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# Expression and serological application of recombinant epitope-repeat protein carrying an immunodominant epitope of Newcastle disease virus nucleoprotein

**Purpose:** The aim of the present study was to develop a serodiagnostic test for differentiation infected from vaccinated animal (DIVA) strategy accompanying the marker vaccine lacking an immunodominant epitope (IDE) of nucleoprotein of Newcastle disease virus (NDV).

**Materials and Methods:** Recombinant epitope-repeat protein (rERP) gene encoding eight repeats of the IDE sequence (ETQFLDLMRAVANS MR) by tetra-glycine linker was synthesized. Recombinant baculovirus carrying the rERP gene was generated to express the rERP in insect cells. Specificity and sensitivity of an indirect enzyme-linked immunosorbent assay (ELISA) employing the rERP was evaluated.

**Results:** The rERP with molecular weight of 20 kDa was successfully expressed by the recombinant baculovirus in an insect-baculovirus system. The rERP was antigenically functional as demonstrated by Western blotting. An indirect ELISA employing the rERP was developed and its specificity and sensitivity was determined. The ELISA test allowed discrimination of NDV infected sera from epitope deletion virus vaccinated sera.

**Conclusion:** The preliminary results represent rERP ELISA as a promising DIVA diagnostic tool.

**Keywords:** Newcastle disease virus, Nucleoprotein, Epitope-repeat protein, Enzyme-linked immunosorbent assay, Differentiation infected from vaccinated animal

## Introduction

Newcastle disease virus (NDV) is the etiological agent of Newcastle disease (ND), one of the most infectious diseases affecting poultry. The virus has an enveloped non-segmented negative stranded RNA genome and is member of the genus *Avulavirus* in the family *Paramyxoviridae*. ND outbreaks cause mortality rate up to 100% in susceptible poultry and is a notifiable disease to the World Organisation for Animal Health (OIE) [1]. The NDV genome consists of 55 nt leader at its 3' end and 114 nt trailer at its 5' end, flanking six essential genes encoding nucleoprotein (NP), matrix protein (M), phosphoprotein (P), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) [2].

Internal structural protein NP is a highly conserved protein involved in virus replication cycle as well as in inducing high level of NDV-specific antibody in chickens [3].

N- and C-terminal regions of the NP serve as B-cell epitopes in host [4,5]. In particular, C-terminal immunological region that forms alpha helical structure is permissible for modification. Thus this epitopic region can serve as negative marker if replaced with a foreign epitope [6]. For example, amino acid sequence ETQFLDLMRAVANSR (aa 444-459) on NDV NP is an epitope, which serves as linear immunodominant epitope (IDE) and can be replaced or deleted [4]. This antigen should facilitate differentiation of vaccinated animal to that of infected during serosurveillance in the case of the marker vaccine applying vaccination program. A multi-epitope protein as a vaccine candidate [7-12] or as diagnostic reagent [13-15] has been explored in multiple instances. Multi-epitope recombinant proteins are advantageous as they can be designed rationally and offers higher specificity and sensitivity [16].

The aim of the present study was to develop a serodiagnostic test for differentiation infected from vaccinated animal (DIVA) strategy accompanying an NDV marker vaccine lacking the NP IDE. For this purpose, an indirect enzyme-linked immunosorbent assay (ELISA) format employing a recombinant protein, which carries multimers of IDE, was developed. The recombinant protein was expressed in baculovirus system. Specificity and sensitivity of recombinant protein was evaluated.

## Materials and Methods

### Design of synthetic gene and tertiary structure prediction

Epitope repeat protein (ERP) gene encoding eight repeats of an IDE sequence containing ETQFLDLMRAVANSR (C-terminal IDE aa 444-459) on NDV NP separated by tetra-glycine linker was synthesized commercially using codon optimization for baculovirus expression. The recognition enzyme sites of *EcoRI* and *HindIII* were built in the upstream and downstream of coding sequence, respectively. The synthetic gene cloned in vector backbone PMK-RQ (KanR) was received as 5 µg of lyophilized plasmid preparation. The conceptual translated sequence was submitted to I-TASSER server for homology modeling [17] and top five predicted structures were visualized with PyMOL software [18].

### Preparation of recombinant bacmid

Synthetic gene was subcloned into pFastBac HT b vector using *EcoRI* and *HindIII* restriction sites. The recombinant plasmid (50 ng) was transformed in *Escherichia coli* DH B10 cells.

Transformed cells were plated onto the Luria-Bertani agar containing kanamycin (50 µg/mL), gentamicin (7 µg/mL), tetracycline (10 µg/mL), Bluo-gal (100 µg/mL), and isopropylthio-β-galactoside (IPTG, 40 µg/mL) and incubated at 37°C for 36 to 48 hours. The high molecular weight bacmid DNA was isolated from the overnight cultures by alkaline lysis purification according to the manufacturer's manual of Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA).

### Generation of recombinant baculovirus

*Spodoptera frugiperda* 9 (Sf9) cells were cultured at 27°C in Sf-900 II serum free medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen), 50 U/mL penicillin and 50 µg/mL streptomycin. Sf9 cells were transfected with recombinant bacmid DNA using Cellfectin II, a cationic lipid for the transfection of the baculovirus particles according to the manufacturer's instructions. Briefly, for each transfection, 2 mL of Grace's Insect Medium, unsupplemented (without antibiotics and serum) was added in each well,  $8 \times 10^5$  cells per well were seeded in a 6-well plate and allowed to attach for 2 hours. The bacmid DNA and Cellfectin II (8 µL of reagent) were diluted separately in 100 µL of Grace's medium, unsupplemented (without antibiotics and serum), then mixed and incubated for 30 minutes at room temperature to form lipid-DNA complexes. The cells were washed with fresh medium, and incubated with lipid-DNA complex at 27°C for 5 hours. The transfection solution was removed and 2 mL supplemented Sf-900 II SFM containing 10% FBS was added. Transfected Sf9 cells were incubated at 27°C for 72 hours for baculovirus production. Recombinant baculovirus production was monitored daily by visualization of the cytopathic effects. Three to four days after transfection, recombinant baculovirus was harvested from the cell culture medium and stored at 4°C. Recombinant viruses were identified by polymerase chain reaction using gene vector specific primers (Invitrogen). The resulting baculovirus was passaged three times by infecting more Sf9 cells.

### Protein expression and purification

Sf9 suspension cultures ( $2 \times 10^6$  cells/mL) grown in 2 L of Sf900 II medium supplemented with 1% of FBS were infected with recombinant baculovirus at multiplicity of infection of 5 plaque-forming unit/cell. Cells were grown for 72 hours and cell pellets were washed in phosphate buffered saline (PBS). As recombinant ERP (rERP) carried 6× His tag at N-terminal,

it was purified by using Ni-NTA spin columns under denaturant condition with 8 M urea according to Ni-NTA spin handbook (Qiagen, Hilden, Germany). The collected cells were lysed in 10 mL buffer B (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 8 M urea, pH 8.0) and centrifuged the lysate at 10,000×g for 30 minutes and collecting supernatant. Loading up to 600 µL of the cleared lysate supernatant containing the 6× His-tagged protein onto a pre-equilibrated Ni-NTA spin column, centrifuged 2 minutes at 700×g and washing the column with 600 µL buffer C (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 8 M urea, pH 6.3) and centrifuged 2 minutes at 700×g, the recombinant protein was finally eluted with buffer E (10 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, 8 M urea, pH 4.3). The recombinant protein was allowed to refold using refolding buffer (50 mM Tris pH 7.5, 0.5 M NaCl, 0.3% CHAPS, 1 mM DTT, 5% glycerol). rERP was dialyzed against refolding buffer (40 mM CHAPS and 10 mM DTT).

### Electrophoresis and Western blotting

Proteins extracted from infected Sf9 cells were fractionated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions. The separated proteins were blotted onto an Immun-Blot polyvinylidene fluoride membrane (Bio-Rad, Richmond, CA, USA) using a wet transfer system (Bio-Rad). The membrane was blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBST) at room temperature for 1 hour. After washing 3 times with PBST, the blocked membrane was subsequently incubated with NDV chicken antiserum (diluted 1:100) for 1 hour, rinsed in PBST, and incubated with alkaline phosphatase-conjugated goat anti-chicken immunoglobulin (diluted 1:1,000, Pierce, Rockford, IL, USA) for 1 hour. Protein bands were visualized by enhanced chemiluminescence using NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, USA).

### Standardization of the indirect ELISA

Optimal dilutions of rERP and sera were determined by a checkerboard titration test with NDV positive and negative sera previously confirmed by hemagglutination inhibition (HI) test. The antigen was coated in 96-well ELISA plates (PolySorb, Nunc, Roskilde, Denmark) ranging in concentration from 2 µg/mL to 0.0625 µg/mL in 50 mM carbonate/bicarbonate buffer (pH 9.6). Reference positive and negative sera were both diluted serially from 1:100-1:6,400 and tested to determine the optimal serum dilution. The dilutions that gave the optimum difference in absorbance at 450 nm between posi-

tive and negative sera were selected to test the sera panel. The working dilution of goat anti-chicken HRP-IgG (Sigma-Aldrich), the reaction temperature, time and other conditions also were optimized.

Ten NDV negative serum samples from specific pathogen free (SPF) chickens were used to determine a cutoff value for the ELISA assay. The mean negative serum optical density (OD) value plus three standard deviations (SD) was used as the cutoff value. Each sample was repeated in triplicate wells and the mean value was calculated. The experiment was repeated two more times. In addition to SPF chicken serum, different hyper-immune chicken sera to NDV, H9N2 avian influenza virus (AIV), infectious bronchitis virus (IBV), and infectious bursal disease virus (IBDV) were tested for specificity of rERP using an indirect ELISA assay. To test the sensitivity of the rERP, the chicken anti-NDV serum was diluted serially (1:100-1:6,400) and the serum with each dilution was used to react with the expressed rERP in an indirect ELISA.

### rERP ELISA

A total of 30 chicken sera comprising three groups with each group of 10 were used in the study. These sera were kept at the OIE reference laboratory for Newcastle disease, Animal and Plant Quarantine Agency (APQA), Korea. First group were sera taken from mock-infected SPF chicken (NDV antibody negative). Second and third groups were sera taken 14 dpi from SPF chickens vaccinated with IDE deleted recombinant Newcastle disease virus (rNDV) [4] and NDV LaSota strain [19], respectively, which kindly supplied by the OIE reference laboratory for ND, Korea. All vaccinated sera were proved to be serologically positive by a HI test using NDV antigen. All sera were available with the laboratory. Microtiter plates were coated with 100 µL of 1 µg/mL rERP protein in 50 mM carbonate/bicarbonate buffer pH 9.6, and incubated overnight at 4°C. Plates were washed three times with PBST and blocked with 150 µL per well PBST with 5% skim milk powder solution at 37°C for 2 hours. The chicken anti-NDV serum (1:400) was added to each. After 1-hour incubation at 37°C, plates were washed three times with PBST. One hundred microliters horseradish peroxidase-conjugated goat anti-chicken IgG (Pierce) at 1:2,000 dilution was added to each well and plates were incubated at 37°C for 1 hour. Then plates were washed three times with PBST and 100 µL TMB (3,3',5,5'-tetramethylbenzidine) was added to each well. After 10-minute incubation at 37°C, reactions were stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and the OD at 450 nm was measured with microplate reader (Sunrise,



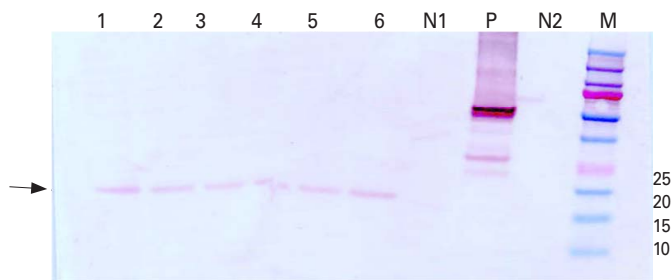


der the transcriptional control of polyhedrin promoter. Recombinant baculovirus bac-rERP infected cells were lysed under denaturing conditions and rERP was purified using His tag purification. The protein was allowed to refold using refolding buffer. To identify the expressed rERP, the purified protein was analyzed by western blot assay. The chicken anti-NDV serum raised by infecting SPF chickens with La Sota strain of NDV [19] was used as the primary antibody in the analysis. The results showed purified rERP with molecular weight of approximately 20 kDa reacted specifically (lanes 1 to 6 in Fig. 2) to the NDV positive serum. No specific band was detected with negative controls (lanes N1 and N2 in Fig. 2), which indicated that the rERP is expressed correctly, and has

good reaction ability with chicken specific anti-NDV serum.

**Specificity and sensitivity of the rERP**

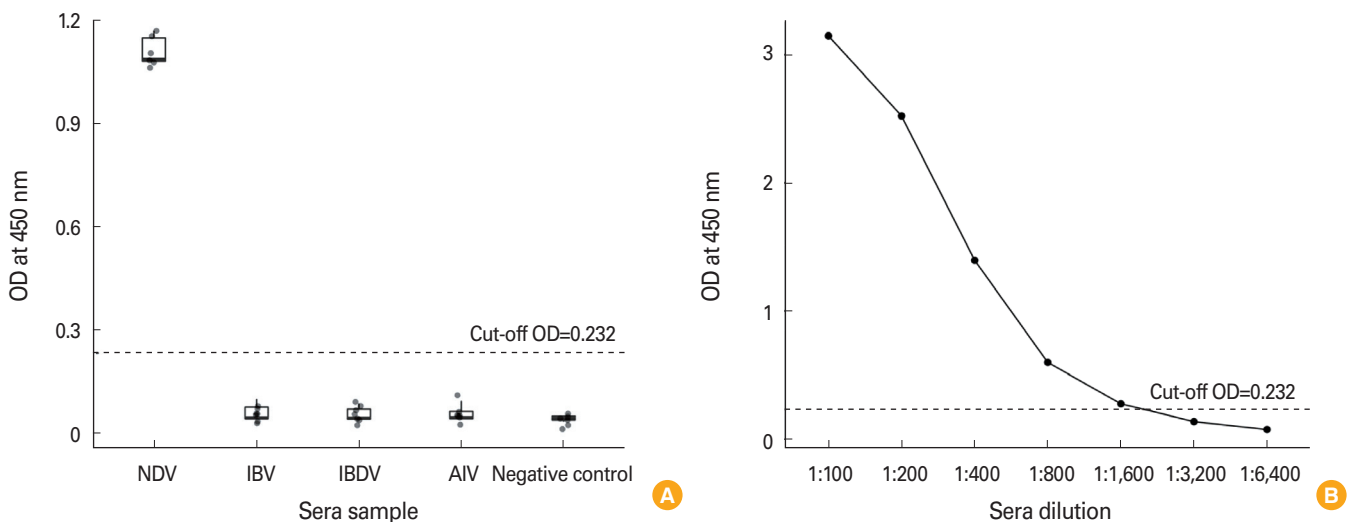
The optimal concentration of the rERP (1 µg/mL) and the dilution rate of sera (1:400) for indirect ELISA assays to evaluate the specificity and sensitivity of the expresses rERP were determined. To set up a cutoff value for indirect ELISA assay, 10 SPF chicken serum samples were analyzed. The mean OD<sub>450</sub> value of these samples as measured by ELISA was 0.231 with a SD of 0.00022. For a 99% confidence interval, the cutoff value determined was 0.232 (mean OD of SPF sera samples+3 SD). Based on the cutoff value, the reactivity of rERP with chicken anti-AIV, anti-IBV, anti-IBDV, and normal sera was measured. The result showed that OD<sub>450 nm</sub> values of non-NDV sera were lower than cutoff value (Fig. 3A). The minimum dilution titer of chicken anti-NDV serum detected was 1:1,600 according to cutoff value of 0.232 (Fig. 3B).



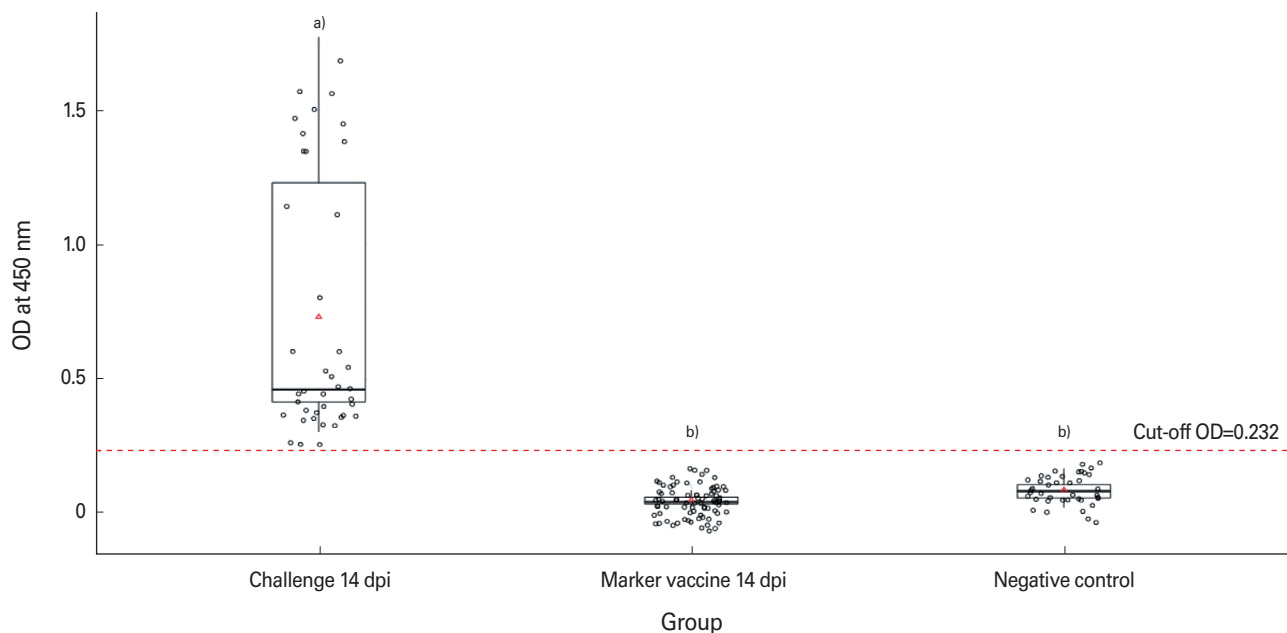
**Fig. 2.** Identification of the recombinant epitope-repeat protein (rERP). Lanes 1 to 6, rERP protein; lane P, positive control (Newcastle disease virus LaSota); lane N1, negative control (cell culture fluid); lane N2, negative control (phosphate buffered saline); M, protein marker.

**rERP ELISA**

A battery of 30 chicken sera samples divided in ten chickens per group in three was tested. Samples in groups of mock infected chicken sera and chicken vaccinated with the IDE deleted rNDV showed OD values below cut-off value. The OD value for samples from wildtype NDV LaSota vaccinated chicken group were in the range of 0.255 to 1.77, which was above determined cut-off value (Fig. 4). The indirect ELISA showed



**Fig. 3.** Specificity and sensitivity of the recombinant epitope-repeat protein (rERP). (A) Specificity of the rERP. Different serum samples of chicken anti-Newcastle disease virus (NDV), anti-infectious bursal disease virus (IBDV), anti-infectious bronchitis virus (IBV), anti-avian influenza virus (AIV), and specific pathogen free serum as negative control were tested by indirect enzyme-linked immunosorbent assay (ELISA). Boxes denote interquartile ranges, with median values shown as horizontal lines inside the box. Whiskers denote ranges. Individual data points are plotted. (B) Sensitivity of the rERP. Serial dilutions of NDV positive serum (from 1:100 to 1:6,400) were tested by an indirect ELISA. The results showed that the minimum detection limit of chicken anti-NDV positive sera was 1:1,600.



**Fig. 4.** Box-and-whisker plot of absorbance values of indirect enzyme-linked immunosorbent assay in three different groups. Boxes denote interquartile ranges, with median values shown as horizontal lines inside the box. Whiskers denote ranges. Triangle represents mean values. All data points from 4 independent experiments of sample size  $n=10$  in each group are plotted. <sup>a),b)</sup>Superscript carrying different alphabets are significantly different ( $p<0.05$ ).

good repeatability through the multiple experiments.

## Discussion

Marker NDV vaccines in conjugation with serological diagnostic test has substantial value in poultry industry for demonstration of the freedom of ND. A serologic marker antigen is a viral protein or an epitope that is absent from the vaccine strains but consistently present in the corresponding wild-type viruses [5]. Therefore, only animals that have been infected with wild-type virus will develop antibodies against the serologic marker antigen while the vaccinated animals will not. The marker antigen should be conserved and immunodominant to ensure that the companion diagnostic test based on this marker antigen will produce reliable diagnostic results [20]. In addition, the marker antigen should be dispensable for the viral life cycle but should not contribute significantly to the overall immunogenicity of the vaccine. Subunit vaccines using F and HN protein [21] can serve as marker vaccines but are less effective than whole virus vaccines [22]. This necessitates NDV marker vaccine based on chimeric live virus.

NDV NP is known to carry three antigenic regions, two of which are located at N terminal [5]. C terminal IDE (aa res-

idues 444-459) ETQFLDLMRAVANSR is a dispensable epitope on NP protein, which can be replaced with foreign epitope [4]. In preliminary study, when chickens were immunized with viable recombinant NDV lacking this IDE with reverse genetics technique, monomeric IDE peptide based ELISA showed poor sensitivity most likely due to small hapten size of 16 aa and immunosteric hindrance to bovine serum albumin conjugated peptide (data not shown). To address this issue, this problem can be solved by using multimers of IDE in the form of recombinant protein antigen.

In the study, we designed, expressed and characterized the rERP in baculovirus expression system. This protein carried tandem repeats of IDE on NDV NP. The protein carried N terminal his tag allowing one-step purification under Ni NTA agar column [23,24]. The construct designed to express recombinant protein was codon optimized for Sf9 cells. Codon optimization involves codon adaptability, mRNA structure, tRNA usage. It significantly increases protein expression [25-27]. We used tetra-glycine linker as linker in between epitopes as it provides flexibility due to lack of  $\beta$ -carbon and is preferred linker in multi-epitope proteins [28]. Computer modeling showed that all the epitopes are freely accessible in three-dimensional space. This rERP was expressed at high concentration in insect cells when titrated by ELISA. We purified pro-

tein under denaturing condition on a Ni-NTA matrix with a high degree of purity [29].

Western blot analysis showed the purified rERP was recognized by chicken anti-NDV serum with a specific band at approximately 20 kDa (lanes 1-6 in Fig. 2), while no specific band was observed when using negative control serum. This indicates that the expressed rERP has the molecular weight as expected. We employed the indirect ELISA to evaluate the specificity and sensitivity of the expressed rERP using confirmed positive and negative sera samples. rERP could react specifically with the anti-NDV serum and showed no reactivity towards anti-IBV, anti-IBDV, anti-AIV, and normal sera. It suggests that rERP can specifically recognize anti-NDV antibodies. The potential of rERP ELISA to serve as discriminatory test in combination with the IDE deleted marker vaccine was investigated using three panels of sera. The test correctly classified the marker vaccinated and SPF chicken sera as antibody negative but sera from wildtype NDV immunized chickens as antibody positive. These results revealed that rERP possessed good reactivity, specificity and sensitivity.

Future work involves testing sera samples under field trials. If proven satisfactory, the recombinant protein represents a promising candidate as companion diagnostic test in NDV DIVA vaccination program. This strategy of epitope-based recombinant proteins as ELISA antigens against modification permissible regions of viruses makes it a highly effective approach in DIVA vaccination.

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