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Research paper

The distinct distribution and phylogenetic characteristics of dengue virus serotypes/genotypes during the 2013 outbreak in Yunnan, China Phylogenetic characteristics of 2013 dengue outbreak in Yunnan, China

Binghui Wang [a], Henglin Yang [b], Yue Feng [a], Hongning Zhou [b], Jiejie Dai [c], Yunzhang Hu [c], Li Zhang [a], Yajuan Wang [a], Zulqarnain Baloch [a], Xueshan Xia [a,*]

[a] Faculty of Life Science and Technology, Center for Molecular Medicine in Yunnan Province, Kunming University of Science and Technology, Yunnan, PR China
[b] Yunnan Research Institute of Parasitic Disease Control, Yunnan, PR China
[c] Institute of Molecular Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, Yunnan, PR China

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ABSTRACT

Since 2000, sporadic imported cases of dengue fever were documented almost every year in Yunnan Province, China. Unexpectedly, a large-scale outbreak of dengue virus (DENV) infection occurred from August to December 2013, with 1538 documented cases. In the current study, 81 dengue-positive patient samples were collected from Xishuangbanna, the southernmost prefecture of the Yunnan province, and 23 from Dehong, the westernmost prefecture of the Yunnan province. The full-length envelope genes were amplified and sequenced. Phylogenetic analysis revealed that nine strains (39.1%) and 14 strains (60.9%) from the Dehong prefecture were classified as genotype I of DENV-1 and Asian I genotype of DENV-2, respectively. All strains from Xishuangbanna were identified as genotype II of DENV-3. Bayesian coalescent analysis indicates that the outbreak originated from bordering southeastern Asian countries. These three epidemic genotypes were predicted to originate in Thailand and then migrate into Yunnan through different routes.

1. Introduction

Dengue fever (DF) is one of the most serious health threats in tropical and subtropical regions of the world, caused by infection with any of the four dengue virus (DENV) serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. The virus is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes, which are present in most parts of the tropical and sub-tropical regions of the world. Moreover, globalization, ease of transportation, and the lack of effective vaccines have aggravated its global prevalence. In the past 50 years, DENV infection has increased 30-fold with 50–100 million new infections being reported each year (WHO, 2012). The rapid increase in the incidence of dengue fever (DF) in recent years has become a serious public health threat to nearly half of the world's population. The outbreaks of dengue have been proposed to have begun in Asia, where the greatest number of dengue cases is currently found. Almost all countries in Southeastern Asia (e.g., Indonesia, Cambodia, Laos, Myanmar, Malaysia, Philippines, Thailand, and Vietnam) have documented epidemic dengue outbreaks (Dorji et al., 2009; Gubler, 1998; Holmes et al., 2009; Jarman et al., 2008; Schreiber et al., 2009; Teoh et al., 2010).

DENV is a member of the *Flavivirus* genus belonging to the Flaviviridae family, with a single stranded, non-segmented, positive-sense RNA genome of approximately 10.7 kb that encodes a single open reading frame for three structural proteins (C, capsid; prM/M, precursor of membrane; and E, envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) (Chambers et al., 1990). Great genetic diversity is a major characteristic of DENV, due to the lack of proofreading capacity of RNA-dependent RNA polymerases. Based on an intergenotypic divergence of at least 6% in the E gene sequence, each serotype of DENV can be classified into several genotypes (Rico-Hesse, 1990). Epidemiological and phylogenic reports have demonstrated a wide range of diversity within each of the four DENV serotypes, which is used for further differentiation of viral genotypes (Rico-Hesse, 1990; Kyle and Harris, 2008; Lanciotti et al., 1997; Twiddy et al., 2002a).

DENV serotypes and genotypes have different epidemic potentials for various geographic locations. As the most widespread serotype, DENV-1 is further classified into five genotypes (I–IV) based on entire or partial sequence of the E gene (Rico-Hesse, 1990; Villabona-Arenas et al., 2013). Phylogeographically, genotype I is prevalent in Southeast

* Corresponding author at: Dept. of Faculty of Life Science and Technology & Center for Molecular Medicine in Yunnan Province, Kunming University of Science and Technology, Yunnan, PR China.
E-mail addresses: binghuiwang@aliyun.com (B. Wang), yanghenglin@yipd.org (H. Yang), fyky2005@163.com (Y. Feng), zhouhn66@163.com (H. Zhou), djj@imbcams.com.cn (J. Dai), huyunzhang@imbcams.com.cn (Y. Hu), 253091425@qq.com (L. Zhang), yajuanwang1990@163.com (Y. Wang), znbalooch@yahoo.com (Z. Baloch), oliverxia2000@aliyun.com (X. Xia).

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Asian countries (Vietnam, Laos, and Myanmar), East Asian countries (China, Japan), and East Africa; genotype II is common in Thailand; genotype III is primarily found in Malaysia; strains of genotype IV are primarily found in Australia, Indonesia, and the Philippines; and all genotype V viruses were isolated in the United States. DENV-2 is classified into six genotypes: Asian I, Asian II, Sylvatic, American/Asian, American, and Cosmopolitan. Except for the sylvatic genotype, the other five genotypes of DENV-2 present unique geographical distributions. Among them, the Cosmopolitan genotype has a wide distribution across the tropical and subtropical regions (Twiddy et al., 2002b); both Asian I and II are currently circulating in Southeast Asia; and both the American and American/Asian genotypes have only been reported in epidemics in America, though the latter is considered to have originated in Southeast Asia (Rico-Hesse et al., 1997). Since the first reported outbreak in the Philippines in 1956 (Hammon et al., 1960), DENV-3 infections have been described worldwide. Of the five genotypes (I to V) of DENV-3, genotypes I, II, and III are the major genotypes circulating in Southeast Asia, the Indian subcontinent, the South Pacific, East Africa, and the Americas (Lanciotti et al., 1994). For DENV-4, three epidemic genotypes (I–III) and a sylvatic genotype have been described. The sylvatic genotype has been isolated from sentinel monkeys in Malaysia, whereas genotypes I–III are circulating in South Asia and Southeast Asia (Chen and Vasilakis, 2011).

Since the first outbreak in Guangdong Province in 1978, dengue epidemics have been reported annually in southern China in regions such as the Guangdong, Guangxi, Fujian, Yunnan, and Hainan provinces and the Special Administrative Regions Macao and Hong Kong (Yi et al., 2003). Except for two large-scale dengue outbreaks in 1980 and 1986 with 452,674 and 118,881 infected cases, respectively, the majority of DENV occurrence was sporadic (Xiong and Chen, 2014). DENV strains of all four serotypes have been identified in China. Surprisingly, the DENV infection has become more serious in recent years, with 44,896 DENV infections being reported during the 2014 dengue outbreak in Guangdong. Moreover, imported cases of DENV infection from Southeast Asian countries were also reported to have increased in the past 30 years because of the rapid increase in trade and travel between these countries and China (Ren et al., 2003).

Geographically, the Yunnan province is located in southeastern China, and neighbors the dengue endemic Southeast Asian countries Laos, Vietnam, and Myanmar (Fukunaga et al., 1983; Thu et al., 2004). In the past decade, only sporadic cases of dengue infection have been typically reported in Yunnan. Unfortunately, a large-scale dengue outbreak occurred in 2013 with 1538 confirmed DENV infections, accounting for 33% of the total reported numbers nationwide. This dengue outbreak was primarily centered in the Xishuangbanna and Dehong prefectures, and both harmed the health of the local population and a resulting panic caused a dramatic reduction in tourism. However, the phylogenetic characteristics of this DENV outbreak and its origins are so far unclear. In current study, using phylogenetic analysis and Bayesian coalescent analysis, we described the molecular epidemiological characteristics of DENV during this dengue outbreak in the Yunnan province, and attempted to illuminate DENV transmission models and even the cause of this outbreak.

2. Materials and methods

2.1. Detection of DENV infection and sample collection

During the dengue outbreak in Yunnan province in 2013, serum samples from suspected clinical cases (fever with a rash and swollen lymph nodes) were subjected to DENV NS1 antigen detection with the One-Step Dengue NS1 RapiDip™ InstaTest kit (Cortez Diagnostics, Inc., Calabasas, CA) and a self-established reverse transcription polymerase chain reaction (RT-PCR) detection protocol. In total, 1538 serum samples have been detected positive, their demographic data were collected for further statistical analysis. Serum samples were collected during the first 5 days of illness and stored at −80 °C.

2.2. DENV E gene amplification and nucleotide sequencing

104 serum samples were randomly selected for phylogenetic and evolutionary analysis from antibody positive serums, including 81 (accounted for 6.1% of total infection) from diagnosed dengue patients at the local hospital of Xishuangbanna, the southernmost prefecture of the Yunnan province, and 23 (accounted for 12.1% of total infection) from the local hospital in Dehong, the westernmost prefecture of the Yunnan province. Their RNA was extracted from 100-μL serum samples using a High Pure Viral RNA kit (Roche, Shanghai, China), according to the manufacturer's instructions. The entire E gene was amplified by a one-step reverse transcription polymerase chain reaction, using the method previous reported (Wang et al., 2015). Serotype was also determined by RT-PCR using these serotype-specific primers reported previously (Foster et al., 2000). PCR products were confirmed by electrophoresis and purified using an Agarose Gel DNA Extraction kit (TaKaRa). Sequencing was then performed by the Invitrogen Company (Beijing, China). All raw sequences obtained were analyzed using the Chromas program (https://www.chroma.com/). DNA fragments encoding the full-length E protein of DENV were submitted to GenBank (accession numbers KJ9394385 to KJ939407 and KR347358 to KR347438).

2.3. Phylogenetic and evolutionary analysis

Sequences of E gene were aligned using ClustalX program (Larkin et al., 2007) and compared with retrieved sequences in the GenBank database. Genotypes were determined by the maximum likelihood (ML) tree which was constructed using the method we previous reported (Wang et al., 2015). The nodal reliability of the ML tree was assessed by bootstrap (BS) with 1000 pseudo-replicates.

Bayesian coalescent analysis was performed to estimate the mean evolutionary rate of the E gene and time of the most recent common ancestor (TMRCA) for the most prevalent group using a BEAST v1.6.1 software package (Drummond and Rambaut, 2007). The data were analyzed using the general time reversible GTR + G4 + I model and a relaxed uncorrelated lognormal molecular clock. Each MCMC analysis was run for at least 50 million generations and sampled every 10,000 generations. Convergence of the MCMC sample on the posterior distribution was defined at an effective sample size (ESS) value >200, which was calculated by the Tracer v.1.4 program (available at http://beast.bio.ed.ac.uk/Tracer). The maximum clade credibility (MCC) tree was constructed using TreeAnnotator v.1.4.8 and then visualized using FigTree v.1.3.1 (available at http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Ethical statement

All the participants were informed at study enrolment and written informed consent was received before sample collection. The research was approved by the Institutional Ethical Committee of Kunming University of science and technology.

3. Results

3.1. The dengue outbreak in 2013 in Yunnan

During the dengue outbreak from July to December of 2013, 1538 cases were confirmed positive in Yunnan. The majority of cases of DENV infection (93.04%; 1431/1538) occurred during 3 months: August, September, and October. The presence of dengue was reported in eight prefectures. Among them, Xishuangbanna, the southernmost prefecture bordering Laos, and Dehong, the westernmost prefecture bordering Myanmar, were the most seriously afflicted regions, with 1320 and 190 cases of DENV infection, respectively. The remaining 28