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Clinical Characteristic and Tumor Necrosis Factor-Alpha (tnf-α) Analysis of Dengue Patient in Bengkulu, Indonesia

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Abstract

Bengkulu Province is one of three provinces with the highest morbidity rate of dengue cases in Indonesia. Tumor necrosis factor alpha is a major cytokine causes plasma leakage in dengue patients. It was the first study about clinical characteristic and cytokine analysis of dengue patients in Bengkulu. This study was conducted to identify the clinical data of dengue patients and analyze TNF-α level in different stages of dengue disease severity. Serum specimens were collected from 30 dengue patients in Bengkulu Hospital, were divided into dengue fever (DF) and dengue hemorrhagic fever (DHF) groups. Patients with dengue clinical symptom were confirmed by dengue NS1 and IgM rapid test, followed by dengue serotype identification trough reverse transcriptase-polymerase chain reaction (RT-PCR) technique. Enzyme-linked immunosorbent assay (ELISA) test was used to measure the levels of TNF-α, continued with statistically analysis using Mann Whitney test. This study was preliminary research in Bengkulu which succeeded in identifying dengue virus serotypes from clinical samples of dengue infection. The clinical characteristic analysis showed that the most symptoms besides fever were nausea and headache. Patients with dengue infection also experienced thrombocytopenia and increased of hemoglobin levels. The mean of TNF-α level in DF patients was 6.38 ± 2,52 pg/ml while in DHF patients was 11.5 ± 9.14 pg/ml. Fever, nausea, and headache were the most common symptoms experienced by dengue patients. Comparing between the two groups showed that DHF patients were higher than DF group in TNF-α levels.

Keywords: Cytokine, disease severity, symptom, hematology, enzyme link immunosorbent assay

Full length article **Corresponding Author, e-mail: mardhatillahs@unib.ac.id*

1. Introduction

Indonesia is a tropical country where almost all regions are endemic with both primary dengue virus vector, *Aedes aegypti* and *Ae. albopictus*. According to epidemiological study, over the 50-year period, the incidence rate of dengue hemorrhagic fever (DHF) in Indonesia was dramatically increase by the year, from 1968 to 2016, the cases increase from 0.05 to 77.96 cases per 100,000 [1]. According to Indonesian Ministry of Health Report in 2020, the incindent rate of DHF in Bengkulu Province is in 63.2 per 100,000 population. Several environmental factors are closely related to dengue risk, like temperature, urbanization, relative humidity, vegetation, and water vapor [2].

The severity of dengue virus infection depends on several factors, including type and number of cytokines secretion. Cytokines secreted during infection are natural and adaptive immune responses that act as inflammatory mediators. Several studies explain that the inflammatory response is related to the irregular production of cytokines which results in the emergence of several severe clinical manifestations, including tumor necrosis factor alpha (TNF-α), interlekuin (IL) 4, IL-6, and interferon gamma (IFN-γ) [3].

One of the cytokines that crucial in increasing the level of disease severity in dengue infection is TNF-α. Prior studies showed that TNF-α levels were found to be higher in DHF patients than in DF patients [4]. This cytokine produced by mononuclear phagocytes causes the aggregation molecules production by endothelium. Several immune responses are activated by dengue virus infection, including monocytes, CD4 T cell helper (Th1 and Th2), which induce TNF-α stimulation and become endothelial cell stressors. The cytokine TNF- α plays an important role in increasing vascular permeability in dengue infection [5].

Changes in quality and quantity happened in the immune system with increasing age. This process is often referred to as immunosenescence. There was a positive correlation between age and circulating pro-inflammatory cytokines like IL-6, TNF-α, and IL-1β [6]. The correlation between cytokines and dengue severity have showed in several studies, althought the specified cytokines involved in dengue infection still identified. This study aim to analyze the clinical data of dengue patients, and to identify TNF–α levels

using enzyme-linked immunosorbent assay (ELISA), which important to get the prognostic indicator of dengue infection.

2. Materials and methods

2.1. Study Design and Population

This study was an observational study with cross sectional design. The collection of blood specimens was carried out in one of Bengkulu Hospital, for thirty dengue patients, who had previously signed the informed consent. Patients were diagnosed with dengue infection based on clinical symptoms and blood laboratory tests. Patients with chronic diseases, malignancies or disorders congenital conditions, and who are taking drugs, such as antiviral, anti hypertension and others were excluded from this study.

2.2. Dengue Virus Identification

Dengue infection was confirmed by NS1 and IgG/IgM rapid test, and viral RNA was identified by RT-PCR technique using SuperScript III Platinum® One-Step *RT-PCR* Kit. The extraction of viral RNA was followed the protocol form Geneaid Viral Nucleic Acid Extraction Kit. The components of RT-PCR were consist of 10X PCR buffer, 5 mM dNTPs, 50 mM $MgCl_2$, RNase inhibitor, SuperScript™ III Reverse Transcriptase, Platinum™ *Taq* DNA polymerase, and 10 mM primer (D1, D2, TS1, TS2, TS3, and TS4) based on Lanciotti et al.,(1992) [7].

2.3. Preparation of Serum Samples

The blood samples were obtained for amount 3-5 ml, then centrifuged at 1000-2000 rpm for 10 minutes. Serum were stored at 2-8^{o} C for immediately use, and were stored at -80^{o} C for using in a longer time. The collection data including details of the physical examinations, clinical features, and laboratory findings.

2.4. Assay for TNF-α Examination

The examination of TNF-α levels was carried out using reagent used the ELISA Quantikine by R&D Systems USA with a minimum detection dose of 0.5-5.5 pg/mL and a minimum detection mean of 1.6 pg/mL.

2.5. Statistical Analysis

Statistical analysis was carried out with SPSS for Windows version 19. Proportions were compared between groups using the chi-square test and the comparison of TNF-α levels between disease severity groups using the Kruskal-Wallis test with a 95% confidence level. The *p*-values < 0.05 were considered statistically significant.

2.6. Ethical Approvals

All subjects in this study were conducted with informed consent. The protocols followed the ethical standards formulated in the Helsinki Declaration of 1964, revised in 2013, and approved by Health Research Ethics Committee Universitas Bengkulu with No. 338/UN30.14.9/LT/2020.

3. Results and Discussions

3.1. Clinical Characteristics

There were 30 patients which confirmed with dengue infection based on clinical symptoms and blo od laboratory tests, followed by NS1, IgG/IgM rapid test and viral RNA identification, which 15 patients were dengue fever (DF) dan 15 dengue hemorrhagic fever (DHF). The characteristics of dengue patients in this study were dominated by male (56.7%). There were two ages group with almost similar proportion between the age range over 18 years old (53.3%) and under 18 years old (46.7%). The median age was 20 years (range from 10 months to 56 years). The Figure 1 shows the several clinical symptoms comparing of the two severity groups of dengue infection. The most common symptoms experienced by both DF dan DHF groups is fever (100%), followed by nauseous, headache and mucle pain.

The four dengue virus serotypes that have been identified in this study can be used as initial epidemiological data in mapping the distribution of dengue virus serotypes in Bengkulu Province. Furthermore, epidemiological studies can be conducted with a larger sample size, which serotype is dominant and further studies can be carried out in relation to clinical characteristics in dengue infection patients in Bengkulu. Fever is the most common clinical symptom experienced by research subjects related to the human immune response against viruses.

Symptoms of fever are also associated with the term “break bone fever” because fever is accompanied by severe muscle and joint pain. This process occurs as a result of the replication of the dengue virus which has spread to several parts of the organ, causing clinical symptoms to appear [8]. As with nausea, retroorbital pain, and headache, these symptoms were non-specific in the DF or DHF groups [9]. The laboratory findings of the two groups can be seen in Table 1. Leucocyte and platelet count were lower in DHF than in DF. Hemoglobin was similar in both DF and DHF groups, but the increase in hematocrit levels was higher in patients with DHF, which indicates that the state of hemoconcentration was more experienced in DHF patients than DF group.

Thrombocytopenia occurs as a result of bone marrow depression in the acute stage of dengue infection or can be caused by direct or indirect interaction of the virus with platelets [10]. Hemoconcentration caused by plasma leakage can be assessed by an increase in the hematocrit value, so that a decrease in platelet value will be accompanied or immediately followed by an increase in the hematocrit value, this usually occurs when the temperature drops or before shock occurs [5]. The highest hematocrit value occurs on the fourth and fifth day, then it will decrease on the next day [11]. The differences that occur in this study can be attributed to one of the factors, namely the time of taking blood for examination of the patient's hematocrit value which was carried out in the early phase of fever because the hematocrit value obtained was still normal. In addition, it is also related to the clinical symptoms of patients at the beginning of the fever experiencing dehydration, high fever, vomiting, anorexia, and poor oral intake which can cause hemoconcentration [12].

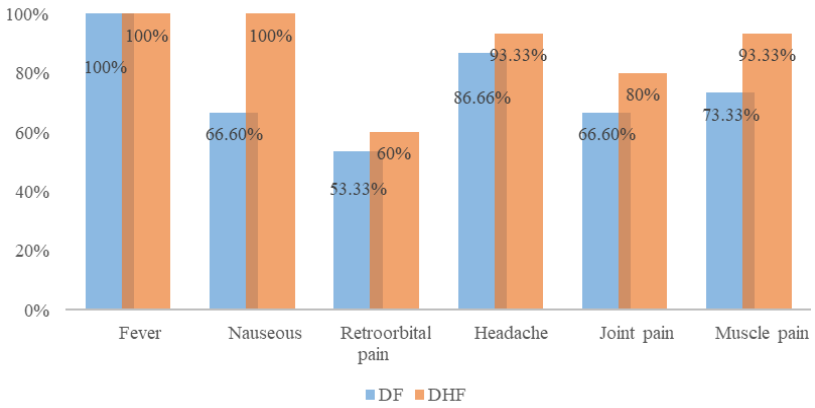

Figure 1. Clinical symptoms of the two dengue patient groups

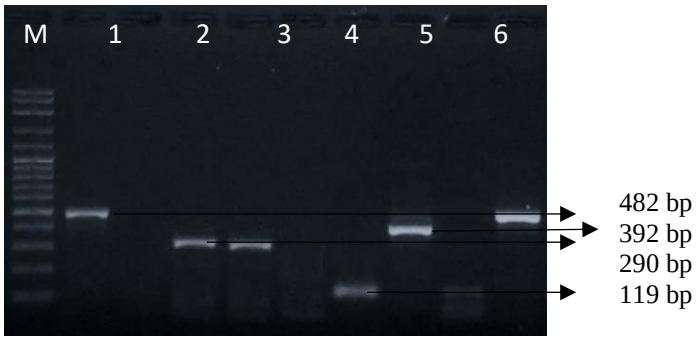

Figure 2: Dengue serotype detection (1-9: dengue serum samples, M: 100 bp DNA ladder,bp: base pair)

Table 1: Laboratory examination of dengue patients

Variable	**Degree of Severity**		***p****
	DF (n=15)	**DHF (n=15)**	
Hemoglobin	13.12 ± 1.67	13.93 ± 2.02	0.710
Hematocrit	37.2 ± 4.73	40.55 ± 5.60	0.336
Leukocytes	7,300 ± 5,210.15	4,368.9 ± 2,609.67	0.061
Platelet	162,667.7 ± 73,408.5	90,200 ± 57,207.9	0.245

Data were reported as means (± SD).

Table 2: TNF-α levels in severity and ages group		
Groups	**TNF-α level (pg/ml)**	***p****
Severity		
DF	6.38 ± 2,5	0.408
DHF	11.5 ± 9.14	
Age		
≤ 18 year	10.23 ± 8.52	0.412
> 18 year	7.83 ± 5.59	

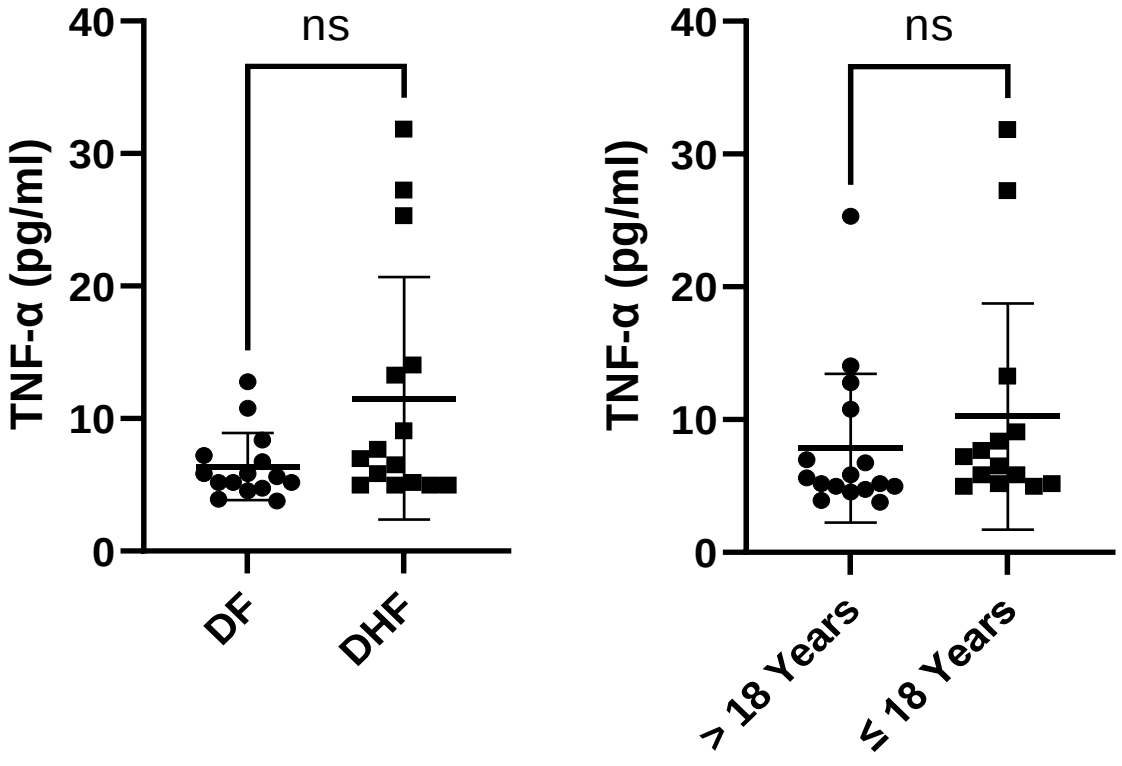

Figure 3. TNF-α levels in severity and ages group (ns: not siqnificant)

Furthermore, hemoconcentration causes a loss of plasma from the vascular compartment to the extravascular tissue. This significant change in vascular permeability will lead to the function of changing cell membranes in maintaining fluid osmotic pressure and ion concentration gradients between intracellular and extracellular compartments which can be characterized by increased levels of hemoglobin in blood vessels [13, 14].

3.2. Dengue Identification and TNF-α Analysis

The four dengue serotypes were identified by viral RNA amplification using RT-PCR method. DNA bands approximately 482 base pair (bp), 119 bp, 290 bp, and 392 bp were determined for dengue serotype 1, 2, 3, and 4 respectively (Figure 2). These positive results of molecular identification proves that all dengue serotypes are circulating in Bengkulu, although it has not shown one of the dominant serotypes. The serum samples for TNF-α level analysis were taken on 1st to 3rd day from clinical onset. The highest levels of TNF-α were found in DHF patients with value of 31.8 pg/ml, 27.26 pg/ml, and 25.30 pg/ml. Both severities and ages variable show that the mean of TNF-α levels were higher in DHF group and at age of less than 18th year. Based on statistical analysis using the Kruskal-Wallis test, the *p*-value was more than 0.05, so there was no significant difference between DF and DHF groups, and also adult and child groups (Table 2, Figure 3).

**Kruskal-Wallis test*

The results of TNF-α levels in this study are in accordance with that reported by Kittigul *et al* [4], TNF-α was higher in DHF patients than in DF patients. The results indicate that the role of TNF-α cytokines in DHF due to the increased of dengue virus viremia, so that many infected macrophages will produce more TNF-α cytokines. The function of the TNF-α cytokine is to stimulate the expression of adhesion molecules on the endothelium of blood vessels and leukocytes which will cause an increase in vascular permeability and inflammatory reactions [15, 16].

Cytokines such as TNF-α produced during viremia are responsible for inflammation and damage to muscle fibers, this is one of the causes of muscle pain in dengue infection [17]. TNF- α is produced by cells such as macrophages, dendritic cells, these cells are found in joint synovial fluid which will cause inflammation resulting in joint pain. At low levels, TNF-α acts on leukocytes to induce acute inflammation. TNF- α at moderate levels plays a systemic inflammatory role, while at high levels TNF-α causes a drop in blood pressure, severe metabolic disorders (sugar levels drop to levels that are not possible to live), intravascular thrombosis, and complications occur in septic shock. The high quantity of TNF-α production can prevent heart muscle contractility, lower blood pressure, intravascular thrombosis, and tissue factor (TF) expression [18].

There was no significant correlation between the two age groups, however the level of TNF-α at the age of children increases in direct proportion to the severity, as evidenced by the study of Kittigul et al [4], which stated that the highest levels of TNF-α in pediatric patients were patients with grade III of DHF, and lower levels in grade I and II of DHF and DF. TNF-α levels in samples of DHF infant patients were significantly higher than samples of healthy control infants, and the same thing was also found in samples of children aged less than 1 year that TNF-α levels in DHF were higher than DF patients [19].

4. Conclusion

This study demonstrated that the increased of cytokine levels, related to disease severity and also by the age. So, this cytokine could be a potential biomarker for prognostic aspects of dengue infection progression and inflammation. Subsequent studies can further analyze the relationship between dengue serotype and several cytokines level, such as TNF-α, IL-6 (interleukin 6), IFN-γ (interferon gamma), and also related to disease severity and prognosis.

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Declaration of Interest Statement

The authors declare that no conflict of interest.

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