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Establishment of Non-Structural V and Structural P Protein-Based Elisas for Detection of Newcastle Disease Virus Infection

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Abstract: Researchers have developed an Enzyme-Linked Immunosorbent Assay (V-ELISA) to detect antibody against the C-terminal region of the Newcastle disease virus non-structural protein V. Another ELISA to detect antibody against a C-terminal polypeptide of structural P protein (P-ELISA) was also developed. Antibody responses to V and P proteins in chicken sera immunized with recombinant proteins were detected by using V-ELISA and P-ELISA with the minimum cross reactions. And using V-ELISA and P-ELISA, antibodies to V and P were also detected in yolk of eggs from chickens immunized with live NDV vaccine. These results indicate that the internal NDV proteins can be used as diagnostic antigens for detection of NDV infection.

Key words: ELISA, Newcastle disease virus, V protein, P protein, NDV

INTRODUCTION

Newcastle disease is a highly contagious and fatal viral disease that affects all species of birds. It is an economically important avian virus disease that can cause huge economic losses to the poultry industry (Alexander *et al*., 1997). The causative agent of the disease is the Newcastle Disease Virus (NDV). NDV is an enveloped, negative-sense, single stranded RNA virus, belonging to the Paramyxoviridae family and genus *Avulavirus* (Mayo, 2002; Alexander and Senne, 2008).

The genomic RNA has been reported to encode for six proteins Namely-Nucleocapsid (NP) protein, Phosphoprotein (P), Matrix Protein (M), Fusion glycoprotein (F), Haemagglutinin Neuraminidase Protein (HN) and Large Protein (L) (Lamb and Parks, 2007). The other proteins V and W were synthesized as a result of mRNA editing at the *P* gene (Steward *et al*., 1993). Analysis of mRNAs produced from the *P* gene transcription showed that 68% were P-encoding mRNA, 29% were V-encoding mRNA and 2% were W-encoding mRNA (Mebatsion *et al*., 2001). The NDV P protein, like the P proteins of other paramyxoviruses, functions in genome replication and transcription (Errington and Emmerson, 1997; Locke *et al*., 2000; Kho *et al*., 2004). The V protein plays a direct role in virus replication and in host range restriction as well as serves as a virulence factor (Mebatsion *et al*., 2001; Huang *et al*., 2003; Park *et al*., 2003a, b). The C-terminal half of the V protein with its cysteine-rich regions is more highly conserved among paramyxoviruses than the N-terminal half (Kattenbelt *et al*., 2006).

In the present study, researchers hypothesized that the V protein of NDV was a suitable diagnostic marker of NDV infection. To detect V protein, researchers developed an Enzyme-Linked Immunosorbent Assay (V-ELISA). For the diagnostic antigen, researchers selected the C-terminal part of the V-protein because it is more highly conserved among paramyxoviruses than the N-terminal half and it is unique to the V-protein (Steward *et al*., 1993). Also, researchers developed another ELISA (P-ELISA) using the C-terminal part of the P protein which is a structural protein of NDV.

To the knowledge, this is the first study that presents a preliminary approach for the essential assessment of seroconversion to the internal proteins of NDV in experimental and field chickens by specific ELISAs.

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MATERIALS AND METHODS

Virus and cell culture: NDV Hitchner B1 (NDV-HB1) was used in the present study. Chicken Embryo Fibroblasts (CEFs) were prepared from 10 day old SPF embryos. NDV-HB1 was inoculated on CEF at m.o.i of 10 EID_{50}/cell. Infected cells were cultivated in α-MEM containing 2.5 μg mL^{-1} trypsin (Sigma, St. Louis, MO, USA) for 3 days at 37°C.

PCR and cloning: PCR protocols for cloning V and P fragments and cloned plasmids were described in another study. In brief, PCR products were digested, purified from gel and cloned into the Glutathione S-Transferase (GST) fusion protein expression vector pGEX-6P-1 (GE Healthcare, UK). Recombinant clones containing P insert or V insert were named pGST-P or pGST-V, respectively. The clones were confirmed by nucleotide sequencing (Doragon Genomics, TAKARA Bio, Mie, Japan).

Protein expression and purification: *E. coli* DH5α was transformed with pGST-P or pGST-V DNA. Recombinant V or P GST fusion proteins were prepared using the standard protocol. Expression of the unique C-terminal part of the V protein in *E. coli* was confirmed by Western blotting as a fusion protein with GST (GST-V) as well as the C-terminal part of the P protein (GST-P). V (NDV-V) and P (NDV-P) proteins were purified by GSTrap FF column affinity chromatography and PreScission protease (GE Healthcare) according to the manufacturer's instructions.

SDS-PAGE and Western blotting assays: Fused or purified protein samples were denatured in SDS sample buffer by boiling for 5 min (Laemmli, 1970). Denatured proteins were separated on a 12% gel and stained with Coomassie brilliant blue R-250 or transferred to a sheet of polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA) (Harlow and Lane, 1988). The blots were blocked with 5% (wt./vol.) non-fat skim milk, incubated for 1 h at 37°C, washed three times in PBS-Tween 20 (0.05%), soaked in sample serum diluted with PBST, incubated for 2 h at 37°C, washed three times in PBST, soaked in one of the two following secondary antibodies (anti-GST antibodies conjugated to horseradish peroxidase (GE Healthcare) or goat anti-chicken IgY conjugated to horseradish peroxidase (Abcam, Tokyo, Japan)), incubated for 2 h at 37°C, washed three times in PBST and incubated in ECL Western blotting detection reagents (GE Healthcare). The membranes were developed by using enhanced chemiluminescence Diamino-Benzidine (DAB; Dojindo).

Immunization of chickens: Three, 2 months old chickens were injected with purified NDV-P or NDV-V at a dose of 10-25 μg/chicken mixed with an equal volume of Complete Freund's Adjuvant (CFA) containing 11 mg mL^{-1} of heat-killed *Mycobacterium tuberculosis* (Difco Laboratories, Detroit MN). Antigen-adjuvant emulsion (0.2 mL) was injected intramuscularly. Booster doses of antigen were mixed with incomplete Freund's adjuvant. The interval between doses was 2 weeks. One chicken was injected with saline as a control.

In another experiment, two, 2 months old SPF chickens were inoculated intraorally or intraocularly with 30 μL/chicken of $10^{8.5}$ EID_{50} live NDV-HB1vaccine (Nippon Biologicals, Inc., Tokyo, Japan). Each received five doses with an interval of 2 weeks between them. The group of chicken is referred to as the live vaccine group. Two chickens were injected with saline as a control. Eggs, whose yolks are an abundant source of polyclonal antibodies (IgY) (Jensenius *et al*., 1981) were collected during the experiment and stored at 4°C for later detection of IgY against NDV. IgY was obtained from the eggs with a chicken IgY purification kit (Pierce, Rockford, IL, USA) according to the manufacturer's protocol.

All experiments were conducted under the guidelines for animal experiments at Gifu University with approval by the Committee of Animal Care and Welfare, Faculty of Applied Biological Science, Gifu University. Specific-pathogen free eggs were obtained from Aoki farm, Tochigi, Japan. The eggs were incubated to hatch in the laboratory. Each group was reared separately with clean food, water and housing conditions.

Field samples: Thirty nine parent white leghorn chickens were randomly selected from a flock of 11,000 chickens (Japan Layer K.K. Company). Another 20 chickens were also randomly selected from a flock of 10,000 chickens at the same farm but from a different house. All chickens received NDV (HB1) live vaccination and inactivated vaccine of NDV as shown in Table 1. Researchers monitored the antibody response from the younger age to the older one. Approximately 10 samples were collected each time.

Indirect ELISA: For V-ELISA and P-ELISA, each antigen (NDV-V and NDV-P) was diluted to 1 μg mL^{-1} with 0.05 M carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and subsequently applied to 96 well plates (F96 Maxisorp, Nunc, Roskilde, Denmark) at 50 μL/well. The plates were incubated at 4°C overnight. The chicken sera were incubated overnight at 4°C with 1% *E. coli* (DH5α) lysate to reduce non-specific reactivity as reported elsewhere (Tumpey *et al*., 2005). Serum samples

Table 1: Chicken field samples obtained from a parent stock of white leghorn variety that received NDV HB1 live and inactivated vaccine of NDV

Age of chickens (days)	The treatment	Number of samples	
		From the first flock	From the second flock
14	Received live NDV (HB1) vaccine	-	-
35	Received live NDV (HB1) vaccine	-	-
42	Blood sampling	10	-
56	Received live NDV (HB1) vaccine	-	-
83	Received live (NDV HB1) vaccine	-	-
92	Blood sampling	10	-
100	Received Inactivated NDV (HB1) v.	-	-
119	Blood sampling	10	-
121	Blood sampling	-	10
155	Blood sampling	9	-
176	Blood sampling	-	10

were serially diluted with blocking buffer (1% (w/v) gelatin in PBST). Diluted sera were added to wells (50 μL/well), incubated for 2 h at 37°C, washed with PBS, incubated with 50 μL/well of a solution of the secondary antibody (goat anti-chicken IgY conjugated to horseradish peroxidase (Abcam, Tokyo, Japan)) for 1 h at 37°C, washed with PBS, mixed with 75 μL/well ABTS (2,2-azino-bis (3-ethlbenzothiazoline-6-sulfonic acid, Sigma-Aldrich, St. Louis, MO) solution (0.05 M citrate buffer, pH 4.0, containing 0.0075% H_2O_2) and incubated at 37°C for 30 min to 1 h. Optical Density (OD) at 405 nm was read with a microplate reader (Model 550, Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

Expression and purification of NDV-P and NDV-V proteins: Expression of GST-V and GST-P was confirmed by Western blotting. The molecular weights of GST-V and GST-P were 40.8 and 55.4 kDa, respectively. Purified NDV-V and NDV-P proteins had sizes of 14.8 and 29.4 kDa, respectively.

Specificities of anti-sera: The specificities of the anti-V and anti-P sera were evaluated by Western blotting using lysates of infected CEF cells and purified NDV-HB1. NDV-HB1 was purified from allantoic fluid of SPF chicken eggs by sucrose density gradient ultracentrifugation. Anti-P sera reacted with the purified virion preparation and infected cell lysates whereas the anti-V sera reacted with the infected cell lysates and only slightly reacted with the purified virion preparation (Fig. 1). These results showed that the anti-V and anti-P serum reactions were specific for V and P proteins, respectively.

Establishment of indirect NDV-V and NDV-P-ELISAs: When V protein was used as an ELISA coating antigen, only serum samples taken from V-immunized chickens reacted to the V-protein in NDV-V ELISA (Fig. 2). Similarly, when P protein was used as an ELISA coating

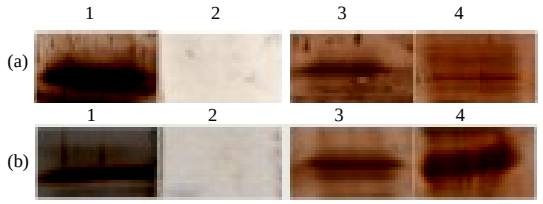

Fig. 1: Western blotting analysis of P and V proteins; a) Lane 1: V protein was detected by sera from V protein immunized chicken (1:100 dilution); Lane 2: V protein against control negative sera (1:100 dilution); Lane 3: infected cell lysate against the sera of V protein immunized chicken (1:100 dilution); Lane 4: purified NDV virions against the sera of V protein immunized chicken (1:100 dilution); b) Lane 1, P protein against the sera of P protein immunized chicken (1:100 dilution); Lane 2: P protein against control negative sera (1:100 dilution); Lane 3: infected cells lysate against the sera of P protein immunized chicken (1:100 dilution). Lane 4: purified NDV virions against the sera of P protein immunized chicken (1:100 dilution)

antigen, only serum samples taken from P-immunized chickens reacted with the P protein in NDV-P ELISA. The immunized chicken serum titer was higher than the non-immunized serum titer. To confirm the specificities of the V- and P-protein-based ELISAs, the chicken sera were tested against the V protein by NDV-P ELISA and were tested against the P-protein by NDV-V ELISA. Neither assay showed any cross-reactions (Fig. 2), demonstrating the specificities of the two ELISAs. Additionally, live vaccine group showed a detectable antibody response to the P and V antigens (Fig. 3).

Evaluation of antibody responses in field chickens: Researchers examined the efficacy of the assay under field conditions. All the samples examined in this study showed seroconversion to the V and P antigens by both

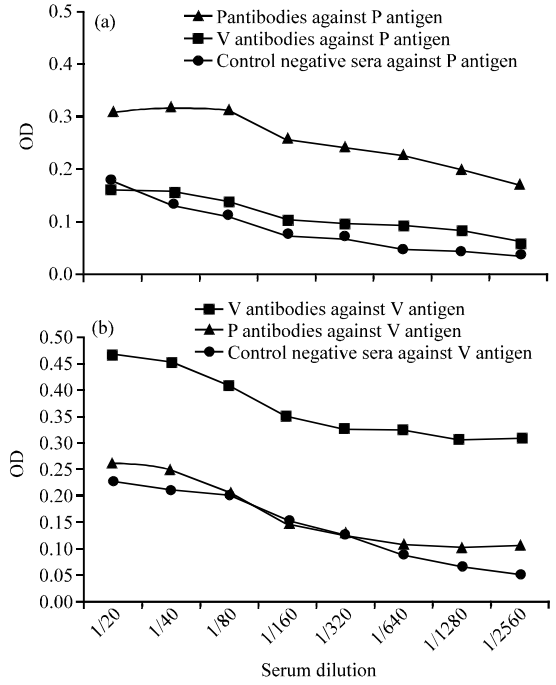

Fig. 2: a, b) Titration curves of antibodies against V and P proteins 2 weeks after immunizing chickens. Each sample was measured in triplicate. Data represent the mean OD value (n = 3 chickens)

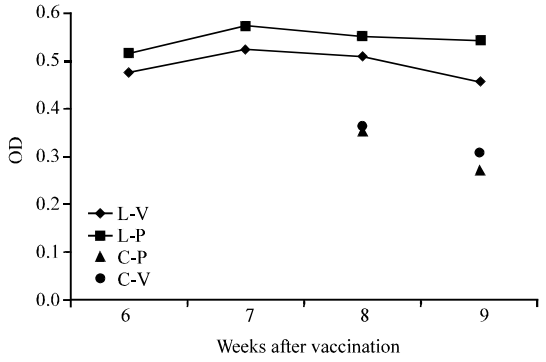

Fig. 3: IgY antibody titers in eggs from the live vaccine group (N = 18) and control group (N = 11). Each sample was diluted 1/100. The V and P antibodies titer of the live vaccine group (named L-V and L-P) were greater than those of the control non-vaccinated chicken (named C-V and C-P) by V and P- ELISAs, respectively. Data represent the mean OD value of total number of eggs examined in each group per week

V and P-ELISAs (Fig. 4). Titers were similar to those obtained when these samples were examined with whole NDV-based ELISAs (Fig. 4a, b).

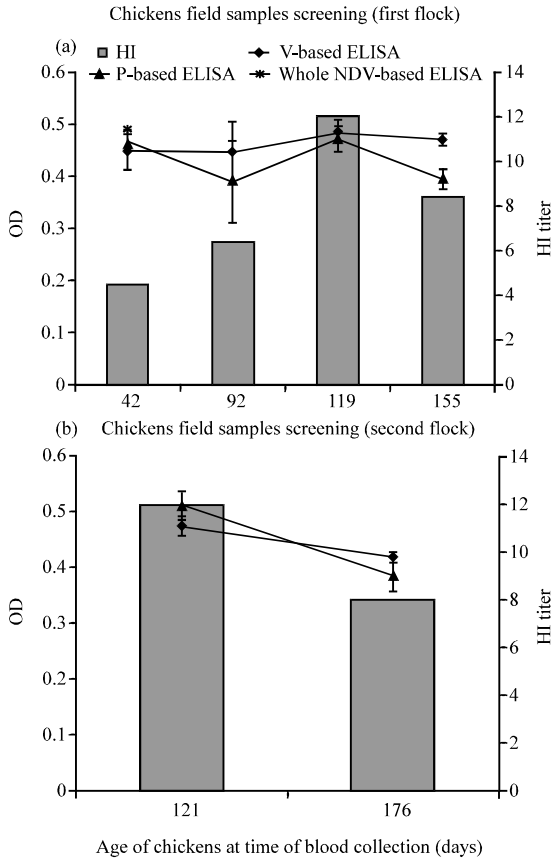

Fig. 4: a, b) Serum titers of HI, V protein and P protein in field chickens vaccinated with live and inactivated NDV vaccines (as shown in Table 1). Data represent the mean OD value of approximately 10 chicken sera±standard deviation

CONCLUSION

Researchers developed an Enzyme-Linked Immunosorbent Assay (V-ELISA) against the C-terminal region of the V protein of Newcastle Disease Virus (NDV), a non-structural protein. Another ELISA against the C-terminal polypeptide of structural protein P (P-ELISA) was also developed.

Safety issues are always a concern when developing a whole virus-based ELISA. NDV P and V-ELISAs represent an indirect method for diagnosis and detection of antibodies against NDV structural or non-structural proteins. V protein might also be useful as a diagnostic marker for paramyxovirus infections since the amino acid sequences of the C termini of V proteins of paramyxoviruses are highly conserved (Matsuoka *et al*., 1991).

Several researchers have reported that an antibody response to a non-structural protein could be used to

differentiate infected chickens from vaccinated chickens such as the non-structural protein of influenza A virus (Tumpey *et al*., 2005). V-ELISA should also be useful for detecting chickens infected by NDV if used as a coating antigen for an indirect ELISA.

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