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Homology Modeling and Expression of Recombinant NS5-RdRp Based on the Indonesian Local Strain of Dengue Virus for Anti-Dengue Drug Development

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Abstract: This study aims to isolate the RNA-dependent RNA polymerase (RdRp) gene of the dengue virus (DENV) isolates from Indonesia, determine the serotype of the local DENV strain through homology modeling, and express the recombinant RdRp protein in *Escherichia coli* BL21 (DE3), as the target for anti-dengue drug development. We utilize reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR for serotyping the DENV isolates, obtained from Hasan Sadikin Hospital, Indonesia. Then, followed by the extraction and purification of the RdRp amplicon for gene sequencing. The resulting RdRp gene sequences are translated into amino acid sequences, which are then used for protein modeling and the construction of a recombinant plasmid for RdRp protein expression. As a result, the RdRp gene, which belongs to the non-structural protein 5 (NS5) of DENV, was successfully isolated and sequenced from DENV RNA samples. Based on serotyping results and homology modeling, the serotype of the isolate DENV was identified as serotype-3. The isolated NS5-RdRp gene from the local strain was then inserted into the pET28a(+) plasmid and transformed into *E. coli* BL21(DE3) as the host cell. Recombinant NS5-RdRp was

predominantly found in the insoluble fraction with a molecular weight of 72 kDa. Future studies on the development of an anti-dengue drug model that confirms the inhibition of the binding domain in the recombinant RdRp protein structure will be required to provide more insight and expand the development of a universal anti-dengue strategy in Indonesia.

Keywords: Homology; modeling; dengue virus (DENV); nonstructural protein 5 (NS5); RNA dependent RNA polymerase (RdRp); recombinant; SDG 3 Good health and well-being

1. Introduction

Dengue virus (DENV) is a cause of acute infectious diseases transmitted by the bite of the *Aedes aegypti* and *Aedes albopictus* mosquitoes[1]. Clinical symptoms of DENV infection begin with flu-like symptoms that sometimes turn into Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (SSD), which can result in death[2]. This disease has spread to 100 countries and infected 40% of the world's population. DHF in Indonesia ranks second highest in the world[3]. This disease has become a serious health problem in Indonesia since 1968, which continues to increase the number of cases and its spread. Until 2015, DHF has spread in 34 provinces and 85% of cities/regencies in Indonesia, with 1,299 deaths from 126,675 sufferers in that year[4].

DENV belongs to the genus Flavivirus of the Flaviviridae family and is divided into four virus serotypes, namely DENV-1, DENV-2, DENV-3, and DENV-4. Dengue is a tropical disease with the fastest growth rate in the world without an effective and specific treatment[5,6]. One serotype infection can have a lifelong immune effect, but a second infection with a different serotype can increase the risk of progression to more severe dengue symptoms due to cross-immunity by different serotypes Cross-immunity to the other serotypes is partial upon recovery and consequently, future infections by any of the other serotypes magnify the risk of developing severe dengue[7,8]. The previous study screened ligands to find a common putative drug candidate against the NS2b/NS3 serine protease of DENV1, DENV2, DENV3 for the development of drugs against DENV[9]. Therefore, the ideal anti-dengue drug must be able to cover all types of serotypes.

The dengue virus genome is a single-stranded RNA consisting of about 11,000 nucleotides which encodes three structural proteins and seven nonstructural proteins. Nonstructural protein 5 (NS5) is a potential target for anti-dengue drugs. NS5 functions as a methyl-transferase and an RNA-dependent RNA polymerase (RdRp), which are needed for virus replication. DENV NS5 RdRp does not have a mammalian counterpart and its protein sequences are the most sustainable than four other nonstructural proteins with more than 65%

homology, which is an opportunity to discover a new universal anti-dengue that can cover all serotypes[10].

The DENV NS5 structural analysis study shows that the location of the RdRp domain is in the C-terminal where there are two sustainable cavities. The three-dimensional crystal structure of NS5 RdRp adopts the classic form of the polymerase enzyme, where there are subdomain fingers, palm, and thumb. The catalytic site of NS5 RdRp is seen from the GDD motif (GLU, ASP, ASP) located on the palm subdomain. These two ASP residues play a role in coordinating with two metal ions which are important in the incorporation of incoming nucleotides[11,12].

In recent years, the discovery of NS5 RdRp inhibitors has been reported to increase[13,14]. Compounds that work on NS5 RdRp are classified into two inhibitor groups i.e. nucleoside inhibitors (NI) and non-nucleoside inhibitors (NNI). NI attaches to the active side of the polymerase and acts as a chain terminator when synthesizing RNA with its metabolite compound, 5-triphosphate. Meanwhile, NNI works on the allosteric active side which inhibits the enzymatic activity of NS5 RdRp[13]. High-throughput screening (HTS) compounds from Novartis® have been carried out using the radioactive scintillation proximity method and fluorescent-coupled test to measure the elongation of the activity of the enzyme[15,16]. As reported by Chen *et al*., anthranilic N-sulfonyl compounds showed activity with an IC50 value of 7.2 μM[17]. Niyomrattanakit *et al*. reported the results of the development of anthranilic N-sulfonic acid compounds with the structure-activity relationship (SAR) method succeeded in increasing its activity to 0.26 μM[15]. However, these development compounds have high lipophilicity and do not provide activity on cell tests.

In 2016, Yokokawa *et al*. developed compounds using the fragmented base X-ray crystallographic screening method followed by rational design, producing anti-dengue compounds with activity in both enzymatic and cell tests[18]. While the X-ray method is very expensive and requires sophisticated equipment, rational design using the Computer Aided Drug Design (CADD) method gives an alternative way to generate anti-dengue candidate compounds. It can minimize the cost and time of modeling a compound and also can determine the physicochemical properties of the resulting model[19]. In addition, NS5 RdRp crystal structure has been available in Protein Data Bank (PDB) which can be used as a basis for a rational design using CADD[11,18]. This study aims to determine the protein structure homology modeling of NS5-RdRp isolated from Indonesia's local strain DENV and the synthesis of recombinant NS5-RdRp to be used in the development of anti-dengue compounds for the needs of anti-dengue drugs against DENV in Indonesia.

2. Materials and Methods

2.1. Materials

DENV RNA samples used were obtained from Hasan Sadikin Hospital, Bandung. Reagents for PCR were nuclease-free water RT-PCR master mix, primer D1 (5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'), primer D2 (5'-TTGCACCAACAGTCAATGTC TTCAGGTTC-3'), primer TS3 (5'-TAACATCATCATGAGACAGC-3'), Taq superscript reverse transcriptase, Go taq master mix, and agarose. GeneJET Gel Extraction Kit (Thermo Scientific) was used for DNA Extraction and Purification. *E. coli* BL21 (DE3) was used as a host cell to express recombinant RdRp. Luria Bertani broth (LB) Medium (Himedia) was used for the host cell culture medium, and isopropyl β-d-1-thiogalactopyranoside (IPTG) (Thermo Scientific) was used for induction for recombinant protein expression. Agarose (Merck) was used in electrophoresis to characterize DNA while sodium dodecyl sulfate poly-acryl amide (Promega) was used in SDS-PAGE to characterize the expressed recombinant protein.

2.2. Serotyping of Dengue Virus

DENV serotyping is carried out in 2 stages. The first stage is the synthesis of complementary Deoxyribonucleic Acid (cDNA) from DENV RNA isolate using reverse transcription polymerase chain reaction (RT-PCR) and the second stage is nested polymerase chain reaction (PCR) followed by DNA identification. RT-PCR on DENV RNA isolate was carried out by preparing the reaction mix consisting of nuclease-free water, 2x RT-PCR reaction mix, primer D1 as forward primer and D2 as a reverse primer, Taq superscript reverse transcriptase, and DENV RNA isolates sample. Then, nested PCR was carried out using cDNA obtained from RT-PCR in the first stage as a DNA template. The reaction mix used in this stage was nuclease-free water, Go taq master mix, primer D1 as forward primer, and primer TS3 as a reverse primer and cDNA from RT-PCR result. For visualization, DNA agarose electrophoresis was carried out to identify the PCR result. The electrophoresis used 2% agarose gel run for 1 h with 100 V, 400 A, and DENV3 Zymo Bunami RNA isolate as a positive control. The results were visualized with ultraviolet (UV) light.

2.3. Isolation and Amplification of NS5-RdRp DENV3 Gene

2.3.1. Primary design for RdRp-DENV3 amplification

Before the primer design, the gene encoding for RdRp as part of NS5 protein from DENV3 was taken from http://www.ncbi.nlm.nih.gov/. Furthermore, the sequence is aligned to determine the sequence polymorphism. The primers were designed and then analyzed

according to the primary requirements such as base length, melting temperature (Tm), percentage of GC, hairpin structure, self-dimer, and cross dimers at https://sg.idtdna.com/calc/analyzer.

2.3.2. Amplification of RdRp DENV3

Amplification of RdRp using one-step RT-PCR enzyme from Invitrogen and carried out with primer RdRp Forward (5'-GGT GGT CAT ATG TCA CAR GGY GAR ACY YTR GGR GAA AAG TGG-3') and primer RdRp Reverse (5'-GGT GGT CAT ATG CAT GTC AAT GCG GAA CCA GAA ACA CCC-3'). The amplicon of the RdRp gene as a result of amplification was identified by agarose electrophoresis for 45 min (80 V/ 400A) with 1% (w/v) agarose gel concentration.

2.3.3. DNA extraction and purification

RdRp Amplicon was purified from gel using the Thermo Scientific GeneJET Gel Extraction Kit to be used in gene sequencing. The RdRp Amplicon band was cut from the electrophoresis gel and transferred into a microtube. A binding buffer with a ratio of 1:1 was added followed by incubation at 50°C for 10 min until the gel was completely dissolved. The dissolved gel in the binding buffer was transferred into the GeneJET purification column. Subsequently, purification was carried out following the GeneJET Gel Extraction Kit procedure. A total of 30 μL of samples were delivered for sequencing analysis to 1st BASE DNA Sequencing Service with primary RdRp forward and RdRp reverse.

2.4. Homology Modeling of RdRp

The sequence quality was evaluated through a blast search at NCBI's BLAST website, which revealed a gap in the reverse and forward sequence. To fill this gap, the top identical sequences were aligned with both the reverse and forward sequences using the Mega-X program. Then, the final sequence was subjected to homology modeling. The homology model was generated and evaluated using the SWISS-MODEL program and MolProbity version 4.4. All structural analyses and visualizations were carried out in Discovery Studio 2020.

2.5. Expression of Recombinant RdRp Protein

2.5.1. Gene synthetic construction of NS5-RdRp DENV3

NS5-RdRp DENV3 gene which encodes 626 amino acids sequence was synthesized and optimized based on *E. coli* codon preference since *E. coli* BL21 (DE3) was used as the host cell. Optimization and synthesis were performed by GenScript Biotech (Figure 1).

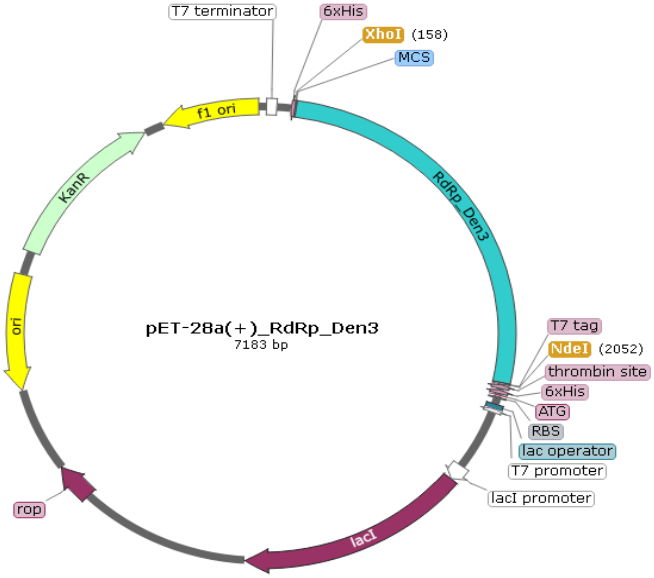


Figure 1. Construction of gene synthetic pET28a(+)-[NS5-RdRp DENV3].

2.5.2. Transformation of E. coli BL21(DE3) with NS5-RdRp DENV3 gene synthetics

A volume of 200µL *E. coli* BL21 (DE3) competent cell was mixtures with 40 ng/ µL synthesis gene pET28a(+)-[NS5-RdRp DENV3] and then was transformed through the heat shock method. The transformant was recovered by 800µL SOC nutrient and incubated shaken at 37ºC, 200 rpm for 1 h. The transformant was selected in LB medium agar containing kanamycin (50µg/mL). Growing transformant colonies on selection medium agar were replicated followed by plasmid isolation. The isolation was carried out using TIANprep Mini Plasmid Kit then the isolate was identified with 1% agarose gel electrophoresis. The isolate of plasmid was sequenced using primer T7 promotor and T7 terminator and aligned using CLUSTAL Omega(1.2.4)multiple sequence alignment and https://blast.ncbi.nlm.nih.gov/.

2.5.3 Expression of NS5-RdRp DENV3

E. coli BL21 (DE3) encodes NS5-RdRp DENV3 was expressed in a 50 mL LB medium containing 50 µg/mL kanamycin at 37ºC, 200 rpm, for 4 h. Then, the culture was induced by 0.5 mM IPTG at OD_{600} 0.5. The culture was harvested by centrifugation at 3.000 g at 4ºC for 20 min. The pellet was dissolved in 20 mM Tris-Cl pH 8 lysis buffer and sonicated for 15 min. The lysate was centrifuged with 5.000 g at 4ºC for 20 min. The supernatant was a soluble fraction while the pellet was an insoluble fraction that needed to be solubilized using 8 M Urea and then was centrifuged with 10.000 g, at 4ºC for 30 min. Soluble and insoluble fractions were characterized with SDS-PAGE.

3. Results

3.1. Serotyping of Dengue Virus

Lanciotti developed a two-step PCR assay to detect dengue virus serotypes. The first step involves RT-PCR using universal primers D1 and D2, which target the capsid and pre-membrane regions, respectively, to amplify all dengue virus serotypes. A band of approximately 511 bp in size indicates the amplicon of the dengue virus (Supplementary materials, Figure S1, Lanes 2 and 3). The second step involves multiplex-PCR to detect specific dengue virus serotypes using D1 and TS3 primers that also target the capsid and pre-membrane regions of DENV-3. Bands corresponding to dengue virus serotypes 1, 2, 3, and 4 are observed at 482 bp, 119 bp, 290 bp, and 392 bp, respectively[20]. The PCR amplification results in a 290 base pair fragment, which aligns with the positive control DENV-3 Zymo Bunami RNA (Figure S1), indicating that the DENV sample is serotype DENV-3 (Lines 7 and 9). The results of the dengue virus serotyping are shown in (Figure S1).

3.2. Isolation and Amplification of RdRp DENV3 Gene

The conserved motifs were commonly present in various RdRP expressed by positive-strand RNA virus identified in NS5, the largest and the most conserved DENV protein. And also, RdRp mediates positive-strand RNA virus replication, flavivirus including dengue virus[21]. It can be seen in (Figure 2), the amplicon band around 1899 bp indicated the isolate was successfully amplificated by specific primers that we designed for the RdRp region. Then, the amplicon sequences were aligned toward reverse and forward-specific primers (Figure 2).

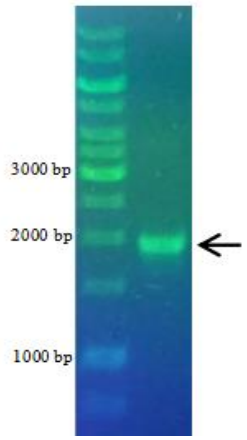


Figure 2. Characterization of PCR result on NS5-RdRp isolate.

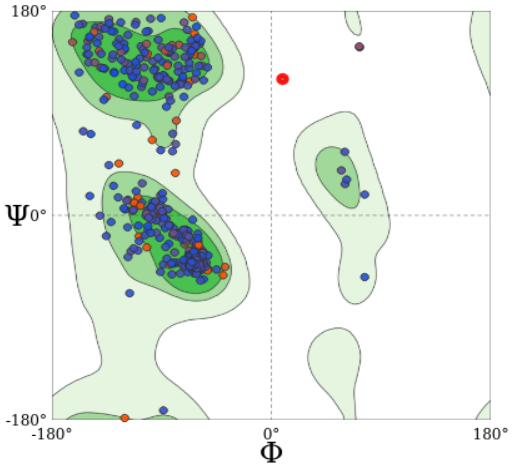
The DENV-3 RNA was subjected to PCR using the RdRp forward and reverse primers. Then the amplicon was sequenced by RdRp primers forward and reverse, yielding sequences of 664 bases and 988 bases, respectively, with a 196 base-gap (Figure S2). The sequences were analyzed using BLAST and found to be 99.5% identical to the AY858045 nucleotide sequence and 100% identical to the AY858041 amino acid sequence, corresponding to the Dengue Virus 3 strain FW06 isolated during the 2004 Indonesian dengue outbreak. These sequences have circulated in Indonesia for many years and the 196 base gap can be filled by referencing these sequences. To ensure the accuracy of this filling, the translated missing nucleotides were blasted against 1000 non-redundant protein sequences in the NCBI database. The blast revealed that the missing sequence was in a conserved region of the DENV3 RdRp, we found 100% identity on 1198 hits sequences (Figure 3), confirming that the sequence was appropriate to fill the gap. Structurally this sequence is located at the interface of the three subdomains[22,23].

Taxonomy	Number of hits	Number of Organisms	Description
⊟Orthoflavivirus denguei	1198	6	
. ⊟Dengue virus	60	6	Dengue virus hits
. . ⊟dengue virus type 3	1133	4	dengue virus type 3 hits
. . . Dengue virus 3 Singapore/8120/1995	1	1	Dengue virus 3 Singapore/8120/1995 hits
. . . Dengue virus 3 China/80-2/1980	1	1	Dengue virus 3 China/80-2/1980 hits
. . . Dengue virus 3 Martinique/1243/1999	1	1	Dengue virus 3 Martinique/1243/1999 hits
. . dengue virus type 1	2	1	dengue virus type 1 hits

Figure 3. Blast Search Results for Dengue Virus Taxonomy. This provides a summary of the blast search results, categorizing dengue viruses by taxonomic level and providing the number of hits and organisms identified for each category.

3.3. Homology Modeling of RdRp

The amino acid sequence of Dengue virus serotype 3 RNA-dependent RNA polymerase (RdRp) was used to predict its 3D structure through protein homology modeling. The crystal structure of the complex between the RdRp compound 27 (PDB ID 5JJS) was selected as a template due to its high sequence identity of 97%. The resulting 3D structure was evaluated using Ramachandran Plots, which showed that the structure was properly modeled with outliers less than 1%, as illustrated in Figure 4. Additionally, QMEANDisCo Global was used to assess the consistency of the CA-CA distance between the model and the template, which scored 0.82, can be seen in Figure 5. However, the template also showed some missing residues in the low confidence or orange area. These missing residues could affect the accuracy of the model, but the QMEANDisCo score indicates that the overall consistency between the model and the template is still high.



MolProbity Score	1.38
Clash Score	1.31
Ramachandran Favoured	96.56%
Ramachandran Outliers	0.33%
Rotamer Outliers	2.45%
C-Beta Deviations	4
Bad Bonds	0 / 5103
Bad Angles	31 / 6904

Figure 4. The Ramachandran plot assessment reveals that the structure of the model is of good quality.

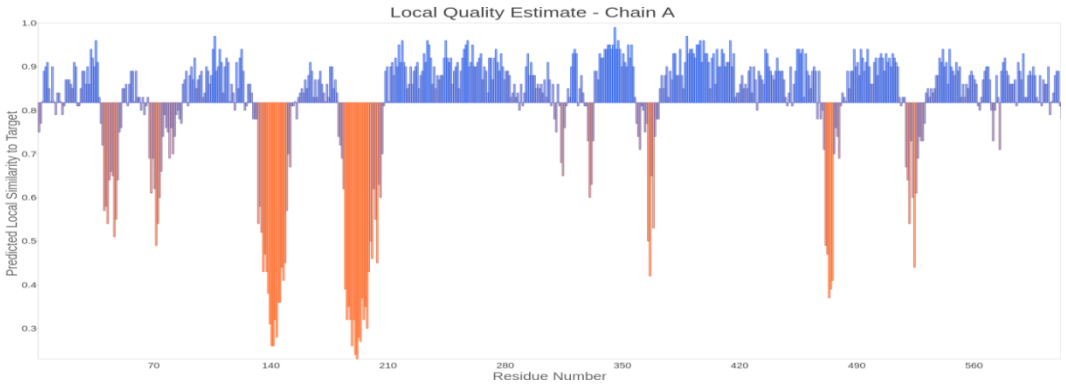


Figure 5. QMEANDisCo of the model, the expected similarity of each residue in the model to the native structure is plotted on the y-axis against the residue number on the x-axis. Residues with scores below 0.6 are considered to be of low quality.

3.4. Expression of recombinant RdRp protein

To investigate the efficacy of recombinant NS5-RdRp protein as a potential drug screening system, the NS5-RdRp cDNA was cloned into pET28a. Transformation of *E. coli* BL21(DE3) with pET28a(+)[NS5-RdRp DENV3] was performed with the heat shock method. *E. coli* BL21(DE3)-pET28a(+)[NS5-RdRp DENV3] transformant was grown on a selection medium with transformation efficiency 861 cfu/mL, presented in (Figure S3). The five colonies were picked and isolated. Then, the plasmid concentration was measured by ImageJ, a densitometry method, to screen the best colony[24]. It can be seen from Figure S4 indicated that colony-2 was the best colony generating 15.9 ng/µL plasmid. The colony-2 plasmid isolate was sequenced using a universal primer T7 to confirm the NS5-RdRp gene sequence (Figure S5). Alignment of RdRp gene sequence with RdRp contig1 and RdRp recombinant sequence result with primer T7 terminator reverse (Figure S5). Then, the NS5-RdRp sequence was aligned to the DENV3 databases. It showed more than 95% identity to polyprotein DENV 3 (Figure 6).

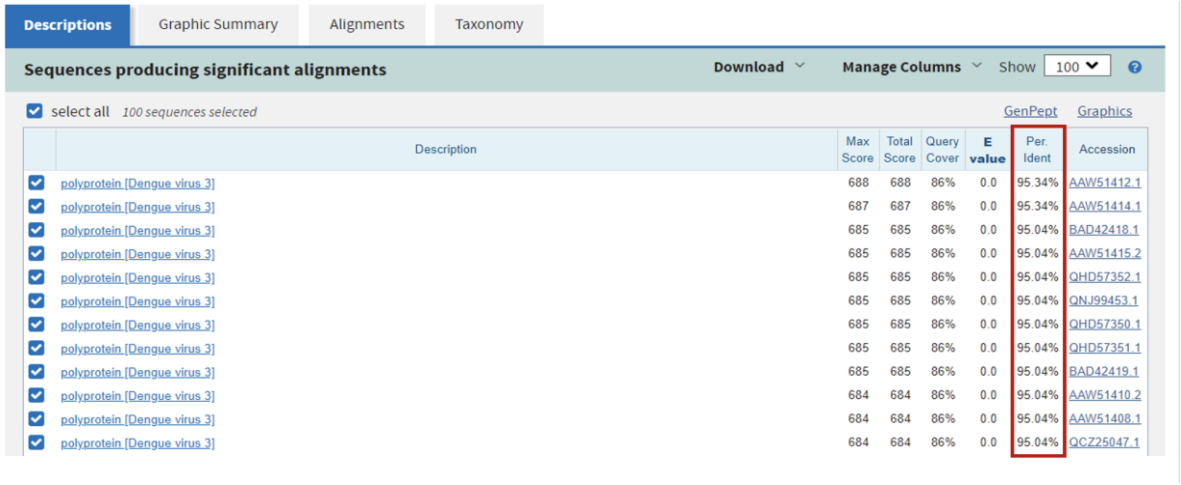


Figure 6. Percent identity of RdRp recombinant sequence toward Polyprotein Dengue Virus 3.

3.5. Expression of NS5-RdRp DENV3

E. coli BL21 (DE3) encodes NS5-RdRp DENV3 was expressed in the condition induced by 0.5 mM IPTG and incubated at 37ºC, 200 rpm, for 4 h. In that condition, NS5-RdRp recombinant was majority expressed in the insoluble fraction or inclusion bodies with molecular weight around 72 kDa and the yield concentration at 2.03 mg/mL, defined by ImageJ densitometry method compared to the Bovine Serum Albumin (BSA) standard (Figure S6). In this circumstance, inclusion bodies are formed during high-level expression of heterologous proteins in the *E. coli* cell host. Although inclusion bodies are seen as undesired, their development can be advantageous since their extraction from cell homogenates is a convenient and effective method mainly for further work like purification[25].

4. Discussion

Flaviviruses are a group of viruses that cause serious illnesses in humans. Some of the most well-known flaviviruses include dengue (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), Yellow Fever virus (YFV), and Tickborne encephalitis virus (TBEV)[26]. Dengue is a viral infection transmitted by mosquitoes that has become a major global problem in recent years. Over half of the world's population, especially in tropical and subtropical areas, is at risk of being infected with dengue, with 50–100 million cases reported each year and over 500,000 severe cases resulting in life-threatening illnesses[27]. The dengue virus (DENV) is the cause of dengue and is divided into four serotypes with 70–80% similarity in their amino acid sequences. Infection by any of the four DENV serotypes can

result in a range of outcomes, including no symptoms, dengue fever, dengue hemorrhagic fever, and dengue shock syndrome[28]. Cross-infection with different serotypes increases the risk of severe dengue, and while there is a vaccine available, it only provides partial protection against all four serotypes. Currently, there are no approved antiviral treatments for dengue or other flaviviral diseases.

The RNA replication of flaviviruses occurs in host cells and is facilitated by a replication complex (RC) consisting of viral NS proteins and host cofactors. The largest and most conserved component of the RC is NS5, a protein composed of 900 amino acid residues. The N-terminal domain of NS5 is a SAM-dependent methyltransferase that methylates the cap of the viral RNA genome, while the C-terminal RdRp domain synthesizes the viral genomic RNA. The linker region between the two domains regulates the interactions between the MTase and RdRp and modulates virus replication. NS5 also inhibits host interferon-mediated signaling in addition to its enzymatic functions[29,30].

Following DENV infection, the RdRp synthesizes viral RNA through a de novo initiation mechanism without a primer strand. The RdRp has a "right-hand-like" architecture with three subdomains: "fingers," "palm," and "thumb." Several conserved amino acid sequence motifs play key roles in RNA and NTP binding, metal ion binding, and catalysis. The DENV RdRp has a "closed" pre-initiation state conformation with a well-ordered priming loop projecting into a narrow RNA binding tunnel[31–33].

The RdRp domain, which is essential for virus replication and not found in humans, is a potential target for antiviral development[34,35]. The thumb subdomain of the RdRp domain has a "priming loop" that is thought to change conformation during replication, and many inhibitors against DENV RdRp bind near this loop. Some inhibitors against HCV RdRp bind to allosteric sites on the thumb subdomain. A fragment was identified that binds to an allosteric site near the enzyme active site in the thumb subdomain of the DENV RdRp, and was used to design potent inhibitors that inhibit DENV replication. Other reports describe potent inhibitors that bind to the site near the finger subdomain[36–38].

The urgency of developing a pan-serotype dengue antiviral drug is evident from our local sequence analysis, which revealed that two binding sites are conserved across all serotypes of dengue. This indicates a high likelihood of creating a pan-serotype antiviral that can reduce dengue-associated morbidity and mortality rates. However, an obstacle to this kind of therapy is the current lack of efficient early diagnostics.

Studies have identified an allosteric pocket, known as the "N pocket," at the interface between the thumb and palm subdomains of the RdRp of the DENV. The N pocket was found through fragment-based screening and X-ray crystallography using the RdRp protein from DENV3 as a target. The N pocket is located near the priming loop of the enzyme and is lined by highly conserved residues across various DENV serotypes and other flaviviruses like the Zika virus. Reports indicate that several N pocket residues play a critical role in the initiation activity of the DENV NS5 polymerase and virus replication[37].

Rational design was used to create inhibitors that target the N pocket and effectively inhibit the initiation activity of DENV1-4 polymerases and virus replication in different cell types. These inhibitors show a high affinity for both the recombinant apo-enzyme and DENV replicon cell lysates. The inhibitory modes of these N pocket inhibitors were found to be non-competitive and uncompetitive during the initiation and elongation phases, respectively, based on competitive experiments with GTP[39].

Two DENV RdRp nonnucleoside inhibitors were characterized as low- to high-micromolar inhibition in DENV RNA polymerization and cell-based assays. They bind in the enzyme RNA template tunnel in X-ray crystallography. NITD-434 induced an allosteric pocket at the junction of the fingers and palm subdomains by displacing residue V603 in motif B. Binding of another compound (NITD-640) ordered the fingers loop preceding the F motif, close to the RNA template entrance. Both sites are essential for polymerase de novo initiation and elongation activities and significant for viral replication. This work provides evidence that the RNA tunnel in DENV RdRp offers appealing target sites for inhibition[40].

RdRp could be employed as a biochemical test in vitro for drug discovery. Compounds were screened using in vitro biochemical assays that measured DENV RdRp elongation and de novo initiation activities. These assays use radioisotope-labeled nucleotides (for a scintillation proximity assay) or fluorescence-labeled nucleotides (for a calf-intestinal phosphatase-coupled fluorescence assay), which may be miniaturized into 384-well or 1536-well plate sizes. In the elongation tests, homopolymeric templates were employed, but in the de novo assays, a mini-genome viral RNA containing the 5’ and 3’ UTR sequences was used. De novo assays using homopolymeric templates and short RNA oligonucleotide from the viral anti-genome 3’ UTR region have also been produced[34].

DENV comprises 5 serotypes, including DENV1, DENV2, DENV3, DENV4, and DENV5. Determination of DENV serotypes can be performed in 2-stages which are the cDNA synthesis stage with RT-PCR PCR and nested PCR[20,41]. In the cDNA synthesis stage using the RT-PCR method, D1 and D2 primers were used which targeted the capsid and pre-

membrane areas which had a size of 511 bp. The primer functions as a barrier for the target DNA fragment to be amplified and to provide a hydroxy group (-OH) at the 3 'end needed for the DNA extension process during PCR. RNA has an unstable nature because it is easily lysed in cells. The enzyme used to convert RNA to cDNA is a reverse transcriptase enzyme derived from Moloney Murine Leukemia Virus (M-MLV) which is more stable and more heat resistant and functions to reduce RNAse H and maintain temperature stability.

The DENV RNA that had been converted into cDNA was then amplified by nested PCR. Nested PCR is a type of PCR which process allows for a reduction of contamination of the product during the amplification of unnecessary primary coalescence. The primers used in the nested PCR method were D1 primers and TS3 primers which targeted the capsid and pre-membrane areas in DENV, respectively. This primer produced PCR amplification products with a fragment length of 290 bp that aligned with control positive DENV3 Zymo Bunami RNA which indicates that the DENV sample is DENV3 (Figure S1). This method has been shown to exhibit greater sensitivity than traditional virus isolation when using identical sample aliquots, and it is commonly utilized for the diagnosis and monitoring of dengue, particularly in Southeast Asian nations[20,42].

DENV3 RNA PCR using primer RdRp forward and primer RdRp reverse produced 1800 bp amplicons (Figure 2). The 1800 bp band was then purified and used as a template for sequencing. It was then aligned with the DENV3 complete genome (accession AY858045) as a reference (data not shown). Alignment results showed that forward primers can amplify the NS5-RdRp isolate gene along 647 bases, whereas reverse primers can amplify genes up to 840 bases. There is a gap of 196 bases that have not been sequenced, the remaining sequence fragments that have not been identified are then taken from AY858045, a part of the conserved area on NS5-RdRp.

The combination of sequence from PCR result and sequence fragment obtained from accession AY858045 gave a complete NS5-RdRp DENV3 sequence. This sequence was translated into amino acid and generated an amino acid sequence. The NS5-RdRp DENV3 sequence with the DENV3 database gave identity around 95% and the rest resulted from the isolates sequencing taken from the Indonesian Dengue Outbreak 2004 (data not shown). The amino acid sequence was used for protein homology modeling to predict the 3D structure of Dengue virus serotype 3 RNA-dependent RNA polymerase (RdRp). The crystal structure of the complex between the RdRp and a new pyridobenzothizole inhibitor called HeE1-2Tyr (PDB ID 5iq6) was used as a template and had 97% sequence identity (Figure 7). Then, the structure was compared to other RdRp-inhibitor complexes to identify additional binding sites.

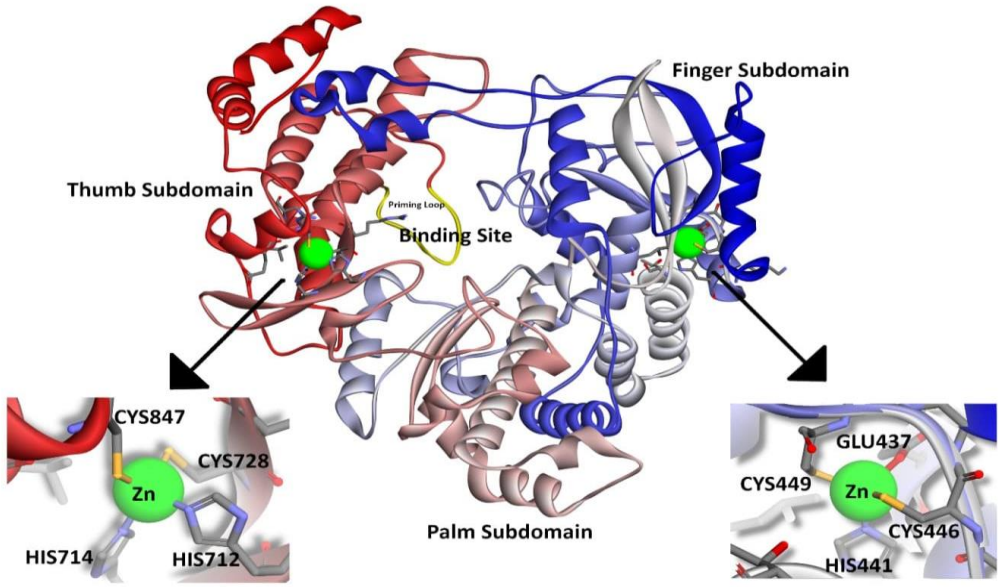


Figure 7. The model structure of Dengue 3 NS5 RdRp. Notes: The structure was divided into three subdomain, finger, palm and thumb. The priming loop (yellow) are located in the middle of the RNA binding site. Zn (green) binding site are located in finger and thumb subdomain, this binding site are made of cysteine, histidine and glutamic amino acid.

The NS5 RdRp has a large central cavity and consists of three subdomains: finger, palm, and thumb (Figure 7). The NS5 RdRp protein was found to have two Zinc (Zn) metal ions, each of which binds to two cysteines. These Zn ions are located in the Finger and Thumb subdomains and are believed to play a role in stabilizing and maintaining the thermal stability of the NS5 RdRp. In the Finger subdomain, Zn is bound to residues GLU437, HIS441, CYS446, and CYS449, while in the Thumb subdomain, it is bound to residues HIS712, HIS714, CYS728, and CYS847. Residues close to HIS712, such as SER710 and ARG729, play a crucial role in binding to incoming nucleotides and thus may be involved in the replication process. These findings suggest that the Zn ions are critical for NS5 RdRp function[11]. NS5 RdRp protein has a large RNA-binding site for viral replication and two known inhibitor-binding pockets. The first pocket is located on the finger subdomain, and the second is on the thumb subdomain[11,14,37,38,43,44]. The pockets are separated by a priming loop that plays a crucial role in conformational activity. Both pockets are highly conserved across all dengue serotypes, making them promising drug targets[11,38]. Benzothiazole scaffolds bind to the finger subdomain pocket, while biphenyl acetic acid scaffolds bind to the thumb subdomain pocket[37,38]. Remdesivir, which effectively inhibits various flaviviruses' RdRp including Dengue virus, has a benzodiazole scaffold similar to benzothiazole, potentially leading to inhibition of the RdRp by binding to the finger subdomain pocket[36]. The binding sites were aligned with the NS5-RdRp sequence from

dengue serotypes 1-4 (Q5UB51, P27909, P12823, and Q2YHF0), showing high conservation of the binding site across all serotypes with identity around 88-97% (Figure S7).

NS5-RdRp DENV3 gene which encodes 626 amino acids sequence was synthesized and expressed in *E. coli* BL21(DE3) to produce recombinant NS5-RdRp DENV3. This recombinant protein will be used in drug discovery for anti-dengue against DENV Indonesia local strain. The gene sequence was optimized based on *E. coli* codon preferences. The optimized RdRp gene was inserted into pET28a plasmid containing kanamycin as a selection marker and *LacI* promoter that can be induced with IPTG generating pET28a(+)[NS5-RdRp DENV3] plasmid to express the protein of interest. Transformation of *E. coli* BL21 (DE3) as host cells with pET28a(+)[NS5-RdRp DENV3] was performed with the heat-shock method shown in Figure S3. The transformant colonies were picked and replicated followed by plasmid isolation. The plasmid was isolated using primer T7 promotor dan T7 terminator and then was characterized by agarose gel electrophoresis, shown in Figure S4. Subsequently, the isolated plasmid was sequenced to confirm the RdRp gene sequence.

The sequence of isolated plasmid was then aligned using *CLUSTAL Omega (1.2.4) multiple sequence alignment* and with a database in NCBI (*https://blast.ncbi.nlm.nih.gov/*). NS5-RdRp DEN3 gene sequence, pET28a_RdRp_Den3 sequence result with primer T7 promoter, and pET28a_RdRp_Den3 sequence result with primer T7 terminator were contigged using online software Prabi (http://doua.prabi.fr/cgi-bin/run_cap3) generating contig1 sequence. The contig1 sequence was aligned with the RdRp gene sequence design using online Clustal omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). There was slight differentiation between the RdRp gene sequence design and the contig1 sequence on position 868-984 bp. This mismatch was re-confirmed with the pET28a_RdRp_Den3 sequence result with primer T7 terminator on that position and resulted that there was no differentiation between RdRp gene sequence design with the pET28a_RdRp_Den3 sequence result with primer T7 terminator (reverse) on position 837-1140. It is concluded that the RdRp gene sequence is identical to the RdRp sequence from pET28a_RdRp_Den3 isolated from the transformant (Figure S5). Alignment of RdRp recombinant sequence from isolated pET28a_RdRp_Den3 with database in NCBI resulted in around 95% identity with polyprotein Dengue Virus 3 (Figure 6) and showed 86% conserved domain with NS5.

E. coli BL21 (DE3)-pET28a(+)_RdRp_Den3 transformant grown in media selection (LB agar medium containing kanamycin) was cultured in LB medium to express recombinant RdRp protein (Supplementary Material, S6). The target protein was expressed after induction with IPTG in transformant soluble and insoluble fractions with a molecular weight of 72 kDa. Nevertheless, an insoluble fraction and a soluble fraction showed a thicker band compared to

the control. The SDS-PAGE results indicate a successful expression of NS5-RdRp. However, further purification steps using His-tag for affinity purification should be demonstrated followed by Western blot analysis to ensure the protein's purity and strengthen the identification of recombinant NS5-RdRp.

5. Conclusions

This study successfully isolated and sequenced the RdRp gene, part of the NS5 protein of DENV, from samples obtained at Hasan Sadikin Hospital, Bandung. The isolated RdRp gene sequence was utilized for homology modeling with the DENV database on NCBI, confirming the sample as serotype DENV-3, the most prevalent local strain in Indonesia. The synthetic RdRp gene was cloned into the pET28a(+) plasmid and introduced into *E. coli* BL21 (DE3). The recombinant RdRp was expressed after induction with 0.5 mM IPTG, predominantly in the insoluble fraction, with a molecular weight of 72 kDa. Future research will focus on developing an anti-dengue drug model to inhibit the binding domain within the recombinant RdRp protein structure.

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