

Prothrombin fragment 1.2 (F1.2) in relation with plasma leakage and thrombocytopenia in dengue infection

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Abstrak

Latar belakang: Manifestasi klinis demam berdarah Dengue (DBD) adalah kebocoran plasma dan trombositopenia. Salah satu teori penyebab kedua hal tersebut adalah kadar trombin yang meningkat akibat aktivasi koagulasi. Kadar trombin dapat diwakili oleh kadar F1.2. Tujuan penelitian ini adalah untuk mengetahui hubungan antara kadar F1.2 dengan kebocoran plasma dan trombositopenia pada infeksi Dengue.

Metode: Desain penelitian ini adalah potong lintang, menggunakan plasma EDTA dari pasien terinfeksi virus *Dengue*. Subyek penelitian adalah 10 subyek dengan kebocoran plasma dan 10 subyek tanpa kebocoran plasma pada infeksi Dengue, 6 sampel berpasangan untuk perbandingan fase kritis dan fase konvalesen, 26 sampel untuk uji korelasi antara kadar F1.2 dengan jumlah trombosit.

Hasil: Penelitian menunjukkan kadar F1.2 pada pasien terinfeksi virus *Dengue* dengan kebocoran plasma (rerata \pm 2SD) $147,4 \pm 105,82$ pg/mL lebih tinggi secara bermakna dibanding tanpa kebocoran plasma $51,3 \pm 39,92$ pg/mL. Kadar F1.2 pada fase kritis dengan median $186,3$ ($108,6$ - $223,2$) pg/mL lebih tinggi secara bermakna dibanding fase konvalesen $46,5$ ($27,4$ - $51,9$) pg/mL. Terdapat korelasi negatif yang bermakna dengan kekuatan sedang antara kadar F1.2 dengan jumlah trombosit, nilai $r = -0,609$.

Simpulan: Terdapat peningkatan aktivasi koagulasi yang ditunjukkan dengan peningkatan kadar F1.2 pada fase kritis, berkaitan dengan kebocoran plasma dan trombositopenia pada pasien terinfeksi virus Dengue. (*Health Science Journal of Indonesia 2016;7:37-43*)

Kata kunci: infeksi *Dengue*, kebocoran plasma, trombin, fragmen protrombin (F1.2), trombositopenia

Abstract

Background: Clinical manifestations of Dengue hemorrhagic fever are plasma leakage and thrombocytopenia. Both manifestations are thought to be caused by an increased thrombin level due to activation of coagulation. The aim of this study is to look for any association between F1.2 level and plasma leakage and also between F1.2 level and thrombocytopenia in Dengue infected patients.

Methods: This study used EDTA plasma from patients infected with Dengue virus. The study design was cross sectional. The thrombin level was represented by the prothrombin fragment 1.2 (F1.2) level. Twenty subjects were enrolled in this study, consisted of 10 subjects with plasma leakage and 10 without plasma leakage, 6 pairs of samples in critical phase and convalescent phase, 26 samples for correlation test between F1.2 level and platelet count.

Results: In this study, it was found that the F1.2 level in patients with plasma leakage (mean \pm 2 SD) 147.4 ± 105.82 pg/mL is significantly higher compared to patients without plasma leakage 51.3 ± 39.92 pg/mL, and the F1.2 level in critical phase has a median of 186.3 (108.6 - 223.2) pg/mL which is significantly higher compared to convalescent phase 46.5 (27.4 - 51.9) pg/mL. Also it was found that a medium negative correlation between F1.2 level and the thrombocyte count existed, $r = -0.609$.

Conclusion: The results of the study suggest that there was increased coagulation activation at critical phase in patients infected with Dengue virus associated with plasma leakage and thrombocytopenia. (*Health Science Journal of Indonesia 2016;7:37-43*)

Keywords: Dengue infection, plasma leakage, thrombin, prothrombin fragment (F1.2), thrombocytopenia

Dengue fever is a disease transmitted by *Aedes aegypti* and *Aedes albopictus* infected with dengue virus of the family Flaviviridae. There are 4 dengue virus serotypes which are DENV-1, DENV-2, DENV-3 and DENV-4.¹ Dengue infection is a dangerous disease, mortality rate is 2.5 %, especially in children less than 15 years old. There are approximately 50 million new cases each year. Of these there are about 500 thousand people with dengue hemorrhagic fever (DHF), which requires hospitalization. WHO report stated there was a rise in dengue cases and number of deaths from 1985 until 2009. In Indonesia in 2009, 156 062 cases of dengue were reported, while the number of deaths from dengue in that year is 1396.²

Clinical manifestations of dengue hemorrhagic fever are plasma leakage and bleeding that can lead to shock and death. There are various theories about the cause of plasma leakage. Plasma leakage is thought to be caused by increased capillary permeability. Increase in capillary permeability can be caused by Dengue virus itself, the immune response associated with the presence of viral proteins that are expressed by infected cells, and the immune responses associated with memory T cells, expressing much proinflammatory cytokines such as TNF- α , IL-1, IL-6, IL-8.^{1,3,4} Increased levels of proinflammatory cytokines in the blood trigger the activation of the vascular endothelial. Activated endothelial will express tissue factor. Further, tissue factor activates coagulation via extrinsic pathway.^{2,5-7}

Thrombin generation occurs after the activation of the coagulation cascade, forming prothrombinase that cuts prothrombin to thrombin and prothrombin fragment 1+2 (F1.2). F1.2 can be used as a marker of activation of coagulation and represented thrombin generation. Thrombin can accelerate its own generation and also can activate endothelial cell and platelet.⁸ Thrombin can activate protease activating receptor (PAR) 1, 3, 4 on endothelial cells. PAR activation will trigger the signal transduction, leading to increased capillary permeability and resulting in plasma leakage.^{9,10} Thrombin can also activate platelets through PAR 1 and 4 on platelets. Platelet activation causes excessive platelet aggregation, which leads to thrombocytopenia.^{3,11} Thrombocytopenia may facilitate bleeding.¹ Free thrombin will soon be neutralized by antithrombin and forms thrombin antithrombin complexes.¹²

The aim of the study was to compare the levels of F1.2 on a group of patients without and with plasma leakage, to compare the levels of F1.2 at a critical

phase and convalescent phase in patients with plasma leakage and to look for correlation between F1.2 levels and platelets number in Dengue virus infected patients. F1.2 represented thrombin generation.

METHODS

Patients

This study was approved by the Research Ethics Committee of the Faculty of Medicine, University of Indonesia and the Cipto Mangunkusumo Hospital institutional review board. The study design was cross-sectional with modification. The study was conducted in Cipto Mangunkusumo Hospital in January-September 2013. Twenty subjects were enrolled in this study, consisted of 10 subjects with plasma leakage and 10 without plasma leakage, 6 pairs of samples in critical phase and convalescent phase, 26 samples for correlation test between F1.2 level and platelet count. The diagnosis of dengue fever or dengue hemorrhagic fever by the clinician were based on WHO criteria 2011, had one positive dengue serology results (or more) (NS1 and or IgM/IgG anti-dengue positive). Dengue hemorrhagic fever was differentiated from dengue fever in the subjects by the presence of plasma leakage and thrombocytopenia. In subjects with dengue hemorrhagic fever there were both plasma leakage and thrombocytopenia presence.² The subjects were over 15 years old and gave informed consent. The study subjects also were examined with Rontgen or albumin level test to determine whether there was any evidence of plasma leakage. Evidence of plasma leakage was shown by at least one of these things : increased hematocrit ≥ 20 % from reference value of average population by age, sex, race or a decrease in hematocrit ≥ 20 % from subject's baseline after fluid replacement therapy / volume, or evidence of pleural effusion and or ascites by Rontgen photo/USG, and or hypoproteinemia , which is expressed with albumin ≤ 3.5 g/dL.¹³ Subjects with infectious diseases other than dengue, taking oral anticoagulants, suffering from malignancy, tissue trauma/ surgery, with pregnancy, were not included in the study. Sampling was performed three times, first sampling conducted between day one until day three of fever/ acute phase, second sampling between day four until six of fever (critical phase), third sampling conducted at day seven of fever or later (convalescent phase). Anticoagulant used was K3 EDTA. Blood with EDTA initially was examined by hematology analyzer to

get hematocrit and platelets count data. Level of F1.2 was analyzed using EDTA plasma which were centrifuged at a speed of 1000 g for 15 minutes at 2-8°C. The plasma was separated within 4 hours since sampled.¹⁴ The plasma was stored at minus 80°C (stable for 6 months). Hemolysis, icteric, and lipomic plasma were excluded from the study.

Plasma which was stored at -80°C, must be put at room temperature at least 30 minutes before analyzed, F1.2 test was done within 2 hours after thawing. Multiple freeze thawed was not allowed. Data of patient's hematocrit value, albumin levels, thoracic X-ray were used to classify patients into groups with and without plasma leakage.

F1.2 level measurement

First, calibration curve was made, then within run precision test was done, after that the F1.2 levels were analyzed. The level of F1.2 was performed using reagents USCN Life Sciences. The principle of F1.2 test was sandwich enzyme immunoassay (ELISA). F1.2 in patient samples formed complex with monoclonal anti- F1.2 antibody precoated in wells. The complex was detected using biotin-conjugated specific F1.2 antibodies. This complex was binded to avidin-conjugated with horse radish peroxidase (HRP). After TMB substrate solution is added, only those wells that contain F1+2, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of F1+2 in the samples is then determined by comparing the O.D. (optical density) of the samples to the calibration curve. The calibration curve was made using Magellan computer program with logarithmic regression techniques.

Hematocrit value analysis

EDTA anticoagulated blood samples were analyzed by automated blood cell analyzer Sysmex XE-2100 within 1 hour of sampling. The instrument used hydrodynamic focusing method and hematocrit value was calculated with pulse height RBC detection method.

Platelet count

EDTA anticoagulated blood samples were analyzed by automated blood cell analyzer Sysmex XE-2100 within 1 hour of sampling. The instrument used

hydrodynamic focusing method and platelet count was calculated by impedance method.

Albumin level measurement

Serum samples were analyzed by automated clinical chemistry analyzer, Cobas C 501 within 2 hours of sampling. The instrument used colorimetric method.

NS1 antigen rapid test

Plasma EDTA was analyzed by immunochromatography method with SD diagnostic NS1 rapid test.

IgG/IgM anti Dengue rapid test

Plasma EDTA was analyzed by immunochromatography method with SD diagnostic IgG/IgM anti Dengue rapid test.

Statistical analysis

The Statistical Product and Service Solutions (SPSS) ver. 17 was used for computation. The demographic data was presented descriptively. A two sided P-value <0.05 was considered statistically significant. Shapiro Wilk test was used to determine the distribution of the data. When the data was normally distributed, F1.2 levels differences between subjects with and without plasma leakage was analyzed by unpaired t test. If the distribution was not normal, it would be analyzed with the Mann - Whitney test. When the data was normally distributed, differences between critical phase, and convalescent phases were analyzed by paired t test. If the distribution was not normal, it would be analyzed with the Wilcoxon test. When the data was normally distributed, the correlation between platelet count and F1.2 levels was analyzed by Pearson correlation test. If the distribution was not normal, it would be analyzed by Spearman correlation test.

RESULTS

As an initial examination, the calibration curve was made from 7 standard levels of F1.2 which is 1000 pg/ml, 333.33 pg/ml, 111.11 pg/mL, 37.04 pg/mL, 12.35 pg/mL, 4.12 pg/mL, and 1.37 pg/mL, plus a blank that contains only standard diluent so that the levels are 0 pg/ mL. Accuracy test was conducted using a sample of patients who were examined five times, CV was 9.5%. According to the insert kit of USCN Life Science, CV < 10 % means that the results can be accepted.

Twenty subjects were enrolled in this study, consisted of 10 subjects with plasma leakage and 10 without plasma leakage, 6 pairs of samples in critical phase and convalescent phase, 26 samples for correlation test between F1.2 level and platelet count. Subject's characteristics could be seen at table 1.

In this study, it was found that the F1.2 level in patients with plasma leakage (number of samples(n)=10, mean ± 2 SD) 147.4 ± 105.82 pg/mL is significantly

higher compared to patients without plasma leakage (n=10) 51.3 ± 39.92 pg/mL (Fig.1.), and the F1.2 level in critical phase (n=6) has a median of 186.3 (108.6-223.2) pg/mL which is significantly higher compared to convalescent phase (n=6) 46.5(27.4-51.9) pg/mL (Fig 2.). Also it was found that a medium negative correlation between F1.2 level and the thrombocyte count existed (n=26), r = - 0.609 (Fig 3).

Table 1. Respondent characteristics

Subject	Number	Male	Female	Ages (year)	Platelet count (cell/ μ L)
Without plasma leakage	10	3	7	Median 30 (15-56)	-
With plasma leakage	10	4	6	Median 25 (15-67)	-
Comparison between critical phase and convalescent phase	6 people, 12 samples	1	5	16-58	-
Correlation between F1.2 level with platelet count	26 samples	8	18	15-67	13 500-320 000

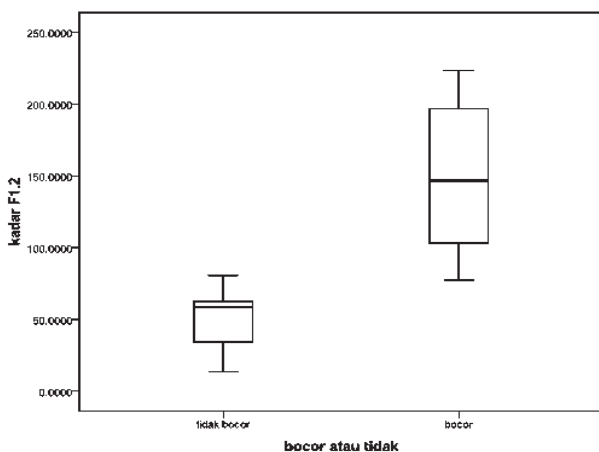


Figure 1. Comparison between F1.2 levels in patient infected with Dengue virus, without and with plasma leakage.

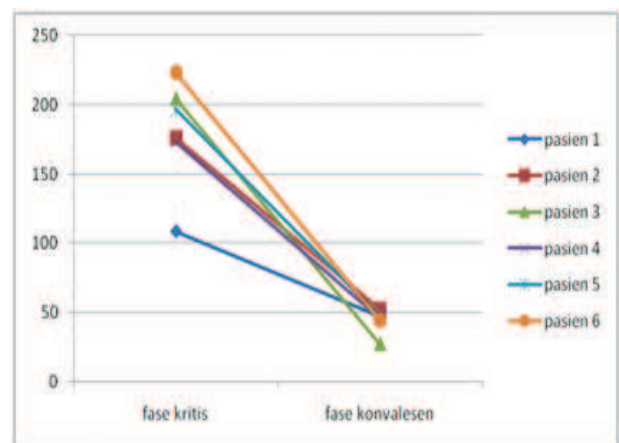


Figure 2. F1.2 level in critical phase and convalescent phase

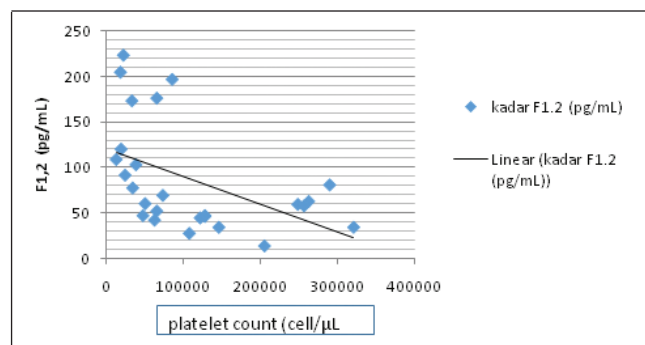


Figure 3. F1.2 level correlation with platelet count in subjects infected with Dengue virus

DISCUSSION

The purpose of the study was to search whether there was association between increased production of thrombin with plasma leakage and thrombocytopenia in patients infected with dengue virus. Thrombin levels were represented by the F1.2 levels because thrombin was bound by anti-thrombin and thrombomodulin rapidly. The hypothesis in this study is when F1.2 levels increased, the plasma leakage and thrombocytopenia occurred in dengue virus infection.

In this study, the F1.2 levels assays within run precision performed 5 times in a row on the same day using one sample. F1.2 levels of test accuracy are not done because the control material for F1.2 is not available. However the assay was done at the same time as the standard, so we can be sure of the accuracy. The coefficient of variation (CV) obtained was 9.5 %, lower than the limit stated in the leaflet (CV is < 10 %), so we concluded that the test results can be accepted.¹⁵ The F1,2 assay was done manually, highly dependent on operator skill, it can caused relatively high CV value.

Initially, we conducted a pilot study with 20 subjects consisting of 10 subjects with plasma leakage and 10 subjects without leakage of plasma to obtain a combined standard deviation . After we calculated sample size for unpaired t test, the required sample size was 38 subjects with plasma leakage and 38

subjects without plasma leakage. The number of samples collected still insufficient. However, the differences in F1.2 levels between the 2 groups in this study were significant.

Most subjects were female. According to WHO report for 6 Asian countries namely Malaysia, Philippines, Singapore, Laos, Cambodia, Sri Lanka, there was a tendency that Dengue virus infection in the population ≥ 15 years is more common in men.¹⁶ But study by Hung et al among 245 infants infected with virus dengue, there is no difference between gender on the incidence of DHF / DSS.¹⁷ The difference in this study is likely due to limited number of subjects. The age of the study subjects was 15-67 years with a mean of 30 years in the group without leakage of plasma, while the median age of the group with plasma leakage is 25 years old.

Patients without plasma leakage usually come to the RSCM at the first day until the fourth day of fever, usually without thrombocytopenia, so the patients weren't hospitalized. Patients with plasma leakage usually come to RSCM on days 4-6, referred from another hospital and were in critical phase, thus we couldn't get acute phase samples from the group with plasma leakage. The convalescent phase sample from the group with plasma leakage was taken on day 7th of fever and thereafter. It was thought that F1.2 levels obtained described condition of the patients when recovered from illness.

Table 2. F1.2 levels comparison between studies

Researcher	Research subjects	N	Method	F1.2 level (nmol/L)
Bauer et al ^{14, 18}	Health population	31	RIA, polyclonal antibody	1,5±0,7
Hurstinget al ¹⁸	Health population <44 yrs old	278	ELISA, monoclonal antibody, Organon	0,21-2,78
Krishnamurtiet al ¹⁹	Children with DF/DHF in Thailand (acute phase samples)	12	ELISA, monoclonal antibody,	DF 2,93±0,46
		6	Enzygnost	DHF1 4,3±0,84
		15		DHF 2 3,6±0,37
Suhartiet al ⁶	Children with Dengue shock syndrome	3		DHF 3 3,82±0,85
		50	ELISA, monoclonal antibody, Enzygnost	At entry
				3,2(1,2-14,2)
				1 st day of fever
This study	DF/DHF patients in Indonesia >15 yrs old			2,6(0,9-40,8)
				2 nd day
				2,7(0,8-21,4)
		10	ELISA, monoclonal antibody, USCN Life Sciences	Without plasma leakage
		10		1,64 (0,36-2,94)
				With plasma leakage (critical phase)
				4,72 (1,33-8,12)
		6		With plasma leakage (convalescens phase)
				1,4 (0,87-1,66)

The mean F1.2 levels in the group with plasma leakage were significantly higher than the group without plasma leakage. This means an increase in coagulation activation in the critical phase of dengue virus infection resulting in leakage of plasma. Although according to the calculation of sample size, the sample is not sufficient, however, because the results showed significant differences that do not need to increase the number of samples.

Comparison of F1.2 levels of this study with other studies can be seen in Table 2 below. Because other researchers discussed the examination F1.2 levels using nmol/L, the results F1.2 levels in this study was converted to nmol/L, they were divided by the molecular weight of 31.2 kDa.

The range of F1.2 levels in the group without plasma leakage was similar to the healthy population. This means that the activation of coagulation in the group without plasma leakage similar to the healthy population. F1.2 levels in the group with plasma leakage in the critical phase was much increased compared to the group without plasma leakage, meaning here was an increase in the activation of coagulation. Then the coagulation activation decreased in convalescent phase, as evidenced by decreased levels of F1.2.

These results were comparable with Krishnamurti's studies in which F1.2 levels in patients without leakage of plasma were lower than those in patients with DHF. Other studies in patients with DHF performed in pediatric patients, whereas this study used subjects aged ≥ 15 years. All research methods using ELISA method, except Bauer et al who used RIA. All the data above proved that there was activation of coagulation resulting in increased levels of thrombin in the critical phase, finally resulting in leakage of plasma. Thrombin can bind to PAR 1,3,4 on vascular endothelial thereby increasing capillary permeability and result in plasma leakage.⁸

There are other studies that seemed to contradict the results of this study. Research by Orsi et al showed thrombin level's mean in Dengue patients with bleeding was 22745 nm, in those without bleeding was 33675 nm and in healthy controls was 37534 nm. The assay of thrombin level was done by kinetic method and read by fluorometer. Orsi et al concluded that there had been a thrombin production disruptions resulting in pendarahan,²⁰ contrary to the conclusions of this study but it should be noted that the study patient samples were taken after the 5th day of fever while in this study, the samples from critically ill

patients were taken on the 4th or 5th day of fever. The explanation, the levels of thrombin decreased after 5th day of fever as evidenced in this study, F1.2 levels decreased in the convalescent phase .

In this study, F1.2 levels in the critical phase was significantly greater than those at convalescent phase proved that in critical phase thrombin production increased and then decreased in the convalescent phase. Increased production of thrombin is likely to be one of the causes of plasma leakage.

Data levels of F1.2 in all groups were analyzed for correlation with platelet counts. Using the Spearman test, negative correlation was found between platelet counts and F1.2 levels significantly. The higher levels of F1.2 meant stronger activation of coagulation occurred and caused lower number of platelets. This provided evidence of the possible influence of thrombin as a cause of thrombocytopenia. This is consistent with the theory about stimulated platelet activation by thrombin via PAR receptors 1 and 4. Activation of platelets causes an increase in platelet aggregation that resulting intrombocytopenia.^{8,21}

In conclusion, the results of the study demonstrated that there was increased coagulation activation at critical phase in patients infected with Dengue virus, as shown by F1,2 as indicator, associated with plasma leakage and thrombocytopenia. We suggest to do research on the strength of association of other factors causing plasma leakage and thrombocytopenia in dengue virus infected patients.

Statement

No conflict of interest.

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