Individual-based dengue virus surveillance in Aedes aegypti mosquitoes concurrently collected 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

DOI: https://doi.org/10.21203/rs.3.rs-56950/v3

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

**Background:** Vector control measures are critical in the prevention and reduction of dengue virus (DENV) transmission. In this context, an effective vector control is reliant not only on the knowledge of mosquito abundance, but also on the timely and accurate detection of mosquito infection. Mosquito-based virus surveillance programs typically rely on a pool-based mosquito testing, although whether individual-based mosquito testing could represent a feasible alternative is not largely studied. Applying an individual-based mosquito testing approach, we conducted a one-month DENV surveillance of adult *Aedes (Ae.) aegypti* mosquitoes around households of suspected dengue patients during the 2015 dengue peak season in Tarlac City, Philippines to more accurately assess the mosquito infection rate and identify the DENV serotypes and genotypes concurrently co-circulating in mosquitoes and patients.

**Methods:** We performed a one-step multiplex real-time reverse transcription-polymerase chain reaction (RT-PCR) assay for the simultaneous DENV detection and serotyping in patients and individual female *Ae. aegypti* mosquito. Additionally, 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 *Ae. aegypti* mosquitoes; of which, we individually tested 359 female mosquitoes for the presence of DENV. Ten (2.8%) among the 359 female mosquitoes were positive for the presence of 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.

**Conclusions:** In this study, we demonstrate the utility of the one-step multiplex real-time RT-PCR assay in the individual-based DENV surveillance of mosquitoes. Our findings reinforce the significance of detecting and monitoring virus activity in local mosquito populations, which is critical for dengue prevention and control activities.

**Background**

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 the tropical and subtropical regions of the world, with the *Ae. aegypti* mosquito as the primary vector [1]. While DENV isolation from patients is vital in dengue disease surveillance, the complementary data from mosquitoes, including viral sequences, mosquito infection rate, and serotype/genotype prevalence, have the potential to provide additional information in understanding the transmission dynamics of DENV. For this reason, virus surveillance in field-collected mosquitoes is useful in tracking virus activity and implementing control measures [2-5].

The detection of DENV remains a challenge owing to the low infection rate (typically approximately 0.1%) observed in adult female *Ae. aegypti* 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 [3-5,7-21]. Current testing of mosquito populations for DENVs has been limited to the RT-PCR of mosquito pools. Pool screening of mosquitoes has been widely utilized in mosquito-based virus surveillance programs owing to its cost-effectiveness, and also, in part, due to the small amount of viral RNA recovered from a single mosquito. However, one significant consideration of pool screening is the choice of pool size, as the inappropriate selection of pool size may lead to an inaccurate estimation of infection rates. Given that no generalized procedure for the size pooling of mosquitoes, indicators used for estimating the 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].
An individual-based approach would be useful to monitor the infection rate with higher precision. The individual-based DENV detection using RT-PCR has been reported to be technically possible using laboratory-infected mosquitoes [7,18]. To date, only two field studies employed an individual-based RT-PCR approach in detecting DENV in mosquitoes [5,8]. Utilizing an individual-based approach not only allows a more accurate infection rate estimation, but also allows a direct DENV RNA sequencing from a single mosquito for further genotypic characterization.

Analyzing DENV sequences from both mosquitoes and patients potentially improves our understanding of the genetic relationships of circulating DENVs. Most studies only emphasize symptomatic infections and do not account for asymptomatic infections, which have been increasingly contributing to the overall burden of dengue. A previous study demonstrated that asymptomatic people can be infectious to mosquitoes despite their lower level of viremia [24], thereby raising the possibility of asymptomatic infections serving as hidden reservoir hosts for mosquito infections [25,26], likely dispersing DENV in the process. Methodologies that account for these undetected infections are, therefore, warranted in dengue surveillance programs. In this context, the viral data from field-collected mosquitoes have the potential to detect these asymptomatic infections [15]. Incorporating the data of infected female *Ae. aegypti* mosquitoes into the current patient-based dengue surveillance system aids in increasing the sensitivity of the surveillance system by enhancing its ability to predict and prevent an outbreak as well as detect silently circulating virus [7,15,19].

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 to assess the distribution of DENVs present in the local mosquito population. We utilized the data of mosquito virus to serve as supporting evidence to the DENVs detected in humans during the same period. Our objectives were two-fold: to (1) provide a more accurate DENV infection rate estimate 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 potential of individual-based mosquito testing in DENV surveillance and the significance of detecting and characterizing DENVs in naturally infected mosquitoes concurrent with dengue patients for inferring local virus activity in a defined time period and area.

**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 a 274.66 km² land area with a total population of 342,493 inhabitants in 2015 [27]. The population density is 1,247 inhabitants 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 [28]. Maps were created using the QGIS 3.6 software and edited in Inkscape (http://www.inkscape.org), with some figures created with BioRender (http://biorender.com). Data for creating the map were acquired from the Philippine GIS Data website (www.philgis.org).

**Recruitment and laboratory diagnosis of patients**

In 2015, a high prevalence of dengue occurred in Tarlac City with a total number of 1,577 dengue cases (no reported deaths). For this study, febrile inpatients within 5 days from the onset of symptoms and suspected of having dengue infection (Dengue fever onset: from August 1 to October 31, 2015) in the Tarlac Provincial Hospital were recruited. After an informed consent was obtained, blood was collected and 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 the serum. A laboratory diagnosis of dengue cases were confirmed based on virus isolation using Vero 9013 (African green monkey) cells. Ten microliters of the serum were inoculated in the Vero 9013 cells in a minimum essential medium supplemented with 10% fetal bovine serum and 100 U/mL of penicillin. Plates were incubated at 34°C and 5% CO₂, and an infected culture fluid (ICF)
was harvested after days 7 and 14 of the incubation period. Viral RNA was extracted from the serum and ICF using the 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 [29]. The RT-PCR amplification of the DENV E gene followed by sequencing was also performed to provide an additional diagnostic evidence.

Mosquito collection

The surveillance of Ae. aegypti mosquitoes was performed in households of dengue suspected cases from August 26 to September 30, 2015. The 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 the Tarlac Provincial Hospital during the mosquito collection period; (category 2) households proximal (< 150 m) to the 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, the selection of barangays was based on the previous dengue epidemiology record provided by the city health office. The 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 a high number of reported cases in the city. All the households were provided with informed consent for their voluntary participation in the mosquito surveillance. In category 1, once participants consented, mosquitoes were immediately collected within 24–48 h after a positive DENV NS1 antigen detection. Direct contact with the head of the household for house visitation and mosquito collection was conducted.

Commercially available mosquito Ultraviolet (UV) light traps (Mosquito Trap®, Jocanima Corporation, Metro Manila, Philippines) were used to collect mosquitoes as previously described [30,31]. The trap emits UV light and generates heat and CO2 gas via a photocatalytic reaction on the TiO2 coated funnel. Decoyed mosquitoes enter the trap through the capture windows and then strongly drawn into the capture net by a strong current produced by the ventilator. The UV light traps collected mosquitoes daily from early afternoon to early morning (14:00 – 07:00) and were installed either inside or outside the premises of the surveyed households. One mosquito trap was installed for each household. The inspection of installed mosquito traps and gathering of trapped mosquitoes were performed every morning daily (07:00 – 11:00). Sampled mosquitoes were sorted, labeled, identified, and separated as male and female based on pictorial keys [32]. The identified Ae. 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 a 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 ISOGN (Nippon Gene Co., Ltd., Toyama, Japan), following the manufacturer's protocol. Crude RNA was then treated with DNase using the TURBO DNA-free Kit (Ambion®, Thermo-Scientific Massachusetts, USA). DNase-treated RNA was eluted in a 30-μL nuclease-free molecular biology reagent water (Sigma-Aldrich Co., Missouri, USA) and stored at -80°C pending analysis. The quantity and quality of the total RNA were verified for each sample with the NanoDrop measurement (Thermo Fisher Scientific, Massachusetts, USA).

A one-step multiplex real-time RT-PCR method [29] was adapted for DENV detection in individual Ae. 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 (Table S1) in this protocol from the original method [29], with few nucleotide bases either revised or deleted based on the consensus sequence of currently major circulating DENV-2 strains. Instead of Texas Red and BHQ2, the DENV-3 probe was labeled with Cy5.5 and BHQ2 (Table S1). All assays were performed using the iTaq Universal Probes One-step Kit (Bio-Rad, California, USA) and conducted in 25 μL reaction mixtures containing 5 μL of total RNA, 1x reaction mix, 200 nM each of DENV-1, DENV-2, DENV-3, and DENV-4 primers, and 180 nM of
each probe. The one-step multiplex real-time RT-PCR assay was performed once in duplicates. The cycling conditions for all primer sets were 50°C and 95°C for 30 and 2 min, respectively, followed by 45 cycles of 95°C and 60°C for 15 sec and 1 min, respectively. 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, a reverse transcription of the total RNA using random primers was conducted using the Superscript® III First-Strand Synthesis SuperMix (Invitrogen, California, USA), and a subsequent PCR amplification of the DENV \( E \) gene using the resulting cDNA as template was performed using the Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA). The 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. The 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 the 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 the BigDye XTerminator Purification Kit (Applied BioSystems) and loaded into Genetic DNA Analyzers 310, 3130, or 3730xl (Applied BioSystems). Bidirectional sequencing was performed using the 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 1,000 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 1,000.

**Phylogenetic analyses**

Mosquito-derived and patient-derived partial \( E \) gene sequences together with DENV reference sequences (Table S4) were aligned using ClustalW 2.1 [33] and manually edited using Mesquite 3.3 [34]. The nucleotide sequences of the DENV isolates were submitted to the GenBank database under the accession numbers MK268743-MK268752 (mosquito-derived sequences) and LC553202-LC553256 (patient-derived sequences). The 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 [35] by the 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). The ML trees were constructed using PhyML 3.1 [36] and the reliability of the analyses was calculated using 1000 bootstrap replications. No outgroups were used, and DENV isolates were grouped accordingly to genotypes as previously described [37]. The trees were visualized and edited in FigTree 1.4.4 [38] 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 tested positive for the presence of the DENV NS1 antigen. Of the 187 patients, 32, who were Tarlac City residents, were detected in September. During the same period, mosquito surveillance was conducted around the residences of the dengue suspected cases. In brief, \( Ae. \) aegypti mosquitoes were collected at 48 households where dengue-
infected mosquitoes were suspected to be present (Figure 1). Of the 48 households, 12, 15, and 21 households were grouped under categories 1, 2, and 3, respectively.

A total of 583 adult *Ae. aegypti* mosquitoes were collected; of which, 383 (65.7 %) were female and 200 (34.3 %) were male mosquitoes. The average number of captured mosquitoes per trap was 1.63 ± 2.66 per day, and the highest number of captured adult *Ae. aegypti* mosquitoes in one day was 31. Of the 383 female mosquitoes, 359 were processed for DENV detection owing to a low RNA quality and quantity of some extracted mosquito samples. Of the 359 female mosquitoes tested, 14 (3.9 %) showed positive multiplex real-time RT-PCR results. When the 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). A clear difference was noted in the Ct values between the 10 validated mosquitoes and the other 4 mosquitoes that were not validated by the RT-PCR amplification and sequencing. The Ct values for the samples that did not yield the DENV partial E gene sequence were ≥ 35, which was generally classified as a negative result.

The DENV infection rate during the one-month mosquito surveillance was calculated to be 27.9 DENV-infected mosquitoes per 1,000 female *Ae. 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 2 DENV-3 cases detected in patients during the study period (data not shown). Notably, 4 DENV-infected mosquitoes were collected from three households of patients who 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).

**Phylogenetic relationships among DENVs isolated from mosquitoes and patients**

Phylogenetic analysis revealed three 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 (Figure 2). Notably, the same serotypes and genotypes were present in the analyzed patient serum samples. DENV sequences among the sampled mosquitoes and patients have especially high nucleotide identities (up to 100 %). High bootstrap values (70 % – 100 %) were also observed to indicate robust support for the tree topology. DENV sequences isolated from the sampled mosquitoes and patients were closely related to reference strains from East Asia (China, Taiwan, Japan) and neighboring Southeast Asian countries (Indonesia and Singapore).

All DENV-1 patient samples (n = 35) and mosquito sample (n = 1) belong to Genotype IV (Figure 2A), which is the only DENV-1 genotype reported to be circulating in the Philippines [39,40]. In DENV-2, all mosquito (n = 6) and patient samples (n = 16) belong to the Cosmopolitan genotype (Figure 2B), which is currently the only circulating DENV-2 genotype in the Philippines displacing the Asian II genotype in the early 2000 since its first isolation in 1998 [40,41]. Lastly, all DENV-4 mosquito (n = 3) and patient samples (n = 4) belong to Genotype II (Figure 2C), which is one of the two DENV-4 genotypes currently co-circulating in the Philippines [39,40]. The other DENV-4 genotype isolated in the country is Genotype I [39,40], which was not detected in this study.

**Discussion**

Mosquito-based virus surveillance is an integral component of dengue disease control as it is a significant tool in monitoring and understanding local virus activity. In this study, we presented the utility of an individual-based DENV surveillance approach in inferring the infection rate and genotypes of circulating DENVs in field-collected mosquitoes. We demonstrated that the detected DENVs in mosquitoes correlated with that of the circulating DENVs in patients, highlighting the significance of mosquito virus data in inferring local virus activity in a defined time period and area.

The major hallmark of this study is the individual-based mosquito testing we employed for the simultaneous detection and serotyping of DENV in the RNA extracts of field-collected mosquitoes. Previous field studies detected the DENV in individual
mosquitoes by using either a semi-nested RT-PCR assay [8] or a commercial duplex real-time RT-PCR dengue kit [5]. In this study, we demonstrated that a one-step multiplex real-time RT-PCR assay [29] could be a potential surveillance tool in DENV monitoring of individual mosquitoes as the method is capable of detecting all 4 DENV serotypes in a single mosquito in one run. This assay detects the presence of viral RNA in mosquito samples in approximately 2 h, eliminating the need to perform gel electrophoresis as fluorescent probes directly detect the amplified target. Based on the Ct cut-off value previously described [29], 14 mosquitoes tested positive using the one-step multiplex real-time RT-PCR assay; however, only 10 mosquitoes were validated to be positive through subsequent RT-PCR amplification and sequencing of the DENV partial E gene. Mosquitoes (n = 4) that were not validated to be DENV-positive showed Ct values ≥ 35, which may indicate a negative result. Alternatively, the high Ct value may also be due to low virus levels present in the mosquito, which was insufficient for detection using a conventional RT-PCR assay for sequencing purposes. In this context, a real-time RT-PCR assay should be used as a screening step and not as an exclusive analytical method in detecting DENV in mosquitoes. We performed serotype-specific RT-PCR amplification and sequencing of the DENV partial E gene to serve as confirmatory steps, thereby facilitating the direct genotypic characterization of DENV in a single mosquito.

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 1,000 female Ae. aegypti mosquitoes in Tarlac City during the one-month DENV surveillance. This infection rate is relatively high compared with the calculated (female) infection rates in previous field studies, which tested mosquito pools and performed mosquito surveillance in longer periods (Table S5). The MIR values in previous studies were mainly low and varied considerably according to the pool size of mosquitoes tested (Table S5). The relatively high infection rate we obtained in this study may be attributed to the targeted surveillance we implemented around residences of dengue suspected cases. Additionally, the individual-based mosquito testing we employed might have contributed to the observed high infection rate. Individual-based mosquito testing is seldom performed in virus surveillance studies primarily owing to logistic and financial reasons. In this study, we opted to employ an individual-based approach to more accurately estimate the infection rate in the study area. Although we were unable to assess the difference in the calculated infection rates between the individual-based and pool-based mosquito testing due to a limited sample volume, we argue that the pooled mosquito testing struggled with lower sensitivity than the individual mosquito testing due to dilution effect, wherein the DENV RNA from an infected mosquito was diluted by uninfected mosquitoes in the pool and can approach the limit of detection for the test. Previous studies reported the inefficiency of pool-based infection rate indicators, such as MIR and MLE, in detecting infection rates as these indicators highly depend on pool size, sample size (number of mosquitoes tested), and disease prevalence in the area [22,23,42]. For instance, MIR is highly efficient during low levels of virus circulation; however, during periods of high transmission, MIR will generally underestimate mosquito infection [42]. Since we collected mosquitoes during the dengue peak season, using MIR would likely underestimate our calculated infection rate. Then again, we also consider the possible effect of the mosquito trapping method we used in calculating the infection rate. Mosquito abundance in traps are not only affected by factors, such as temperature [43], rainfall [44], and structure of urban landscapes [45,46], but also with the trapping method used. In this study, we utilized a commercial mosquito UV light trap (that is able to generate CO₂) because it is easy to use, easy to purchase, inexpensive, and uses electricity from within a home. Although previous field studies used the same trapping method [30,31], no definitive study has shown the efficiency of UV light traps (baired with CO₂) in collecting Ae. aegypti mosquitoes. Ae. aegypti mosquitoes are diurnal species that occupy distinct time-of-day niches, and conventionally, these species are non-specifically attracted to UV light, thus, it is possible that the type of trapping method we utilized affected the number of collected Ae. aegypti mosquitoes. Because not all mosquitoes were equally captured, the trapping method we employed may have introduced a bias that affected the relationship between the actual mosquito infection prevalence and the estimated mosquito infection rate [42].

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 Ae. albopictus mosquitoes and viremic patients in Catalonia, Spain [47]. Sequencing and phylogenetic analyses showed that the detected DENVs were 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. Furthermore, these results are 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, which may be attributed to the low number of DENV-3 infected individuals in the study area in 2015
(data not shown). In the Philippines, the persistence of a single genotype of DENV-1 (Genotype IV) is exhibited since
1974 [40]. DENV-2, on the other hand, exhibited a genotypic shift from Asian II to Cosmopolitan genotype in the early 2000,
and the Cosmopolitan genotype has since persisted thereafter [40,41], while DENV-4 displayed a temporal domination of
Genotype II, with minor Genotype I co-circulation during the last 10 years [40]. Our results, therefore, suggest that there is a
continuous circulation of the same DENV genotypes in the Philippines, implying that DENV genotype distributions remained
unchanged. Since 1956, dengue has been considered as a notifiable disease [48] in the Philippines. A national program
directed toward community-based prevention and control has been implemented nationwide in 1998 to combat dengue [49].
While a notable increase in the reported incidence of dengue has been observed through the years, the amount of published
dengue research in the country remains limited [50]. 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 residences of dengue suspected cases in the Philippines. Our results
underline the need for an enhanced DENV surveillance to monitor the DENV transmission dynamics in the Philippines.

A noteworthy result in this study is the difference we observed 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 [51-53]. This
result corroborates with the notion that DENV transmission is likely driven by the movement of infected humans, rather than
infected mosquitoes [54,55]. Considering the role of asymptomatic infections in DENV transmission, asymptomatic
infections may also have significant implications to these results. Individuals residing in the same household with dengue
patients may be asymptomatic and may have reported the same serotype to that of the mosquito collected in the same
household. To detect asymptomatic dengue infections, people in the same household of dengue cases must also be tested
for DENV [56], highlighting the need for a more detailed surveillance and contact tracing of dengue index cases [57].

Our study provides useful insights regarding the feasibility of an individual-based mosquito testing in the DENV surveillance;
however, some limitations should be considered. First, the mosquito surveillance we conducted proved to be challenging
owing to a limited access to residences of patients. We only tested a small subset of mosquitoes and patients; therefore, our
current findings suggest areas for further study and future application of mosquito-based virus surveillance around
residences of dengue suspected cases conducted with larger sample size and longer time scales to fully establish the
impact of mosquito viral data in the prevention of human dengue cases. Moreover, our study would have provided additional
novelty in understanding the dynamics of DENV transmission had the whole genomes been sequenced from individual
mosquitoes and patients. Owing to a limited sample volume and variations in amplification efficiency, we were only able to
sequence the partial E gene for DENV genotyping in mosquitoes. Additionally, we were not able to record the blood meal
status of the mosquitoes. We indiscriminately analyzed both blood-fed and unfed mosquitoes. The detection of virus from a
blood-fed female mosquito may not represent an actual infection occurrence, but may only indicate that the mosquito had
ingested viremic blood [58]. Since our results demonstrated a mismatch in the DENV serotypes of mosquitoes and patients
residing in the same household, it is possible that the blood-fed mosquitoes fed on the blood of asymptomatic individuals.
This may represent a significant parameter to be determined in future studies. Lastly, although the focus of this study is on
Ae. aegypti mosquitoes, it would also be relevant to address the role of Ae. albopictus in the transmission of DENV. Both
mosquito species are reported to co-exist in the country [59–62]. Considering the vector competence of Ae. albopictus for
DENV [63], checking the role of this mosquito species in the maintenance of DENV circulation in peri-urban municipalities,
like Tarlac City, would be a vital point to examine in future studies.

Conclusions

In conclusion, we demonstrated in this study that an individual-based mosquito testing using a one-step multiplex real-time
RT-PCR assay could be a potential tool in a mosquito-based DENV surveillance. Using this approach, we identified the DENV
genotypes and serotypes concurrently co-circulating in mosquitoes and patients and revealed that a high DENV infection rate was present in the local *Ae. aegypti* population during the 2015 dengue peak season in Tarlac City, Philippines. While we have provided evidence for the continued circulation of the same DENV genotypes in the Philippines, a mosquito and patient surveillance conducted in a larger population and broader setting is needed to fully understand the dynamics of circulating DENV genotypes in the country. Taken together, our results reinforce the significance of DENV surveillance in field-collected mosquitoes, especially in the evaluation of local virus activity in a defined time period and area. Phylogenetic similarities of circulating DENVs in a particular geographic region 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.

**Abbreviations**

*Ae. aegypti*: *Aedes aegypti*

*Ae. albopictus*: *Aedes albopictus*

**cDNA**: complementary DNA

**DENV**: dengue virus

**E** gene: envelope gene

**ML**: maximum likelihood

**MLE**: maximum likelihood estimation

**MIR**: minimum infection rate

**NS1**: nonstructural protein 1

**PCR**: polymerase chain reaction

**RT-PCR**: reverse transcription-polymerase chain reaction

**RNA**: ribonucleic acid

**UV**: ultraviolet

**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. A written informed consent was received before sample collection. This study was approved by the Ethics Committee of the 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. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

**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.

**Acknowledgments**

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 Titus Tan and 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. JCB is a recipient of the Japanese Government (Monbukagakusho) Scholarship from the Ministry of Education, Science Sport and Culture of Japan.

**References**


### Tables

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

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Barangay</th>
<th>Household category</th>
<th>Total RNA concentration (ng/μl)</th>
<th>Mean Ct value</th>
<th>Detection</th>
<th>Validation</th>
<th>Mosquito DENV serotype</th>
<th>Patient DENV serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI5-5</td>
<td>San Isidro</td>
<td>Category 3</td>
<td>43.4</td>
<td>23.46</td>
<td>+</td>
<td>+</td>
<td>DENV-1</td>
<td>-</td>
</tr>
<tr>
<td>AS2-2</td>
<td>San Miguel</td>
<td>Category 1</td>
<td>131.1</td>
<td>23.26</td>
<td>+</td>
<td>+</td>
<td>DENV-2 DENV-1/DENV-3</td>
<td>-</td>
</tr>
<tr>
<td>SI1-1</td>
<td>San Isidro</td>
<td>Category 2</td>
<td>83.0</td>
<td>15.40</td>
<td>+</td>
<td>+</td>
<td>DENV-2</td>
<td>-</td>
</tr>
<tr>
<td>SI6-2</td>
<td>San Isidro</td>
<td>Category 3</td>
<td>8.1</td>
<td>34.66</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SI6-3</td>
<td>San Isidro</td>
<td>Category 3</td>
<td>77.1</td>
<td>20.69</td>
<td>+</td>
<td>+</td>
<td>DENV-2</td>
<td>-</td>
</tr>
<tr>
<td>SI4-4</td>
<td>San Isidro</td>
<td>Category 3</td>
<td>107.5</td>
<td>34.76</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB6-6</td>
<td>San Sebastian</td>
<td>Category 3</td>
<td>36.6</td>
<td>35.97</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SB4-22</td>
<td>San Sebastian</td>
<td>Category 3</td>
<td>7.8</td>
<td>35.47</td>
<td>+</td>
<td>+</td>
<td>DENV-2</td>
<td>-</td>
</tr>
<tr>
<td>SB4-12</td>
<td>San Sebastian</td>
<td>Category 3</td>
<td>36.3</td>
<td>34.82</td>
<td>+</td>
<td>+</td>
<td>DENV-2</td>
<td>-</td>
</tr>
<tr>
<td>SB4-53</td>
<td>San Sebastian</td>
<td>Category 3</td>
<td>113.6</td>
<td>31.23</td>
<td>+</td>
<td>+</td>
<td>DENV-2</td>
<td>-</td>
</tr>
<tr>
<td>SB3-30</td>
<td>San Sebastian</td>
<td>Category 3</td>
<td>8.5</td>
<td>36.11</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AS10-49</td>
<td>Maliwalo</td>
<td>Category 1</td>
<td>32.6</td>
<td>29.14</td>
<td>+</td>
<td>+</td>
<td>DENV-4 DENV-1</td>
<td>DENV-1</td>
</tr>
<tr>
<td>AS10-29</td>
<td>Maliwalo</td>
<td>Category 1</td>
<td>106.7</td>
<td>26.36</td>
<td>+</td>
<td>+</td>
<td>DENV-4 DENV-1</td>
<td>DENV-1</td>
</tr>
<tr>
<td>AS12-4</td>
<td>Dalayap</td>
<td>Category 1</td>
<td>122.8</td>
<td>36.20</td>
<td>+</td>
<td>+</td>
<td>DENV-4</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 2** Detected DENV in field-collected female *Aedes aegypti* from selected households in Tarlac City (August 26 – September 30, 2015).
<table>
<thead>
<tr>
<th>Household category</th>
<th>No. of households</th>
<th>No. of households with DENV-positive mosquitoes</th>
<th>Female mosquitoes collected</th>
<th>Female mosquitoes analyzed</th>
<th>DENV-positive mosquitoes</th>
<th>Infection rate per 1000</th>
<th>Distribution of serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Category 1</td>
<td>12</td>
<td>3</td>
<td>163</td>
<td>146</td>
<td>4</td>
<td>27.4</td>
<td>0 1 0 3</td>
</tr>
<tr>
<td>Category 2</td>
<td>15</td>
<td>1</td>
<td>43</td>
<td>43</td>
<td>1</td>
<td>23.3</td>
<td>0 1 0 0</td>
</tr>
<tr>
<td>Category 3</td>
<td>21</td>
<td>3</td>
<td>177</td>
<td>170</td>
<td>5</td>
<td>29.4</td>
<td>1 4 0 0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>48</td>
<td>7</td>
<td>383</td>
<td>359</td>
<td>10</td>
<td>27.9</td>
<td>1 6 0 3</td>
</tr>
</tbody>
</table>