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# Research Article
# Relationship of Viral Load toward Platelet Count and Hematocrit Level in DENV-2 Infection

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## Abstract

**Background and Objective:** Dengue is an infection caused by the dengue virus transmitted by the bite of infected *Aedes* mosquitoes. It evolves rapidly over a short time course and displays a wide range of clinical manifestations. Thrombocytopenia is commonly observed in patients with dengue virus infection and may contribute to complication bleeding and plasma leakage. This study aimed to identify the correlation of viral load with the platelet count and hematocrit levels in DENV infection. **Materials and Methods:** This study combined the molecular examination for determining the serotype of dengue virus and viral load with qPCR and hematology parameters to count of platelet and hematocrit. The correlation between the number of viral load with platelet count and hematocrit level was analyzed with Pearson's correlation test. **Results:** There were significant correlations between the number of viral load with platelet count and hematocrit level ($p = 0.000$, $r = 0.729$, $p = 0.029$, $r = 0.369$, respectively). **Conclusion:** Platelet count and hematocrit level can be applied to predict viremia and disease prognosis in dengue infection patients.

Key words: Viral load, platelet, hematocrit, DENV-2, qRT-PCR

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Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Dengue virus (DENV) belongs to the family *Flaviviridae*, genus *Flavivirus* and is transmitted to humans by *Aedes* mosquitoes, mainly *Aedes aegypti*. Based on neutralization assay data, four serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) can be distinguished[1]. Infection with any of the DENV serotypes may be asymptomatic in the majority of cases or may result in a wide spectrum of clinical symptoms[2]. The infection is capable of causing disease with a wide spectrum of clinical manifestation, ranging from an undifferentiated fever in a mild clinical form to the severe clinical and potentially fatal[3]. The disease is now with very high case fatality rate and children are the most affected age group world wide[4]. Dengue is small spherical particles with diameter of 50 nm, lipid enveloped, ss-RNA viruses with approximately 11,000 nucleotides[5-8].

Thrombocytopenia is commonly observed in a patient with dengue virus infection and may contribute to bleeding complication and plasma leakage[9]. Bleeding and shock are the most dreaded complications in children with dengue leading to high mortality. The mechanism for bleeding manifestation is multifactorial in dengue and the factor such as thrombocytopenia, coagulation defects, vasculopathy and hepatic derangement act synergistically[4,10].

Platelet contributes to increased vascular permeability by inflammation to depend on the release of IL-1β. A rapid decrease in platelet count, concomitant with a rising hematocrit is suggestive of progression to plasma leakage[11]. Thrombocytopenia in dengue might result from reducing of bone marrow function, decreased platelet production, increased platelet destruction and increased platelet consumption[12].

The enhance in vascular permeability is associated with vascular leakage, resulting in accumulation of fluid in pleural and peritoneal cavities and the reduction in blood pressure and pulse pressure, resulting in poor organ perfusion[13]. Rapid fluid loss into tissue spaces causes the hemoconcentration and hypotension that can result in mortality[14].

A high number of virus in the blood is assumed to cause vascular fragility, together with infection of endothelial cells and high levels of cytokines and other soluble mediators, may result in bleeding[7]. The high dengue viremia titter was associated with increased disease severity. Peak of viral titters were 100 to 1000-fold higher in patients with dengue shock syndrome (DSS) than those with dengue fever (DF). Viral load is also a contributing factor in the development of dengue hemorrhagic fever (DHF)/DSS[15]. Higher viral loads have been associated with disease severity in both primary and secondary dengue and with different serotype[16].

To date, DENV-2 is the common genotype circulating in Indonesia and has been found in several capital cities, such as Jakarta, Surabaya, Semarang, Makassar and others[17]. Secondary infection particularly with DENV-2 was significantly associated with severe dengue[7]. Previous studies found the serotype of DENV-2 predominantly in their studies[8,18]. Viral factors include both the infecting serotype of the virus, with certain genotype considered more virulent than the others and have been linked to outbreaks of severe disease[16].

Dengue virus infection can be diagnosed by using cell culture, serology, viral NS1 protein test or a PCR based method. A real-time RT PCR was performed in the quantitative PCR system to detect the dengue virus in acute-phase serum samples[19,20]. Several real-time PCR based methods for the detection of DENV have been reported in the last decade. These assays have targeted the 3′ UTR, NS5, core and the envelope gene sequences. PCR is considered as the gold standard for dengue diagnosis (80-90% sensitivity and 95% specificity), if it was applied in the adequate time window. The molecular detection of dengue RNA offers a sensitive, rapid and simple ways[21-23]. This study aimed to identify the viral load correlation with the platelet count and hematocrit levels in DENV infection.

## MATERIALS AND METHODS

**Location and population of study:** A total of 119 samples was eligible in this study, samples were collected from five Clinical laboratories at public hospitals in West Sumatera (Dr. M. Djamil General Hospital, Padang; Dr. Ahmad Muchtar Regional Public Hospital, Bukit Tinggi; Regional Public Hospital Padang Panjang, Regional Public Hospital Pariaman and Regional Public Hospital Painan). This study was conducted on January 2018 to October 2019 with a purposive sampling technique. Molecular analysis was conducted in Biomedical Laboratory, Faculty of Medicine, Andalas University, Padang.

**Preparation of samples:** A blood sample was collected from each patient who visited Clinical Laboratory for the serologic dengue test and whole blood exam. Blood was taken from mediana cubiti vein using an aseptic procedure by trained personnel, using a 3cc syringe. All sample was collected within the first five days of illness, previously screened using NS1 and/or IgM and IgG anti-dengue detection.

The serum was separated and stored at -80°C. All subject was briefed on the study including the objectives, risks and benefits of the study and informed consent was conducted via viva voce. The study was approved by the Ethics Committee of Medical Faculty, Andalas University, Padang, Indonesia No.: 268/KEP/FK/2019.

**Viral RNA extraction and cDNA synthesis:** RNA viral extraction was extracted from 140 µL serum samples using QIA Amp viral RNA Mini Kits (Qiagen, Germany) according to the manufacturer's instructions and then stored at -80°C for further analysis. cDNA was converted in 20 µL reaction mixture (11 µL RNA, 4 µL 5x trans-Amp buffer, 1 µL reverse transcriptase and 4 µL DNAse/RNAse free water (Bioline cat no. Bio 65053)).

**Detection of DENV RNA by qRT-PCR:** The RT-PCR was performed using the nested-qPCR method, the capsid gene was amplified which first round of running used Dengue outer primer F (5' to 3'): GAGAAACCGCGTGTCAAC and Dengue R: TCCTGCTTGCTGACTATCATG, furthermore the second used four specific primers DENV-1 (5' to 3'): TTCTTTCTTGAAACTCCGTAGC, DENV-2 (5' 'to 3'): GCGGGATTGTTAGGAAACGA, DENV-3 (5' to 3'): CTTTTTCCGTCTGTTGATAATGC, DENV-4 (5' to 3'): GACCTATCTCCTTCCTGAATCCAA. The primers were derived from the positive screening of DENV. The DENV was amplified and sequenced to obtain the whole sequences. The BLAST (Basic Local Alignment Search Tool) analysis was needed to confirm the result of isolation. The primer was designed by Primer 3 (version 0 .4.0). The size of the nested PCR product was 205 bp for DENV-1, 125 bp for DENV-2, 244 bp for DENV-3 and 212 bp for DENV-4.

**Statistical analysis:** Statistical analysis was performed using SPSS vers.15.0. The correlation between parameters was analyzed by Person's correlation test. Pooled data were presented as the average value. Data were compared using Student t-test, p-value<0.05 was considered statistically significant.

## RESULTS

In this study, a total of 119 samples; 91 samples were positive dengue infection after PCR amplification (76.47%) and 28 samples (23.36%) were negative. Most of sample was DENV-2 (n = 36, 39.56%). Multiple dengue infections were found in sample screening (n = 47, 51.64%) (Table 1).

The viral load count showed that DENV-2 (8.01±0.79) log/mL was the highest serotype number than DENV-4 and DENV-1 (7.89±1.01, 7.57±0.61, respectively) (Table 2). Data of viral load for DENV-3 were not analyzed because only two samples were positive. The viral load for DENV-2 and DENV-1 showed significant difference (p<0.05), while DENV-4 was not significantly different (p>0.05). The hematology parameter such as thrombocyte was to decrease (61,000µL$^{-1}$ ±30,490) and hematocrit (39.62% ±5.76) (Table 1).

The correlation between viral load infected by DENV-2 and platelet count in Fig.1a was a significant inverse

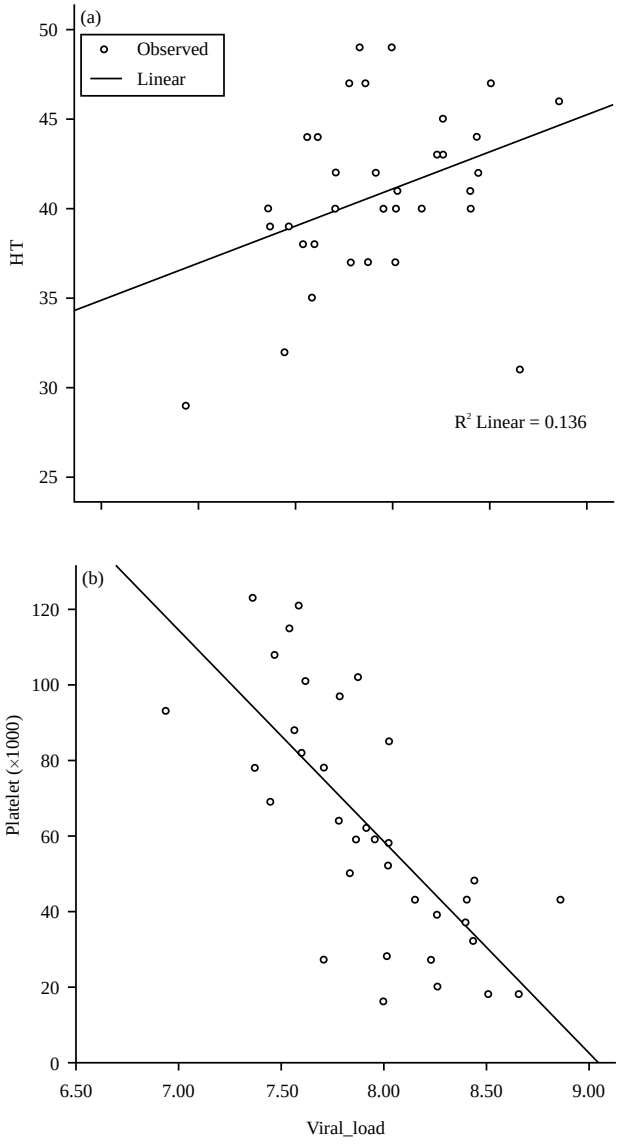


Fig. 1(a-b): (a) Correlation between viral load and hematocrit (r = 0.369, p = 0.029) and (b) Correlation between viral load and platelet count (r = -0.729, p = 0.000)

Table 1: Molecular screening for DENV serotype

| Sample | N | % |
|---|---|---|
| Positive DENV infection | 91 | 76.47 |
| Negative DENV infection | 28 | 23.36 |
| **DENV serotypes** | | |
| DENV-1 | 5 | 4.49 |
| DENV-2 | 36 | 39.56 |
| DENV-3 | 1 | 1.09 |
| DENV-4 | 2 | 2.19 |
| Multiple dengue infections | 47 | 51.64 |

Table 2: The average of several parameters on dengue patients

| Parameter | Mean (SD) | Minimum | Maximum | p-value |
|---|---|---|---|---|
| **Viral Load (log mL$^{-1}$):** | | | | |
| DENV-2 | 8.01±0.79 | 6.93 | 11.59 | 0.000 |
| DENV-1 | 7.57±0.61 | 7.00 | 11.00 | 0.138 |
| DENV-4 | 7.89±1.01 | 7.14 | 11.15 | |
| **Hematology parameters:** | | | | |
| Platelet (/μL) | 61,000±30,490 | 15,000 | 132,000 | |
| Hematocrit (%) | 39.62±5.76 | 21 | 49 | |

relationship (p = 0.000, r = -0.729), indicated a negative linear effect. The higher the number of viruses, the lower the platelet count. While the higher of viral load, will increase the number of hematocrits. The correlation between viral load and hematocrit was significant (p = 0.029, r = 0.369), indicated a relationship with positive liner (Fig. 1b). So the risk of a patient to lead into shock syndrome and fatal risk will be greater.

## DISCUSSION

Dengue is the most important mosquito-borne viral infection to infect humans, it causes a high burden of disease and mortality across tropical and subtropical regions[2,5]. Dengue now affects larger areas because of environmental changes, deforestation, transportation and lack of an effective vaccine. The determining factors of the severity of dengue infection are vascular permeability, thrombocytopenia and coagulopathy. These result in intravascular volume depletion, hypotension and shock which result in high mortality[24].

In endemic areas, primary DENV infections occur early in life and are usually mild and often undiagnosed. Primary infections in older children and adults can result in dengue fever[25]. Antibody response to the first virus infection may be non-neutralizing and can enhance the entry of the second serotype into mononuclear cells, resulting in increased activation of complements and rapid production of a pro-inflammatory type-1 cytokine such as IFN-γ and TNF-α. These cytokines probably directly affect vascular endothelial cells to cause plasma leakage. The cytokine storm has a direct effect on the vascular endothelial cells by increasing capillary permeability. Cytokines also exhibit synergism where for example TNF-α, IFN-ɣ and IL-1 together can increase the capillary permeability compared to when the cytokine acts lonely[26,27]. An in vitro study revealed a cross-reaction of pro-inflammatory mediators such as TNF-alpha and anti NS1 antibodies with surface protein on endothelial cells causing apoptosis of these cells and subsequently plasma leakage[1].

There was a significant correlation between the numbers of viral load of DENV-2 infected patients with platelet counts because the mechanism by which thrombocytopenia is caused by the dengue virus is complex. Previous studies suggested that the virus probably contributes to bone marrow suppression and platelet destruction[5]. There are several hypotheses to explain thrombocytopenia such as an infected megakaryocyte by the virus, peripheral destruction and the cross reaction of antibodies against platelets. The platelets of dengue infected patients had mitochondrial dysfunction which activated the apoptosis cascade and leads to cell death, cytokines decreased megakaryopoiesis, sequestration of platelets by dengue infected endothelial cells. In dengue patients, the transient suppression of hematopoiesis was showed within 3-4 days of infection then the host inflammatory response which occurred to eliminated infected cells[1,9,10].

Thrombocytopenia is an early and consistent feature of dengue virus infection and dengue complications are usually preceded by a rapid drop in platelet count. It has been noted in some studies that thrombocytopenia is a risk factor for bleeding manifestations. Hemorrhagic tendencies included a positive tourniquet test, skin bleeding (petechial, ecchymosis, or purpura), mucosal bleeding (epistaxis, gum bleeding, or other sites), hematemesis, or melena[9,18,28]. Since thrombocytopenia is seen in both DF and DHF patients, a platelet count of 60,000 per mm serves as a better cut-off in identifying more severe cases[29].

The result of the study found several declines of hematocrit value below normal value (data not shown) may be due to the fluid management in the ward where given fluid quotas may have caused some dilution of the plasma or a drop in hematocrit as a sign of severity especially among patients with internal bleeding in the area such as gastrointestinal tract[5,11]. Overall, this study concerned to calculating the number of thrombocyte and hematocrit level in order to be used to follow up the progression of patients and to determine the prognosis of dengue infection.

## CONCLUSION

In conclusion, this study confirmed that most of the sample was DENV-2 (DENV serotype-2). The highest number of viral load was also DENV-2. The correlation between DENV-2

viral loads with platelet count showed a negative linear effect, whereas the correlation between DENV-2 viral load with hematocrit level was the positive linear effect. The higher the number of viruses, the lower the platelet count. While the higher of viral load will increase the number of hematocrits. The correlation between viral loads with platelet and hematocrit was important to predict viremia in dengue infection and prognosis of patients. Because of the more number of viral load, the more high risk of dengue infection.

## SIGNIFICANCE STATEMENT

This study discovered that DENV-2 was the highest serotype number than DENV-4 and DENV-1 that can be beneficial for predicting the viremia and disease prognosis. This study will help the researcher to uncover how endothelial and cytokine factors which suppress thrombocyte production in the bone marrow that be able to explore further. Thus, the theory of dengue virus infection may be arrived at the more number of viral load, the more high risk of DHF/DSS. The parameter can be investigated by the high number of hematocrit and the low number of thrombocyte.

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