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Elevated Serum Levels of Matrix Metalloproteinase-9 (MMP-9) in Severe Dengue Virus Infection at Sanglah Hospital Denpasar, Bali - Indonesia

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ABSTRACT

Background: Dengue virus - infected dendritic cells along with cytokines overproduction trigger endothelial barrier dysfunction and plasma leakage through matrix metalloproteinase (MMP)-9 overproduction. The event is responsible for more severe dengue virus infection's manifestation. The study objective is to measure the association of MMP-9 serum level with disease severity of dengue virus infection.

Method: Prospective study of 70 participants hospitalized in Internal Medicine ward of Sanglah Hospital Denpasar, during 1 July 2011 – 31 December 2011 and diagnosed as dengue virus infection were followed during their hospital stay. Baseline demographic were obtained on admission; serum level of MMP-9 was measured once at third – fifth day from fever onset. Independent-samples t-test was used to compare the MMP 9 levels among diagnosis groups and exploration of variables were using logistic regression analysis.

Result: There was significant higher level of MMP-9 serum level on DHF (median [range]) compared to DF (367.78 ng/mL [81.16 – 797.79] vs.128.67 ng/mL [41.79 – 327.32]; p< 0.0001). After adjusted with baseline hematocrit, for every 10 ng/mL elevation of MMP-9 serum contributes to 1.159 increased risk of DHF with optimal threshold MMP-9 level is 330 ng/mL (100 % sensitivity, 64% specificity).

Conclusion: MMP-9 level is associated with dengue severity. The measurement was able to predict the DHF among those infected with dengue virus infection. This may lead to a novel marker of dengue hemorrhagic fever predictor.

Keywords: MMP-9, Dengue Fever, Dengue Hemorrhagic Fever, severity risk

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INTRODUCTION

Dengue viruses' infection are one of emerging arthropod – borne viral diseases, confined as a group of four serologically distinct but related genus *Flavivirus*, family Flaviviridae.[1-4] Current estimates of 50 to 100 million infections worldwide each year and over 2.5 billion individuals at risk for dengue infection especially in the many part of tropics and subtropics where dengue is endemic.[5,6]

Dengue virus infection results in wide spectrum of clinical manifestations, ranging from asymptomatic to dengue hemorrhagic fever (DHF) with plasma leakage as the distinguished hallmark leading to dengue shock syndrome as its severe form.[7,8] Dengue cases traditionally classified as dengue fever or dengue hemorrhagic fever by World Health Organization (WHO) 1997 scheme. Newest WHO guidelines divided the cases of dengue virus infection based on the presence of warning sign which more practical use in clinical decision.[9-11]

The exact mechanisms of endothelial barrier dysfunction that lead to increased vascular permeability and plasma leakage in DHF are poorly understood. Endothelial cells with positive viral antigen in tissue had minimal or without morphological damage, usually limited to swelling and occasional intercellular gaps.[12-14] The increased vascular permeability leading to plasma leakage develops suddenly during viral clearance phase in early symptoms recovery by the time of significant immune activation. Plasma leakage initially occurs focal and predominantly in the pleural and peritoneal spaces but also maybe extensive in various tissue spaces and body cavities.[15-17] The most favorable theory for increased vascular permeability is the enhancing antibodies and memory T-cells in a secondary infection resulted in cytokine tsunami and transient compromise of endothelial barrier.[18-20]

Matrix metalloproteinase 9 (MMP-9), a peri/extracellular Calcium dependent–Zinc containing endopeptidase, is excreted by a variety of connective tissue and pro – inflammatory cells including fibroblast, osteoblast, endothelial cells, macrophages, neutrophils and lymphocytes. Like other proteolytic enzymes, MMP-9 is first synthesized as inactive proenzyme. The activation is mediated by plasminogen activator/plasmin system, and the regulation of its activity is controlled by Tissue Inhibitors of Metalloproteinase-3 (TIMP-3).

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MMP-9 expression is regulated by several cytokines and growth factors. MMP-9 primary function is degradation of proteins in extracellular matrix, so physiologically as well as in pathological process, MMP-9 in coordination with other MMPs play role in tissue remodeling and degradation of extracellular matrix events.[12]

Dengue viruses target endothelial cells and monocyte-derived cells such as dendritic cells as antigen presenting cells. The dengue infected dendritic cells and uninfected but dengue exposed dendritic cells will undergo activation which triggers their maturation followed by released of pro-inflammatory cytokines and chemokines. Those dendritic cells migrating trans-endothelial to lymph nodes and prime T-cells through Major Histocompatibility Complex (MHC) type 1 and 2, initiating the adaptive immune responses. Dengue virus infected dendritic cells also secreting matrixmetalloproteinases especially MMP-9 and in less extent MMP-2, and along with up regulation expression of MMP-9 by connective tissue, endothelial cells and other pro inflammatory cells during cytokine tsunami, will enhance the extracellular remodeling. The event will be followed by reduced expression of the endothelial adhesion molecule 1 (PECAM-1) and vascular endothelium (VE)-cadherin cell adhesion molecules, causing a redistribution of F-actin fibers and the main effect be resulted in compromised endothelial permeability.[19-21]

Very few studies exist in this area and among those have had conflicting results, some suggesting that MMP-9 overproduction present during critical phase of dengue virus infection[20-22], whereas others found that MMP-9 have no role in pathogenesis of dengue related illness.[23] To address this problem, we documented the MMP-9 serum level during critical phase of dengue infection among adult patient. This study focused on assessing correlation and role of MMP-9 serum level with manifestation of DV infection among adults.

MATERIAL AND METHODS

We enrolled a cohort of patients with acute fever, hospitalized during 1 July 2011 – 31 December 2011 and diagnosed with dengue virus infection, based on 1997 WHO Dengue Guidelines. Patients were subsequently followed during their hospital stay. The study was approved by the ethical committee of Medical Faculty – Udayana University/Sanglah Hospital on the Ethics of Human Research. Written informed consents were signed before enrollment. The patients who fulfilled the inclusion and exclusion criteria were enrolled as participants. The inclusion criteria were: (1) male or female patients confined in Internal Medicine wards, aged >12 year - 60 years old who were hospitalized due to acute fever during the study period; (2) patients fulfilling the case definition for dengue virus; acute febrile illness lasting 2 – 7 days with two or more the following symptoms': headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestation, leucopenia and seropositive serology; and (3) understanding of the study and signing the inform consent form; for participant aged < 18 year old, the consent signed by parents or legal guardian. The exclusion criteria included (1) prior medical history, investigator review and physical examination according to standard practice, with concomitant condition(s) of malignancy, recent myocardial infarction, congestive heart failure, neurologic disorder and concomitant use of medication for treating dyslipidemia; (2) psychiatric disorder by investigator interview and physical examination according to standard practice that in the judgment of the investigator would interfere with ability to give informed consent were excluded.

Dengue hemorrhagic fever was defined as: cases with fever, or history of acute fever, lasting 2–7 days, occasionally biphasic and hemorrhagic tendencies, evidenced by at least one of the following: (1) a positive tourniquet test; (2) petechiae, ecchymosis or purpura; (3) bleeding from the mucosa, gastrointestinal tract, injection sites or other locations; (4) hematemesis or melena; Thrombocytopenia (100,000 cells per mm3 or less) and evidence of plasma leakage due to increased vascular permeability, manifested by at least one of the following: (1) a rise in the HCT equal to or greater than 20 percent above average for age, sex and population; (2) a drop in the HCT following volume-replacement treatment equal to or greater than 20 percent of baseline; or (3) signs of plasma leakage such as pleural effusion, ascites and hypoproteinemia. We ascertained the participants' baseline characteristic with a written questionnaire. Evaluation of vital sign and monitoring the complete blood count were evaluated according to the local protocol. CBCs were drawn within an interval at least 12 hours or sooner depend on participants' clinical condition.

Serum level of MMP-9 was obtained from venous blood sampling drawn on the third, fourth or fifth day from fever onset. MMP-9 was measured by ELISA using *Quantikine Human MMP-9 (total) Immunoassay DMP900, R&D Systems Inc., Minneapolis United State of America* with minimal detected value 0.156 ng/mL. Anti-dengue Ig-G and Ig-M serology obtained from venous blood sampling was drawn on the seventh day from onset of fever. The anti-dengue serology was measured

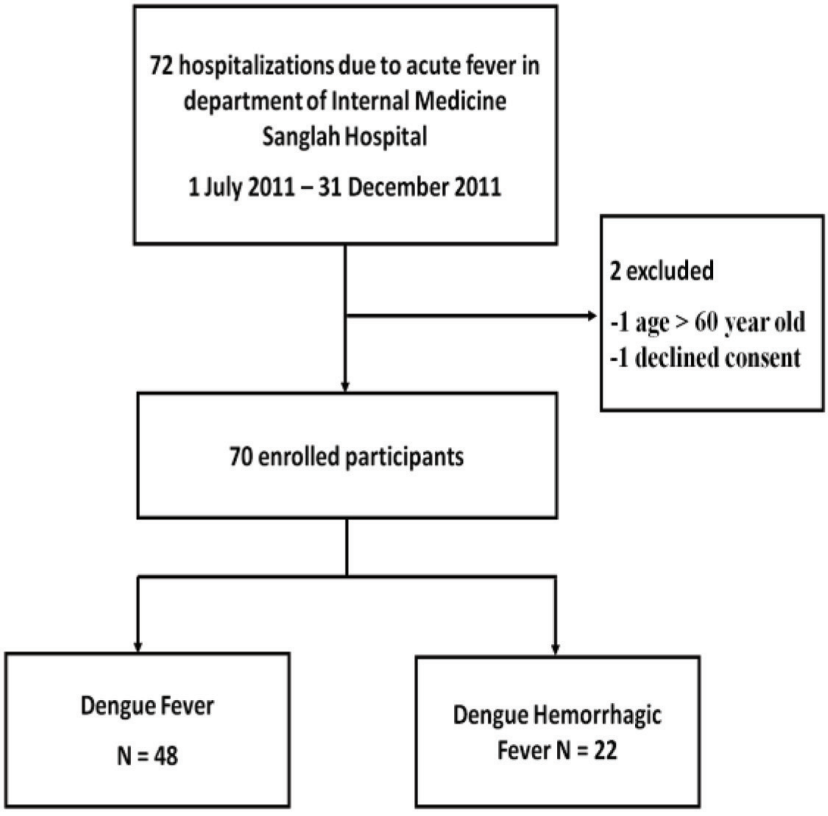

Figure 1 Selection of study sample

by using immunochromatography method. Descriptive analyses were assessed with frequency histograms and statistical analysis for normality. An independent-sample t test was used to compare the MMP 9 levels among DF vs DHF groups and the strength of prediction calculated using logistic regression analysis by IBM SPSS Statistics for Windows (version 21) (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Descriptive analysis

From 72 cases with acute fever, we enrolled 70 participants (Figure 1.) with median onset of fever 4 days (3 – 5 days) prior to the admission, and of those 40 (57%) were male. The age (mean ± SD) of participants was 30.50 ± 10.78-year-old. On admission, 80% (56/70) participants presented with positive tourniquet test and 20% (14/70) with spontaneous bleeding. The spontaneous bleeding was manifested as petechiae (3; 21.43%), epistaxis/gum bleeding (6; 42.86%), vaginal bleeding (4; 28.57%) or gastrointestinal bleeding (1; 7.14%). During the hospital stay, the median of lowest recorded platelets was 72.500/µL (3.000/µL – 115.000/µL), occurred on median day 5 (3 – 7) from fever onset. All participants were followed during their hospital stay with median length of stay 5 days (3 – 8). Based on serology results drawn on day 7 from fever onset, the final assessment revealed more than one third of cases were DHF (22/70; 31.40%) of which almost two thirds (52/70; 74.30%) were secondary infection (Table 1).

Table 1 Demographic Features of 70 Dengue Virus Infection Cases

Variables	Value
Malen/total (%)	40/57.1
Age in year; mean ± SD	30.5 (12~59)
Onset of fever in day; median (range)	4 (3~5)
Length of hospital stay in day; median (range)	5 (3~8)
Bleeding manifestation/total (%)	
Positive tourniquet	56/80
Spontaneous bleeding	14/20
Spontaneous petechiae	3/4.3
Epistaxis, gum bleeding	6/8.6
GI bleeding	1/1.4
Vaginal bleeding	4/5.7
MMP-9 Level in ng/mL; median (range)	154.8 (41.8~797.8)
Baseline Hematocrit in %; mean ± SD	41.2 (33.3~52.1)
Lowest platelet count /µL; median (range)	72.5 (3~115)
Time of lowest platelet count in day; median (range)	5 (3~7)
Final assessment/total (%)	
DF	48/68.6
DHF	22/31.4
Nature of infection/total (%)	
Primary	18/25.7
Secondary	52/74.3

During median observation of 5 days, the participants diagnosed with DHF had significantly higher MMP 9 serum level (median [range]) compared with DF participants (367.78 ng/mL [81.16 – 797.79] vs.128.67 ng/mL [41.79 – 327.32]; p< 0.0001) (Figure 2; Table 2).

Bivariate and Multivariate Analysis

The bivariate analysis predicts that the odds for DHF are 3.698 higher for males than they are for females with probability 42.52% of male participants being diagnosed with DHF and 16.67% probability for

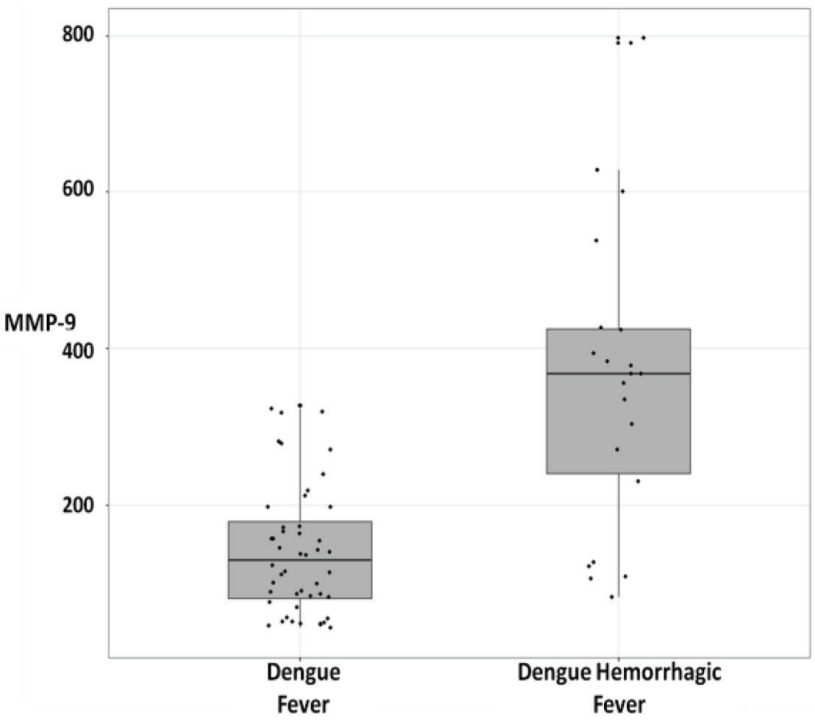

Figure 2 Participant with Dengue Hemorrhagic Fever have higher MMP-9 compared to Dengue Fever. The difference was significant.

Table 2 Variable comparison among Dengue Fever Vs Dengue Hemorrhagic Fever

Variable	DHF	DF	P value
MMP-9 (ng/mL)	367.78 (81.16–797.79)	128.67 (41.79–327.32)	<0.0001
Baseline HCT (%)	43.39 ± 4.48	40.20 ± 4.37	0.0063
Gender *	0.77	0.48	0.0212
Age (year)	30.6 ± 9.87	30.4 ± 11.27	0.9435
Nature of Infection*	0.36	0.21	0.1675

*Percentage of Male; Percentage of primary infection;

Table 3 Independent Predictors of Dengue Hemorrhagic Fever among 70 Participants

	Bivariate analysis			Multivariate analysis		
Predictors*	**OR**	**95% a**	**P**	**OR**	**95% CI**	**P**
MMP-9 level (per 10 ng/mL)	1.130	1.065-1.198	<0.0001	1.159	1.072-1.253	0.0002
Baseline H(T (per 1 %)	1.177	1.019—1.332	0.0101	1333	1.065-1.668	0.0121
Gender Male (vs Female)	0.271	0.II86-0.852	0.0255			
Age (per 1 year old}	1.002	0.956-1.050	0.9124			
2 DV infection (vs. 1 infection)	2.172	0.713-6.612	0.1722			

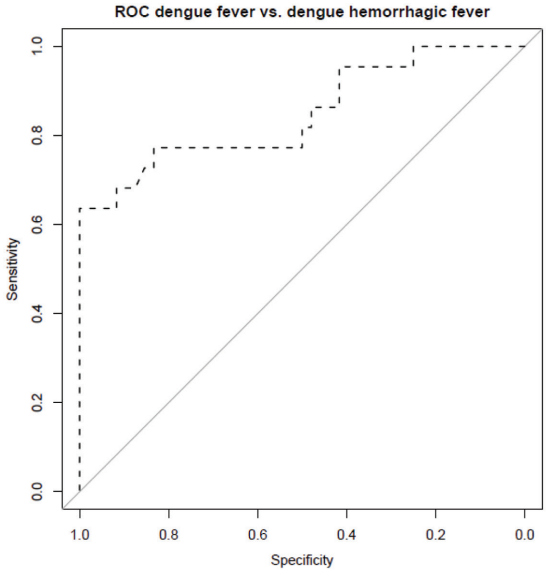

Figure 3 The ROC curve of Dengue virus infection

females. Every 10 ng/mL elevation of MMP-9 serum level is associated with 1.130 higher risks for DHF with overall predictive accuracy 84.3% (sensitivity 91.7%, specificity 68.62%). Analysis on initial Hematocrit serum level showed that 10% increase in HCT is associated with 5.10 fold higher risks for DHF (Table 3).

*MMP-9, Matrix Metalloproteinase 9; HCT, Hematocrit; DV, Dengue Fever; OR, Odds Ratio; CI, Confidence Interval; p, significance value

Serum MMP-9 levels were associated with the risk for DHF manifestation (OR = 1.159; 95% CI = 1.072 – 1.253; p = 0.0002) adjusted with baseline Hematocrit level. The results indicate that every 10 ng/mL elevation of MMP-9 associated with 1.159 times increase in risk for DHF (Table 3). Optimal sensitivity and specificity for MMP-9 serum level's threshold is 330 ng/mL (sensitivity 100%, specificity 64%) (Figure 3).

DISCUSSION

Conventionally, pathogenesis of dengue hemorrhagic fever involves activation of innate and adaptive immune response which results in production of several kinds of cytokines. Among those cytokines produced by immune cells are IL-1β, IL-8, TNF-α, and MIP-1β. These cytokines cause vasodilatation and increased permeability of capillary endothelium. However, recent studies shown that at some point, there are non-cytokines factors that take part in DHF pathogenesis especially in plasma leakage.[22,23]

Particular attention was focused on MMP-9, a proteolytic enzyme secreted in inflammatory response. Several studies had pointed to its

potential contribution in pathogenesis of DHF especially in plasma leakage process.[21,23,24] Kubelka et al. reported that production of MMP-9 was elevated in dengue fever.[24] It also shown that MMP-9 level was associated with disease severity in adult patients. On the other hand, Vorapani et.al found no significant association between serum MMP-9 levels with disease severity in children.[25] Apparently, there is a difference between DHF pathogenesis in adult and children. However, the number of samples in Vorapani study is considered too small so the result is needed to interpret carefully.

Meanwhile, in this study, we found that plasma MMP-9 was strongly associated with DHF diagnosis. Furthermore, MMP-9 appear to be independent risk factors albeit with only with slightly but significant increase in risk of hemorrhagic. The theoretical basis of this phenomenon is although MMP-9 contribute significantly to increased permeability of endothelium, it need to work in conjunction with other factors such as pro-inflammatory cytokines and complement system in order to efficiently increase vascular permeability.[23] The evidences of this interaction are highlighted in several researches.[21-24] However, based on ROC curve, MMP-9 appears to had fairly good predicting value to predict the occurrence of plasma leakage and, hence, differentiate dengue fever from dengue hemorrhagic fever.

Despite the promising result of our study, we identify several limitations of our study namely frequency of MMP-9 serum measurements and the number of participants. MMP-9 serum measurements were made only once, since we did not have sufficient resources to make more frequent measurements. Larger samples should be enrolled over one-year period in order to better describe the seasonal effect on dengue viral infection and to understand the role of MMP-9 on different stages of DHF severity.

CONCLUSION

Our study indicate that clinicians should consider the use of MMP-9 serum evaluation in patient presenting with acute fever as early as the third day of fever onset for early detection of more severe form of dengue infection. MMP-9 measurement provides an opportunity for early identification of more severe form of DV infection and will provide better tools for patient assessment and management. Further studies needed to validate the finding, including more cases with multiple time measurement of MMP-9.

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