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Complete genome analysis of dengue virus type 3 isolated from the 2013 dengue outbreak in Yunnan, China

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ABSTRACT

In the past few decades, dengue has spread rapidly and is an emerging disease in China. An unexpected dengue outbreak occurred in Xishuangbanna, Yunnan, China, resulting in 1331 patients in 2013. In order to obtain the complete genome information and perform mutation and evolutionary analysis of causative agent related to this largest outbreak of dengue fever. The viruses were isolated by cell culture and evaluated by genome sequence analysis. Phylogenetic trees were then constructed by Neighbor-Joining methods (MEGA6.0), followed by analysis of nucleotide mutation and amino acid substitution. The analysis of the diversity of secondary structure for E and NS1 protein were also performed. Then selection pressures acting on the coding sequences were estimated by PAML software. The complete genome sequences of two isolated strains (YNSW1, YNSW2) were 10,710 and 10,702 nucleotides in length, respectively. Phylogenetic analysis revealed both strain were classified as genotype II of DENV-3. The results indicated that both isolated strains of Xishuangbanna in 2013 and Laos 2013 stains (KF816161.1, KF816158.1, LC147061.1, LC147059.1, KF816162.1) were most similar to Bangladesh (AY496873.2) in 2002. After comparing with the DENV-3SS (H87) 62 amino acid substitutions were identified in translated regions, and 38 amino acid substitutions were identified in translated regions compared with DENV-3 genotype II stains Bangladesh (AY496873.2). 27(YNSW1) or 28(YNSW2) single nucleotide changes were observed in structural protein sequences with 7(YNSW1) or 8(YNSW2) non-synonymous mutations compared with AY496873.2. Of them, 4 non-synonymous mutations were identified in E protein sequences with (2 in the β-sheet, 2 in the coil). Meanwhile, 117(YNSW1) or 115 (YNSW2) single nucleotide changes were observed in non-structural protein sequences with 31(YNSW1) or 30 (YNSW2) non-synonymous mutations. Particularly, 14 single nucleotide changes were observed in NS1 sequences with 4/14 non-synonymous substitutions (4 in the coil). Selection pressure analysis revealed no positive selection in the amino acid sites of the genes encoding for structural and non-structural proteins. This study may help understand the intrinsic geographical relatedness of dengue virus 3 and contributes further to research on their infectivity, pathogenicity and vaccine development.

1. Introduction

The dengue virus (DENV) is an arthropod-borne virus belonging to the *Flaviviridae* family, genus *Flavivirus*, and occurs as four antigenically related but distinct serotypes designated as DENV-1, 2, 3 and 4 (Nogueira et al., 2001). Currently, DENV-3 has five distinct genotypes (I to V) found in the following regions: Southeast Asia, the Indian subcontinent, the South Pacific, East Africa and the Americas (Aquino et al., 2009; Lanciotti et al., 1994; Wittke et al., 2002). The genome of DENV is a single-stranded RNA approximately 11 kb in length including untranslated regions (5′ UTRs, 3′ UTRs) and a single open reading frame (ORF). Translation of the single ORF generates one polyprotein

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that is cleaved by host- and virus-derived proteases to encode three structural proteins (capsid, premembrane, envelope) and seven nonstructural proteins (NS1, NS2A,NS2B,NS3,NS4A,NS4B,NS5) (Lindenbach et al., 2007; Weaver and Vasilakis, 2009).

Dengue fever is the most important and rapidly spreading vector-borne viral disease in Southeast Asia, Central and South America, and Africa (Moi et al., 2010; Undurraga et al., 2013). China has seen an increase in human cases of dengue fever in recent years. Since 1978, dengue fever occurred epidemically every 4–7 years in China, commonly affecting people between the ages of 20–60 years old (Xiong and Chen, 2014; Yang et al., 2014). Dengue is an emerging disease in China, for instance, from 2009 to 2014, a total of 52749 cases of dengue fever and six deaths were reported, according to the China National Notifiable Disease Surveillance System (Chen and Liu, 2015). In 2013, more than 4500 DENV infection cases were reported in mainland China. Of these, more than 2800 cases were reported in Guangdong Province, and both DENV1 and DENV2 were reported by local CDC. Meanwhile, 37 cases were reported in Zhejiang, 36 cases in Henan, and 33 cases in Fujian (Hang Zhou et al., 2015). A total of 1331 cases were identified as DENV by dengue fever symptoms and positive DENV nonstructural protein (NS) 1 antigen detected with the Dengue Ag Rapid Test in Yunnan Province. Severe haemorrhage was seen in 28 patients, and severe plasma leakage in 27 patients (Hu et al., 2017; Lao et al., 2014). However, there were no death patients in this outbreak. Of all the DENV cases in Yunnan Province were mainly DENV serotype 3 and small amount of DENV serotype 1, 2, 4 (Hu et al., 2017). A lack of complete genome information was a major hindrance in understanding the DENV circulating in Yunnan. In this study, DENV-3 strains were isolated from patients serum samples collected during the outbreak in Yunnan Province. Two strains were selected for full-length gene sequencing followed by phylogenetic and selection pressure analyses. Further analyses of base substitutions in coding genes, amino acid mutations in structural proteins (C, prM and E) and nonstructural proteins protein molecules (NS1, NS2, NS3, NS4, NS5) were completed to better understand the DENV-3 circulating in this area.

2. Materials and methods

2.1. Ethics statement

Written consent was obtained from each participant. The study protocol was approved by the Institutional Ethics Committee (Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College) and was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2000).

2.2. Virus isolation

During the dengue outbreak in the Yunnan Province in 2013, the serum samples were collected from viremic human patients at Xishuangbanna Dai Autonomous Prefecture People's Hospital (XDAPPH) in August. Ten strains were isolated from those serum samples, and two strains were selected randomly to be sequenced. These two strains were isolated from a 51 year old male patient and a 52 year old female patient. Both patients had mild symptoms and no record of going abroad. A few days before sample collection, these two patients developed symptoms of fever, with body rush and fatigue. Patient serum samples were diluted with RPMI 1640 culture medium using the ratio 1:10, and then 100 μl of the diluted serum was inoculated into 25 cm^2 bottle with a monolayer of C6/36 cells. After 2 h of adsorption, the supernatant was discarded and the cells were cultured in RPMI 1640 culture medium with 2% serum for 7d, followed by sub culturing three times. Detection of DENV type was conducted by RT-PCR (data not shown). Two isolated strains, at passage level 4 in C6/36 cells, were selected for complete genome sequencing.

2.3. Extraction of viral RNA

Viral RNA was extracted from 140 μl of dengue virus-infected culture supernatant using the QIAamp viral RNA mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions. Finally, RNA was eluted in 60 μl of nuclease-free water and stored at −80 °C.

2.4. Primer design

A total of 22synthetic oligo nucleotide primer pairs were designed to amplify overlapping fragments of sizes between 500 and 800 nt spanning the entire viral genome of DENV-3, based on the strain 98TW407 (GenBank accession no. DQ675528).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

A total of 22 overlapping amplicons spanning the complete genomic region were amplified using 44 primers. RT-PCR was carried out in one step with the following protocol: initial reverse transcription at 50 °C for 30 min; denaturation at 94 °C for 2 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 37 s; and a final elongation step at 72 °C for 5 min. PCR products were confirmed by agarose gel electrophoresis and sent to Sangon Biotech (Shanghai, China) for sequencing.

2.6. Genome synthesis for amplification and sequencing

Total RNA was extracted from infected C6/36 cells. One-step PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China) was used to amplify the virus in 22 overlapping fragments by RT-PCR and directly sequence the amplicons.

2.7. Genetic analysis

The 22 sequences were assembled using DNASTAR version 7.0. The assembled nucleotide sequences and translated amino acid sequences were analyzed by BioEdit. The phylogenetic analysis, based on the complete envelope glycoprotein (E) protein genes, was conducted using the Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0 (the neighbor-joining method) (Lao et al., 2014).The reference viral sequences used to construct the distinct phylogenetic branches were collected from the GenBank sequence database under the following country accession numbers: Fiji(L11422.1); Thailand (L11440.1, L11441.1, GQ868593.1); Viet Nam (EU482460.1, EU482461.1, FJ562103.1); Bangladesh (AY496873.2, DQ401691.1); China (GU363549.1); India (L11424.1); Puerto Rico (L11434.1); Indonesia (L11428.1); Tahiti(L11439.1); Malaysia (L11429.1); Samoa (L11435.1); Haiti (KT279761.2, KX702403.1); Brazil (KT794007.1); H87 (M93130.1); Viet Nam (EU482460.1, EU482461.1, FJ562103.1); Lao 2013 (KF816161.1, KF816158.1, LC147061.1, LC147059.1, LC147060.1, KF816162.1); China 2013 (KJ622198.1, KJ622191.1, KJ622195.1, KJ622194.1, KM651781.1, KF954947.1, KF954949.1, KF824903.1, KF824902.1); Solomon Islands 2013 (KU053467.1, KU053466.1); Indonesia 2013 (KF709425.1, KF709426.1); Singapore 2013 (KP685235.1, KR779787.1, KX224291.1, KX224280.1, KX224271.1); Tahiti 2013 (KU053472.1, KU053471.1); Paraguay 2013 (JF808129.1); Viet Nam 2013(KP176712.1, KP176711.1, KP176710.1, KP893717.1); Thailand 2013 (KP176108.1); India 2013 (KU216209.1); Pakistan 2013 (KM217133.1, KM217159.1).

2.8. Selection pressure analysis

To assess the selection pressure acting on individual codons of the ORF (C-prM/M, E, NS1, NS2, NS3, NS4, NS5), a dataset comprising of other close related genotypes DENV-3 (n = 20) was prepared (S8 Table). The non-synonymous to synonymous substitution rate ratio

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