Molecular characterization of structural protein genes of dengue virus serotype 1 epidemic in Yunnan, Southwest China, in 2018

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Abstract
A dengue virus serotype 1 (DENV-1) epidemic occurred from October to December 2018 in Xishuangbanna, Yunnan, Southwest China, neighboring Myanmar, Laos, and Vietnam. In this study, we investigated the molecular characteristics, evolution, and potential source of DENV from Xishuangbanna. The C (capsid), prM (premembrane), and E (envelope) genes of DENV isolated from 87 serum samples obtained from local patients were amplified and sequenced, and the sequences were evaluated by identification of mutations, phylogenetic and homologous recombination analysis, and secondary structure prediction. Phylogenetic analysis showed that all of the epidemic DENV strains from Xishuangbanna could be grouped in a branch with DENV-1 isolates, and were most similar to the Fujian 2005 (China, DQ193572) and Singapore 2016 (MF314188) strains. When compared with DENV-1SS (the standard strain), there were 31 non-synonymous mutations, but no obvious homologous recombination signal was found. Secondary structure prediction showed that some changes had occurred in a helical region in proteins of the MN123849 and MN123854 strains, but there were few changes in the disordered region. This study reveals the molecular characteristics of the structural genes of the Xishuangbanna epidemic strains in 2018 and provides a reference for molecular epidemiology, infection, and pathogenicity research and vaccine development.

Abbreviations
DENV dengue virus
DF Dengue fever
E Envelope
M Membrane
C Capsid
prM Premembrane
DENV-1SS DENV-1 standard strain
RT-PCR Reverse transcription polymerase chain reaction
ML Maximum likelihood
NCBI National Center for Biotechnology Information
RDP Recombination Detection Program

Introduction
Dengue virus (DENV) is a ssRNA virus belonging to the family Flaviviridae that is mainly transmitted by Aedes aegypti and Aedes albopictus mosquitoes [1–4]. The DENV genome encodes three structural proteins (C, prM and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [5–7]. There are four different serotypes of DENV, and infection with any of the four serotypes can result in extremely severe manifestations [8–10]. The serotypes are antigenically distinct although they have up to 65% nucleotide sequence identity [11–13]. Dengue was classified as the most important mosquito-borne viral
disease worldwide by WHO in 2012. DENV infection usually causes a relatively mild disease called dengue fever (DF), but severe disease, including dengue shock syndrome and dengue hemorrhagic fever, can also occur [6, 8]. DF is mainly prevalent in tropical and subtropical areas, especially in urban and suburban areas. The worldwide incidence of dengue infection has been increasing over the past decades, and about half of the world’s population is currently at risk of DENV infection. However, no effective treatment or vaccine is available for DF [14–16].

Dengue-like illnesses are common in China, and the first record of DENV infection dates back to 1978 in the city of Foshan in Guangdong Province [17–19]. Since then, several large outbreaks of DF have occurred in the southeastern part of China, including Zhejiang, Fujian, Taiwan, Hainan, Yunnan, Guangxi, and Guangdong [17]. Sporadic outbreaks or imported cases of dengue were identified from the 1990s to the 2010s in China [19]. However, in 2012, the number of DENV infections began to increase and peaked in 2014 [9, 10]. Located in southwestern China, Yunnan province, neighboring Myanmar, Laos, and Vietnam, was reported to have an outbreak of 56 cases in 2008 [20], which was the first outbreak of the disease in this region since 1949 [20, 21]. Dengue outbreaks or epidemics were reported annually in Yunnan province from 2013 to 2015. In 2013, there were 1319 confirmed cases of DENV-3 in Xishuangbanna for the first time [22, 23]. Since then, there have been two large-scale dengue outbreaks in 2015 and 2017, with 1132 and 1348 confirmed cases, respectively [24, 25].

In 2018, DF cases were reported in some of the Southeast Asian countries neighboring China, including Vietnam, Laos, Cambodia, Myanmar, Thailand, and Malaysia, and DF cases were also reported in the Chinese cities of Yunnan, Hunan, and Guangzhou. Therefore, understanding the potential sources, molecular characteristics, and evolution of DENV is critical for preventing future outbreaks of DF.

In this study, we investigated the potential sources of the causative agent and analyzed the evolution and characteristics of the structural genes of the epidemic DENV strains from 2018.

**Materials and methods**

**Identification and serotyping of DENV**

Serum samples were collected from DF patients in Xishuangbanna, Yunnan, China, followed by viral RNA extraction. Dengue NS1 antigen was detected using a DF NS1 test kit (Blue Cross, Beijing, China). Viral RNA was extracted from 150 µL of serum using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The viral RNA was used for amplification the structural protein genes of DENV.

**Reverse transcription PCR (RT-PCR) and sequencing of structural protein genes**

A PrimeScript One Step RT-PCR Kit (Takara, Dalian, China) was used according to the manufacturer’s instructions to synthesize cDNA. Standard DENV serotyping primers were then used for PCR, followed by sequencing. A NCBI-BLAST search was used for serotyping. In addition, primer pairs were used to amplify the overlapping fragments from nt 96 to 2420 of the DENV-1 genome. All PCR procedures were performed using Golden Star T6 Super PCR Mix (TSINGKE, Kunming, China) using previously described primers [24]. The cycling parameters were as follows: 98 °C for 2 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 56 °C for 10 s, and extension at 72 °C for 40 s, and then a final extension at 72 °C for 5 min. The PCR product was analyzed by 1% agarose gel electrophoresis, stained with SYBR Safe DNA Gel Stain Dye (Invitrogen, Eugene, USA), and visualized under UV light. Nucleotide sequences were determined by direct sequencing using an ABI 3730XL sequencer (Macrogen, Seoul, Korea). Briefly, the PCR product was excised from the gel and purified, BDT sequencing was performed, and the sequencing results were analyzed. The resulting sequences were compared to sequences in the GenBank database using NCBI BLAST. The sequences were also compared to that of standard strain DENV1-SS (GenBank® ID:EU848545.1).

**Phylogenetic analysis**

A multiple sequence alignment of the region encoding the structural proteins was performed using Clustal X, using reference sequences from the GenBank database. The total number of reference strains was 51, and their countries of origin and accession numbers are as follows: standard strains (DV-1SS: EU848545, DENV-2SS: M29095, DENV-3SS: M93130, and DENV-4SS: AF326573); China (KF864667, FJ196845, AY835999, FJ176780, DQ193572, EU359008, MF405201, KC762652, KF971869, KX458014, KY672941, and MG679800); Brazil (JX669474, KP188547 and GU131863); Japan (AB074760); Singapore (EU081262, KX380806, MF314188, and GQ357692); Thailand (JQ922547, AY732477, AY732483, and JN638341); Vietnam (JQ45668 and JQ45660); French Polynesia (DQ672559); Malaysia (JN697057 and EF457905); India (JQ922548 and KJ755855); Indonesia (MH823207); Laos (KC172829 and KC172832); Myanmar (AY726550, AY726553, JF459993, and MG679801); Cambodia (AF309641, HM488255 and KU509260); USA (FJ562106 and EU482591); Haiti (KT279761 and KU509264) and...
Dengue virus serotype 1 in Yunnan, China

Argentina (KC692514 and KC692495). Phylogenetic trees were constructed using MEGA software (version 6.0) by the maximum-likelihood (ML) method with 1000 bootstrap replications.

**Molecular characterization**

The sequences of the structural protein genes of the Xishaungbanna 2018 DENV-1 epidemic strain were uploaded to the National Center for Biotechnology Research (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the accession numbers MN123810-MN123861. The BioEdit program was used to analyze nucleotide substitutions, and the Sequence Manipulation Suite (SMS, http://www.bio-soft.net/sms/index.html) was used to analyze amino acid sequence mutations. SimPlot and RDP version 5.5 (https://web.cbio.uc.ac.darren/rdp.html) were used to identify sites where homologous recombination might have occurred in the structural protein genes of epidemic strains in Xishaungbanna. The detailed procedure for RDP analysis was as follows: open an MEG file, left click the “open” button, click the “options” button at the top of the page, then enter the general page to select the corresponding parameters, and left click the “run” button on the main page to start the reorganization analysis. PredictProtein (https://www.predictprotein.org/) was used to predict the secondary structure of the structural proteins of the DEN-1SS and Xishaungbanna 2018 DENV-1 epidemic strains. Potential protein binding sites and the exposed and buried regions in the structural proteins were also predicted. Potential helical structures were also identified.

**Results**

**Geographical location of the dengue fever epidemic of 2018 and the study design**

Since the first large-scale outbreak of DF in 2013, there has been an outbreak of this disease with different serotypes every other year in Xishaungbanna. In 2018, there was no large-scale outbreak, only a small-scale epidemic, and DF cases were also reported in some Southeast Asian countries neighboring China (Fig. 1). During the DENV epidemic from October to December 2018 in Xishaungbanna, a total of 87 serum samples from patients were collected at Xishaungbanna Dai Autonomous Prefecture People’s Hospital. All samples were confirmed to be NS1-positive using a colloidal gold test. Viral RNA was successfully extracted from 52 of the samples, followed by gene amplification and sequencing of the region containing the DENV C, prM, and E genes. In order to analyze the origin and genetic relationships of the DENV isolates from Xishaungbanna, phylogenetic analysis was performed and mutations, potential sites of homologous recombination, and secondary structures were predicted.

**Phylogenetic analysis**

A phylogenetic tree was constructed based on 52 DENV structural protein gene sequences. Reference sequences obtained from the GenBank database, including representatives of the four serotypes of DENV, Chinese epidemic strains, and other epidemic strains from Southeast Asian countries and the rest of the world. The results indicated that all 52 Xishaungbanna epidemic DENV strains were of the DENV-1 serotype and belonged to one branch of the tree. They were most similar to the Fujian 2005 (China, DQ193572) and Singapore 2016 (MF314188) strains. DENV-1 strains have been divided into six genotypes, and the results showed that these epidemic strains belonged to genotype I (Fig. 2). These results indicated that the DENV-1 epidemic in Xishaungbanna in 2018 was caused by a DENV strain circulating in Southeast Asia or a domestic strain.

**Analysis of mutations and recombination sites**

All 52 sequences of structural proteins genes were compared to those of the four standard DENV reference strains using BLAST, and it was found that all of them were most similar to DENV-1SS. Since all the sequences were similar, two representative sequences (MN123849 and MN123854) were selected randomly to perform mutation and recombination analysis based on DENV-1SS. A total of 151 nucleotide
Fig. 2  Phylogenetic analysis of DENV-1 epidemic strains in Xishuangbanna, Yunnan, China, 2018. The phylogenetic tree was constructed by the maximum-likelihood method with 1000 bootstrap replicates. ○, DENV epidemic strains from Xishuangbanna, Yunnan, China in 2018; The structural protein sequences of the reference strains were obtained from the NCBI database.
variations and 31 amino acid sequence differences were observed in the DENV structural protein genes. The mutation rate in the C/prM region was 2.58%, and in the E gene, it was 4.09%. To identify possible sites of homologous recombination in the structural proteins of the two Xishuangbanna 2018 DENV-1 epidemic strains (MN123849 and MN123854), these sequences were compared to that of DENV-1SS using both Simplot and RDP, but no obvious homologous recombination signals were found (Fig. 3).

**Secondary structure analysis of structural proteins**

Potential secondary structure elements in the structural proteins of the MN123849 and MN123854 strains were predicted and compared with those predicted in DENV-1SS. There were 41 and 42 predicted protein binding sites in MN123849 and MN123854, respectively. Of these, 21 (aa 87, 93, 97, 120, 157, 184, 198, 199, 200, 357, 376, 377, 503, 505, 507, 508, 509, 595, 622, 623 and 642) were found in MN123849, MN123854 and DEN-1SS. However, 9 and 10 sites were lacking in MN123849 (aa 5, 7, 158, 195, 197, 365, 366, 429 and 621) and MN123854 (aa 5, 7, 40, 41, 123, 195, 429, 510, 594 and 596), respectively, compared with DENV-1SS. Two potential nucleotide binding sites (aa 10 and 17) were lacking in MN123849 and MN123854, compared with DEN-1SS. In addition, one nucleotide binding site (aa 98) only found in the MN123849 and MN123854 strains, and two sites (aa 204 and 205) are present only in MN123854. A few changes were observed in disordered regions. Some differences in a predicted helical transmembrane region were observed between DEN-1SS, MN123849, and MN123854 (Fig. 4).

**Discussion**

In this study, 87 serum samples were collected from febrile patients in Xishuangbanna. However, only 52 of these samples were successfully amplified by PCR and sequenced. This might have been due to samples being collected at different time points. Phylogenetic analysis was performed based on the structural protein genes (C/prM/E), which are critical for viral adsorption, penetration, replication, and release [26–28], and these genes were compared with reference sequences from all over the world. Our results showed that all 52 strains were DENV-1 and belonged to one cluster. They were evolutionarily close to the Fujian (2005) and Singapore (2016) strains. Previous studies have shown that imported cases in China were primarily from Asian countries, and Laos and Myanmar were the main sources of the
DF epidemic in Yunnan Province [22, 23, 29, 30]. In addition, our previous study showed that the first DENV-1 outbreak in Xishuangbanna in 2017, including imported cases from Myanmar, was caused by viruses that were closely related to domestic epidemic strains (Guangzhou, JQ048541 and Hubei, KP772252) and Southeast Asia epidemic strain (Laos, KC172834). Compared to the DENV-1 outbreak in 2017, the DENV epidemic in Xishuangbanna in 2018 was small in scale, but the viral strains were again closely related to a domestic epidemic strains (Fujian, DQ193572) and an epidemic strain from Southeast Asia (Singapore, MF314188). This suggests that the outbreak of DF might have been caused by viruses imported from neighboring countries in Southeast Asia or by circulating domestic epidemic strains.

Genetic recombination is a vital mechanism by which viruses evolve and gain genetic variation, and the rate of gene recombination is higher than that of mutation and natural selection alone [31, 32]. To assess whether genetic recombination had occurred in the region encoding the structural proteins of the Xishuangbanna 2018 epidemic strains MN123849 and MN123854, their sequences were compared with that of DENV-1SS, and no evidence of recombination was found.

Although DENV has spread rapidly worldwide and led to millions of people being infected each year, licensed vaccines or effective antiviral treatments are not available to control DENV. Therefore, it is urgent to develop a safe and effective vaccine against DENV infection. Structural proteins, especially the E protein, serve as the main targets in the development of subunit vaccine candidates [33]. The E protein has three domains (I-III). Domain III contains serotype-specific epitopes that can elicit neutralizing antibodies and a host cell receptor recognition site that allows viral attachment and entry into the host cell [34–36]. This domain is widely used in vaccine development because it can induce antibodies against DENV infection [36–38]. Kawano et al. reported that conversion of the amino acid residue located at position E155 in domain I of the DENV-4 E protein from T to I altered the virulence of the virus [39]. In our previous
study, we made a similar observation [24]. In this study, we also observed mutations at amino acid residue 155 (nt position: 435, from T to S) in the E proteins of Xishuangbanna epidemic strains. However, there were no related clinical characteristics of dengue hemorrhagic fever or dengue shock in these patients, almost all of whom had only DF. Therefore, it remains to be determined whether the mutation of amino acid residue 155 (from T to S) affects the virulence of the endemic virus.

Due to geographical location and national economic policy, most Southeast Asian countries have experienced DF epidemic, so it is difficult to find the exact source of the Xishuangbanna 2018 epidemic strains. This study may provide the basis for further study on the molecular epidemiology, infection, pathogenicity, and vaccine development of DENV.

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Availability of data and materials The sequences of the structural protein genes of DENV from Xishuangbanna in this study were uploaded to the GenBank database with the accession numbers MN123810-MN123861.

Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

Ethical approval and consent to participate Written consent was obtained from each participant or the patient’s parents or legal guardians before serum collection in this study. Ethical approval was granted by the Institutional Ethics Committee (Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College), and the entire study protocol was in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2000).

Consent for publication All authors reviewed and approved the manuscript for publication.

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