Individual-based dengue virus surveillance in Aedes aegypti mosquitoes collected concurrently with suspected patients in Tarlac City, Philippines

Jean Claude Balingit
Ehime University
Thaddeus M. Carvajal
Ehime University
Mariko Saito-Obata
Tohoku University
Maribet Gamboa
Ehime University
Amalea Dulcene Nicolasora
Research Institute for Tropical Medicine
Ava Kristy Sy
Research Institute for Tropical Medicine
Hitoshi Oshitani
Tohoku University
Kozo Watanabe (watanabe_kozo@cee.ehime-u.ac.jp)
Ehime University

Research

Keywords: dengue virus, mosquito-based virus surveillance, Aedes aegypti, multiplex real-time RT-PCR, phylogenetic analysis, Philippines

Posted Date: August 11th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-56950/v1

Version of Record: A version of this preprint was published on November 25th, 2020. See the published version at https://doi.org/10.1186/s13071-020-04470-y.

Abstract

Background Dengue virus (DENV) infection continues to be a major public health concern throughout tropical and subtropical regions of the world where Aedes aegypti mosquitoes, its primary vector, dwell. In the context of DENV transmission, effective control is reliant not only on knowledge of mosquito abundance, but also on mosquito infection. In the 2015 dengue season, we conducted a one-month entomological surveillance of adult Aedes aegypti mosquitoes around households of suspected dengue patients in Tarlac City, Philippines to assess the DENV infection rate in the local mosquito population, and to identify the DENV genotypes and serotypes concurrently co-circulating in mosquitoes and patients. Methods We performed a one-step multiplex real-time RT-PCR assay for the simultaneous detection and serotyping of DENV in patients and in individual female Aedes aegypti mosquito. Consequently, we performed sequencing and phylogenetic analyses to further characterize the detected DENVs in mosquitoes and patients at the genotype level. Results We collected a total of 583 adult Aedes aegypti mosquitoes, of which we tested 359 female mosquitoes individually for the presence of the DENV. Ten mosquitoes (2.8%) from amongst 359 female mosquitoes were confirmed to be positive for the presence of the DENV. We detected DENV-1, DENV-2, and DENV-4 in the field-collected mosquitoes, which were consistent with the serotypes concurrently infecting patients. Sequencing and phylogenetic analyses of the detected DENVs based on the partial envelope (E) gene revealed three genotypes concurrently present in the sampled mosquitoes and patients during the study period, namely: DENV-1 genotype IV, DENV-2 Cosmopolitan genotype and DENV-4 genotype II. Notably, we observed a random geographic distribution of DENVs in the study area suggesting the occurrence of active DENV transmission within and outside the vicinities of Tarlac City. Conclusions In this study, we demonstrate the utility of an individual-based DENV surveillance in field-collected mosquitoes and the importance of incorporating mosquito virus data in phylogenetic studies. Analyzing virus sequences from vector and host could potentially improve our understanding of the dynamics of DENV transmission.

Introduction

The increasing incidence and expanding geographical range of dengue virus (DENV) infections are causes for international concern. DENVs are transmitted through a human-to-mosquito-to-human cycle throughout tropical and subtropical regions of the world, with the *Aedes aegypti* mosquito as the primary vector [1]. While DENVs isolated from patients is vital in dengue disease surveillance, the complementary data from mosquitoes, including viral sequences, mosquito infection rate and serotype/genotype prevalence, has the potential to provide additional information in understanding the transmission dynamics of the DENV. For this reason, virus surveillance in field-collected mosquitoes is useful in tracking virus activity and in implementing control measures [2, 3, 4, 5].

Detection of DENV in adult female *Aedes aegypti* mosquitoes remains a challenge due to the low infection rate (typically about 0.1%) observed in adult female mosquitoes [6]. However, recent advancements in molecular virus detection techniques, particularly nucleic acid amplification tests such as RT-PCR and real-time RT-PCR assays, have enabled researchers to directly detect DENV RNA in field-collected mosquitoes [3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. The sensitivity, specificity and speed of nucleic acid amplification tests provide an alternative to the traditional method (i.e. virus isolation by cell culture) of detecting DENV. Current testing of mosquito populations for DENVs has been limited to RT-PCR of mosquito pools. Pool screening of mosquitoes has been widely utilized in mosquito-based virus surveillance programs due to its cost-effectiveness, and also, in part, due to the small amount of viral RNA recovered from a single mosquito. However, one important consideration of pool screening is the choice of pool size as the inappropriate choice of pool size may lead to inaccurate estimation of infection rates. Given that there is no generalized procedure for size pooling of mosquitoes, indicators used for estimating levels of mosquito infection, minimum infection rate (MIR) and maximal likelihood estimation (MLE), cannot warrant the accurate estimation of proportions of infected mosquitoes [22, 23].

In order to monitor the infection rate in field-collected mosquitoes with higher precision, an individual-based approach would be useful. Individual-based DENV detection using RT-PCR has been reported to be technically possible using laboratory-infected mosquitoes [7, 10]. Thus far, only one field study employed an individual-based RT-PCR approach in detecting DENV in local mosquitoes [8]. Utilizing an individual-based approach not only allows a more accurate estimation of infection rate, but also allows the direct sequencing of DENV RNA from a single mosquito that could potentially improve the robustness of constructing DENV phylogenetic trees.

By utilizing DENV sequences from mosquitoes and patients, taxa-inclusive phylogenetic analysis comprising both vector and host [24] could be obtained potentially improving our understanding of how DENV moves in a human-to-mosquito-to-human cycle. Knowledge of spatial patterns of DENV infection is generally limited because most studies only emphasize symptomatic infections and do not account for asymptomatic infections [25]. These asymptomatic infections are possibly an important component of the overall burden of dengue because these infections may likely serve as hidden reservoir hosts for mosquito infection [26] as asymptomatic people can be infectious to mosquitoes despite their lower level of viremia [27]. Viral data from field-collected mosquitoes has the potential to detect these asymptomatic infections [15] providing additional information in understanding DENV dynamics. In this context, incorporating data on infected female *Aedes aegypti* mosquitoes into the current patient-based dengue surveillance system might aid in increasing its sensitivity by enhancing its

ability to predict and prevent an outbreak as well as detect silent virus [7, 15, 19]. Moreover, analyzing DENV sequences isolated from mosquitoes and patients has the potential to reveal whether DENV is geographically constrained in the household- or community-level.

To this end, we conducted a one-month DENV surveillance in mosquitoes collected around households of suspected dengue patients during the 2015 dengue peak season in Tarlac City, Philippines in order to assess the distribution of DENVs present in the local mosquito population. We utilized mosquito virus data to serve as supporting evidence to the DENVs detected in humans during the same period. Our objectives were two-fold: (1) provide a more accurate estimate of DENV infection rate in mosquitoes by employing an individual-based one-step multiplex real-time RT-PCR assay, and (2) assess the DENV serotype and genotype distribution circulating in mosquitoes and patients during the same period. In this study, we highlight the importance of detecting and characterizing the DENV in a naturally infected mosquito and the potential of mosquito virus data alongside patient virus data in inferring DENV transmission dynamics.

Methods

Study area

This study was conducted in Tarlac City, the provincial capital of Tarlac province located in Central Luzon, Philippines. The city is situated at the center of Tarlac province and is a densely populated peri-urban area that encompasses 274.66 km² with a total population of 342, 493 inhabitants in 2015 [28]. The population density is 1,247 habitants per square kilometer. The city is composed of 76 *barangays* (i.e. village equivalent); of these, 19 *barangays* comprise the urban area as defined by the 2000 Census of Population and Housing [29]. Maps were created using the QGIS 3.6 software and data were acquired from the Philippine GIS Data website (www.philgis.org).

Patients' recruitment and laboratory diagnosis

In 2015, high prevalence of dengue occurred in Tarlac City with a total number of 1577 dengue cases (no reported deaths). For this study, febrile inpatients within 5 days from onset of symptoms and suspected of having dengue infection (Dengue fever onset: August 1 to October 31, 2015) in the Tarlac Provincial Hospital were recruited. After the informed consent was obtained, blood was collected and then serum was separated. The presence of DENV NS1 antigen was initially tested using PanBio® Dengue Early Rapid Kit (Alere Medical Co. Ltd., Massachusetts, USA) using serum. Laboratory diagnosis of dengue cases were confirmed based on virus isolation using Vero 9013 (African green monkey) cells. Ten microliters of serum were inoculated in Vero 9013 cells in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml of penicillin. Plates were incubated in 34^{o}C, 5% CO_2 and infected culture fluid (ICF) was harvested after day 7 and 14 of incubation period. Viral RNA was extracted from serum and ICF using QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) based on the manufacturer's protocol. DENV detection and serotyping were performed using a multiplex real-time RT-PCR method [30]. RT-PCR amplification of the DENV *E* gene followed by sequencing was also done as additional diagnostic evidence.

Mosquito collection

Surveillance of *Aedes aegypti* mosquitoes was purposely carried out in households of dengue suspected cases from August 26 to September 30, 2015. Households were categorized using the following conditions: (category 1) households of patients who tested positive for DENV NS1 antigen using the PanBio® Dengue Early Rapid kit (Alere Medical Co. Ltd., Massachusetts, USA) at Tarlac Provincial Hospital at the time of the mosquito collection period, (category 2) households proximal (< 150 m) to households of patients from category 1, and (category 3) households of suspected dengue patients reported by *barangay* health workers 15 days prior to the commencement of the mosquito collection period. For category 3, selection of *barangays* was based on the previous dengue epidemiology record provided by the city's health office. Identified *barangays* were San Isidro, San Miguel, San Sebastian, Maliwalo, Dalayap, San Rafael, San Nicolas, Ligtasan, San Vicente, Binauganan and Matatalaib. Based on previous years, these *barangays* had high number of reported cases in the city. All households were provided with informed consent for their voluntary participation in the mosquito surveillance. In the case of category 1, once participants agreed, mosquitoes were immediately collected within 24–48 hours after positive DENV NS1 antigen detection. Direct contact with the head of the household for house visitation and mosquito collection was done.

Commercially available mosquito UV-light traps (Mosquito Trap®, Jocanima Corporation, Metro Manila, Philippines) were used to collect mosquitoes daily from early afternoon to early morning (14:00–07:00), and the traps were installed either inside or outside the premises of the surveyed households. Inspection of installed mosquito traps and gathering of trapped mosquitoes were done daily during the morning (07:00–11:00). Sampled mosquitoes were sorted, labeled, identified and separated as male and female based on pictorial keys [30]. Identified *Aedes aegypti* mosquitoes were individually kept in a 1.5 ml tube containing 1.0 mL of RNAlater® (Ambion®, Invitrogen, California, USA), and stored at -20 °C until processed.

DENV detection in mosquitoes

Individual female mosquitoes were manually homogenized with a sterile plastic pestle in 200 µl of 1X phosphate-buffered saline (Takara Bio Inc., Shiga, Japan) in a 1.5 mL microcentrifuge tube. Total RNA was subsequently extracted from the homogenate using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan), following the manufacturer's protocol. The crude RNA was then treated with DNase using the TURBO DNA-free Kit (Ambion®, Thermo-Scientific Massachusetts, USA). DNAse-treated RNA was eluted in 30 µl nuclease-free molecular biology reagent water (Sigma-Aldrich Co., Missouri, USA) and stored at -80 °C pending analysis. Total RNA quantity and quality were verified for each sample with NanoDrop measurement (Thermo-Scientific, Massachusetts, USA).

A one-step multiplex real-time RT-PCR method [31] was adapted as the method for detection of DENV in individual *Aedes aegypti* mosquitoes. The assay was performed using the Bio-Rad CFX96 Touch™ Deep Well Real-Time PCR Detection System (Bio-Rad, California, USA). Primer and probe sequences for DENV-2 were modified, and the DENV-3 probe was labeled with Cy5.5 and BHQ2, instead of Texas Red and BHQ2 (Table S1). All assays were performed using the *iTaq* Universal Probes One-step Kit (Bio-Rad, California, USA) and carried out in 25 µl-reaction mixtures containing 5 µl total RNA, 1X reaction mix, 200 nM each of DEN-1, DEN-2, DEN-3 and DEN-4 primers, and 180 nM of each probe. Cycling conditions for all primer sets were 50 °C for 30 min, and 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 sec and 60 °C for 1 min. Negative template controls consisted of water as template. A sample was defined as positive if the average threshold cycle (Ct) value of the sample replicates was above cycle 15 and below cycle 37.

DENV nucleotide sequencing

The DENV *E* gene of both mosquito (partial sequence) and patient (full-length) samples were amplified using the primers described in Table S2. Briefly, reverse transcription of the total RNA using random primers was carried out using Superscript® III First-Strand Synthesis Supermix (Invitrogen, California, USA), and subsequent PCR amplification of the DENV *E* gene using the resulting cDNA as template was carried out using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA). RT-PCR and gene-specific PCR were performed using the Bio-Rad T100 Thermal Cycler (Bio-Rad, California, USA).

Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), according to instructions from the manufacturer. Purified PCR products of mosquito samples were sent to Eurofins Genomics, Tokyo, Japan for Sanger sequencing. For the patient samples, cycle sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA, USA) in the TaKaRa PCR Thermal Cycler Dice. Sequencing reactions were purified using a BigDye XTerminator Purification Kit (Applied BioSystems) followed by loading into Genetic DNA Analyzers 310, 3130, or 3730xl (Applied BioSystems). Bidirectional sequencing was performed using primers listed in Table S3 to resolve the full-length DENV *E* gene.

DENV infection rate in mosquitoes

The number of DENV positive mosquitoes per 1000 mosquitoes was determined from the DENV partial *E* gene PCR and sequencing results. The infection rate was calculated as the number of DENV-positive female mosquitoes divided by the total number of female mosquitoes analyzed in the study area multiplied by 1000.

Phylogenetic analyses

Mosquito-derived and patient-derived partial *E* gene sequences together with DENV reference sequences (Table S4) were aligned using ClustalW 2.1 [32] and were manually edited using Mesquite 3.3 [33]. Nucleotide sequences of the DENV isolates were submitted to the GenBank database under accession numbers MK268743-MK268752 (mosquito-derived sequences) and LC553202-LC553256 (patient-derived sequences). Phylogenetic analyses of DENV-1, DENV-2 and DENV-4 isolates were conducted using the Maximum Likelihood (ML) method. The best-fit substitution model was determined using the jModeltest [34] by Bayesian Information Criterion. ML trees were inferred using the TN93 + G parameters for DENV-1 (300 bp) and DENV-2 (258 bp), and GTR + I parameter for DENV-4 (486 bp). ML trees were constructed using PhyML 3.1 [35] and the reliability of the analyses was calculated using 1000 bootstrap replications. No outgroups were used, and DENV genotypes were grouped accordingly [36]. Trees were visualized and edited in FigTree 1.4.4 [37] and Inkscape (http://www.inkscape.org).

Results

Mosquito collection and DENV detection

In this study, 421 patients were screened for DENV NS1 antigen at the Tarlac Provincial Hospital from August to October 2015. Of the 421 patients, 187 patients tested positive for the presence of the DENV NS1 antigen. Amongst the 187 patients, 32 patients, who were residents of Tarlac City, were detected in September. During the same period, mosquito surveillance was conducted around dengue suspected cases. In brief, *Aedes aegypti* mosquitoes were collected at 48 households where dengue-infected mosquitoes were suspected to be present (Fig. 1). Of the 48 households, 12 households were grouped under category 1, 15 households were grouped under category 2, and 21 households were grouped under category 3.

A total of 583 adult *Aedes aegypti* mosquitoes were collected; of which, 383 (65.7%) were females whereas 200 (34.3%) were males. Out of the 383 female mosquitoes, 359 were processed for DENV detection due to low RNA quality and quantity of some extracted mosquito samples. Of the 359 female mosquitoes tested, 14 mosquito samples (3.9%) showed positive multiplex real-time RT-PCR results. When RT-PCR amplification and sequencing of the DENV partial *E* gene were performed, 10 mosquito samples (2.8%) were validated to be DENV-positive (Table 1). There was no clear difference in the Ct values or total RNA concentrations between the 10 validated mosquitoes and the other 4 mosquitoes that were not validated by RT-PCR amplification and sequencing.

The DENV infection rate during the one-month mosquito surveillance was calculated to be 27.9 DENV-infected mosquitoes per 1000 female *Aedes aegypti*. Six out of the 10 DENV-positive mosquitoes harbored DENV-2, 3 mosquitoes harbored DENV-4 and 1 mosquito harbored DENV-1 (Table 2). No DENV-3 was detected in the analyzed mosquito samples as there were only two DENV-3 cases detected in patients during the study period (data not shown). Notably, 4 DENV-infected mosquitoes were collected from 3 households of patients tested positive for DENV NS1 antigen, and the DENV serotypes detected from all 4 mosquitoes did not coincide with the serotypes of the patients residing in the same household where the mosquitoes were collected (Table 1).

Table 1
Mosquito samples validated to be DENV-infected using RT-PCR amplification and sequencing of the DENV partial *E* gene.

Sample code	*Barangay*	Household category	Total RNA concentration (ng/µl)	Mean Ct value	Detection	Validation		Mosquito DENV serotype	Patient DENV serotype
					One-step multiplex real-time RT-PCR	RT-PCR of partial E gene	Sequencing of partial E gene		
SI5-5	San Isidro	Category 3	43.4	23.46	+	+	+	DENV-1	-
AS2-2	San Miguel	Category 1	131.1	23.26	+	+	+	DENV-2	DENV-1/DENV-3
SI1-1	San Isidro	Category 2	83.0	15.40	+	+	+	DENV-2	-
SI6-2	San Isidro	Category 3	8.1	34.66	+	-	-	-	-
SI6-3	San Isidro	Category 3	77.1	20.69	+	+	+	DENV-2	-
SI4-4	San Isidro	Category 3	107.5	34.76	+	-	-	-	-
SB6-6	San Sebastian	Category 3	36.6	35.97	+	-	-	-	-
SB4-22	San Sebastian	Category 3	7.8	35.47	+	+	+	DENV-2	-
SB4-12	San Sebastian	Category 3	36.3	34.82	+	+	+	DENV-2	-
SB4-53	San Sebastian	Category 3	113.6	31.23	+	+	+	DENV-2	-
SB3-30	San Sebastian	Category 3	8.5	36.11	+	-	-	-	-
AS10-49	Maliwalo	Category 1	32.6	29.14	+	+	+	DENV-4	DENV-1
AS10-29	Maliwalo	Category 1	106.7	26.36	+	+	+	DENV-4	DENV-1
AS12-4	Dalayap	Category 1	122.8	36.20	+	+	+	DENV-4	-

Table 2
Detected DENV in field-caught female *Aedes aegypti* from selected households in Tarlac City (August 26 – September 30, 2015).

Household category	No. of households	No. of households with DENV-positive mosquitoes	Female mosquitoes collected	Female mosquitoes analyzed	DENV-positive mosquitoes	Infection rate per 1000	Distribution of serotype			
							DENV-1	DENV-2	DENV-3	DENV-4
Category 1	12	3	163	146	4	27.4	0	1	0	3
Category 2	15	1	43	43	1	23.3	0	1	0	0
Category 3	21	3	177	170	5	29.4	1	4	0	0
TOTAL	**48**	**7**	**383**	**359**	**10**	**27.9**	**1**	**6**	**0**	**3**

Phylogenetic relationships among DENVs isolated from mosquitoes and patients

Phylogenetic analysis revealed 3 serotypes and genotypes co-circulating in the local mosquito population during the study period, namely: DENV-1 genotype IV, DENV-2 Cosmopolitan genotype and DENV-4 genotype II (Fig. 2). Notably, the same serotypes and genotypes were present in the analyzed patient serum samples.

All DENV-1 patient samples (n = 35) and mosquito sample (n = 1) belong to Genotype IV (Fig. 2A). The only DENV-1 mosquito sample (MOS.SI5-5) isolated from San Isidro showed high nucleotide identity with DENV-1 patient samples from San Isidro, Sapang Maragul and Matatalaib within Tarlac City. In the case of DENV-2, all mosquito samples (n = 6) and patient samples (n = 16) belong to the Cosmopolitan genotype (Fig. 2B). All DENV-2 mosquito samples clustered together. One DENV-2 mosquito sample (MOS.AS22) from San Miguel formed a different cluster with a DENV-2 patient sample from San Vicente. The other 5 DENV-2 mosquito samples from San Sebastian (MOS.SB412, MOS.SB453, and MOS.SB422) and San Isidro (MOS.SI11 and MOS.SI63) formed a different cluster with a DENV-2 patient sample from San Isidro. Notably, all DENV-2 mosquito samples from San Sebastian were collected from the same household, and all showed high nucleotide similarity. Lastly, all DENV-4 mosquito samples (n = 3) and patient samples (n = 4) belong to Genotype II (Fig. 2C). The DENV-4 mosquito samples from Dalayap (MOS.AS124) and Maliwalo (MOS.AS1029 and MOS.AS1049) clustered together with the DENV-4 patient samples from the municipalities of Paniqui, Gerona and Santa Ignacia, which are 21 km, 14 km, 26 km away from Tarlac City, respectively. The DENV-4 mosquito samples from Maliwalo (MOS.AS1029 and MOS.AS1049) were collected from the same household and showed high nucleotide similarity with each other. Interestingly, the DENV-4 mosquito sample from Dalayap (MOS AS124) showed high nucleotide similarity with the patient sample from Paniqui (HUM O294), a municipality 21 km away from Tarlac City.

Phylogenetic analysis revealed no geographical segregation of DENV sequences within the survey sites in Tarlac City, with some sequences from different *barangays* or municipalities outside Tarlac City clustering together in the tree (Fig. 2) and have especially high nucleotide identities (up to 100%). Furthermore, reference strains from East Asia (China, Taiwan and Japan) and Southeast Asia (Indonesia and Singapore) were closely related to the DENV detected in mosquitoes and patients from Tarlac City.

Discussion

We have presented in this paper the utility of an individual-based DENV surveillance scheme in inferring serotype and genotype distributions in local mosquito populations and the significance of utilizing virus sequences from both vector and host in generating DENV phylogenetic trees.

In this study, we have shown that an individual-based virus detection approach using a one-step multiplex real-time RT-PCR assay can directly detect and serotype DENV in the RNA extracts of field-collected mosquitoes. This method is capable of detecting all 4 DENV serotypes in a single mosquito in one run. Our results from the RT-PCR amplification and sequencing of the DENV partial *E* gene revealed an infection rate of 27.9 DENV-infected females per 1000 female *Aedes aegypti* mosquitoes in Tarlac City during the one-month DENV surveillance. This result was comparable to the calculated (female) infection rates in previous studies where pooled mosquitoes were analyzed and mosquito collections were done in longer periods (Table S5). The relatively high infection rate we obtained may be attributed to the purposive surveillance of mosquitoes we implemented around dengue suspected cases, and also to the individual-based DENV detection approach we employed in this study. Individual-based virus detection in field-collected mosquitoes is seldom performed in mosquito surveillance studies due primarily to financial costs. However, in this study, we opted to employ an individual-based virus detection approach in order for us to more accurately estimate the infection rate in the study area, and to assess the circulating genotypes and serotypes among mosquitoes and patients at the individual level.

Our findings showed concurrent co-circulation of similar serotypes and genotypes in mosquitoes and patients, which is similar to the results of a previous study that detected the DENV in both *Aedes albopictus* mosquitoes and viraemic patient in Catalonia, Spain [38]. Sequencing and phylogenetic analyses showed that the detected DENVs grouped primarily into three genotypes, namely: (1) DENV-1 genotype IV, (2) DENV-2 Cosmopolitan genotype, and (3) DENV-4 genotype II, suggesting the hyperendemicity of dengue in Tarlac City, Philippines. These results are also consistent with the reported multiple genotypes currently co-circulating in the Philippines [39]. No DENV-3 was detected in mosquitoes at the time of the study period and this may be attributed to the low number of DENV-3 infected individuals in the study area in 2015 (data not shown). Our results suggest that there is continuous circulation of the same DENV genotypes in the Philippines over the last years since their first reported isolation implying that DENV genotype distributions remained unchanged. Since 1956, dengue has been considered a notifiable disease [40] in the Philippines, and a national program directed towards community-based prevention and control has been implemented nationwide in 1998 to combat dengue [41]. While there has been a notable increase in the reported incidence of dengue through the years, the amount of published dengue research in the country is still limited [42]. Moreover, to date, no report has been published about the circulating DENV serotypes and genotypes in local mosquito populations in the Philippines. To our knowledge, our study is the first report of a mosquito-based virus surveillance around dengue suspected cases in the Philippines. Our results underline the need for enhanced DENV surveillance to monitor the DENV transmission dynamics in the Philippines.

The major hallmark of the phylogenetic analyses we conducted in this study was the overall clustering of DENVs from various geographical locations (i.e. *barangay*, municipality) (Fig. 2). Notably, genetically related DENVs detected from mosquitoes and patients were not geographically segregated. Rather, these DENVs were randomly distributed within and outside the vicinities of Tarlac City during the same period suggesting that there is active movement of DENV in the area that may involve the transfer of both infected humans and mosquitoes. This hypothesis was further supported by the mismatch in the detected DENV serotypes between mosquitoes and patients residing in the same household (Table 1). A study conducted in Brazil harbored the same result [12] as our study indicating that most infections are obtained at other houses or public spaces such as schools or workplaces [43, 44, 45]. However, we also consider that asymptomatic infections may have significant implications to our results. Individuals residing in the same household with dengue patients may be asymptomatic and may have harbored the same serotype to that of the mosquito collected in the same household. In order to detect asymptomatic dengue infections, people in the same household of dengue cases must also be tested for DENV [46], highlighting the need for a more detailed surveillance and contact tracing of dengue index cases [47].

Tarlac City is the primary urban center of business and trade within the Tarlac province, with high concentration of commercial activities, the city likely attracts more people on a regular basis, creating hubs for disease transmission and spatial spread [48]. However, since only partial DENV *E* gene sequences were analyzed and only a subset of mosquitoes and patients in Tarlac City were used in this study, further investigation is necessary to confirm our hypothesis. A wider geographic analysis of the phylogeny of DENV in both vector and host is needed as this has considerable epidemiological significance.

We were able to show how mosquito-based virus surveillance around dengue suspected cases can be useful in the evaluation of local virus activity patterns in a defined time period and area. However, our study's small sample size is not enough to permit definitive conclusions regarding our hypothesized active transmission of DENV in Tarlac City. Also, the mosquito surveillance we conducted proved to be challenging due to limited access to patients' residences. For these reasons, our current findings suggest areas for further study and future application of mosquito surveillance around dengue suspected cases conducted with larger sample size and longer time scales to fully establish the impact of mosquito-based virus surveillance data in the prevention of human dengue cases. We were unable to obtain complete DENV *E* gene sequences from all mosquito samples due to variations of their amplification efficiency. It should also be noted that we analyzed both blood-fed and unfed mosquitoes. Detection of virus from a blood-fed female mosquito may not represent an actual occurrence of infection, but may only indicate that the mosquito had ingested viremic blood [49].

Conclusions

In conclusion, we identified the DENV genotypes and serotypes concurrently co-circulating in mosquitoes and patients and revealed that there was high infection rate of DENV in the local *A*edes *aegypti* population during the 2015 dengue peak season in Tarlac City, Philippines. Taken together, our results highlight the importance of DENV surveillance in field-collected mosquitoes, especially in evaluating local virus activity patterns in a defined time period and area. Integrating mosquito and patient viral data for phylogenetic analysis could potentially improve our understanding of how DENV moves in a human-to-mosquito-to-human cycle in a specific locality. Further studies utilizing data from naturally infected mosquitoes may provide additional information in understanding DENV transmission dynamics. In particular, DENV transmission and the evolution of DENV RNA populations may be better described by considering not only viruses from severe cases (hospitalized), but also from mild cases (outpatients), asymptomatic infections, as well as viruses from mosquitoes.

Declarations

Ethics approval and consent to participate

All participants were informed of the aims of the study and procedures involved in study participation at enrolment, and written informed consent was received before sample collection. This study was approved by the ethics committee of Tohoku University Graduate School of Medicine (2020-1-098) and the institutional review board of the Research Institute for Tropical Medicine of the Philippines (2013-017).

Consent for publication

Not applicable

Availability of data and material

All data generated or analyzed during this study are included in this published article and its supplementary files. All generated sequences are available in GenBank with accession numbers: MK268743-MK268752 (mosquito-derived sequences) and LC553202-LC553256 (patient-derived sequences).

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported in part by the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid Fund for the Promotion of Joint International Research (Fostering Joint International Research (B)) under grant number 19KK0107; the Japan Initiative for Global Research Network (J-GRID) from Japan Agency for Medical Research and Development (AMED) under grant number JP19fm0108013 and JPwm0125001, the Leading Academia in Marine and Environment Pollution Research (LaMer), Ehime University (Grant number: 30-04), the JSPS Core-to-Core Program B. Asia-Africa Science Platforms, and the Endowed Chair Program of the Sumitomo Electric Industries Group Corporate Social Responsibility Foundation.

Authors' contributions

JCB, TMC, MG, MO, HO and KW conceptualized and designed the experiments. TMC collected and identified the adult mosquito samples for the study. ADN and AKS conducted the virus detection process in patients. JCB conducted the virus detection process in mosquitoes, performed the data analysis, and wrote the original draft of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We are grateful to the patients for their participation in this study and the involved households for granting us permission to collect mosquitoes. We also extend our gratitude to Cecille Lopez-Zuasula (public health nurse of the Tarlac Provincial Hospital), to the health practitioners of the Tarlac Provincial Hospital, and to the staff of the Local Government Unit of Tarlac City for their help and support in the hospital-based patient surveillance and mosquito surveillance. Our deepest thanks also go to the Tohoku-RITM Collaborative Research Group for their assistance in the virus isolation, detection and sequencing of DENV in patient samples as well as their helpful comments in the detection and characterization of DENV in field-collected mosquitoes. We are also grateful to Katherine Viacrusis for her technical assistance with the mosquito surveillance.

Affiliations

Center for Marine Environmental Studies (CMES), Ehime University, Matsuyama, Ehime, Japan

Jean Claude Balingit (Email: jcpbalingit@gmail.com), Thaddeus M. Carvajal (Email: tads.carvajal@gmail.com), Maribet Gamboa (Email: gamboa@cee.ehime-u.ac.jp) & Kozo Watanabe (Email: watanabe.kozo.mj@ehime-u.ac.jp)

*JCB is currently affiliated to the Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Graduate School of Science and Engineering, Ehime University, Matsuyama, Ehime, Japan

Jean Claude Balingit (Email: jcpbalingit@gmail.com), Thaddeus M. Carvajal (Email: tads.carvajal@gmail.com), Maribet Gamboa (Email: gamboa@cee.ehime-u.ac.jp) & Kozo Watanabe (Email: watanabe.kozo.mj@ehime-u.ac.jp)

*JCB is currently affiliated to the Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan.

Biological Control Research Unit, Center for Natural Science and Environmental Research, De La Salle University, Taft Avenue, Manila, Philippines

Thaddeus M. Carvajal (Email: tads.carvajal@gmail.com) & Kozo Watanabe (Email: watanabe.kozo.mj@ehime-u.ac.jp)

Department of Virology, Graduate School of Medicine, Tohoku University, Sendai, Miyagi, Japan

Mariko Saito-Obata (Email: saitom@med.tohoku.ac.jp) & Hitoshi Oshitani (oshitanih@med.tohoku.ac.jp)

Tohoku-RITM Collaborative Research Center on Emerging and Reemerging Infectious Diseases, Muntinlupa City, Metro Manila, Philippines

Mariko Saito-Obata (Email: saitom@med.tohoku.ac.jp) & Amalea Dulcene Nicolasora (Email: amaleanicolasora@gmail.com)

Virology Department, Research Institute for Tropical Medicine, Muntinlupa City, Metro Manila, Philippines

Ava Kristy Sy (Email: avakristysy@gmail.com)

References

1. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science. 1988;239:476-481.
2. Victor TJ. Detection of dengue viral infections in *Aedes* mosquitoes: an essential tool for epidemiological surveillance. Indian J Med Res. 2010;129:634-636.
3. Guedes DRD, Cordeiro MT, Melo-Santos MAV, Magalhaes T, Marques E, Regis L, et al. Patient-based dengue virus surveillance in *Aedes aegypti* from Recife, Brazil. J Vector Borne Dis. 2010; 47:67-75.
4. Chen CF, Shu PY, Teng HJ, Su CL, Wu, JW, Wang, JH, et al. Screening of dengue virus in field-caught *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) by one-step SYBR green-based reverse transcriptase-polymerase chain reaction assay during 2004-2007 in southern Taiwan. Vector Borne Zoonotic Dis. 2010;10:1017-1025.
5. de Castro MG, Nogueira, RMR, Filippis, AMB, Ferreira, AA, Lima, MRQ, Faria, NRC, et al. Dengue virus type 4 in Niteói, Rio de Janeiro: the role of molecular techniques in laboratory diagnosis and entomological surveillance. Mem Inst Oswaldo Cruz. 2012;107:940-945.
6. Lambrechts L, Ferguson NM, Harris, E, Holmes EC, McGraw EA, O'Neill SL, et al. Assessing the epidemiological effect of Wolbachia for dengue control. Lancet Infect Dis. 2015;15:862-866.
7. Chow VT, Chan YC, Yong R, Lee KM, Lim LK, Chung YK, et al. Monitoring of dengue viruses in field-caught *Aedes aegypti* and *Aedes albopictus* mosquitoes by a type-specific polymerase chain reaction and cycle sequencing. Am J Trop Med Hyg. 1998;58:578-586.
8. Chung YK, Pang FY. Dengue virus infection rate in field populations of female *Aedes aegypti* and *Aedes albopictus* in Singapore. Trop Med Int Health. 2002;7:322-330.
9. Lourenço-de-Oliveira R, Honório NA, Castro MG, Schatzmayr HG, Miagostovich MP, Alves JCR, et al. Dengue virus type 3 isolation from *Aedes aegypti* in the municipality of Nova Iguaçu, State of Rio de Janeiro. Mem Inst Oswaldo Cruz. 2002;97:799-800.
10. Liotta DJ, Cabanne G, Campos R, Tonon SA. Molecular detection of dengue viruses in field-caught *Aedes aegypti* mosquitoes from northeastern Argentina. Rev Latinoam Microbiol. 2005;47(3-4):82-87.
11. Urdaneta L, Herrera F, Pernalete M, Zoghbi N, Rubio-Palis Y, Barrios R, et al. Detection of dengue viruses in field-caught *Aedes aegpyti* (Diptera: Culicidae) in Maracay, Aragua state, Venezuela by type-specific polymerase chain reaction. Infect Genet Evol. 2005;5:177-184.
12. Garcia-Rejon JE, Loroño-Pino, MA, Farfan-Ale, JA, Flores-Flores, L, Rosado-Paredes, EDP, Rivero-Cardenas, N, et al. Dengue virus-infected *Aedes aegypti* in the home environment. Am J Trop Med Hyg. 2008;79:940-950.
13. García-Rejón JE, Loroño-Pino MA, Farfán-Ale JA, Flores-Flores L, López-Uribe MP, Najera-Vazquez MR, et al. Mosquito infestation and dengue virus infection in *Aedes aegypti* females in schools in Merida, Mexico. Am J Trop Med Hyg. 2011;84:489-496.
14. Sánchez-Casas RM, Alpuche-Delgado, RH, Blitvich, BJ, Diaz-Gonzalez, EE, Ramirez-Jimenez, R, Zarate-Nahon, EA, et al. Detection of dengue virus serotype 2 in *Aedes aegypti* in Quintana Roo, Mexico, 2011. Southwest Entomol. 2013;38:109-117.
15. Méndez-Galván J, Sánchez-Casas RM, Gaitan-Burns A, Díaz-González EE, Ibarra-Juarez LA, Medina de la Garza CE, et al. Detection of *Aedes aegypti* mosquitoes infected with dengue virus as a complementary method for increasing the sensitivity surveillance: Identification of serotypes 1, 2, and 4 by RT-PCR in Quintana Roo, Mexico. Southwest Entomol. 2014;39:307-316.
16. Paingankar MS, Gokhale MD, Vaishnav KG, Shah PS, 2014. Monitoring of dengue and chikungunya viruses in field-caught *Aedes aegypti* (Diptera: Culicidae) in Surat City, India. Curr Sci. 2014;106:1559-1567.
17. Lau SM, Vythilingam I, Doss JI, Sekaran SD, Chua TH, Sulaiman WYW, et al. Surveillance of adult *Aedes* mosquitoes in Selangor, Malaysia. Trop Med Int Health. 2015;20:1271-1280.

18. Peña-García VH, Triana-Chávez O, Mejía-Jaramillo AM, Díaz FJ, Gómez-Palacio A, Arboleda-Sánchez S. Infection rates by dengue virus in mosquitoes and the influence of temperature may be related to different endemicity patterns in three Colombian cities. Int J Environ Res Public Health. 2016;13:734.
19. Pérez-Castro R, Castellanos JE, Olano VA, Matiz MI, Jaramillo JF, Vargas SL, et al. Detection of all four dengue serotypes in *Aedes aegypti* female mosquitoes collected in a rural area in Colombia. Mem Inst Oswaldo Cruz. 2016;111:233-240.
20. Medeiros AS, Costa DMP, Branco MSD, Sousa DMC, Monteiro JD, Galvao SPM, et al. Dengue virus in *Aedes aegypti* and *Aedes albopictus* in urban areas in the state of Rio Grande do Norte, Brazil: Importance of virological and entomological surveillance. PLoS ONE. 2018;13:e0194108.
21. Hoyos-Lopez R, Atencia-Pineda MC, Gallego-Gomez C. Phylogenetic analysis of dengue-2 serotypes circulating in mangroves in Northern Cordoba, Colombia. Rev Soc Bras Med Trop. 2019;52.
22. Gu W, Novak RJ. Short report: detection probability of arbovirus infection in mosquito populations. Am J Trop Med Hyg. 2004;71:636-638
23. Gu W, Unnasch TR, Katholi CR, Lampman R, Novak, RJ. Fundamental issues in mosquito surveillance for arboviral transmission. Trans R Soc Trop Med Hyg. 2008;102:817-822.
24. Rico-Hesse R. Microevolution and virulence of dengue viruses. Adv Virus Res. 2003;59:315-341.
25. Liebman KA, Stoddard, ST, Morrison, AC, Rocha, C, Minnick, S, Shihuincha, M, et al. Spatial dimensions of dengue virus transmission across interepidemic and epidemic periods in Iquitos, Peru (1999-2003). PLoS Negl Trop Dis. 2012;6:e1472.
26. Grange L, Simon-Loriere E, Sakuntabhai A, Gresh L, Paul R, Harris E. Epidemiological risk factors associated with high global frequency of inapparent dengue virus infections. Front Immunol. 2014; 5: 280.
27. Duong V, Lambrechts L, Paul RE, Ly S, Lay RS, Long KC, Huy R, Tarantola A, Scott TW, Sakuntabhai A, Buchy P. Asymptomatic humans transmit dengue virus to mosquitoes. PNAS. 2015; 112: 14688-14693.
28. Philippine Statistics Authority. 2016 Philippine Statistical Yearbook. 2016. https://www.psa.gov.ph/sites/default/files/PSY_2016.pdf. Accessed 09 April 2018.
29. National Statistics Office. 2000 Census of population and housing, Report no. 4 urban population. 2000. https://psa.gov.ph/sites/default/files/2000%20CPH-Report%20No.4%20Urban%20Population.pdf. Accessed 09 April 2018.
30. Rueda L. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission. Zootaxa. 2004;589:589.
31. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR. J Clin Microbiol. 2005;43:4977-4983.
32. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673-4680.
33. Maddison WP, Maddison DR. MESQUITE: a modular system for evolutionary analysis Version 3.61. 2019. http://mesquiteproject.org. Accessed 17 July 2019.
34. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest2: more models, new heuristics and high performance computing. Nat Methods. 2015;9:772.
35. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst Biol. 2010;59:307-321.
36. Chen R, Vasilakis N. Dengue – Quo tu et quo vadis? Viruses. 2011;3:1562-1608.
37. Rambaut A, Drummond AJ. FigTree version 1.4.4. 2009. http://tree.bio.ed.ac.uk. Accessed 17 July 2019.
38. Aranda C, Martínez MJ, Montalvo T, Eritja R, Navero-Castillejos J, Herreros E, et al. Arbovirus surveillance: first dengue virus detection in local *Aedes albopictus* mosquitoes in Europe, Catalonia, Spain, 2015. Euro Surveillance. 2018;23:pii=1700837.
39. Rabaa MA, Girerd-Chambaz, Y, Hue, KDT, Tuan, TV, Wills, B, Bonaparte, M, et al. Genetic epidemiology of dengue viruses in phase III trials of the CYD tetravalent dengue vaccine and implications for efficacy. Elife. 2017;6:e24196.
40. Dominguez NN. Current DF/DHF prevention and control programme in the Philippines. Dengue Bull. 1997;21:41-47.
41. Tomayao A. People's knowledge and practice and Aedes aegypti infestation in Cebu City, Philippines and implications for community-based dengue control. Philipp J Sci. 2000;37:79-83.
42. Bravo L, Roque VG, Brett J, Dizon R, L'Azou M. Epidemiology of dengue disease in the Philippines (2000-2011): A systematic literature review. PLoS Negl Trop Dis. 2014;8:e3027.
43. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Paz-Soldan V, Kochel TJ, Kitron U, et al. The role of human movement in the transmission of vector-borne pathogens. PLoS Negl Trop Dis. 2009;3:e481.

44. Stoddard ST, Forshey BM, Morrison AC, Paz-Soldan VA, Vazquez-Prokopec GM, Astete H, et al. House-to-house human movement drives dengue virus transmission. Proc Natl Acad Sci USA. 2013;110:994-999.
45. Zarate-Nahon EA, Ramirez-Jimenez R, Alvarado-Moreno MS, Sanchez-Casas RM, Laguna-Aguilar M, Sanchez-Rodriguez OS, et al. *Aedes aegypti* mosquitoes at nonresidential sites might be related to transmission of dengue virus in Monterrey, Northeastern Mexico. Southwest Entomol. 2013;38:465-476
46. Mammen MP, Pimgate C, Koenraadt CJM, Rothman AL, Aldstadt J, Nisalak A, et al. Spatial and temporal clustering of dengue virus transmission in Thai villages. PLoS Med. 2008;5:e205.
47. Carrington LB, Simmons CP. Human to mosquito transmission of dengue viruses. Front Immunol. 2014;5:1-8.
48. Pepin KM, et al. Utility of mosquito surveillance data for spatial prioritization of vector control against dengue viruses in three Brazilian cities. Parasit Vectors. 2015;8:98.
49. Eldridge BF. Evolutionary Relationships of Vectors and Viruses. In: Morse SS, editor. Emerging Viruses. New York: Oxford University Press, 1993. p. 252-259

Figures

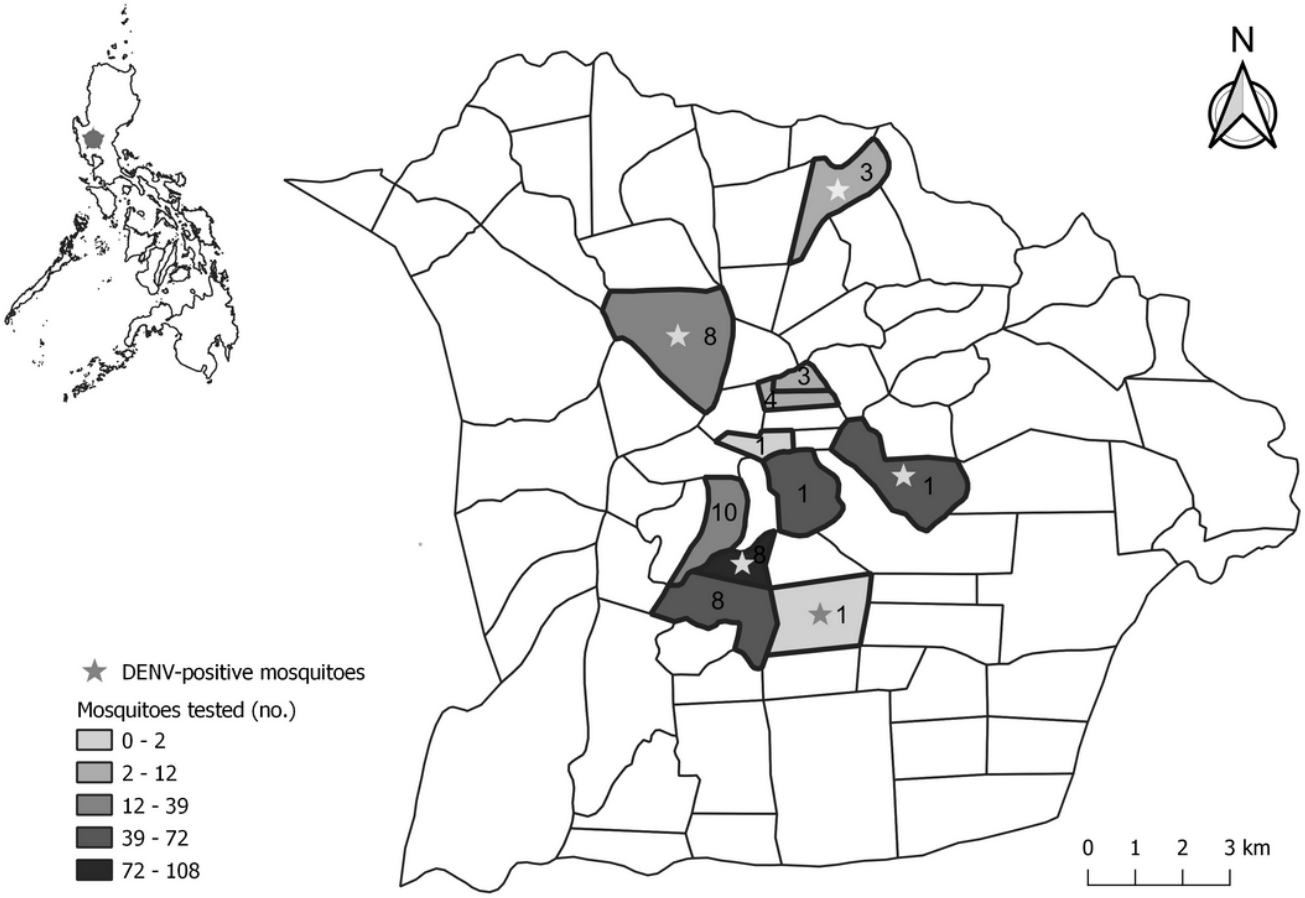

Figure 1

Location of Tarlac City in the Philippine map (upper left) and choropleth map of Tarlac City. The surveyed barangays (n = 11) where female Aedes aegypti mosquitoes were collected were shown with bold boundary with the number of surveyed households (n = 48). Star mark indicates the barangay where the virus positive mosquitos were detected.

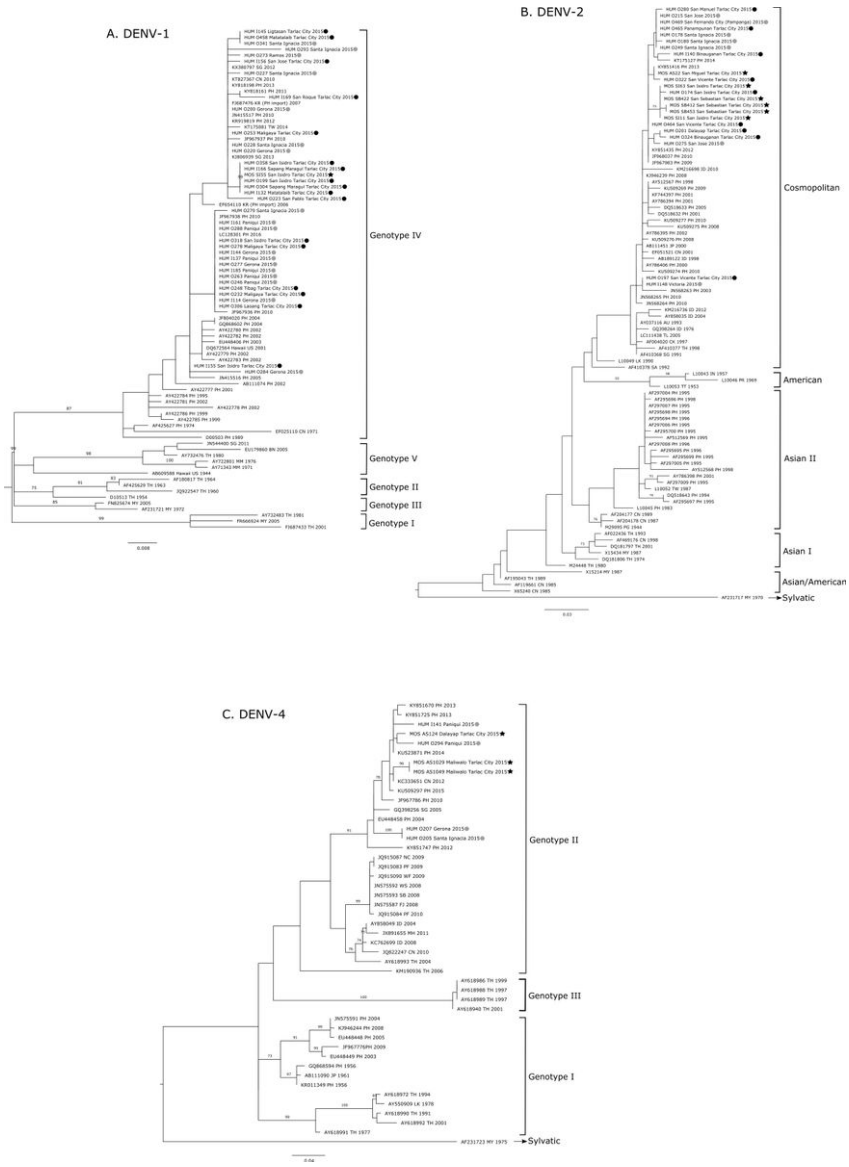

Figure 2

Phylogenetic trees of the partial E gene of DENV-1 (A), DENV-2 (B) and DENV-4 (C). The trees were inferred with the maximum likelihood criterion. Node support was evaluated with 1000 bootstrap replicates. Bootstrap values more than 70% were shown on the branches. DENV mosquito (black star) and patient sequences from Tarlac City (black circle) and other neighboring municipalities (grey circle) collected from August 1 to October 31, 2015 were included in the tree. Reference sequences are labeled by their NCBI accession numbers, two-letter ISO country code, and corresponding year of isolation. Scale bar indicates nucleotide substitutions per site.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx
- TableS2.docx
- TableS3.docx
- TableS4.docx
- TableS5.docx