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# Genotype Variants Of Dengue Virus On Dengue Hemorrhagic Fever (Dhf) Suspect: Cross Sectional Study In Health Facilities In Semarang City, Indonesia

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***Abstract: Semarang has been reported as an endemic area of dengue hemorrhagic fever (DHF), yet continuous data of molecular epidemiology related to dengue fever is not available. The aim of this study was to analyze the characteristics of virus strains, host suspected dengue, and the dynamics of dengue fever in Semarang City. The design of this study was observational description with cross-sectional design. Seventy three DHF suspected patients from two hospitals and seven health centers in Semarang City were sampled during 2017- 2018. Reverse Transcription Polymerase Chain Reaction (RT-PCR) method was used to examine serum suspected dengue and sequence serotypes and genotypes of dengue virus. The results showed that the highest frequency to be exposed by DHF was infant and children; while, the number of male and female with suspected dengue was relatively the same. The dominant serotype was DENV-3, DENV-1, and mix of DENV-2 and DENV-3; while, the circulation of DENV-1 genotype I and DENV-3 genotype I were identified as Indonesian local endemic strain causing epidemics in Indonesia.***

***Keywords: RT-PCR, dengue hemorrhagic fever, serotype, genotype***

## 1. INTRODUCTION

As an arthropod-borne disease, dengue hemorrhagic fever (DHF) has caused health problems to Indonesian. DHF is triggered by the bite of the Aedes sp mosquito infected by dengue virus that further results in clinical manifestations ranging from mild dengue (DD) to more severe, Dengue Hemorrhagic Fever (DHF), and Dengue Shock Syndrome (DSS).[1,2]

Semarang City has been categorized as an endemic area of DHF, as dengue cases are to be found every year. Previous studies has identified a diversity of dengue virus serotypes that dominate in particular infected areas.[3] Molecular surveillance in Semarang has proved the existence of all serotypes with DENV-3 as the dominant one.[4,5] During 2011 to 2012, a study by Fahri et al. identified the circulation of DENV-2 and DENV-3. In 2017, Hiayati, et al. revealed that from 54 serum samples of patients with suspected DHF, 22.2% tested to be positive of being exposed by dengue virus infection, of which 91.7% were infected by DENV-3 strains and 8.3% by DENV-1.[6]

Virology studies have identified the possibility that genetic evolution of viruses have been in progress and produced more virulent variants (epidemic strains). In this stage, variations in immunogenic properties or viruses' virulence might spread throughout Indonesia, and changes in virulent properties will affect clinical pattern of virus infection following viral serotypes and genotypes. Therefore, a study that put emphasis on molecular characteristic suspects needs to be carried out by conducting continuous surveillance on the serotype of dengue virus and the genomics of DENV in order to determine dengue potential pathogenesis in the population from epidemiology perspective.

At present, detailed data on the continuous molecular epidemiology of DHF in the city of Semarang are not available; in fact, changes in serotype and genotype of the dengue virus in an area always occur. Having been identified the strain of the dengue virus, management and strategy of controlling dengue disease in endemic areas, especially the city of Semarang, might be set up and developed.

## 2. RESEARCH METHODS

This study was an observational descriptive with cross sectional design. A 73 dengue suspected patient from several hospitals and health centers in Semarang City was sampled during 2017-2018. Serum taken from patients was conducted in the following stages:

*Patient Screening*

The dengue suspected patients were determined based on WHO 2009 criteria stated that a patient is found to have a sudden fever that continuously happen for three days or more accompanied by one or more bleeding manifestations, such as positive tourniquet test, appearance of spots (petechial), bruising (ecchymosis), rash (puerperal), mucosal bleeding (gum bleeding), nosebleeds, and shock (weak and fast pulse). Patients were screened by anamnesis and clinical examination was conducted by physicians in the hospitals and health centers. Patients participated in this study were those who had received informed consent.

*Serum collection*

The research specimen was collected from a part of serum used for routine examination of DHF suspected patients in the hospitals and health centers. Serum was taken from venous blood by hospital laboratory personnel as many as 5 ml for adult and 3 ml for children. From this amount, 2 -3 ml was used for routine laboratory examinations, and 1 – 2 ml was used for this study. The blood was stored in tubes and they were left for 15 minutes at room temperature, then they were stored in a refrigerator at $4^0$C for 20 minutes. Next, they were centrifuged at the speed of 1500 rpm for 15 minutes at room temperature. The serum produced was around 500 – 1000 µl was stored at $-20^0$C until examination.

*RT-PCR examination*

Examination by Real Time Polymerase Chain Reaction (RT-PCR) was carried out at the Laboratory of the Institute of Tropical Disease Surabaya. Isolation of Viral RNA was conducted using RNA isolation kits (Qiagen). Copies of cDNA from capsid and prM were obtained by amplification using primers (TS1, TS2, TS3, and TS4) that stick to the four types of dengue serotypes. After that, a second amplification with a specific primer (TS1, TS2, TS3, TS4, and D1) was carried out for each serotype. The results of the second amplification were analyzed using 1% agarose gel stained with ethidium bromide. The interpretation of the results of electrophoresis is DENV-1 if the DNA band size is 482 bp, DENV-2 if the DNA band size is 119 bp, DENV-3 if the DNA band size is 290 bp, and DENV-4 if the DNA band size is 392 bp.[7]

*Sequencing of the Dengue Virus*

The DNA sequencing reaction was carried out by Dig Dye Dideoxy Terminator Sequencing Kit and the product was analyzed using a DNA sequencer ABI 3500xL Genetic Analyzer. DNA sequencer 3500xL, using 3500 series data collection software 3 (Applied Bio system), could be used to determine nucleotide sequences of region envelope that could reach nucleotide lengths up to 1400 nucleotide bases. The determination of the nucleotide sequence of region envelope used 2 pairs of primary sense and antisense. The expanding sequences to map nucleotide base arrangements was conducted using DNASIS program.

*Dengue Virus Genotype Analysis*

The sequence results were carried out by multiple alignments on various sequences of DENV-2 referents for DENV-2 serotypes and so for DENV-3. All data sequences carried out in this study were accessed from Gen Bank. Sequences that had been multiple alignments with the Clustal X program were phylogenetic analyzed. PAUP software version 4.0 was used to determine the phylogenetic tree. Based on the phylogenetic tree, the genotype pattern of each serotype was obtained.

## 3. RESULTS

This study analyzed 73 DHF suspected samples visiting hospitals and health centers in Semarang city; Semarang is an endemic area of DHF and outbreaks often occur. Respondents were diagnosed as suspected DHF and were being treated at a hospital or health center in the city of Semarang. Male respondents (54.8%) and women (46.2%) were comparable and distributed almost evenly to age group. Meanwhile, based on clinical conditions, the majority of the dengue suspected patients had thrombocyte levels of ≥100,000 cells/$mm^3$, which were equal to 80.8% (Table 1).

Table 1. Characteristics of Dengue Suspects in the City Of Semarang

| **Characteristic (n=73)** | **n** | **Percentage (%)** |
|---|---|---|
| **Gender** | | |
| Male | 40 | 54.8 |
| Female | 33 | 45.2 |
| **Age** | | |
| Toddler (0-5 years) | 13 | 17.8 |
| Childhood (5-11 years) | 11 | 15.1 |
| Adolescent (12-25 years) | 21 | 28.8 |
| Adult (26-45 years) | **20** | **27.4** |
| Elderly (46-65) | 8 | 10.9 |
| **Thrombosis Level** | | |
| <100.000 cell/$mm^3$ | 14 | 19.2 |
| ≥100.000 cell/$mm^3$ | 59 | 80.8 |
| **Result of PCR** | | |
| Positive | 25 | 34.2 |
| Negative | 48 | 65.8 |
| **Virus Strain (n=25)** | | |
| DENV-1 | 3 | 12.0 |
| DENV-2 | 0 | 0.0 |
| DENV-3 | 20 | 80.0 |
| DENV-4 | 0 | 0.0 |

| | | |
|---|---|---|
| Mix DENV-2, DENV-3 | 2 | 8.0 |

RT-PCR was applied to analyzed 73 samples whether the sample was infected with a dengue virus or other virus. Serotyping examination in dengue cases with RT-PCR was confirmed by standard serotyping of RT-PCR method of Lanciotti et al., (1992). [7] Among 25 samples examined with PCR and identified of being positive to be infected to dengue virus infection, the dominant strain of the dengue virus was DENV-3, which was found in 20 samples (80%); while, 3 samples (12.0%) were exposed to DENV-1 virus strain, and 2 samples (8.0%) were exposed to Mix of DENV-2 + DENV-3.

*Dengue Virus Genotype in Suspected Dengue Patients in Semarang City*

After being examined with PCR sequencing, 7 samples were identified to be positive with flavivirus primers, and 3 samples with primary envelope. The 10 samples then were sequenced to see the base composition of the dengue virus. There were 3 samples detected to have a long base arrangement that its appearance was influenced by various factors, such the accuracy of annealing temperature during PCR sequencing, viral titter, and contamination. These samples (1 sample of DENV-1 and 2 samples of DENV-3) detected were analyzed by phylogenetic trees.

The phylogenetic analysis was carried out by selecting the 30 most closely related DENV NS5 gene sequences from each dengue sample to be the reference of the genotypes classification. The phylogenetic tree produced for grouping the genotypes is explained in Figure 1. Based on the classification of the DENV-1 genotype by Goncalvez et al., observation upon the circulation of genotype I in Semarang City was conducted. The samples of SEM 55 isolates genotype I together with viruses from Cambodia, China, and Singapore were group.

The grouping of samples of SEM 55 isolates genotype I along with viruses from Cambodia, China, and Singapore.

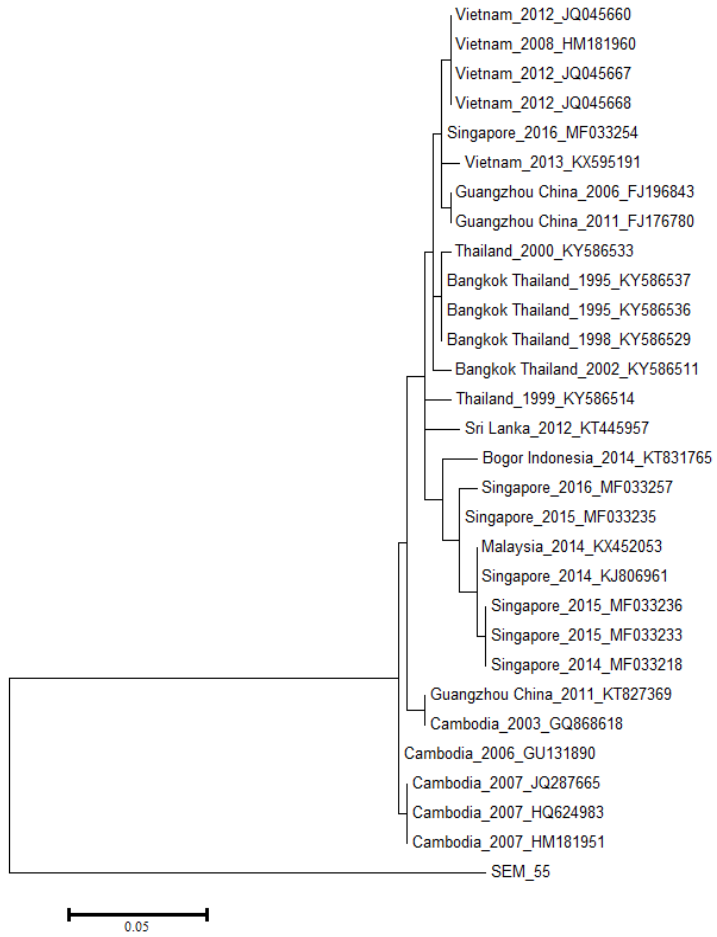


Figure 1. Phylogenetic Trees of NS5 Gen Samples SEM-55 DENV-1 Genotype I

The SEM 55 samples clustered, thus they probably had a similar bloodline to the sample from Cambodia. Meanwhile, the genotype I samples of Semarang were group together with viruses from Vietnam (in 2008, 2012, 2013), Singapore (in 2014, 2015, 2016), China (in 2006, 2011), Thailand (in 1995, 1998, 1999, 2000, 2002), Sri Lanka (in 2012), Malaysia (in 2014), Bogor City of Indonesia (in 2014), and isolation of the Cambodia's DHF outbreak in 2003, 2006, 2007.


Figure 2. Phylogenetic Genes of NS5 Gen Samples SEM-58 and SEM-59 DENV-3 Genotype

The phylogenetic analysis was conducted on DENV-3 virus genome 2 isolated from Semarang and compared it with DENV-3 genome available in Gen Bank, as shown in Figure 2. Being phylogenetic analysis, 2 DENV-3 genomes from Semarang was identified as genotype I according to the Lanciotti et al. classification, then 2 isolates of Semarang were grouped with DENV-3 isolates from other countries. The isolates were most closely related to isolates of Makassar Indonesia in 2008.

## 4. DISCUSSION

The sample of this study was DHF suspected patients visiting hospital and health center in the city of Semarang. Generally, suspects visiting hospital and health center for having a treatment during 2-3 days of clinical period of high fever. Medical personnel diagnosed the clinical fever suspects and advised them to carry out laboratory tests.

The incident was identified to occur more on male belong to toddlers and children; although, some studies showed female were more often to suffer from DHF than male were. Some researchers stated that women are more likely to be easily infected by the dengue virus, as the risk of women being more easily infected by dengue virus is associated with capillary walls in women more likely than that in men.[8] .

Based on the age group, 32.9% of sample characteristics belonged to toddler and children. Age is one of the characteristics of hosts that can affect individuals' health, as

exposure to risk factors increases.[9] Yanuar's finding showed that respondents with <5 years of age are more than 14 years.[10] Toddler is the age group susceptible to DHF because of low immunity, factors in nap habits, and exposure to mosquito bites.[11]

Specific antibody formation to antigens affects body's immunity against viral infections . Thus, DHF incidence found in 0-5 years' age group is due to the immune response to specificity and immunological memory stored in dendritic cells and imperfect lymph nodes. In addition, the function of macrophages and the formation of specific antibodies against certain antigens are still lacking. As a result, the secretion of cytokines by macrophages due to viral infections is lack as well leading to the lack of interferon production (IFN) that functions to inhibit viral replication and prevents the spread of infection to unexposed cells.[10]

Thrombocyte level was one of the criteria used to determine the classification of dengue fever. Pathophysiological theory categorized dengue suspected patients, patients suspected of having dengue infection, into DD, DHF, or DSS. In dengue fever infection, dengue suspected patients have possibility to be accompanied with or without bleeding that is directly related to thrombocyte levels.[12]

The results of this study indicated that most respondents had thrombocyte levels of ≥100,000 cells/$mm^3$. Similarly, this finding was in accordance with the research conducted at Roemani Hospital in Semarang that more than half DHF patients has thrombocyte levels of ≥100,000 cells/mm3 which is equal to 58.1%.[13] On the other hand, a study conducted in General Hospital Dr. M. Djamil Padang showed that 100% patients suffer from thrombocytopenia[14]; while, in Semarang City, 79.6% DHF suspected patients suffer from thrombocytopenia.[6]

The results of those studies were probably caused by the thrombocyte examination was conducted during the fever period. The value of thrombocyte begins to decrease from the normal value (150,000/mm3 – 450,000/mm3) during the fever period and reach the lowest point in shock period.[15] The level of declining in thrombocyte value varies in various clinical degree of DHF.[16] This finding is in accordance with the theory of thrombocyte levels from WHO that the initial examination of thrombocyte on the 1st to 3rd fever day, thrombocyte levels are still relatively normal.[17] The pattern of the number of thrombocyte in patients was reported to be starting to decrease at the beginning of the fever period, which was in the 3rd day of the illness. However, the number of thrombocyte that are still within the normal limits before the 3rd day of the fever cannot be used to rule out the possibility to be exposed of DHF.[18]

*Serotypes and Genotypes of Dengue Virus*

Four serotypes of dengue virus have been found to circulate in Indonesia.[19] Research conducted in several regions in Indonesia during 1994 – 2012 have identified all types of dengue virus serotypes, of which some regions have been dominated by DENV-3 serotypes.[20] The most dominant serotypes is different in every city; however, recent study showed that the most dominant serotype circulated are DENV-1, DENV-2, and DENV-3, while the least one is DENV-4.[21] The dominant serotypes could vary according to time and place that caused by the nature of the dengue virus infection. A person who has been infected with one of the serotypes will be immune for life to that serotype, but is still vulnerable to other serotypes.[3]

Different dominance of serotypes according to time proved to occur in Semarang city, in which DENV-3, according to studies in 2007 and 2017, has been identified to be the dominance. [6,5] However, different finding from a research conducted in 2012 showed that the distribution of serotypes in the city of Semarang is dominated by DENV-1 by 35.5%, followed by mix infection (29%), DENV-2 and DENV-3 (12.9%), and DENV-4 (9.7%) as the least.[22] In this research, DENV-3 was the highest serotypes found in Semarang city. The

DENV-3 serotype was reported to affect severe clinical symptoms that often cause death; thus, the most contagious was DENV-3 and the least one was DENV-1.

The implications was that the population of Semarang city might in danger of being infected by the widespread of DENV-3, as this serotype was one of the most infectious ones due to its nature of viral infection. Clinically, DENV-3 is the dengue virus serotype that mostly occurs without showing its clinical manifestations of rash, but severe cases of the infection virus occur.[8] The appearance of DENV-3 that has spread out in many regions in Indonesia potentially cause secondary heterotypic infections leading to more severe symptoms. [23]

At present, DENV-2 and DENV-3 serotypes originating from Asia have been associated with epidemics and severe dengue infection. Recent study has found the primary target cells (dendritic cells) of humans in mosquitoes; as a result, DENV-2 and DENV-3 serotypes produce higher viral titers compared to other serotypes. However, the ability of some dengue virus serotypes to have a higher replication compared to that of other serotypes had not been conclusive and understood. Therefore, it was necessary to monitor the transmission of dengue virus serotypes that have highly potential virulence. The purpose is to determine the influence of host genetic factors and immune status that is currently known to be related to the host's ability to resist virus attacks.[24,35]

Genotype is subtypes of serotypes based on viral envelope of gene sequences. Genotypes may cause epidemics in a particular region. The genotype division can be used to determine the origin of the virus that causes epidemic. The gene sequence can be conducted by nucleotide sequencing process; a technique used to determine the nucleotide sequence directly from a DNA fragment.[26] The DENV sequencing of nucleotides isolated from different locations have provided several variations in genetic diversity.

The sequence of nucleotide can be seen from phylogenetic tree, which is a two-dimensional graph, showing the relationship between organisms or more specifically the gene sequences of viruses.[27] Phylogenetic studies proved that DENV can move both long distance between continents and short distance between neighboring countries.[28,28] In addition, the theory in phylogenetic studies shows the relationship between genotype and disease severity. Genetic analysis of each strain is also thought to provide variation in viral virulence.[30]

Research on dengue virus genotypes carried out in various countries and regions in Indonesia showed a diversity of different genotypes. Phylogenetic studies using full genome sequencing method carried out in Surabaya proved the existence of genotypes I and IV on DENV-1. The result of a study conducted in Surabaya suggested a more recent introduction of genotype I virus compared to genotype IV, which was more endemic.[31] In 2012, DENV-2 cosmopolitan genotypes and DENV-3 genotype I was identified in Semarang City.[22] Meanwhile, the circulation of genotype I and genotype III on DENV-3 was identified in Colombia.[22] In contrast, the findings in Malaysia showed that DENV-3 was grouped into genotypes II and I, whose its isolates was similar to Thailand's from 1962 to 1987.[29]

Following the classification of Goncalvez, DENV-1 was analyzed by observing the presence of genotype I circulating in the area of Semarang City.[32] Geographically, the closest regions reported to store genotype I are Singapore, Thailand, and Surabaya.[36,37,38] in this study, it was found that the isolates were closely resemble to viruses from Cambodia, China and Singapore. This was consistent with the theory of phylogenetic studies that DENV can travel long distances between continents and short distances between neighboring countries.[28,29]

The detection of genotype I in DENV-1 in Semarang City in 2018 was different from the findings of genotypes I and II in Semarang City in 2012. The study in 2018 concluded that genotype I has been circulating and a shifting from genotype II to genotype I, which is more

virulent, is possible to happen.[22] In addition, phylogenetic analysis concluded that DENV-3 isolates from Semarang City were also grouped in genotype I.

This finding was similar to the one of previous study in Semarang City in 2012.[22] the isolates found was similar to the ones from Makassar in 2008 and Philippines in 1964 1955, and genotype was the same as the Indonesian strains (Sumatra, Jakarta, Makassar) from 1998, 2004, 2008, and 2010. This genotype had been described as the cause of four epidemics in the region in the past. This grouping shows the endemic genotype of the DENV-3 in Indonesia, and the strong temporal relationship among the DENV-3 viruses in Indonesia. This virus strain is most likely a local Indonesian endemic strain that has been circulating for more than three decades.[34]

This study contributed to new information about genotypic distribution in DENV-1 and DENV-3 in Semarang City and the implications of the genotypes distribution that was closely related to the causes of epidemics causing fatal cases of dengue. The changing of dengue virus genotypes in Semarang City meant that the malignant strains could replicate and be transmitted faster to humans, outperforming viruses that are less malignant and causing more cases of disease; thus, ecologically, they could replace low severity strains of dengue fever.[36]

## 5. CONCLUSION

The characteristic of suspected DHF shows that the number of suspected DHF in male and in female as well as age under five and children are not much different, most have thrombocyte level of ≥100,000 cells/mm$^3$, because many respondents come to visit the hospital when entering the initial clinical period.

The predominant type of dengue virus serotype that infects suspected dengue is Den-3. The types of dengue virus genotypes detected from the patients of the dengue suspected isolates in Semarang City are DENV-1 genotype 1 and DENV-3 genotype 1; genotypes often found in the territory of Indonesia.

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