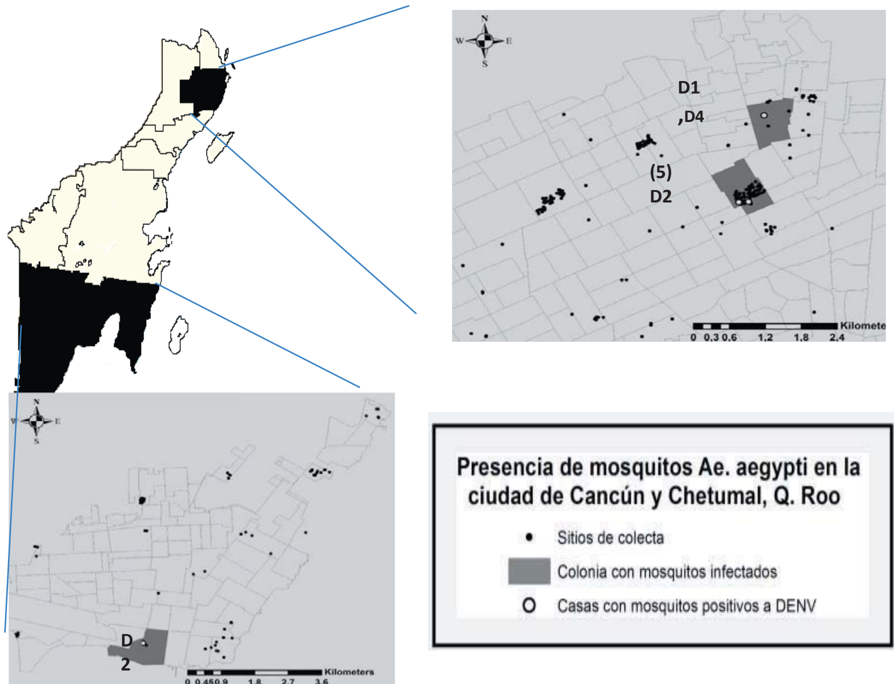

Fig. 1. (a) Map of Mexico, the state of Quintana Roo, and the municipalities of Benito Juarez and Othon P. Blanco, Quintana Roo, Mexico. (b) Spatial distribution of houses where infected *Ae aegypti* mosquitoes with DENV-1, 2, and 4 were found (ArcGis10 Software, CA, USA).

We captured *Ae. aegypti* indoors to validate the potential use of identifying infected female mosquitoes as an adjunct to the current clinical surveillance system. The collection period was from October to December 2012. Using a motorized CDC-type aspirator (Clark et al. 1994), 569 households (480 at Cancun, Benito Juarez, and 89 at Chetumal, Oton P. Blanco) were sampled. Resting adult mosquitoes were sought in and outside the home, e.g., interior, walls, furniture, closets, curtains, blinds, and dark, moist places where mosquitoes rest. Outside collections included aspirations in gardens, vegetation, and fences. The average time spent collecting mosquitoes at each house was 25 minutes per visit. The trapped mosquitoes were placed in 2-ml microtubes with screw caps and stored at −180˚C in a tank of liquid nitrogen. At the end of the collection time they were transported in refrigeration at −80˚C in solid carbon dioxide by air to the Emerging Pathogens and Vectors Unit of the Center for Research and Development in Health Sciences (CIDICS), of the Universidad Autónoma de Nuevo León in Monterrey, México. Taxonomic keys were used to identify mosquitoes to species and sex (Darsie and Ward 1981); this was done on a cold plate to prevent degradation of the viral genetic material. Only female *Ae. aegypti* were used and stored at −80°C until processing. Molecular identification of DENV and its serotypes was done by reverse transcription polymerase chain reaction (RT-PCR). For extraction of viral ribonucleic acid, mosquitoes were placed in 0.2-ml Eppendorf tubes with L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 2% fetal bovine serum (Hyclone, Logan, UT), penicillin (100 U/ml), streptomycin (100 mg/ml), and amphotericin B (0.25 µg/ml), and homogenized for 30 seconds using a cordless electric macerator with pestle (Daigger, Vernon Hills, IL). One hundred microliters of each homogenate was mixed with 0.5 ml of Trizol (Invitrogen) (Chomezynski and Sacchi 1987), and RNA was extracted following instructions of the manufacturer.

Complementary DNA was generated using SuperScript III reverse transcriptase (Invitrogen), and PCR was done using Taq polymerase (Invitrogen) and primers specific for the 470-bp region of the NS3 gene of the four serotypes of dengue virus (Seah et al. 1995a,b). RT-PCR products were stained with ethidium bromide and visualized on 2.0% agarose gel (Promega Corp., Madison, WI). This molecular technique was used in variable groups of female *Aedes aegypti*; e.g., 6, 8, 9, 10, 11, and 17, depending on the number of mosquitoes collected per household. The group positive in the first analysis by RT-PCR was analyzed again to obtain the infected mosquito and associate the house where it was collected and its geographical distribution. The serotype diagnosis in individuals initially positive was obtained with a semi-nested PCR (Seah 1995b).

Number and rate of viral infection of female *Ae. aegypti* per house were analyzed. Data on houses, locations, and infection rates were compared and analyzed using the SPSS 18.0 Statistics® statistical package (SPSS Inc., Chicago, IL). Spatial distribution of mosquitoes was graphed using ArcGIS 10 Software.

Results

During 3 months, a total of 569 households (Table 1) was sampled. The largest group of 480 (84.4%) was at Benito Juarez, with only 89 (14.6%) at Othon P. Blanco. Houses positive for infected *Ae. aegypti* totaled 392 (78.2%). Overall, the percentage was high at the two locations: 81.7% at Benito Juarez and 59.6% at Othon P. Blanco. In total, 4,751 female and male *Ae. aegypti* were collected, with 4,197 (88.3%) from Benito Juarez and 554 (11.7%) from Othon P. Blanco. Of the

Table 1. Distribution of *Aedes aegypti* Females Collected in Houses in the Municipalities of Benito Juarez and Othon P. Blanco, Quintana Roo, Mexico, September to November 2012

Municipality	Homes sampled	Homes with *Ae . aegypti*	*Ae. aegypti* collected	Female *Ae. aegypti* collected	Number of females/total homes
Othon P. Blanco	89	53 (59.5%)	554	295	3.31 ± 4.48
Benito Juarez	480	392 (81.6%)	4,197	1,849	3.85 ± 5.91
Total	569	445 (78.2%)	4,751	2,144	3.77 ± 5.71

total number of mosquitoes collected, 2,144 (45.1%) were female. Infection of *Ae. aegypti* females collected per household averaged 3.77 ± 5.71 at both locations. Arithmetic means were similar at Othon P. Blanco and Benito Juarez, 3.31 ± 4.48 and 3.85 ± 5.91, respectively. Comparison of means by X^2 analysis revealed no significant difference (t = 15.731, df = 88,479, P < 0.01). However, the average values did not show the aggregate distribution of *Ae. aegypti*. Interestingly, the scatter diagram of Fig. 2 shows one half (49.5%) of females (1,061) were obtained only in 57 (10.0%) of the sampled homes. The number of female *Ae. aegypti* in these houses ranged from 11 to 58 (Fig. 2).

When mosquitoes were processed by RT-PCR, five (2.3%) of the 220 groups were positive for DENV (Table 2, Fig. 3). The greatest number of positive groups, four (2.2%), was 185 groups from Benito Juarez municipality and only one (2.9%) of 35 groups from Othon P. Blanco.

Mosquitoes of each positive group were individually analyzed with the same RT-PCR technique. With this procedure, we related the house where the mosquitoes were collected and their likely density association with viral infection. We obtained eight (3.6%) mosquitoes infected with DENV: seven (3.2%) at Benito

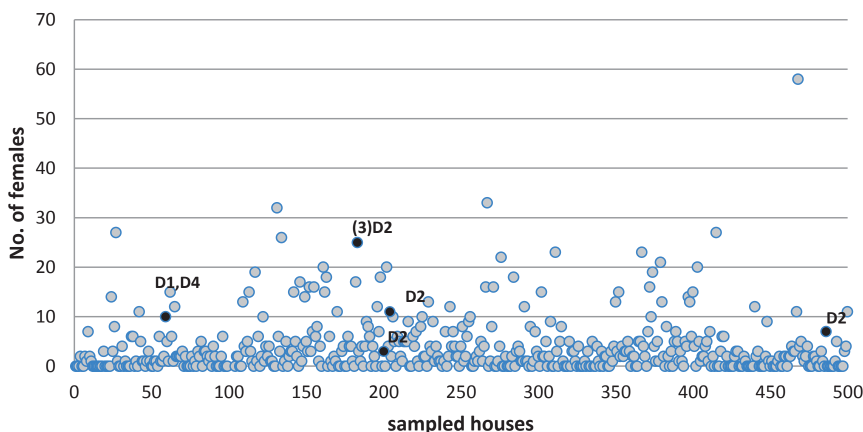

Fig. 2. Female *Aedes aegypti* in homes monitored with a CDC backpack aspirator at Benito Juarez and Othon P. Blanco, Quintana Roo, Mexico, from September to November 2012.

Table 2. Groups (%) of Female *Ae. aegypti* with Dengue Virus at Benito Juarez and Othon P. Blanco, Quintana Roo, Mexico, from September to November 2012

Municipality	Groups	Groups positive for DENV	Mosquitoes positive for DENV	Houses with infected mosquitoes	Serotype
Othon P. Blanco	35	1 (2.9%)	1 (0.5%)	1 (0.2%)	DENV2
B. Juarez	185	4 (2.2%)	7 (3.2%)	4 (0.9%)	DENV1 DENV2 DENV4
Total	220	5 (2.3%)	8 (3.6%)	5 (1.1%)	DENV1,2,4

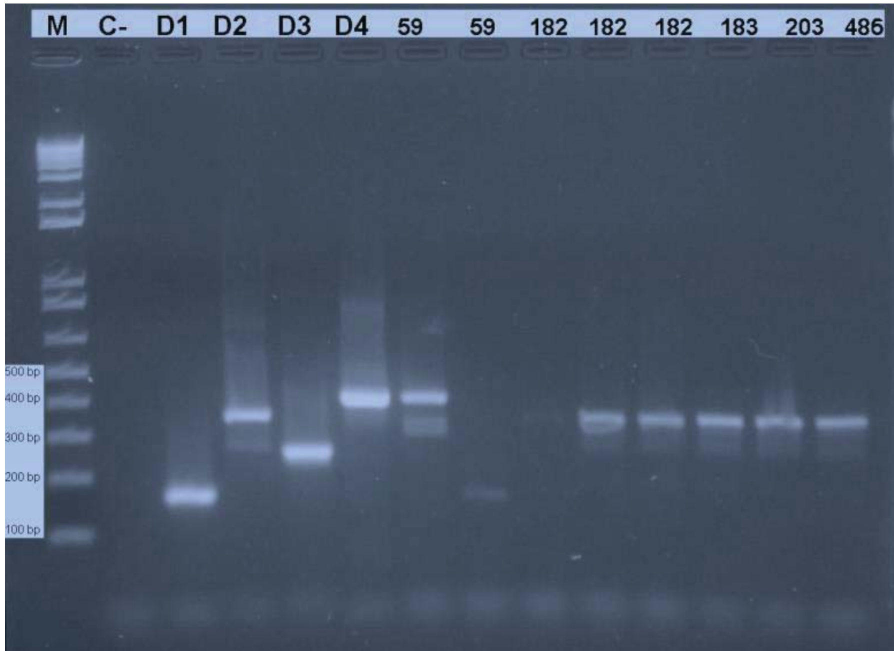

Fig. 3. Electrophoresis of RT-PCR products on 2.0% agarose gel. M: molecular marker, C(-), D1, D2, D3, D4, samples (eight) of infected female mosquitoes collected.

Juarez and one (0.5%) at Othon P. Blanco. To determine what serotypes corresponded to the DENV in individual mosquitoes, we used a second nested RT-PCR. Three serotypes were found at Benito Juarez, i.e., DENV-1, DENV-2, and DENV-4 in 1, 5, and 1 mosquitoes, respectively (Fig. 2). Finding two serotypes, DENV-1 and DENV-4, was documented in two mosquitoes at this location. Additionally, one home had three mosquitos with DENV-2 infection. Regarding the results at Chetumal, in Othon P. Blanco, one house with a mosquito infected with DENV2 was in the downtown area (Fig. 1).

The percentage of viral infection of mosquitoes collected in households in both municipalities was 0.37%. Similar percentages were found at Othon P. Blanco and Benito Juarez, 0.34 and 0.38%, respectively.

In analysis of the vector densities relative to DENV viral infection, we observed most infected mosquitoes were collected in homes where female *Ae. aegypti* were more abundant. For example, three infected mosquitoes were collected in a house with 17 female *Ae. aegypti*. Similarly, two infected mosquitoes were captured in a house with 10 female mosquitoes. An infected mosquito was found in another house with 25 females. According to previous findings, 75% (six) of infected mosquitoes were associated with homes with more mosquitoes than the mean of 3.77 ± 5.71 females per house. Only two (25%) infected mosquitoes were caught in two houses where the number of female mosquitoes was near the mean, three and four, respectively (Fig. 2).

Discussion

Results of this study showed 78.2% of houses at the two study sites with *Ae. aegypti* (Table 1). This finding is consistent with reports by Mendez et al. (2006) in Colombia and Garcia-Rejon et al. (2008) at Merida, Yucatan, who found large numbers of adult female mosquitoes in 20 to 80% of houses in endemic/epidemic areas. Relevant to epidemiological risk was the aggregated pattern of distribution of *Ae. aegypti* mosquitoes in dwellings. Mosquitoes were abundant in some households while the overall mean per household was 3.77 ± 5.71. This explains why almost half (49.5%) of the 1,061 females were obtained in only 10.0% (57) of the sampled households (Fig. 2). Scott et al. (2000) reported the same pattern of aggregation for both vectors of dengue in Thailand. Similar findings have been documented in Brazil and in a study in Quintana Roo (Sánchez-Casas et al. 2013).

As mentioned, the rate for dengue virus was 0.4% in female *Ae. aegypti* in this study. Similar infection rates have been documented for *Ae. aegypti* females in other epidemic/endemic dengue areas (Mendez et al. 2006). García-Rejon et al. (2011) reported an infection rate of only 1.8% in *Ae. aegypti* females collected from March 2007 to February 2008 in the interior of houses at Merida, Yucatan State, Mexico. The minimum infection rate for mosquitoes collected during October-December 2012 was 0.4%.

During the epidemic period of September-November 2012, the clinical surveillance system reported 375 cases, 177 of dengue fever and 198 of dengue hemorrhagic fever (CENAVECE 2013). The method used in our research detected virus simultaneously in transmitting mosquitoes in the same endemic locations (Seah et al. 1995a,b). The molecular technique also identifed serotypes in mosquito vectors. From a total of 2,144 mosquitoes collected indoors at Othon P. Blanco and Benito Juarez, eight (3.6%) had viral infection of serotypes DENV-1, DENV-2, and DENV-4 (Tables 1, 2). The minimum rate of viral infection was 0.4%, near the 1.8% reported by García-Rejon et al. (2011) in home environments at Merida. However, there were several differences between the two studies; all households sampled at Mérida had patients diagnosed with dengue virus and the mosquitoes were processed in groups of 1-30. Guedes et al. (2010) identified a higher minimum infection rate (10.2%) in *Ae. aegypti* females collected during 18 months in the homes of dengue patients in Brazil. Sanchez-Casas et al. (2013) reported 1.4% in *Ae. aegypti* females in the municipality of Benito Juarez during 2011 after Hurricane Alex; the time of collection in this study was only 1 month.

These results are consistent with other studies in Colombia and Venezuela that recommend the use of virological surveillance by RT-PCR to detect infected *Ae. aegypti*. The data have the potential to be used in an early warning system for dengue outbreaks. Similarly, Urdaneta et al. (2005) reported in areas with a prevalent epidemic in Venezuela, eight (5.2%) of 154 groups of mosquitoes collected in homes of dengue patients and 18 (12%) of 142 groups collected in neighboring houses had serotypes DENV-1, DENV-3, and DENV-4. In Colombia, Mendez et al. (2006) captured 4,964 mosquitoes, 292 and 30 groups of *Ae. aegypti* and *Ae. albopictus* (Skuse), respectively. They reported 37 (12.7%) positive groups of *Ae. aegypti*, providing information of DENV-1, DENV-2, and DENV-4 serotypes.

Other advantages of a potential surveillance system in infected mosquitoes complementary to the current epidemiological surveillance system are its ability to anticipate an outbreak as well as detect silent virus. In Venezuela, infected *Ae.* mosquitoes were identified 8 weeks before the peak of the epidemic was evident (Urdaneta et al. 2005). Although the clinical manifestations in patients disappear, the virus continues to circulate in mosquitoes at these houses. Garcia-Rejon et al. (2008) identified dengue virus in mosquitoes collected as long as 27 days after the clinical case was reported. Also, García-Rejon et al. (2011) used the viral surveillance system for mosquitoes at schools at Mérida, Yucatán, Mexico, and found an infection rate of 4.8 per 100 *Ae. aegypti* females. There are reports of infected *Ae. aegypti* with co-circulation of serotypes not yet reported by the clinical surveillance system. Thus, Guedes et al. (2010) in Brazil found circulating serotypes DENV-1, DENV-2, and DENV-3 in *Ae. aegypti* while the dominant serotype in the human population was DENV-3. In our study, we identified DENV-4 in infected mosquitoes 10 weeks before it was reported by the official clinical surveillance system (Table 2). Monitoring to identify virus in mosquitoes and spatial distribution would enhance the efficiency of targeted and timely implementation of control.

The Official Mexican Standard (NOM-032-SSA2-2010) and the international guideline of the World Health Organization (2009) indicated that epidemiological surveillance of dengue cases is supported by clinical diagnosis and entomological surveillance of larval indices. However, silent circulation in asymptomatic patients is responsible for a disturbing underreporting of 80% of cases (Kyle and Harris 2008). It is obvious the mosquito population is a reservoir of the virus and is responsible for subsequent outbreaks. Similarly, larval indices poorly reflect predictive power in correlation between vector densities and human cases. Increased awareness of the dengue surveillance system is a priority in Mexico and endemic countries. Other vector-borne diseases such as West Nile Virus have more robust surveillance systems. In addition to clinical monitoring, three indicators of virus circulation are included, both in vertebrate hosts and transmitter mosquitoes, e.g., vectors, horses, and birds (CDC 2013). The magnitude of the epidemics of dengue demands a more sensitive surveillance system in addition to diagnosis of clinical cases. Like other Latin American authors, our results suggest the implementation of viral infection monitoring in populations of *Ae. aegypti* as an additional indicator to strengthen the current surveillance system by the Mexican Official Standard.

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