

# Recombinant fusion protein expression of Indonesian isolate Newcastle disease virus in *Escherichia coli* BL21(DE3)

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**Abstract.** Wulanjati MP, Witasari LD, Wijayanti N, Haryanto A. 2021. Recombinant fusion protein expression of Indonesian isolate Newcastle disease virus in *Escherichia coli* BL21(DE3). *Biodiversitas* 22: 3249-3255. Newcastle disease is a major problem in poultry industry due to high mortality of susceptible chicken. New vaccine agents are important to be developed to eliminate the disease threat. This study aimed to examine the expression of fusion (F) protein of Newcastle Disease Virus (NDV) from Indonesian isolates in *E. coli* BL21(DE3) by IPTG induction. The sample was a part of F gene of NDV from Galur, Kulon Progo, Yogyakarta, Indonesia (0663/04/2013) with a molecular size of 600 bp that was synthesized and inserted into pBT7-N-His expression vector. The recombinant F protein with molecular weight of 25.6 kDa was successfully expressed in *E. coli* BL21(DE3), purified using Ni-NTA magnetic silica beads, and confirmed by western blotting. Optimization of expression showed that recombinant F protein was optimally expressed by induction of 1.0 mM IPTG when the cells reached OD<sub>600</sub> = 0.6. The induction duration was 8 h. B-cell epitopes prediction showed that F protein possessed four epitopes that possibly recognized by B-cell. Since recombinant F protein was considered to possess immunogenicity, its potency as a candidate of NDV vaccine agent should be investigated in the future.

**Keywords:** *Escherichia coli* BL21(DE3), fusion protein, gene expression, newcastle disease virus

## INTRODUCTION

Newcastle disease is a highly contagious disease in poultry. The disease is characterized by lesions in the respiratory, gastrointestinal, nervous, and reproductive systems in susceptible chickens (Dimitrov et al. 2017). Moreover, the infection caused highly mortality rate in susceptible chickens (Mariappan et al. 2018). The disease can lead to a significant economic loss and become a threat to the progression of the poultry industry in many countries, especially in Indonesia (Etriwati et al. 2017; Shofa et al. 2018).

The etiological agent of Newcastle disease (ND) is Newcastle disease virus (NDV), also known as avian paramyxovirus serotype-1 (APMV-1), a family member of *Paramyxoviridae* (Mariappan et al. 2018). The NDV genome is single-stranded, negative-sense RNA, with a size of 15,186 nt. It consists of six genes encoding nucleoprotein (NP), phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) protein, and RNA polymerase (L) protein (Dimitrov et al. 2019).

Fusion (F) protein is a surface glycoprotein mediating virus fusion into the plasma membrane of the host cell, which is a critical protein for virulence. It becomes active

when the precursor F<sub>0</sub> is proteolytically cleaved, resulting in disulfide-linked F<sub>1</sub> and F<sub>2</sub> polypeptides (Wang et al. 2017). F protein is a crucial antigen that induced a protective immune response (Kang et al. 2016).

Nowadays in Indonesia, Newcastle disease is controlled by vaccination programs using the live vaccine and the inactivated vaccine. The commercial vaccines used are LaSota and B1 strains. However, there was an outbreak in Indonesia, in the year 2009-2010, leading to mortality of 70-80% from total chicken (Xiao et al. 2012). Xiao et al. (2012) studied that there were genetic variances between the virus in the field and vaccine agents commercially used. Besides, Wulanjati et al. (2018) studied the phylogenetic analysis and the genetic variations between Indonesian isolates compare to NDV vaccine strain. Indonesian isolates NDV were belonged to genotype VII class II, while LaSota and B1 vaccine agents were concluded in genotype II class II.

A genotype-matched vaccine is highly needed to overcome the vaccination problem (Izquierdo-Lara et al. 2019). A previous study showed that using a vaccine that homologous with a virus in the field increased specific antibodies, decreased clinical signs, and improved survival rates after challenge assay compared to the heterologous vaccine (Cardenas-Garcia et al. 2015). Consequently,

developing an NDV vaccine based on Indonesian NDV isolates for the Indonesia poultry industry is important by formulating recombinant protein as a vaccine agent. This study aimed to examine the expression of recombinant F protein of NDV isolate from Galur, Kulon Progo, Yogyakarta, Indonesia in *E. coli* BL21(DE3) cells.

**MATERIALS AND METHODS**

**Synthesis fusion (F) gene in pBT7-N-His plasmid**

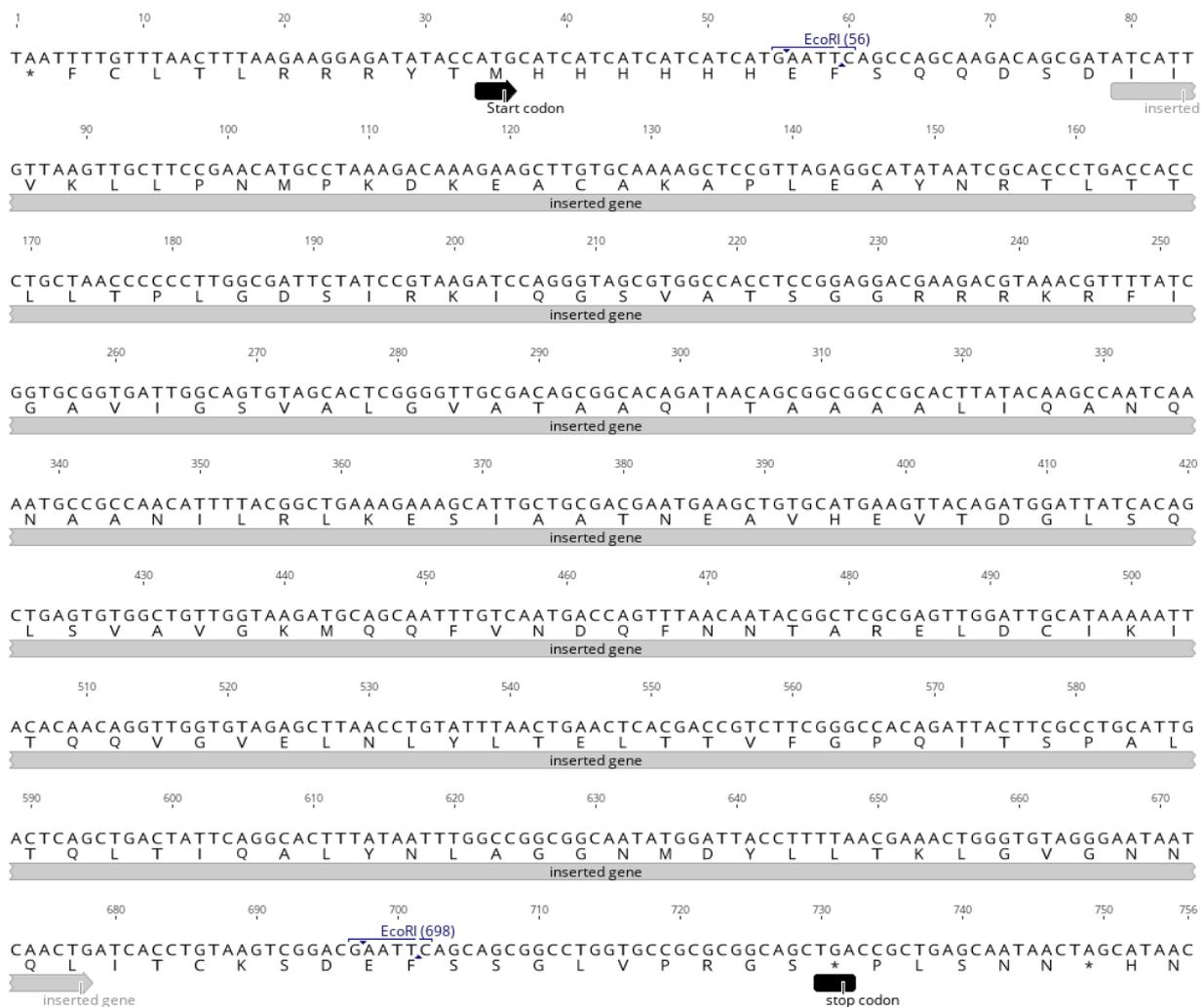
The F protein-encoding gene of NDV isolates from Galur, Kulon Progo, Yogyakarta, Indonesia (0663/04/2013), was sequenced by the previous study (Wulanjati et al. 2018). This sequence was submitted to Genbank with accession MW084963. A part of the F gene with 600 bp length (sequence no. 181-780) was selected to be expressed. After codon optimization, a 4,601 bp-long construct containing F gene sequence (600 bp) in pBT7-N-His vector was generated by Bioneer (Daejeon, Korea) (Figure 1). The pBT7-N-His vector has N-terminal His-tag sequence, T7 promoter, LacI, and T7 terminator.

**BLAST analysis and epitope prediction**

The sequence of F gene (600 bp) and its translated protein (200 amino acids) were analysed using BLAST to determine nucleotide and protein homology with database in Genbank NCBI. The multiple alignment of protein sequences was generated using CLC Sequencer Viewer 7. Meanwhile, B cell epitopes of F protein (200 amino acids) were predicted by IEDB with Bepipred Linear Epitope Prediction 2.0 and a threshold value of 0.5 (Hasan et al. 2013).

**Transformation recombinant plasmid pBT7-N-His-F into *E. coli* BL21(DE3)**

The recombinant plasmids were transformed into *E. coli* BL21(DE3) competent cells by heat shock method. The cells were inoculated on LB medium with ampicillin (50 µg.mL<sup>-1</sup>), then incubated at 37°C for 18 h. The colonies were verified by plasmid isolation using the Presto Mini Plasmid isolation kit (Geneaid). The plasmids were digested using *EcoRI* restriction enzyme. The whole and digested plasmids were electrophoresed in 1.5% agarose gel.



**Figure 1.** Sequences of inserted synthetic F gene (600 bp) in pBT7-N-His

### Expression of recombinant F protein in *E. coli* BL21(DE3) cells

The recombinant *E. coli* BL21(DE3) cells were cultured in LB broth with ampicillin ( $50 \mu\text{g}\cdot\text{mL}^{-1}$ ) at  $37^\circ\text{C}$ , 150 rpm. When the cells reached  $\text{OD}_{600} = 0.6$ , cells were induced by 1.0 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at  $37^\circ\text{C}$  for 8 h. The cells were harvested by centrifugation at 3,000 rpm,  $4^\circ\text{C}$  for 20 min. The cell pellets were resuspended in phosphate-buffered saline (PBS) and sonicated in an ice bath. The supernatants and the cell pellets were separated by centrifugation at 12,000 rpm, 5 min. Protein production was optimized by variation of induction duration (2, 4, 8, 18, and 24 h) and IPTG concentration (0.1, 0.2, 0.5, 0.8, 1.0, and 1.5 mM). The optimization of induction duration was performed with IPTG induction of 1.0 mM, while the optimization of IPTG concentration was conducted with induction duration of 8h.

To determine the molecular weight and the solubility of the recombinant F protein, the supernatant protein and cell pellet were separated by SDS PAGE with 12% resolving gel. The PageRuler Prestained Protein Ladder (Thermo Scientific) was used as a protein marker. The staining solution of Coomassie Brilliant Blue R250 (Sigma) was applied to visualize the protein bands.

### Purification of recombinant F protein

Protein purification in denatured conditions was performed using *MagListo* His-tagged protein purification kit (Bioneer). Binding/washing buffer and elution buffer were added with 8 M urea. The cell pellets were resuspended in binding/washing buffer and centrifuged at 12,000 rpm, 10 min. The Ni-NTA magnetic silica resin was equilibrated with 1 mL binding/washing buffer and placed on magnet, then supernatant was removed. The lysate sample 500  $\mu\text{L}$  was loaded into Ni-NTA magnetic silica resin and the loading waste (unbound fraction (UF)) was collected. The binding/washing buffer was added into tube, then the loading waste was collected as wash fraction (W1). To obtain the purified protein, elution buffer with 0.5 M imidazole and elution buffer with 0.75 M imidazole was added, sequentially. The eluents were collected as E1 and E2, respectively. The unbound fraction, wash fraction, and eluent fractions were analyzed by SDS PAGE.

### SDS-PAGE and western blotting

The expressed recombinant F protein of NDV was visualized by SDS-PAGE continued by Western blotting. At first, all of samples were denatured at  $95^\circ\text{C}$  for 5 min, then they were loaded 5  $\mu\text{l}$  on 5% stacking gel and separated on 12% resolving SDS-PAGE gel. The SDS-PAGE gel was run at 100 volts for 1 h. After that, the SDS-PAGE gel was then electrotransferred onto the polyvinylidene difluoride (PVDF) membrane to detect the recombinant F protein. Western blotting preparation was performed by blocking PVDF membrane using blocking solution (1% BSA dissolved in 0.5% Tween-PBS). The PVDF membrane was then incubated in room temperature for 1 h. After washing three times in washing solution (0.05% Tween-PBS), the PVDF membrane was probed

with a mouse monoclonal antibody anti-6X His tag antibody (Thermo Scientific Canada) in 1:2,000 dilution the primary antibody. The membrane was then incubated and shaken overnight at  $4^\circ\text{C}$ . After three-time wash in washing solution, PVDF membrane subsequently incubated with Alkaline Phosphatase (AP) conjugated goat anti-mouse IgG antibody (Thermo Scientific Canada) with 1:15,000 as the secondary antibody. Then the PVDF membrane was incubated and shaken in room temperature for 1 h. After washing with washing buffer two times, the immunological reaction was visualized by adding NBT-BCIP (NBT: p-nitroblue tetrazolium chloride, BCIP: 5-bromo-4-chloro-3-indolyl phosphate) substrate onto the PVDF membrane.

## RESULTS AND DISCUSSION

### BLAST analysis

BLAST analysis of F gene (600 bp) had 85.05% homology with Avian orthoavulavirus 1 isolate layer/Indonesia/GK-SR2-I7/978/2013 (MN557407.1), Avian orthoavulavirus 1 isolate layer/Indonesia/Jatim3-I6/977/2014 (MN557406.1), Avian orthoavulavirus 1 isolate layer/Indonesia/GK-SR2-I5/976/2013 (MN557405.1), and Avian avulavirus 1 isolate layer/Indonesia/Jatim3-I6/977/2014 (MK006004.1), and 85.02% homology with Avian avulavirus 1 isolate NDV/chicken/Badung/06140948/2014 (MK408593.1).

Meanwhile, BLAST F protein sequences (200 amino acids) showed 99.50% identity with fusion protein of Avian orthoavulavirus 1 NDV/chicken/Belitung/03150041/2015 (AZU96873.1), Avian orthoavulavirus 1 broiler/Indonesia/Muntilan-1P-II/972/2014 (QCX35374.1), Avian orthoavulavirus 1 layer/Indonesia/Jatim3-I6/977/2014 (QCX35375.1), Avian orthoavulavirus 1 NDV/chicken/Badung/06140948/2014 (QEE80204.1), and Avian orthoavulavirus 1 chicken/Banjarmasin/010/10 (AEV40792.1). There was a difference in amino acid at number 114, which was arginine (R) from this F protein instead of glutamine (Q) in other F protein (Figure 2). The strategy for developing the virus vaccine is determining the conserved region of the protein that protects the organism population against virus infection (Jazayeri and Poh, 2019). BLAST analysis of F protein sequence (200 amino acids) against the database in Genbank showed that the protein contained high conserved region with others F proteins.

**Table 1.** B-cell epitopes prediction of NDV F protein

Amino acid no.	Sequence	Length
7-26	PNMPKDKEACAKAPLEAYNR	20
41-54	KIQGSVATSGRRR	14
162-173	GPQITSPALTQL	12
190-197	LTKLGVGN	8

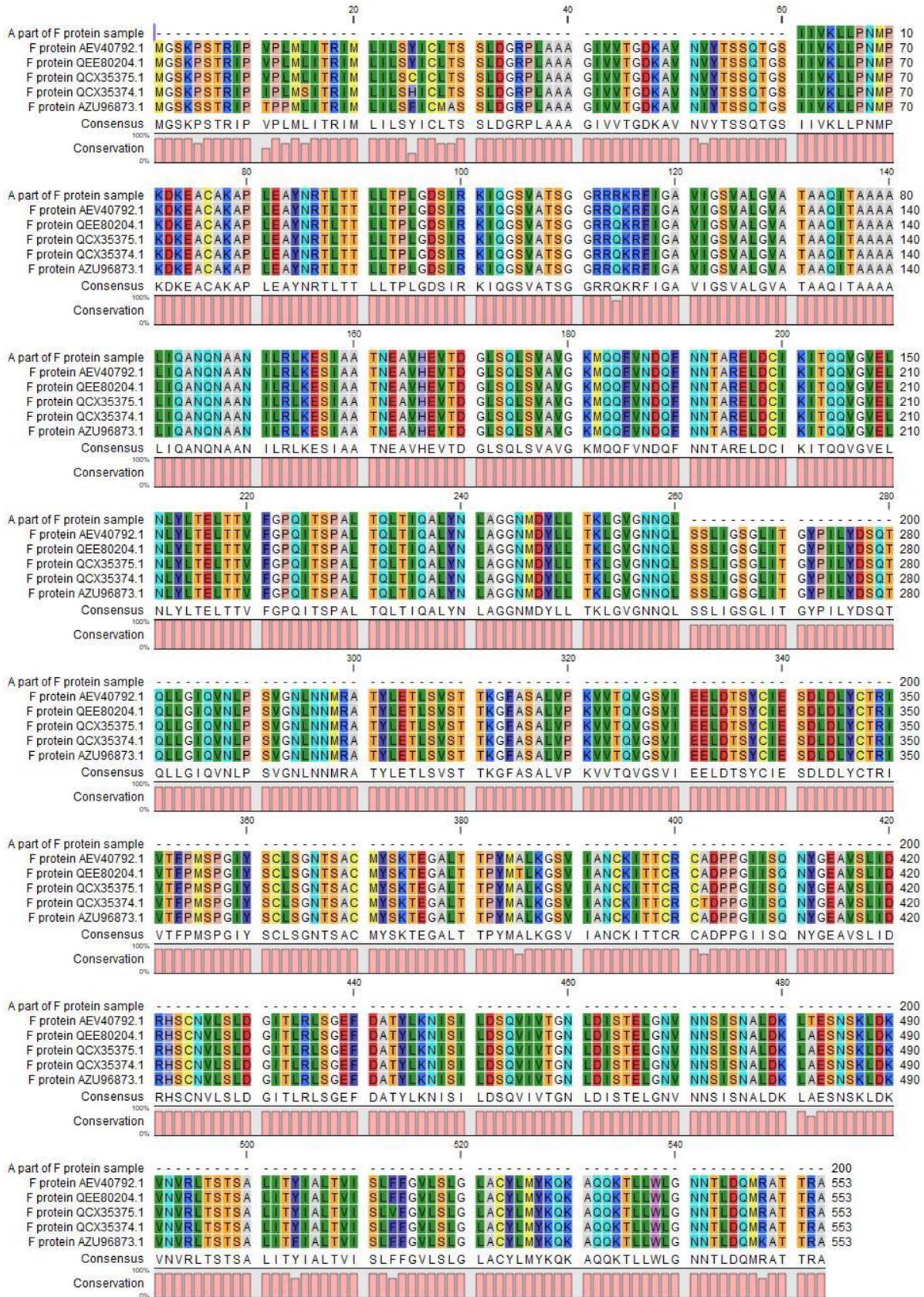


Figure 2. Multiple alignments of a part of fusion protein sample with other fusion protein from Indonesia

### B-cell epitopes prediction

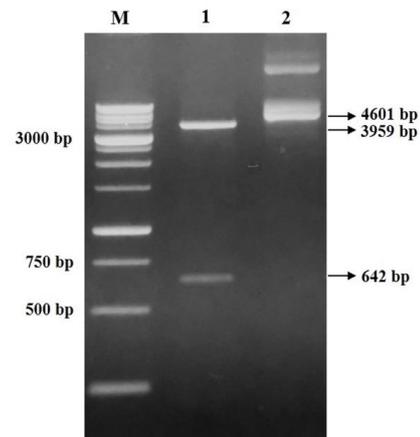
Table 1 presented the B cell epitopes prediction of F protein (200 amino acids). B-cell epitopes of F protein were predicted in four positions. Prediction of B-cell revealed that the protein was possibly recognized by B-cell and produced immune responses. Although the target protein was a partial protein of NDV F protein, it was still predicted to possess high immunogenicity because some epitopes were recognized by epitope prediction (Murwantoko et al. 2016).

### Production and purification of recombinant F protein in *E. coli* BL21(DE3)

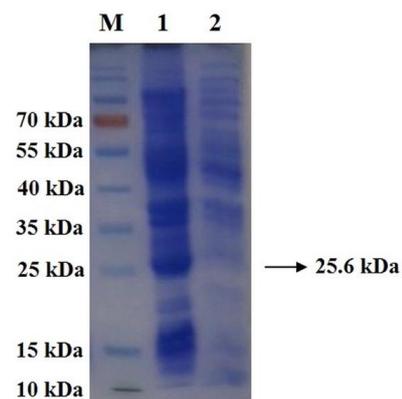
Digesting of the recombinant plasmid pBT7-N-His-F showed that recombinant plasmid was digested at Eco RI restriction sites generated two bands at 3,959 bp and 642 bp, based on the construct (Figure 3). It stated that the recombinant plasmid could be successfully transformed into *E. coli* BL21(DE3).

The F protein was expressed in *E. coli* BL21(DE3) cells by IPTG induction. The determination of F protein solubility showed that protein was expressed in cell pellets with a molecular weight of 25.6 kDa (Figure 4). It revealed that F protein was expressed in insoluble protein. Insoluble proteins or inclusion bodies are protein aggregates that are formed due to protein misfolding (Trimpin and Brizzard, 2018). The inclusion bodies occur as the impact of an unbalanced equilibrium between protein aggregation and solubilization so that driving to form protein aggregate (de Marco et al. 2019). However, the inclusion bodies have several advantages, including that they are not easily degraded by cell protease, are inactive form so that they do not harm bacterial cell, are easy to isolate, and have high yield (Jonasson et al. 2002). Although the protein is expressed as an insoluble protein, it can be solubilized to become a soluble protein by denaturing with denaturing agents, such as 6 M guanidine HCl and 8 M urea, or by solubilizing with non-denaturing solubilization agent, such as sarcosyl, 5% DMSO, or 5% n-propanol (Singhvi et al. 2020).

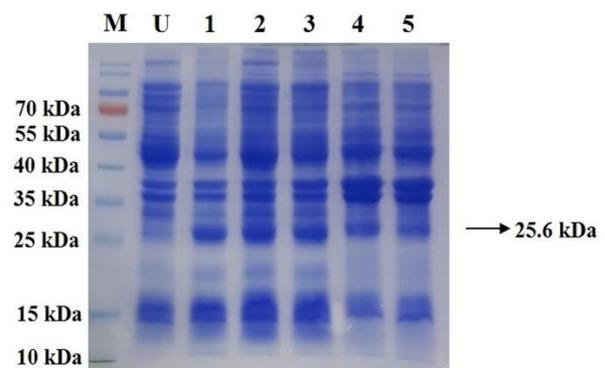
The efficiency of recombinant protein expression is directly affected by expression vector, compatible host, and culture condition (Kaur et al. 2018). Besides that, recombinant protein expression productivity can be increased by performing optimization of external factors, such as bacterial growth temperature, level of expression inducer, and induction duration (Larentis et al. 2014). The optimization of induction duration exhibited that the optimal induction duration was 8 hours, with the thickest protein band compared to other protein bands (Figure 5). The optimal IPTG concentration for F protein expression was 1.0 mM (Figure 6). Higher concentration and longer duration of IPTG induction lead to a significant decrease in bacterial growth and induces protease expression so that decreasing the amount of recombinant protein (Larentis et al. 2014).



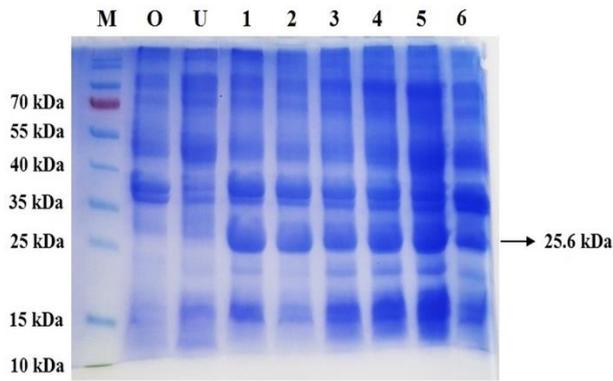
**Figure 3.** Visualization of the pBT7-N-His-F recombinant plasmid in 1.5% agarose gel electrophoresis. Lane M: DNA ladder 1 kb (Geneaid). Lane 1: digested recombinant plasmid by *Eco*RI restriction enzyme with two bands at 3,959 bp and 642 bp. Lane 2: undigested recombinant plasmid at 4,601 bp



**Figure 4.** SDS PAGE visualization of determination of recombinant F protein solubility. Lane M: protein marker. Lane 1: cell pellet. Lane 2: supernatant



**Figure 5.** SDS PAGE visualization of optimization of recombinant F protein production by variation of induction duration. Lane M: protein marker, Lane U: without IPTG induction, Lane 1-5: induction duration of 2, 4, 8, 18, and 24 h, respectively. The samples were induced by 1.0 mM IPTG.

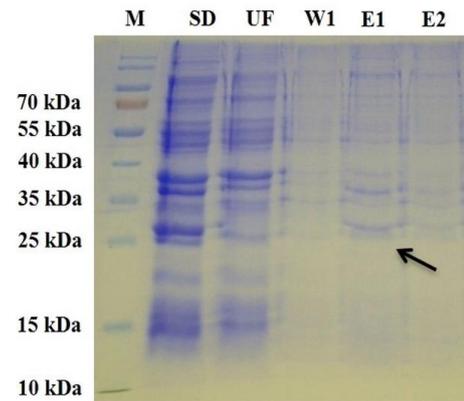


**Figure 6.** SDS PAGE visualization of optimization of recombinant F protein production by variation of IPTG concentration. Lane M: protein marker. Lane O: cell without recombinant plasmid. Lane U: without IPTG induction. Lane 1-6: induction time of 0.1, 0.2, 0.5, 0.8, 1.0, and 1.5 mM, respectively. The samples were induced for 8 h

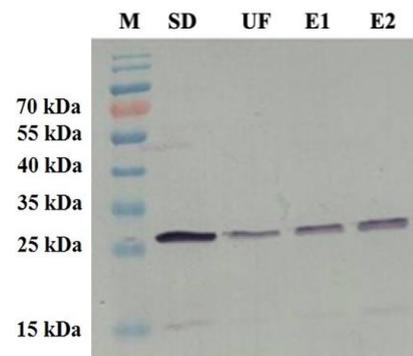
Protein purification assay indicated that recombinant F protein was successfully bound to Ni-NTA magnetic silica beads (Figure 7). The target protein was eluted in 0.5 M and 0.75 M imidazole. By western blotting analysis, the recombinant F protein was confirmed through reaction with specific monoclonal anti-6X His tag primary antibody and AP-conjugated goat anti-mouse IgG secondary antibody. The color development by incubation in NBT-BCIP substrate confirmed that recombinant F protein was expressed with molecular weight of 25.6 kDa (Figure 8).

In this work, the recombinant F protein of NDV had successfully in-vivo expressed into *E. coli* BL21(DE3) as a protein fragment with a molecular weight of 25.6 kDa under an optimal condition by 1.0 mM IPTG induction when the  $OD_{600}$  of the cell was 0.6 and an induction duration of 8 h. The results of this study were in line with Putri and Haryanto (2019), who carried out the in-vitro expression of NDV recombinant F protein from *E. coli* C-1a clone and Haryanto et al. (2020), who successfully conducted in-vitro expression of the recombinant F protein of NDV from local Indonesian isolates by using a cell-free protein expression system. This finding also was concurrent with another researcher group Astuti et al. (2020), who have successfully expressed and purify the recombinant F protein of local isolate NDV and studied the antibody response to recombinant F protein in broiler chicken post-vaccination.

Fusion (F) protein of NDV is a choice to be a recombinant vaccine agent because of its immunogenicity in susceptible chickens (Firouzmandi et al. 2016). The F protein of NDV isolates from Galur, Kulon Progo, Yogyakarta (0663/04/2013) had amino acid sequences no. 112-RRRKRF-117, indicating it is highly virulent, because of the basic amino acids in the cleavage site (Haryanto et al. 2016). The selection of recombinant vaccine agents from highly virulent isolates is expected to overcome the infection of highly virulent viruses because of the high mortality effect in susceptible chickens.



**Figure 7.** Recombinant F protein purification using Ni-NTA magnetic silica beads. Lane M: protein marker. Lane SD: supernatant denatured. Lane UF: unbound fraction. Lane W1: washing fraction. Lane E1: elution fraction with 0.5 M imidazole. Lane E2: elution fraction with 0.75 M imidazole. The arrow indicates the recombinant F protein band



**Figure 8.** Visualization of the recombinant F protein of NDV by SDS-PAGE and Western blotting. The recombinant F protein of NDV showed a single protein fragment in size of 25.6 kDa. Lane M: protein marker, lane SD supernatant denatured, UF: unbound fraction, lane E1: elution fraction with 0.5 M imidazole. Lane E2: elution fraction with 0.75 M imidazole.

It can be concluded that recombinant F protein (25.6 kDa) was expressed in *E. coli* BL21(DE3). The recombinant F protein was optimally produced by induction of 1.0 mM IPTG for 8 h. For further study, the immunogenicity of recombinant F protein in chicken should be tested to investigate the potency of recombinant F protein as a candidate of NDV vaccine agent. This study might help to develop strategy to eradicate NDV.

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## REFERENCES

- Astuti RW, Wijayanti N, Haryanto A. 2020. Expression of recombinant fusion protein from local isolate of Newcastle disease virus and antibody response to recombinant fusion protein in broiler chicken post-vaccination. *J Indon Trop Anim Agric* 45 (2): 78-90. DOI: 10.14710/jitaa.45.2.78-90.
- Cardenas-Garcia S, Diel DG, Susta L, Lucio-Decanini E, Yu Q, Brown CC, Miller PJ, Afonso CL. 2015. Development of an improved vaccine evaluation protocol to compare the efficacy of newcastle disease vaccines. *Biologicals* 43 (2): 136-145. DOI: 10.1016/j.biologicals.2014.11.003.
- de Marco A, Ferrer-Miralles N, Garcia-Fruitos E, Mitraki A, Peternel S, Rinas U, Trujillo-Roldan MA, Valdez-Cruz NA, Vazquez E, Villaverde A. 2019. Bacterial inclusion bodies are industrially exploitable amyloids. *FEMS Microbiol Rev* 43 (1): 53-72. DOI: 10.1093/femsre/fuy038.
- Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, Berg M, et al. 2019. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. *Infect Genet Evol* 74: 103917. DOI: 10.1016/j.meegid.2019.103917.
- Dimitrov KM, Afonso CL, Yu Q, Miller PJ. 2017. Newcastle disease vaccines—A solved problem or a continuous challenge?. *Vet Microbiol* 206: 126-136. DOI: 10.1016/j.vetmic.2016.12.019.
- Etriwati, Ratih D, Handharyani E, Setiyaningsih S. 2017. Pathology and immunohistochemistry study of Newcastle disease field case in chicken in Indonesia. *Vet World* 10 (9): 1066-1071. DOI: 10.14202/vetworld.2017.1066-1071.
- Firouzmandi M, Moeini H, Hosseini D, Bejo MH, Omar AR, Mehrbod P, Ideris A. 2016. Improved immunogenicity of Newcastle disease virus inactivated vaccine following DNA vaccination using Newcastle disease virus hemagglutinin-neuraminidase and fusion protein genes. *J Vet Sci* 17 (1): 21-26. DOI: 10.4142/jvs.2016.17.1.21.
- Haryanto A, Wati V, Pakpahan S, Wijayanti N. 2016. Molecular pathotyping of Newcastle disease virus from naturally infected chickens by RT-PCR and RFLP methods. *Asian J Anim Sci* 10 (1): 39-48. DOI: 10.3923/ajas.2016.39.48.
- Haryanto A, Wihadmadyatami H, Wijayanti N. 2020. In vitro expression of the recombinant fusion protein of Newcastle disease virus from local Indonesian isolates by using a cell-free protein expression system. *Indonesian J Biotechnol* 25 (2): 69-75. DOI: 10.22146/ijbiotech.54703.
- Hasan A, Hossain M, Alam J. 2013. A computational assay to design an epitope-based peptide vaccine against Saint Louis Encephalitis virus. *Bioinform Biol Insights* 7: 347-355. DOI: 10.4137/BBI.S13402
- Izquierdo-Lara R, Chumbe A, Calderón K, Fernández-Díaz M, Vakharia VN. 2019. Genotype-matched Newcastle disease virus vaccine confers improved protection against genotype XII challenge: The importance of cytoplasmic tails in viral replication and vaccine design. *PLoS One* 14 (11): e0209539. DOI: 10.1371/journal.pone.0209539.
- Jazayeri SD, Poh CL. 2019. Development of universal influenza vaccines targeting conserved viral proteins. *Vaccines* 7 (4): 169. DOI: 10.3390/vaccines7040169.
- Jonasson P, Liljeqvist S, Nygren PA, Ståhl S. 2002. Genetic design for facilitated production and recovery of recombinant proteins in *Escherichia coli*. *Biotechnol Appl Biochem* 35 (2): 91-105. DOI: 10.1042/ba20010099.
- Kang X, Wang J, Jiao Y, Tang P, Song L, Xiong D, Yin Y, Pan Z, Jiao X. 2016. Expression of recombinant Newcastle disease virus F protein in *Pichia pastoris* and its immunogenicity using flagellin as the adjuvant. *Protein Expr Purif* 128: 73-80. DOI: 10.1016/j.pep.2016.08.009.
- Kaur J, Kumar A, Kaur J. 2018. Strategies for optimization of heterologous protein expression in *E. coli*: Roadblocks and reinforcements. *Int J Biol Macromol* 106: 803-822. DOI: 10.1016/j.ijbiomac.2017.08.080.
- Larentis AL, Nicolau JFMQ, Esteves GS, Vareschini DT, de Almeida FVR, Reis MG, Galler R, Medeiros MA. 2014. Evaluation of pre-induction temperature, cell growth at induction and IPTG concentration on the expression of a leptospiral protein in *E. coli* using shaking flasks and microbio-reactor. *BMC Res Notes* 7 (1): 671. DOI: 10.1186/1756-0500-7-671.
- Mariappan AK, Munusamy P, Kumar D, Latheef SK, Singh SD, Singh R, Dhama K. 2018. Pathological and molecular investigation of velogenic viscerotropic Newcastle disease outbreak in a vaccinated chicken flocks. *Virus disease* 29 (2):180-191. DOI: 10.1007/s13337-018-0445-5.
- Murwantoko, Fusiando CK, Triyanto. 2016. Gene cloning and protein expression of Koi Herpesvirus ORF25. *Hayati J Biosci* 23 (3): 143-149. DOI: 10.1016/j.hjb.2016.10.001.
- Putri CN, Haryanto A. 2019. Fusion recombinant protein expression of Newcastle disease virus from *Escherichia coli*-Cloned C1a using accurapid TM protein expression kit. *IOP Conf Ser Earth Environ Sci* 355: 012026.
- Shofa M, Wibawan IWT, Zarkasie K, Setiyaningsih S. 2018. Molecular analysis of Newcastle disease virus isolated from a vaccinated layer farm in Indonesia. *IOP Conf Ser Earth Environ Sci* 197 (1): 012044.
- Singhvi P, Saneja A, Srichandan S, Panda AK. 2020. Bacterial inclusion bodies: A treasure trove of bioactive proteins. *Trends Biotechnol* 38 (5): 474-486. DOI: 10.1016/j.tibtech.2019.12.011.
- Trimpin S, Brizzard B. 2018. Analysis of insoluble proteins. *BioTechniques* 46 (6): 409-419. DOI: 10.2144/000113135
- Wang Y, Bi Y, Yu W, Wei N, Wang W, Wei Q, Wang X, Zhang S, Yang Z, Xiao S. 2017. Two mutations in the HR2 region of Newcastle disease virus fusion protein with a cleavage motif “RRQRRL” are critical for fusogenic activity. *Virol J* 14 (1): 185. DOI: 10.1186/s12985-017-0851-0.
- Wulanjati MP, Wijayanti N, Haryanto A. 2018. Phylogenetic analysis of Newcastle disease virus from Indonesian isolates based on DNA-sequence of fusion protein-encoding gene. *Biotechnol* 17 (2): 69-74. DOI: 10.3923/biotech.2018.69.74.
- Xiao S, Paldurai A, Nayak B, Samuel A, Bharoto EE, Prajitno TY, Collins PL, Samal SK. 2012. Complete genome sequences of Newcastle disease virus strains circulating in chicken populations of Indonesia. *J Virol* 86 (10): 5969-5970. DOI: 10.1128/JVI.00546-12.