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Immunogenicity and reactivity of recombinant Zika virus NS1 inclusion bodies from an Indonesian isolate

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Abstract. *Mulyaningrum U, Subronto YW, Nugrahaningsih DAA, Wijayanti N. 2024. Immunogenicity and reactivity of recombinant Zika virus NS1 inclusion bodies from an Indonesian isolate. Biodiversitas 25: 2028-2034.* Zika virus (ZIKV) still poses a significant global health hazard, with the potential for its dissemination to additional nations and the re-emergence of epidemics in previously impacted regions. Proper laboratory testing during surveillance can prevent future outbreaks. Non-Structural 1 (NS1) protein has been identified as a promising biomarker for detecting ZIKV infection. Therefore, this study produces and assesses the immunogenicity of the full-length recombinant ZIKV NS1 protein (rNS1$_{FL}$) from an Indonesian isolate. It also studies its reactivity against other flavivirus members, especially the Dengue Virus (DENV). The cDNA fragment comprising the ZIKV NS1 sequence was synthesized and cloned into the pET28a plasmid. Subsequently, the recombinant plasmid was introduced into BL21(DE3)-competent *Escherichia coli* cells, and the protein was purified through affinity chromatography. Female BALB/c mice (*Mus musculus*) were intraperitoneally immunized with purified rNS1$_{FL}$. An Enzyme-Linked Immunosorbent Assay (ELISA) evaluates the immunogenicity of rNS1$_{FL}$ and its cross-reactivity with the DENV NS1 monoclonal antibody. rNS1$_{FL}$ protein was expressed as insoluble fractions (inclusion bodies) at the expected size of approximately 48 kDa. The ELISA showed that immunization of BALB/c mice with purified rNS1$_{FL}$ from inclusion bodies led to a substantial triggering of NS1-specific antibodies. Furthermore, the test also revealed limited recognition of rNS1$_{FL}$ by the DENV NS1 monoclonal antibody. The findings indicated that rNS1$_{FL}$ inclusion bodies have immunogenic solid properties and did not display any cross-reactivity with the DENV NS1 monoclonal antibody. These observations showed the potential of rNS1$_{FL}$ as a serological diagnostic material for detecting ZIKV infection.

Keywords: Biomarker, *Flavivirus*, rNS1$_{FL}$, serological diagnostic, ZIKV

INTRODUCTION

Zika virus (ZIKV) is a primary arthropod-borne virus (arbovirus) of the *Flavivirus* genus and Flaviviridae family (Simmonds et al. 2017). This virus is a single serotype with two distinct lineages, namely African and Asian strains (Haddow et al. 2012). It possesses a genome of a positive-sense, single-stranded RNA that spans about 11 kilobases in length. The genome is structured as 5′UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-2K-NS4B-NS5-3′UTR. This arrangement consists of Untranslated Regions (UTR) that flank a region responsible for coding proteins (Ramaiah et al. 2017).

The ZIKV gained worldwide attention during outbreaks in the Pacific Islands, such as Yap Island in 2007 (Duffy et al. 2009), and then continued in French Polynesia in 2013 (Cao-Lormeau et al. 2014). The rapid global spread of ZIKV, particularly in Latin America and the Caribbean, along with its potential association with microcephaly and central nervous system disorders, prompted the World Health Organization (WHO) to declare it a Public Health Emergency of International Concern (PHEIC) in February 2016 (Gulland 2016). Although there has been a decrease in ZIKV cases worldwide since 2018, the transmission of this virus continues to persist at a low level in several American and endemic countries (PAHO 2023). According to the WHO (2022), the presence of this virus has been verified in 89 countries globally. The findings emphasize the need for continuous post-epidemic vigilance in monitoring diseases and ensuring global readiness to detect and respond to potential threats, including monitoring potential risks, implementing measures to control the vectors, and investing in the development of diagnostic tools, vaccines, and treatments (WHO 2022).

Indonesia has not yet reported any ZIKV infection outbreaks. However, numerous studies have confirmed the virus's presence in the country, with the first serological detections occurring in the late 1970s in Klaten, Central Java (Olson et al. 1981), and the Lombok region (Olson et al. 1983). ZIKV has also been identified in Australian tourists who previously visited Indonesia (Kwong et al. 2013; Leung et al. 2015). A retrospective study revealed 9.1 and 9.2% seroprevalences of ZIKV infection in children aged 1-4 years and 5-9 years across 14 provinces, respectively (Sasmono et al. 2018, 2021). Following the successful isolation of ZIKV in Jambi, Sumatra, in 2014, phylogenetic analysis revealed that the virus, JMB-185 isolate, along with strains from Thailand, Cambodia, and the Philippines, belongs to the Asian lineage (Perkasa et al. 2016). Yudhaputri et al. (2017) conducted whole genome sequencing of the JMB-185 isolate to explore its genetic properties. Molecular evolution analysis from this study indicated that this ancient lineage of the isolate could have been present in Southeast Asia in the 2000s, explaining its distinctiveness from the isolates responsible for the outbreaks in French Polynesia and Brazil. Additionally, Amino Acid (AA) analysis of this isolate identified nine distinct alterations in the viral polyprotein, not found in other published genomes from the Asian-American lineage. These unique variations were found in prM, M, NS1, NS2A, NS2B, 2K, and NS4B.

Laboratory confirmation of ZIKV infection is essential to identify and differentiate ZIKV infection from other arboviruses (Chan et al. 2016). Serological examinations, such as antibody detection through ELISA, provide a broad timeframe for the confirmation of ZIKV infection, even after the virus has been eliminated from the body of a patient (Goncalves et al. 2018). To carry out this diagnostic procedure, an antigen recognized by a ZIKV-specific antibody without cross-reacting is required (Landry and St George 2017). The NS1 protein is a common marker for diagnosing acute and convalescent flavivirus infection. This protein is highly conserved among flaviviruses and comprises approximately 352 amino acids, with a molecular mass ranging from 46 to 55 kDa (Muller and Young 2013). Previous investigations reported that NS1-derived protein microarrays exhibit considerable sensitivity and specificity in detecting IgG and IgM antibodies against medically relevant flavivirus infection, with minimal cross-reactivity, suggesting the potential of NS1 as a suitable antigenic substrate for diagnostic purposes (Cleton et al. 2015).

Prior studies used strains from epidemic regions, such as French Polynesia (Viranaicken et al. 2017a), Haiti (Liu et al. 2017), Brazil (Viranaicken et al. 2017b; Kanno et al. 2020), and Puerto Rico (Roldán et al. 2020), to generate ZIKV NS1 recombinant protein. No previous research has been conducted on producing this recombinant protein using isolates from Indonesia. Hence, this study aimed to produce a recombinant ZIKV NS1 protein based on an Indonesian isolate and evaluate its immunogenicity. Additionally, we determined these recombinant proteins' reactivity, particularly against DENV, a flavivirus member that often causes outbreaks in Indonesia.

MATERIALS AND METHODS

Construction and transformation of ZIKV NS1 recombinant plasmid

For efficient expression of the NS1 protein in *E. coli* cells, the cDNA fragment comprising the complete sequence of ZIKV NS1 (JMB-185 Indonesian isolate, GenBank: KU179098.2) was codon optimized, chemically synthesized, and cloned into the pET28a plasmid by GenScript. The recombinant plasmid was named pET28a-NS1$_{FL,}$ containing the gene that codes for a 352-residue full-length protein with one histidine tag at the N-terminus. The recombinant plasmids were introduced into One Shot^{TM} BL21(DE3)-competent *E. coli* cells (ThermoFisher Scientific) through the heat shock technique, which included a 45 s exposure to 42°C. Subsequently, these cells were cultured at 37°C overnight on Luria-Bertani (LB) agar with 50 µg/mL of kanamycin sulfate (Gibco). Polymerase chain reaction (PCR) was conducted to verify the colonies using GoTaq Green Master Mix (Promega). The forward primer was the T7 promoter, while the reverse was the T7 terminator. Then, the PCR products were run on a 1% agarose gel.

Expression and purification of recombinant NS1$_{FL}$ protein (rNS1$_{FL}$)

E. coli BL21(DE3) bacterial cultures deployed for protein production were grown at 37°C with continuous shaking (170 rpm) in an LB medium containing 50 µg/mL of kanamycin sulfate. Isopropyl-ß-D-1-Thiogalactopyranoside (IPTG) (Merck-Omnipur) was added to the colonies once a 0.5-0.6 Optical Density (OD) was reached at 600 nm to stimulate protein expression. Several IPTG concentrations (0.5 and 1.0 mM) and induction times (4 h and overnight) were used to enhance protein synthesis. The cells were harvested by 5000 rpm centrifugation for 5 min at 4°C. The cell pellets were cleaned three times with PBS, resuspended in it again, and subjected to six rounds of sonication, each comprising 30 s on and 30 s off. The cell pellets and supernatants were separated by 12,000 rpm centrifugation for 5 minutes at 4°C. These were analyzed through SDS-PAGE with a 12% resolving gel to assess the molecular weight and solubility of rNS1$_{FL}$ protein.

The HisPurTM Ni-NTA Purification Kit (ThermoFisher Scientific) was used to purify rNS1$_{FL}$ under native conditions. After resuspending the cell fragments in PBS, protein extract was mixed with equilibration buffer (10 mM imidazole) to create the sample. The Ni-NTA spin column was equilibrated with two resin-bed volumes of equilibration buffer. Next, the sample was applied to the column and washed with a wash buffer (25 mM imidazole). rNS1$_{FL}$ was extracted using an elution buffer (250 mM imidazole). The eluents were collected and kept in storage at -20°C. rNS1$_{FL}$ was also purified using the electroelution technique, similar to a previous study (Kusuma et al. 2021).

SDS-PAGE and western blot

ZIKV rNS1$_{FL}$ was subjected to analysis through SDS-PAGE, followed by western blotting. The protein suspensions in sample buffer were heated at 95°C for 5 min, and then loaded onto a 12% resolving SDS-PAGE gel, which was run at 80 volts for 2 h. After electrophoresis, it was transferred onto a nitrocellulose membrane. It was rinsed three times with washing solution (0.05% TBS-Tween) before being blocked with 1% BSA in 0.5% TBS-Tween for 60 min at room temperature. Furthermore, it was incubated for one night at 4°C with a 6X-His Tag Monoclonal Antibody (ThermoFisher Scientific) in a 1:1000 dilution (5% BSA in 0.1% TBS-Tween) and washed three times. This was again exposed to 1:500 (1% BSA in 0.05% TBS-

Tween) AP-conjugated goat anti-mouse IgG antibody (Abbkine) as the secondary antibody for 60 min at room temperature with agitation. The reaction was visualized by applying NBT-BCIP substrate to the nitrocellulose membrane after three additional washes.

Immunizations of mice and serum sample collection

Ethical approval for laboratory animal use was from the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (Ref: KE/FK/0892/EC/2022). Immunization was carried out at the Animal House, Department of Pharmacology and Therapy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada. The experimental process included 20 female BALB/c mice (*Mus musculus*) aged 6-8 weeks, based on good health, absence of physical abnormalities, and no history of previous usage. Mice were acclimatized to the laboratory environment and dieted for one week before commencing the study. These subjects were placed in a well-ventilated room with a 12:12 light-dark cycle.

The mice were divided into five groups (n=4). These included K1, K2, K3, K4, and K5 groups, which were treated with PBS, 5 µg of rNS1$_{FL}$ purified through Ni-NTA resin, 20 µg of rNS1$_{FL}$ purified through Ni-NTA resin, 5 µg of rNS1$_{FL}$ purified by electroelution, and 20 µg of commercial ZIKV recombinant NS1 protein (MyBioSource), respectively. All mice were intraperitoneally injected with the antigen emulsified in complete Freund's adjuvant, except for those in K1. Following the initial immunization, booster injections were administered on days 14 and 28 at the exact dosage as the primary injection but with incomplete Freund's adjuvant.

Blood samples were drawn from the retroorbital plexus on days 7, 21, and 35 following the first, second, and third injections. The collected blood was placed in microtubes and centrifuged; the serum was extracted and stored at -20°C.

Immunogenicity and reactivity of ZIKV rNS1$_{FL}$

An ELISA evaluates the ZIKV anti-NS1 IgG antibody in the blood samples of rNS1$_{FL}$-immunized mice. Purified rNS1$_{FL}$ proteins were prepared in coating buffer (0.2 M Na_2CO_3; 0.2 M $NaHCO_3$) and used to coat each well of a 96-well plate (MaxiSorp-Nunc) at a concentration of 5 µg/mL. The plate was blocked for 2 h with 1X PBS containing 1% BSA and cleaned three times with washing buffer (0.15 M NaCl; 0.05% Triton X-100; 0.02% NaN_3). Immunized mice sera, diluted at 1:200 in incubation buffer (0.1 M NaCl; 50 mM Na_3PO_4; 0.05% Triton X-100; 0.02% NaN_3), were introduced into the plate and incubated for 60 min at 37°C. Following three cleansing steps, the bound NS1 antibody was detected by applying AP-conjugated anti-mouse IgG (Sigma) at a 1:1000 dilution for 60 min. The plate was three times cleansed, and 4-Nitrophenylphosphate in substrate buffer (0.1% Diethanolamine; 0.5 mM $MgCl_2$) was added, then incubated at 37°C for 15-30 min. Measurement was conducted using a microplate reader (Zenix) at 405 nm. Furthermore, rNS1$_{FL}$ was assessed for cross-reactivity with DENV type 1-4 NS1 monoclonal antibody (ThermoFisher Scientific) through the previously described protocols.

Data analysis

All quantitative data were given as mean±Standard Deviation (SD), where each dataset normality distribution was evaluated by applying the Shapiro-Wilk test, and the variance homogeneity was obtained with Levene's test. A one-way ANOVA was performed to compare antibody concentration values between groups at each time point for parametric data (collected on days 7 and 35). The Kruskal-Wallis test was used for non-parametric data (obtained on day 21). In cases of significant differences, post-hoc tests such as LSD or Mann-Whitney were carried out. Differences were considered statistically significant at $p<0.05$. Statistical analyses were conducted using GraphPad Prism 8 software.

RESULTS AND DISCUSSION

Production of ZIKV rNS1$_{FL}$

Prior to protein expression, NS1$_{FL}$ gene insert was verified in competent *E. coli* BL21 (DE3) cells growing on LB agar through PCR, using T7 promoter and T7 terminator primers. Figure 1 shows the results of a colony PCR experiment, showing a correlation between the size of the gene insert band and the target position, which is ~1300 bp.

After induction tests, SDS-PAGE analysis confirmed the ~48 kDa molecular mass rNS1$_{FL}$ expression in *E. coli* BL21(DE3), as in Figure 2. A substantial portion of the expressed recombinant protein was found in the insoluble fraction of the cell extract, showing the presence of Inclusion Bodies (IBs). Alterations in bacterial culture conditions, such as varying IPTG concentrations and induction times, did not lead the protein soluble fraction to rise.

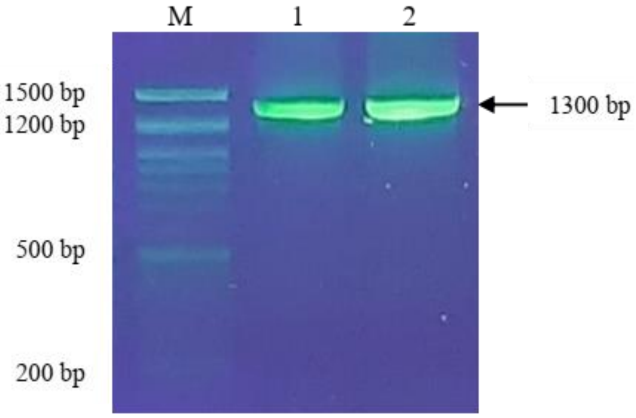

Figure 1. Gel electrophoresis of a colony PCR experiment to verify the correct transformation of the pET28a-NS1$_{FL}$ recombinant plasmid into *E. coli* BL21(DE3). Lane (M): DNA ladder; lanes (1) and (2): The ~1300 bp bands corresponded to NS1$_{FL}$ gene from two *E. coli* BL21(DE3) colonies, as shown by the arrow

rNS1$_{FL}$, isolated from IBs, was purified with Ni-NTA affinity chromatography. Figure 3.A shows protein bands with a molecular weight of ~48 kDa in the eluted fractions on a 12% SDS-PAGE gel, confirming the success of the purification process. Subsequent verification through western blotting, applying an anti-polyhistidine monoclonal antibody, obtained same band corresponding to the target NS1 protein, as presented in Figure 3.B.

Immunogenicity and reactivity of ZIKV rNS1$_{FL}$ in mice model

An assessment was conducted to determine whether the purified rNS1$_{FL}$ IBs could stimulate antibody production in mice. To achieve this, BALB/c mice were immunized with rNS1$_{FL,}$ and the serum samples were analyzed using ELISA following administrations on days 7, 21, and 35. The antibody titers were represented as mean±SD OD values. The results of the one-way ANOVA (days 7 and 35) and the Kruskal-Wallis test (day 21) indicated significant differences in anti-NS1 antibody production between the K1-K4 groups (all $p<0.05$). Figure 4 shows that serum samples from K2, K3, and K4, immunized with rNS1$_{FL}$, exhibited significantly more robust antibody responses than K1 (PBS) after the first administration (all $p<0.05$; LSD test) and subsequent booster injections (all $p<0.05$; Mann-Whitney test for day 21 and LSD test for day 35). These results demonstrate that anti-NS1 antibody concentrations in the treatment groups were significantly higher than in the control group. Additionally, the mean antibody levels in the high-dose group (K3) were not significantly different from those in the low-dose groups (K2 and K4) at each sample collection time (all $p>0.05$).

rNS1$_{FL}$ was also tested using mice sera that had been injected with commercial ZIKV recombinant NS1 protein (K5) derived from the Brazilian SPH2015 strain or commercial DENV NS1 monoclonal antibody (DEN$_{mAb}$) to assess reactivity. As depicted in Figure 5, after the third and final administrations, one-way ANOVA analysis demonstrated a significant difference among K3, K5, and DEN$_{mAb}$ ($p<0.05$). Following a post-hoc LSD test, K5 showed robust reactivity against rNS1$_{FL}$, with no significant differences compared to K3 ($p>0.05$). Meanwhile, rNS1$_{FL}$ presented minimal reactivity with DEN$_{mAb}$ and showed highly significant differences from K3 and K5 (all $p<0.05$).

Discussion

ZIKV has attracted noteworthy attention since the 2010s due to its correlation with adverse outcomes such as microcephaly and other neurological complications (Lee et al. 2021). The emergence of ZIKV in areas with high DENV transmission triggered the pressing necessity for simple serological assays to distinguish infections caused by both viruses (Chao et al. 2019). Previous studies have found that the NS1 protein of flavivirus is a crucial target for immune responses and could be used to create diagnostic tests for both the antigen itself and the antibodies produced against it (Ceconi et al. 2023). Here, we report the production of the recombinant NS1 protein in a bacterial system using the ZIKV NS1 sequence from the JMB-185 Indonesian isolate. Furthermore, the immunogenicity and reactivity of this protein were evaluated.

This study successfully transformed the recombinant plasmid pET28a-NS1$_{FL}$, containing NS1$_{FL}$ gene, into *E. coli* BL21 (DE3) competent cells. Despite optimizing the expression of NS1$_{FL}$ by varying IPTG concentrations and induction times (Figure 2) and culture temperature (data not shown), the expressed recombinant protein was found in the pellet fraction. These results showed the insolubility of rNS1$_{FL}$ as seen in previous reports (Viranaicken et al. 2017a; da Silva et al. 2018; Magalhães et al. 2021). *Escherichia coli* is a commonly used host for recombinant protein production due to its well-characterized genetics, numerous available cloning vectors and mutant host strains, and ability to proliferate rapidly on inexpensive substrates (Peternel and Komel 2011). However, producing heterologous proteins in *E. coli* is susceptible to protein stability and suitability issues because the produced protein tends to form IBs, which are insoluble aggregates (Lebendiker and Danieli 2014). Recent studies have reported IBs containing a considerable amount of correctly folded protein components, indicating that aggregation does not necessarily lead to protein inactivation (García-Fruitós et al. 2005; Schwaighofer et al. 2020; Chura-Chambi et al. 2022). Inclusion bodies enriched with correctly folded proteins can be extracted in biologically active forms under non-denaturing conditions without requiring a renaturation procedure (Peternel et al. 2008; Trinh et al. 2021). Since the attempts to produce soluble rNS1$_{FL}$ failed, this recombinant protein was directly purified from IBs through affinity chromatography under native conditions and then administered to BALB/c mice for immunization. This approach of using IBs as an immunogen in animals has been successfully applied in several investigations, whether performed with other ZIKV NS1 antigens (Viranaicken et al. 2017a) or other pathogen antigens (Yang et al. 2011; Rivera and Espino 2016; Nurjayadi et al. 2019; Rachmania et al. 2021).

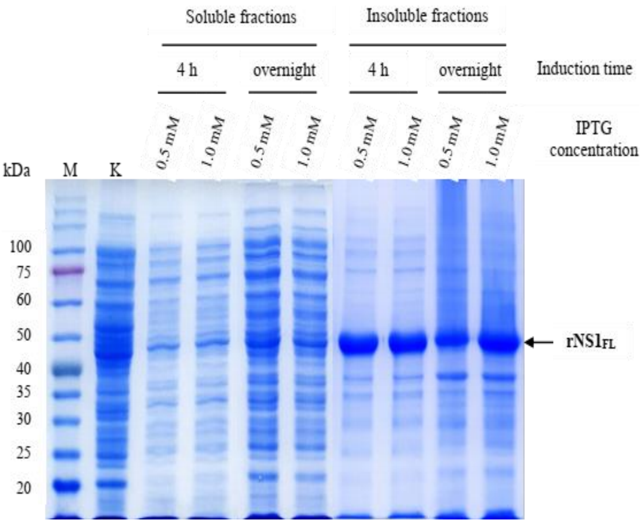

Figure 2. SDS-PAGE results of rNS1$_{FL}$ expression optimization. Protein expression was induced at 37ºC using different IPTG concentrations and induction times. Lane (M): protein marker; lane (K): *E. coli* BL21(DE3) without the pET28a-NS1$_{FL}$ recombinant plasmid. The arrow shows the protein band corresponding to rNS1$_{FL}$

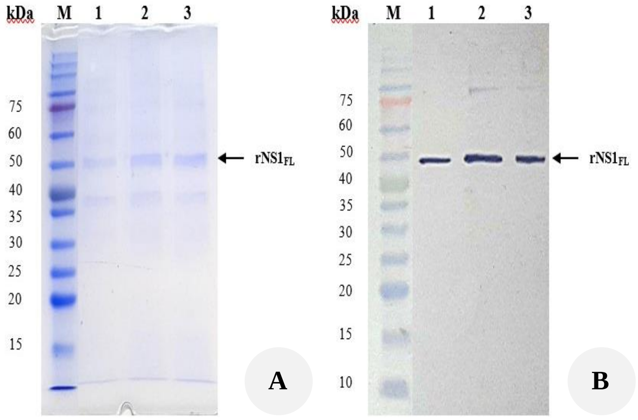

Figure 3. Analysis of purified rNS1$_{FL}$ by: A: SDS-PAGE; and B: Western Blot using anti-his monoclonal antibody. Lane (M): Protein marker; lanes (1), (2), and (3): Eluted fractions

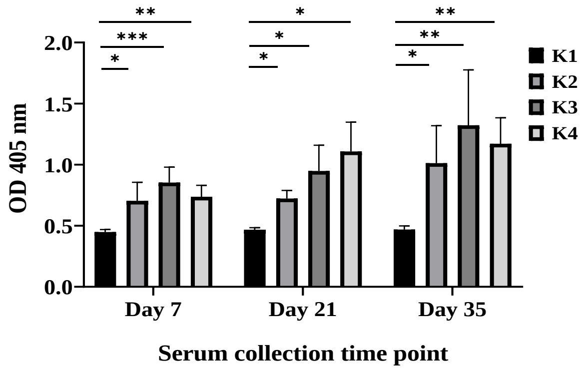

Figure 4. Serum antibody levels in rNS1-immunized mice. Results are presented as mean±SD OD values. *p<0.05; **p<0.005; ***p<0.001. The obtained data were subjected to ANOVA with the LSD test for days 7 and 35, as well as the Kruskal-Wallis and Mann-Whitney test for day 21

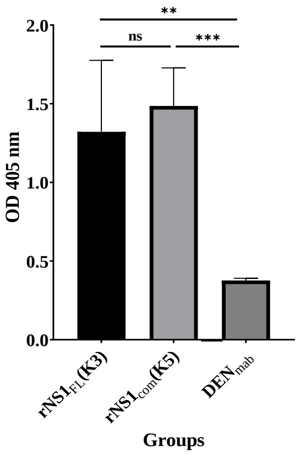

Figure 5. Reactivity of ZIKV rNS1$_{FL}$ to mouse sera following the third immunization and DENV type 1-4 NS1 monoclonal antibody (DEN$_{mAb}$). K3 received 20 µg of ZIKV rNS1$_{FL,}$ while K5 was given 20 µg of commercial ZIKV NS1 protein. Results are presented as mean±SD OD values. **p<0.005; ***p<0.001; ns = not significant (p>0.05). The data were determined by applying ANOVA with the LSD test

Following administration of the purified rNS1$_{FL}$, this study showed the effectiveness of this antigen in inducing antigen-specific antibody responses. Prior research has documented that both the N-terminal region (Viranaicken et al. 2017a) and the C-terminal region (Kanno et al. 2020) of ZIKV NS1 can induce the formation of anti-NS1 antibodies. NS1 is a viral antigen with immunogenic solid qualities. This protein could stimulate antibody production through its immunogenic properties and interaction with the host immune system (Cavazzoni et al. 2021). Despite the aggregation in IBs, rNS1$_{FL}$ successfully triggered an antibody response. Antigen aggregation enhanced immunogenicity by promoting the cross-linking of B-cell receptors. This cross-linking increased B-cell activation, efficient antigen internalization into lysosomes, and improved antigen presentation to T cells (Snapper 2018).

Our study also showed that the rNS1$_{FL}$ IBs derived from an Indonesian isolate could recognize ZIKV anti-NS1 antibodies from different strains, such as the Brazilian type. Meanwhile, ZIKV rNS1$_{FL}$ presented minimal reactivity with the DENV NS1 monoclonal antibody. It showed highly significant differences from ZIKV anti-NS1 antibodies from immunized mice, suggesting the absence of cross-reactivity between ZIKV and DENV. Previous studies on ZIKV-positive patient sera reported mapping a substantial ZIKV NS1 epitope to residues 118-147, where DENV sera had no cross-reaction with this epitope (Lee et al. 2018). The enhanced NS1 specificity can be primarily attributed to the electrostatic surface potential of the residue within the protein (Song et al. 2016; Stettler et al. 2016). ZIKV-NS1 possesses varying surface features and distinct electrostatic potential, leading to unique binding properties with host factors and the stimulation of protective antibodies different from those found in other flaviviruses (Xu et al. 2016; Song et al. 2016).

The use of recombinant NS1 protein shows promising applications in diagnosing ZIKV infections, as using recombinant antigens in ELISA tests offers advantages over whole viruses by improving specificity and reducing cross-reactivity in flavivirus diagnostics (Chávez et al. 2010). Compared to other ZIKV proteins, NS1 provides higher specificity and has been used experimentally to differentiate ZIKV infections (Sornjai et al. 2019). In our work, rNS1$_{FL}$ can potentially be employed in indirect ELISA to detect anti-NS1 antibodies. Although we did not utilize serum from patients infected with ZIKV, rNS1$_{FL}$ could identify serum from mice immunized with a commercial recombinant NS1 ZIKV protein from a different strain. This finding suggests that the rNS1$_{FL}$ could lead to the development of universal diagnostic tests. Previous studies have shown that recombinant NS1 protein resembles the native NS1 protein, allowing it to bind to ZIKV-specific antibodies present in the serum of infected patients (Roldán et al. 2020). The similarities between ZIKV and DENV pose difficulty in distinguishing diseases due to evidence of a high degree of cross-reactivity (Lanciotti et al. 2008; Cao-Lormeau et al. 2016; Priyamvada et al. 2016). Nevertheless, rNS1$_{FL}$ emerged as an essential protein for potentially differentiating between ZIKV and DENV. This is a significant finding, as ZIKV and DENV often co-circulate because they are transmitted by the same mosquito vectors, *Aedes aegypti* and *Aedes albopictus*, in endemic regions (Carrillo-Hernández et al. 2018). We also confirmed that $rNS1_{FL}$ had good immunogenicity. Hence, this antigen holds promise for generating both polyclonal and monoclonal antibodies. These antibodies could facilitate the development of an NS1 capture diagnostic assay, as they could detect secreted NS1 in either ZIKV-infected cells or the sera of ZIKV-infected patients, as evidenced by prior research (Zhang et al. 2019; Beddingfield et al. 2021; Magalhães et al. 2021).

In conclusion, ZIKV $rNS1_{FL}$ was successfully generated and characterized from an Indonesian isolate as inclusion bodies. It induced the production of antibodies specific to the ZIKV NS1 protein and did not cross-react with the DENV NS1 monoclonal antibody. These findings suggest that $rNS1_{FL}$ is a promising antigen for ZIKV serological testing. Consequently, further investigation is required to ascertain the reactivity of $rNS1_{FL}$ to sera from individuals infected with the ZIKV.

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