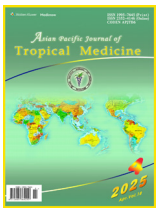

Letter to Editor

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Three serotypes of dengue virus circulated in hospitalized adult patients in an endemic metropolitan city of Northern Vietnam

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Dengue virus (DENV) is a positive-sense single-stranded RNA virus belonging to the genus *Flavivirus* within the Flaviviridae family. Four serotypes, DENV 1-4, are distributed globally[1]. Hanoi metropolitan city is an endemic hotspot for DENV transmission in Vietnam[2,3]. The largest outbreak occurred in 2017, with more than 36 000 cases and 7 deaths reported, causing by all four serotypes with the predominance of DENV1, following by DENV2[4,5]. During the following dengue season, we collected 390 blood and serum samples from 197 hospitalized patients in a national hospital in Hanoi city, Northern Vietnam to identify the circulating DENV serotypes responsible for the 2018-2019 outbreak.

This retrospective case-control study was a continuation of previously published work[6] that was conducted in Bach Mai hospital, one of the largest medical healthcare facility in Vietnam. The patients were recruited among people living in Hanoi and hospitalized at the Department of Infectious Diseases, Bach Mai hospital for dengue-like symptoms. All procedures performed in this research involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical approval for this research was obtained from the Ethical Committee of Bach Mai hospital No. 690/QD-BM on 15 September 2018. Both written and oral informed consents were collected before taking blood samples. The respondents participated voluntarily in the study after being explained clearly about the research. All information was handled anonymously, research data was only approached by the research team. The laboratory and sequencing results were last analyzed in December 2022.

98 patients with dengue and 99 patients without dengue who were living in Hanoi city were recruited between September 2018 and January 2019. In our study, only patients aged 16 years old and above were recruited. The definitions for dengue case and control patients are given in a previous publication[6]. Briefly, according to the case definition in Decision 458/QD-BYT and its guideline issued by the Vietnam Ministry of Health in 2011, the case group (patients with dengue) included in-patients who were clinically diagnosed with dengue fever and confirmed positive by rapid tests for detection

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of DENV NS1 antigen and/or DENV-specific IgM. According to hospital routines, only a diagnosis of dengue fever was done, with no further classification. The control group (patients without dengue) included out-patients or in-patients who were not diagnosed with dengue fever and confirmed negative by a rapid test.

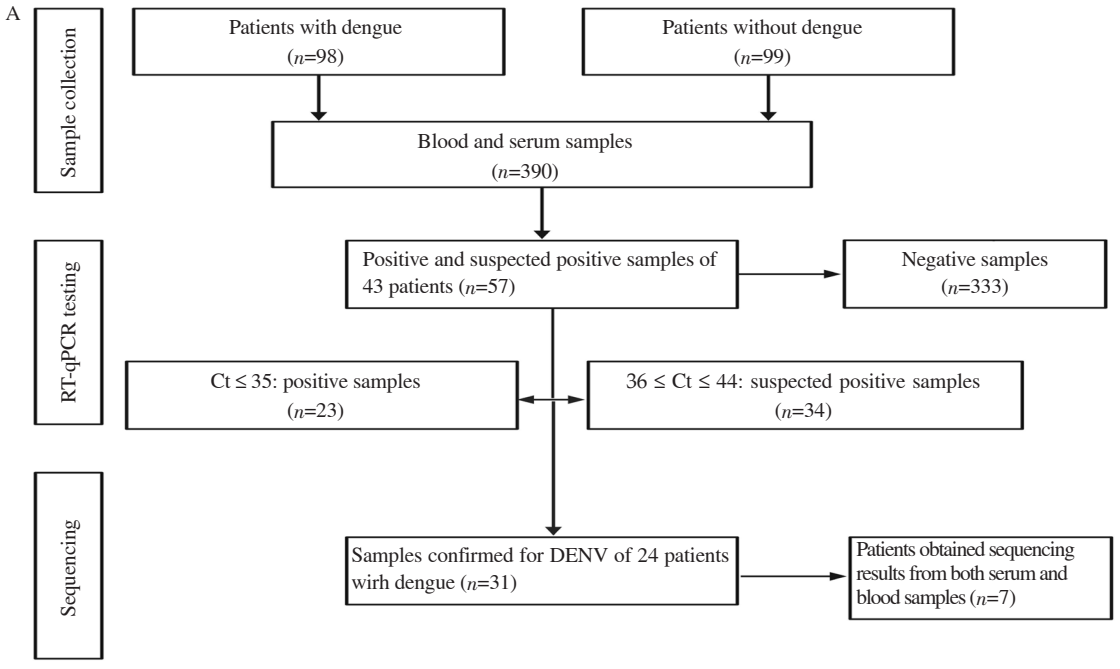
At patient admission, blood drawn was conducted one time for running the assay in our study. Total ribonucleic acid (RNA) was extracted from patient blood/serum samples using QIAamp viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's manual. The extracted RNA was immediately stored at −80 ℃ until further use. The analyses were performed by BioRad thermocycler using a SYBR Green-based Pan-Flavi One-step quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay targeting the flavivirus *NS5* gene modified from Patel *et al*[7]. In each reaction, 2X QuantiTect SYBR® Green RT-PCR kit master mix (Qiagen, Germany, Catalogue No. 204245), 0.4 μM of each primer, 1X QuantiTect RT mix, RNase free H_2O in addition to 5 μL of RNA creating a total volume of 25 μL were included. The PCR amplification conditions were as following: reverse transcription at 50 ℃ for 30 min, initial activation at 95 ℃ for 15 min, followed by 45 cycles of denaturation at 94 ℃ for 15 sec, annealing at 60 ℃ for 30 sec and extension at 72 ℃ for 30 sec, and followed by a melting curve analysis.

Both samples from patients with and without dengue were included in the molecular testing to ensure there had been no misdiagnosis of the control patients. A blood/serum sample was considered positive if it had a cycle threshold value (Ct-value) below 35 with a correct melting temperature (Supplementary Table 1). The cut-off point was validated based on the protocol of Patel *et al*.[7] at Zoonosis Science Center in Uppsala University. The samples with Ct-value higher than 35 and lower than 45 were regarded as suspected positive. All positive and suspect positive samples were sent for Sanger sequencing (Macrogen, Amsterdam, Netherlands) for further confirmation and identification of serotype.

Sequencing results were analyzed by using Geneious Prime v.2019.2.1. The sequences of good quality were further searched in the NCBI Refseq virus database. DENV reference sequences were download from Genbank (https://www.ncbi.nlm.nih.gov/genbank/). Multiple sequence alignments were obtained by MAFFT v7.490, and the phylogenetic tree was reconstructed by MrBayes version 3.2.0. All computational calculations were performed using the computational resource UPPMAX from Uppsala University (https://www.uppmax.uu.se/).

Based on the RT-qPCR results, 333 out of 390 samples were negative. The Ct values of the 57 positive or suspected positive samples ranged from the lowest value of 23 to the highest value of 44, and the blood samples exhibited higher positivity than the serum samples. There were 23 positive samples with Ct values ≤ 35. The remaining 34 samples with Ct values ranging from 36 to 44 were regarded as suspected positive for DENV. To confirm the RT-qPCR results, the amplicons of 57 positive and suspected positive samples from 43 patients were sent for sequencing. The sequencing data confirmed DENV in 24 patients, all belonging to the group of patients with dengue. In 7 patients with dengue, we obtained sequencing results from both the serum and the blood sample (Figure 1A).

Phylogenetic analysis revealed three co-circulating serotypes during the study period, namely DENV-1, DENV-2, and DENV-4 (Figure 1B). Nineteen patients were infected by DENV-1, four patients had a DENV-2 infection, and only one patient was infected



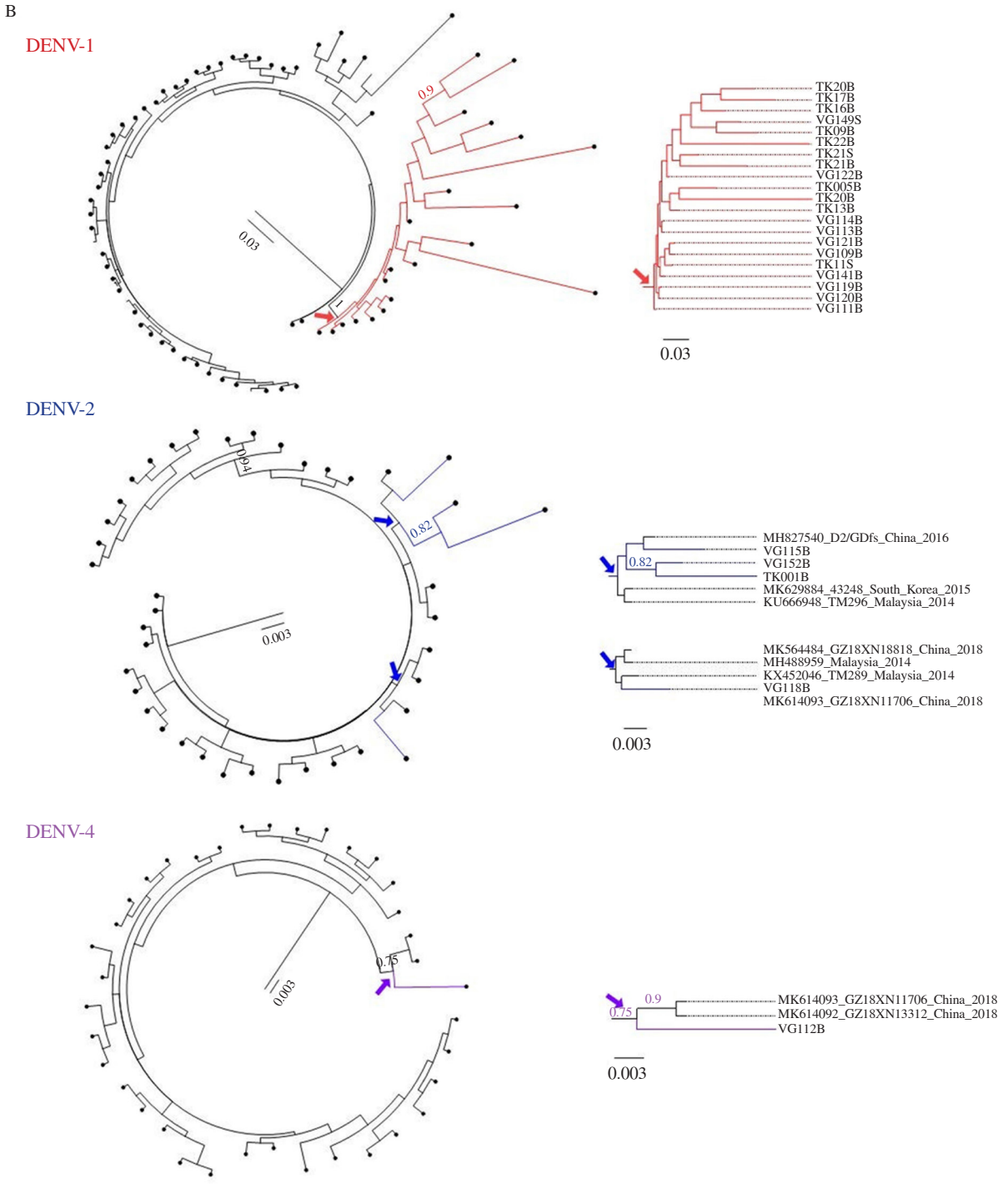


Figure 1. (A) Flowchart of study process and findings. (B) Bayesian phylogenetic trees of DENV based on partial NS5 sequences. Three serotypes DENV-1 (red), DENV-2 (blue) and DENV-4 (purple) co-circulate in Hanoi.

by DENV-4. Interestingly, two different strains of DENV-2 have been identified. TK001B, VG152B, and VG115B cluster together with the strain D2/GDfs, from Guangdong, China, 2016[8], while VG118B has a closer relationship with TM289, from Malaysia, 2014 (Genbank Accession No.KX452046). Both strains belong to DENV-2 Genotype [] Cosmopolitan according to Dengue Virus Typing Tool (https://www.genomedetective.com).

Our findings were consistent with the 2017 outbreak's characterization findings[4,5]. In our study, DENV-1 was the dominant serotype, consistent with global patterns, while DENV-4 was rare, detected in only one patient. It is noted that the distribution of circulating DENV serotypes can vary by region and season[9,10]. One explanation of our finding could be that DENV-1 was responsible for the outbreak in Hanoi city in 2018, patients with other DENV serotypes may have been infected elsewhere, or from a smaller cluster of people living in peripheral and peri-urban areas of Hanoi city. We have traced back the patients with dengue and confirmed that three out of four patients infected by DENV-2 and

the single patient infected by DENV-4 were living in peripheral and peri-urban districts. However, it must be awared that the number of samples were small, and it is possible that a larger sample size may also have found DENV-3 if this circulated at a low prevalence.

In conclusion, this study highlights the simultaneous circulation of three DENV serotypes during the 2018-2019 outbreak in Hanoi. These findings contribute to understanding the genetic diversity of circulating serotypes and provide valuable data for health policymakers to improve strategies for dengue prevention and control, particularly in Hanoi, the capital of Vietnam.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Authors' contributions

J.F.L and T.N.T: conceptualization; T.N.T, J.L, T.D.D and J.F.L: methodology; T.N.T, D.D.C, L.X.L: ethics; T.N.T, J.F.L and C.D.D: data collection supervisor; T.N.T, A.N.B, T.D.D, J.L and J.F.L: data analysis; T.N.T: writing original draft version; D.G, H.N.V, A.L and J.F.L: supervision; all the authors: review and editing.

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Supplementary Table 1. Melting temperature of different flaviviruses generated during the development of the Pan-Flavivirus RT-qPCR protocol.

Virus type	Melting temperature (℃)
West Nile virus	79.0
Zika	81.0
DENV1	79.0
DENV2	81.0
DENV3	80.5
DENV4	80.5
Japanese encephalitis virus	81.5
Yellow fever virus	81.5
Negative control (primer)	74.5