Dengue in China: Comprehensive Phylogenetic Evaluation Reveals Evidence of Endemicity and Complex Genetic Diversity

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Abstract. Despite the increasing threat of dengue outbreaks in China, it is still considered as an imported disease and its introduction and/or circulation patterns remain obscure. On the basis of the most extensive phylogenetic analysis to date, we showed highly complex genetic diversity of dengue viruses (DENVs) in south China with up to 20 different clades/lineages from multiple serotypes co-circulating in the same year. Despite that most of these clades/lineages were resulted from imported cases, evidence of local persistence of DENV serotype 1 (DENV-1) was observed, indicating its potential endemicity in Guangdong province. This study, therefore, provided an overview of DENV genetic diversity and evolutionary dynamics in China, which will be useful for developing policies to prevent and control future dengue outbreaks in China.

INTRODUCTION

Since 1978, dengue outbreaks have been frequently reported in the southern provinces of China, including Guangdong, Hainan, Guangxi, Fujian, Zhejiang, and so on.¹ Despite a relatively quiet period without major epidemics for the past two decades, dengue returned to prominence with its scale and speed of Chinese outbreaks in 2014, including the Guangdong epidemic, infecting over 40,000 people within 2 months.² Understanding the circulation pattern of dengue viruses (DENVs) in China has therefore become an urgent and essential task for the design of control and prevention strategies.

With its location in tropical and subtropical regions and hot, humid climate, as well as highly populated communities with widely distributed populations of the DENV mosquito vectors *Aedes albopictus* and *Aedes aegypti*, southern China is ideal for dengue propagation and maintenance.^{3,4} However, contrasting opinions exist on whether DENVs have developed endemic transmission in mainland China.^{5,6} Unfortunately, all these conclusions were supported by insufficient and biased data sampling, which failed to represent the enormous genetic diversity and complicated transmission network of DENVs.^{7,8} This under-sampling precluded accurate determination of Asian DENV origins and transmission routes, leading to poorly supported conclusions.^{5,6} Indeed, the vast number (thousands for each serotype) of sequences in GenBank with their metadata often obscure, makes data analysis an even more challenging task. Designing DENV phylogenetic studies and interpreting the corresponding results therefore require extraordinary caution.

To provide an overview of DENV genetic diversity and evolutionary dynamics in China, we conducted the most extensive DENV phylogenetic analyses to date, based on all available E gene sequences in GenBank as of June 2015. Selecting E gene sequences rather than complete genomes allowed us to maximally sample DENV genetic diversity. On the basis of this extensive phylogenetic study, our results for the first time showed clear evidence of endemic dengue circulation in Guangdong province, revealed surprisingly complex genetic

diversity of DENV in southern China, and spotted potential pitfalls in interpreting phylogenetic patterns. This study will therefore not only significantly affect dengue-control policies in China but also shed light on future phylogenetic studies to identify dengue outbreak origins and further address endemicity.

MATERIALS AND METHODS

All sequences for each DENV serotype as of June 2015 were downloaded from GenBank. Sequences shorter than 1,000 nucleotides and those from nonstructural protein gene, related to patents, or from artificial clones were first eliminated from the datasets. Each dataset representing a single serotype was then manually aligned using Se-Al (available at <http://tree.bio.ed.ac.uk/software/seal/>) according to amino acid sequences. Nucleotide sequences from the complete E gene region were used for further analysis. To trim the datasets, a guide Neighbor-Joining tree was first built for each serotype, based on empirical nucleotide substitution models from previous analysis,⁷ using the PAUP* v4.0b package.⁹ On the basis of these guide trees, excessively sampled strains from each cluster of the same country (other than China) and year were then removed to preserve genetic diversity. This led to final datasets of 687 (DENV-1), 526 (DENV-2), 660 (DENV-3), and 478 (DENV-4) sequences. Phylogenetic trees were constructed by both maximum-likelihood method implemented in PAUP* and an approximately maximum-likelihood method implemented in FastTree 2,¹⁰ with clade credibility evaluated by bootstrap and Shimodaira–Hasegawa (SH) tests, respectively (see details in Supplemental Material).

RESULTS AND DISCUSSIONS

First, our phylogenetic trees suggested that there are about 50 individual clades (including those represented by single strains) of DENV-1, over 20 of DENV-2 and a few of DENV-3 and DENV-4 in mainland China (shaded in bright green; Figure 1A and B, Supplemental Figures 1–4; summarized in Table 1). It must be noted that these do not nearly reflect the number of imported DENV lineages/clades given the limited DENV sampling in China. On the basis of sequences of recent years when surveillance and sequencing were more active, there were multiple lineages, often from multiple serotypes, co-circulating each year in China, particularly

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FIGURE 1. Maximum-likelihood trees of DENV-1 (A, C) and DENV-2 (B, D). Geographic regions are reflected by different colors.

in Guangdong province. For example, both DENV-1 and DENV-2 were identified during the 2014 Guangdong outbreak, each involving two lineages reflecting two and three origins, respectively (Figure 1A and B). Similarly, all four serotypes were present in 2013, including even more lineages and reflecting about 20 different origins. In contrast to the single

origin of recent dengue outbreaks in some South American and African countries, the genetic diversity of dengue exhibited in Guangdong province is surprisingly large.^{11,12} Clearly, annual co-circulation of multiple DENV serotypes poses a high risk for dengue hemorrhagic fever and therefore demands further public health attention.^{13,14} On one hand, frequent

TABLE 1
Summary of phylogenetic positions and genetic diversity of sequenced DENV strains from mainland China by year

Year	DENV-1		DENV-2		DENV-3		DENV-4	
	Lineage	Province (no. of clade*)	Lineage	Province (no. of clade)	Lineage	Province (no. of clade)	Lineage	Province (no. of clade)
2014	I	GD	Cosmopolitan	GD (2)				
	IV	GD	Asian II	GD				
2013	I	GD (6); ZJ (2)	Cosmopolitan	GD (4)	II	GD; YN; Henan	I	ZJ
	IV	GD (2); ZJ (2)	Asian II	GD	III	GD		
	V	ZJ						
2012	I	GD (4)	Cosmopolitan	GD	II	GD	II	GD
			Asian II	GD				
2011	I	GD (2)						
	IV	GD						
2010	I	GD (3)	Cosmopolitan	GD; ZJ	I	GD	II	GD
	IV	GD			III	GD (2)		
	V	GD						
2009	I	GD (2)			III	GD; ZJ		
	IV	GD			V	GD		
2008	I	GD (2)						
2007	I	GD	Cosmopolitan	GD				
	V	GD						
2006	I	GD (4)			I	GD		
2005	I	GD						
2004	I	GD (2); ZJ	Cosmopolitan	ZJ	II	ZJ		
	V	GD						
2003	V	GD						
2002	V	GD						
2001	I	GD	Cosmopolitan	GD				
1999					II	GD		
1998			Asian II	GD				
1995	V	GD						
1993			Cosmopolitan	GD (2)				
1991	V	GD						
1990							I	GD
1989			SA/A	HN				
			Asian I	(1)†				
1987			SA/A	GD; ZJ; HN				
			Asian I	(1)				
1986			SA/A	GD				
1985	I	GD	SA/A	HN				
1980	I	GD			V	GX		
1979	I	GD						
1978							II	GD

DENV = dengue viruses; GD = Guangdong; GX = Guangxi; HN = Hainan; SA/A = southeast Asian/American; YN = Yunnan; ZJ = Zhejiang.

*Number is omitted when there is only 1 clade.

†Province information missing.

lineage replacements may reflect not only the changing genetic diversity of source strains but also the establishment of herd immunity, indicating underestimated dengue infections; on the other hand, the ability of Guangdong Province to harbor such complex DENV genetic diversity indicates its highly competent eco-environments for dengue circulation. This, together with its pivotal role in Asian commerce as well as its historical role in severe acute respiratory syndrome (SARS) transmission,¹⁵ indicating the potential of its residents to disseminate viruses via human movement, makes Guangdong province a potential source location for DENV exportation.

In addition, although their immediate sources were not always apparent, DENV strains from mainland China were mostly clustered with those from southeast Asia countries, with occasional cases from the Indian subcontinent, south Pacific islands, Latin America, and Somalia. These findings indicate that southern China is closely related to the southeast Asia DENV transmission cycle.

Strikingly, the grouping of Guangdong DENV strains in consecutive years in the same cluster was observed in a few cases, suggesting local persistence (endemicity). This was most

obvious for DENV-1 (shown in Figure 2), with groupings ranging from 2006 to 2014, 2009 to 2014, 2010 to 2013, and 2002 to 2004. Interestingly, some clusters also included strains from other countries, such as shown in Figure 2B, confounding this interpretation. Two scenarios could explain this phylogenetic pattern: 1) some DENV lineages are indeed persisting endemically in Guangdong province and the external strains are the result of exported cases, and 2) DENVs were imported from other parts of southeast Asia or other countries each year from the same source lineage, but these external sources were not always sampled and were thus missing from the tree. Given the uneven sampling in southeast Asia (Table 2), especially in recent years when the sequence of the actual source strains might not yet be available, we cannot exclude the possibility of that some source strains are missing. However, without further evidence suggesting otherwise, we should sufficiently consider the likelihood that there is indeed endemic transmission of DENV1 in China. Nonetheless, even if the second scenario is true, we may still consider Guangdong as an integrated part of the DENV transmission cycles in southeast Asia. In fact, there are frequent movements of DENV-1

FIGURE 2. Signs of local persistence of DENV-1 in China. Phylogenetic structures are based on maximum-likelihood tree. Bootstrap values and Shimodaira-Hasegawa-like local support values (shown in parenthesis) are labeled next to selected major clades.

and DENV-2 among southeast Asian countries, manifested by many lineages/clades of mixed country origins, leading to constant lineage reshuffling and sometimes replacement (Figure 1C and D, Supplemental Figures 5 and 6, Table 2). This kind of lineage replacement has also been reported in Mexico, where DENVs are considered endemic.¹⁶ Therefore, annual replacement of lineages in Guangdong by imported DENVs does not necessarily exclude the possibility that this part of China is an integrated part of the southeast Asia and/or south Asia endemic region if transmission is two way. Indeed, we observed potential signs of such two-way transmission, such as in the example shown in Figure 2B, where Singapore and Malaysia strains from 2013 and 2014 were grouped with

Chinese strains from the same years. This clade may have originated from Chinese strains in 2009. Because of the high sequence similarity, the E gene sequences are not informative enough to fully resolve the phylogenetic relationships among these DENV strains. So, complete genomic sequences could provide more robust data to address the question of Chinese endemicity. With collaborative effort of a more balanced surveillance strategy with southeast Asia countries, we could better evaluate the role of Guangdong province, the hotspot of dengue in China, in global transmission.

Moreover, rapid DENV movement has been frequently observed among different cities of Guangdong province, including Guangzhou, Zhongshan, Zhuhai, Dongguan, Shenzhen,

TABLE 2
Sample distribution of DENV1 in southeast Asia and China since 2001

Year	Country								
	MY	SG	ID	VN	LA	KH	TH	PH	CN
2014	B,C*	B,C,L							D,L
2013	A,B,C,E,F, J,L,N	A,B,C,D,E, F,G,L				H	F,H,J		D,E,F,H, J,L,N
2012	C,E,G,L	L							E,H
2011			A,B,M	G,H,I	F				H,I,L
2010	C,L,O		A,B,C,K,M,N,O	I	E,F,H	E,H	A,E,F,G	N	C,F
2009		F	E	H,I		H	E,F		A,D,H,L
2008		A,B,C,L	A	B,F,G,H,I,K	E,F	G,H	D,E,F,J		F,K
2007		F	M	E,G,I,K	F	F,G,H	E,F,J	N	A,N
2006	A	B		A,G,I,K,N		H,I	F,J		D,F,G
2005	A	A,B	L,M,O			H	F	N	B
2004	C,O	B		K				N	B,C,F,M
2003		A,M	N	E,K		E,I	E,F		M
2002		C,I	M			K	F	N	M
2001						K	F,I,J	N	F

CN = China; ID = Indonesia; KH = Cambodia; LA = Laos; MY = Malaysia; PH = Philippines; SG = Singapore; TH = Thailand; VN = Vietnam.

*A total of 15 sub-lineages coded from A to O are annotated in Supplemental Figure 5. The presence of sample from a particular country and year is listed in the table. Notably, the presence of a clade in multiple years in a country does not necessarily correspond to endemic transmission. It can be caused by repeated introduction from other countries.

Foshan, Jiangmen, and so on (such as shown in Figure 2A). Similarly, rapid transmission of DENVs among different provinces was observed a few times. For example, transmission of DENV-3 was observed among Guangdong, Yunnan, and Henan provinces in 2013, manifested by the cluster of these strains in the same clade, along with those from Laos 2013 (Supplemental Figure 3). The ability to rapidly spread and cause outbreaks in inner provinces such Henan indicates that DENVs have the potential to spread to broader regions in China.

In conclusion, the complicated DENV genetic diversity, long-term local persistence of DENV-1 in Guangdong province, and the rapid movement of DENVs as well as its widespread vector *Ae. albopictus* suggest that China is facing a substantial dengue threat with potential invasion into broader areas. After the 2014 Guangdong epidemic, it is likely that the etiologic DENV strain may persist locally at a low level. Through June 2015, more imported cases have been observed compared with the first half of 2014 (data from Guangdong Health Department). Will the local DENV strain prevail? Or will imported strains replace it due to less effective herd immunity? We will keep a close eye on the coming season.

Received July 25, 2015. Accepted for publication August 31, 2015.

Published online October 12, 2015.

Note: Supplemental material and figures appear at www.ajtmh.org.

Acknowledgments: We thank Scott Weaver for reviewing this article and Mengfeng Li for his instructive discussion.

Financial support: This work is supported by the Institute for Human Infections and Immunity at University of Texas Medical Branch at Galveston. Guan-Zhu Han is supported by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions.

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