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Antibody response to Newcastle disease virus recombinant fusion protein in post-vaccinated laying hens

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ABSTRACT

This study was aimed to analyze the antibody response in laying hens post-vaccination to Newcastle disease virus (NDV) recombinant fusion (F) protein, which has been successfully expressed using the F gene of local isolates from Kulon Progo Strain (0663/04/2013), Yogyakarta, Indonesia. The F gene was cloned into an expression vector plasmid pBT7-N-His. Two types of NDV vaccine, a concentrated and pure F recombinant protein, were used for vaccination. The concentrated recombinant F protein was collected from centrifugal ultrafiltration, while the pure sample was obtained from the electroelution process. Recombinant F protein of NDV was successfully expressed, purified, and visualized by Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining with a molecular weight of 28 kDa. The two types of recombinant F protein and a commercial live vaccine as a positive control were injected two times at 14 and 18th weeks of age in laying hens to analyze the antibody response in serum. In comparison with a commercial live NDV vaccine, indirect Enzyme-linked Immunosorbent Assay (ELISA) revealed that antibody responses were high in both recombinant F protein vaccinated groups. Therefore, the recombinant F protein has the potential to be developed as a vaccine candidate to obtain a higher antibody response in laying hens compared to commercially available live NDV vaccines.

Keywords: ELISA; Laying hens; NDV; Post-vaccinated, Recombinant F protein

INTRODUCTION

The World Organization for Animal Health reported, Newcastle disease virus (NDV) is a virulent strain that causes Newcastle disease (ND) in Poultry. It was first identified in 1900 and has caused global outbreaks four times. The first outbreak was reported in Indonesia, England, Korea, the Philippines, India, Japan, Australia, and Kenya, while the second occurred in Palestine, Syria, French, Congo, Africa, Europe, and the United States. The last two outbreaks were reported in Europe, Canada, Mexico, Central and South America, as well as China from 2006 to 2009. ND is the fourth top-ranked infectious disease in terms of loss in livestock and several bird species, after highly pathogenic avian influenza, avian infectious bronchitis, and low pathogenic influenza (Dimitrov *et al*., 2017).

NDV is a member of Genus Avulavirus, family Paramyxoviridae, and included in type 1 Avian Paramyxovirus (APMV-1) which is capable of infecting more than 240 bird species. Therefore, it is a serious problem for the poultry industry and also contributes to significant economic losses worldwide (Dortmans *et al*., 2012). NDV is an antisense RNA virus and based on virulence, four major strains namely viscerotropic velogenic, neurotropic velogenic, mesogenic, and lentogenic are highly responsible for morbidity and mortality as opposed to other strains which are highly virulent in the poultry industry. The distribution depends on the occurrence of the disease (Ecco *et al*., 2011; Moura *et al*., 2016). Furthermore, pathotyping of NDV can be carried out by a combination method of RT-PCR amplification and RFLP using Hin-fI restriction enzyme to differentiate the virulent or avirulent NDV strain from naturally infected chickens (Haryanto *et al*., 2016).

The NDV genome consists of six transcriptional units namely 3′-N–P-M-F–HN-L-5′. The F unit is the spike protein on the surface envelope of the virus and synthesized as an inactive precursor (F0). This precursor is cleaved by protease into two biologically active F1 and F2 subunits which facilitate the entry of the virus inside the host cell. The F protein sequence is well characterized and is the major determinant of NDV pathogenicity in chickens (Kim *et al.,* 2013).

Several reports stated, vaccination is a highly effective method of preventing the spread of ND. Various commercially available inactivated NDV and attenuated live vaccines have been used for many years to prevent and control ND (Kang *et al*., 2016). However, failure still occurs due to potential incompatibility between the vaccine and the virus which exists in different forms.

Nucleoprotein (N) hemagglutinin-neuraminidase (HN) in NDV is commonly used to induce hormonal response (Mohan *et al*., 2006). Meulemans *et al*. (1986) reported that monoclonal antibodies against the F protein of NDV are more effective than HN antibodies in protecting chickens against viral infection. This suggests that the use of F protein as a vaccine might be effective against Paramyxovirus. Therefore, using genetic engineering, F protein can be selected and cloned to be expressed as protein and for vaccination purposes against NDV in birds.

The F gene of the local isolate NDV strain Kulon Progo (0663/04/2013), Yogyakarta, Indonesia, has been successfully expressed in the vector pBT7-N-His plasmid (Wulanjati *et al*., 2018). Astuti *et al*. (2020) then reported the results of vaccination using this recombinant F protein in broiler chickens. Therefore, this study aims to express the recombinant F protein and analyze the anti-NDV antibody response compared to commercial live ND vaccine in laying hens.

MATERIAL AND METHODS

Sample Preparation

The F protein NDV gene used in this study was obtained from a local isolate of Kulon Progo

Strain (0663/04/2013), Yogyakarta, Indonesia. It was cloned using recombinant plasmid pBT7-N-His (Wulanjati *et al*., 2018). Meanwhile, *E. coli* BL21 (DE3) isolates carrying pBT7-N-His-F were grown according to the protocol of Astuti *et al*. (2020).

Plasmid Isolation and *Eco*RI Digestion

The Presto Mini Plasmid Kit was used to isolate the plasmid (Geneaid Biotech Ltd, Taiwan), while the endonuclease restriction digestion was performed using the *Eco*RI enzyme for isolated plasmid and visualized on a 1.5% agarose electrophoresis gel.

In-vitro Protein Expression

The Accu RapidTM Protein Expression Kit was used to express the NDV recombinant F protein (Bioneer, South Korea). In the native state, the 6x histidine-tagged recombinant F protein was collected using the Ni-NTA Spin Kit (Qiagen, USA). Furthermore, Nanosep centrifugal apparatus (Pall Corporation, USA) was used to concentrate the recombinant F protein, then it was purified through electroelution. The concentrated and pure F protein was visualized on Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) with Coomassie Brilliant Blue staining. To determine the concentration of recombinant F protein, Spectrophotometer DU-65 was used (Beckman, USA).

Laying Hens Vaccination

A total of 15 hens were used and divided into three groups, each consisting of 5 samples. Group A was injected with concentrated recombinant F protein of 10 µg/hen, emulsified with complete Freund's adjuvant (Sigma, USA) that consists of 0.15 ml mannide monooleate and 0.85 ml paraffin oil in a total volume of 1 ml. Furthermore, group B was vaccinated with purified recombinant F protein with the same adjuvant as Group A, while Group C was vaccinated with a live ND commercial vaccine (killed NDV Vaccine). The second vaccination was carried out using incomplete Freund's adjuvant (Sigma, USA) as a mixture for the recombinant F protein. The first vaccination was administered to 14 weeks old hens and the second was given at week 18 through intramuscular injection.

Blood Sampling

The first blood sample was collected when hens were at the age of 14th weeks old before the

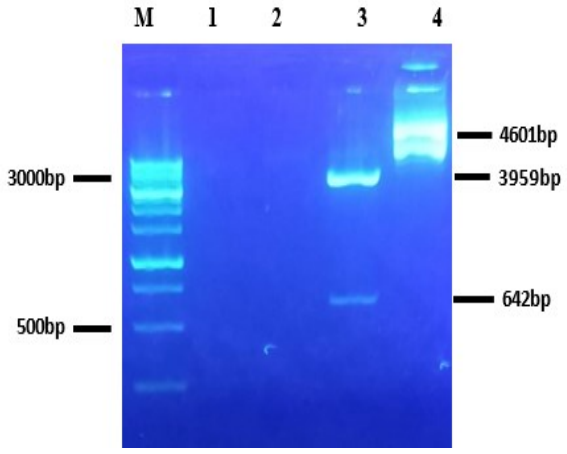


Figure 1. Digestion of recombinant plasmids using *Eco*RI on 1% agarose gel. (M) DNA Marker 1kb, (1, 2) is empty, (3) pBT7-N-His-F recombinant plasmid cut with *Eco*RI enzyme, yielding sizes of 642 and 3959 bp, (4) nondigested pBT7-N-His-F.

first dose of vaccination, while the second was collected at the 16th week. After the booster vaccination, blood sample collection was again carried out in the 20th week or 15 days after the 2nd vaccination. The serum was collected from all the blood samples and stored at −20°C for further processing.

Indirect ELISA

The analysis of anti-NDV protein from the sera was performed using 10 µg/ml of each recombinant F protein or commercial vaccine as an antigen coated on the plate. Sera dilution was optimized using a ratio of 1:100 and secondary antibodies (Goat Anti-Chicken IgY (H+L) were incubated on the plate at a ratio of 1:5000. Alkaline Phosphatase (Novex, USA) of 100 µL/ well was linked with enzyme and yielded results upon reaction with 100 µL/well of 4-Nitrophenylphosphate dinatriumsalz substrate (Merck, Germany). The OD of the reaction was measured with an ELISA reader (Zenix, USA) at a wavelength of 405 nm.

Statistical Analysis

The average ± Standard Deviation (SD) was used to represent Enzyme-linked Immunosorbent Assay (ELISA) results for serum samples. Meanwhile, the statistical difference between data from the three different groups that were vaccinated at various times was analyzed using the two-way ANOVA and Tukey's test with p-values less than 0.05 (*) considered significant. All statistical analyses were performed with the GraphPad Prism 9 computer program.

RESULTS AND DISCUSSION

The NDV F protein plays a crucial role in viral-induced membrane fusion, which is required for virus penetration and propagation. It also has important implications for the prevention of infection transmission, and antibodies. According to a previous study, the F glycoprotein can be used as a vaccine to inhibit the spread of NDV infections (Merz *et al*., 1980).

Isolation and Confirmation of Recombinant Plasmid pB T7-N-His-F

The recombinant F protein of NDV was expressed in the expression vector pBT7-N-His that had been previously prepared by Wulanjati *et al*. (2018). The NDV Fusion gene was obtained from local isolates of Kulon Progo Strain (0663/04/2013). Its pathotype has been

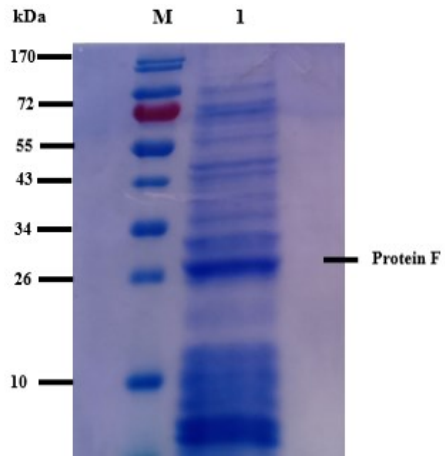


Figure 2. Concentrated F recombinant protein by using Ni-NTA spin kit (Qiagen). Visualized on 12% SDS-PAGE gel with Coomassie Brilliant Blue staining. (M) Protein Marker, (1) Concentrated F recombinant protein with a target size of 28 kDa.

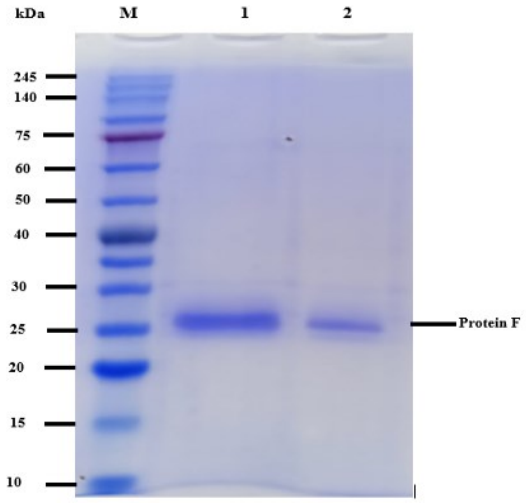


Figure 3. Pure F recombinant protein from electroelution. Coomassie Brilliant Blue staining. (M) Protein Marker, (1, 2) Pure F recombinant protein with a size of 28 kDa.

previously characterized by Haryanto *et al*., (2016). Plasmid isolation and restriction digestion with *Eco*R1 enzyme were used to confirm the presence or absence of pBT7-N-His-F recombinant plasmid from *E. coli* as shown in Figure 1. Digestion results with *Eco*RI have the same pattern as reported by Wulanjati *et al*. (2018) and Astuti *et al*. (2020). There are two bands, each at 642 and 3959 bp, indicating that the plasmids used are the same.

Expression of NDV F Recombinant Protein

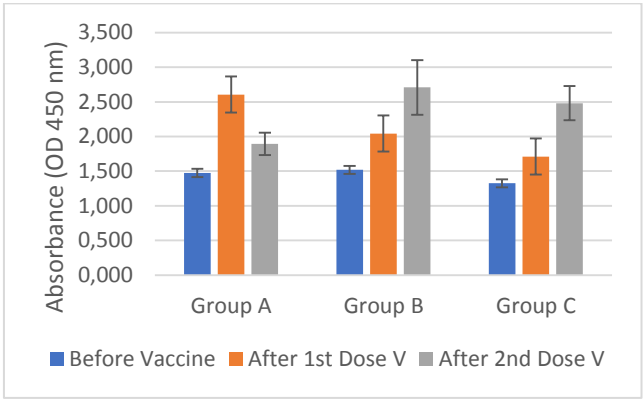
The expressed recombinant F protein was concentrated, purified, and visualized on 12 % SDS-PAGE using Coomassie Brilliant Blue staining and a total molecular weight of 28 kDa was obtained (Figure 2). The concentrated and pure F recombinant protein shown in Figures 2 and 3, respectively, were used as vaccine immunization for laying hens at the age of 14th weeks, and booster vaccine immunization was given at the 18th week. Compared to Astuti *et al*. (2020), the 14 th days old broiler chickens were vaccinated only with the concentrated F recombinant vaccine. Furthermore, the electroelution was performed to obtain a single band of pure recombinant F protein with a molecular weight of 28 kDa and used for vaccination as shown in Figure 3.

Antibody Response after Recombinant F Protein Vaccination

ELISA is one of the most effective methods that can detect antigen antibodies interaction. In this test, N HN in NDV is commonly utilized to coat antigens (Mohan *et al.,* 2006). Monoclonal antibodies against the F protein of NDV are more effective than HN antibodies in protecting hens against viral infection. This suggests that the use of F protein as a vaccine might be effective against paramyxovirus. Therefore, using genetic engineering, F protein can be selected and cloned to the expressed as a protein and for vaccination purposes against NDV in birds (Meulemans *et al.,* 1986). According to Arora *et al*. (2010), the F protein in NDV has a higher ELISA titer value than HN, suggesting that the F protein is involved in protecting against NDV infection.

Serological testing of blood samples from vaccinated laying hens was carried out using an ELISA kit coated with the antigen of the concentrated, pure F recombinant protein, and commercial live NDV antigen. Three different antigens were coated in three distinct plates. The serum of all three different groups was tested and cross-hybridization for antibodies titer was evalu-

A



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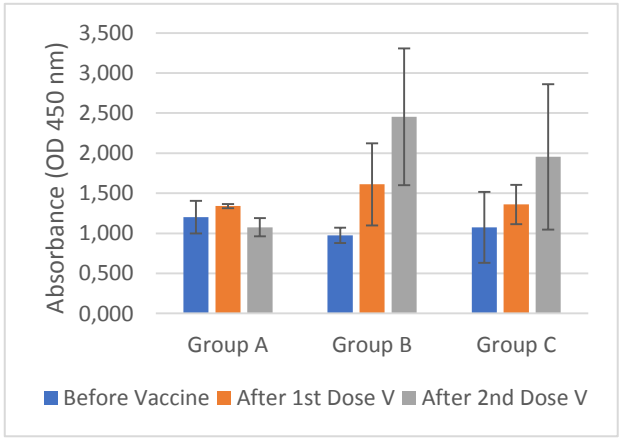

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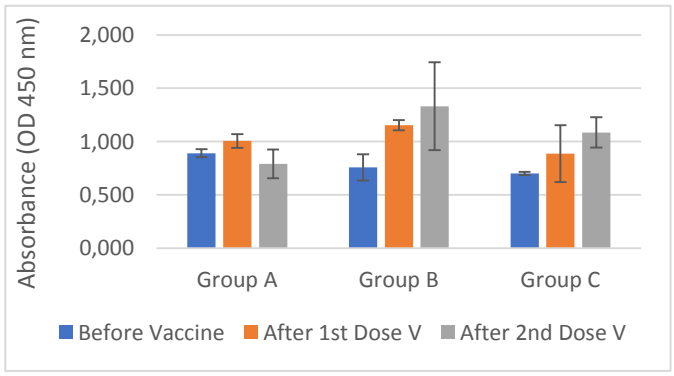

Figure 4. Antibody titer in sera sample from laying hens. Antibody titer response against three different antigens namely (A) Concentrated, (B) pure F recombinant antigen, and (C) commercial live NDV antigen. Group A, B, and C represent hens that were immunized with Concentrated, pure F recombinant, and commercial live NDV vaccined, respectively. Sera were tested for all three types of antigens in ELISA to determine the antibody titers.

ated.

The antibody titer was higher after the first vaccine was administered in all three groups of hens, indicating that all antigens induced humoral immune response. After the second vaccination, only pure recombinant F protein showed an increase in antibody production, similar to the commercial vaccine (Figure 4). This suggests that the pure recombinant F protein is more potent to induce antibodies than the concentrated. This result differs from data obtained after the vaccination of broiler chickens (Astuti *et al*., 2020) where antibody production increased along with the vaccination time.

The results showed that laying hens vaccinated with both recombinant F proteins have a similar humoral immune response to those vaccinated with commercial inactivated vaccines. Based on the ELISA test, both recombinant F proteins recognize the NDV antigen developed from the commercial inactivated vaccine (Jawad, 2021). Meanwhile, humoral immune responses play an important role in protection against NDV infection. The antibodies generated both ways either naturally or by vaccination, have the potential to neutralize infections and keep viruses from adhering to receptors on the surface of host cells. These antibodies can be used as a biomarker to evaluate the interaction of hens host with viral infectious agents through antibody production response (Jeurissen *et al*., 2000). Furthermore, the indirect ELISA shows that the recombinant F protein has the potential to be developed as a vaccine due to its high antibody production and can be commercialized after 6x his-tag is removed.

Based on the results, the future perspective is that the recombinant protein expressed from the pBT7-N-His-F plasmid has the potential to be used as a vaccine candidate against NDV. This is because the antibody production in vaccinated laying hens was at the same level as those vaccinated with commercial inactivated vaccines. Furthermore, antibody production in laying hens induced by pure recombinant F protein of NDV was reportedly higher than those induced with the concentrated antigen. To effectively and optimally increase the antibody titer in laying hens vaccinated with recombinant protein, the part of the recombinant protein containing his-tags must be removed enzymatically using the enzyme Tobacco Etch Virus (TEV) protease as reported by Goh *et al*., (2017).

CONCLUSION

The recombinant F protein of NDV derived from a local isolate (Kulon Progo strain, Indonesia) was successfully expressed, purified, and visualized through SDS-PAGE using Coomassie Brilliant Blue staining with a molecular weight of 28 kDa. The vaccinated laying hens showed similar anti-NDV antibody production to commercial ND vaccines. This suggests that recombinant F protein has the potential to be developed as an NDV vaccine candidate after truncating the 6x His-tag portion.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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