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Short communication

The origin of dengue viruses caused the DF outbreak in Guangdong province, China, in 2006

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ABSTRACT

Phylogenetic analysis suggested that the DENV1 strains isolated in the DF outbreak in Guangdong province in 2006 were likely to be imported from Southeast Asian. Specifically, viruses isolated from Shantou and Chaozhou were imported from Singapore; viruses isolated from Guangzhou, Yangjiang, and Foshan were imported from Thailand/Vietnam. Therefore, importation of DENV1 from Southeast Asia was an important contributory factor of the 2006 DF outbreak in Guangdong province.

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1. Introduction

Dengue viruses (DENVs) belong to the family *Flaviviridae* and genus *Flavivirus*. Four closely related but antigenically distinct serotypes (DENV1, DENV2, DENV3, and DENV4) have been described. They are distributed worldwide and infection in humans results in a variety of clinical manifestation, ranging from mild, flu-like dengue fever (DF) to life-threatening dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS). In the last decades, DF and DHF/DSS have emerged as one of the most important public health problems in the tropical and subtropical regions. It's estimated that 50–100 million infections occur annually worldwide, and more than 2.5 billion people are at risk (Gubler, 2002, 2004).

DF re-emerged in China in 1978 after disappeared for more than 30 years. And Guangdong province was the most severe epidemic region, with 66 190 infected cases from 1978 to 1989 (He et al., 2002). But cases in this province dropped dramatically since 1990, with 374, 371, 2, 359, 4, 6812, 0, 632, 488, 304, 401, 344, 1348, 42, 47, 6, and 1010 cases from 1990 to 2006, respectively (He et al., 2002; Liang et al., 2007). Strikingly, infected cases were mainly caused by DENV1 since 1990, such as in 1995, 1997, 2000, 2002, 2003, 2004, and 2006. The 3rd largest DF outbreak in Guangdong province in 2006 only occurred in Guangzhou, Yangjiang, Foshan, Chaozhou and Shantou city, and most cases were distributed in Guangzhou (765/1010) and Shantou (177/1010) (Wang et al., 2009; Yao et al., 2007; Luo et al., 2007; Fan et al., 2007; Liu et al., 2007). Therefore, we attempted to address the origin of DENV1 caused this DF outbreak.

2. Materials and methods

2.1. Virus strains

Eight DENV1 strains were isolated from the sera of 38 acute-phase dengue patients admitted to Guangzhou No. 8 hospital, Guangdong province, China, during the DF epidemic that broke out in 2006. The viruses were recovered in C6/36 cells and identified using serological assays, RT-PCR (Lanciotti et al., 1992), and sequences analysis. All virus strains used in this study were listed in Table 1.

2.2. RNA extraction, RT-PCR and sequencing

Viral RNA was extracted from the supernatant of the virus-infected C6/36 cell culture by using the Qiagen RNeasy mini kit. To amplify E/NS1 fragments, RT-PCR was performed by using primers ES1 and ES2 (Table 2). To determine the full-length genomic sequences, 9 overlapping PCR fragments spanning the whole genome were amplified using a combination of primers (A1 and A2, B1 and B2, C1 and C2, D1 and D2, E1 and E2, F1 and F2, G1 and G2, H1 and H2, I1 and I2, Table 2). We performed 5′ and 3′ rapid amplification of cDNA ends (RACE) by using the SMART™ RACE cDNA amplification kit and gene specific primers (GSP1 and GSP2, Table 2). The PCR products were purified from agarose gels and sequenced. To ensure accuracy of the sequenced fragments, each nucleotide was sequenced in at least 3 independent sequencing reactions to avoid sequencing errors.

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Table 1
DENV1 strains used in this study.

No.	Virus strain	Year of isolation	Country of origin	Sequence	GenBank accession number
1	DEN1/GZ/OY (in this study)	2006	Guangdong, China	Genome	FJ176779
2	DEN1/GZ/XNC (in this study)	2006	Guangdong, China	Genome	FJ176780
3	GZ061707	2006	Guangdong, China	Envelope	EF508203
4	GZ16/2006	2006	Guangdong, China	Envelope	EF113152
5	GZ17/2006	2006	Guangdong, China	Envelope	EF113153
6	D060117	2006	Guangdong, China	Envelope	EF508207
7	D06098	2006	Guangdong, China	Envelope	EF508206
8	D06068	2006	Guangdong, China	Envelope	EF508205
9	D06045	2006	Guangdong, China	Envelope	EF508204
10	A04137	2004	Guangdong, China	Envelope	EF508202
11	D02031	2002	Guangdong, China	Envelope	EF508201
12	GZ01/02	2002	Guangdong, China	Envelope	DQ855296
13	71/02GZ	2002	Guangdong, China	Genome	EF025110
14	GZ/218/2002	2002	Guangdong, China	Envelope	EF079826
15	D01048	2001	Guangdong, China	Envelope	EF508200
16	D99020	1999	Guangdong, China	Envelope	EF508199
17	GD05/99	1999	Guangdong, China	Genome	AY376738
18	D98039	1998	Guangdong, China	Envelope	EF508198
19	GD14/97	1997	Guangdong, China	Genome	AY376737
20	GZ01/95	1995	Guangdong, China	Envelope	DQ855297
21	GD23/95	1995	Guangdong, China	Genome	AY373427
22	GZ02/03	1993	Guangdong, China	Envelope	DQ211349
23	DENV-1/VN/BID-V4036/2008	2008	Vietnam	Genome	GU131793
24	DENV-1/VN/BID-V3895/2008	2008	Vietnam	Genome	HM181964
25	DENV-1/VN/BID-V3864/2008	2008	Vietnam	Genome	GU131699
26	DENV-1/VN/BID-V957/2007	2007	Vietnam	Genome	EU482502
27	DENV-1/VN/BID-V2805/2007	2007	Vietnam	Genome	FJ882554
28	DENV-1/VN/BID-V2824/2007	2007	Vietnam	Genome	GQ199829
29	DENV-1/VN/BID-V1324/2006	2006	Vietnam	Genome	EU660394
30	DENV-1/VN/BID-V807/2006	2006	Vietnam	Genome	EU482801
31	DENV-1/VN/BID-V2687/2006	2006	Vietnam	Genome	GQ199771
32	DENV-1/VN/BID-V817/2006	2006	Vietnam	Genome	EU482811
33	SG(EHI)DED149408	2008	Singapore	Envelope	GQ357686
34	SG(EHI)DED80208	2008	Singapore	Envelope	GQ357682
35	D1/SG/06K2236DK1/2006	2006	Singapore	Genome	EU081280
36	D1/SG/05K4142DK1/2005	2005	Singapore	Genome	EU081257
37	S418/05	2005	Singapore	Envelope	EU069594
38	D1/SG/05K2916DK1/2005	2005	Singapore	Genome	EU081234
39	T3196/04	2004	Singapore	Envelope	EU069624
40	T3179/04	2004	Singapore	Envelope	EU069619
41	SG(EHI)D1209Y03	2003	Singapore	Genome	FJ469907
42	S159/03	2003	Singapore	Envelope	EU069598
43	S144/02	2002	Singapore	Envelope	EU069600
44	D1/Thailand/0707aTw	2007	Thailand	Envelope	EU448394
45	DenKor-05	2005	Thailand	Envelope	EF654108
46	D1/Thailand/03-135	2003	Thailand	Envelope	HM134239
47	D1/Thailand/0310aTw	2003	Thailand	Envelope	EU448393
48	ThD1_0075_02	2002	Thailand	Envelope	AY732398
49	ThD1_0102_01	2001	Thailand	Genome	AY732479
50	ThD1_0049_01	2001	Thailand	Genome	AY732482
51	DENV-1/TH/BID-V2273/2001	2001	Thailand	Genome	FJ850068
52	01A00082	2001	Thailand	Envelope	EU117312

Table 2
Oligonucleotide primers used in this study.

	Upstream primers			Downstream primers		
	Name	Position	Sequences (5′–3′)	Name	Position	Sequences (5′–3′)
1	ES1	2183–2206	ctttggttctataggaggagtgtt	ES2	2625–2648	ttcaagtaagatgtgattcagttc
2	A1	298–321	agaagaatggagcgatcaaagtgc	A2	1646–1623	tgtcttaaatgtcaccagcaaatc
3	B1	1448–1469	cgactacggagctcttacattg	B2	2692–2670	atcccagcaacatctcctacaac
4	C1	2547–2567	aaggcatgggaggagggtgtg	C2	3805–3782	tccacagatgccactagactcaat
5	D1	3657–3677	gacaggatggggatgggaacg	D2	4890–4867	ctagggcaatggctccaacttcac
6	E1	4678–4701	tcatgtatcaagggaagagactgg	E2	5987–5965	catttttgcttccgtccagtgag
7	F1	5760–5779	cgggccgacagggtgataga	F2	7180–7201	gcttttgcttgcagtccaggtc
8	G1	6917–6940	agcttcagcctggaccctctatgc	G2	8183–8161	tgggtttcgcactagcattcctc
9	H1	8027–8047	agaggaaggaagaacgctacg	H2	9383–9363	taagccataagttccgacctg
10	I1	9169–9192	gggatacaaggataacagaggatg	I2	10497–10476	agtctgctaccccatcgctaca
11	GSP2	10337–10311	gtcaggccggattaagccatagtacgg	GSP1	580–606	ctggttccgcctcagtgattcgagggc

Positions were given relative to the published sequence of the strain Cambodia (AF309641).

2.3. Sequence analysis

Nucleotide and amino homology analysis was performed by using MEGA4 (Tamura et al., 2007). The ML tree, based on the full-length envelope gene, was inferred by using TREE-PUZZLE program (Schmidt et al., 2002).

3. Results

3.1. Full-length viral genome sequences

Pairwise comparisons of 8 DENV1 strains recovered in this study showed nucleotide homology of 100% between 7 strains in the E/NS1 region, except 1 strain which had nucleotide divergence of 2.6% with above 7 strains. Therefore, full-length genomic sequences of strain DEN1/GZ/XNC (the most divergent strain) and DEN1/GZ/OY (1 of the mentioned 7 strains) were determined. Both viral genomes had 10 735 nucleotides with no insertions and deletions. The genome sequences of DEN1/GZ/OY and DEN1/GZ/XNC had been submitted to GenBank and assigned the accession numbers FJ176779 and FJ176780.

3.2. Phylogenetic analysis

The ML tree (Fig. 1) showed that all DENV1 strains isolated in Guangdong province in the past 17 years were grouped into 2 genotypes (I and IV). But all 9 strains (GZ061707, DEN1/GZ/XNC, D06098, D060117, D06045, DEN1/GZ/OY, GZ16/2006, GZ17/2006, and D06068) isolated in this province in 2006 were classified as genotype I. To determine the origin of DENV strains caused the DF outbreak in Guangdong province in 2006, blast analysis in GenBank based on the complete envelope genes was performed and the 9 strains were confirmed to be highly homologous to those strains circulated around 2006 in Southeast Asian. Strikingly, the 9 strains in 2006 were classified as 3 different clades (A, B, and C). GZ061707 (Guangzhou) and DEN1/GZ/XNC (Guangzhou) were grouped into clade A formed by strains isolated between 2006 and 2008 from Vietnam. D06098 (Shantou) and D060117 (Chaozhou) were grouped into clade B formed by strains isolated between 2002 and 2006 from Singapore. D06045 (Yangjiang), DEN1/GZ/OY (Guangzhou), GZ16/2006 (Guangzhou), GZ17/2006 (Guangzhou), and D06068 (Foshan) were grouped into clade C formed by strains isolated between 2001 and 2007 from Thailand/Vietnam.

4. Discussion

Because there were not enough complete DENV1 viral genomes available, especially for strains isolated in Guangdong province in 2006, the ML tree constructed in this study was solely based on the envelope gene sequence. Thus phylogenetic inference from this tree might have missed intraserotype recombination events that would have been revealed by using complete genome sequences.

According to results described previously (Zheng et al., 2009), D06098, D060117, D06045, D06068, and GZ061707 were circulating strains, and strain GZ061707 was imported from Cambodia. But in our opinion, the DF outbreak in Guangdong province in 2006 was unlikely caused by local strains. As shown in Fig. 1, the viruses isolated from Shantou and Chaozhou (D06098 and D060117) were similar to those from Singapore. Also, 2 strains from Guangzhou (GZ061707 and DEN1/GZ/XNC) were similar to those from Vietnam; 3 strains from Guangzhou (DEN1/GZ/OY, GZ16/2006, and GZ17/2006), 1 strain from Foshan (D06068), and 1 strain from Yangjiang (D06045) were similar to those from Thailand/Vietnam. In addition, nucleotide and amino acid homology were very high in the envelope or the whole coding region between the strains in Guangdong province in 2006 and epidemic strains in Southeast Asian in corresponding clades (Table 3). Therefore, we concluded that DF outbreak in Guangdong province in 2006 were likely caused by imported cases, i.e., viruses isolated from Shantou and Chaozhou were imported from Singapore; viruses isolated from

Fig. 1. The maximum likelihood (ML) phylogenetic tree based on the full-length envelope gene. Taxon names corresponded to "strain name + origin + year of isolation". Main bootstrap values were shown on the key nodes of the tree. The tree was rooted by strain 43 of DENV2 (AF204178). Strains isolated from Guangdong province were shown as bold fonts.

Table 3
Nucleotide and amino acid homology analysis.

Strain pairs	Sequences	Nucleotide homology (%)	Amino acid homology (%)
DEN1/GZ/XNC DENV-1/VN/BID-V2687/2006	Envelope	99.1	100
	Coding region	99.2	99.8
DEN1/GZ/OY DENV-1/VN/BID-V817/2006	Envelope	99.2	99.8
	Coding region	98.8	99.4
D06098[a] D1/SG/06K2236DK1/2006	Envelope	99.1	99.8
	Coding region	ND	ND

[a] The genome sequence was not available. ND: not done.

Guangzhou, Yangjiang, and Foshan were imported from Thailand/Vietnam (Fig. 2). To strain D01048 isolated in Guangdong province in 2001, it was imported from Thailand. But to strain A04137 isolated in Guangdong province in 2004, it was previously deemed that it was introduced from Micronesia (Zheng et al., 2009). But we deduced that it was more likely introduced from Singapore.

Meanwhile, Fig. 1 showed that strains (genotype I), which were isolated in 1998, 1999, 2001, 2004, 2006, were imported from Thailand, Vietnam, and Singapore. However, other strains, which were isolated in 1993, 1995, 2002, fell into genotype IV. And strains isolated in 2002 were likely introduced from Indonesia (Zheng et al., 2009). In addition, envelope nucleotides diversities were more than 4.7% between strains isolated in 1995 and strains isolated in 1993 and 2002. Therefore, the DF outbreak in Guangdong province in 2006 was unlikely to be caused by local strains, and importation of DENV1 from Southeast Asia was an important contributory factor.

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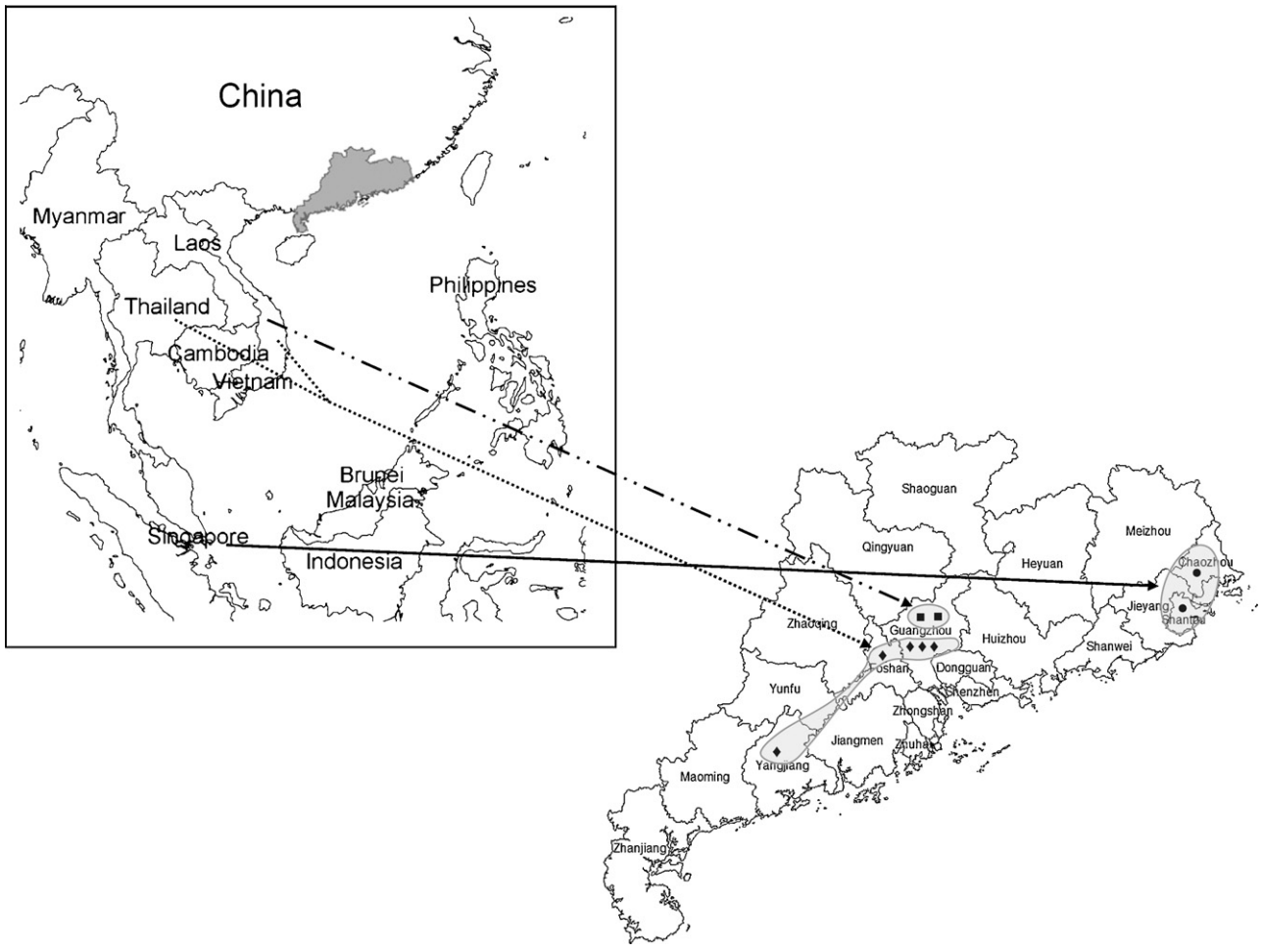

Fig. 2. The origin of DENV1 strains isolated in Guangdong province in 2006. The map in the box showed Southeast Asian, and the grey region in Southeast China was Guangdong province. The right map was an enlargement of Guangdong province. "●" denoted DENV1 strains isolated in Shantou (D06098) and Chaozhou (D060117), which were introduced from Singapore. "■" denoted DENV1 strains isolated in Guangzhou (GZ061707 and DEN1/GZ/XNC), which were introduced from Vietnam. "◆" denoted DENV1 strains isolated in Guangzhou (DEN1/GZ/OY, GZ16/2006, GZ17/2006), Foshan (D06068), and Yangjiang (D06045), which were introduced from Thailand/Vietnam.

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