NS2A-NS2B SEQUENCE ANALYSIS OF DEN-4 VIRUS STRAINS

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Abstract. To study the genetic variability of DEN-4 Chinese isolates, and to trace the origin of DV4 Chinese isolates, we cloned and sequenced the NS2a-NS2b junction of 5 isolates and prototype DV4 (H-241). Our results show that isolates from the 1990 Guangdong epidemic, which were isolated in the early, middle, and late periods of the epidemic, share the same sequence in the NS2a-NS2b junction. The sequence similarity between isolates from the Guangdong epidemic in 1990 and DV4 H-241 is 96%; these isolates can be grouped into genotype I. The sequence similarity between the isolate from the Guangdong epidemic in 1987 and Dominica strain 814669 is 96%; this isolate can be grouped into genotype II. For the first time, our results show that there are also 2 DV4 genotypes in the Guangdong area of southern China, and these isolates perhaps were introduced from other epidemic areas outside of China.

Dengue virus (DEN) is a mosquito-borne flavivirus and the most prevalent arbovirus in tropical and subtropical countries. Dengue virus occurs as 4 distinct serotypes, DEN-1, DEN-2, DEN-3, and DEN-4. Dengue virus infection can cause dengue fever (DF); its severe form is dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), the mortality rate of which is very high, especially in young children (Gubler, 1998).

Dengue viruses are single-stranded positive–sense RNA virus, which are transmitted between humans, mainly by *Aedes aegypti*. The genome consists of a single open reading frame which encodes a precursor polyprotein. Proteolytic cleavage of the polyprotein results in the formation of core (C), membrane (M) and envelope (E) proteins and the non-structural proteins NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5. Studies of the molecular evolution and epidemiology of DEN-1, DEN-2 and DEN-3 viruses using genome sequence relatedness have demonstrated the occurrence of genotype groupings among these viruses. They can cause epidemics in most tropical and subtropical areas of the world, including the Americas, Africa, Asia, and South Pacific regions. It is estimated that there are some 100 million cases of dengue fever, 500,000 cases of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) and 25,000 deaths attributable to dengue, annually (Pervikov, 2000).

Variation analysis can be used to define genetic variations between strains of the same serotype, follow the genetic geographic movement of virus strains, and facilitate the identification of the sources of virus strains in new outbreaks. This method has been successfully used in the variation analysis of DEN-1 (Chungue *et al*, 1995), DEN-2 (Ruiz *et al*, 2000), DEN-3 (Chungue *et al*, 1993), and DEN-4 (Chungue *et al*, 1995; Lanciotti *et al*, 1997). With the help of these analyses, many valuable findings have been made. Even intra-type recombination of dengue virus has been found (Holmes *et al*, 1999; Tolou *et al*, 2001; Uzcategui *et al*, 2001). This finding will improve our understanding of dengue virus virulence variation. At the same time, these results remind us that more attention should be given to research into recombination within dengue viruses, this kind of recombination may explain why the occurrence rate is rising and epidemic areas are enlarging. In this article, for the first time, we characterized some DV4 Chinese isolates, and compared these isolates' NS2a-NS2b sequence with isolates' sequences from other parts of the world.

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MATERIALS AND METHODS

Virus isolates and cell line

Five DV4 Chinese isolates from the 1990 epidemic in Guangdong, and one from the 1978 epidemic in Guangdong were chosen for analysis (Table 1). These isolates were taken from sera of DF patients using routine methods in the C6/36 cell line. Their serotypes were determined by indirect immuno fluorescence assay (IFA). The 3 isolates from the 1990 epidemic were from DF patients in the early, middle and later stages of the epidemic. The GDA63 strain was isolated from *Aedes albopictus* in the early stage of the 1990 epidemic. GD7856B2 was isolated from a DF patient in the Guangdong epidemic, in 1987. DV4 H-241 is the prototype of DV4, which was isolated from a DF patient in Philippines, in 1956.

RNA extraction and RT/PCR amplification of viral RNA

Viral RNA was extracted directly from virus stock and RT was performed according to the method of Yao *et al* (1997). The primers used in RT and PCR are as follows:

Upstream primer: D4S, 5′-CCATTATGGCT GTGTTGTTT-3′, 3973nt---3992nt. Downstream primer: D4C , 5′-TTCATCCTGCTTCACTTCT-3′, 4370nt---4352nt.

PCR amplification was done in 50 μl reaction volume (Yao *et al*, 1997), the only difference being that 2.5 units of Pfu DNA polymerase (Gibco) was used in the PCR reaction to increase the fidelity of the PCR products.

Cloning of the PCR product

Specific PCR products were purified with a Geneclean II kit (HB101, Inc). Then, this product was inserted into pCR 4Blunt-TOPO vector (Invitrogen) according to the protocols in the kit. Briefly, the product was ligated with the pCR 4Blunt-TOPO vector at appropriate ratio, and kept at 4ºC overnight. Two microliters of ligation mixture was transformed into a DH5α electrocompetent cell by electrotransfection method (25 μF, 2.5 kV, 200 Ω), then, plated onto LB plate containing ampicillin (100 μg/ml).

Identification of correct clones

White colonies were picked and identified with enzyme digestion (with Eco RI) and PCR.

DNA sequencing

Nucleic acid sequencing was performed in an automatic ABI PRISM 377 DNA Sequencer with Bigdye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The protocols used were depicted in the kit's manual.

DNA sequence analysis

Strain 814669 (Dominica strain of dengue 4 virus, isolated in 1981) and GZB5 (isolated in Guangzhou China, in 1993) were also included in the analysis for comparison (Table 1). The Clustal V program (Baylor College of Medicine) was used to align the above sequences.

RESULTS

Sequences of the cDNA of the NS2a –NS2b region of the 6 strains, GZB5 and 814669, are listed in Fig 1. The 1990 isolates were taken from DF patients who were infected at the beginning, middle and end of the epidemic. No difference was observed among the isolates collected in the same epidemic (strains GD9006A1, GD9033A1, GD9049A2, GDA63). There was sequence difference between the isolates from 1990 and from 1987 (similarity between them 93%). Homology analysis of nucleotide sequences showed that the similarity is 96% between DV4 H-241 and 4 isolates from 1990. The similarity between DV4 H-241 and GZB5 96%. The similarity between DV4 H-241 and 814669 93%; between DV4H-241 and GD7856B2 93%, and between strain 814669 and GD7856B2 96%. The similarity among the above strains for amino acid level is ≥96%. Most of the mutation occurred in the third position of the codon, and mainly are transitions (T ↔ C, G ↔ A). The result of sequences alignment is shown in Fig 1.

DISCUSSION

DF/DHF/DSS are still important health problems in many tropical and subtropical areas. Although there were dengue epidemics in the 1940s in China, no major outbreak of DF was reported until 1978, which was the time when China began to open up to the outside world. The present

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DV4H-241     1 CCATTATGGCTGTGTTGTTTGTGGTCACACTCATTCCTTTATGCAGGACAAGCTGTCTTC
GDA63        1 ..............................................................C.
GD9006A1     1 ..............................................................C.
GD9033A1     1 ..............................................................C.
GD9049A2     1 ..............................................................C.
GZB5         1 ....A...........A..............................................C.
GD7856B2     1 ........................................G.....................
814669       1 ........................................G.....................

DV4H-241    61 AAAAGCAGTCACATTGGGTAGAAATAACAGCACTCATCCTGGGAGCCCAGGCTCTGCCAG
GDA63       61 ....A.........................................................
GD9006A1    61 ....A.........................................................
GD9033A1    61 ....A.........................................................
GD9049A2    61 ....A.........................................................
GZB5        61 ....C.........................................................
GD7856B2    61 ....A.....T........G.....................A............T......
814669      61 ....A.....T............................A........A..........

DV4H-241   121 TGTACCTAATGACTCTCATGAAAGGAGCTTCAAAGAGATCTTGGCCCCTTAACGAGGGTA
GDA63      121 ............................C.....A.....C...................
GD9006A1   121 ............................C.....A.....C...................
GD9033A1   121 ............................C.....A.....C...................
GD9049A2   121 ............................C.....A.....C...................
GZB5       121 ............................C.....A.....C...................
GD7856B2   121 .....T......................C....GA...........T...........C.
814669     121 ................T...........C....GA...........T...........C.

DV4H-241   181 TAATGGCTGTGGGTTTGGTCAGTCTCTTGGGAAGCGCCCTCCTAAAGAATGATGTCCCTT
GDA63      181 ..............C...............................................C.
GD9006A1   181 ..............C...............................................C.
GD9033A1   181 ..............C...............................................C.
GD9049A2   181 ..............C...............................................C.
GZB5       181 ..............C...............................................C.
GD7856B2   181 ............C...........T............T..TT...................
814669     181 ................A..T........A.........T..TT...................

DV4H-241   241 TAGCTGGCCCAATGGTGGCAGGAGGCTTACTCCTGGCAGCCTATGTGATGAGTGGTAGCT
GDA63      241 .G..............................T.............................TG
GD9006A1   241 .G..............................T.............................TG
GD9033A1   241 .G..............................T.............................TG
GD9049A2   241 .G..............................T.............................TG
GZB5       241 .G..............................T.............................TG
GD7856B2   241 ..............................T.....G..T..C...................
814669     241 ..............................T.....G..T..C...................

DV4H-241   301 CAGCAGACCTGTCACTAGAGAAGGCCGCCAATGTGCAGTGGGATGAGATGGCAGACATAA
GDA63      301 ............................................................
GD9006A1   301 ............................................................
GD9033A1   301 ............................................................
GD9049A2   301 ............................................................
GZB5       301 ............................................................
GD7856B2   301 ........................T.....................A.............
814669     301 .......T..................................A.............

DV4H-241   361 CAGGCTCAAGCCCAATCACAGAAGTGAAGCAGGATGAA
GDA63      361 ...................T....................
GD9006A1   361 ...................T....................
GD9033A1   361 ...................T....................
GD9049A2   361 ...................T....................
GZB5       361 ...................T....................
GD7856B2   361 ...................T....................
814669     361 ...................T....................
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Fig 1–Sequences alignment of the NS2a-NS2b region of eight dengue type 4 strains from China, Dominica and prototype DEN-4 strain. GZB5 and 814669 were included for comparison.

Table 1
Description of DV4 virus isolates compared by sequence analysis.

Strain	Receiving date	Geographical origin	Source	GeneBank accession No.
GDA63	9/15,1990	China	*Aedes albopictus*	Y19171
GD9006A1	9/13,1990	China	DF patient	Y19172
GD9033A1	9/30,1990	China	DF patient	Y19173
GD9049A2	10/23,1990	China	DF patient	Y19174
GD7856B2	1978	China	DF patient	Y19175
GZB5	1993	China	-	AF289029
DV4 H-241	1956	Philippines	DF patient	Y19176
814669	1981	Dominica	-	M14931

study of DEN-4 isolates from patients and mosquitos aimed to find the relationship between these isolates, and their origin.

A short nucleotide sequence of 398 bp, the NS2a-NS2b junction of six DEN-4 strains (one is prototype of DEN-4, DV4 H-241) were studied by RT/PCR and sequencing, and they were compared with strain 814669 (isolated in Dominica, in 1981). Lanciotti's results (Lanciotti *et al*, 1997) show that DV4 can be classed into two genotypes. Genotype I is formed mainly by Asian isolates, Thailand, Sri Lanka, and the Philippines. Genotype II includes isolates from Indonesia, Tahiti and the Caribbean islands, central and south America. DV4 H-241 was isolated in 1956 in the Philippines, belonging to genotype I. As the similarity between DV4 H-241 and isolates from the 1990 Guangdong epidemic are 96%, isolates GD9006A1, GD9033A1, GD9049A2, GDA63 are grouped into genotype I. GZB5 can also be grouped into genotype I. Strain 814669 is a Dominican strain isolated in 1981; the nucleotide homology between 814669 and strain GD7856B2 is 96%, so GD7856 B2 belongs to genotype II.

GD7856B2 was the first isolate that caused dengue fever in southern China after nearly 40 years' silence, so we deduce that this strain was imported from one of the above genotype II areas. This is supported by the fact that China started opening up to the outside world at that time. This strain was perhaps imported to southern China by trade or travelers. GD9006A1, GD9033A1, GD9049A2, GDA63 have come from one of the genotype I areas. As it is impossible for dengue virus to survive the cold weather in southern China, and there was no DV4 epidemic in 1989 in China, these strains probably came from other countries, and most likely were brought into China by travelers. As GD9006A1, GD9033A1, GD9049A2, and GDA63 share the same sequence (at least in the sequenced region), this reveals that the epidemic in southern China in 1990 was caused by the same DV4 strain. GDA63 is a strain isolated from *Aedes albopictus* in an epidemic area, and, through sequence comparison, it shares the same sequence with the three strains from patients. This may indicate that the disease was transmitted to humans from mosquitos. Although we did not sequence the whole sequence of the four strains from 1990, it seems that dengue viruses were stable in this epidemic. Singh *et al* (1999) also sequenced the 455 bp E-NS1 region in nine dengue type 2 virus isolates from India, in 1996, and found that there were some mutations among the nine isolates in this region, but mainly in the 3rd position of a codon. Because these isolates were collected from a DF patient in the 1996 epidemic, and the intervals were very short, these isolates may indeed be different strains co-circulating in a specific area. It is also possible that the E-NS1 region has more mutation opportunities. At any rate, the results show that the epidemic strains were relatively stable in one epidemic.

Homology analyses of the above strains' amino acid sequences show that the homology among these strains is 96%; these results are in accordance with the conclusion of Lanciotti *et al*

(1997). As there are little DV4 NS2a-NS2b sequence data in GeneBank, we could not compare our strains with others with the help of a phylogenetic tree. However, our data, provides a good basis to surmise, for the first time, the possible origin of DF in China. These strains are imported into China from elsewhere. Although the precise source of the DF in China remains unknown, these data can help us to take some preventive measures against DF.

In recent years, DF epidemics in southern China have been very frequent. The interval was about 2-3 years, but it is becoming shorter, and the epidemic areas are growing larger. Concurrent DF infections (DEN-4 and DEN-2) were also found in southern China, in 1993 (Liao *et al*, 1996). Our recent data show that nearly 1/3 of DF patients in southern China were secondary dengue infections, and that 2/3 of patients had primary dengue infections (Yao *et al*, 2000). Dengue has thus become a serious health problem in southern China. The epidemic pattern in China is in accord with the world-wide epidemic pattern. Therefore, continued surveillance of DEN-4 virus movement and molecular analysis of emerging viruses are still a challenge for public health workers.

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