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Summary

Dengue remains a major public health problem in the Philippines. Here, we determined the circulating serotypes in the Philippines during the 2015-2017 outbreaks by using a total of 678 serum samples that were collected from 537 individual dengue patients. Following an increase in the percentage of DENV-4 patients in recent years, we conducted a comprehensive molecular epidemiology analysis on the DENV-4 strains isolated recently in the Philippines. While two genotypes of DENV-4 have been isolated in the Philippines since 1956, the GI and GIIa which were the DENV-4 strains isolated in this study were closely related to a distinct group of GIIa strains that were isolated from the Philippines since 2004. A majority of the isolates of this sub-group has been identified in the Philippines, suggesting that the lineage may have been introduced and evolved in the Philippines to form the distinct sub-group within GIIa strains. The increase in DENV-4 activity also coincided with the appearance of GIIa sub-group and the phasing-out of GI lineage in the Philippines. Overall, our study demonstrates a shift in DENV-4 genotype and epidemic dynamics in a hyperendemic region, suggesting the importance of DENV genetic evolution in establishing and sustaining transmission.
Introduction

Dengue fever is the most prevalent arthropod-borne viral disease in humans. There are approximately 390 million infections per year accounting to approximately 96 million of clinically apparent cases (1). The virus causes various clinical symptoms ranging from mild fever without warning signs to fever with warning signs and severe dengue as defined by the WHO (2). The agent dengue virus (DENV) is a member of the genus Flavivirus of the family Flaviviridae, which can be antigenically divided into four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). Any of these serotypes can cause mild febrile illness that is self-limiting which can then progress to bleeding manifestations leading to severe dengue (2).

Dengue is endemic in the Philippines where all the causative agents–the four dengue virus serotypes–are circulating. It has been a notifiable disease in the Philippines since 1958 (3) and is one of the country’s eight pervasive infectious diseases (4). In 1954, an epidemic characterized by symptoms of fever and hemorrhages among infants and children which led to death of some infected individuals was reported in the Philippines (5). It was in 1956 when the dengue viruses were associated with hemorrhagic fever in an outbreak that occurred in Manila where there were over 750 cases and approximately 10% mortality (6). Although not the most prevalent isolated serotype, DENV-4 was reported to be associated with an outbreak for the first time. In 1964, another outbreak of mosquito-borne hemorrhagic fever occurred in Manila and nearby areas (7). This was the only time when DENV-4 was reported to be the most prevalent serotype during an outbreak in the Philippines. Circulation of the dominant serotypes has demonstrated distinct cyclic patterns in the Philippines, with a cycle of 2-4 years for each major circulating serotype. Previously, DENV-1 and DENV-2 have been reported to be the major circulating serotypes.
towards the end of the 20th century and the beginning of the 21st century with DENV-3 predominating towards the end of the first decade of the 21st century (8). While DENV serotypes have played an important role in DENV outbreaks in the Philippines, DENV-4 has been consistently the least detected serotype (9) which may amount to only 7% of reported cases (7). Globally, DENV-4 has been re-emerging in various parts of the world. DENV-4 is stratified into 8 lineages with unique spatiotemporal characteristics and limited genetic recombination (10).

Previous studies have suggested the important role of the introduction of new genotypes and serotypes in changing the dynamics of DENV outbreaks. Notably, between 1995-2002 in the Philippines, the predominant serotype shift to DENV-2 coincides with the DENV-2 genotype replacement from the Asian-2 genotype to the Cosmopolitan genotype (11). Between 2015-2017, we identified an increase in DENV-4 infections (DENV-1=26.0%, DENV-2=19.8%, DENV-3=35.7%, DENV-4=18.3%) (Table 1). In the present study, phylogenetic analyses of the full-length virus genome by using next-generation sequencer was conducted to better understand the molecular epidemiology of the recent DENV-4 emergence. Here, we show the emergence and circulation of DENV-4 genotype IIa and, demonstrate the recent changes in DENV-4 circulation dynamics in a hyperendemic region.
Materials and Methods

Clinical samples, patient data and ethics statement

A total of 678 serum or plasma samples obtained from 537 febrile patients (145 in-patients and 392 out-patients) who sought medical treatments at St. Luke’s Medica Center (SLMC) and San Lazaro Hospital from 2015-2017, were analyzed in this study. Blood specimens were collected from all patients at ≤5 days after onset of fever with written consents from patients or guardians. Subsequent blood specimens mostly from in-patients were collected 6 days or more after onset of fever. Patients were confirmed to have dengue infection by the in-house IgM capture ELISA at SLMC (12) and detection of dengue virus gene by real-time RT-PCR (13, 14). Primary and secondary type of infection was determined by using the DENV IgG ELISA kit according to manufacturer’s instructions (Panbio, Australia) (15). Virus isolation was done by inoculating 10 µl serum or plasma sample onto a confluent monolayer of *Aedes albopictus* C6/36 cells following a previous protocol (16). The study obtained its ethical clearances from SLMC Institutional Ethics Review Committee (EC Reference number : 14018) and Institute of Tropical Medicine, Nagasaki University (no. 20144004).

Virus serotying and sequence analyses

Virus serotyping was performed either by conventional RT-PCR (17) or by using fluorogenic Taqman primers that are specific to each serotype (14, 18). For conventional sequencing methods, the E region was amplified by Qiagen One-step RT-PCR kit (Qiagen, Hilden, Germany) by using primers flanking the whole envelope gene. The resulting 2014 bp product
was then sequenced by using sequencing primers according to the methods previously described (19).

To determine the whole sequence of dengue virus by NGS, cDNA libraries were synthesized with Super Script III first strand synthesis system (Invitrogen USA) by using random primers according to manufacturer’s instructions. Second strand was synthesized by using Ultra II Non-directional RNA second strand synthesis module (NEB, Ipswich, MA, USA). The amplified double stranded (ds) DNA was quantified and normalized by using Qubit 2.0 fluorometer (Invitrogen USA). The amplicons were fragmented with transposome according to manufacturer’s instructions (Illumina, USA). Library was then generated by using Illumina Nextera XT library preparation kit (Illumina, USA) and purified by using Agencourt AMPure XP beads (Beckman Coulter Genomics, USA). The pooled library was then quantified by Qubit 2.0 fluorometer. Pooled libraries were denatured with 0.2 M NaOH and the prepared sample was loaded into Miseq v2 kit (500 cycles) for paired-end sequencing on the Illumina Miseq platform. Image processing and base calling were generated as FASTQ files. Quality assessment was performed using the Miseq control software, in which samples with a Q score of >30 were used for further analyses (20).

**Phylogenetic Analyses and Molecular Dating**

Low quality sequences were removed by FASTX-Toolkit Ver 0.0.14 (21) from the input data file. Before and after the quality trimming, sequence quality was assessed by using FastQC Ver 0.11.7 (22). Trinity Ver 2.8.4 (21) was used for de novo assembly. Using Entrez-edirect (23) and
BLASTN Ver 2.7.1 (24), consensus sequence was chosen from INSDC. Reads from trimmed fastq data set were mapped by BWA Ver 0.7.17 (25) to the reference sequence. Using Varscan Ver 2.4.3 (26) and Samtools Ver 1.9 (27), consensus sequence was then constructed. Due to limited full length genome analyses of DENV-4, sequences from the E-gene was used for the phylogenetic analyses (table 2). The sequences of E protein coding region were aligned using MAFFT Ver 7.407 (28). Substitution model was selected by JModeltest Ver 2.1.10 (29) and bModelTest 1.1.2 (30). Bayesian Markov Chain Monte Carlo (MCMC) analysis was conducted using the GTR substitution model by BEAST Ver 1.10.4 (31) and BEAGLE Ver 3.1.2 (32). The MCMC length of chain was for 100,000,000 generations. The Maximum Clade Credibility (MCC) tree was generated and annotated with posterior probability by TreeAnnotator Ver 1.10.4 (31). Phylogenetic tree was drawn by Figtree Ver 1.4.4. Gaussian Markov random field (GMRF) Skyride plot was constructed using Tracer Ver 1.7.1 (33).

**Statistical analyses**

Data were described using descriptive statistics such as mean and standard deviation. Student’s *t* test was used to compare the means and difference between means was considered to be significant when the P-value was less than 0.05.
Results

A total of 537 DENV cases (678 serum and plasma samples) confirmed by real-time RT-PCR and anti-DENV IgM ELISA was used in this study. A total of 261 patients were confirmed to have secondary DENV infection (N_{out-patient}= 204/347 (58.8%) and N_{in-patient}=57/131 (43.5%)). DENV was isolated from 141 out of 167 samples. Among the DENV cases, patients that demonstrated signs of severe dengue were admitted as “in-patient” and other patients who did not require hospitalization were grouped as “out-patient”. Most of the in-patient cases were of DENV-3 infection (N=5) while one case was of DENV-4 infection. There was no DENV-1 and DENV-2 cases in the in-patient group. In contrast, 28.0%, 28.0%, 21.0% and 23.0% of out-patient samples were positive for DENV1, DENV2, DENV3 and DENV4, respectively. While there was an increase in the percentage of patients with DENV-4, the clinical signs including body temperature and blood chemistry were not significantly different those of other serotypes (data not shown).

Following an increase in the percentage of DENV-4 patients in recent years, we conducted a comprehensive molecular epidemiology analysis on the DENV-4 strains isolated in the Philippines over several decades. In addition to the 15 DENV-4 isolates obtained from this study, along with 97 published strains isolated from the Philippines between 1956 to 2016 available from public databases, a phylogenetic analysis was done. A neighbor joining (NJ) phylogeny revealed two DENV-4 genotype lineages (GI and GII) in the Philippines (figure 1). DENV-4 GI was associated with two different major lineages, one which consist of strains isolated in 1956, and a second group of GI lineages that is distinct from that of the strains in the 1950s. The GI
group consist of more recent DENV-4 isolates, and these strains were isolated co-incidentally with a subsequent wave of increased DENV-4 activity, involving a 10-year span of 2003-2013.

Following a period of relatively low levels of DENV-4 activity between 2006-2011 (Table 1), a new genotype (GIIa) was detected from early 2000s. Subsequently, a majority of the isolates from the lineage (L3, figure 2a) was identified recently between 2012-2016. The GIIa lineage from the Philippines formed a distinct and well supported monolineages that originated from strains isolated in East Timor in 2001 (KY275251, KY275252). Of the recent isolates in the GIIa group, the MCC analyses revealed a distinct lineage (L3), that consist of strains from early 2000s to present. The L2 lineage, sub-groups in the L3-1-1a and the L3-1-1b lineage was however, no longer detected in recent years, demonstrating a pattern of active sub-lineage replacements between 2010-2015.

Interestingly, while there was an increase of GIIa isolates, there were no DENV-4 GI isolates beyond 2013. The turnover from GI to GII also coincides with a period of relatively high DENV-4 activity between 2012-2016. Additionally, the mechanisms between the genotype shift and DENV-4 activity in the Philippines were further examined by using the GMRF Skyride plot (figure 2b). The GMRF Skyride plot suggests that the DENV-4 in the Philippines appears to have a phase of exponential increase of $N_e$ during 2000-2010. This inference is consistent with the appearance of GIIa lineage and, the phasing-out of GI lineage in the Philippines. Out of a total 84 strains that clustered within the distinct GIIa lineage of the Philippines (figure 1), 73 were isolated from the Philippines (2004-2015), while the remaining strains were isolated from Indonesia (2016), East Timor (2001), Singapore (2005, 2013-2014), China (2012) and Thailand (2014). Because most of the isolates of this GIIa lineage have been identified in the Philippines,
the lineage may have been introduced and evolved in the country to form a distinct group within GIIa strains.
Discussion

Two genotypes of DENV-4 have been isolated in the Philippines since 1956; GI and GIIa. The DENV-4 strains isolated in this study from 2015-2017 were closely related to previous GIIa strains isolated from the Philippines since 2004. Although all 4 serotypes co-circulate in the Philippines, there are limited data on outbreaks associated with DENV-4 and evolutionary trajectory. Here, we present a comprehensive analysis of an important DENV serotype, DENV-4, in the Philippines over the recent decades.

Phylogenetic analyses on the DENV-4 serotype isolated since 1956 demonstrated several lineage turnover, associated with changes in DENV serotype dynamics. By using analyses of all strains isolated in the Philippines, we found that introduction of distinct lineages were followed by large DENV-4 outbreaks, and this incident of higher DENV-4 activity occurred between 2004-2006 and 2012-2016. While the number of proportion of DENV-4 patients increased, there was no significant increase in the number of DENV-4 patients with severe dengue. Additionally, clinical signs of the DENV-4 patients including blood chemistry and viremia levels did not differ significantly in comparison to that of patients with other serotypes among the DENV-4 hospitalized patients during our study period. While our results suggest that higher DENV-4 activity may not be consistent with an increase in the number of severe dengue cases, further studies are needed to define the virus pathogenicity in association with enhanced transmission efficiency.

Whereas GI lineage has been isolated from the Philippines since 1956 and persisted up until at least 2013, the GI group was replaced with GIIa domination since 2011. The emergence of GIIa in the Philippines was estimated to be between 2004 to 2007, and the genotype turnover, as
defined in a switch in dominance, took at most 7 years. Introduction of GIIa in the Philippines also coincided with the period in which there was an increase in DENV-4 GIIa outbreaks in neighboring regions including Malaysia (34), while further studies are needed to determine the patterns of genotype shift in the region. Interestingly, the GIIa isolated from the Philippines forms a distinct group among the global GIIa strains (figure 1), suggesting that the strain may have evolved independently in the Philippines and is unique to the region.

Several shifts in the predominant DENV-4 genotype lineages occurred in the Philippines during 2004-2016. In concurrence with other studies in which genotype and lineage turnover coincides with changes in epidemic activity (19, 35 - 38), our study demonstrated an increase in DENV-4 patients during the turnover period. While the GIIa lineage from the Philippines forms a distinct lineage, processes of genotype replacement may have been a result of introduction strains and on-going local evolution, with the dominance of lineage L3, and replacement of lineage L2 and sub-lineages within L3 (L3-1-1b). One possible factor that is associated in driving the turnover may be immune escape, in which positive selection could confer selective advantage over other strains (39, 40). Based on the high number of DENV-4 cases that coincided with the genotype turnover, the GIIa virus may also possess enhanced ability to replicate or transmit at the population level in the Philippines, or possess greater fitness as compared to prior strains. While this study did not include any participants from the recent national dengue vaccination program, further studies on viral dynamics and herd immunity would clarify the factors associated in DENV evolutionary trajectory. Further studies on the viral dynamics in the region would also be of importance, particularly the introduction of strains between hyperendemic regions, to better understand the factors that drive epidemic dynamics.
DENV remains a major public health problem in the Philippines. Based on the DENV-4 sequence evolution study spanning over 6 decades, we found that DENV-4 has been evolving rapidly in recent years in this DENV hyperendemic region, with a major genotype turnover to GIIa and the subsequent disappearance of GI. GIIa strains associated with the 2004 outbreaks and beyond were marked with local evolution in the Philippines, rapid and frequent inter-genotype lineage turnover and increased DENV-4 epidemic activity. Overall, our study demonstrated a shift in DENV-4 genotype and epidemic dynamics in a hyperendemic region, suggesting the importance of DENV genetic evolution in establishing and sustaining transmission.
Funding information

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Acknowledgments

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Conflicts of interest

The authors declare that there are no conflicts of interest.
References


Table 1. Dengue virus (DENV) serotypes in Metro Manila, 2003-2017†

<table>
<thead>
<tr>
<th>Year</th>
<th>DENV-1</th>
<th>DENV-2</th>
<th>DENV-3</th>
<th>DENV-4</th>
<th>Total by year</th>
</tr>
</thead>
<tbody>
<tr>
<td>2003</td>
<td>16 (80**)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>2 (10)</td>
<td>20</td>
</tr>
<tr>
<td>2004</td>
<td>3 (20)</td>
<td>5 (33)</td>
<td>2 (13)</td>
<td>5 (33)</td>
<td>15</td>
</tr>
<tr>
<td>2005</td>
<td>7 (16)</td>
<td>9 (20)</td>
<td>17 (39)</td>
<td>11 (25)</td>
<td>44</td>
</tr>
<tr>
<td>2006</td>
<td>4 (3)</td>
<td>6 (5)</td>
<td>120 (90)</td>
<td>3 (2)</td>
<td>133</td>
</tr>
<tr>
<td>2007</td>
<td>0 (0)</td>
<td>1 (7)</td>
<td>12 (86)</td>
<td>1 (7)</td>
<td>14</td>
</tr>
<tr>
<td>2008</td>
<td>3 (5)</td>
<td>24 (36)</td>
<td>38 (58)</td>
<td>1 (2)</td>
<td>66</td>
</tr>
<tr>
<td>2009</td>
<td>3 (1)</td>
<td>198 (92)</td>
<td>11 (5)</td>
<td>3 (1)</td>
<td>215</td>
</tr>
<tr>
<td>2010</td>
<td>15 (17)</td>
<td>35 (41)</td>
<td>30 (35)</td>
<td>6 (7)</td>
<td>86</td>
</tr>
<tr>
<td>2011</td>
<td>8 (73)</td>
<td>1 (9)</td>
<td>2 (18)</td>
<td>0 (0)</td>
<td>11</td>
</tr>
<tr>
<td>2012</td>
<td>12 (52)</td>
<td>1 (4)</td>
<td>2 (9)</td>
<td>8 (35)</td>
<td>23</td>
</tr>
<tr>
<td>2013</td>
<td>4 (16)</td>
<td>11 (44)</td>
<td>2 (8)</td>
<td>8 (32)</td>
<td>25</td>
</tr>
<tr>
<td>2014</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>2015</td>
<td>54 (32)</td>
<td>44 (26)</td>
<td>32 (19)</td>
<td>38 (23)</td>
<td>168</td>
</tr>
<tr>
<td>2016</td>
<td>17 (19)</td>
<td>21 (23)</td>
<td>36 (40)</td>
<td>16 (18)</td>
<td>90</td>
</tr>
<tr>
<td>2017</td>
<td>28 (23)</td>
<td>10 (8)</td>
<td>67 (56)</td>
<td>15 (13)</td>
<td>120</td>
</tr>
</tbody>
</table>

| Total by serotype | 174 (17) | 367 (36) | 372 (36) | 117 (11) | 1030 |

* Number in brackets indicate percentage of total serotype by year.

**Underline indicates the serotype with the highest percentage in the specific year.

† Data source from SLMC database.

ND indicates no data.
Table 2. DENV isolates characterized in the present study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Sex</th>
<th>Age</th>
<th>Days of Fever</th>
<th>Date of sample collection</th>
<th>DENV-4 real-time PCR (ct values)</th>
<th>GenBank accession number</th>
</tr>
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<tr>
<td>173</td>
<td>F</td>
<td>13</td>
<td>4</td>
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<td>29.8</td>
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</tr>
<tr>
<td>310</td>
<td>F</td>
<td>12</td>
<td>3</td>
<td>2015/8/10</td>
<td>32.4</td>
<td>MN027546</td>
</tr>
<tr>
<td>322</td>
<td>M</td>
<td>18</td>
<td>3</td>
<td>2015/8/14</td>
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<td>MN027547</td>
</tr>
<tr>
<td>334</td>
<td>M</td>
<td>35</td>
<td>4</td>
<td>2015/8/18</td>
<td>28.3</td>
<td>MN027548</td>
</tr>
<tr>
<td>336</td>
<td>F</td>
<td>7</td>
<td>4</td>
<td>2015/8/18</td>
<td>32.0</td>
<td>MN027549</td>
</tr>
<tr>
<td>337</td>
<td>M</td>
<td>30</td>
<td>3</td>
<td>2015/8/17</td>
<td>31.8</td>
<td>MN027550</td>
</tr>
<tr>
<td>363</td>
<td>M</td>
<td>27</td>
<td>4</td>
<td>2015/8/22</td>
<td>31.4</td>
<td>MN027551</td>
</tr>
<tr>
<td>379</td>
<td>M</td>
<td>27</td>
<td>4</td>
<td>2015/8/25</td>
<td>35.1</td>
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</tr>
<tr>
<td>380</td>
<td>F</td>
<td>7</td>
<td>4</td>
<td>2015/8/25</td>
<td>31.0</td>
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</tr>
<tr>
<td>382</td>
<td>M</td>
<td>32</td>
<td>3</td>
<td>2015/8/23</td>
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<tr>
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<td>M</td>
<td>35</td>
<td>4</td>
<td>2015/8/29</td>
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<tr>
<td>405</td>
<td>M</td>
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<td>2015/8/29</td>
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<tr>
<td>444</td>
<td>M</td>
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<td>6</td>
<td>2015/9/15</td>
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</tr>
<tr>
<td>573</td>
<td>F</td>
<td>61</td>
<td>4</td>
<td>2015/11/15</td>
<td>33.0</td>
<td>MN027558</td>
</tr>
<tr>
<td>991</td>
<td>F</td>
<td>20</td>
<td>5</td>
<td>2016/10/21</td>
<td>N.D.</td>
<td>MN027559</td>
</tr>
</tbody>
</table>

N.D. indicates no data for the sample.
Fig. 1. Phylogenetic tree of global DENV-4 strains. The tree was constructed by the neighbor-joining (NJ) method (Seaview version 4.7) with 100 bootstrap replicates, by using 1683 global DENV-4 sequences. Branches of the tree that were color-coded green indicate bootstrap values of >80. DENV-4 strains from the Philippines are color-coded as red.
Fig 2. Molecular clock analysis of DENV-4 genotype IIa sequences in the Philippines. (a) Bayesian maximum clade credibility (MCC) phylogenetic tree estimated by using BEAST Ver 1.10.4 and BEAGLE Ver 3.1.2. The MCMC length of chain was for 100,000,000 generations, in which the generated MCC tree was annotated with percentage of highest posterior density (95% HPD) by TreeAnnotator Ver 1.10.4. The 95% HPD is indicated adjacent to nodes. Strains isolated from this study are indicated as solid red diamonds. (b) Gaussian Markov random field (GMRF) Skyride plot showing the demographic history of DENV-4 GI and GII as constructed by using Tracer Ver 1.7.1. Blue area indicates variations in effective population size (Y axis, Ne) whereas blue line indicates estimated mean of Ne variations.