

Vascular Endothelial Growth Factor Levels in Single- and Multiple-Serotype Dengue Virus Infections

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Vascular endothelial growth factor (VEGF) is recognized as a cytokine that is generated by endothelial cells. It contributes to inflammation and increased vascular permeability. VEGF has been correlated with disease severity, plasma leakage, and outcome in dengue. Serotype detection in dengue infections is crucial since secondary infections with heterologous serotypes can result in potentially fatal DHF and DSS. This study was conducted to determine the multiple dengue serotype infection by RT-qPCR and to identify VEGF levels in single and multiple serotype dengue infections. VEGF levels in this study were assessed by ELISA, whereas serotype was identified by molecular analysis. Data were analyzed with Student t-test. A total of 60 positive samples were analysed. Multiple-serotype dengue infections were detected in 20 samples (33.3%), while 40 samples (66.7%) had single-serotype infections. The mean VEGF level in multiple-serotype dengue infections (324.48 pg/mL) was not significantly different from that in single-serotype dengue infections (300.68 pg/mL), with a p-value of 0.709. Multiple-serotype dengue infections were identified in one-third of the study population. Patients with multiple-serotype infections showed higher mean VEGF levels than those with single-serotype infections; however, no statistically significant difference was observed. These findings suggest that VEGF may not differ substantially between single- and multiple-serotype dengue infections in the studied population.

Keywords: Dengue virus, VEGF, Multiple serotype infection, RT-qPCR, Endothelial permeability, Dengue hemorrhagic fever.

Dengue virus (DENV-1 to DENV-4), a member of the genus *Flavivirus*, is a mosquito-borne virus that causes dengue infection. The virus is approximately 50 nm in diameter and possesses a single-stranded positive-sense RNA genome. The genome encodes seven non-structural (NS) proteins and three structural proteins, namely the capsid or core protein (C), membrane protein (M), and envelope protein (E), which constitute the viral particle. The viral genome is approximately 11,644 nucleotides in length.¹⁻³

Dengue outbreaks have been reported worldwide and are caused by four distinct dengue virus serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). In endemic areas, these serotypes often circulate simultaneously, allowing individuals to be exposed to different serotypes throughout their lifetime. Indonesia is considered a dengue hyperendemic country, where the co-circulation of all four serotypes contributes to the high burden of dengue infection.^{3,4}

Infection with one dengue virus serotype generally provides long-term immunity against the same serotype but only limited protection against the others. As a result, individuals living in endemic areas may experience subsequent infections with heterologous serotypes. Such secondary infections have been associated with a higher risk of severe clinical manifestations, including dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).⁴ Therefore, identifying circulating serotypes is important not only for epidemiological surveillance but also for improving our understanding of disease severity and transmission patterns.^{2,4-6}

The presence of multiple dengue virus serotypes in a single host may induce a more complex immune response than infection with a single serotype. Enhanced immune activation and cytokine production have been implicated in endothelial dysfunction and increased vascular permeability, which are key features of severe dengue. Therefore, biomarkers associated with endothelial activation may provide additional information regarding the biological consequences of multiple-serotype infections. VEGF plays a crucial role in endothelial cell activation through its interaction with specific cell-surface receptors. It is essential for wound healing and development and acts as a proangiogenic factor that promotes the growth of new blood vessels from pre-existing vascular networks.⁷ Endothelial injury may stimulate VEGF production, which increases vascular permeability and may contribute to plasma leakage during dengue infection.⁸

Vascular endothelial growth factor (VEGF) is a cytokine involved in endothelial cell activation and vascular permeability. By binding to specific cell-surface receptors, VEGF promotes angiogenesis and regulates endothelial function.⁸ Increased VEGF expression has been associated with plasma leakage and disease severity in dengue infection.^{10,12,21} However, it remains unclear whether VEGF levels differ between patients with single-serotype and multiple-serotype dengue virus infections.

Therefore, this study was conducted to detect multiple-serotype dengue virus infections using RT-qPCR and to compare VEGF levels between patients with single-serotype and multiple-serotype dengue infections.

MATERIAL AND METHOD

This study included 60 samples in total, which were taken from clinical laboratories at public hospitals (Military Hospital, Solok, and Dr. M. Djamil General Hospital, Padang). The study was carried out in 2020 between January and October. Purposive sampling was employed in this study to examine the numerous dengue infections in each participant. All samples were collected within the first five days after the onset of symptoms. NS1 antigen and/or anti-dengue IgM and IgG assays were used as the initial screening tests for all samples in Biomedical Laboratory, Faculty of Medicine, Universitas Andalas, Padang, Indonesia. All ELISA and molecular analyses were performed at the Biomedical Laboratory, Faculty of Medicine, Universitas Andalas, Padang, Indonesia.

Patients who underwent laboratory testing for suspected dengue infection were eligible for inclusion in this study. Full blood count and serological dengue testing were performed for all participants. Trained professionals used a 3-cc syringe to draw blood aseptically from the median cubitus vein. After centrifugation, serum samples were stored at -80°C until further analysis. Viral RNA was extracted from serum samples obtained from patients with dengue infection. QIAamp Viral RNA Mini Kits (Qiagen, Germany) were used for RNA extraction. Viral RNA was isolated from 140 iL of serum according to the manufacturer's instructions, stored at -80°C until further analysis. After using DNase Amp Grade (Invitrogen, USA) to denature the isolated RNA for 10 minutes at 65°C, and 20 iL a reaction mixture transcribed to cDNA. The reaction was permitted to continue for five minutes at 25°C, twenty minutes at 46°C, and one minute at 95°C to inactivate the enzyme.

Detection of DENV RNA by RT-qPCR

The capsid gene was amplified by nested qPCR method. The initiate cycle was amplified by outer primer Dengue_F (5' to 3'): GAGAAACCGCGTGTCAAC and Dengue R: TCCTGCTTGCTGACTATCATG; furthermore, the second cycle of PCR used four specific primers DENV-1 (5' to 3'): TTCTTTCTTGAAACTCCGTAGC, DENV-2 (5' to 3'): GCGGGATTGTTAGGAAACGA, DENV-3 (5' to 3'):CTTTTCCGTCTGT TGATAATGC, and DENV-4 (5' to 3'):

GACCTATCTCCTTCCTGAATCCAA. The primers came from DENV positive screening. To get the whole sequencing, the DENV was amplified and sequenced. The isolation result was then verified using the Basic Local Alignment Search Tool. Primer3 (version 0.4.0) created the primer. For DENV-1, DENV-2, and DENV-3, the nested PCR result was 205 bp, 125 bp, 244 bp, and 212 bp in size, respectively. The following reaction conditions were used when doing DNA amplification: 30 seconds of denaturation at 95°C, followed by five cycles of 5 seconds of denaturation at 95°C and 5 seconds of annealing at 65°C, followed by 11 cycles of touchdown (1°C/cycle) denaturation at 95°C for 5 seconds and 5 seconds of annealing at 65°C to 55°C. Furthermore, 19 cycles of denaturation at 95°C for 5 s, annealing at 55°C for 5 s, and the melt curved at 65°–95° for 5 s (every 5 s the temperatures raised 0.5°C).

In the initial RT-qPCR step, 1 iL of cDNA was mixed with 5 iL of EvaGreen (Biorad, USA), 0.5 iL of Dengue-F and 0.5 iL of Dengue-R primers, and 3 iL of nuclease-free water in a 10 iL reaction mixture. After external PCR, four serotype-specific primers (DEN-1, DENV-2, DENV-3, and DENV-4) and the primer Dengue-F were used in a second round of nested PCR. The external PCR's amplified product was diluted 1:100. A nested PCR mixture of 10 uL was made by mixing 1 iL of diluted

external PCR product with 5 uL of EvaGreen (Biorad, USA), 0.5 iL of Dengue-F primer, 0.5 iL of each of the DENV-1, DENV-2, DENV-3, or DENV-4 primers, and 3 iL of nuclease-free water. Standard ELISA kits (Ray Biotech) were used to assess VEGF. The tests were conducted in compliance with the manufacturer's instruction manual.

Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD). Comparisons between groups were analysed using Student's t-test. A p-value of < 0.05 was considered statistically significant.

RESULTS

RT-qPCR analysis confirmed dengue virus infection in all 60 samples included in this study. A single-serotype dengue infection was detected in the majority of samples ($n=40$, 66.6%), while 20 samples (33.4%) had multiple serotype dengue infections. The mean hematocrit level was $39.88 \pm 3.52\%$, the mean leukocyte count was $5,342 \pm 404$ cells/mm³, the mean hemoglobin level was 13.39 ± 2.29 g/dL, and the mean platelet count was $62,222 \pm 47,339$ cells/mm³. This investigation revealed that among the study participants, 34 (56.7%) were male and 26 (43.3%) were female. All characteristic data were presented in Table 1.

Table 2 presents the comparison of VEGF levels between patients with single-serotype and multiple-serotype dengue infections. The mean VEGF level was higher in the multiple-serotype infection group (324.48 ± 212.75 pg/mL) than in the single-serotype infection group (300.68 ± 192.87 pg/mL). However, the difference was not statistically significant ($p = 0.709$), indicating that VEGF concentrations were comparable between the two infection groups.

Table 1. Characteristics of study participants

Variable	Value
Male, n (%)	34 (56.7)
Female, n (%)	26 (43.3)
Platelet count (/mm ³)	$62,222 \pm 47,339$
Hematocrit (%)	39.88 ± 3.52
Leukocyte count (/mm ³)	$5,342 \pm 404$
Hemoglobin (g/dL)	13.39 ± 2.29

Note: Data are presented as mean $\pm$ SD.

Table 2. Comparison of VEGF levels between infection groups

Infection type	n (%)	VEGF (pg/mL), mean $\pm$ SD	p-value
Multiple-serotype infection	20 (33.3)	324.48 ± 212.75	0.709
Single-serotype infection	40 (66.7)	300.68 ± 192.87	

Note: Data are presented as mean $\pm$ SD. Comparisons between groups were performed using Student's t-test. A p-value < 0.05 was considered statistically significant.

DISCUSSION

By isolating the virus, detecting its genomic sequence using RT-qPCR, and detecting the NS1 antigen with matching IgM and IgG antibodies using enzyme immunoassay and immunochromatographic testing, dengue is diagnosed. For potentially deadly and epidemic-prone illnesses, the latter are straightforward, quick, and simple diagnostic tests that are great tools. Other than these, the platelet count is the only laboratory test that may be obtained in remote locations to support a dengue illness diagnosis^{24,25}. Initial laboratory test alterations include a reduction in white blood cell counts, platelet counts, and metabolic acidosis.

Multiple dengue cases have become more common; according to Almurdi *et al.*'s study, in West Sumatra, Indonesia, 51.64% of positive test findings are multiple-serotype dengue infections.³ In 2010, Indonesia emerged as the top-ranked nation with the most dengue cases and hyperendemic conditions.⁹ Locations where two or more dengue serotypes are concurrently circulating are referred to as dengue hyperendemic areas.¹² The severity of an illness can also be increased when two or more serotypes infect a single person.²

In the present study, multiple-serotype dengue infections were detected in 33.3% of dengue-positive samples. Although this proportion was lower than that reported previously in West Sumatra, it indicates that concurrent infection with more than one dengue virus serotype remains relatively common in the study area. The co-circulation of multiple dengue virus serotypes is a characteristic feature of hyperendemic regions such as Indonesia and may increase the likelihood of multiple-serotype infections. These findings highlight the continuing importance of molecular surveillance for monitoring dengue virus circulation and understanding the epidemiology of dengue infections in endemic settings.

A major contributor to disease and mortality, the dengue virus causes dengue fever as well as the more severe conditions dengue hemorrhagic fever and dengue shock syndrome, which are linked to alterations in vascular permeability.⁶ The primary target cells for DENV are dendritic cells and monocytes/macrophages that release various chemokines and cytokines upon

infection, which can activate the endothelium and are thought to play a major role in DENV-induced vascular permeability.¹⁴

Endothelial cells produce and secrete vascular endothelial growth factor (VEGF).^{10,11} As a physical barrier, the endothelium regulates circulation and is involved in vascular tone, blood vessel growth, and the regulation of thrombosis and thrombolysis.^{15,16} Therefore, elevated VEGF levels have been associated with severe dengue manifestations, including circulatory shock and plasma leakage. VEGF has been linked to dengue outcome, plasma leakage, and disease severity. Protein and plasma loss increase as a result of alterations in endothelial microvascular permeability and thermoregulatory mechanisms.²³ Recent research, however, shows that DENV also replicates in endothelial cells, and that endothelial cells infected with DENV may directly aid in viremia, immunological activation, vascular permeability, and endothelium-specific immune targeting.¹⁷

Severe dengue patients had significantly greater levels of VEGF than both dengue patients and healthy controls. VEGF may be involved in inflammation and coagulation in addition to its function in enhancing endothelial permeability and proliferation. Consequently, it might play a crucial part in the pathophysiology of dengue.^{18–20} Clinical outcomes are linked to thrombocytopenia, which is frequently seen in both moderate and severe dengue illness. Thrombocytopenia may result from bone marrow suppression, immune-mediated platelet destruction, and increased peripheral consumption. It could be a factor in plasma leakage and bleeding problems.¹⁹ Dengue has a complex mechanism for bleeding manifestation, with thrombocytopenia, coagulation abnormalities, vasculopathy, and hepatic derangement all working in concert. Platelets rely on the production of IL-1 to contribute to enhanced vascular permeability through inflammation. A sharp decline in platelet count accompanied by an increase in haematocrit indicates the onset of plasma leakage.²¹

In the present study, the mean VEGF level was slightly higher in patients with multiple-serotype dengue infections (324.48 pg/mL) than in those with single-serotype infections (300.68 pg/mL). However, this difference did not reach statistical significance ($p = 0.709$). This finding

suggests that VEGF expression may be influenced more strongly by endothelial activation and disease severity than by the number of infecting dengue virus serotypes. Previous studies have demonstrated that VEGF plays an important role in vascular hyperpermeability and plasma leakage during dengue infection and is frequently associated with severe clinical manifestations rather than specific viral serotypes.^{10,12,17,21} Therefore, the absence of a significant difference in VEGF levels between single- and multiple-serotype infections in the present study may indicate that serotype multiplicity alone is insufficient to alter VEGF expression substantially.

The present findings are consistent with previous studies reporting that VEGF levels are more closely associated with disease severity and plasma leakage than with viral serotype. Teo *et al.* and Fiestas Solórzano *et al.* suggested that endothelial activation and host inflammatory responses play a more prominent role in VEGF regulation during dengue infection than viral factors alone.^{10,21} Accordingly, the absence of a significant difference observed in the present study may indicate that VEGF expression is driven primarily by host immune responses and endothelial activation rather than by the number of infecting dengue serotypes. The inflammatory response induced by dengue infection is highly heterogeneous among individuals and may overshadow the effect of serotype multiplicity on VEGF production.^{15,17,21}

One possible explanation for this finding is the wide variation in VEGF concentrations observed in both groups, as reflected by the large standard deviations. VEGF production is regulated by a complex interaction between viral factors and host immune responses, including cytokine release, endothelial activation, and inflammatory mediators.^{10,17} In addition, patients were not stratified according to disease severity, which has been reported to influence circulating VEGF levels.¹² Consequently, potential differences attributable to multiple-serotype infections may have been masked by interindividual variations in clinical and immunological responses.

CONCLUSION

Multiple-serotype dengue infections were

detected in 33.3% of dengue-positive samples. Although the mean VEGF level was higher in the multiple-serotype group, the difference was not statistically significant compared with single-serotype infections. Further studies involving larger sample sizes and disease severity stratification are required to clarify the relationship between dengue serotype patterns and VEGF expression.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

Ethical approval for the study was granted by the Ethics Committee of the Faculty of Medicine, Universitas Andalas, Padang, Indonesia (Approval No. 557/UN16.2/Kep-FK/2021).

Informed Consent Statement

All participants were informed about the objectives, potential risks, and benefits of the study, and verbal informed consent was obtained before participation.

Clinical Trial Registration

This research does not involve any clinical trials.

Permission to reproduce material from other sources

Not Applicable

Author Contributions

Almurdi Almurdi: Conceptualization, data collection, laboratory investigation, and manuscript preparation; Rauza Sukma Rita: Collected the literatures and manuscript revision; Dessy Arisanty: Designed the research, analytic tools and analysed the result of study; Zelly Dia Rofinda: Contributed to revise the manuscript, analysing the result of study.

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