



In vitro expression of the recombinant fusion protein of Newcastle disease virus from local Indonesian isolates by using a cell-free protein expression system

Aris Haryanto¹, Hevi Wihadmadyatami², Nastiti Wijayanti^{3,*}

¹Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Jl. Fauna No. 2, Karangmalang, Yogyakarta 55281, Indonesia

²Department of Anatomy, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Jl. Fauna No. 2, Karangmalang, Yogyakarta 55281, Indonesia

*Corresponding author: nastiti_wijayanti@ugm.ac.id

SUBMITTED 6 March 2020 REVISED 23 April 2020 ACCEPTED 8 June 2020

ABSTRACT The aim of this work was the *in vitro* expression of the recombinant fusion (F) protein of Newcastle disease virus (NDV). The pBT7-N-His-Fusion-NDV expression plasmid which carries the recombinant F protein encoding gene from local Indonesian isolates, was prepared and transformed into *Escherichia coli* BL21 (DE3). To detect bacterial colonies carrying the recombinant plasmid, a restriction endonuclease analysis was performed using the EcoRI restriction endonuclease. These results showed that the pBT-N-His-Fusion-NDV plasmid was successfully isolated with a size of 4.601 bp, and three recombinant plasmids carrying the gene coding for the recombinant F protein of NDV were obtained. Selected recombinant plasmids were then *in vitro* by using a cell-free protein expression system followed by visualization of the recombinant F protein on a 12% SDS-PAGE gel both by Coomassie Brilliant Blue staining and Western blotting. Recombinant F protein was successfully *in vitro* expressed by using a cell-free protein expression system as indicated by a specific single protein band with a molecular mass of 25.6 kDa.

KEYWORDS cell-free protein expression; *in vitro* expression; Newcastle diseases virus (NDV); recombinant F protein

1. Introduction

Newcastle disease (ND) is a poultry infectious viral disease which has become a major problem in the poultry industry in developing countries. Office International des Epizooties (OIE) categorizes ND in the A list of animal diseases, which includes infectious diseases with rapid and serious spreading and affecting public health and socio-economic communities ([OIE] Office International of Epizootics 2015). As other infectious viral disease outbreak which had been reported in Indonesia poultry industry, such as avian influenza (Njoto et al. 2018), infectious bursal disease (Parede et al. 2003), and avian encephalomyelitis (Haryanto et al. 2016). An outbreak of ND can cause a devastating effect as it causes almost 100% mortality in susceptible poultry (Alexander et al. 2012). ND was also reported to have a significant economic effect on the poultry industry in Indonesia (Dharmayanti et al. 2014). Newcastle disease is caused by avian paramyxovirus serotype-1 (APMV-1) virus, which is also known as Newcastle disease virus or NDV (Alexander 2003). NDV belongs to the genus Avulavirus, subfamili Paramyxovirinae, famili Paramyxoviridae, and order *Mononegavirales* (King et al.

2012). NDV genome is a negative sense single stranded RNA (ssRNA) encoding six viral proteins, namely nucleocapsid (N), phosphoprotein (P), matrix (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA polymerase (L) (De Leeuw and Peeters 1999; King et al. 2012). NDV infection on the host cell is mediated by two surface viral glycoproteins, the hemagglutinin-heuraminidase (HN) protein and fusion (F) protein (Chang and Dutch 2012).

F protein in NDV plays an essential role in the virulence of the virus (Dortmans et al. 2011). F protein can also induce protective immunity against NDV infection (Arora et al. 2010). Kim et al. (2013) reported that the F protein of NDV is a major part of protective immunity in developing genotype-matched vaccine. NDV F protein consists of 553 amino acids which are synthesized as F₀ inactive precursor (Morrison 2003). F₀ protein can be cleaved by cellular protease resulting in active F₁ and F₂ proteins. F protein is processed in trans Golgi in a mammalian cell, which produces the active form of F₁-F₂ protein with a disulfide bond (Lamb and Parks 2007). The specificity of cleavage F protein is determined by the amino acid sequence at the region of cleavage sites and varied between strain types.

In the cleavage site of F protein, low virulence NDV has one or two base amino acids. Therefore, low virulence NDV is not sensitive to intracellular protease enzyme and depends on extracellular protease in the respiratory and enteric systems of the host cells. Highly virulence NDV has multi base amino acids in the cleavage site so that it can be recognized by intracellular protease enzyme. Amino acid sequence at the cleavage site of F₀ protein is one of the factors that determines the systemic spreading and virulence of NDV. Highly virulence NDV contains lysine (K) and arginine (R) amino acids with ¹¹²R-R-Q-R/K-R¹¹⁶ motive at C terminus F₂ and phenylalanine (F) amino acid at 117 position at N terminus protein F₁ (Ganar et al. 2014).

Newcastle disease outbreak was reported in 2009-2010 in poultry farms in Indonesia, which caused death on 70-80% of the total number of chickens (Dharmayanti et al. 2014). Analysis on the amino acid identity of F and HN protein sequences from eight isolates NDV, which caused the outbreak indicated antigenic differences from La Sota and B1 vaccine strains (Xiao et al. 2012). A vaccination program has been conducted to prevent and overcome ND ([OIE] Office International of Epizootics 2012). However, ND outbreaks still continue to happen in Indonesia. The outbreak is a result from antigenic differences between NDV strain causing the outbreak in the field with NDV strain used in the available commercial vaccine. Therefore, the vaccination program did not provide optimal protection in the poultry post vaccination (Xiao et al. 2012; Dharmayanti et al. 2014).

A recombinant vaccine is a vaccine containing antigen of a pathogenic agent that can induce the host immunity against the pathogenic agent (Grand et al. 2012). The recombinant vaccine is safer and more stable compared to conventional live vaccines. A recombinant vaccine is created by cloning a certain encoding gene of an immunogenic protein, expressed and purified the recombinant protein, and formulated it to be a vaccine (Nascimento and Leite 2012). Regardless of the type of ND vaccines in use, all vaccinated birds were fully protected from disease. All vaccinated and challenged birds show significant seroconversion after 14 days of challenge. However, some vaccinated birds were reported to shed the challenge virus from their oro-pharynx and cloaca, even though they are significantly lower in titers than unvaccinated challenged control birds (Jeon et al. 2008).

The development of NDV vaccines based on the phylogenetic similarity to NDV in the time of the outbreak can provide better protection against ND (Miller et al. 2007). Therefore, one of the attempts to prevent ND on poultry in Indonesia is developing an ND vaccine based on local isolates originated in Indonesia. This work aimed to express recombinant F protein of NDV from pBT7-N-His-Fusion-NDV expression vector which carries the F protein encoding gene from NDV local isolate by a cell-free protein expression system. Recombinant NDV F protein obtained from the expression is expected to be a candidate for the recombinant F vaccine to prevent ND on poultry in Indonesia.

2. Materials and Methods

2.1. Preparation of pBT7-N-His-Fusion-NDV recombinant plasmid

The expression vector used in this work had been prepared by Haryanto et al. (2016) and Wulanjati et al. (2018) by subcloning the F protein encoding gene of NDV from local isolate in Kulon Progo, Indonesia (0663/04/2013) into pBT7-N-His expression vector. The design of the recombinant plasmid vector is shown in Figure 1.

2.2. Transformation of recombinant plasmid into *Escherichia coli* BL21 (DE3)

Transformation of DNA plasmid pBT7-N-His-Fusion-NDV was carried out using competent *Escherichia coli* BL21 (DE3) strain by carefully transferring DNA plasmid into a tube containing competent cells. Plasmid DNA and competent cells were mixed gently and placed on ice for 20 min. Then, the cells were heat-shocked at 42 °C for 60 s. The mixture of plasmid DNA and competent cells were placed immediately on ice for 3 min. The mixture was then put into the culture medium, 10 mL of Luria Bertani (LB) (Oxoid) supplemented with 100 µg/mL ampicillin (Gold Biotechnology), and incubated at 37 °C overnight. The transformation of the recombinant plasmid into *E. coli* generated eight growing colonies of *E. coli* carrying recombinant plasmid. The eight colonies of bacteria were extracted using Presto Mini Plasmid kit (Geneaid) to obtain the recombinant plasmid DNA.

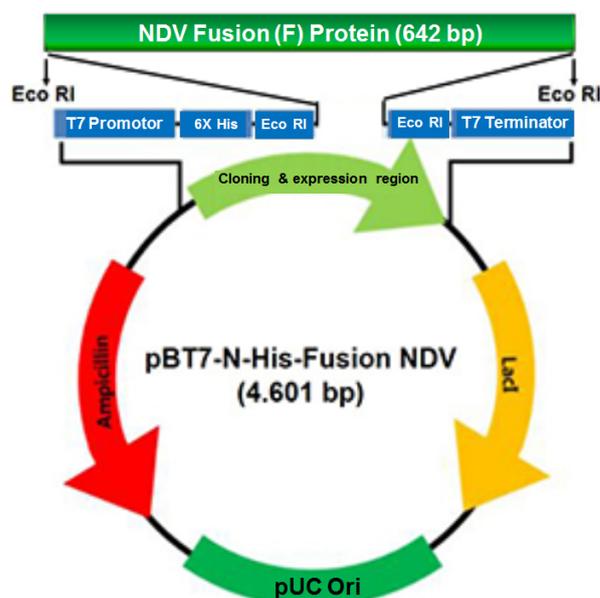


FIGURE 1 Design of recombinant plasmid pBT7-N-His-Fusion NDV (4,601 bp). F protein encoding gene of NDV (642 bp) was subcloned into the Eco RI restriction site

2.3. Restriction endonuclease analysis of recombinant plasmid vector by *EcoRI* enzyme

The screening of recombinant plasmid pBT7-N-His-Fusion-NDV was carried out by digestion of recombinant plasmid by *EcoRI* enzyme (Thermo Fisher Scientific) in a total volume of 10 μ L. The samples were then incubated at 37 °C for 3 h. The digestion product was then visualized on a 1.5% agarose gel electrophoresis with FluorSAFE (Genetika Science Indonesia) staining. The restriction product was analyzed under UV light in the dark room.

2.4. *In vitro* expression of recombinant F protein of NDV by the cell-free protein expression system

The *in vitro* expression of recombinant F protein of NDV from Indonesian local isolates was performed by a cell-free protein expression system using the AccuRapid protein expression kit (Bioneer) according to the standard manual procedure. The expressed recombinant F protein of NDV was then visualized by electrophoresis on an SDS-PAGE gel followed by Coomassie Brilliant Blue R-250 (Thermo Fisher Scientific) staining.

2.5. SDS-PAGE with Coomassie Brilliant Blue staining

Before SDS-PAGE, the samples were denatured at 95 °C for 5 min, then put into ice for about 30 min. Each sample (5 μ L) was loaded on a 5% stacking gel and separated on a 12% resolving SDS-PAGE gel. The protein concentration of each sample was as follow 1.39 μ g/ μ L for sample 1, 0.13 μ g/ μ L for sample 2, 0.88 μ g/ μ L for sample 3, and 1.52 μ g/ μ L for positive control. The SDS-PAGE gel was run at 100 Volt for 1 h, and then it stained with Coomassie Brilliant Blue R-250.

2.6. Western blotting

The expressed recombinant F protein of NDV was visualized by Western blotting. As a positive control, *Aequore courulescens* GFP fluorescent protein (AcGFP) with a molecular weight of 28.0 kDa on lane with a concentration of 1.52 μ g/ μ L was used. The recombinant F protein was separated by a 12% resolving SDS-PAGE gel. They were then electrotransferred onto polyvinylidene difluoride (PVDF) membrane (Merck) for detecting the recombinant F protein. Western blotting preparation was performed by blocking the membrane by the blocking solution [1% BSA (Thermo Fisher Scientific) dissolved in 0.5% Tween-PBS (Sigma-Aldrich)]. The PVDF membrane was then incubated at room temperature for 1 h. After washed three times in washing solution (0.05% Tween-PBS), the PVDF membrane was probed with a mouse monoclonal antibody, anti-6x histidine (his)-tag antibody (Thermo Fisher Scientific) as the primary antibody in a 1:2,000 dilution. The membrane was then incubated and shaken overnight at 4 °C. After three time washing, the PVDF membrane was subsequently incubated with the secondary antibody, alkaline phosphatase (AP)-conjugated goat antimouse IgG antibody (Thermo Fisher

Scientific) in a 1:15,000 dilution. The PVDF membrane was then incubated and shaken at room temperature for 1 h. After two time washing, the immunological reaction was visualized by adding NBT-BCIP (Thermo Fisher Scientific) substrate onto the PVDF membrane.

3. Results and Discussion

3.1. Transformation plasmid pBT-7-N-His-Fusion NDV into *E. coli* BL 21 and visualization of recombinant NDV fusion protein expression

The transformation of pBT7-N-His-Fusion-NDV recombinant plasmid was performed initially on *E. coli* BL21 (DE-3) competent cell. The transformation result indicated that there were eight colonies of *E. coli* grew on agar LB medium (Figure 2). To confirm the result of the recombinant plasmid transformation, three from the eight colonies were selected for DNA isolation of recombinant. The next process was restriction endonuclease analysis by digestion recombinant plasmid pBT7-N-His-Fusion-NDV using *EcoRI* restriction enzyme. Recombinant plasmid digestion resulted two DNA bands in size of 3,959 bp (plasmid) and 642 bp (insert gene), respectively, while the undigested recombinant plasmid was observed to be 4,601 bp in size (Figure 3). The undigested recombinant plasmid which was run by electrophoresis in agarose gel showed three bands of DNA because it had three forms of conformation: relaxed circular form, linearized form, and superhelix form (De Mattos et al. 2004).

In Figure 4, electrophoresis using SDS-PAGE of recombinant F protein on the agarose gel 12% with Coomassie Brilliant Blue staining showed that recombinant F protein of NDV could be expressed as a thick pro-

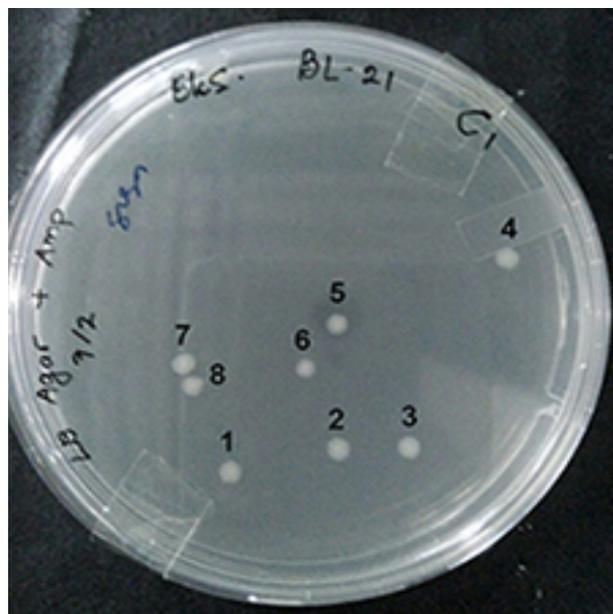


FIGURE 2 Eight growing colonies of *E. coli* BL21 carrying recombinant plasmid pBT7-N-His-Fusion-NDV in LB agar medium with ampicillin.

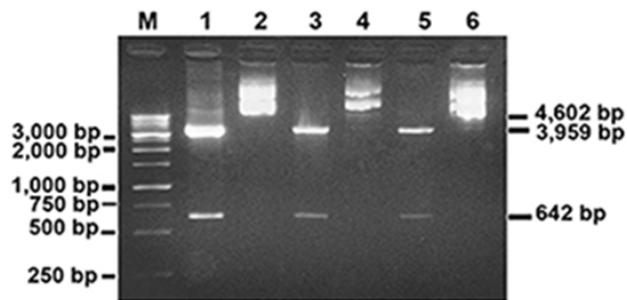


FIGURE 3 The digestion result of recombinant plasmid pBT-N-His-Fusion-NDV by using Eco RI enzyme. No. 1 is recombinant plasmid of Colony-1 (C-1) was digested by Eco RI, No. 2 is an intact recombinant of C-1, No. 3 is recombinant plasmid of Colony-2 (C-2) was digested by Eco RI. No. 4 is an intact recombinant plasmid of C-2, No. 5 is recombinant plasmid of Colony-3 (C-3) was digested by Eco RI, No. 6 is an intact recombinant plasmid of C-3.

tein band with the molecular weight of 25.6 kDa (lane 1, 2, 3). Meanwhile, AcGFP with the molecular weight of 28.0 kDa on lane (+) appeared as the positive control. The recombinant F protein of NDV which expressed using cell-free protein expression system found in the supernatant after incubated for 3 h at 30 °C. It indicated that the expressed recombinant F protein of NDV in this expression system is a soluble protein. This soluble protein can be re-activated and followed by a refolding process with dilution or dialysis method in refolding buffer to obtain active soluble protein (Middelberg 2002; Burgess 2009; Yang et al. 2011).

3.2. Western blotting

Western blotting using a mouse antihistidin-tag antibody as the primary antibody and goat antimouse antibody as the secondary antibody was conducted to assure the result of recombinant F protein of NDV expression. The result of Western blotting can be seen in Figure 5. It showed a

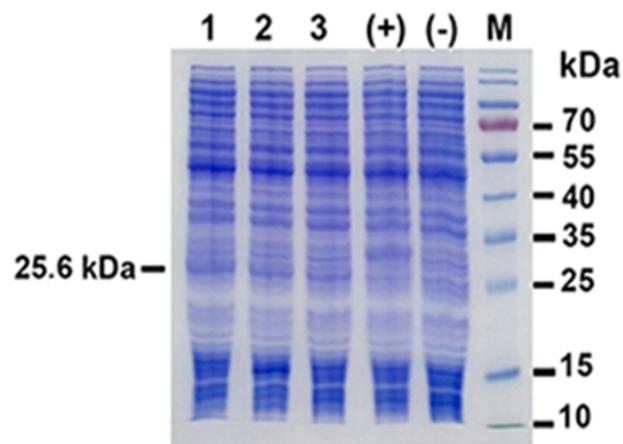


FIGURE 4 SDS-PAGE with Coomassie Brilliant Blue staining of the expressed recombinant F protein of NDV into *E. coli* extract. No. 1 is a recombinant plasmid of Colony-1 (C-1), No. 2 is a recombinant plasmid of Colony-2 (C-2). No. 3 is a recombinant plasmid of Colony-3 (C-3), (+) is positive control, (-) is negative control

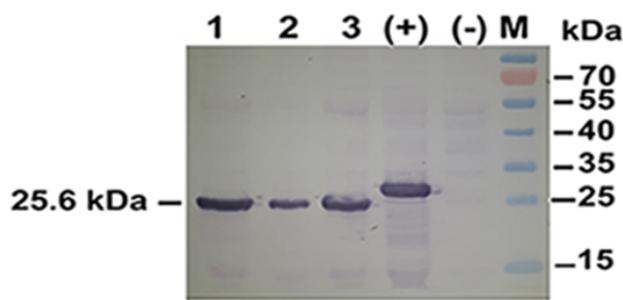


FIGURE 5 Western blotting of the recombinant F protein of NDV M = protein marker. No. 1, 2, and 3 are Colony-1 (C-1), Colony-2 (C-2), and Colony-3 (C-3), respectively. (+) = positive control. (-) = negative control.

specific protein band with a molecular weight of 25.6 kDa at lane 1, 2, 3. The positive control AcGFP protein with a molecular weight of 28.0 kDa could be observed in lane (+). Both recombinant F protein of NDV in lane 1, 2, 3 and AcGFP in lane (+) were fusion protein with 6x his-tag at the N-terminus end.

3.3. Discussion

The recombinant expression plasmid that we designed pBT7-N-His-Fusion-NDV (4,601 bp) was derived from pBT7-N-His plasmid which having the origin of replication (ORI) from pUC plasmid. This plasmid carried the F protein encoding gene from NDV which was isolated from Indonesian local isolate. Recombinant F protein expressed from pBT7-N-His-Fusion-NDV will be prepared as a candidat vaccine for viral diseases, ND, in poultry based on local isolate virus. The expression of recombinant F protein from pBT7-N-His-Fusion-NDV plasmid vector was controlled by T7 promoter (Bioneer 2016). The expression system was controlled by T7 promoter, which could be induced through inducing lactose analog compound, namely isopropyl β-D-thiogalactoside (IPTG) (Rosano and Ceccarelli 2014). pBT7-N-His-Fusion-NDV has a multi cloning site (MCS) and stop codon (TGA) areas with nucleotide sequences that can be digested by certain endonuclease restriction enzymes. In this plasmid, there is a ribosome binding site (RBS) that facilitates the translation process. This vector plasmid also has *pUC Ori* which is an initial area for DNA replication. In addition, there is a β-lactamase encoding gene which functions in the resistance against ampicillin, which can be used in for selection of recombinant *E. coli*.

The expression of recombinant protein is a translation process from gene into protein through the processes of transcription and translation. Expression of protein could be performed using a prokaryotic cell system with *E. coli* or eukaryotic cells using yeast, filamentous fungi, and unicellular algae. The selection of the host system depended on the target protein which was going to be expressed. In this research, the expression of recombinant NDV F protein was performed by using *E. coli* extract in a cell-free protein expression system. This system was selected because a prokaryotic cell is one of the selected organisms to

produce a recombinant protein (Wilson and Walker 2010). In pBT7-N-His-Fusion-NDV expression plasmid, recombinant F protein is expressed as a fusion protein with 6x his-tag (tag polyhistidine) at the N-terminus end. After the purification process, the expressed recombinant F protein of NDV would be used as a candidate of recombinant vaccine against ND based on Indonesian local isolate of NDV strain. The existence of 6x his-tag in the recombinant F protein of NDV did not affect the structure and function of the recombinant protein (Ramos et al. 2004). Polyhistidine tag can function as the epitope for protein detection in Western blot. Polyhistidine tag also functioned for binding with Ni²⁺ in the protein purification process (Sambrook and Green 2012). The benefit of using a polyhistidine tag was the recombinant F protein could be purified in the denatured condition because the interaction between histidine residue and Ni-NTA in the purification column could be stable with the existence of strong protein denaturant as guanidine HCl and urea. The purified recombinant protein can be renatured by slowly removing the denaturant (Terpe 2003).

In order to obtain high gene expression, the expression can be performed on *E. coli* since it grows fast, the enriched growth medium for *E. coli* is affordable, and the process of DNA transformation on *E. coli* is fast and easy (Qiagen 2001). *E. coli* BL21 (DE3) is a prokaryotic cell used for gene expression regulated by T7 promoter. This strain carries lysogen λ DE3 containing *lacI* gene, RNA polymerase T7 RNA controlled by *lacUV5* promoter, and a small part of *lacZ* gene (Wulanjati 2016). The expression of the recombinant F protein of NDV could be influenced by several factors, including vector plasmid, target protein, and strain bacteria used in the protein expression. Various internal factors of the plasmid which could influence the expression are promoter, initiation region, terminator region, enhancer, ribosome binding site, origin of replication, resistance marker against antibiotic, and the number of plasmid copy. Meanwhile, other factors of target protein which could influence the expression are nucleotide sequences, amino acid sequences, secondary structures of the protein, and the suitable codon usage (Makrides 1996; Rosano and Ceccarelli 2014). Besides, the productivity of the recombinant protein expression could be enhanced by optimization of external factors such as the temperature of bacterial growth and the level of expression-inducing compound (Chen et al. 2007; Volontè et al. 2008; Larentis et al. 2011, 2014).

Expression of NDV F protein encoding gene have been conducted by some researchers. For example, Chen et al. (2001) conducted cloning and full-length expression of cDNA from NDV F gene isolate V₄ (Queensland/66strain) on mammal's cell in Chinese Hamster Ovary (CHO-K1) and its mutant line cell Lec- 3.2.8.1. Park et al. (2014) developed a virus-like particles (VLP) vaccine by expressing NDV F protein with Sf9 cell to obtain recombinant protein of baculovirus (rBV). In this work, we used local isolates of NDV from Kulon Progo, Yogyakarta, Indonesia, whose pathotype characterization

has been identified by Haryanto et al. (2015). The result of this work showed that the recombinant F protein of NDV was successfully expressed by a cell-free protein expression system and visualized by using SDS-PAGE electrophoresis with Coomassie Brilliant Blue staining and Western blot as a specific protein with a molecular weight of 25.6 kDa. This result was in line with the recombinant protein study performed by other researchers, who have successfully expressed the recombinant F protein of NDV from *E. coli* clone of C-1a (Putri and Haryanto 2019).

The cell-free protein expression system is one of *in vitro* protein expression methods to study biological reactions in a cell using a living cell system that reduces complex interactions on living cells, so the transcription, translation, and cell metabolism processes occur in an open environment. This system aims to understand, utilize, and extend natural biological system capability without using living cells (Hodgman and Jewett 2012). The expressed recombinant F protein of NDV which had been purified, had the potential as a candidate of recombinant F vaccine for NDV based on the local isolate.

4. Conclusions

The fusion protein encoding gene of NDV subcloned into the pBT7-N-His expression vector has been successfully *in vitro* expressed by a cell-free protein expression system. Visualization by using Coomassie Brilliant Blue staining on SDS-PAGE gel and Western blot confirmed that the recombinant F protein of NDV expressed as a specific recombinant protein with a molecular weight of 25.6 kDa.

Acknowledgments

We would like to thank the head office of the Biochemistry Department, Faculty of Veterinary Medicine, Universitas Gadjah Mada (UGM), Yogyakarta, Indonesia and the head office of Inter-University Center for Biotechnology UGM, Yogyakarta, Indonesia for the opportunity and for allowing us to use the laboratory facilities to finish this work.

Authors' contributions

AH was responsible for the overall stage of research (recombinant plasmid preparation, laboratory works, drafting, and completing the final manuscript). HW did samples preparation, performed the laboratory works, and analyzed data. NW analyzed final data, drafted, and revised the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare no competing interest.

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