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

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Abstract

Background: Vector control measures are critical in the prevention and reduction of dengue virus (DENV) transmission. In this context, effective vector control is reliant not only on knowledge of mosquito abundance, but also on the timely and accurate detection of mosquito infection. Mosquito-based virus surveillance programs commonly rely on pool-based mosquito testing, but 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 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 to identify the DENV serotypes and genotypes 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 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 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.

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

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 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, 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 [2-5].

Detection of DENV in adult female *Ae. 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 [3-5,7-21]. 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 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 [7,18]. So

far, 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 estimation of infection rate, but also allows the direct sequencing of DENV RNA 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. Majority of literatures only emphasize symptomatic infections and do not account for asymptomatic infections, which are increasingly contributors 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] 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, viral data from field-collected mosquitoes has the potential to detect these asymptomatic infections [15].

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 potential of individual-based mosquito testing in DENV surveillance and the importance of detecting and characterizing DENVs in naturally infected mosquitos in concert 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 274.66 km² with a total population of 342, 493 inhabitants in 2015 [27]. 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 [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).

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 [29]. RT-PCR amplification of the DENV *E* gene followed by sequencing was also done as additional diagnostic evidence.

Mosquito collection

Surveillance of *Ae. 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 as previously described [30,31]. The trap emits UV light, and generates heat and CO_2 gas via a photocatalytic reaction on the TiO_2 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 mosquito 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. For each household, one mosquito trap was installed. Inspection of installed mosquito traps and gathering of trapped mosquitoes were performed daily each morning (07:00-11:00). Sampled mosquitoes were sorted, labeled, identified and separated as male and female based on pictorial keys [32]. 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 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 [29] was adapted as the method for detection of DENV 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 a few nucleotide bases either revised or deleted based on the consensus sequence of currently major circulating DENV-2 strains. 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. The one-step multiplex real-time RT-PCR assay was performed once in duplicates. 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 [33] and were manually edited using Mesquite 3.3 [34]. 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 [35] 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 [36] and the reliability of the analyses was calculated using 1000 bootstrap replications. No outgroups were used, and DENV isolates grouped accordingly to genotypes as previously described [37]. 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 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 (Figure 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 *Ae. aegypti* mosquitoes were collected; of which, 383 (65.7%) were females whereas 200 (34.3%) were males. The average number of captured mosquitoes per trap was 1.63 ± 2.66 per day, and the highest number of captured mosquitoes in one day is 31 adult *Ae. aegypti* mosquitoes. 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 a clear difference in the Ct values between the 10 validated mosquitoes and the other 4 mosquitoes that were not validated by RT-PCR amplification and sequencing. The Ct values for the samples that did not yield the DENV partial E gene sequence were

The DENV infection rate during the one-month mosquito surveillance was calculated to be 27.9 DENV-infected mosquitoes per 1000 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 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).

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 (Figure 2). Notably, the same serotypes and genotypes were present in the analyzed patient serum samples. DENV sequences among sampled mosquitoes and patients have especially high nucleotide identities (up to 100%). High bootstrap values (70-100%) were also observed indicating robust support for the tree topology. DENV sequences isolated from 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 the case of DENV-2, all mosquito samples (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 samples (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 an important 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 correlate to that of the circulating DENVs in patients highlighting the importance 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 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 hours, eliminating the need to perform gel electrophoresis as fluorescent probes directly detect the amplified target. Based on the Ct value cut-off described previously [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. The 4 mosquitoes that were not validated to be DENV-positive showed Ct values of ≥35, which is generally considered to be negative in most laboratories. This particular result may also be due to the low viral titer present in the infected mosquito, which can only be detected by real-time RT-PCR, but not sufficient enough to be detected by conventional RT-PCR for sequencing purposes. In this context, real-time RT-PCR 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 1000 female *Ae. aegypti* mosquitoes in Tarlac City during the one-month DENV surveillance. This infection rate is relatively high in comparison to 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 are mainly low and vary considerably according to pool size of mosquitoes tested (Table S5). The relatively high infection rate we obtained in this study may be attributed to the purposive 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 due primarily 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 individual-based and pool-based mosquito testing due to limited sample volume, we argue that Then again, we also consider the possible effect of the mosquito trapping method we used in this study 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 CO_2) because it is easy to use, easy to purchase, inexpensive and it uses electricity from within a home. Although previous field studies used the same trapping method in collecting *Ae. aegypti* [30,31], there is still no definitive study showing the efficiency of UV-light traps (baited with CO_2) in collecting *Ae. aegypti* mosquitoes. *Ae. aegypti* mosquitoes are diurnal species that occupy distinct time-of-day niches, and the conventional knowledge is that these species are non-specifically attracted to UV light, hence it is possible that the type of trapping method we utilized affected the number of *Ae. aegypti* mosquitoes we collected. Additionally, because not all mosquitoes are 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 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). In the Philippines, persistence of a single genotype of DENV-1 (Genotype IV) is exhibited since 1974 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.

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 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 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 [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 individual-based mosquito testing in DENV surveillance; however, some limitations should be considered. First, the mosquito surveillance we conducted in this study proved to be challenging due to limited access to patients' residences. We only tested a small subset of mosquitoes and patients; hence our current findings suggest areas for further study and future application of mosquito-based virus surveillance around 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. Due to 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 mosquitoes' blood meal status. Since our results demonstrated a mismatch in the DENV serotypes of mosquitoes and patients residing in the same household, it is possible that blood-fed mosquitoes fed on the blood of asymptomatic individuals. This may represent an important parameter to be determined in future studies. Lastly, although the focus of this study is on *Ae. aegypti* mosquitoes, it would also be important 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 the DENV [63], checking the role of this mosquito species in the maintenance of DENV circulation in peri-urban municipalities, like Tarlac City, would be an important point to consider in future studies.

Conclusions

In conclusion, we demonstrated in this study that individual mosquito testing using a one-step multiplex real-time RT-PCR assay could be a potential tool in mosquito-based DENV surveillance. Using this approach, 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 *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, mosquito and patient surveillance conducted in a larger population and wider setting is needed to fully understand the dynamics of circulating DENV genotypes in the country. Taken together, our results reinforce the importance 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 estimate

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

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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.

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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.

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Tables

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-collected 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**

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 Ae. aegypti mosquitoes were collected were shown with bold boundary with the number of surveyed households (n = 48). Detected DENV serotypes in mosquitoes and patients were shown in the map.

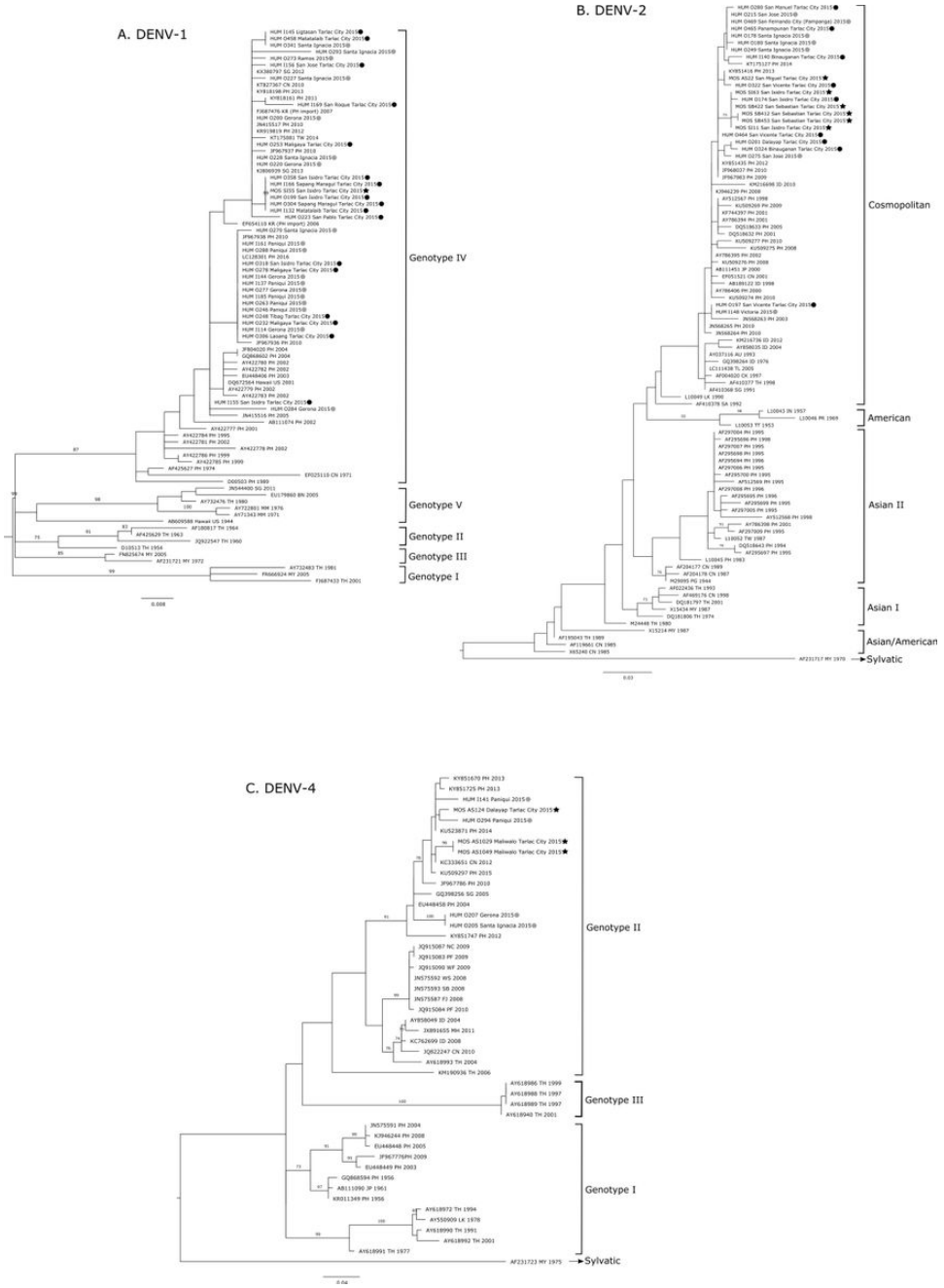

Figure 2

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) isolated in this study 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

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