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Comparison of Results of Atypical Lymphocyte Test, RT-PCR and ELISA Using Recombinant Multivalent Envelope Protein Domain III (ED-III) Dengue Virus in Dengue Fever Patients

Nurul Inayati[1a], Fihiruddin[1b*], Sri Ernawati[1c], Lalu Unsunnidhal[2d], Raudatul Jannah[3e]

[1] Department of Medical Laboratory Technology, Politeknik Kesehatan Kemenkes Mataram, Mataram, West Nusa Tenggara, Indonesia
[2] Food Technology Study Program, Faculty of Food Technology and Agroindustry, University of Mataram, Mataram, West Nusa Tenggara, Indonesia
[3] Midwifery Study Program, INKES Yarsi Mataram, Mataram, West Nusa Tenggara, Indonesia

[a] Email address: inayati250@gmail.com
[b] Email address: fihir.analis79@gmail.com
[c] Email address: sriernawati392@gmail.com
[d] Email address: lalu.unsunnidhal@unram.ac.id
[e] Email address: raudatul.unsun.10desember2017@gmail.com

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Abstract
Prevention of the transmission of dengue hemorrhagic fever (DHF) is carried out by breaking the chain of dengue transmission and administering vaccines, but to date, this has not achieved the expected target. Dengue virus tests using RT-PCR require skills and relatively expensive equipment. Serological test of IgM and IgG often shows false negatives or false positives, especially in dengue-endemic areas. The antibody test against NS1 using the ELISA method has weaknesses because anti-dengue IgM is often not detected in secondary infections. The development of serodiagnostic tests for rapid, affordable, sensitive, and specific detection of dengue virus infection is very necessary. Recombinant multivalent envelope proteins domain III (ED-III) dengue virus is a biomarker that has the potential to be developed to detect all dengue virus serotypes. One of the proteins that has high antigenicity is glycoprotein E which is found in the envelope of the dengue virus and is the most antigenic part of the virus. This research aims to combine several parts of the antigenic protein found in all dengue virus serotypes as immunoserodiagnostic material. This research is an analytical survey research, that compares the results of the atypical lymphocyte test, RT-PCR, and ELISA using the multivalent ED-III antigen. The number of samples used was 26 samples obtained from patients who were diagnosed with dengue fever using an accidental sampling technique. The results of the atypical lymphocyte examination showed 14 positive samples, while the results of the RT-PCR and ELISA examinations were 23 and 24 positive respectively. The average Optical density (OD) of examination using the ELISA method was 1.902 with sensitivity and specificity levels of 92% and 96%. There is no difference result of the RT-PCR compared with the ELISA test. Therefore, recombinant multivalent envelope protein domain III (ED-III) dengue virus can be used as a diagnostic tool to detect dengue fever infection.

Keywords: ED-III Recombinant Proteins, Dengue Virus, Diagnostic Test.

Corresponding Author:

Fihiruddin
Department of Medical Laboratory Technology, Politeknik Kesehatan Kemenkes Mataram, Mataram, West Nusa Tenggara, Indonesia
Email: fihir.analis79@gmail.com

1. INTRODUCTION

Dengue Hemorrhagic Fever (DHF) is an arboviral disease transmitted by the Aedes aegypti mosquito. Based on the serotype, dengue viruses are classified into four types: DEN-1, DEN-2, DEN-3, and DEN-4 (Nguyen et al., 2019). This virus is endemic in over 125 countries and causes approximately 390 million infections annually, of which 100 million exhibit symptoms and 25,000 result in fatalities, particularly among children. The incidence of dengue has increased thirtyfold over the past fifty years (Ahmad & Poh, 2019; Laiton-Donato et al., 2019; Sholihah, 2020). Indonesia has the highest number of Dengue Hemorrhagic Fever cases on the Asian continent, accounting for more than 57% of cases and nearly 70% of dengue-related deaths (Syamsir & Pangestuty, 2020). The Incidence Rate (IR) or new dengue cases in Indonesia tends to increase yearly. 2014, there were 100,347 cases, which rose to 129,650 in 2015 and 204,171 in 2016. The number then dropped to 68,407 in 2017, decreased to 53,075 in 2018, and was 13,683 in 2019 (Indonesian Health Ministry, 2019). In West Nusa Tenggara, there were 535 dengue cases in 2018, with an Incidence Rate (IR) of 10.7% (Provincial Health Department, 2018).

Laboratory tests for detecting dengue virus infection include serological examinations, Real Time-Polymerase Chain Reaction (RT-PCR), and virus culture. The presence of atifical lymphocytes in peripheral blood is only a supporting test for the diagnosis of dengue fever. Detection using molecular methods, like RT-PCR, is possible only 5 days after symptoms appear, and the virus's presence in serum is brief, requiring specialized skills and costly equipment (Nguyen et al., 2019). The anti-dengue IgM serological test often fails to detect secondary dengue virus infections, while the NS1 test is sensitive for identifying primary infections but less effective for secondary infections (Resna et al., 2014). Using a single dengue serotype to detect all serotypes is challenging due to variations in protein sequences produced by each serotype (Rathore et al., 2019).

The diagnosis of dengue fever relies on clinical history and laboratory tests. The Enzyme-Linked Immunosorbent Assay (ELISA) is the most commonly used diagnostic test, measuring dengue IgM or IgG antibodies. This test can detect dengue antibodies 3-4 days after symptoms begin. Serological diagnosis is based on the rise in IgM or IgG antibody titers (Hidayati, 2019). Previous studies have examined changes in cellular immune responses, discovering atypical lymphocytes characteristic of dengue infection, specifically blue plasma lymphocytes. These lymphocytes are closely associated with dengue fever, with 88.4% of confirmed dengue fever patients showing atypical lymphocytes. Dengue fever patients have more blue plasma lymphocytes than those with other fever types (Cardinal & Alba, 2017). Blue plasma lymphocytes can also help predict the progression of severe dengue fever, with patients suffering from severe dengue having significantly more atypical lymphocytes than those with mild dengue (Thanachartwet et al., 2015).

There is a need for a fast, affordable, and accurate diagnostic method for dengue virus infection. The dengue virus domain III multivalent fusion protein is a biomarker with the potential to detect all dengue virus serotypes (Shukla et al., 2020). The main component of the dengue virus is the Envelope protein (E protein), which includes a specific region capable of binding to host cells and acts as the dominant antigen. This protein can elicit responses such as virus-neutralizing antibodies, haemagglutinin-inhibiting antibodies, anti-fusion antibodies, and virus-enhancing antibodies (Chiang et al., 2016). This study aims to compare the examination results of atypical lymphocytes, RT-PCR, and ELISA using the dengue virus domain III (ED-III) multivalent envelope protein fusion antigen. The ED-III antigen is derived from combining the envelope (E) protein sequence, which is antigenic across all dengue virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4).

2. RESEARCH METHOD

This analytical observational study compares the results of atypical lymphocyte (Blue Plasma Lymphocyte) examination, RT-PCR, and ELISA using the dengue virus ED-III recombinant protein multivalent antigen. Blood samples were collected from 26 patients diagnosed with dengue fever who were examined at Praya Regional General Hospital (RSUD). Serum samples from healthy people were obtained from previous researchers' collections. Ethics Clearance was obtained from the Mataram Health Polytechnic Ethics Commission, number LB.01.03/6/3037/2022. Sampling was conducted using purposive random sampling. The samples were divided into three parts: the first for atypical lymphocytes (blue plasma lymphocytes) examination, the second for RNA extraction using the Zymo RNA extraction kit, and the third for serum separation for ELISA examination. The recombinant multivalent envelope proteins domain III (ED-III) dengue virus was produced by expressing competent E. coli cells containing the pET15b plasmid. The pET15b plasmid with the multivalent insertion sequence of dengue virus domain III (ED-III) protein was previously transformed into competent E. coli BL21 cells using the heat shock method at 42°C for 30 seconds. Large-scale bacterial isolation involved preparing 400 ml of LB medium in 4 sterile Erlenmeyer flasks, each with 100 ml of LB medium containing 10 µg/ml Ampicillin. Ten ml of each bacterial suspension was added to 100 ml of liquid LB medium and incubated for 4 hours until an OD of 0.6 was achieved. One mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to all LB media. The resulting pellet was sonicated, and the recombinant protein was purified using the Protino™ Ni-NTA System.

Examining atypical lymphocytes involves preparing slides from the blood of dengue fever patients. The slides were fixed with methyl alcohol for 5 minutes and then stained with Giemsa for 30 minutes before being examined under a microscope. RT-PCR (Reverse transcriptase PCR) was performed using MyTaq One-Step Mix with a final volume of 25 µl. The reverse transcription was conducted for 1 hour at 45°C, followed by polymerase activation for 1 minute at 95°C. Denaturation occurred for 30 seconds at 95°C, annealing for 1 minute at 55°C, extension for 2 minutes at 72°C, and post-extension for 10 minutes at 72°C. The amplification results were electrophoresed on 1% agarose, and the electrophoresis bands were visualized using UV light. The band sizes for each virus serotype were 482 bp (DEN-1), 119 bp (DEN-2), 290 bp (DEN-3), and 392 bp (DEN-4). Antibody examination in patients was conducted using the Enzyme-Linked Immunosorbent Assay (ELISA) method, employing a recombinant multivalent fusion protein domain III (ED-III) dengue virus. The results were expressed by reading the test results' optical density (OD) and comparing it with the cut-off value (COV).

3. RESULTS AND DISCUSSION

The purification of the recombinant multivalent envelope proteins domain III (ED-III) dengue virus from the sonication results was performed using a nickel affinity chromatography column, as the recombinant protein is a fusion protein with polyhistidine at the amino end. The purification procedure followed the protocol of a modified Protino™ Ni-NTA System column at the elution volume. SDS-PAGE confirmed the success of the purification, and a band with a size of 52 kDa indicated successful purification (figure 1).

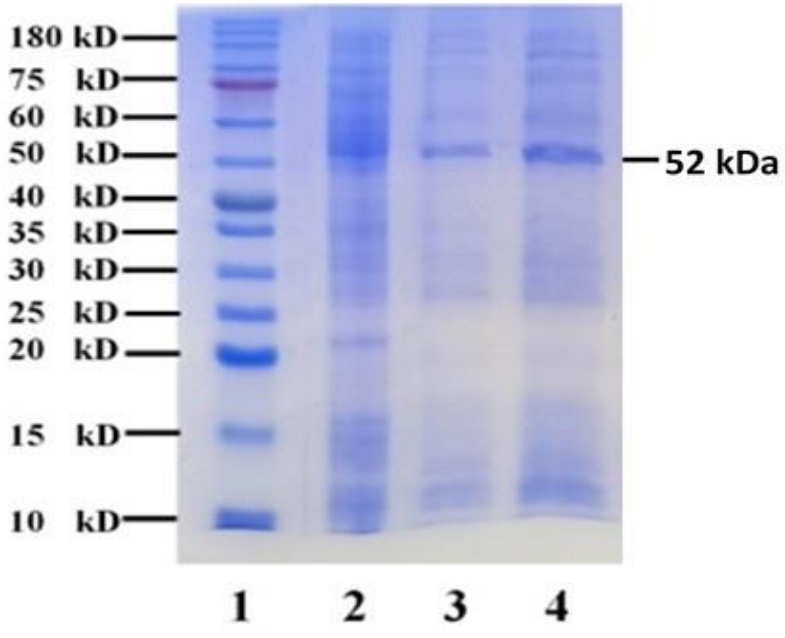

Figure 1. SDS-PAGE results Recombinant multivalent envelope proteins (ED-III) dengue virus. line 1: DNA ladder, line: 2, 3 and 4: Multivalent proteins ED-III dengue virus, each run with volumes: 5 µL (line 2), 10 µL (line 3) and 15 µL (line 4).

Microscopic examination of blue plasma lymphocytes revealed medium to large cells with slightly basophilic cytoplasm. The nuclei of these lymphocytes are generally more oversized and irregular in shape compared to normal lymphocytes. These cells contain dense chromatin; in some, nucleoli are easily visible. Other lymphocytes, resembling lymphoblasts, exhibit more cytoplasm and prominent nucleoli, often two or more, and are observed separately.

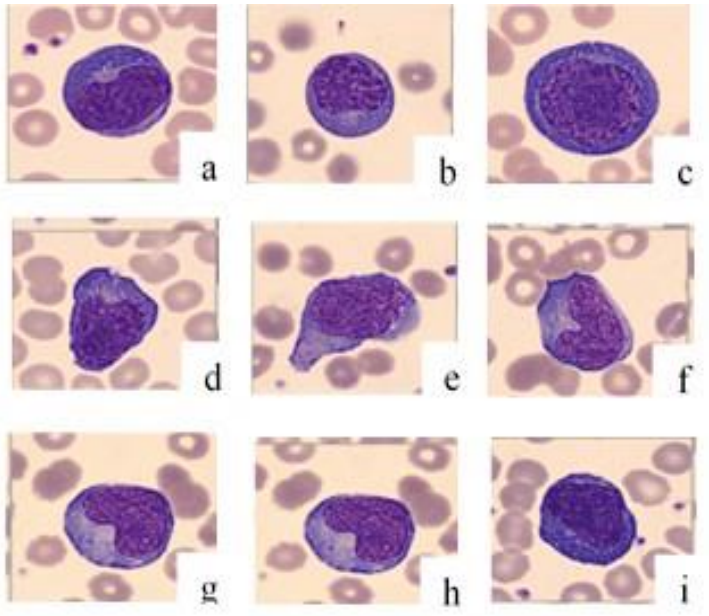

Figure 2. Atypical monocytoid lymphocytes (a,c,g and h); atypical lymphocytes with plasmacytoid features (d,e and f); atypical blastoid lymphocytes (b and i) using Giemsa staining (Microscope magnification: 1000x)

Table 1. Results of atypical lymphocytes Examination in Dengue Hemorrhagic Fever (DHF) Patients showing reactive IgG and IgM.

Variable	IgM Test		IgG test		Blue Plasma lymphocyte	
	Reaktif	Non-Reaktif	Positif	Non-Reaktif	Positive	Negative
Man	1	11	12	0	6	6
Women	0	14	14	0	8	6
Amount	1	25	26	0	14	12

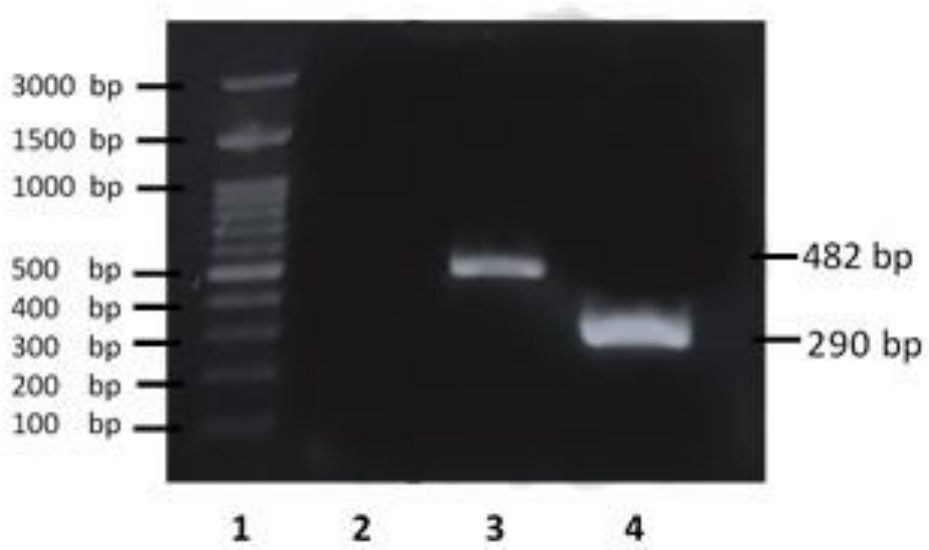

Figure 3. Results of PCR from blood sample of dengue fever patients. Line 1: DNA ladder, line 2: Sample negative, line 3: Dengue virus serotype-1 (482 bp), and line 4: Dengue virus serotype-3 (290 bp).

Based on the results of the RT-PCR test of 26 dengue fever patients, it was found that 8 people (30%) had dengue serotype-1 virus 17 people (65%) had dengue serotype-3 virus and 1 sample showed negative results (4.1%). The results of this study are in accordance with research conducted by Tatontos et al (2021) which found that the dominant dengue virus serotype in Aedes sp mosquitoes is DENV-3.

Table 2. RT-PCR and ELISA examination results using recombinant multivalent envelope protein domain III (ED-III) of dengue virus in patients with dengue fever

Variable	ELISA (Dengue virus ED-III recombinant protein)		RT-PCR	
	Positive	Negative	Positive	Negative
Man	12	1	12	0
Women	11	2	13	1
Amount	23	3	25	1

Table 3. Results of ELISA test using recombinant multivalent envelope protein (ED-III) dengue virus.

Variable	Positive	Negative	Sensitivity/Specificity
Dengue fever patients	24 people	2 people	92 %
Healthy people	1 people	25 people	96 %

DISCUSSION

The study results indicated that atypical lymphocytes were more abundant in dengue fever patients with IgG reactivity than those with IgM. IgM is the initial antibody produced by the body during an infection, detectable from the first week of illness or fever. IgM appears 1-2 days after fever symptoms onset, while IgG appears after 5-10 days of fever. Atypical lymphocytes can be detected from the second day, increasing in number from day 3 to 7 and then decreasing. Early in the fever, leukocyte levels can be normal or predominantly neutrophils, decreasing between the third and eighth day. In severe shock, leukocytosis with leukopenia and thrombocytopenia can occur, with a significant presence (20%-50%) of atypical lymphocytes (blue plasma lymphocytes), especially in secondary infections (Hidayati, 2019). These atypical lymphocytes are mononuclear cells with smooth, dense nuclear chromatin and relatively wide, dark blue cytoplasm (Arruan et al., 2015).

The presence of atypical lymphocytes correlates closely with the symptoms of dengue fever, where higher clinical severity corresponds to increased numbers of LPB (Irianti et al., 2019). Research findings revealed that not all dengue fever-positive samples exhibited atypical lymphocytes. Of the patients, 14 showed positive atypical lymphocytes, accounting for 37%. This variability in findings may be attributed to differences in sampling times and the duration of fever affecting the presence of atypical lymphocytes (Ayu & Karima, 2019).

The RT-PCR analysis of viral RNA from blood samples indicated positive results in 25 samples, with only one sample testing negative. The examination identified two dengue virus serotypes: DEN-2 in 10 (40%) and DEN-3 in 15 samples (60%) samples. Notably, DEN-1 and DEN-4 were absent in the samples analyzed. The predominant serotype in this study was DEN-3, consistent with previous research by Sumarno, which observed alternating dominance between DEN-2 and DEN-3 serotypes (Prasetyowati et al., 2010). Dengue virus serotypes typically circulate year-round in an area, contributing to increased infections. While serotype distribution influences endemicity levels, environmental factors also play a crucial role in the transmission dynamics of dengue virus infections in the Central Lombok district.

The ELISA results indicated that among the 26 samples tested, 24 showed positive outcomes, while 2 were negative. Similarly, when testing serum from healthy individuals using the EDIII recombinant protein antigen in ELISA, 25 out of 26 samples yielded negative results. These healthy samples' average optical density (OD) value was 0.34. The calculated cut-off value (COV) was determined to be 0.62, establishing it as the negative threshold. Table 3 illustrates that the lowest OD value for positive samples was 0.957, exceeding the COV of 0.62. Positive results from serum samples signify the presence of specific antibodies against the dengue virus. ELISA serological testing for IgG and IgM antibodies in dengue patients is crucial for diagnosing the disease, as these antibodies typically appear 5-7 days after infection. Negative results may occur if the test is conducted early in the infection. IgG antibodies are diagnostically significant when accompanied by supportive symptoms of dengue fever. Furthermore, IgG and IgM testing aids in distinguishing between primary and secondary infections (Va´zquez et al., 2003).

Primary infection occurs in individuals without a previous history of dengue virus exposure. IgG antibodies develop slowly and often exhibit low titers in these cases, sometimes resulting in shallow OD readings in ELISA dengue tests. Secondary infection, on the other hand, occurs in individuals with a prior history of dengue virus exposure. Immunity to the same or closely related dengue virus persists throughout life, but subsequent infections may occur with different serotypes over time. During secondary infection, IgG antibodies typically appear early in the fever phase as part of a memory response from immune cells (Va'zquez et al., 2003). The serological profile indicative of secondary infection involves the detection of IgG alone, necessitating additional clinical observation and comprehensive blood laboratory examinations based on WHO criteria for confirmation. Detection of IgG may be challenging in secondary infections due to significantly lower levels compared to primary infections, leading to

undetectable levels in some cases. Sharp increases in IgG levels from previous primary infections may suggest infection with a different dengue virus serotype (Lidya et al., 2014). In this study, 26 serum samples yielded 24 positive ELISA results. Whether these infections were primary or secondary was impossible because both IgG and IgM antibodies were detected simultaneously using the recombinant protein antigen employed in the tests.

The recombinant antigen's sensitivity and specificity testing results were 92% and 96%, respectively. These findings demonstrate that the recombinant protein employed in this study exhibits strong antigenicity and specificity and is suitable for serological examinations using the ELISA method. In serological tests for dengue diagnosis, the sample collection timing significantly impacts test results' accuracy because the presence of dengue antigens or antibodies in the body follows a specific timeframe. Typically, the duration of fever serves as a standard for determining the appropriate time for blood collection in dengue diagnosis. Failure in timing can lead to false negative results. The optimal time for conducting serological tests is between days 3 and 5 of fever when antibody production has commenced. During the initial 0-2 days of fever, patients are still in the acute phase, where diagnostic tools do not detect hemodynamic changes and antibody formation (Lidya et al., 2014).

Research conducted by Rockstroh et al. (2019) utilized the ELISA method with recombinant envelope protein to detect IgM antibodies, revealing that the predominant serotype was detected in 91.1% of tourist samples and 86.5% of samples from endemic populations. PCR analysis confirmed 97.1% concordance with the serotypes identified by ELISA (Rockstroh et al., 2019). Due to its high sensitivity and specificity, the ELISA method has become widely adopted for serological testing in dengue virus diagnosis. It is a valuable tool for monitoring dengue, hemorrhagic, and dengue shock syndrome (Rockstroh et al., 2019; Charisma et al., 2020).

The research findings indicated that positive dengue IgG antibodies were highest in patients with fever around day 6. These results closely align with another study conducted by Andreas, where positive dengue IgG antibodies were frequently detected in urine samples from respondents showing fever indications on days 6-7, accounting for 52% of cases (51 out of 98 samples). However, a higher frequency of positive dengue IgG was observed among respondents tested during the second week post-infection, totaling 75.3% (64 out of 85 samples), followed by 61.7% (37 out of 60 samples) during the third week. This research involved serial sampling and tracking respondents' disease progression (prognosis) for up to 3 months post-infection, highlighting the significant influence of sample collection timing on dengue IgG serology results. These findings differ slightly from previous research by other investigators, which reported positive dengue IgG results predominantly in patients with fever lasting 5 days (Va´zquez et al., 2003).

4. CONCLUSION

Examination of the serum of dengue fever sufferers with recombinant multivalent envelope proteins (ED-III) dengue virus using the ELISA method showed the result is not different from with RT-PCR test which sensitivity and specificity levels were 92% and 96% respectively.

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