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Original Article

Emergence of Genotype I of Dengue Virus Serotype 3 during a Severe Dengue Epidemic in Sri Lanka in 2017

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SUMMARY: During the 2017 outbreak of severe dengue in Sri Lanka, dengue virus (DENV) serotypes 2, 3, and 4 were found to be co-circulating. Our previous study of 295 patients from the National Hospital Kandy in Sri Lanka between March 2017 and January 2018 determined that the dominant infecting serotype was DENV-2. In this study, 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 and 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. Complete genome analysis determined that DENV-3 isolates belonged to 2 different clades of genotype I and were genetically close to strains from Indonesia, China, Singapore, Malaysia, and Australia. There were 16 amino acid changes among DENV-3 isolates, and a greater number of changes were found in nonstructural proteins than in 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 and genotypes is needed to determine their effects on future outbreaks and 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 manifests as a mild to severe illness such as dengue fever, dengue hemorrhagic fever, or dengue shock syndrome (2). The different degrees of dengue severity were reclassified in 2009 by the World Health Organization 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 4 distinct serotypes, respectively designated DENV-1, DENV-2, DENV-3, and DENV-4 (4). Each serotype of DENV has 4 to 6 geographically distinct genotypes. Five genotypes of DENV-3 exist, namely I, II, III, IV, and V; however, complete genome sequences are available for only the I, II, III, and V genotypes (5,6).

Dengue has emerged as a major public health burden in Sri Lanka, and all 4 serotypes of DENV are present in the country (7). During the epidemic outbreaks between 1965 and 1968 (8), DENV-1 and DENV-2 were the circulating serotypes, and in 1978, DENV-4 was detected (9). In the 1990s and early 2000s, all 4 serotypes of DENV were in circulation, with DENV-3 as the dominant serotype, the genotype of which belonged to 2 distinct clades of genotype III (9,10). During the 2009 epidemic, DENV-1 was the predominant serotype, and 35,008 cases with 346 deaths were reported (11). During this period, an Asian genotype I of DENV-1 was first observed. During the epidemics of 2012 and 2013, DENV-1 and DENV-4 were in circulation (12). In 2017, the largest dengue outbreak occurred in Sri Lanka, during which over 185,000 clinical cases with at least 250 fatalities were recorded, and many young people were infected (13). We reported the co-

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circulation of DENV-2, DENV-3, and DENV-4 and the presence of unusual manifestations of SD in Sri Lanka during this outbreak based on the 295 patients whom we studied between March 2017 and January 2018 (14). The patients were from the National Hospital Kandy in Sri Lanka. Overall, 219 DENV-2, three DENV-3, and three DENV-4 strains were isolated from these patients. Two of the three DENV-3 strains were isolated together with DENV-2. We found that a new clade of the DENV-2 cosmopolitan genotype caused this unprecedented outbreak (14). In the present study, we aimed to characterize the three DENV-3 strains from serum samples of non-severe and SD patients in this previous study population.

MATERIALS AND METHODS

Patients, clinical records, laboratory test results, and ethical clearance: Clinical records and laboratory test results of 3 patients previously identified as being infected with DENV-3 (14) were obtained from the National Hospital Kandy in Sri Lanka (Fig. 1). We considered laboratory tests, such as the dengue nonstructural protein 1 (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 described in our previous report (14). 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 of DENV was performed as described below. Ethical approval for this study was provided by the Institutional 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 (Qiagen, Hilden, Germany). A volume of 5 µL of RNA was used for quantitative RT-PCR (qRT-PCR) with TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Foster, CA, USA), in accordance with a protocol described in a previous report (15). The viral genome levels were expressed as $\log_{10}$ genome copies/mL.

Whole-genome sequencing of DENV-3: To amplify the full-length viral genome in accordance with the manufacturer's instructions, whole-transcriptome libraries (Ion Total RNA-Seq Kit v2, Life Technologies) were synthesized using RNA extracted from culture fluids of C6/36 cells that were previously infected with serum samples (14). Sequencing was conducted using an NGS Ion Proton apparatus (Life Technologies). The sequences of the full-genome coding region were aligned using MAFFT v. 7.407 (16). Maximum-likelihood phylogenetic trees were constructed using PhyML v. 3.2.0 (17). Bootstrap values were obtained after 1,000 replications. The substitution model was selected using jModelTest v. 2.1.10 (18).

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

RESULTS

According to previous records, two (N-189 and N-100) of the three DENV-3 infected patients had DwoWS and were concurrently infected with DENV-2, while the third patient (N-204) had SD and was infected only with DENV-3. DENV-3 infection 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 successful sequencing 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 evaluations.

The patient with code N-189 was a 30-year-old woman with DwoWS. Upon admission (second day of illness), she presented with fever, muscle pain, headache, mild abdominal pain, and nausea for 2 days, as well as a white blood cell (WBC) count of 6.9 × 10^3/mm^3, platelet (PLT) count of 110 × 10^3/mm^3, and hematocrit (HCT) of 37.7%. Mild gum bleeding was observed on the 3rd day of her illness. The liver was not palpable, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels remained within the normal range. Although a lowest WBC count of 4.9 × 10^3/mm^3, lowest PLT count of 32.9 × 10^3/mm^3, and < 20% increase in HCT were observed on the 4th day of illness and the disease was clinically managed as dengue fever without any complications, an increase in the WBC and platelet counts occurred afterward, and the patient was discharged on the 6th day of illness.

Patient N-204, who had SD, was a 20-year-old man presenting with fever, muscle pain, joint pain, severe upper abdominal pain, headache, and nausea. He had vomited several times within a 4-day period. Upon admission (4th day of illness), his WBC count was

3.1 × 10^3/mm^3, PLT was 67.9 × 10^3/mm^3, and HCT was 37.8%. Ultrasonic and biochemical investigations revealed tender palpable liver, free fluid in the abdomen and pelvic area, pleural effusions, and elevated serum AST and ALT levels, while he also developed difficulty in breathing on the 5th day of the illness. On the 6th day, bleeding from the cannula site, vomiting of blood, and features of acute kidney injury requiring hemodialysis were observed. Although his lowest WBC count was 2.8 × 10^3/mm^3, lowest PLT count was 19.6 × 10^3/mm^3, and highest HCT level was 44.9% on the 5th day of illness, the patient's condition gradually improved over 9 days post-infection upon treatment. The patient was discharged 14 days post-infection.

Both patients were DENV NS1 positive. Laboratory results revealed that the DwoWS patient (N-189) was DENV-IgM negative with primary infection and that her serum viral genome RNA levels were at 2.02 and 6.82 log10 copies/mL for DENV-2 and DENV-3, respectively. The SD patient (N-204) was DENV-IgM positive with secondary infection, and his serum DENV-3 RNA level was determined to be 7.22 $\log_{10}$ copies/mL.

The phylogenetic trees of DENV-3 included the strains from the 2 patients (N-189 and N-204) examined in this study, as well as other strains from Sri Lanka, its neighboring countries, and other countries, as found in GenBank. The phylogenetic trees were constructed based on the full coding region of the E gene (Fig. 2) and the whole genome (Fig. 3) to determine the relationships and genotypes of the strains examined in this study. Both of 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 2 different clades. According to the phylogenetic tree based on the E gene, DENV-3 isolated strains in our study were closely related to 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, and KY921906) (Table 1), after which 30 amino acid changes were detected. Sixteen amino acid changes were observed between the DENV-3 strains from patients N-189 and N-204. A greater number of amino acid differences were detected in nonstructural proteins (10 positions) than in structural proteins (6 positions). Two amino acid changes in the NS5 protein (A3018V, P3312S) had not been reported previously, whereas 6 uncommon amino acid changes, namely G620E (E protein), Y948H (NS1), I1245V (NS2), I1664M, A1829T (NS3), and T3318A (NS5), were found based on the complete genomes of other strains recorded in GenBank.

DISCUSSION

In 2017, a SD epidemic of unprecedented magnitude occurred in Sri Lanka, and the cosmopolitan genotype of DENV-2 was dominant in the most affected district (19). In our previous study on this outbreak (14), we detected the cosmopolitan genotype of DENV-2 (74%) and genotype I of DENV-4 (1%). In addition, we reported the isolation of a neurotropic DENV-2 cosmopolitan genotype from the cerebrospinal fluid of patients with encephalitis (20). In the present study, we described the emergence of genotype I of DENV-3, which was first observed in Sri Lanka during the 2017–2018 period. One patient (with DwoWS clinical manifestation) concurrently infected with 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 of primary infection. Our findings are in line with those of previous studies in Brazil and India that reported mild dengue manifestation caused by concurrent infections with DENV-2 and DENV-3 (21–23). However, conflicting outcomes have been documented in which co-infections with DENV serotypes may lead to either mild or SD manifestation (24). The co-infection rate caused by DENV-2 and DENV-3 infection was lower than that caused by other serotype combinations (25). Although a higher virus titer of DENV-3 over DENV-2 was recorded, both viruses were isolated from the patient with DwoWS. It is possible to acquire co-infections via the bite of a single mosquito that has two DENV serotypes or be bitten by 2 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 to be a severe manifestation of secondary infection. It has been observed in several studies that secondary infection with heterologous serotypes is more severe than primary infection, which may be explained by an antibody-dependent enhancement mechanism (27–29). In previous studies, genotype III of DENV-3 found in Sri Lanka formed 2 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 2 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 differences in evolutionary patterns, we compared the E gene and the whole genome by phylogenetic analysis. The DENV-3 strains in this study were determined to be genetically close to those found in almost the same set of countries in both gene analyses. Although new and uncommon amino acid changes were found in the two strains, the effect of these changes on clinical severity is unknown. Further studies are warranted to characterize the two DENV-3 isolates in vivo and their tropism in vitro.

Our study site, Kandy Hospital, is situated in the central part of Sri Lanka, which has a high altitude (500 m 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 infections in Kandy and the central part of the country. In summary, co-infection with DENV-2 and DENV-3, and mono-infection with DENV-3, were observed as non-severe and severe manifestations in 2 patients, respectively,

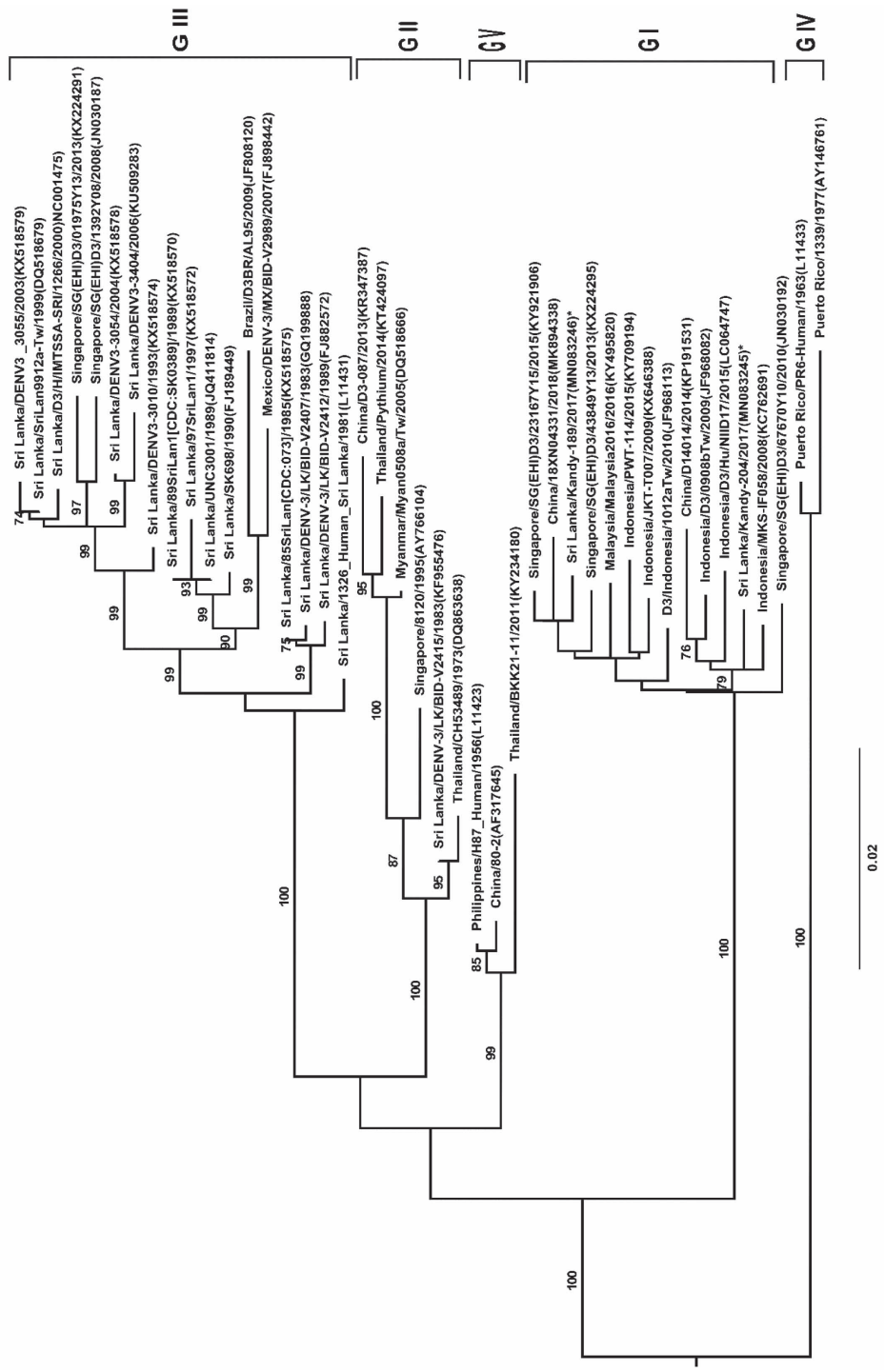

Fig. 2. DENV-3 phylogenetic tree. Phylogenetic tree was constructed on the basis of whole E gene of DENV-3. It showed the relationship of 41 virus strains from different sources and the 2 strains (*) of DENV-3 from this study. The representative strains of each genotype obtained from GenBank were named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

and both patients were infected in the latter part of the 2017 epidemic. Although a new clade of a cosmopolitan genotype of DENV-2 caused the unprecedented dengue outbreak in 2017, a new genotype introduction of DENV-3 might be in the pipeline to lead future outbreaks in Sri Lanka. According to data released by the Epidemiology Unit of the Ministry of Health in Sri Lanka, the number of suspected dengue cases was reported to be 51,659 in 2018, and 70,092 between January and November 2019 (31). Based on preliminary findings in our new study population, we detected genotype I of DENV-3 among dengue patients during the 2018 and 2019 dengue outbreaks. Currently, the increase in dengue cases could be based on circulation of multiple serotypes, along with serotype and genotype shifting, and further studies are necessary to understand the molecular epidemiology of dengue in the 2018–2019 outbreaks.

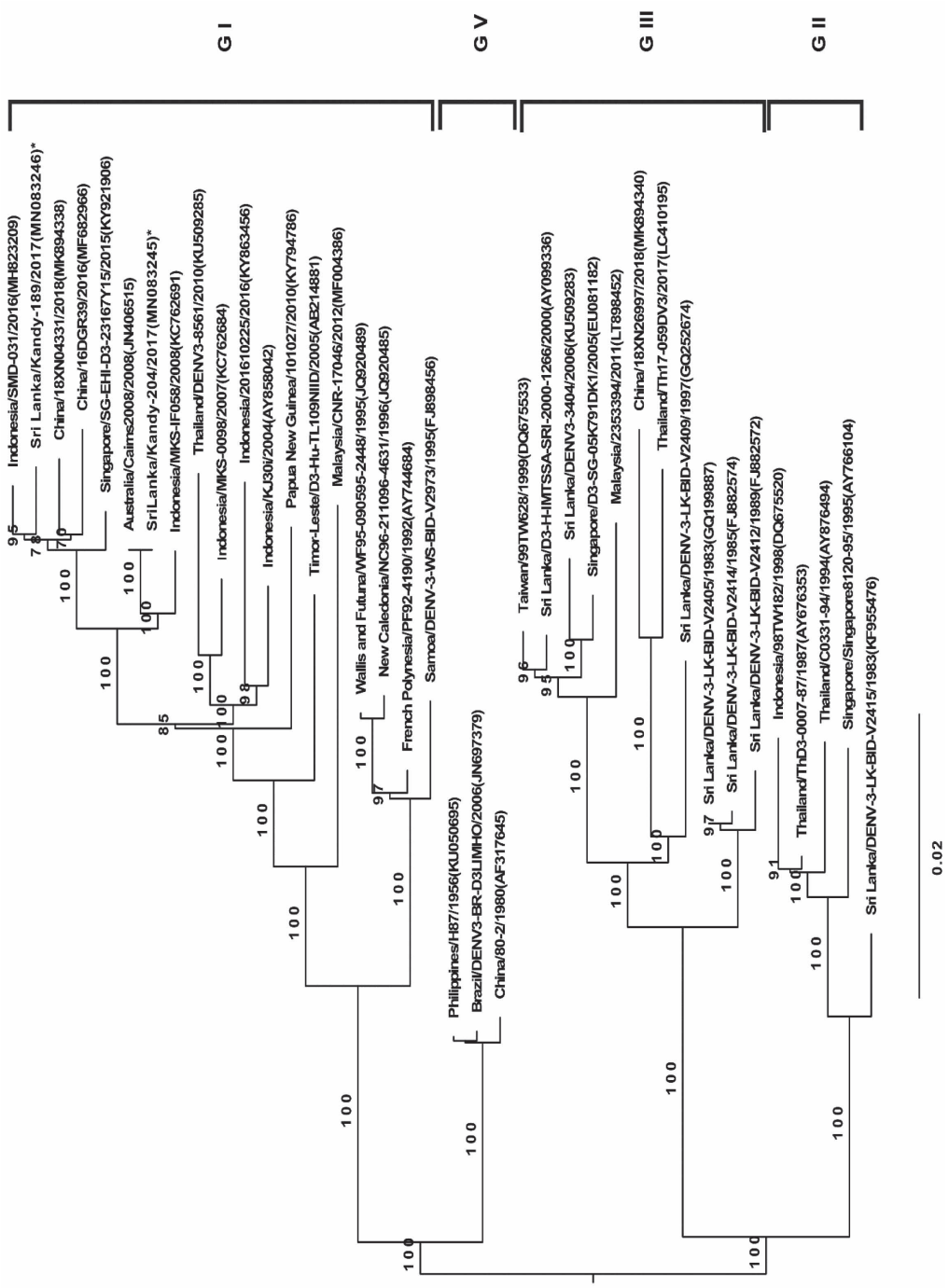

Fig. 3. DENV-3 phylogenetic tree. Phylogenetic tree was constructed on the basis of full coding regions of whole genome of DENV-3. It showed the relationship of 36 virus strains from different sources and the 2 strains (*) of DENV-3 from this study. The representative strains of each genotype obtained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

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Conflict of interest None to declare.

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

DENV-3 gene	polyprotein position	Amino acid residue				
		189 isolate	204 isolate	MK 894338 (China_2018)	KC 762691 (Indonesia_2008)	KY 921906 (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
NS1	779	V	I	V	I	I
	813	K	K	K	R	K
	867	T	I	I	I	I
	948	**H**	Y	Y	Y	Y
	1066	T	T	T	A	T
NS2	1245	I	**V**	I	I	I
	1390	T	T	A	T	T
	1437	I	I	V	I	I
NS3	1533	**H**	Y	Y	Y	Y
	1664	I	**M**	I	I	I
	1829	**T**	A	A	A	A
NS4	2185	G	G	G	G	C
NS5	2537	R	R	K	R	R
	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

Boldface types indicate amino acid substitutions in DENV-3 isolates.

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