Research Article

MCP-1 Levels and Atypical Lymphocytes in Early Fever of Dengue Virus Infection with Non-Structural Protein 1 (NS-1) Antigen Test in Dr. Darsono Hospital, Pacitan

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ABSTRACT

Dengue infection caused by DENV and transmitted by mosquitoes Aedes aegypti and Aedes albopictus is a major health problem in the world, including Indonesia. Clinical manifestations of dengue infection are very widely, from asymptomatic until dengue shock syndrome (DSS). DENV will attack macrophages and dendritic cells (DC) and replicate them. Monocytes are macrophages in the blood (± 10% leukocytes). Macrophages produce cytokines and chemokines such as monocyte chemotactic protein-1 (MCP-1)/CCL2. The monocytes that are infected with DENV will express MCP-1, which will increase the permeability of vascular endothelial cells so that they have a risk of developing DHF/DSS. Macrophages and DC secrete NS1 proteins, which are the co-factors that are needed for viral replication and can be detected in the early phase of fever. The increased MCP-1 levels in dengue infection followed by an increase in the number of atypical lymphocytes indicate the arrival of macrophages and monocytes to the site of inflammation which triggers proliferation rather than lymphocytes. This is an observational analytical study with a cross-sectional design to determine the MCP-1 level in dengue infection patients with 1st until the 4th day of fever and the presence of a typical lymphocytes. Dengue infection was determined by rapid tests NS1 positive or negative and MCP-1 levels were measured using by ELISA sandwich method.MCP-1 level of sixty patients dengue infection NS-1 rapid positive or negative with 2nd until 4rt fever were significantly higher than healthy subjects (420.263 ± 158,496 vs 29, 475 ± 23.443;p=0.000), but there was no significant difference in subjects with DF, DHF or DSS (436,47 ± 225,59 vs 422,77 ± 170,55 vs 448,50 ± 117,39; p =0.844). A typically lymphocytes differs significantly in healthy subjects than subjects infected with DENV an average of 2% (p= 0,000). In conclusion, this shows the arrival of macrophages and monocytes to the site of inflammation, which triggers the proliferation of lymphocytes.

Keywords: MCP-1, Atypical lymphocytes, NS-1, Hematology parameter, Pacitan

ABSTRAK

Infeksi dengue disebabkan DENV ditularkan oleh nyamuk Aedes aegypti dan Aedes albopictus yang masalah kesehatan utama di dunia, termasuk Indonesia. Manifestasi klinik infeksi dengue sangat bervariasi, dapat asimptomatik sampai dengue shock syndrome (DSS). DENV akan menyerang makrofag juga sel dendritik (DC) dan akan bereplikasi. Monosit merupakan makrofag dalam darah (±10% leukosit). Makrofag memproduksi sitokin dan kemokin seperti MCP 1. MCP-1 terekspresi oleh monosit terinfeksi DENV yang dapat meningkatkan permeabilitas sel endotel vaskular sehingga memiliki risiko mengalami DHF/ DSS. Makrofag dan DC mengeluarkan protein NS1 merupakan co-factor yang dibutuhkan untuk replikasi virus dan dapat dideteksi pada fase awal demam.

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Adanya peningkatan kadar MCP-1 pada infeksi dengue diikuti dengan peningkatan jumlah limfosit biru menunjukkan datangnya makrofag dan monosit ke tempat terjadinya inflamasi yang memicu proliferasi daripada limfosit. Penelitian ini merupakan penelitian observasional analitik dengan desain potong lintang untuk mengetahui kadar MCP-1 pada subyek terinfeksi dengue dengan NS-1 positif pada hari demam ke 1-4 dan adanya limfosit plasma biru. Subyek penelitian adalah pasien dewasa terinfeksi dengue dengan hasil rapid tes NS-1 positif dan negatif. Kadar MCP-1 pada serum/ plasma diukur menggunakan metode ELISA sandwich. Subyek penelitian terdiri 50 pasien infeksi dengue dengan NS-1 rapid tes positif dengan demam hari kedua sampai hari keempat memiliki kadar MCP-1 lebih tinggi dibandingkan subyek sehat (420.263 ± 158,496 vs 29, 475 ± 23.443;p=0.000), dan tidak ada perbedaan bermakna kadar MCP-1 subyek dengan DF, DHF dan DSS (436,47 ± 225,59 vs 422,77 ± 170,55 vs 448,50 ± 117,39; p =0.844). Limfosit plasma biru berbeda bermakna pada subyek sehat dengan subyek terinfeksi DENV rata-rata 2% (p= 0,000). Kesimpulannya, hal ini menunjukkan datangnya makrofag dan monosit ke tempat terjadinya inflamasi yang memicu proliferasi daripada limfosit pada infeksi dengue.

Kata kunci: MCP-1, Limfosit Plasma Biru, NS-1, Hematology parameter, Pacitan

INTRODUCTION

Dengue virus (DENV) is a Flaviviridae family and the Flavivirus genus. DENV is a positive RNA virus that contains envelopes with genomes ~10.7 kb in four serotypes (DENV1-4). DENV infection is an acute disease caused by one of the four viral serotypes of the genus Flavivirus, Flaviviridae family, transmitted by mosquito bites Aedes aegypti and Aedes albopictus[1,2] and in some cases developed until dengue hemorrhagic fever (dengue hemorrhagic fever/ DHF) or even until dengue shock syndrome (DSS).[3]

Dengue virus infection cause is asymptomatic or symptomatic infections, from undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS) and expanded dengue syndrome.[4,5] Dengue disease may manifest with gastrointestinal symptoms which may make diagnosis and treatment difficult and wrong.[6] In Indonesia, there were 68,407 cases of dengue hemorrhagic fever in 2017 compared to 2016 with 204,171 cases with the highest cases in the provinces of West Java, Central Java, and East Java. The highest mortality dengue in 2017 in the East Java province with the death rate or Case Fatality Rate (CFR) of 2017 DHF by 1.3%.[7] Pacitan in 2016 there were 1,338 DHF sufferers with a population of 552,327 DHF (Incidence Rate = IR) morbidity amounted to 242.3 per 100,000 population and there was 1 death from dengue fever.[8] This prospective cohort study in West Java provides several important findings on the epidemiology of dengue virus infections in adults living in an endemic area. First, the dengue virus is a major etiology of febrile illness (12.4%) in adults in Bandung, West Java, Indonesia.[9]

DENV nonstructural protein (NS)-1 is a diagnostic marker to early detection of DENV compared to serological tests because it is detected in serum Patients infected with DENV as early as one day after the appearance of day post onset symptoms (DPO). DPO up to 18 at a concentration of NS-1 up to 50 µg/mL.[10] The NS-1 is a glycoprotein with two glycosylation sites that are conserved among flaviviruses. It is synthesized in the ER as a hydrophilic monomer but exists as a more hydrophobic homodimer. The NS-1 dimer is transported to the Golgi apparatus where it undergoes carbohydrate trimming. The role of NS-1 in virus replication is unknown but is believed to facilitate viral infection and DENV pathogenesis. NS-1 is in addition secreted from infected cells (SNS-1) and has been shown to be immunologically important.[1]

During natural dengue infection in humans, the mosquito delivers virus in skin epithelium where it infects and replicates in the cells of mononuclear lineage like monocytes, dendritic cells, macrophages, and Langerhans cells.[11] Dengue virus later attached to monocytes through the receptor factor and into monocytes. In this

situation, there is an afferent mechanism in which the virus has been developed through unification and attachment of several gene segments and receptor factors are developed. Furthermore, efferent mechanisms occur, namely monocytes containing distributed viruses.[12] These infected monocytes carry the virus to the lymph nodes where it replicates resulting in viremia followed by systemic infections of the liver, lungs, and spleen.[11] The migration of these infected cells around the lymphatic system triggers the production of cytokines and the recruitment of other immune cells. These include monocytes and macrophages, which are the primary target of infection and the main site of DENV replication.[13]

Two mechanisms of immunity are considered responsible the first occurrence of dengue hemorrhagic fever is *a nonneutralizing antibodies* produced from previous infections believed to increase viral replication by connecting the virion with the Fc receptor on the surface of the target cell, which then carries it into the cytoplasm, thereby increasing the number of infected cells, the number of virus particles entering each cell and the release of cytokines and other vasoactive mediators. Second, CD8+ T reactive memory cells can attack monocytes and macrophages that express viral epitopes on their surface, triggering an explosive inflammatory response.[14]

Dengue infection induces the overexpression of many chemokines and cytokines in monocytes, such as *Tumor Necrosis Factor* (TNF)-α, IFN-γ, IL-1β, IL-8, IL-12, *Macrophage inflammatory protein* (MIP)-1α, MCP-1/ CCL(Chemokine(C-C motif)ligand)2, and RANTES (regulated upon activation, normal T-cell expressed and secreted). MCP-1 levels in the plasma of DF and DHF patients were increased significantly.[15] *Monocyte Chemoattractant Protein(MCP)-1/ CCL2* is chemokine which regulates the movement of monocytes/macrophages.[16] The production of MCP-1 and *monokine induced by gamma interferon* (MIG) from monocytes or macrophages could be induced by interferon (IFN)-γ upon DENV infection in order to recruit more leucocytes to the site of infection for viral clearance.[17] DENV-infected monocytes

enhance functional regulation of caspase-1 mRNA and activation of procaspase-1 in late response to infection responsible for excretion of interleukin(IL)-1β and pyroptosis from DENV-infected monocytes. Late activation of caspase-1 in monocytes infected with DENV can contribute to pro-inflammatory results that may play a role in the immunopathogenesis of dengue.[18]

MCP-1 causes openings of tight endothelial cell connections in vitro[19] and expression of VEGF-induced MCP-1 in vascular endothelial cells increases changes in endothelial permeability in vivo.[20] Recombinant (RH)MCP-1 and MCP-1 containing conditioned media from DENV-infected monocytes increased the permeability of vascular endothelial cells and also clarified that MCP-1 but not VEGF in DENV-infected monocyte culture media increased endothelial permeability.[19]

MCP-1/ CCL2 s Elain recruit and direct the movement of leukocytes also may affect T-cell and CCL2 enhances the secretion of IL-4 by T-cells. Other chemokines and their receptors will be associated with specific T-helper cell responses.[21] CCL2, -7, -8, and-13 (MCP -1 to -4) are strong chemotactic factors for colonization of inflamed target tissues not only for T-cells but also for NK cells and immature dendritic cells. In virus IFN-γ infection produced locally at the site of infection by Th1 effector cells or NK cells, it is responsible for the formation of chemokines CXCL10 and CXCL9, and this then dances c-cytotoxic effector T cells or cells expressing CXCR3.[22]

T-cells may be the source of atypical lymphocytes for their activation and proliferation of T-cells that are seen in most of the viral infection. It is possible that atypical lymphocytes can also represent cell-mediated immune responses host for dengue virus.[23]

The aim of this study based on the levels of MCP-1 and the NS1 protein in the serum or plasma from whole blood with K2EDTA (*Dipotassium Ethylene Diamine Tetra Acetate*) anticoagulant of DENV infection patient and control healthy control, where increasing the level of MCP-1 from healthy people can not describe the possibility of prognosis more severe dengue

hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DENV infection increased MCP-1 levels will increase Monocyte activation towards the inflammatory area and the presence of atypical lymphocytes in DENV infection.

MATERIALS AND METHODS

Study Population

This study was done in dr. Darsono Pacitan hospital and Faculty of Medicine laboratory, Gajah Mada University, Yogyakarta in February 2019. This study included 60 patients with dengue fever and 10 healthy people as the controls who were selected from dr. Darsono Hospital, Pacitan Indonesia. Patients who were selected were in accordance with the criteria of inclusion in patients with clinical symptoms had on set 1st until 4th fever day and ages limit 18 - 55 years. Patients who did not enter the inclusion and exclusion criteria were excluded from the study.

The group control with 10 healthy subjects with comparable age characteristics, no positive history of dengue infection, no fever for 1 month before the study and CBC levels within normal limits.

The clinical disease severity was classified according to the 2011 World Health Organization (WHO) dengue diagnostic criteria. In patients with fever where the sufferer experiences with a fever between 39-40°C and headache or lasting 5-7 days in the majority of cases and the other common symptoms include anorexia were classified as DF. The DHF grade I if there are plasma leakage obtained tourniquet test positive and thrombocytopenia equal to 100.000/m³ and hematocrit greater than 20% of the baseline. If there is spontaneous bleeding, is DHF grade II, and if hypotension and the patient are nervous, is DHF grade III, and DHF grade IV infection DSS that is Shock was defined as having cold clammy skin, along with a narrowing of pulse pressure of 20 mmHg[4].

The aim of this study was to determine the relationship between MCP1 and Atypical lymphocytes levels to the risk of DHF / DSS. The difference between MCP-1 and atypical lymphocyte from a patient with DS or DHF and DSS by counting the mean of SD.

Ethics Statement

The ethical agreement was obtained from the *Ethical Review Committee* Faculty of Dentistry, Universitas Airlangga. All research subjects used *informed consent*.

Blood Samples

The blood sample of the study was taken from patients on fever day 1st-4th, accommodated in 2 types of tubes namely tubes 3ml without anticoagulants and tubes 2ml with K2-EDTA anticoagulants. To obtain a blood sample serum in a tube without anticoagulants, it is waiting to clot for 20-30 minutes and centrifuges 5-15 minutes 1500-3000 rpm, then the serum is separated in a tube sample of 1 mL each. Blood samples in tubes with anticoagulants were thoroughly examined and blood smears were made which were colored with Wright's stain for atypical lymphocyte examination.[24,25] To get plasma blood samples in tubes with anticoagulants rotated 5-15 minutes 1500-3000 rpm, then plasma was separated in a tube sample of 1 mL each. Samples are stored at -20°C (stabilized 1 month) before MCP-1(Insert Kit) is examined and the NS-1 examination of the rapid test method was immediately carried out at that time.

Laboratory diagnosis

The examination of NS-1 on the subject serum was using Dengue Early Rapid Tests Panbio with the ICT (*immunochromatography test*) method with results expressed in positive or negative (Insert Kit). Examination of atypical lymphocyte is counting in 100 leukocytes on a blood smear stained by Wright's and the results expressed in percent (%)[23]. Examination of MCP-1 levels with the Sandwich-ELISA method from the *Elabscience* kit according to the insert kit procedure and the results are expressed in pg/mL.[26]

Statistical analysis

In this study, we analyzed MCP-1 levels, atypical lymphocytes and NS1 in patients with

dengue fever are determined clinically and then use the *Mann-Whitney* test and *Kruskal–Wallis test* to see the difference between dengue infection with fever on fever day 1- 4 and healthy subject. Quantitative variables not following a normal distribution such as MCP-1 levels and atypical lymphocytes were compared using a non-parametric test (Kruskal-Wallis test) to see the difference between dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). *Statistic Package for Social Sciences* (SPSS) was used for data entry, processing and statistical analysis at the end of the study. P-values less than 0.05 were considered significant.[9]

RESULTS AND DISCUSSION

Study population

This study was 60 patient and 10 healthy subjects as control without dengue infection from various ages, 60 patient has infected dengue with 10 patient NS-1 negative and 50 patient NS-1 positives.

Table 1 gives the age and gender distribution of the participants and fever day when was sampling. The age grouping used was based on the Ministry of Health (2009), Grouping between the ages of 17-35 years and 36-55 years. The majority of dengue infection are between the age group of 17 to 35 years (50%), and at the age of 36-55 years is 3,3%. Patient dengue infection with the NS-1 positive at the age of 17-35 years was 50% (55% males) and patients with dengue infection with the NS-1 negative at the age of 36-55 years 7% (5% males), the males were found more than females. There various fever days at sampling, 2nd fever day until 4rd.

NS-1 test

NS-1 was Examined in the serum of dengue-infected patients performed on the condition of subjects with a fever between 2nd until 4th. The method used for the NS-1 examination uses the Panbio Rapid Test. Of the 60 serum samples of dengue fever patients, 50 NS1 samples were positive and 10 NS-1 samples were negative. From positive NS-1 sufferers sampling on 2nd feverday = 4 (6.7%), 3rd fever day = 20 (33.3%), 4th fever day=16 (26.7%) and in patients with NS-1 negative sampling at fever day 2nd=1 (1.7%), 3rd= 5 (8.3%), and 4th= 4 (6.7%).

MCP-1 Levels

MCP-1 levels Examined in serum or plasma of patients with sampling Table 1 gives time at 2nd-day fever until 4th-day fever. Table 2 gives the MCP-1 levels in patients dengue infection increased significantly ($p = 0,000$; $p<0.05$) from healthy subjects, in healthy subjects the average emission value MCP-1 levels is 29.475 ± 23.443 pg/mL and in samples infected with dengue NS-1 negative average 471,290 pg/mL higher than patients NS-1 positive who averaged 420.262 pg/mL, but MCP-1 levels were no significant difference in 60 patients dengue-infected with NS-1 positive and NS-1 negative.

Table 1. Age and Gender Distribution of the Participants (n = 70)

	CONTROL n (%)	NS-1 Post (+) n (%)	NS-1 Neg (-) n (%)
Age			
17-35	8	30 (50%)	3 (5 %)
36-55	2	20 (33%)	7 (11.7 %)
Gender			
Man	6	33 (55%)	5 (8, 3 %)
Women	4	17 (28.3%)	5 (8, 3 %)
Fever Day at the sampling	0	Day 2: 4 (6.7%) Day 3:20 (33.3%) Day 4: 16 (26.7%)	Day 2: 1 (1.7%) Day 3: 5 (8.3%) Day 4: 4 (6.7%)

Table 2. Mean Difference of MCP-1 Level in Research Subjects

	Sampel n	Average MCP-1 level (pg / mL Average ± SB	p
Healthy Subjects	10	29,475 ± 23,443	<0.05
Dengue NS-1 (+)	50	420,263 ± 158,496	
Dengue NS-1 (-)	10	471,290 ± 266,386	

Mann-Whitney test

Table 3. Mean Difference of MCP-1 Level based on Fever Day

	Sampel n	Average MCP-1 level (pg / mL Average ± SB	p
2nd Day Fever	6	503,869 ± 149,632	= 0.562
3rd Day Fever	30	428,165 ± 195,446	p > 0.05
4th Day Fever	24	410,744 ± 165,490	

Kruskal–Wallis test

Table 4. Mean Differences in MCP-1 Levels based on the Diagnosis of Dengue Infection Patients

Variable	N (%)	Average MCP-1 level Average ± SB	p
DF	15 (25%)	436,467 ± 225,585	=0,884
DHF	39 (65%)	422,770 ± 170,548,803	> 0.05
DSS	6 (10%)	448,499 ± 117,391	

Kruskal–Wallis test

Table 3 gives the average MCP-1 levels were examined by sampling time when 2nd fever days until 4th no significant differences $p= 0,562$ ($p>0,005$).

Table 4 gives the average MCP-1 levels were examined by clinical development of patients both DF, DHF and DSS are no significant differences ($p = 0.884$) ($p > 0.005$).

Atypical Lymphocyte

Atypical Lymphocyte examined in blood smear of dengue infected patients with sampling time 2nd fever day until 4th. The percentage of Atypical Lymphocyte in patients with dengue infection increased significantly ($p <0.05$) from the percentage of Atypical Lymphocyte healthy subjects as controls not found Atypical Lymphocyte, the average levels Atypical Lymphocyte patient dengue-infected with NS-1 positive is equal to 3 ± 2% in 100 leucocytes and the samples were patient dengue-infected with NS-1 negative average 1 ± 1% in 100 leucocytes.

Table 5. Differences in Mean Atypical Lymphocytes in Research Subjects

Variable	Sample n	Atypical Lymphocyte % (in 100 leucocytes) Average ± SB	p
Healthy Subjects	10	Not found / Zero	<0,005
Dengue NS-1 (+)	50	3 ± 2	
Dengue NS-1 (-)	10	1 ± 1	

Mann-Whitney test

MCP-1 levels tend to increase in all patients with clinical dengue disease and also the presence of Atypical Lymphocyte with an average of 2% in 100 leucocytes ($p = 0,000$), but there is no significant difference in sufferers of dengue infection with NS1 positive and NS1 negative (pMCP-1 =0, 744; p LPB=1,000) ($ρ> 0.05$).

Table 5 gives the highest Atypical Lymphocyte number was 6% of patient NS-1 positive and the highest MCP-1 level was found in patients with negative NS-1 781,494 pg/ml.

Table 6. Differences in MCP-1 Levels, Hematology Variables and Fever Day When Sampling the Dengue Infection Severity

Variable	Infection Dengue			P
	DF	DHF	DSS	
MCP-1 levels	436,47 ± 225,59	422,77 ± 170,55	448,50 ± 117,39	0,844
Hemoglobin	13,8 ± 1,48	13,9 ± 1,87	13,7 ± 2,60	0,836
Hct	39,5± 4,1	39,4 ± 4,66	38,7 ± 5,7	0,706
PLT	171±93	136±62	94 ± 37	0,131
% Monosit	7,42 ± 2,37	8,25 ± 3,32	8,43 ± 3,45	0,915
% Lymphocyte	27,0±13,2	21,2±10,4	25,4±13,4	0,122
Atypical lympocyte	2±1	2±1	3±1	0,313
Feverday on the sampling time	3±1(2-4)	3±1(2-4)	3±1(2-4)	0,579

Kruskal–Wallis test

Hematology Parameters

Table 6 gives the laboratory investigations are evaluated in our study, the finding shows that hemoglobin levels, hematocrit, monocyte, and lymphocyte were not a significant difference from healthy subjects. Platelet level in 1st until 4th every day decreased from healthy subjects. Leukopenia was mainly found in NS-1 seropositive patients. The hematology result of this study differs from the Kauser study in 2014.[27]

DISCUSSION

Dengue fever can be caused by one of four distinct dengue virus (DENV) serotypes that cocirculate in many parts of the world. They're suggesting that infection to DENV does not provide lifelong immunity and a person can be infected with the same virus.[28] The importance of plasma leakage as a key feature of DHF facilitated the development of clinical management guidelines that successfully reduced dengue-related morbidity and mortality. Recognition of the predominant infection of monocytic cells, the increased risk for DHF associated with the circulation of multiple DENV serotypes and secondary DENV infections and the association of DHF with enhanced cytokine production in vivo guided development of disease models, diagnostic tests and candidate therapeutics.[2]

Proinflammatory cytokines were secreted to initiate the inflammation and to control the DENV replication especially at the early stage of infection. However, dysregulation of these cytokines was also considered an important reason in dengue pathogenesis, especially in DHF and DSS.[29] The occurrence of DHF/DSS is thought to result from a complex interplay between the virus, host genetics, and host immune factors.[30] DENV infection of DCs resulted in CCL2, CCL3, and CCL4 expression, cytokines IL-6, TNFα, and IFN-γ and chemokines CCL2, CCL3, and CCL4 have been associated with disease severity, endothelial dysfunction, and vasodilation.[31]

This study showed that in patients with dengue NS-1 test and obtained from 60 samples of patients with dengue fever found 50 patients with NS-1 positive and 10 patients with NS-1 negative, it homogeneous sample in this study is done by limiting the sampling time of patients with fever day 1-4. Some studies have found that NS-1 antigen levels, especially during days 4–8 of illness, were lower in patients with more severe forms of illness.[20]

DENV nonstructural protein 1 (NS-1) is a unique diagnostic marker for early detection of DENV compared to serological tests (i.e., anti-DENV IgM) because it is detected in the serum of DENV-infected patients as early as one day post onset of symptoms (DPO) to 18 DPO at NS-1 concentration up to 50 µg/mL and it is a confirmatory test. This ELISA was sensitive and specific to DENV-4 with no cross-reactivity to other three DENV (1–3) serotypes and other heterologous flaviviruses.[10] The secondary infection with a different serotype occurs, the immune response can lead to the presentation of dengue fever is more severe in some cases.[32] The incubation period ranges from 3 to 14 days and symptoms usually develop between 4 and 7 days after vector bites.[33]

Dengue infection is usually confirmed by viral genomic RNA identification, antigen, or the antibodies it causes. An antigen detection test based on NS-1 detection has been used to detect viral NS-1 proteins released from dengue that infect and appear early in the bloodstream. Rapid tests such as the NS-1 enzyme-linked immunosorbent assay (ELISA) are commercially available for DENV with relatively good sensitivity and specificity.[10]

Clinical diagnosis of dengue can be challenging, depending largely on what stage in the infection process a patient presents. Depending on the geographic region of the world, there can be a number of disease-causing pathogens or disease states that can mimic the disease spectrum arising from dengue infection. In the early stages of clinical disease, dengue can present as a mild undifferentiated "flu-like" fever with symptoms similar to those of other diseases such as influenza, measles, Zika, chikungunya, yellow fever, and malaria.[34]

Dengue infection is usually confirmed by viral genomic RNA identification, antigen, or the antibodies it causes. An antigen detection test based on NS-1 detection has been used to detect viral NS-1 protein released from dengue that infects cells and appears early in the bloodstream. NS-1 Rapid detection tests or *enzyme-linked immunosorbent assay* (ELISA) is available commercially for DENV was sensitive and specific to DENV-4 with no cross-reactivity to other three DENV (1–3) serotypes and other heterologous flaviviruses.[10] Ordering a dengue NS-1 antigen assay within the first week of symptom onset is one of the initial investigations recommended by the Ministry of Health, Singapore, for dengue infections.[35]

NS1 test detects at the same time as viral RNA and before an antibody response, so the time to do the best examination is day 0 to day 4 and can be detected before the decrease in platelets. our study included several patients with negative NS-1 results when examined using the Panbio rapid test. In, patients with 1-4 day dengue fever with NS-1 positive showed that there was dengue infection. Conversely, dengue fever patients with negative NS-1 did not rule out dengue infection but NS1 was detected at a low level that caused false negatives and further examination was needed. To detect viral proteins, a sufficient level of virus is needed, whereas in the initial stage there is not enough virus, but if it takes samples after the appearance of antibodies, the level of the dengue virus will also decrease.[34]

The NS-1 protein itself is secreted from infected cells and is found in serum at detectable levels that overlap with peak viremia (and RNA detection). These NS-1 levels also coincide with the onset of detectable IgM in acute primary cases and IgG in acute nonprimary cases. It has been found that elevated levels of serum NS-1 directly indicate increased viral burden and further establish a positive correlation between viremia and NS-1 profiles.[36] The rapid tests and qRT-PCR had high sensitivity for dengue diagnosis. Both tests correlated well with the serological diagnosis for case definition (positive NS-1 ELISA) and the overall performance of the method was satisfactory when compared with NS-1 ELISA.[37] Immunochromatography based *rapid diagnostic tests* (RDTs) can detect NS-1 dengue antigen because they may provide a rapid (POCT) point-of-care test in high specificity.[38]

The advantage of the NS-1 antigen rapid test for dengue diagnosis has been widely documented. Although the WHO has recommended the NS-1 rapid test as one of the diagnostic tests for dengue infection, the use of the test is still limited due to its high cost and low sensitivity. In general, the result of the NS1 antigen rapid test should be carefully interpreted because its accuracy can change over the course of illness following the dynamics of viral antigen and antibody levels. Therefore, clinical information of patients must be considered along with the NS1 test result.[39]

Dengue fever is a disease that is difficult to treat at the clinical level, mostly because of the late manifestation of severe disease in some patients.[36] Early in the acute febrile period of the disease, dengue fever presents with the same clinical symptoms as primary dengue. Later, during defervescence, patients can rapidly deteriorate, progressing to hemorrhage with or without a vascular leak. During this period, patients can experience bleeding, thrombocytopenia with <100.000 platelets/µL, ascites, pleural effusion, increased hematocrit concentrations, severe abdominal pain, restlessness, vomiting, and sudden reduction in temperature with profuse perspiration and adynamia.[34]

The sensitivity of NS-1 detection depends on the technique used and whether the sample corresponds to a primary or secondary infection. In primary infection, the sensitivity can exceed 90%, while in secondary infection, the sensitivity is lower and ranges from 60% to 80%.[40,41,42] A longer duration of NS-1 antigenemia than that of viremia in primary DENV infection makes the NS-1 detection method an advantage over DENV nucleic acid detection technique for dengue diagnosis during the acute phase of infection. In secondary DENV infection, however, a decreased sensitivity of the NS-1 Ag Strip test was observed.[43]

The roles of DENV NS-1 antigen and lipid mediators such as (Platelet-Activating Factor) PAF in causing vascular leak are emerging DENV

NS-1 are likely to be helpful in reducing disease pathogenesis due to NS-1, drugs that block PAF receptors or the pathways in which PAF is generated may be helpful in the treatment of acute illness.[20] PAF was previously found to be an important contributor to vascular leak and PAF receptor blockade was found to inhibit the effects of acute dengue sera on the expression of the tight junction protein ZO-1, and in the reduction of trans-endothelial resistance.[15]

Endocytosed DENV like particles has been proven by ultrastructural analysis of platelets from patients with dengue. In vitro studies identified the mechanisms of DENV binding and internalization by platelets requiring DC-SIGN and heparan sulfate proteoglycans for viral attachment. When isolated platelets are infected with DENV in vitro, positive- and negative-sense viral RNA, as well as DENV NS-1, accumulate in platelets, indicating replication and translation of viral genome.[44]

Although thrombocytopenia is more common in DHF than DF, a significant fraction of DF patients also develop thrombocytopenia. Thrombocytopenia is not an early indicator for DHF as the platelet counts during the early febrile phase of DF and DHF are not significantly different. As such, platelet counts serve as a monitoring tool for disease progression rather than an early indicator of severe disease. Platelet counts are rarely low enough to cause spontaneous hemorrhage in DHF patients but may contribute to the hemorrhagic tendency in cases complicated with plasma leakage and shock.[2]

MCP-1/CCL2

Dengue Hemorrhagic Fever has unique pathogenesis which is a consequence of the evolution of the virus into four different serotypes. DENV infects a variety of cell types in vitro including epithelial cells, endothelial cells, hepatocytes, muscle cells, dendritic cells, monocytes, and mast cells.[45] The pathological basis of dengue fever lies in a complex series of immunological responses resulting in a rapid increase in the levels of cytokine and other

chemical mediators that are central to the severe manifestations of dengue hemorrhagic fever, such as plasma leakage, shock, and bleeding.[46] Primary dengue infection causes unpleasant but rarely fatal, diseases such as influenza resulting from the temporary release of proinflammatory cytokines from monocytes and macrophages that are infected with the virus. A pathogenic role for an aberrant inflammasome and monocyte activation in the development of the severe form of dengue disease.[47]

Chemokine plays an important role in the immune response, CCL2, -7, -8, and -13 (MCP-1 to -4) are strong chemotactic factors for colonizing target tissue that is inflamed not only for T cells. Chemokines are cytokines that stimulate the migration of cells that are attracted to the sites with a higher concentration of ligands. [22] Chemokines share the common function of attracting leukocytes to sites of an inflammatory or immune response. A standardized nomenclature in which chemokines were given numerical names, like the interleukins and the chemokine receptors, was proposed more than a decade ago and is now widely used.[48] The *monocyte chemoattractant protein-1* (MCP-1) is secreted from macrophages, monocytes, endothelial cells, epithelial cells, and fibroblasts after stimulation with microbial products or cytokines, primarily attracts monocytes and T cells.[49]

The inflammatory response against DENV is believed to play an important role in its pathogenesis. The different manifestations between mild and severe dengue patients indicate that inflammatory response may differ substantially. Many studies have demonstrated that levels of inflammation mediators such as TNF-α, IFN-γ, IP-10, IL-8 are elevated in dengue patients and higher levels in severe cases.[50] Rothman and colleagues reviewed how innate and adaptive immune responses contribute to promoting severe DHF manifestations, also reviewing specific cytokines and chemokines, TNF-α, *Vascular Endothelial Growth Factors* (VEGF-A), IL-6, IL-10, IL-8, IL-8, CCL2 and CXCL10, and how they promote the production of clinical presentation DHF cellular and molecular contributor cytokine storm but will likely on

targeting single soluble mediators and focus over common in the inflammatory cascade.[51]

Peaks of pro-inflammatory cytokines and peaks of anti-inflammatory cytokines coexist. MCP-1 is a potent monocytes chemoattractant and has been reported that increased levels correlated with severe dengue symptoms. The results in this study also proved that inflammation in severe dengue patients resolved differently from mild cases.[50]

Oliveira *et al* showed expression profiles of 36 cytokines and chemokines in 44 acute serum samples acute-phase DF patients (n=25), DHF (n=19) and healthy controls (n=6). All 36 proteins were expressed at a higher level in patients infected with DENV compared to healthy controls.[52] Huang *et al* 2018 in his study that the CCL2 CX CL9, IP-10, CXCL11, IL-8, and IL-10 serum levels were significantly higher in the group of patients infected DENV during the first two weeks compared to the control group.[29]

The majority of DENV-infected patients make a full recovery after the febrile period and do not enter the critical phase of the disease. However, patients that do enter the critical phase may develop warning signs that indicate increased capillary permeability leading to plasma leakage. Generally, patients worsen at the time of defervescence (from illness day 4th) when their temperature drops to 37.5°C–38°C, and it is during this period that early symptoms of vascular leakage may be seen.[34] The initial prediction of severe dengue in patients without warning signs who can later develop severe DHF is very important to choose the right intensive supportive therapy. Severe responses to dengue include activation and apoptosis of T-cells and B-cells, *cytokine storm*, hematological disorders, and complement activation. Cytokines, complement and other unknown factors can temporarily work on the endothelium and change the normal fluid barrier function of endothelial cells and cause plasma leakage. The elevated levels of cytokine in severe dengue make them good predictors of the severity of dengue fever. Cytokine estimation at presentation can provide us a clue whether a patient is likely to develop severe manifestations of dengue or not.[46] Differences in levels of

cytokines and chemokines were found when sera/plasma samples from Dengue *Hemorrhagic Fever* (DHF) and *Dengue Fever* (DF) patients were compared.[53,54]

There has been limited evidence of endothelial injury measured as apoptosis or structural changes. These findings together with the transient nature of plasma leakage and rapid recovery suggest that transient perturbation of vascular barrier integrity is the main mechanism underlying plasma leakage in DHF and that the activation of endothelial cells and the coagulation system is likely mediated by cytokines produced by the innate and adaptive immune system. In vitro studies demonstrated the production of antiviral and proinflammatory cytokines by these cells when exposed to DENV. These cytokines include type I IFNs and chemotactic factors such as *Migration Inhibition Factor* (MIF), *Monocyte Chemotactic Factor* (MCP), and IL-8. Infection of dendritic cells by DENV also induces the production of MMP-2 and MMP-9 which may facilitate the migration of dendritic cells to the local lymph nodes where virus further replicates and subsequently enters the circulation. Most studies to date have described the production of Th1 cytokines and minimal production of Th2 cytokines by DENV specific T-cells. IL-17 and IL-21 secretion by DENV specific T-cells is just beginning to be described and therefore the roles of these cytokines in dengue pathogenesis are currently unknown.[45] Malagive et al 2018 have found that IL-10, IL-1β, *monocyte chemoattractant protein* (MCP)-1 and IL-8 levels were associated with severe dengue and that monocytes were likely to be the predominant source of IL-10. Apart from interaction with monocytes, platelets are also known to contribute to vascular permeability due to the production of IL-1β by platelet microparticles. Other mediators that are known to cause vascular leak include bradykinins, complement proteins C3a and C5a, IL-33, fibrin products, prostaglandins E2, F2a, and D2.[15]

Hemorrhagic manifestation in Dengue virus Infection patients are not common and within mild to severe. Skin hemorrhage, including petechiae and purpura, are the most common,

along with gum bleeding, epistaxis, menorrhagia, and gastrointestinal bleeding.[55]

Atypical lymphocytes

The atypical lymphocyte is a non-malignant leukocyte seen in the peripheral blood. It is a reactive lymphocyte of lymphoid origin and produced in a variety of disorders. It appears to be a nonspecific response to stress from a variety of stimuli. A small lymphocyte becomes larger in size and capable of dividing.[56] Atypical lymphocytes or reactive lymphocytes on peripheral blood smear in dengue which the morphology of reactive lymphocytes often reported.[57]

In this study, it was found that subjects with dengue infection with NS-1 positive and with NS-1 negative percentage of atypical lymphocyte 2-6%. But there is no difference in patients who subsequently experience DF, DHF or DSS.

Cardinal and Joseph Alba showed in the Philippines that are 155 confirmed cases of dengue fever, a total of 137 (88.4%) patients had atypical lymphocytes and 18 (11.6%) were found to be negative. The positive and negative predictive values of atypical lymphocytes were 86.2% and 86.9%, respectively. However, no differences were noted when the proportion of atypical lymphocytes was compared across all dengue severity. Lymphocytes plasma blue or atypical lymphocytes are predictors were significantly dengue based on an analysis logistic regression showed that the risk of patients with atypical lymphocytes was 41.16 times higher for dengue than those who did not have atypical lymphocytes.[58]

In the 2007 Jampangern study also found a significant increase in the absolute number of atypical lymphocytes on the incubation day and one day after incubation during acute dengue virus infection, especially in DHF patients. Of the 49 dengue *hemorrhagic fever* (DHF), 25 *dengue fever* (DF), and 26 *dengue fever* (DFS) cases. Atypical 10% or higher lymphocyte count is a good indicator of dengue infection (50% sensitivity and 86% specificity).[23]

A disease is atypical as a special hematological finding in patients with dengue fever, and although it is not a classic specific finding of the disease, their concentration is significantly higher in these patients, especially in the form of the severe disease. There may be a relationship between the presence of atypical lymphocytes and dengue virus infection, but the intensity and usefulness of these findings require further study and analysis.[59]

Patients who have > 300 cells/μL absolute atypical lymphocytes can be used to predict the development of severe dengue because patients with severe dengue have a greater level of absolute atypical lymphocytes than patients with dengue fever who do not severe. This finding is similar to previous. After a secondary dengue infection, atypical lymphocytes could indicate an augmented immune response attempting to control the spread of dengue-infected cells. Simultaneously, these antibodies could enhance the entry of the dengue virus into macrophages and dendritic cells whereupon the virus would replicate. Previous reports have also indicated that patients with higher dengue viremia have higher disease severity.[60]

The presence of atypical lymphocytes is due to the T-cell activation should be considered as a useful screening parameter for dengue infection.[61]

CONCLUSION

In conclusion, this study analyzed MCP-1 levels and atypical lymphocytes inpatient dengue-infected which detecting use NS-1 rapid test. The correlation MCP-1 levels and atypical lymphocytes were found an increase in MCP-1 levels was followed by an increase of atypical lymphocytes. This shows the arrival of macrophages and monocytes to the site of inflammation which triggers proliferation rather than lymphocytes.

Until now, biomarkers cannot act as predictors of cytokine storm in dengue infection patients. From the results of this study, there were no significant different parameters between DF, DHF, and DSS.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest for this research.

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