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Molecular detection and analysis of dengue virus genetic diversity in North Sulawesi, Indonesia during 2022

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Abstract. *Datu AM, Natzir R, Yustisia I, Wahid I, Soraya GV, Kadir S. 2023. Molecular detection and analysis of dengue virus genetic diversity in North Sulawesi, Indonesia during 2022. Biodiversitas 24: 3407-3413.* Dengue is a highly infectious disease caused by the dengue virus, consisting of four serotypes (DENV1-DENV4). The existence of diverse serotypes and genotypes in an area significantly impacts the degree of virulence in humans, clinical manifestation, and the epidemiology of the disease. Studying virus diversity in an area is pivotal, especially in dengue-endemic areas such as North Sulawesi. This study was an explorative research analyzing dengue virus serotypes and sequencing on the C-PrM protein region to determine the genetic diversity. This study was conducted on children and adults in 3 health centers in North Sulawesi, Indonesia, in 2022. Of 137 sera samples from febrile patients with dengue-like symptoms, ten were confirmed positive for dengue virus. Out of the ten positive samples, DENV1 was the dominant serotype, which was 70%. This was followed by 20% DENV2 and 10% DENV4. The genotype of each serotype, DENV1, DENV2, and DENV4, was respectively classified into genotype I, cosmopolitan genotype, and genotype I as a new genotype of DENV4 reported in North Sulawesi. This study provides the latest data on the serotypes and genotypes of dengue viruses in North Sulawesi. Molecular surveillance of the dengue virus in North Sulawesi must be continuously conducted due to the circulation of multiple serotypes and genotypes in this province.

Keywords: Dengue, genotype, sequencing, serotype

Abbreviations: C-PrM: Capsid-premembrane; DENV: Dengue Virus; RT PCR: Reverse Transcriptase-Polymerase Chain Reaction

INTRODUCTION

Dengue is a highly endemic infectious disease in tropical regions caused by the dengue virus transmitted by Aedes mosquitoes. The symptoms can range from mild fever to severe conditions like Dengue Hemorrhagic Fever (DHF). The dengue virus is classified into four serotypes (1, 2, 3, and 4) based on antigenic differences (Harapan et al. 2020). Each serotype has diverse genotypes with variations in geographical distribution, epidemic potential, and nucleotide sequences. Genotypes within each serotype consist of different variants, namely DENV1 (Genotypes I-V), DENV2 (Genotypes Asian I, Asian II, Cosmopolitan, American, Asian/American), DENV3 (Genotypes I-IV), and DENV4 (Genotypes I-IV) (Poltep et al. 2021). The circulation of different dengue viruses can impact the alternation of serotypes, genotypes, or clades in the endemic cycle and may lead to immune reactions between serotypes. The evolution of the dengue virus significantly influences its virulence in humans and dengue epidemiology. Infections with different serotypes can increase the risk of severe manifestations, such as DHF and Dengue Shock Syndrome (DSS) because of antibody-dependent enhancement (ADE) (Harapan et al. 2020).

Monitoring the circulation of diverse strains of the dengue virus is highly required as it may provide insight into the dynamics of the virus and guide the impact of specific serotypes/genotypes in local epidemics (Hamel et al. 2019). The phylogenetic analysis of various dengue virus isolates may provide an understanding of the evolutionary and migratory processes of dengue viruses, which in turn will provide a better understanding of the epidemiology of the disease (Drumond et al. 2016). Changes in each dengue serotype's nucleotide and protein sequences lead to genetic changes that increase genotypic diversity leading to endemic and epidemic (Khan et al. 2012). Data on the genotype diversity of the dengue virus in a region is fundamental in disease surveillance and epidemiology and valuable in vaccine development (Zeng et al. 2018).

Monitoring of the dengue virus in Indonesia primarily focuses on epidemiology, clinical aspects, vectors, and virological aspects. However, there is still a gap in genomic aspects (Yohan et al. 2018). Research conducted in Malaysia in 2018 revealed the association between specific serotypes and genotypes with distinct clinical manifestations. The DENV2 cosmopolitan genotype was significantly linked to severe dengue cases, while the DENV1 genotype

I was not associated with severe cases (Suppiah et al. 2018). A study carried out in Singapore between 2005 and 2011 showed that DENV1 infection was more severe compared to DENV2, possibly due to specific genotypes at the molecular level, such as DENV1 genotype I and DENV2 cosmopolitan genotype (Yung et al. 2015). During an outbreak in Nepal in 2010, DENV1 and DENV2 were predominantly responsible, with severe dengue fever mainly caused by secondary infection with DENV2 (Dumre et al. 2017). In Guangzhou, China, the invasion of DENV1 genotype I in 2006 resulted in a significant outbreak, while DENV1 genotype II was the primary genotype discovered in 2003. Another outbreak in 2013 introduced a new genotype known as genotype III (Ma et al. 2021). The high mutation rate of the dengue virus leads to genetic diversity, highlighting the significance of genomic data in virus management, including surveillance, pathogenesis, diagnostics, drug design, and vaccine development (Yohan et al. 2018).

In mid-2022, the Indonesian Ministry of Health reported that North Sulawesi had one of the highest Incidence Rates (IR) of Dengue Hemorrhagic Fever (DHF) among the ten provinces in Indonesia. The circulation of multiple serotypes in North Sulawesi is believed to contribute to the high number of cases. This study is an updated research on the circulation of dengue viruses in North Sulawesi. Conducted in 2022, the study aimed to investigate the diversity of serotypes and genetics of dengue viruses in North Sulawesi, covering all age groups. Due to limited research on variations of the dengue virus, there is a need to gather additional data on the variety of serotypes and genotypes in this region. This data is essential for early warning of dengue virus outbreaks, assisting in disease surveillance and epidemiology, and providing guidance for vaccination. The findings are expected to contribute to the appropriate and effective management of dengue outbreaks in North Sulawesi and Indonesia as a whole.

MATERIALS AND METHODS

Study design

This study was an exploratory study. The study population included residents in three dengue sentinel community health centers (Puskesmas) in the North Sulawesi Province, including Puskesmas Sario, Puskesmas Tuminting, and Puskesmas Bitung Barat. The Indonesian Ministry of Health designates sentinel health centers to conduct epidemiological surveillance in limited areas and populations in order to detect health problems in the broader area. Sera samples were collected from patients with symptoms of fever for 1-5 days, accompanied by other symptoms such as retro-orbital pain, joint pain, muscle pain, ulcer pain, vomiting, rash, shock/injury, and positive Ag NS1 test results. Collected serum was stored at -20ºC and immediately delivered to the BTKL PP Kelas I Manado Laboratory (no more than seven days) for further analysis. This research has obtained ethical approval. All eligible patients or legal guardians of patients have signed informed consent prior to sample collection. Serum samples delivered to the BTKL PP Kelas I Manado Laboratory, Indonesia, were immediately stored at -80ºC for extended storage. This sample collection was conducted based on the standard operating procedures of the Indonesian Ministry of Health's arboviruses surveillance. A total of 137 serum samples collected in this study were then analyzed using conventional RT PCR. The results indicated 10 positive samples and 127 negative samples. The ten positive samples were further characterized for their serotype and genotype. The workflow of sample collection and processing is depicted in Figure 1.

Virus isolation

The RNA of the dengue virus from the patient's serum was extracted using a Geneaid Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan) following the instructions. Furthermore, the obtained RNA was analyzed to determine the type of serotype of the sample.

Serotyping and amplification

Molecular analysis and serotyping were performed using the CFX96 Real-Time PCR machine (Bio-Rad, USA) and Superscript III One-Step RT-PCR Kit with Platinum Taq Lot No. 2327775 (Invitrogen, USA). The serotyping process was carried out with the conventional RT PCR method utilizing specific primers. Forward primer was D1 (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3') with a product size of 511 bp and the reverse primers were TS1 (5'-CGTCTCAGTGATCCGGGGG-3'), TS2 (5'-CGCCACAA GGGCCATGAACAG-3'), TS3 (5'-TAACATCATCATG AGACAGAGC-3'), and TS4 (5'-CTCTGTTGTCTTAAA CAAGAGA-3'), with product size of 482 bp, 119 bp, 290 bp, and 392 bp, respectively. The analysis was performed based on Lanciotti et al. for each of the four dengue virus serotypes (Lanciotti et al. 2005). Conventional RT-PCR protocol was performed at 48ºC for 45 minutes and 94ºC for 2 minutes, followed by 40 cycles of 94ºC for 15 seconds, 56ºC for 30 seconds, 68ºC for 1 minute, and 68ºC for 5 minutes and hold at 4ºC.

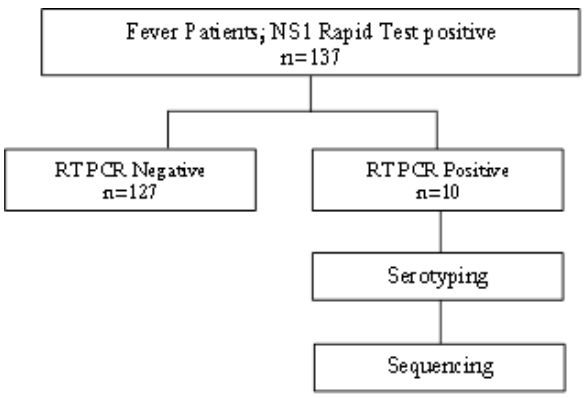

Figure 1. The workflow of sample collection and processing

Genome sequencing

Genome sequencing was performed by the Sanger sequencing method on the amplification products of the C-PrM protein region. This procedure uses specific primers DENV1-DENV4 according to the Lanciotti method. Genome sequencing was performed at the first BASE Laboratory.

Phylogenetic analysis

Dengue virus sequences, along with reference genomes of all genotypes, were downloaded from NCBI (https://www.ncbi.nlm.nih.gov). Representative genotypes of each DENV serotype were taken from several previous studies, and isolates from this study were compared to several isolates from Indonesia, several Asian countries, and other dengue-endemic countries. Sequence alignment was performed on MEGA 11 program. A phylogenetic tree was constructed based on the maximum likelihood method employing the model selection function of MEGA 11 program with a bootstrap test (1000 replicates). Evolution was calculated utilizing the Tamura-Nei model (Mo et al. 2018; Du et al. 2021; Umair et al. 2023).

Ethics approval

This study has been approved by the Research Ethics Committee of Universitas Hasanuddin, Wahidin Sudirohusodo Hospital Makassar (392/UN4.6.4.5.31/PP36/ 2022), Indonesia. All eligible patients or legal guardians of patients have signed informed consent prior to sample collection.

RESULTS AND DISCUSSION

Serotyping and amplification

Serotyping process showed ten positive samples. The diversity of serotypes and genotypes leads to multiple immune responses and different abilities in infecting target cells, consequently leading to varying dengue disease severity. Serotype analysis of the dengue virus is crucial in order to facilitate the development of appropriate epidemiological control measures for dengue virus transmission. Moreover, molecular detection of the dengue virus RNA is one of the most sensitive and rapid techniques in dengue virus analysis (Chien et al. 2006; Sasmono et al. 2014). Conventional RT PCR uses specific primers which bind in the particular regions of the dengue virus, and thus minimizing false-negative results caused by spontaneous mutations during viral RNA replication. The sensitivity of the conventional RT PCR method is 48.4-100%, and the specificity is 100% (Tang and Ooi 2012).

In this study, DENV1 was detected as the most dominant serotype (70%), followed by DENV2 (20%) and DENV4 (10%) (Table 1). On the other hand, DENV3 serotype was not identified in this study. In contrast, another study conducted in North Sulawesi in 2019 reported that serotype 3 was the dominant serotype detected during the outbreak in that province, followed by serotype 2, serotype 4, and serotype 1, which occurred in early 2019 (Tatura et al. 2021). This could be attributed to differences in timing, and the objects sampled, where the samples analyzed in 2019 were obtained from the serum of DHF pediatric patients at Prof. Dr. Kandouw Hospital in North Sulawesi. Samples used in this study were collected from DHF suspects in children and adults in 3 sentinel health centers in North Sulawesi. In addition, in many populations, the annual transmission of DENV occurred periodically, which results in outbreaks every 3-5 years (WHO 2009). The occurrence of this serotype shift can serve as an early warning of a potential outbreak in North Sulawesi.

Clinical symptom characteristics were more commonly identified in DENV2, where most clinical symptoms, such as fever accompanied by retro-orbital pain, joint pain, muscle pain, ulcer pain, vomiting, and rash, were observed in both confirmed DENV2 samples. DENV4 was the serotype with the mildest clinical symptoms, presenting only as fever. DENV1 also exhibited various symptoms, including fever along with joint pain, muscle pain, and rash. Clinical symptoms of shock or hemorrhage were not found in the positively confirmed patients (Table 2).

Table 1. Serotyping results of serum samples of dengue suspect patients from 3 sentinel health centers. The + indicates the positive result and the blank column indicates the negative result

Sample ID	Age (YO)	Conventional RT PCR results		
		DENV1	DENV2	DENV4
MND004	4	+		
MND020	8	+		
MND030	3	+		
MND034	10	+		
MND035	11			+
MND042	11	+		
MND050	9		+	
MND052	3	+		
MND102	11	+		
MND119	1		+	

Table 2. Clinical characteristics of dengue-positive patients

Characteristics of positive sample	Dengue positive (n=10)		
	DENV1 (n=7) (%)	DENV2 (n=2) (%)	DENV4 (n=1) (%)
AgNS1 positive test	57	50	100
Fever	100	100	100
Retro-orbital pain	-	50	-
Joint pain	29	50	-
Muscle pain	29	50	-
Ulcer pain	-	50	-
Vomiting	-	50	-
Rash	14	50	-
Shock/injury	-	-	-

All positive samples in this study were detected from patients between the ages of toddlers and adolescents. This result showed a similar result to previous studies that have reported that dengue cases are more highly detected in children than adults (Utama et al. 2019). A study conducted in Jakarta from 2009 to 2010 suggested that a single DENV4 infection resulted in dengue fever, while mixed infections of DENV4 with other serotypes caused more severe clinical symptoms (Dewi et al. 2014). The severity of dengue fever during the outbreak in Nepal in 2022 was predominantly caused by DENV2 infection, which commonly occurred in the male and pediatric populations (Rimal et al. 2023).

Electrophoresis results on agarose gel showed that DENV1-DENV4 were amplified with a length of 482 bp, 119 bp, 290 bp, and 392 bp, respectively. This is in accordance with the size of the amplification products of the primers used. Representation of serotyping and amplification results visualized on gel electrophoresis can be seen in Figure 2.

Phylogenetics and genetic diversity

Genotype grouping was performed and compared based on genotype data obtained from previous studies. Based on phylogenetic analysis, each serotype in this study was grouped as follows: (i) The seven DENV1 samples (MND004, MND020, MND030, MND034, MND042, MND052, MND102) were grouped into genotype I (Figure 3), (ii) DENV2 samples (MND050, MND119) were grouped into cosmopolitan genotypes (Figure 4), and (iii) DENV4 samples (MND035) were grouped into genotype I (Figure 5).

Each serotype of the dengue virus has antigenic and genetic differences, but they share similarities in terms of epidemiology, leading to similar symptoms upon infection in humans. Furthermore, each serotype of the dengue virus comprises several genotypes (Drumond et al. 2016), with each serotype having 3-5 distinct genotypes classified based on genetic divergence. Genotypes are commonly separated according to geographic locations. Phylogenetic analysis enables the monitoring of the spread or increased diversity of dengue virus genotypes, as well as the tracking of genotype shifts and changes in virulence within a region (Hamel et al. 2019). Additionally, epidemiological and molecular studies of the dengue virus can provide information on the extinction of genotypic and sub-genotypic lineages during periods of epidemiological and endemic transmission, although the precise driving factors behind such shifts are not yet fully understood (Santiago et al. 2019). Phylogenetic analysis conducted on dengue viruses can provide insights into the evolutionary processes and migration patterns of the dengue virus. Through phylogenetic analysis, a better understanding of dengue virus epidemiology can be achieved (Drumond et al. 2016). The results of a study undertaken in Surabaya in 2018 showed that DENV1 was grouped into genotypes I and IV (Yohan et al. 2018). Similar findings were also observed in a study carried out in Makassar from 2007 to 2010, where genotypes I and IV were identified for DENV1 in that region. The study also classified genotypes for each of DENV2, DENV3, and DENV4 into cosmopolitan, genotype I, and genotype II, respectively (Sasmono et al. 2015).

In general, the circulating genotypes in Indonesia for DENV1 consist of genotypes I and IV, the cosmopolitan genotype for DENV2, and genotype II for DENV4 (Herman et al. 2016). The DENV4 genotype I that we detected in this study differed from the commonly distributed DENV4 genotype in Indonesia; this genotype is circulating in neighboring countries such as Malaysia, Thailand, Cambodia, Sri Lanka, and the Philippines. This genotype is commonly observed in Asian countries, particularly in Southeast Asia (Harapan et al. 2020).

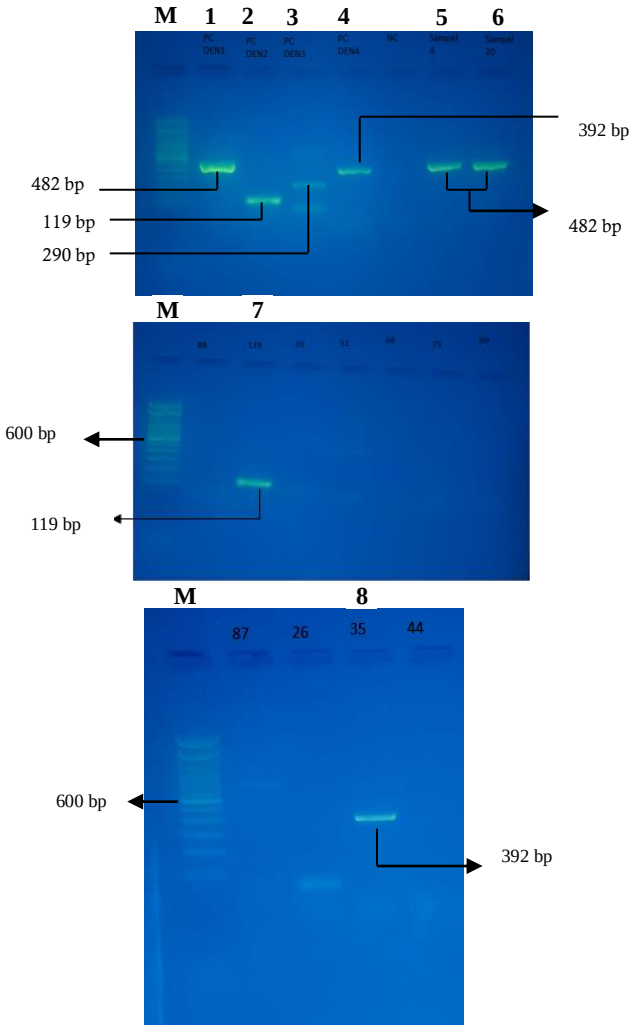

Figure 2. Representation of electrophoresis results of serotyping and amplification processes. Note: 1. Positive DENV1 Control (482 bp), 2. Positive DENV2 control (119 bp), 3. Positive DENV3 Control (290 bp), 4. Positive DENV4 Control (392 bp), 5. Sample MND004 DENV1 Positive (482 bp), 6. Sample MND020 DENV1 Positive (482 bp), 7. Sample MND035 DENV4 Positive (392 bp), 8. Sample MND119 DENV2 Positive (119 bp)

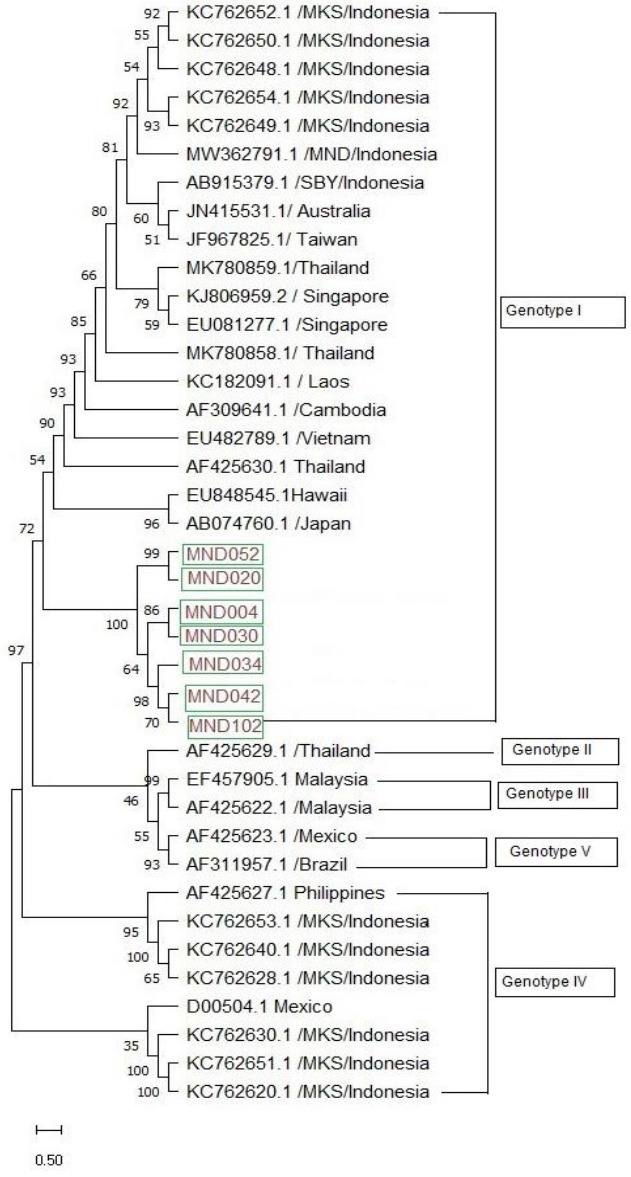

Figure 3. DENV1 Phylogenetic Tree of Samples along with DENV1 Sequences from NCBI website. The phylogenetic tree was constructed with the maximum likelihood tree model. The number of nodes indicates bootstrap values. Research sample codes, namely: MND004, MND020, MND030, MND034, MND042, MND052, and MND102

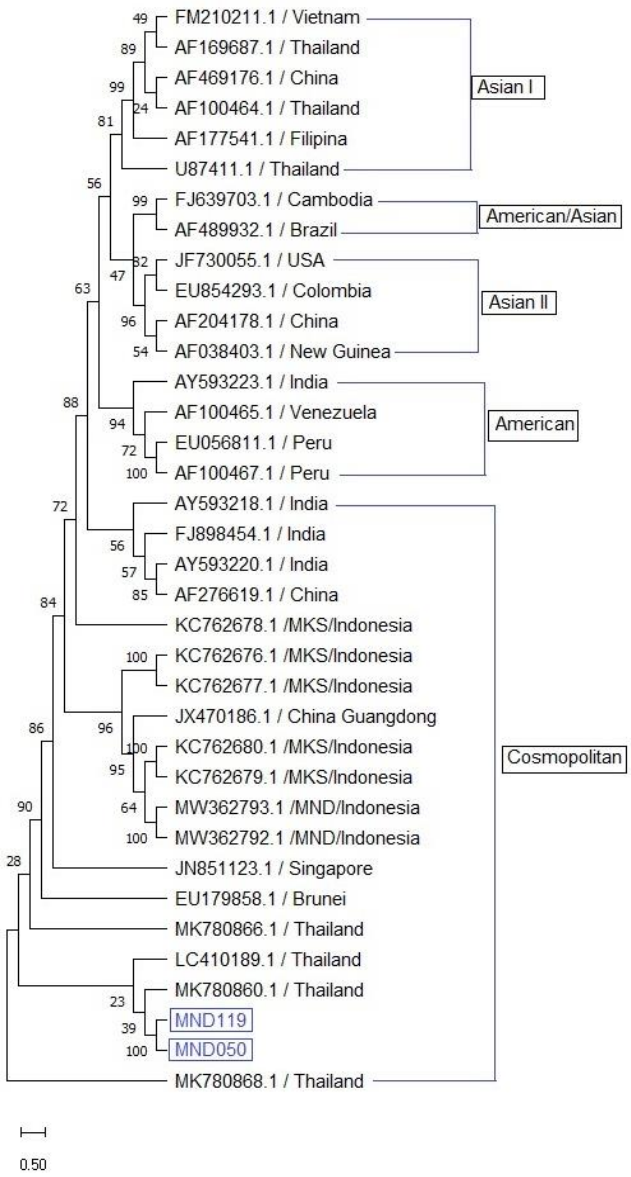

Figure 4. DENV2 Phylogenetic Tree of Samples along with DENV2 Sequences from NCBI website. The phylogenetic tree was constructed with the maximum likelihood tree model. The number of nodes indicates bootstrap values. Research sample codes, namely: MND050 and MND119

The invasion of this genotype into North Sulawesi is entirely possible, considering the geographical location of North Sulawesi bordering the Philippines. The dengue virus spreads rapidly across different regions, regardless of large geographic distances. The introduction of new serotypes and genotypes in a region holds significant importance and requires comprehensive analysis. Such introductions often result in a shift in the dominant circulating virus strain within that particular region (Holmes and Burch 2000). A study conducted in Guangzhou, China, spanning from 2003 to 2013, demonstrated that the invasion of different DENV1 genotypes led to an outbreak (Ma et al. 2021). Despite studies suggesting that DENV4 may exhibit milder clinical symptoms, the circulation of DENV4 with various serotypes can still cause severe illness and outbreaks, as observed in India in 2007. The presence of DENV3 and DENV4 was detected during the outbreak, with DENV4 genotype I being the prevailing type (Neeraja et al. 2013).

The high mutation rate in dengue viruses leads to significant genetic changes, including in virus serotypes and genotypes. The accumulation of these mutations can result in wider genetic variations in the dengue virus population. Studies on the genetic evolution of DENV have shown that the average mutation rate of DENV is relatively higher than other RNA viruses. Phylogenetic analysis of DENV1 during the outbreak in Vietnam in 2017 revealed complex genetic variations. The high mutations in the virus genome were mentioned as the cause of the outbreak in that region in 2017 (Dang et al. 2020). Infection of *A. aegypti* by different viruses during outbreaks and epidemics leads to high levels of viral replication. This phenomenon could lead to genetic diversity in the dengue virus, which has serious consequences such as increasing virulence and pathogenic properties (Bona et al. 2012). DHF and DSS cases are severe in dengue virus infection, most of which are caused by secondary infection by heterologous viral serotypes or primary infection with highly virulent genotypes. Some genotypes are reported to have a high level of viremia and greater transmissibility, which may lead to tremendous epidemic potential (Dash et al. 2004).

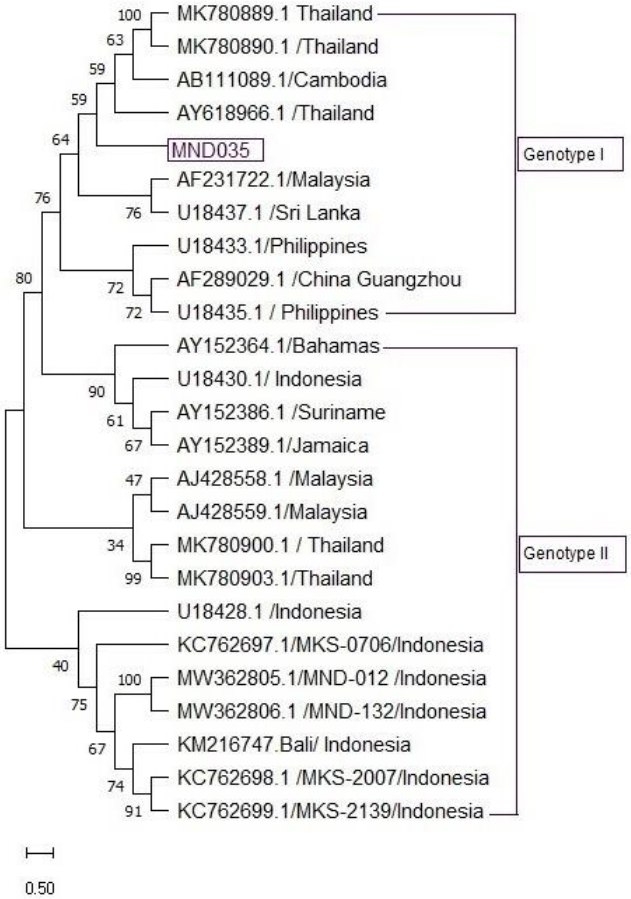

Figure 5. DENV4 Phylogenetic Tree of Samples along with DENV4 Sequences from NCBI website. The phylogenetic tree was constructed with the maximum likelihood tree model. The number of nodes indicates bootstrap values. Research sample code, namely: MND035

In summary, three types of dengue virus serotypes were found in this study, which consisted of DENV1 in 7 samples, DENV2 in 2 samples, and DENV4 in 1 sample. Interestingly, there was no DENV3 detected. Genotype types DENV1, DENV2, and DENV4 are respectively categorized as genotype I, cosmopolitan, and genotype I. Since all four dengue virus serotypes and their corresponding genotypes are circulating in North Sulawesi, monitoring changes in serotype circulation and dominant genotypes is crucial. The circulation changes of the dengue virus may lead to dengue outbreaks. The population used in this study may not yet be representative of the entire population in North Sulawesi. Still, this data is precious regarding disease control and early detection of dengue fever outbreaks in this province. This data can also serve as a foundation for the government regarding vaccination. Continuous surveillance of the dengue virus circulation may assist in predicting the occurrence of dengue cases. Active surveillance, epidemiological investigation, mosquito control, and improvement of public health infrastructure contribute to reducing outbreaks.

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