Molecular characterization of the viral structural protein genes in the first outbreak of dengue virus type 2 in Hunan Province, inland China in 2018

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Abstract

Background: An unexpected dengue outbreak occurred in the Hunan Province in 2018. This is the first dengue outbreak in this area of inland China, and 172 cases were reported.

Methods: To verify the causative agent of this outbreak and investigate gene characterization, the structural protein C/prM/E genes of viruses isolated from local residents were sequenced followed by mutation and phylogenetic analysis. The recombination, selection pressure, potential secondary structure and three-dimensional structure analysis were also performed.

Results: Phylogenetic analysis revealed that all epidemic strains were classified as the cosmopolitan DENV-2 genotype, closest to the Zhejiang strain (MH010629, 2017) and then Malaysia strain (KJ806803, 2013). Compared with the DENV-2SS, 151 base substitutions were found in 89 sequences of isolates, which resulted in 20 non-synonymous mutations, of which 17 mutations existed among all samples (two in capsid protein, six in prM/M, and nine in envelope proteins). Moreover, amino acid substitutions at 602^{th} (E322:Q→H) and 670^{th} (E390: N→S) may enhance virulence of the epidemic strains. One new DNA-binding site and five new protein binding sites were observed. Two polynucleotide-binding sites and seven protein binding sites were lost compared with DENV-2SS. Meanwhile, five changes were found in helix regions. Minor changes were observed in helical transmembrane and disordered regions. The 429^{th} amino acid of E proteins was switch from histamine (positively charged) to asparagines (neutral) in all 89 isolate strains. No recombination events or positive selection pressure sites were observed. To our knowledge, this study is the first one to analyze the genetic characteristics of epidemic strain in the first dengue outbreak in Hunan Province, inland China.

Conclusions: The causative agent is likely to come from Zhejiang Province, a neighbouring Province where dengue fever broke out in 2017. This study may help to understand the intrinsic geographical relatedness of DENV-2 and contributes to further research on pathogenicity and vaccine development.

Background

Dengue fever (DF) is an ancient disease with a history of approximately 200 years, which is caused by four different but closely related dengue viruses (DENV-1, DENV-2, DENV-3, and DENV-4) and is mainly transmitted by female *Aedes aegypti* or female *Aedes albopictus* [1, 2]. It occurs in tropical and subtropical urban and semi-urban areas around the world. The global dengue epidemic has spread quickly in recent decades, from 9 endemic countries before 1970 to 128 in 2012 [3]. The incidence of dengue has also increased dramatically, from 1.2 million in 2008 to 3.9 million in 2015 [4, 5]. In 2016, there were more than 2.38 million cases in the Americas alone, of them Brazil accounting for about 1.5 million cases. Meanwhile, more than 375000 cases were reported in Western Pacific region, including 176000 cases in the Philippines and 100000 cases in Malaysia [6]. However, the number of dengue cases reported in the Americas was 580,000 in 2017, approximately 78.9% fewer than last year. According to the data provided by the World Health Organization (WHO, the number of cases in the first quarter of 2018

decreased by 27% compared with the same period of 2017, mainly occurring in countries such as Paraguay, Argentina, Bangladesh, Cambodia, India, Myanmar, Malaysia, Pakistan, Thailand, Yemen, and China, and mainly caused by DENV-1 and DENV-2 serotypes [7].

DF has become a serious public health problem in China, according to data provided by the Chinese Center for Disease Control (CCDC), there were 757,243 people infected in the past 42 years [8, 9], largely occurring in Hainan [10], Guangdong [11, 12], Zhejiang [13, 14], Fujian [15], Taiwan, and Yunnan [16-19]. In 2018, an unexpected dengue outbreak occurred for the first time in Hunan Province, an inland Province of China. According to data provided by the Center for Disease Control (CDC), the earliest dengue fever case was reported on September 2. To October 6, 172 infected individuals were confirmed as NS1-positive, with one death in Hunan Province. Qiyang County as the most serious area of the epidemic, 73 cases were confirmed during September 8 to September 14, accounting for 76.04% of the total confirmed cases in this area. The ratio of female and male infected patients was 1.04 to 1 (49:47), with an average age of 49.5 (ranging from 11 to 84 years old). It should be noted that no dengue case was found in this area from 2000 to 2013, and there were only five imported cases were reported during 2014-2017 with no local cases in Qiyang County.

This was the first dengue outbreak in Hunan, interior province of China. It provided us an early warning that the dengue fever has gradually spread inland from China's coastal and border regions and highlighted the urgent need to monitor the cross-border and cross-regional spread of dengue virus. The purpose of this study was to verify the causative agent and analyze the molecular characteristics of the epidemic strain in this outbreak.

Methods

The geographic analysis of Hunan Province and study design

The geographical distribution map of dengue fever in China over the years was created by Chinese mapping and drawing software. Blood samples of patients were collected from two local hospitals responsible for the treatment of DENV patients (Qiyang People's Hospital and the Nongshan Hospital) during the 2018 dengue outbreak. The dengue fever epidemic situation in the surrounding areas of Hunan Province was also included in this analysis..

Dengue virus NS1 antigen detection

The DENV NS1 antigen was detected by colloidal gold method according to the manufacturer's instructions (Dengue NS1/IgG/IgM Test Cassette, GuangZhou Biological Products, GuangZhou, China). When there are red bands in both the quality control area and the sample area, the results are judged as positive.

Viral RNA Extraction, Dengue virus identification and sequencing of structural protein genes

Viral RNA was extracted from 140 μl of serum of patient using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany; No.52906) and then reverse transcribed into cDNA using the Prime Script TM II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan; No.6210A). Universal primers of dengue virus and the specific primers of the four serotypes (Table S1) were used for polymerase chain reaction (PCR), and the serotype was identified. The PCR reaction conditions were denaturation at 95℃ for 5 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and elongation at 72℃ for 30 s, with a final elongation step at 72℃ for 7 min. The PCR products were confirmed by agarose gel electrophoresis and sequenced at Sangon Biotech Co., Ltd. (Shanghai, China). Both forward and reverse sequencing were done.

Primer design

A total of three synthetic oligonucleotide primer pairs F1/R1, F2/R2, and F3/R3 (Table S2) were designed to amplify overlapping fragments with sizes of 2,325 nucleotides spanning the entire structural protein genes of DENV-2. All primers were designed using SnapGene software (version 3.2.1), based on the reference strain (GenBank accession no. M29095). All primers were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China).

Molecular characteristics analysis

A total of 89 nucleotide sequences were assembled using BioEdit 7.1.3 (http://www.mbio.ncsu.edu/bioedit/bioedit.html) then uploaded to the National Center for Biotechnology Research (NCBI) GenBank®database (http:// www.ncbi.nim.nih.gov/GenBank/index.html) (GenBank ID: MK543451-MK543470, MK543472-MK543478, MK543480-MK543492, MK949396-MK949438) by Sequin Application (version15.50). Next, the mutations in nucleotide sequences and translated amino acid sequences of the structural proteins of these 89 strains were analyzed with BioEdit and Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0. The secondary structure of the structural proteinswere predicted by the Predict Protein server (https://www.predictprotein.org/) for both epidemic and reference strains.

Phylogenetic analysis

Sequence of the structural protein genes (C/prM/E) were aligned by MEGA 7.0 and compared with 133 DENV reference strains, including four serotypes of standard strains (Table S3), which were collected from websites (https://www.viprbrc.org). Phylogenetic analysis was performed using MEGA 7.0 through the ML phylogeny test with a bootstrap of 1,000 replications.

Recombination and selection pressure analysis

For detection of recombination, the Genetic Algorithm (GARD) [20] online server of Datamonkey [21] was used for automatic analysis of reorganization events of the structural protein genes of 130 DENV-2 reference sequences in Table S4 and 89 epidemic strains in our study. The phylogenies server was used for analysis of selection pressure. In this research, four methods, the Fixed Effect Likelihood (FEL) [22],

the Internal Fixed Effect Likelihood (IFEL) [23], the Mixed Effect Evolution Model (MEEM) [24], and the Rapid Unbiased Bayesian Approximation (FUBAR) [25], were adopted to estimate the locus specific selection pressure. If at least three of the four methods meet the requirement of $\omega > 1 (\omega = \beta/\alpha)$, and the p-value < 0.1 or Posterior Prob ($\alpha < \beta$) >0.9, then the positive selection of this site can be inferred.

Results

The Geographic Analysis of Hunan Province and Study Design

The geographic relationships between Hunan Province and the DENV outbreak areas in China were analyzed. The results showed that Hunan had become a central area of the DENV epidemic, which is surrounded by Yunnan, Guangdong, Guangxi, Hainan, Fujian, Zhejiang and the other dengue outbreak areas (Fig. 1) (The map in the figure was drawn by ourselves. Part of the data in the figure is cited from Zhao, et al. [26], and part of the data was provided by Centers for Disease Control and Prevention of Hunan Province).

During the DENV outbreak in Qiyang County, Hunan, from September 2018, a total of 260 serum samples of fever patients were collected, and 96 cases were confirmed to be NS1-positive by colloidal gold testing. Seven strains were proliferated in C6/36 cells for over 6 days to build a viral seeds pool of Hunan DENV. Eighty-nine viral RNAs were successfully extracted directly from 96 NS1-positive serum samples, followed by gene sequencing of the DENV structural protein C/prM/E genes. The phylogenetic analysis, recombination and selection pressure analysis, potential secondary structure prediction based on structural gene sequences originating from epidemic strains were performed to understand the genetic characterization, potential source, and evolution. The study design is shown in Fig. 2.

Phylogenetic analysis

The E protein gene sequences of 123 representative DENV-2 strains and four serotypes of standard strains were selected to construct phylogenetic trees with MEGA software version 7.0. The result showed that all 89 strains in this study were cosmopolitan DENV-2 genotypes. The closest relative was the Zhejiang epidemic strain (MH010629, 2017), followed by strains isolated from Malaysia (KJ806803, 2013), Bali (KT806318, 2014), Indonesia (KT781561, 2014), and the Philippines (KU517847, 2015) (Fig. 3). Among the neighboring provinces of Hunan Province, Zhejiang, Yunnan and Guangdong provinces had more than 1000 reported cases of dengue fever in each province in 2017, and all four serotype have been detected in each provinces [15, 20]. There were also reported cases in Fujian Province in 2017. Since only one amino acid mutation (I431V/A) was observed in all 89 epidemic strains compared with the nearest related strain from Zhejiang (MH010629, 2017). These data suggest that the causative agent of DENV outbreak in Hunan Province in 2018 may come from the epidemic strains in Zhejiang Province in 2017.

Bases and amino acid mutations

Three structural protein-overlapped fragments of epidemic strains were obtained by PCR amplification. After sequencing, the proteins were effectively spliced, and the length of coding nucleotide sequences was 2,325 nt, encoding 775 amino acids. The homology between isolates was 99.7-100%, and the amino acid (AA) sequence of E protein was highly conserved. By comparison, the comparability of nucleotide and amino acid sequences between the 89 epidemic strains and DENV-2SS were 93.5 and 97.8%, respectively. Compared with DENV-2SS, two hundred fifteen bases had mutations in the structural protein region of epidemic strains, among which 195 were synonymous mutations and 20 were non-synonymous mutations, leading to 17 AA substitutions (Fig. 4). Two AA substitutions at 104^{th} (C104: M→I) and 108^{th} (C108: L→M) were observed in protein C in isolate strains, six amino acid mutations including 143^{th} (M29: D→N), 166^{th} (M52: K→N), 196^{th} (M82: T→A), 241^{th} (M127: I→V), 262^{th} (M148: H→Y), and 266^{th} (M152: A→V) occurred in the structural protein prM/M, and nine amino acid mutations including 332^{th} (E52: Q→H), 351^{th} (E:71 D→A), 406^{th} (E126: K→E), 409^{th} (E129: V→I), 429^{th} (E149: H→N), 444^{th} (164: I→V), 602^{th} (E322: I→V), 670^{th} (E390: N→S), 742^{th} (E462: I→V) were observed in structural protein E (Fig. 4).

Potential secondary structure of the structural protein region

The protein secondary structure among DENV-2 standard strain KM204118 and three randomly selected sequences (HNQY2018014, 021, and 028) from the 89 isolate strains were predicted. Compared with DENV-SS, Hunan epidemic strains had missed one nucleotide-binding site (site 6) and one DNA-binding site (site 18), as well as one protein binding region (sites: 4 and 5) in the capsid protein (Fig. S1), while one new DNA-binding site (site 74) and two new protein binding sites (19 and 29) were observed in isolate strains. Moreover, variations were found in the disordered region among Hunan epidemic strains, DENV-2SS and Zhejiang/2017 (Fig. S1). In the prM/M region, which contained 166 amino acids, the protein secondary structure of the epidemic strains was highly consistent with that of the Zhejiang/2017 strain (Fig. S2). However, compared to the DENV-2SS, three protein binding regions disappeared in Hunan epidemic strains (sites: 122, 133, and 220), and one novel protein binding region emerged (site 144). Additionally, one helical transmembrane region of the isolates visibly differed from the DENV-2SS, and eight significant changes were observed in the buried and exposed region, while no noticeable variation was found in the strand and helix region (Fig. S2). Three protein binding sites (sites: 584, 596, and 642) disappeared at the 495AA locus of E protein, one protein binding location (site 377) was observed in Hunan isolates, four considerable alterations were also observed in exposed and buried regions, and minor changes were found in the helical transmembrane and disordered region (Fig. 5). Meanwhile, there were 22 changes in strand regions. Of them, 11 were new (120, 166, 192, 309, 334, 347, 446, 455, 512, 582-584, 591), 11 were missing (101, 102, 124, 141, 207, 290, 294, 553, 607, 636, 651, 692-695), and nearly 70% of changes occurred in E proteins. Nevertheless, compared with the Zhejiang 2017 strain, there was no significant change in protein binding region and polynucleotide-binding region in structural protein (C, prM/M, and E) (Fig. 5, Fig. S1, and Fig. S2).

Possible three-dimensional structure of the structural protein E genes

The possible three-dimensional structure of structural proteins of the representative epidemic strains (HNQY2018014, 021, and 028) were predicted and compared with DENV2-SS and Zhejiang/2017 strain. Homology modeling revealed that five strains had the same three-dimensional structure. In addition, binding sites were also predicted by the 3DLigandSite ligand binding site prediction server, four protein binding sites (HIS429, ALA430, THR435, and GLY436) were observed in DENV-2SS (Fig. 6E). Hunan epidemic strains and the Zhejiang/2017 strain have the same binding sites at ASN429, THR435, and GLY436) (Fig. 6D). HNQY2018028 has two different binding sites (429 and 430) compared to DENV-2SS (Fig. 6) and one diverse binding site (429) compared to Zhejiang/2017.

Recombination and election pressure analysis

RDP4 software was used to analyze potential recombination events among HNQY2018001-HNQY2018089 and other representative DENV-2 virus strains. Preliminary analysis results showed that no recombination event occured in these DENV-2 strains ($p<0.05$). The structural proteins of 202 strains were analyzed, including 113 representative strains of DENV-2 and 89 isolate strains. The results showed that the MEME method identifies the maximum number of actively selected sites (n = 16). However, the FEL, IFEL and FUBAR methods indicated that all 775 sites were under negative pressure (Table 1). Therefore, no significant evidence of positive selection was presented in at least three different methods, so the positive sites of selection pressure at these sites cannot be determined.

Discussion

In mainland China, Dengue fever mainly occurs in Guangdong, Hainan, Zhejiang, Fujian, Guangxi, and other coastal regions, or in Yunnan Province and Southeast Asian countries adjacent to Yunnan Province. Only scattered cases have been reported in inland China, but no large-scale dengue epidemic has been reported in the inland area to date. Hunan is an inland province of China, located near 30 degrees north latitude. The climate is warm and humid from June to November, which provides a natural environment for the breeding of *Aedes albopictus*. Hunan Province is located near Guangdong, Guangxi, Zhejiang, and other areas with high incidence of dengue fever. The total number of dengue infections in China was 5106 in 2018, including 3,250 cases in Guangdong Province, 217 cases in Zhejiang, and 172 cases in Hunan. This was the first dengue outbreak in Hunan, an inland province of China, which provides us with early warning that dengue fever has gradually spread from coastal and border areas to inland areas of China, highlighting the urgent need to monitor the cross-border and cross regional transmission of dengue viruses.

In this study, we collected serum from 260 patients with fever in Qiyang County, Hunan Province, and 96 cases were confirmed to be NS1-positive. Of them, 89 viral RNAs were extracted and structural protein gene fragments (HNQY2018001-089) were obtained by amplification of overlapping fragments with a length of 2,325 nucleotides. Phylogenetic tree analysis showed that all isolate strains were cosmopolitan DENV-2 genotypes, and all belonging to one cluster of the ML tree and were closely related to the Zhejiang strain (2017, MH110588). Additionally, it is also closely related to the following four strains:

Malaysia (KJ806803, 2013), Bali (KT806318, 2014), Indonesia (KT781561, 2014) and the Philippines (KU517847, 2015). Although all four serotypes of dengue virus were prevalent in Zhejiang Province in 2017, the vast majority of the epidemic strains were still cosmopolitan DENV-2 genotype, and the coding protein of this epidemic strain has only one amino acid different from the epidemic strain in Hunan Province in 2018. This result suggested that the DENV-2 epidemic in Hunan possibly imported from Southeast Asian countries, such as Malaysia, Indonesia or the Philippines, passed through Zhejiang Province, and then further spread to Hunan Province.

Compared with standard strains, 17 amino acid substitutions were observed in structural protein C/prM/E of all 89 epidemic strains. The prM-E protein is the main structural protein of flavivirus, which is related to virulence, host affinity, virus adsorption, penetration, and cell fusion [27]. Hydrophobic amino acids play an important role in maintaining the tertiary structure of proteins due to their hydrophobic interactions and may impact the virulence of the virus. Tamm et al. found that hydrophobic domains affect the virulence potential of Yersinia enterocolitica [28]. Sainz et al. determined that single hydrophobic amino acids play an important role in transcriptional activation in vivo [29]. In our study, three hydrophobic amino acids in CDS region mutated into hydrophilic ones at 196^{th} (M82: T→A), 262^{th} (M148: H→Y), and 351^{th} (E71: D→A). In addition, neutral amino acids became basic amino acids at 332^{th} (E52: Q→H), and two positive electricity amino acids converted into negative ones at sites 406^{th} (E126: K→E) and 429^{th} (E139: H→N), mutations in these amino acids have not been reported, and changes in polarity or charge of amino acids may affect the functions of prM and E proteins, however further studies are needed to confirm these hypotheses. DENVE protein domain III (E295~E395) is the immunoglobulin G (lgG) immunoglobulin-like folding and plays an important role in mediating the fusion of virus and host receptor [30]. In this study, there were two amino acid changes in EDIII domain, 602^{th} (E322: I→V) and 670^{th} (E390: N→S). It has been reported that the mutation of E390 from N amino acid to S amino acid can enhance the replication ability of virus [31], but the influence of E322 amino acid mutation remains to be further confirmed.

The change of protein secondary structure will affect the enzyme activity. Compared with DENV-2 standard strain (KM204118), eight protein binding sites (4, 5, 122, 120, 133, 584, 596, and 642) and two polynucleotide-binding sites (6 and 18) were missed. Simultaneously, four new protein binding sites (19, 29, 144, and 377) and one polynucleotide-binding site (74) emerged. Furthermore, approximately eight obvious changes were observed in buried area and exposed region. All of the above changes may lead to the diversification of protein structure domain, further influencing the protein function. Homologous modeling and prediction of the possible 3D structure of structural proteins showed that both epidemic strains and DENV2-SS had the similar 3D structure and 4 predicted protein binding sites, olny one protein binding (429^{th}) was different (DENV2-SS: His 429; Zhejiang/2017 and Hunan epidemic strain: ASN 429).

The analysis showed that there was no recombination event among the Hunan epidemic strains and 130 DENV-2 reference strains, and no distinct positive selection site in structural protein, which contained 775 amino acids, suggesting that these structural protein coding genes were conservative.

Conclusions

This study described the characteristics of the structural protein coding sequences in DENV-2 originating from the 2018 outbreak in Hunan Province, inland China. This will benefit to the follow-up study of DENV in China and Southeast Asia. Our finding also indicatesd that the transmission region of DENV has gradually spread from China's border and coastal areas to inland China. It provided us a warning that the dengue fever epidemic in China is becoming increasingly serious and difficult to control and emphasized the urgent need to monitor the cross-border spread of DENV.

Abbreviations

AA: Amino acid; CCDC: Chinese Center for Disease Control; DENV: Dengue virus; DENV-1/2/3/4: Dengue virus serotype 1/2/3/4; DF: Dengue fever; DENV-2SS: Dengue virus serotype 2 standard strain; FEL: Fixed Effect Likelihood; FUBAR: Rapid Unbiased Bayesian Approximation; GARD: Genetic Algorithm Recombination Detection; IFEL: Internal Fixed Effect Likelihood; IgG: Immunoglobulin G; MEEM: Mixed Effect Evolution Model; MEGA: Molecular Evolutionary Genetics Analysis; NCBI: National Center for Biotechnology Information; NS1: Non-structural protein 1; NT: Nucleotides; PCR: Polymerase Chain Reaction; WHO: World Health Organization.

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from all participants and each one was informed of the purpose of the study, and written informed consent was obtained from parents or guardians if the participant was children (under 16 years old). The study protocol was approved by the Institutional Ethics Committee (Institute of Medical Biology, Chinese Academy of Medical Sciences, and Peking Union Medical College).

Consent for publication

Not applicable.

Availability of data and material

All the data supporting our findings is contained within the manuscript. The structural protein gene sequences of 89 epidemic strains in Hunan Province in 2018 have been uploaded to the National Biotechnology Research Center 89 gene sequences of structural protein have been uploaded to National Center for Biotechnology Information (NCBI) GenBank®database (http://www.ncbi.nim.nih.gov/GenBank/index.html) (GenBank ID: MK543451-MK543470, MK543472-MK543478, MK543480-MK543492, MK949396-MK949438)

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

QS, and SF contributed to the design of the study. JG was responsible for data collection, analysis and manuscript writing. MQ, and ZH provided software technology support. XD, and XG provided samples collection support. SD, JC, YP, JC and YY provided experiment technology support. All the authors agree to the final version of the submission.

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Table

Table 1. Selection pressure analysis of the structural protein of DENV-2 (n = 202) using FEL, IFEL, MEME, and FUBAR.

Serial number	AA position	FEL		IFEL		MEME		FUBAR	
		ω	p-Value	ω	p-Value	ω	p-Value	ω	Posterior Prob(α<β)
1	11	/	/	/	/	>100	0.083	/	/
2	19	/	/	/	/	>100	0.027	/	/
3	35	/	/	/	/	>100	0.066	0.132	0.963
4	207	/	/	/	/	>100	0.061	0.174	0.916
5	209	0.049	0.000	0.114	0.012	>100	0.078	0.068	1.000
6	228	/	/	/	/	>100	0.056	/	/
7	332	/	/	/	/	>100	0.032	/	/
8	400	/	/	/	/	>100	0.004	/	/
9	451	0.070	0.014	0.000	0.011	>100	0.069	0.082	0.998
10	463	/	/	/	/	>100	0.037	/	/
11	464	0.164	0.008	0.000	0.001	>100	0.000	0.182	0.988
12	474	/	/	/	/	>100	0.089	/	/
13	475	0.153	0.078	0.000	0.047	>100	0.053	0.130	0.983
14	488	/	/	/	/	>100	0.053	0.164	0.928
15	506	/	/	/	/	>100	0.052	/	/
16	521	/	/	/	/	>100	0.053	0.133	0.976

Note: Criteria to consider sites with significant evidence of positive selection: p-value <0.1 in FEL, IFEL, and MEME, Posterior Prob (α<β) >0.9 in FUBAR, and omega should be greater than 1. Sites that were found to be positive by at least one method are included in the list. “/”: represents that the site was not selected by the corresponding method as a positive or negative selection. “AA”: represents amino acids.

Figures

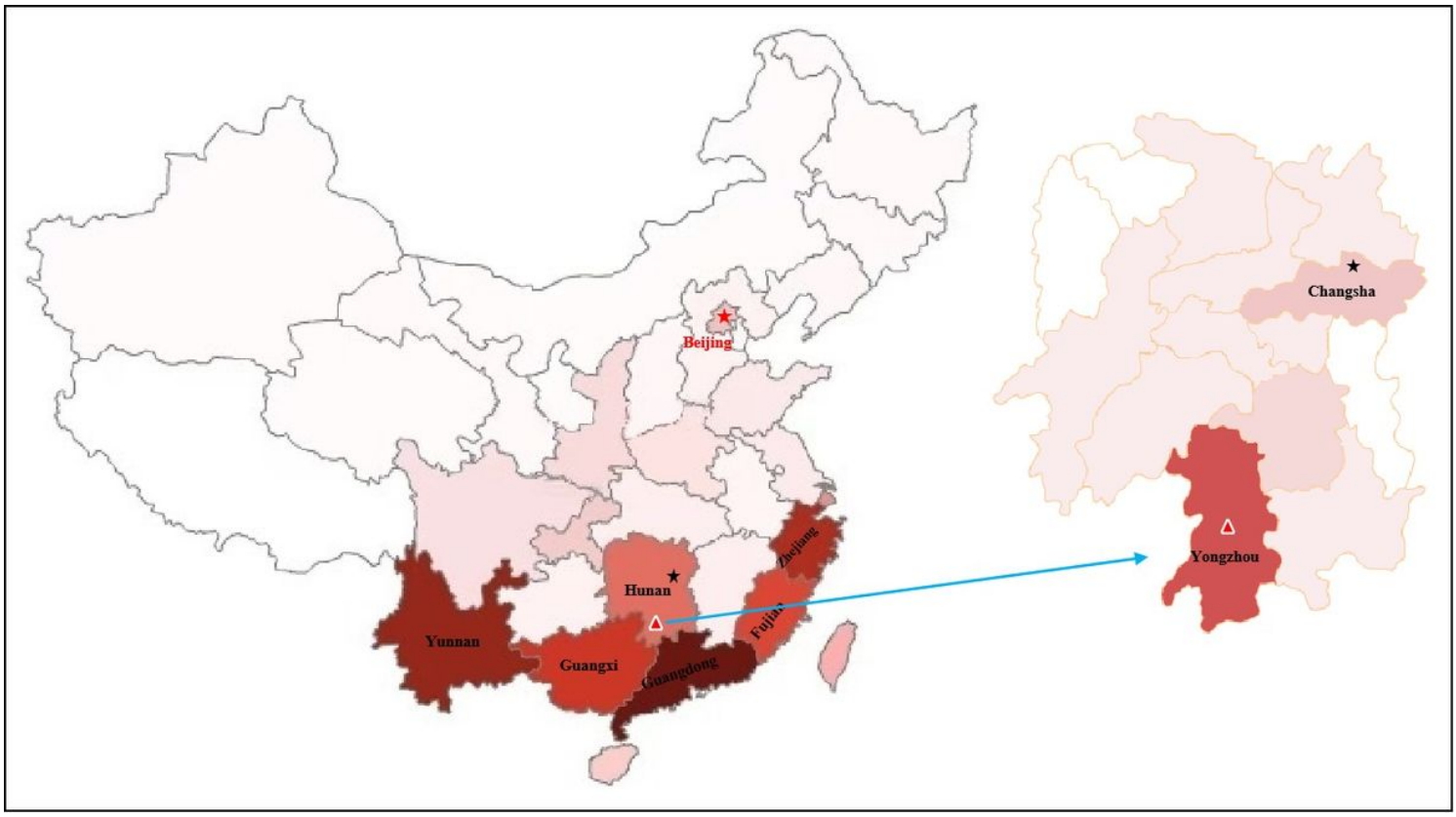
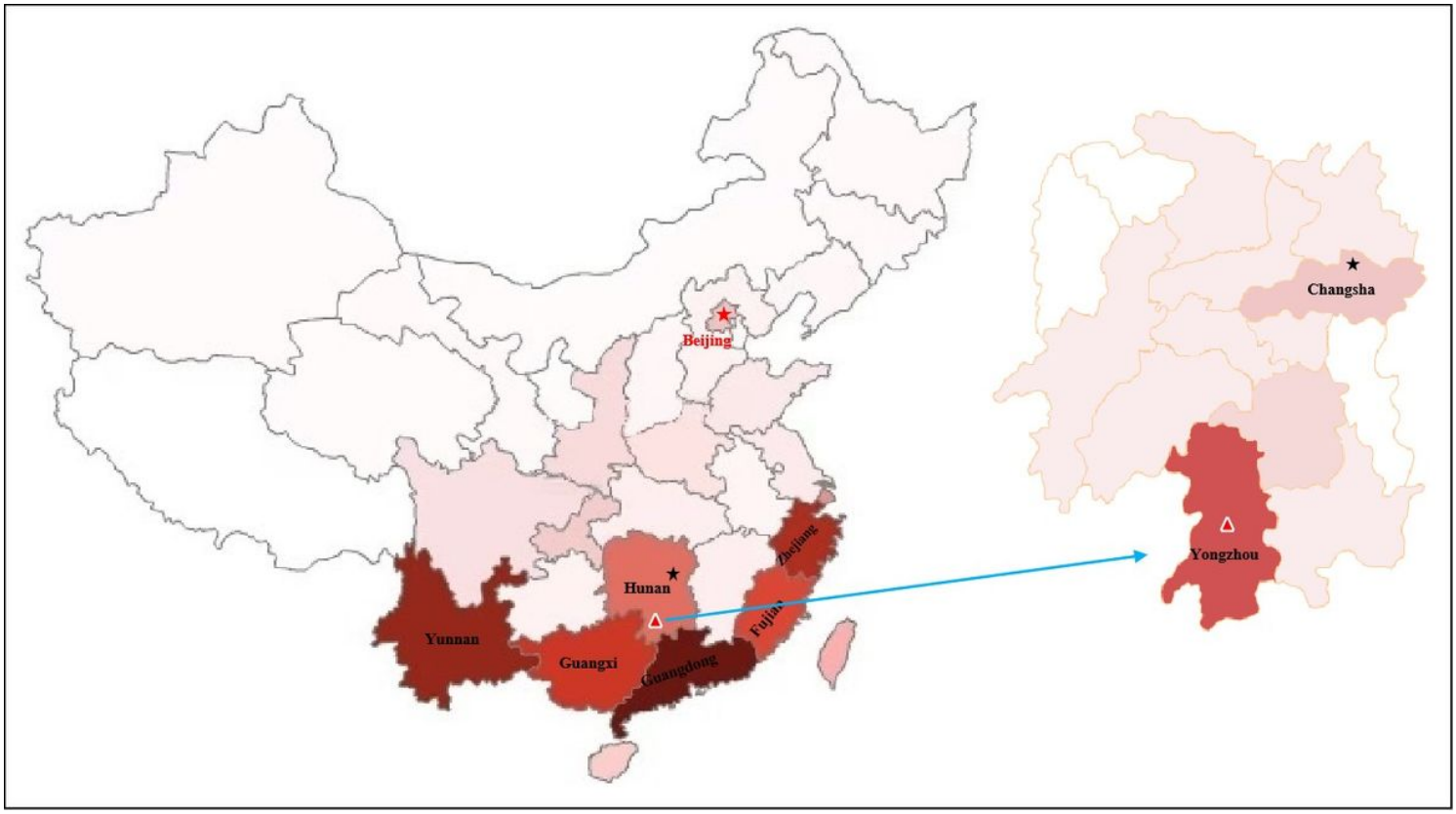


Figure 1

Geographic relationships between Hunan Province and other dengue outbreak areas in China. The intensity of the color in the figure depends on the amount of dengue cases, the darker the color is, the more dengue cases there are in the area. And the map of China shows the distribution of dengue cases in China in the past 15 years (from 2004 to 2018), the map of Hunan Province shows the distribution of dengue cases in 2018. Visible from the figure, Hunan Province is surrounded by areas with high incidence of dengue fever, such as Yunnan, Guangxi, Guangdong, Fujian, Zhejiang, Hainan and Taiwan. Moreover, the central area of this dengue outbreak is Qiyang County in Hunan, which is the most closely location in Hunan adjacent to Guangxi and Guangdong geographically. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.



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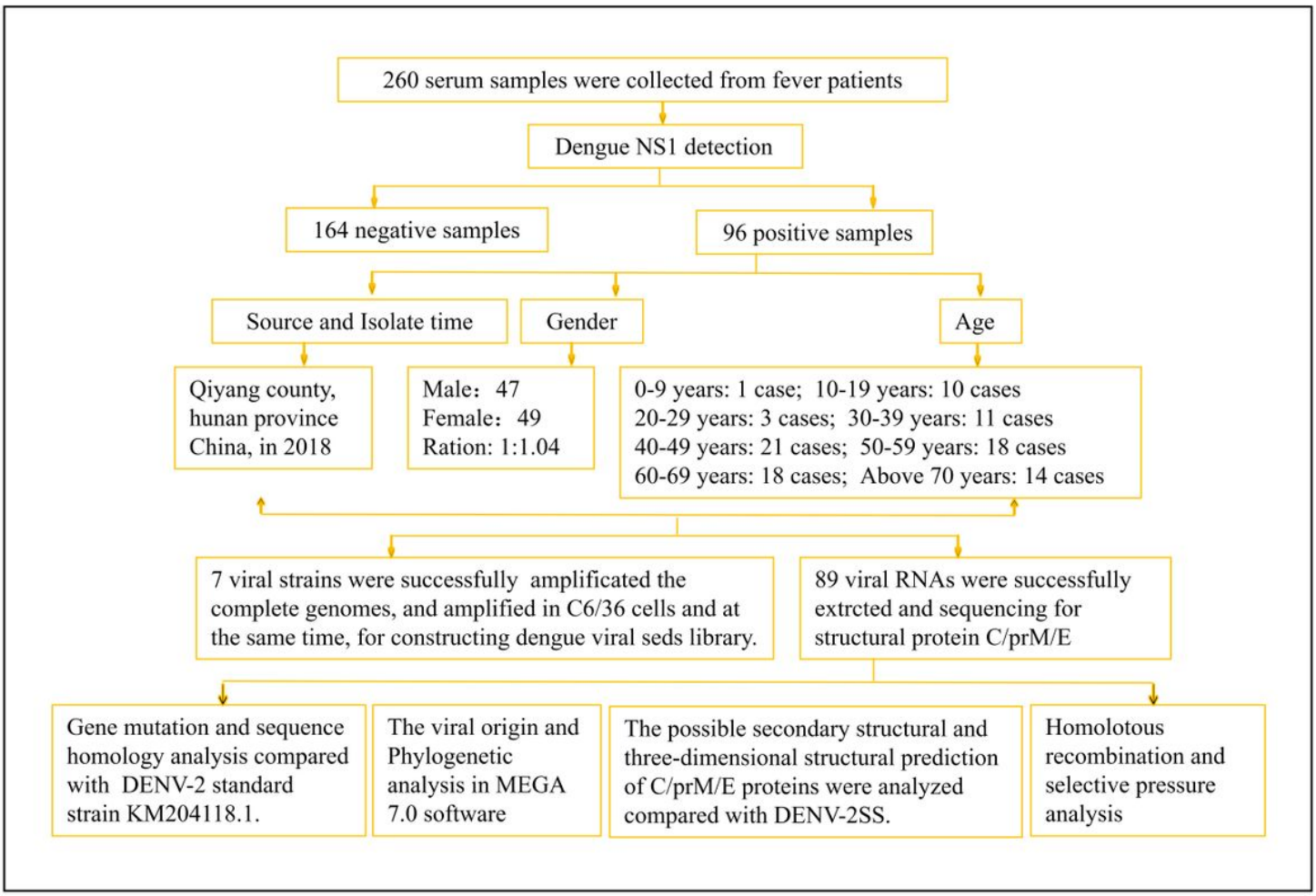
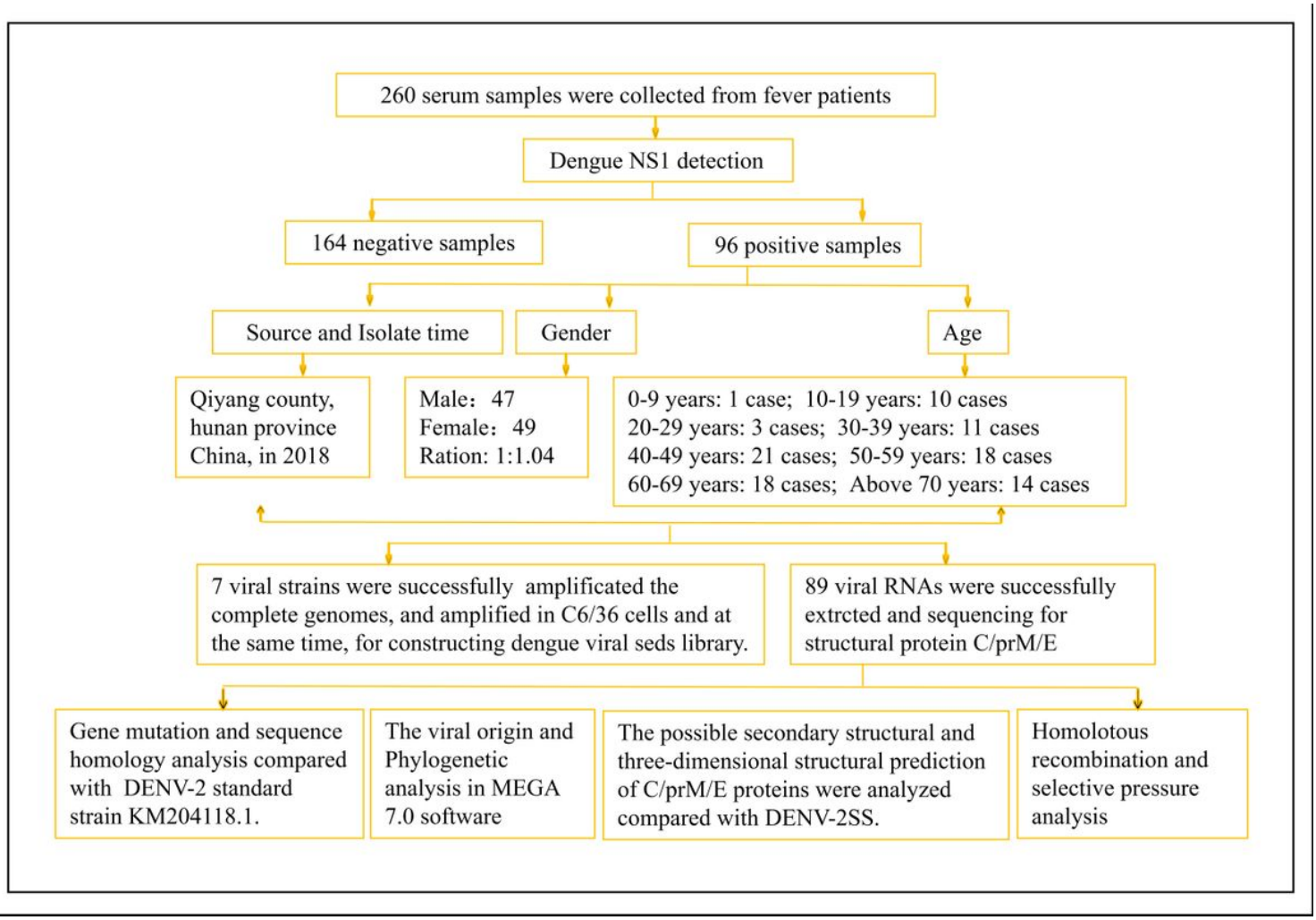


Figure 2

The study design and the following disposition of study subjects. Two hundred and sixty fever patients were recruited in our study; among them, 96 cases were identified as dengue NS1-positive. NS1-positive serum samples were collected for virus amplification and viral RNAs extraction. Phylogenetic analysis was then conducted to characterize the origin of DENV in Qiyang, Hunan during 2018.



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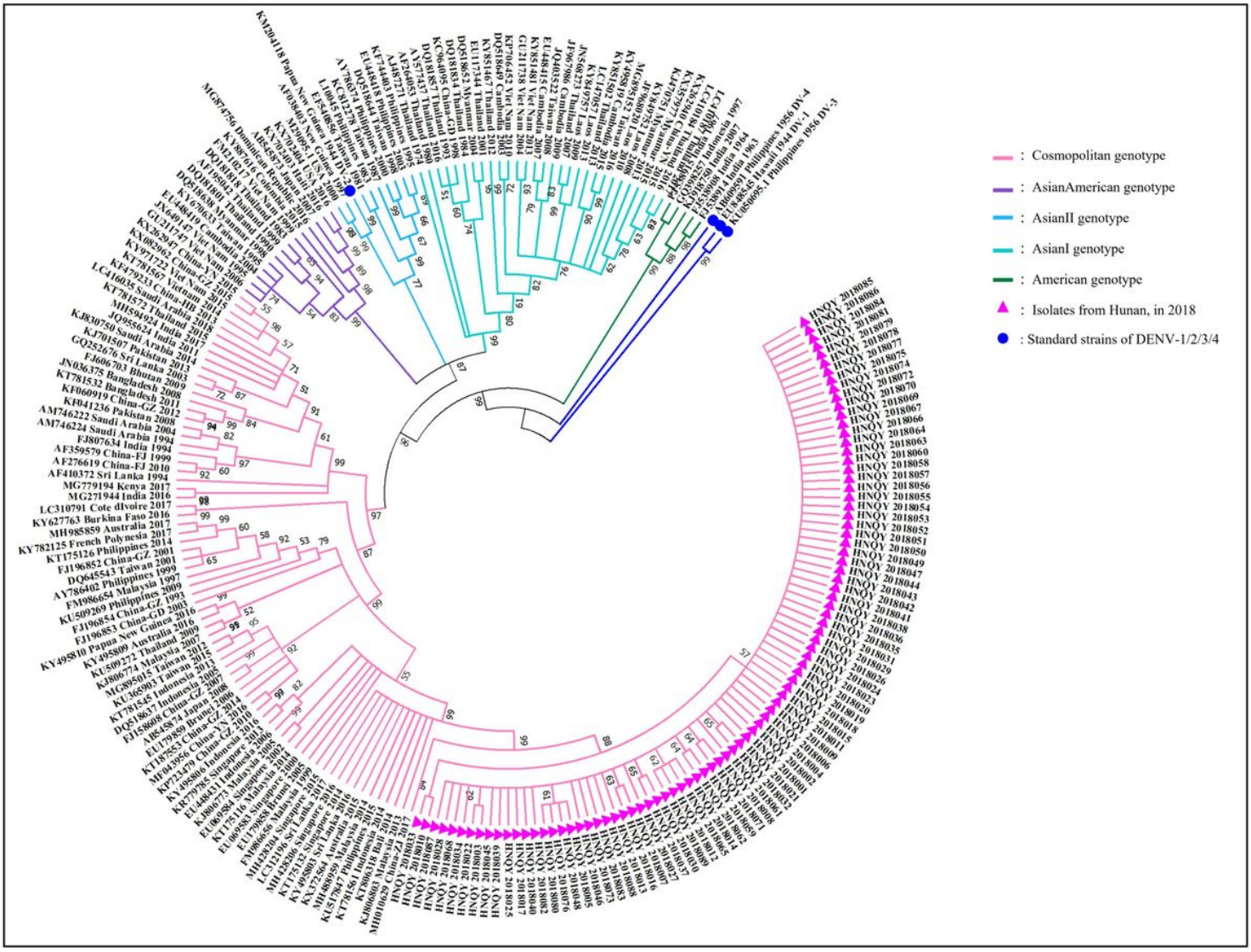

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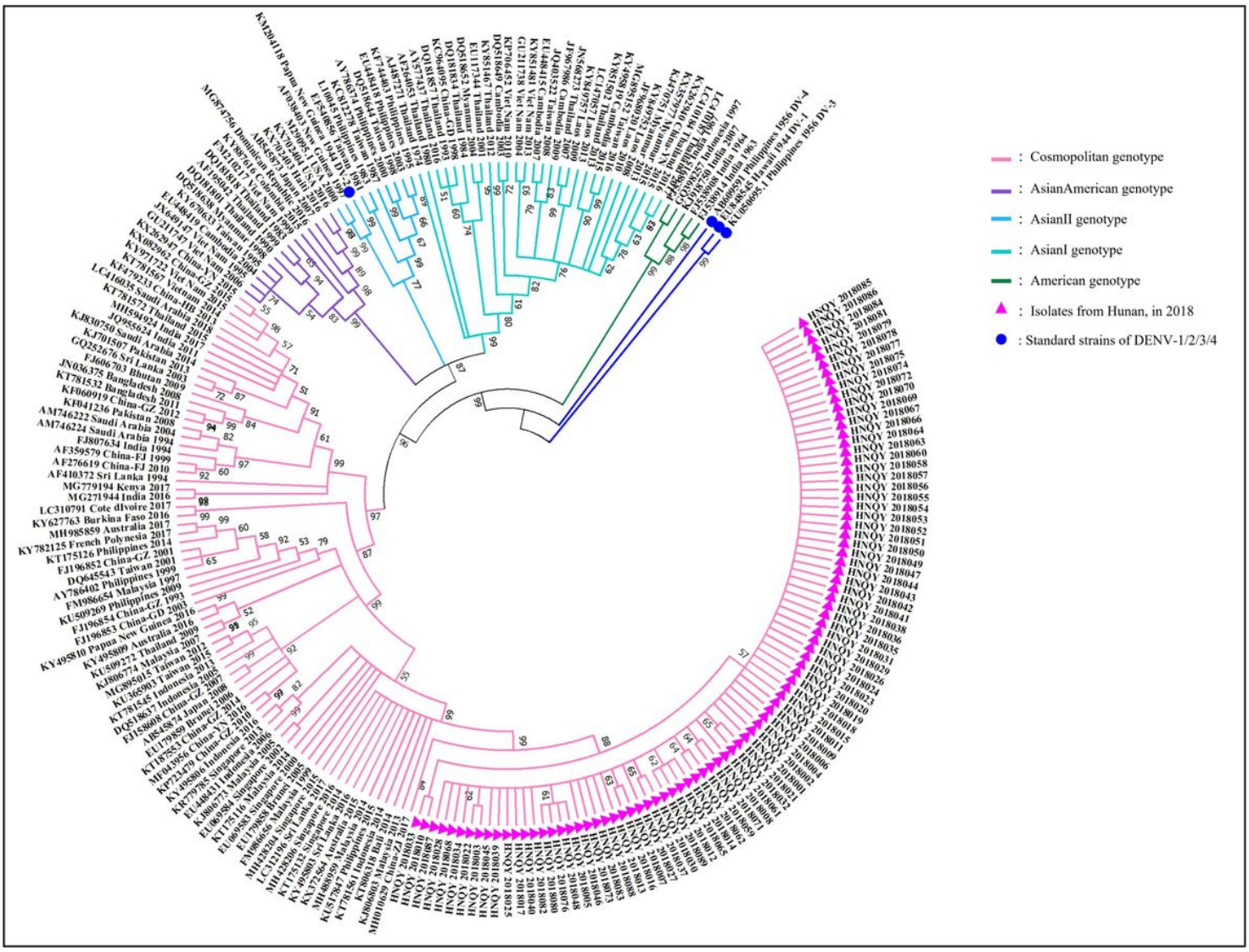
Phylogenetic tree of E protein of DENV-2 epidemic strains in Hunan Province, China in 2018. The phylogenetic trees were constructed by the maximum-likelihood method with a Kimura 2 parameter model using MEGA 7.0 software (https://www. megasoftware. net). The rose red triangles in the picture represent 89 epidemic strains of Hunan; the blue dots represent the standard strains (DENV-1/2/3/4).



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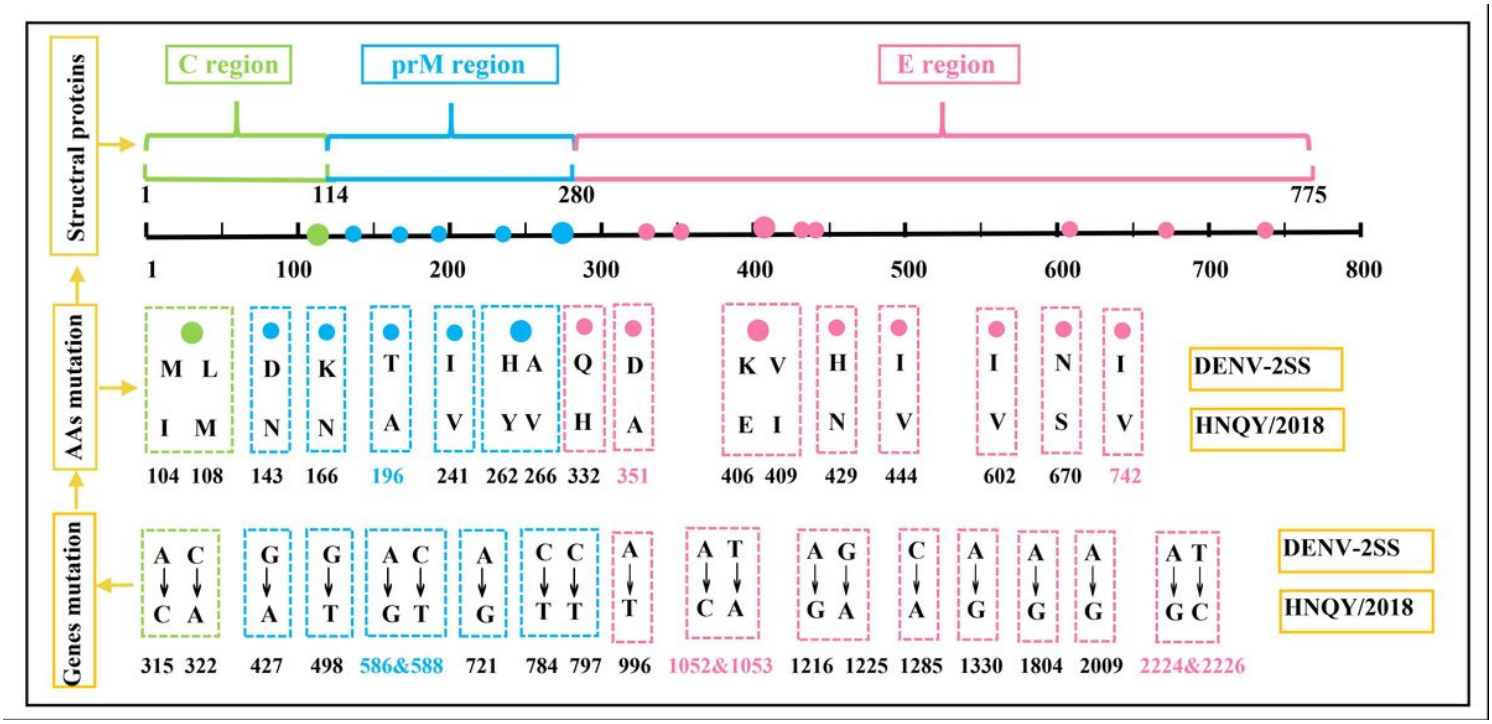
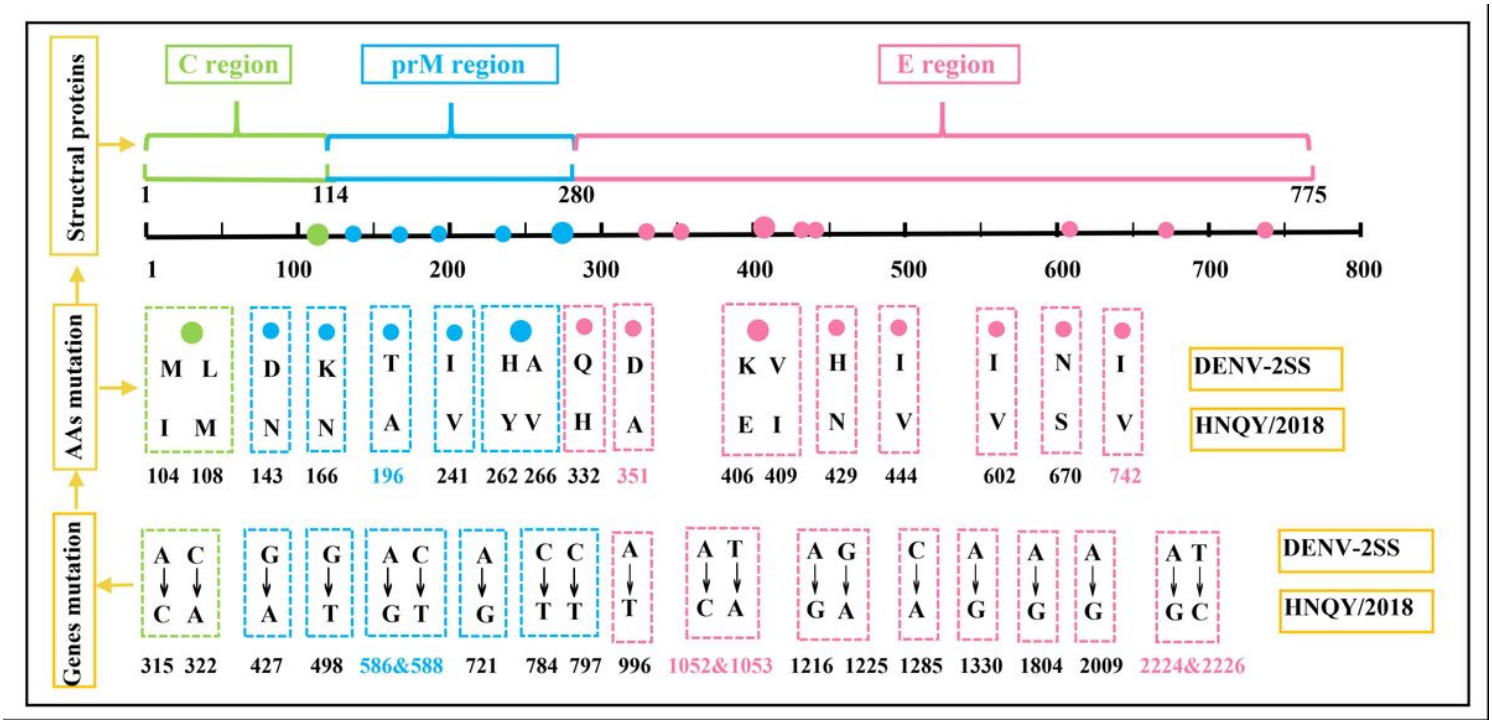


Figure 4

The gene and amino acid mutation site map of structural proteins of epidemic strains from Hunan (HNQY2018001-2018089) compared to the DENV-2 standard strain KM204118.1.



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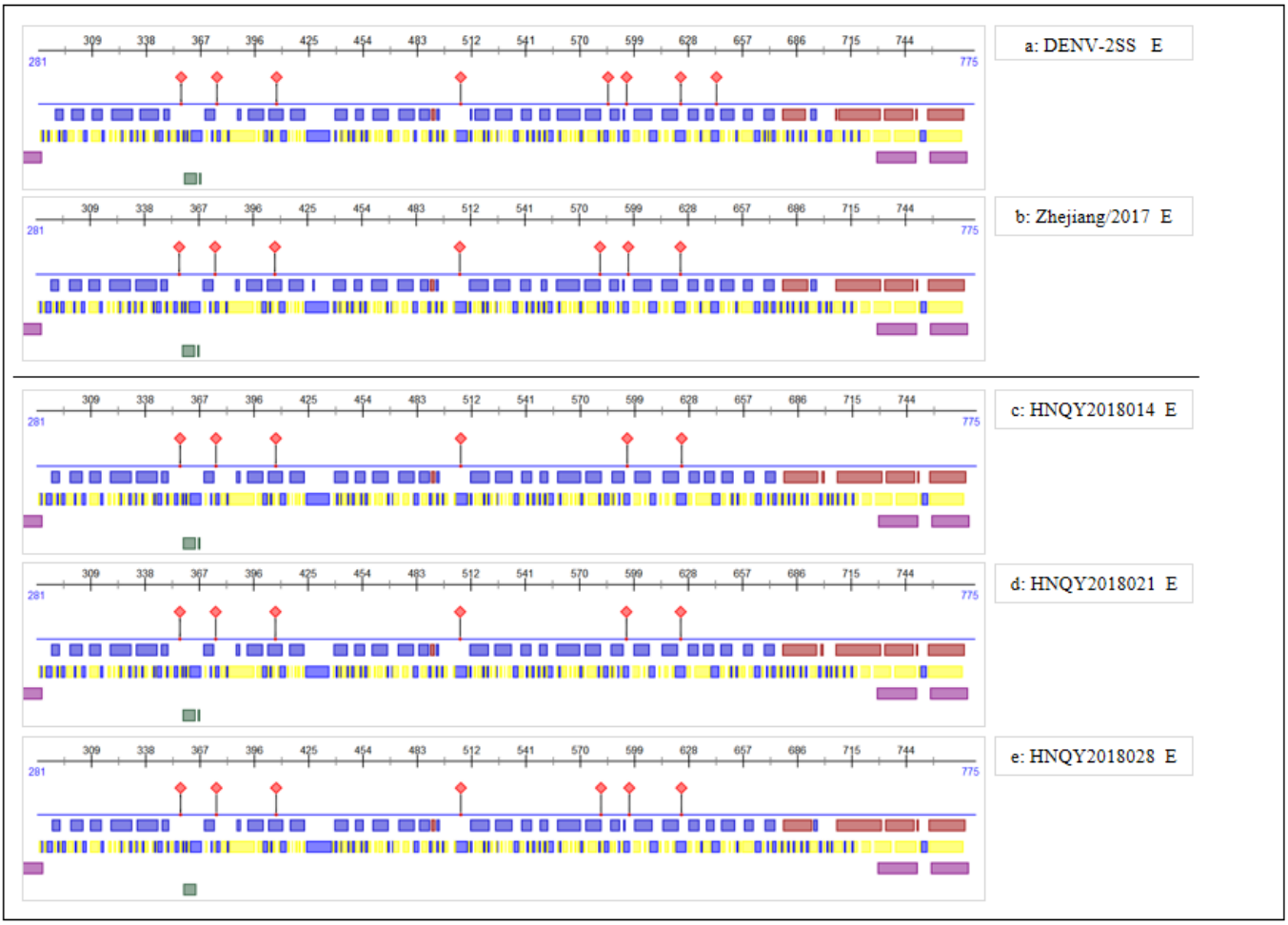

Figure 5

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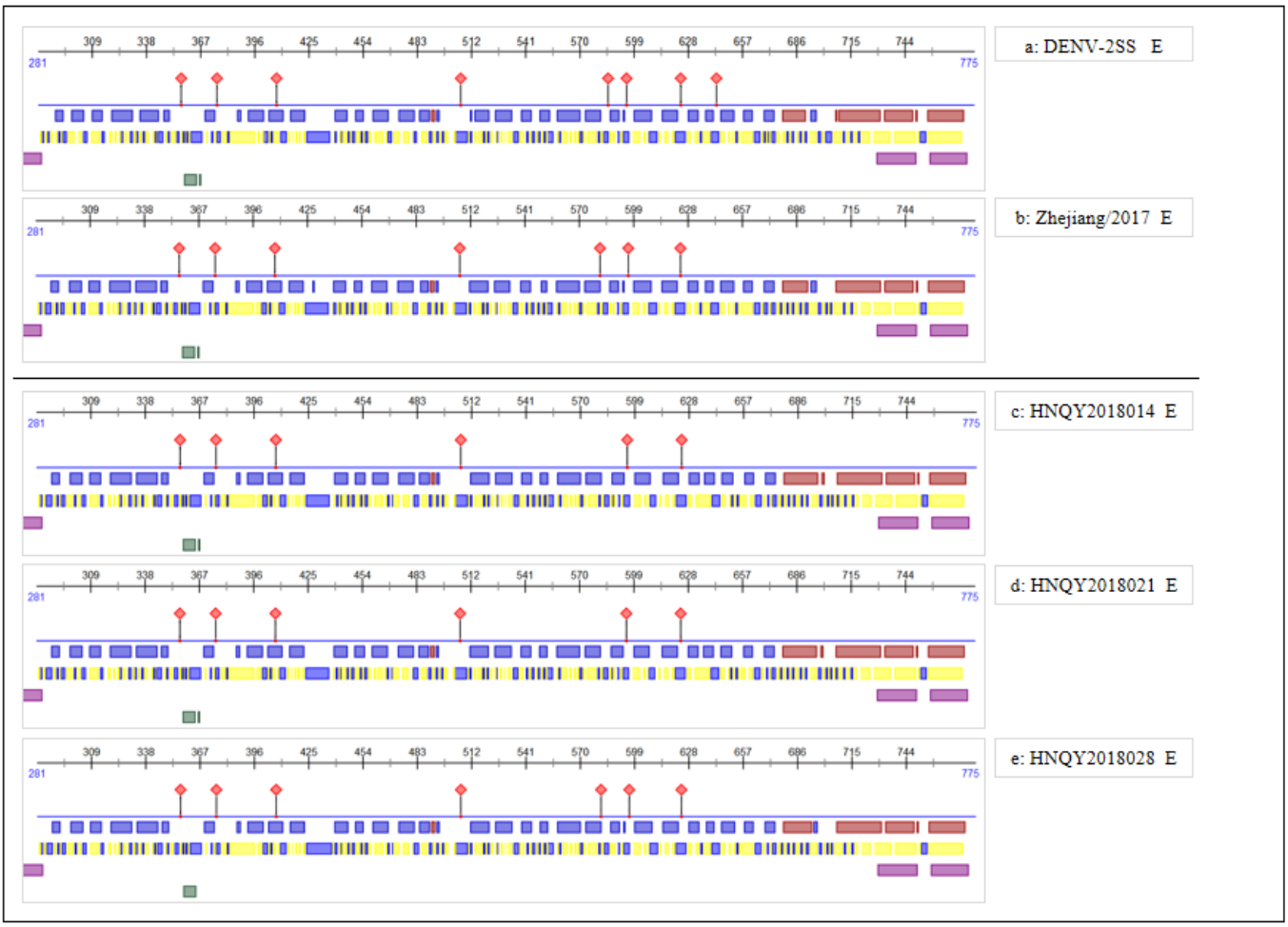

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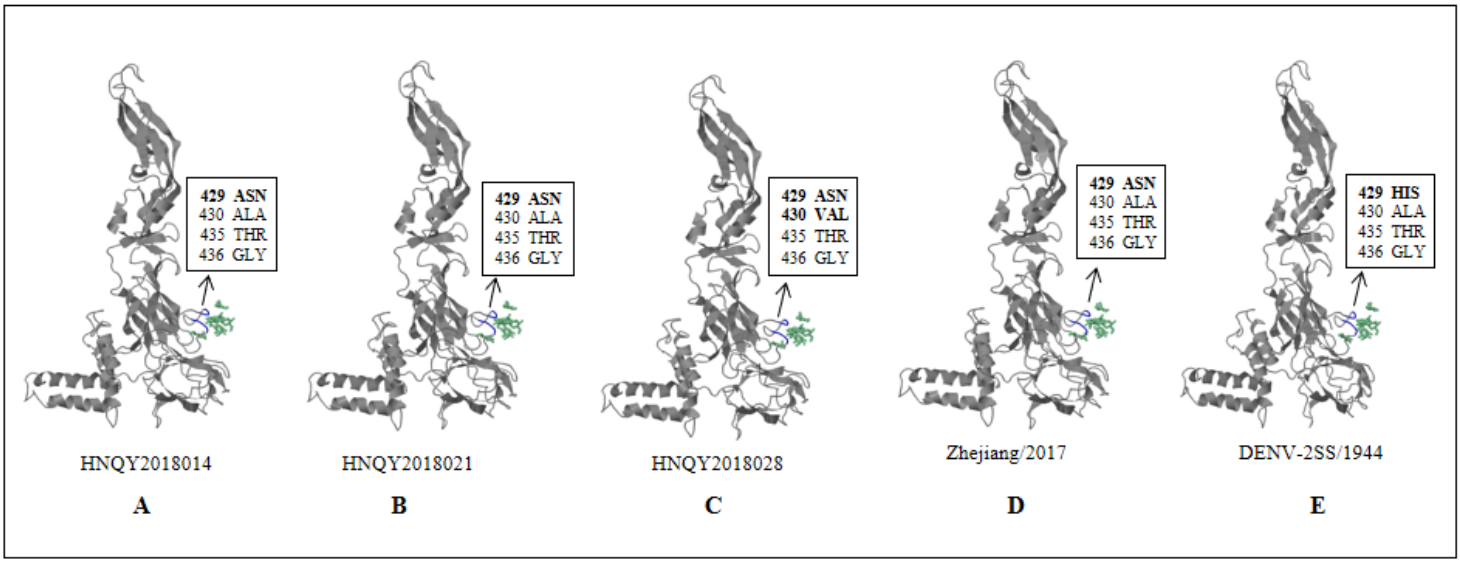
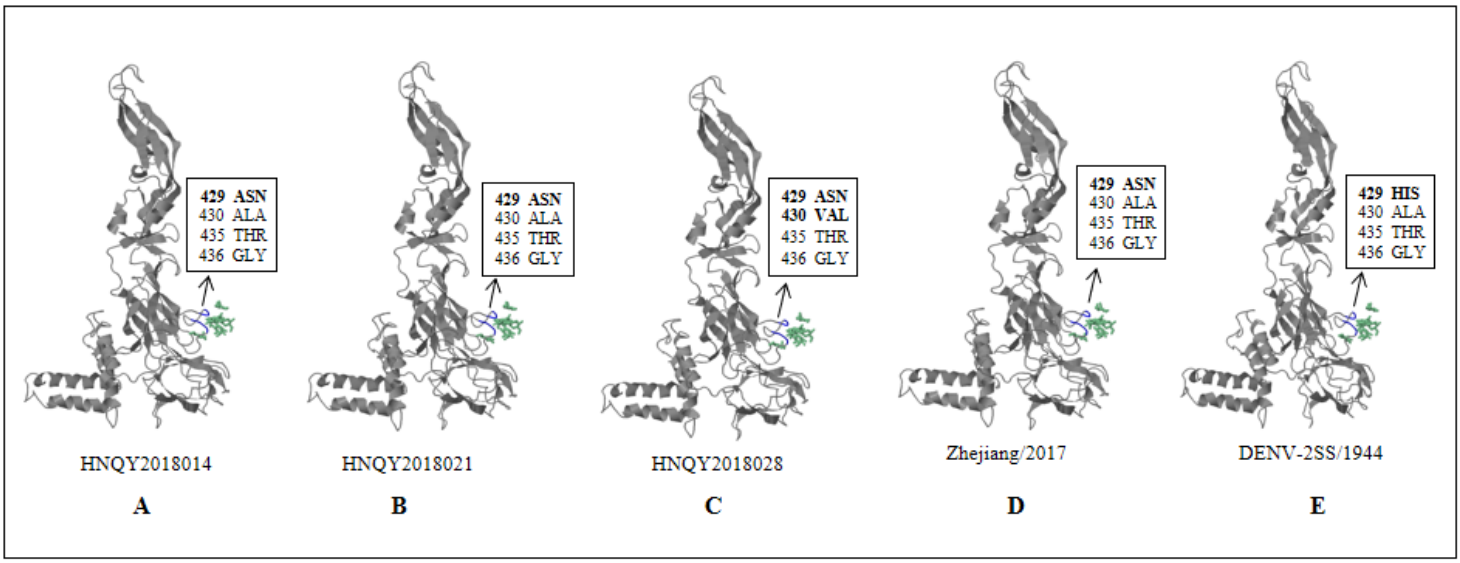


Figure 6

Predicted possible three-dimensional structure of structural protein E genes of three representative 2018 Hunan epidemic strains (HNQY2018014, HNQY2018021, and HNQY2018028), the closest strain (Zhejiang/2017, MH010629), and DENV-2 standard strain (DENV2-SS, KM204118). Blue indicates predicted protein binding sites. There are 4 possible binding sites in the E protein region of these five strains.



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