

Short Communication

Sequence Variation of Dengue Type 2 Virus Isolated from Clinical Cases in Thailand

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SUMMARY: Dengue fever (DF) and dengue hemorrhagic fever (DHF) are caused by mosquito-borne dengue virus (DENV) infection leading to death in tropical and subtropical countries. In Thailand, all 4 serotypes of DENV are circulating. The most severe cases of DF and DHF are primarily introduced by secondary infections. Epidemiological studies have demonstrated that approximately 20% of the primary infection cases were caused by DENV-1 and -3, while the cases of DENV-2 or -4 accounted for less than 3%. For this reason, DENV-2 and -4 from primary infections have not been well studied. In this study, the sequence diversity of the envelope gene of 8 DENV-2 clinical isolates from primary/secondary infections was analyzed. DENV-2 from primary infections were highly heterogeneous in individual patients, whereas those from secondary infections were homogeneous. Phylogenetic analysis demonstrated that the heterogeneous population of DENV-2 from primary infections was composed of closely related quasispecies. Homogenous DENV-2 could be derived from selection of a particular viral population in secondary infections. The degree of sequence diversity of DENV-2 varied, and thus quasispecies may be involved in the progression of DENV infection.

Dengue virus (DENV) is the causative agent of dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), which remain major health concerns in tropical and subtropical regions (1,2). Recent reviews of DENV infections indicate that there is an increase in the number of patients, an expansion of epidemic areas, and an increase in the incidence of severe clinical manifestations, all of which are major health concerns (2). It is well known that DHF and DSS occur more frequently as secondary infections, suggesting that host immune responses may play an important role in the pathogenesis of DENV infection (3,4). In fact, several groups have proposed that mediators in the host immune system, such as cytokines and the complement system, may play direct roles in the occurrence of plasma leakage, which is the defining feature of DHF (5,6). In addition, it has been hypothesized that differences in virulence among DENV serotypes and genotypes also contribute to disease severity (7,8).

Although DF and DHF are main manifestations of secondary or serial infections, approximately 20% of cases in Thailand occur as primary infections, primarily by DENV-1 and DENV-3 (9). Although all 4 serotypes are capable of causing DF, DHF, or DSS by secondary

infections, DENV-2 and DENV-4 rarely cause disease in individuals with primary infections (approximately 2%–3% of cases) (9,10). For this reason, DENV-2 and -4 isolated from primary infections have not been studied. Therefore, the aim of this study was to elucidate virological differences between DENV-2 isolates derived from primary and secondary infections.

Recent observations suggest that DENVs circulate as a population of closely related genomes, which are referred to as quasispecies (12–14). However, DENV-2 quasispecies derived from primary infections have not been previously studied because of the relatively small number of cases. Therefore, we analyzed the sequence diversity of DENV-2 isolated from both primary and secondary infections. Peripheral blood specimens were collected for diagnostic purposes from 8 confirmed cases of DENV-2 infection of patients who presented to hospitals in the cities of Nakhon Ratchasima, Lampang, and Ratchaburi in Thailand. Serotypes were determined by reverse transcription polymerase chain reaction (RT-PCR). Levels of DENV-specific immunoglobulin (Ig) M and IgG were measured in all plasma specimens using antibody-capture enzyme-linked immunoabsorbent assay (ELISA), as described previously (9,11). Anti-DENV IgM was considered positive by a binding index (BI) ≥ 40 U, whereas anti-DENV IgG was considered positive by a BI of ≥ 80 U. Four cases (N99-012, N01-212, L04-225, and L04-153) with an IgM:IgG ratio of $\geq 1.8:1$ were diagnosed as primary infections, whereas the other four cases (R05-624, R05-552, R05-135, and R06-018) with a ratio of $< 1.8:1$ were

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Sequence Variation of DENV-2 Isolated in Thailand

Table 1. Sequence diversity in E gene of DENV-2 isolates in this study

Patient	Clone number isolated	Substitution number (NS/S) ¹⁾	Mean diversity (%) ²⁾		<i>p</i> -distance (%) ³⁾			
							Nucleotide	
			Nucleotide	Amino acid	Mean	Range	Mean	Range
Primary								
N99-012	10	7 (7/0)	0.094	0.141	0.115	0-0.269	0.346	0-0.808
N01-212	10	19 (10/9)	0.195	0.202	0.371	0-0.010	0.450	0-0.808
L04-225	10	33 (10/23)	0.222	0.202	0.549	0-1.886	0.404	0-1.021
L04-153	10	10 (7/3)	0.067	0.141	0.145	0-0.269	0.314	0-0.606
Average			0.145	0.172	0.295		0.379	
Secondary								
R05-624	11	5 (1/4)	0.037	0.018	0.061	0-0.135	0.037	0-0.202
R05-552	13	1 (1/0)	0.010	0.015	0.010	0-0.067	0.031	0-0.202
R05-135	15	3 (1/2)	0.018	0.013	0.053	0-0.202	0.027	0-0.202
R06-018	17	2 (2/0)	0.016	0.024	0.016	0-0.135	0.048	0-0.404
Average			0.020	0.018	0.035		0.036	

¹⁾: Total nucleotide substitutions identified. The number of the substitutions of nonsilent (NS) and silent (S) substitutions are shown at left and right in parenthesis, respectively.

²⁾: The mean diversity is the number of substitutions divided by the total number of nucleotides or amino acids sequenced.

³⁾: *p*-distances were calculated by pairwise comparison of nucleotide sequences between clones using the program MEGA 5.0 software.

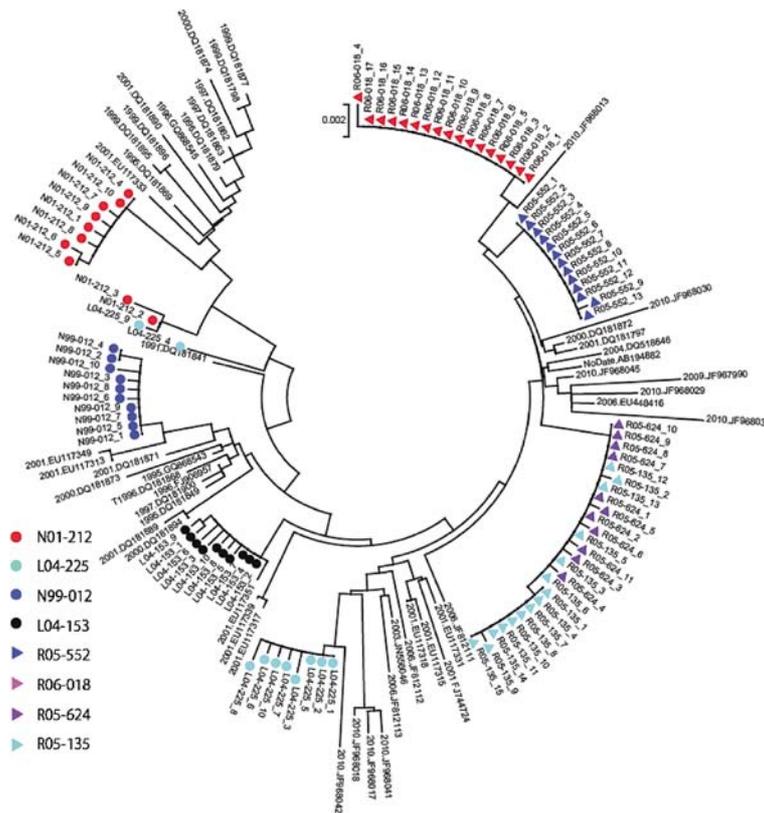


Fig. 1. (Color online) Phylogenetic tree showing the evolutionary relationships of the E gene among 146 sequences from 96 clonal sequences of 8 DENV-2 isolates and 50 Thai isolates. The years of virus isolations and the accession numbers were indicated in each virus branch. The branch length of the trees was drawn to scale.

diagnosed as secondary infections.

Viral RNA was extracted from culture fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA), in accordance with the manufacturer's instructions. For the synthesis of viral cDNA, RT-PCR was performed using the SuperScript III Reverse Transcriptase Kit with random hexamer primers (Invitrogen,

Carlsbad, Calif., USA). The RT-PCR products were cloned into a pCR-Blunt plasmid and transformed in *Escherichia coli* TOP10 cells (Invitrogen). Ten to 17 clones from each specimen were subjected to sequencing analysis of the E region, which was performed using an ABI PRISM 3130XL genetic analyzer (Applied Biosystems, Foster City, Calif., USA). The DENV se-

quences identified in this study were submitted to GenBank and assigned the accession numbers AB587891–AB587986. At the nucleotide level, DENV-2 isolates from primary infections exhibited a high mean diversity (0.067%–0.222%), whereas those from secondary infections exhibited a low mean diversity (0.010%–0.037%) (Table 1). At the amino acid level, clearer differences were observed. DENV-2 isolates from primary infections showed higher diversity than those from secondary infections (0.141%–0.202% vs. 0.013%–0.024%) (Table 1). A similar feature was observed in the pairwise *p*-distance at both the nucleotide and amino acid levels. The mean *p*-distances for primary and secondary infections at the nucleotide level were 0.005–0.549 and 0.010–0.061, respectively, and 0.314–0.450 and 0.027–0.048, respectively, at the amino acid level (Table 1). These results suggest that the differences in DENV-2 isolates from primary and secondary infections may be due to different environments or immunological states of the hosts.

To determine the evolutionary history of the DENV-2 isolates identified in this study, the full-length nucleotide sequences of the E genes were compared with those of previously published DENV-2 E gene sequences available in the Molecular and Genetic Bioinformatics Facility database. Phylogenetic trees were constructed of the 96 clonal sequences obtained from the 8 viral isolates of this study and 50 DENV-2 sequences of isolates obtained in Thailand (Fig. 1). Most of the clonal sequences formed a tight cluster, suggesting that they are closely related quasispecies. Some clones from L04–225 (L04–225_9) and N01–212 (N01–212_2 and _3), derived from primary infections, formed a separate cluster. One clone (L04–225_4) from L04–225 did not belong to any cluster. Presumably, the viral population in this patient had more than 2 origins and not just quasispecies derived from 1 strain. Reportedly, co-infections with 2 distinct DENV serotypes often occur (15).

There are 2 possibilities to explain the homogeneous DENV-2 population in secondary infections. First, it may be the result of higher fitness of the viruses to pre-existing enhancing antibodies (so-called ADE antibodies). The virus population with the highest fitness against non-neutralizing antibodies may become the predominant strain in secondary infections. Second, it may be a result of escape from pre-existing neutralizing antibodies or other immunological selection pressure originating from a previous infection with another serotype.

Taken together, the results of this study demonstrated that the degree of diversity varies between DENV-2 in primary and secondary infections. There were 2 limitations to this study: the small sample size and the fact that the specimens were collected from different provinces in Thailand. Nonetheless, our observations

suggest the possible involvement of quasispecies in the progression of DENV infection.

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Conflict of interest None to declare.

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