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Emergence of genotype I of dengue virus serotype 3 during severe dengue epidemic in Sri Lanka, 2017

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Running head; New genotype I of DENV-3 in Sri Lanka

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Summary

During the 2017 outbreak of severe dengue in Sri Lanka, dengue virus (DENV) serotypes 2, 3 and 4 were co-circulating. Based on our previous study on the 295 patients from the National Hospital Kandy in Sri Lanka between March 2017-January 2018, the dominant infecting serotype was DENV-2. Here, we aimed to characterize the DENV-3 strains from non-severe and severe dengue patients from our previous study population. Patients' clinical records and previous laboratory tests including dengue-specific nonstructural protein 1 antigen rapid test, IgM-capture and IgG enzyme-linked immunosorbent assays, were analyzed together with the present results of real-time reverse transcription polymerase chain reaction, and next-generation sequencing of DENV-3. Based on complete genome analysis, DENV-3 isolates belonged to two different clades of genotype I and were genetically close to the strains from Indonesia, China, Singapore, Malaysia and Australia. There were sixteen amino acid changes among DENV-3 isolates, and the greater number of changes was found in nonstructural than structural proteins. The emergence of DENV-3 genotype I was noted for the first time in Sri Lanka. Continuous monitoring of this newly emerged genotype and other DENV serotypes/genotypes are needed to determine their effects on future outbreaks and to understand the molecular epidemiology of dengue.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne disease that remains a major global public health concern in many parts of the world, including Southeast Asia and the Americas (1). Infection with DENV may present as an asymptomatic infection or as a symptomatic infection that is manifested from mild to severe illness: dengue fever, dengue hemorrhagic fever, and dengue shock syndrome (2). The different degrees of dengue severity were reclassified in 2009 by the World Health Organization (WHO) into dengue without warning signs (DwoWS), dengue with warning signs (DwWS), and severe dengue (SD) (3). DENV belongs to the genus *Flavivirus* of the family *Flaviviridae* and contains a single stranded, positive sense RNA genome of approximately 11 kb in length. The dengue virus exists as four distinct serotypes, designated DENV-1, DENV-2, DENV-3, and DENV-4 (4). Each serotype of DENV has four to six geographically distinct genotypes. For DENV-3, there are five genotypes namely I, II, III, IV, and V, however, complete genome sequences are available for only four genotypes, namely I, II, III, and V (5, 6).

Dengue has emerged as a major public health burden in Sri Lanka, and all four serotypes of DENV are present in the country (7). In the epidemic outbreaks between 1965 and 1968 (8), DENV-1 and -2 were the circulating serotypes and in 1978, DENV-4 was detected (9). In the

1990s and early 2000s, all four serotypes of DENV were in circulation with DENV-3 as the dominant serotype, the genotype of which belonged to two distinct clades of genotype III (9, 10). During the epidemic in 2009, DENV-1 was the predominant serotype, and 35,008 cases with 346 deaths were reported (11). During this period, Asia genotype I of DENV-1 was first observed. In the epidemics in 2012–2013, DENV-1 and -4 were in circulation (12). In 2017, the largest dengue outbreak occurred in Sri Lanka, where over 185,000 clinical cases with at least 250 fatalities were noted and many young people were infected (13). We reported the co-circulation of DENV-2, -3 and -4 and the presence of unusual manifestations of severe dengue in Sri Lanka during this outbreak based on the 295 patients that we studied between March 2017 and January 2018 (14). The patients were from the National Hospital Kandy in Sri Lanka. Two hundred nineteen DENV-2, three DENV-3 and three DENV-4 strains were isolated from among these patients. Two of the three DENV-3 strains were isolated together with DENV-2. We noted that a new clade of DENV-2 cosmopolitan genotype caused this unprecedented outbreak (14). In our present study, we aimed to characterize the three DENV-3 strains from serum samples of non-severe and severe dengue patients of this previous study population.

MATERIALS AND METHODS

Patients, clinical records, laboratory test results and ethical clearance

Clinical records and results of laboratory tests of three patients identified previously to be infected with DENV-3 (14) were obtained from the National Hospital Kandy in Sri Lanka (Fig.1). Also, we took into consideration the laboratory tests, such as Dengue NS1 antigen rapid test, IgM capture ELISA and IgG ELISA, the results of which were included to provide a general characterization of the study population in our previous study (14). The DENV-3 as well as the co-infecting DENV-2 (if present) from the serum samples of these patients were subjected to quantification of

DENV genome levels while whole genome sequencing DENV was done using described below. Ethical approval for this study was provided by the Institution Ethical Committee on Medical Research and Review, General Hospital (Teaching) Kandy, Sri Lanka (THK/ERC/73/2017) and the Institute of Tropical Medicine Ethical Committee, Nagasaki University, Japan (180608200).

Quantification of DENV genome levels (qRT-PCR)

To quantify the DENV genome RNA level from serum, viral RNA was directly extracted from 140 µl of patient serum using a viral RNA mini kit (Quigen, Hliden, Germany). A volume of 5 µl of RNA was employed for quantitative RT-PCR (qRT-PCR) using TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Foster, USA), following the protocol from a previous report (15). The viral genome levels were expressed as log₁₀ genome copies/ml.

Whole genome sequencing of DENV-3

To amplify the full-length viral genome following the manufacturer's instructions, whole-transcriptome libraries (Ion Total RNA-Seq Kit v2, Life Technologies, California, USA) were synthesized using RNA extracted from culture fluids of C6/36 cells that we infected with serum samples in our previous experiment (14). Sequencing was conducted using an NGS Ion Proton apparatus (Life Technologies, California, USA). The sequences of the full-genome coding region were aligned using MAFFT v 7.407 (16). Maximum-likelihood phylogenetic trees were constructed with PhyML v 3.2.0 (17). Bootstrap values were obtained after 1000 replications. The substitution model was selected via jModelTest v 2.1.10 (18).

RESULTS

Based on previous records, two (N-189 and N-100) of the three DENV-3 infected patients had DwoWS and infected concurrently with DENV-2, while the third patient (N-204) had SD and

infected only by DENV-3. Infection with DENV-3 was observed between November 2017 and January 2018. Complete clinical records and previous laboratory tests of only two DENV-3 infected patients, N-189 and N-204, were analyzed because of the successful sequence of the genome of the DENV-3 that infected them. The genome of the DENV-3 from patient (N-100) could not be fully sequenced, thus, this patient was excluded from further study.

The patient, with code N-189, was a 30-year-old female characterized to have DwoWS. On admission (second day of illness), she came presenting with fever, muscle pain, headache, mild abdominal pain, and nausea for 2 days; her white blood cells (WBC) at $6.9 \times 10^3/\text{mm}^3$, platelet count (PLT) at $110 \times 10^3/\text{mm}^3$, hematocrit (HCT) at 37.7% were noted. Mild gum bleeding was observed on the third day of her illness. Liver was not palpable, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels remained within normal range. Although the lowest WBC at $4.9 \times 10^3/\text{mm}^3$, lowest PLT at $32.9 \times 10^3/\text{mm}^3$, and $< 20\%$ increase of HCT were noted on the fourth day of illness and the disease was clinically managed as dengue fever without any complications, a rise in WBC and platelet counts occurred afterwards, thus, the patient was discharged on the sixth day of illness.

The patient (N-204), who had SD, was 20-year-old male (N-204) presenting with fever, muscle pain, joint pain, severe upper abdominal pain, headache and nausea. She had vomited several times within 4 days. On admission (fourth day of illness), WBC at $3.1 \times 10^3/\text{mm}^3$, PLT at $67.9 \times 10^3/\text{mm}^3$, HCT at 37.8% were noted. Ultrasonic and biochemical investigations revealed that he had tender palpable liver, free fluid in the abdomen and pelvic area, pleural effusions, and elevated serum AST and ALT; while also developing difficulty in breathing on the fifth day of the illness. On the sixth day, bleeding from the cannula site, vomiting of blood, and features of acute kidney injury requiring hemodialysis were recorded. Although the lowest WBC count at $2.8 \times$

$10^3/\text{mm}^3$, lowest PLT count at $19.6 \times 10^3/\text{mm}^3$, and highest HCT level at 44.9% were noted on the fifth day of illness, because of treatment, the patient's condition gradually improved from 9 days post-infection (pi). The patient was discharged 14 days pi.

Both patients were DENV NS1 positive. Laboratory results revealed that the DwoWS patient (N-189) was DENV-IgM negative with primary infection and the serum viral genome RNA levels were at 2.02 and 6.82 $\log_{10}$ copies/ml of DENV-2 and DENV-3, respectively. The SD patient (N-204) was DENV-IgM positive with secondary infection and the serum DENV-3 RNA levels was detected at 7.22 $\log_{10}$ copies/ml.

The phylogenetic trees of DENV-3 that included the strains from the two patients (N-189, N-204) in this study and other strains from Sri Lanka, neighboring, and other countries as found in GenBank were constructed based on the full coding region of the E gene (Fig. 2) and whole genome (Fig. 3) to determine relationships and genotypes of the strains in this study. Both the DENV-3 isolates in the present study belonged to genotype I. This genotype was detected for the first time in Sri Lanka. Interestingly, based on the whole genome, the two DENV-3 genotype I isolates belonged to two different clades. According to the phylogenetic tree based on the E gene, DENV-3 isolated strains in our study were closely related to the strains from Indonesia, China, Singapore and Malaysia (Fig. 2). Similarly, the phylogenetic tree based on the whole genome indicated that the two DENV-3 strains in our study were genetically close (99%) to the strains circulating in Indonesia, China, Singapore, and Australia (Fig. 3). We, then compared amino acid changes between DENV-3 strains from patients N-189 and N-204 and the closely related reference strains (MK 894338, KC762691, KY921906) (Table 1), thirty amino acid changes were detected. Sixteen amino acid changes were observed between DENV-3 strains from patients N-189 and N-204. The greater number of amino acid differences was detected in nonstructural (10

positions) than in structural proteins (6 position). Two amino acid changes in NS5 protein (A3018V, P3312S) have not yet been reported previously, while the six uncommon amino acid changes G620E (E protein), Y948H (NS1), I1245V (NS2), I1664M, A1829T (NS3), T3318A (NS5) were found based on the complete genomes of other few strains recorded in GenBank.

DISCUSSION

In 2017, an unprecedented magnitude of severe dengue epidemic occurred in Sri Lanka and cosmopolitan genotype of DENV-2 was dominant in the most affected district (19). In our previous study on this outbreak (14), we reported the detection of cosmopolitan genotype of DENV-2 (74%) and genotype I of DENV-4 (1%). In addition, we also reported the isolation of a neurotropic DENV-2 cosmopolitan genotype from the cerebrospinal fluid of encephalitis patients (20). In the present study, we described the emergence of genotype I of DENV-3 that was first seen in Sri Lanka during 2017-2018. One patient (with DwoWS clinical manifestation) infected concurrently by DENV-3 (determined in this study as genotype 1) and DENV-2 cosmopolitan genotype as determined in our previous study (14) was observed to have a mild manifestation with primary infection. Our findings are in line with previous studies in Brazil and India that reported mild dengue manifestation caused by concurrent infections of DENV-2 and DENV-3 (21-23). However, conflicting outcomes were documented in which co-infections of DENV serotypes may lead to either mild or severe dengue manifestation (24). The co-infection rate caused by the DENV-2 and DENV-3 infection was lower than other serotype combinations (25). Although a higher virus titer of DENV-3 over DENV-2 was recorded, both viruses were isolated from the DwoWS patient. It may be possible to acquire co-infections by the bite of a single mosquito that have two DENV serotypes or being bitten by two different mosquitoes carrying different serotypes within a short period (25, 26).

Clinical manifestation caused by mono-infection with DENV-3 in the SD patient was found as a severe manifestation with secondary infection. It has been observed in several studies that secondary infection with heterologous serotypes is more severe than primary infection and it may be explained by antibody dependent enhancement mechanism (27-29). In previous studies, genotype III of DENV-3 from Sri Lanka formed two distinct clades and was linked to mild (genotype IIIA) and severe (genotype IIIB) dengue from 1989 to 2000 (30). Moreover, a new 2003–2004 clade appeared that was distinct from clade IIIB (9). Notably, genotype I of DENV-3 was newly introduced in this outbreak, and two distinct clades appeared with one clade containing the DENV-3 of the DwoWS patient N-189 and the other clade with DENV-3 of the SD patient N-204. To highlight the importance difference in evolutionary patterns, we compared E gene and whole genome by phylogenetic analysis. The DENV-3 strains in this study were genetically close to almost the same countries in both gene analysis. Although new and uncommon amino acid changes were found in the two strains, the effect of those changes in clinical severity is unknown. In addition, further studies are warranted to characterize the two DENV-3 isolates *in vivo* and its tropism *in vitro*.

Our study site, Kandy Hospital, is situated in the central part of Sri Lanka, which has a high altitude (500 meters above sea level) compared with other parts of the country. However, global warming, increased globalization, trade, travel, and rapid urbanization have contributed to increasing DENV infection in Kandy and the central part of the country. In summary, co-infection of DENV-2 and DENV-3 as well as mono-infection of DENV-3 among patients were observed as non-severe and severe manifestations, respectively, and both patients were infected in the latter part of the 2017 epidemic. Although a new clade of cosmopolitan genotype of DENV-2 caused the unprecedented dengue outbreak during 2017, a new genotype introduction of DENV-3 might

be in the pipeline to take the lead in future outbreaks in Sri Lanka. According to data released by the Epidemiology Unit of the Ministry of Health in Sri Lanka, suspected dengue cases were reported as 51659 for the year 2018 and 70092 up to November 2019 (31). Based on preliminary findings in our new study population, we detected genotype I of DENV-3 among dengue patients during 2018 and 2019 dengue outbreaks. Currently, the rise in dengue cases could be based on multiple serotype circulation along with serotype and genotype shifting, and further studies are necessary to understand the molecular epidemiology of dengue in the 2018–2019 outbreaks.

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Conflict of interest

None to declare.

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Figure Legends

Fig 1. Location of National Hospital Kandy (circle) in Sri Lanka where collection of serum samples from dengue confirmed patients took place.

Fig. 2. DENV-3 phylogenetic tree. Phylogenetic tree was constructed on the basis of whole E gene of DENV-3. It showed the relationship of 41 virus strains from different sources and the 2 strains (*) of DENV-3 from this study. The representative strains of each genotype obtained from GenBank were named by country-origin, strain name, year of isolation, and GenBank accession number.

Fig. 3. DENV-3 phylogenetic tree. Phylogenetic tree was constructed on the basis of full coding regions of whole genome of DENV-3. It showed the relationship of 36 virus strains from different sources and the 2 strains (*) of DENV-3 from this study. The representative strains of each genotype obtained from GenBank were named by country-origin, strain name, year of isolation, and GenBank accession number.

Table 1. Amino acid differences between DENV-3 isolates and the reference strains (MK 894338, KC 762691, KY 921906) based on complete genome analysis. Boldface indicates amino acid substitutions in DENV-3 isolates.

DENV-3 gene	polyprotein position	Amino acid residue				
		189 isolate	204 isolate	MK 894338 (China_2018)	KC 762691 (Indonesia_2008)	KY921906 (Singapore_2015)
C	97	R	K	R	R	R
M	145	I	I	T	I	I
	234	I	V	I	V	I
E	404	S	L	L	L	L
	481	N	N	N	D	N
	502	A	A	A	A	T
	558	A	A	V	A	A
	578	A	A	A	T	A
	620	G	E	G	G	G
	642	L	P	L	P	L

	660	I	I	S	I	I
	666	R	K	R	K	R
	779	V	I	V	I	I
	813	K	K	K	R	K
NS1	867	T	I	I	I	I
	948	H	Y	Y	Y	Y
	1066	T	T	T	A	T
	1245	I	V	I	I	I
NS2	1390	T	T	A	T	T
	1437	I	I	V	I	I
	1533	H	Y	Y	Y	Y
NS3	1664	I	M	I	I	I
	1829	T	A	A	A	A
NS4	2185	G	G	G	G	C
	2537	R	R	K	R	R
NS5	3018	V	A	A	A	A

3043	I	T	T	T	T
3129	P	L	P	L	P
3139	N	T	N	T	N
3318	T	A	T	T	T

Fig. 1.

Fig. 1.

Fig. 2.