Original Article

Genomic Characterization of Travel-Associated Dengue Viruses Isolated from the Entry-Exit Ports in Fujian Province, China, 2013–2015

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SUMMARY: Over the past decade, indigenous dengue outbreaks have occurred occasionally in Fujian province in southeastern China because of sporadic imported dengue viruses (DENV). In this study, 3 DENV-2 and 2 DENV-4 strains were isolated from suspected febrile travelers at 2 ports of entry in Fujian between 2013–2015. Complete viral genome sequences of these new isolates were obtained with Sanger chemistry. Genomic sequence analyses revealed that these strains belonged to genotypes of 2-Cosmopolitan and 4-II. Consistent with the patients’ travel Information, phylogenetic analyses of the complete coding regions also indicated that most of the new isolates were genetically similar to the circulating strains in Southeast Asia rather than previous Chinese strains that were available. Therefore, phylogenetic analyses of the imported DENV demonstrated that multiple introductions of DENV emerged continuously in Fujian, and highlighted the importance of dengue surveillance at entry-exit ports in the subtropical regions of southern China.

INTRODUCTION

Dengue fever is caused by 4 distinct serotypes of dengue viruses (DENV-1 to DENV-4) that belong to the genus Flavivirus of the family Flaviviridae (1,2). As the leading cause of illness and death in the tropical and subtropical regions, dengue fever has been gradually expanding its global geographic distribution during the past 6 decades, thus the incidence of dengue has increased dramatically. It was estimated that nearly half of the world’s population from more than 100 countries are at risk for dengue virus infection (1,3).

Dengue viruses are positive-sense single-stranded RNA viruses. The full-length genome of approximately 10.7 kb encodes a single polyprotein flanked by 2 untranslated regions (5’ and 3’ UTRs). The precursor polyprotein is subsequently cleaved by host and virus-derived proteases into 3 structural proteins (C, M, and E) and 7 nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5), respectively (2,4). Dengue viruses are transmitted by bites of infected Aedes mosquitoes. In particular, Ae. aegypti and Ae. Albopictus are primary vectors for the urban transmission cycle associated with human infections (1,2). About 75% of infections with any of the DENV serotypes may be asymptomatic; however, dengue viral infections cause clinical manifestations ranging from mild flu-like illness (known as dengue fever) to severe, life-threatening dengue hemorrhagic fever (1,3).

Based on partial and/or complete genomic sequences, phylogenetic analyses on the 4 genetically distinct serotypes of DENV resulted in the identification of 4 to 6 genotypes within each serotype (4–6). Comparisons of either nucleotide or amino acid sequences have contributed to elucidating the origins, genetic diversity, transmission dynamics, and viral evolution among circulating DENV strains worldwide. Indeed, some genotypes are distinguishable by their geographical distributions, whereas some genotypes contain a complex of DENV strains from diverse locations (4–6).

In Fujian, one of the subtropical provinces in southern China, several autochthonous outbreaks have occurred during the recent decade via the introduction of sporadic imported cases. Therefore, it is essential to sequence the partial or complete viral genome whenever a DENV strain is isolated from non-endemic regions regardless of whether they are from imported or indigenous dengue cases. In this study, since the arrival time of the travelers ranged from February to August, partially overlapping with the domestic dengue season, a returning short-term traveler might have been infected prior to his/her departure from China as well. Therefore, we sequenced and analyzed full-length genomes of 5 DENV strains isolated from febrile travelers who had just returned from abroad to Fujian province between 2013–2015.

MATERIALS AND METHODS

Patients and viral strains: Between 2013–2015, the 5 febrile patients, arriving at ports-of-entry in Fujian province with abnormal body temperatures were initially suspected with DENV infection based on their travel history as summarized in Table 1. Blood samples were collected at the ports of entry and were preliminarily detected to be positive for DENV-specific RNA by real-time reverse transcription polymerase chain reaction (rRT-PCR). Serum specimens were subsequently inoculated on monolayer C6/36 cells for 7–10 days until cytopathic effects were observed. Cultured supernatants
containing propagated viruses were stored in liquid nitrogen until further analysis.

Written consent forms were obtained from each participant included in the study. This study was reviewed and approved by the Ethics Committee of Fujian International Travel and Healthcare Center.

**Serotyping and sequencing:** Total RNA was extracted from cultured supernatants using the RNA EZ1 Virus Mini Kit v2.0 (Qiagen, Hilden, Germany) according to the manufacturer’s recommended protocol. Serotypes were determined by the nested RT-PCR assay established previously (7). Nine pairs of primers were designed for each serotype based on relevant genome sequences of the reference strains. Consequently, 9 overlapping fragments for each isolate were amplified by conventional one-step RT-PCR and purified using QIAquick PCR Purification kits (Qiagen, Valencia, CA, USA). Amplicons were sequenced at commercial DNA sequencing facilities with Sanger chemistry.

**Sequence analysis:** Sequence data were initially assembled into contigs using Lasergene package version 7.0 (DNASTAR Inc., Madison, WI, USA). Sequence homology with the reference strains were compared using the MegAlign software for nucleotides and putative amino acid sequences, respectively. The draft genome was searched for closely related strains on the BLAST servers hosted by the National Center for Biotechnology Information. Genome sequences of the closest reference strain, representative strains of known genotypes, and previous isolates from China were retrieved from the GenBank database for genotyping and phylogenetic analyses. Full-length genomes, except for the manually trimmed non-coding sequences at both ends, were aligned by Clustal W (version 1.83), and phylogenetic trees were constructed with the entire coding regions in MEGA 6.06 using a maximum likelihood method based on the Tamura-Nei model (8), outgrouped by the genome of the DENV-1 reference strain Hawaii (GenBank: KR011349).

**RESULTS**

**Serotypes and sequencing of the new DENV isolates:** Between 2013–2015, 5 DENV strains were isolated from suspected febrile travelers at 2 ports of entry in Fujian province. The 2 ports of entry were located in Changle and Jinjiang of Fujian, and the origins of departure of the 5 travelers are as followed: one was from Papua New Guinea, 2 were from the Philippines, and 2 were from Indonesia. According to the nested RT-PCR serotyping scheme, 3 DENV-2 and 2 DENV-4 isolates were detected, which are listed in detail in Table 1. In order to investigate these imported DENV strains, 9 overlapping fragments covering the full-length genome were subsequently amplified by one-step RT-PCR. The amplicons were sequenced with the same primers used in the amplification step.

**Complete genome sequence and BLAST search:** The assembled genomes for the 3 DENV-2 strains were consistently 10,723 bp (GenBank: KU517845-517847) in length, while the DENV-4 isolates showed minor variations at the 3’UTR region with 10,589 bp for the strain PH-CN08-14 (GenBank: KU523871) and 10,653 bp for the strain ID-CN27-15 (GenBank: KU523872). Compared with the DENV-2 New Guinea C reference strain (GenBank: KM204118), the coding regions of the new DENV-2 isolates harbored 93.1–93.4% for nucleotide sequence identity, while the similarity was 97.8–98.1% for relevant amino acid sequences. Furthermore, the coding regions of the 2 DENV-4 isolates had a 97.5% nucleotide and 98.6% amino acid sequence homology with the DENV-4 reference strain H241 (GenBank: K0011349).

Preliminary BLAST searches revealed that PG-CN10-13, one of the new DENV-2 isolates, shared a high homology with some strains isolated from a dengue outbreak at Makassar, Indonesia, in 2007 (9). Another DENV-2 isolate, ID-CN18-14, shared homology with some Singaporean isolates from 2012–2013. The other DENV-2 isolate, PH-CN77-15, shared the highest homology with the Singaporean strains from 2012. As for the new DENV-4 isolates, ID-CN27-15 shared a high homology with the isolates from Makassar, Indonesia, from 2007. The other DENV-4 isolate, PH-CN08-14, shared high homology with GZ06K-2012, SG06K-2270DK1/2005, and ThD4_0734_00. GZ06K-2012 (GenBank: KC333651) was isolated from Guangzhou, Guangdong province, China in 2012; SG06K-2270DK1/2005 (GenBank: GQ398256) was isolated from Singapore in 2005; and ThD4_0734_00 (GenBank: AY618993) was isolated from Thailand in 2000.

**Genotyping and phylogenetic analyses:** To address the genetic relationship of these isolates with domestic and global circulating DENV strains, dozens of reference genomes were retrieved from the GenBank database for multiple genome alignment and phylogenetic analyses. By considering the top-hits from the BLAST
searches as well as the representative reference strains of each genotype and previous Chinese isolates with genome sequences available, 2 sets of databases composed of 69 DENV-2 strains and 50 DENV-4 strains were established. From the maximum likelihood (ML) tree based on complete coding regions for the DENV-2 strains (Fig 1), the 3 DENV-2 isolates in this study were classified into the genotype of 2-Cosmopolitan, while the 2 DENV-4 isolates were designated to the genotype 4-II. (Fig 2).

Fig 1 shows that the 3 DENV-2 strains PG-CN10-13, ID-CN18-14, and PH-CN77-15 had genetic similarities to the Indonesian or Singaporean isolates from 2007–2013, but none of the 3 new isolates has similarities with a subset of the 27 DENV-2 isolates from China. Notably, the phylogenetic tree indicated that these 27 Chinese isolates from 1985–2015 belonged to 4 genotypes, namely, 2-Cosmopolitan, 2-Asian-I, 2-Asian-II, and 2-AsianAmerican. Moreover, FJ-10 and FJ11/99 (GenBank: AF276619 and AF359579, respectively), 2 DENV-2 strains isolated from the first indigenous outbreak in Fuzhou, Fujian province in 1999, demonstrated considerable genetic distance with the new isolates by presenting at a lower branch of the 2-Cosmopolitan

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Fig 1. Phylogenetic analysis of DENV-2 strains by Maximum Likelihood method. The tree is constructed with coding regions of 69 DENV-2 strains, including 27 Chinese DENV-2 strains (labeled with triangles) with full-length genome sequences available at the GenBank database. The 3 new isolates were labeled with diamonds. Representative strains of each genotype were denoted with GenBank accession number, strain name and country.
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On the ML tree for the DENV-4 strains (Fig 2), the PH-CN08-14 strain showed high genetic similarity with the Chinese isolate GZ/9809/2012 (GenBank: KC333651), which was possibly imported from Southeast Asia (10). Their close neighbor was another Singaporean strain SG/06K2270DK1/2005 (GenBank: GQ398256) isolated in 2005. Similarly, strain ID-CN27-15 shared homology with some Singaporean and Indonesian strains. There were 7 DENV-4 strains from China between 1978–2012 with genome sequences available, and the remaining 6 Chinese strains, except for GZ/9809/2012, were distinct from the new DENV-4 isolates, although they belong to the identical genotype of 4-II.

DISCUSSION

Located on the humid and warm southeast coast of China, Fujian has been verified province-wide for the presence of Ae. Albopictus, a major mosquito vector in DENV transmission, which is active from May to October each year. Since the first reported indigenous dengue outbreak in 1999 in Fuzhou, the capital of Fujian, at least another 5 outbreaks associated with multiple domestic or international origins of introduction sources have been reported during the past decade within Fujian province (11,12). Therefore, according to the National Dengue Surveillance Protocol since 2005, it is critical to implement active surveillance and intervention to suspected febrile travelers at entry-exit ports in Fujian, so as to reduce the probability of indigenous dengue outbreaks caused by viremic cross-border travelers. Consequently, 5 DENV strains in this study were recovered from serum specimens of febrile travelers at 2 ports of entry in Fujian during 2013–2015. According to their travel records, these patients departed from Papua New Guinea, the Philippines, or Indonesia where dengue...
has been endemic in recent years (3,4). Overall, Pacific Islands of Oceania and Southeast Asian countries were major sources for the introduction of DENV in Fujian province in the past 3 years.

These new isolates were classified as the genotype 2-Cosmopolitan in DENV-2, and the genotype 4-II in DENV-4 serotype. DENV-2 strains caused the first indigenous outbreak of more than 1,000 cases in 1999 in Fuzhou, and re-emerged occasionally in Fujian during the past decade (11,12). However, none of DENV-4 strains has been associated with autochthonous cases in Fujian thus far (13,14). Therefore, the genetic relationship of the new isolates with domestic or international circulating DENV strains was investigated based on viral genome sequences. BLAST search and phylogenetic analyses with complete coding regions of the DENV-2 isolates in this study, i.e., PG-CN10-13, ID-CN18-14, and PH-CN77-15, were genetically closer to some Indonesian and Singaporean strains than previous DENV-2 strains from China (Fig 1). On the other hand, the DENV-4 isolates, such as the ID-CN27-15 strain, showed high similarity with the Indonesian strain SW38i (GenBank: AY858050), whereas one DENV-4 strain, GZ/9809/2012 (GenBank: KC333651) from China, was listed at the top, among the closest neighbors for the PH-CN08-14 strain in a BLAST search. In fact, strain GZ/9809/2012, isolated in 2012 in Guangzhou, the capital of Guangdong province, possibly originated from Southeast Asia (10). Moreover, similar isolates such as strain SG/06K2270DK1/2005 (GenBank: GQ398256) have been isolated from Singapore since 2005 (Fig 2).

To clarify the possible genetic relationship of these travel-related DENV isolates with previous strains from China, 2 subsets of 27 DENV-2 and 7 DENV-4 strains with available complete genome sequences were integrated in the phylogenetic analyses. Consistent with results from other groups (13,14), the majority of the 27 Chinese DENV-2 strains belonged to the 2-Cosmopolitan genotype, while the remaining DENV-2 strains were assigned to the 2-AsianAmerican, 2-Asian-II, and 2-Asian-I genotypes (Fig 1). The 3 new DENV-2 isolates, flanked by some outbreak-associated strains in 2014 at the upper branches and some historical strains from Guangdong at the lower branches within the 2-Cosmopolitan genotype in the context of ML tree, were distinct from previous DENV-2 strains from China. Even the 2 strains isolated during the 1999 outbreak at Fuzhou, FJ-10 and FJ11/99 (GenBank: AF276619 and AF359579, respectively), were assigned to the lower branch of the 2-Cosmopolitan clade on the ML tree, suggesting their distant genetic relationship. Likewise, except for the aforementioned strain GZ/9809/2012, the remaining 6 DENV-4 strains from China also demonstrated considerable genetic distance from the new DENV-4 isolates on the ML tree (Fig 2). Thus, it is implied from genomic analyses that most of the 5 new isolates in this study were not genetically related to previous Chinese DENV strains that had genome sequences available. However, it is worthy to note that not all of the current dengue virus isolates are accessible with complete genome sequence records in public databases, and such gaps may lead to inaccurate phylogenetic trees. Therefore, there is no doubt that more viral strains, in addition to the several isolates in this study, would be helpful in revealing the real genetic relationships between the new isolates and the globally circulating strains.

In summary, 5 DENV strains were isolated from suspected febrile travelers at ports-of-entry in Fujian between 2013–2015. Phylogenetic analyses and epidemiological information indicated that these travel-associated strains originated from the Pacific Islands and Southeast Asia. These results also suggest that viremic travelers from dengue-endemic regions may be an ongoing source of dengue virus that is introduced into areas such as Fujian, highlighting the importance of routine surveillance of suspected febrile travelers at ports of entry in southern China.

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Conflict of interest None to declare.

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