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Production of recombinant nucleocapsid protein of Newcastle disease virus in *Escherichia coli* for a diagnostic ELISA

Hyun-il Kim[1,†], Kyoung-Phil Park[1,‡], Chan-Hee Park[1], Hyun-Ah Cho[1], Ho-Suk Yang[1], Tae-Wook Hahn[2,*]

[1]*Lab of Clinical Pathology, Institute of Cheilbio, Ansan 457-7, Korea*
[2]*School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea*
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Abstract : Transmission of avian viruses both bird-to-bird and from birds to non-avian species is a major health concern. Newcastle disease virus (NDV) is an economically important avian virus that poses substantial risks to the poultry industry. Rapid and sensitive diagnostic methods, such as the enzyme-linked immunosorbent assay (ELISA), are required to track such infections. To develop an ELISA for detecting anti-NDV antibody in avian sera, the nucleocapsid protein (NCP) gene of the NDV La Sota strain was cloned and expressed in *Escherichia coli* and the 513-amino acid recombinant NCP was purified by Ni-NTA affinity chromatography. To evaluate its ability to replace NDV whole virus antigen as a coating antigen, NCP-coated and whole NDV-coated ELISAs were tested and compared using a panel of NDV positive antisera from chickens. Results using purified NCP were highly correlated with those obtained using whole NDV (r = 0.927), demonstrating that recombinant NCP expressed in *Escherichia coli* is a suitable substitute antigen for whole NDV in a diagnostic ELISA.

Keywords : *E. coli* expression, ELISA, Newcastle disease virus, nucleocapsid protein

Introduction

Newcastle disease virus (NDV) is an economically important avian virus that poses substantial risks to the poultry industry [1]. The most widely used methods for detection of anti-NDV antibody in birds have been serum neutralization and hemagglutination inhibition (HI) tests, but the enzyme-linked immunosorbent assay (ELISA) has emerged as a convenient method for monitoring both immune status and viral infection [9, 14]. Several commercial ELISA kits are available for detection of NDV-specific antibodies. Most of these kits use whole, inactivated NDV as a coating antigen. However, propagation and purification of NDV is time-consuming and expensive. Unlike full virions, recombinant proteins obtained via gene engineering are safe, and both easy and inexpensive to produce.

NDV is an enveloped virus with a single-stranded, negative-sense RNA genome. The total genome is about 15 kb in length and encodes 6 structural proteins: hemagglutinin-neuraminidase (HN), fusion (F) protein, matrix protein, nucleocapsid protein (NCP), phosphoprotein, and large protein [1]. Of these, NCP has been the antigen of choice for anti-NDV titer diagnostic systems [3]. Nucleocapsid proteins in general are highly immunogenic in nature and have been used as antigens for detection of other viruses, including measles virus [13] and avian influenza virus [16]. The NCP subunit is a single polypeptide of 489 amino acids with a molecular weight of about 53 kDa. The viral RNA is located at the core of the virus particle surrounded by 2,200 to 2,600 NCP subunits [2].

HI test is still the most widely used serological method for measuring antibodies to NDV and is considered standard method for ND. However, ELISA seems to be more convenient than HI when very large number of sera is examined. It was also reported that sera from other species tend to show high incidence of false-positive results [14].

NDV ELISA using NCP antigen expressed in baculovirus systems were found to perform just as well as the HI test or a commercial ELISA [3, 6]. Unfor-

*Corresponding author: Tae-Wook Hahn
School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea
[Tel: +82-33-250-8671, Fax: +82-33-244-2367, E-mail: twhahn@kangwon.ac.kr]
Present address: †Optifarm solution center, Seoul 135-937, Korea. ‡Asan Pharmaceutics, Seoul 130-070, Korea

tunately, NCP production in baculovirus suffers from low yield, and is expensive and time-consuming. Recently, NCP antigen of NDV tagged with histidine residue was produced in *Escherichia* (*E.*) *coli* and purified by histidine residue affinity chromatography [10, 11]. However, ELISA using the purified NCP has not been compared with whole NDV coated ELISA.

The purpose of the present study was to express the NCP gene of the NDV La Sota strain in *E. coli* and to evaluate an ELISA based on the recombinant NCP and compare NCP based ELISA with whole NDV based ELISA.

Materials and Methods

Virus and RNA extraction

NDV strain La Sota was propagated in 9- to 10-day-old specific pathogen free chicken embryonated eggs. Total RNA was extracted from 100 μl of allantoic fluid using a MagExtractor nucleic acid purification kit (Toyobo, Japan) and was dissolved in 30 μl of diethyl pyrocarbonate (DEPC)-treated distilled water (DDW). For preparation of whole NDV antigens, the collected allantoic fluid was centrifuged at 38,000 × *g* for 2 h at 4°C. The precipitated virus was purified through a discontinuous sucrose gradient (50%, 30% and 20%) and the purified virus was inactivated with 0.3% formalin for 30 min. The protein concentration of the purified virus was determined by Lowry's method [5].

Reverse transcriptase - polymerase chain reaction (RT-PCR)

Complementary DNA was synthesized from genomic NDV RNA using random hexamer included in the cDNA synthesis kit (Promega, USA).

Total NDV RNA was incubated with 2 μg of random hexamer (Promega, USA) at 72°C for 10 min and the mixture was immediately chilled on the ice. The synthesis of the first strand cDNA was carried out in a 20 μl of reaction mixture containing 0.3 mM of each deoxynucleoside triphosphate (dNTP; Promega, USA), 10 U AMV reverse transcriptase (Promega, USA), 20 U of Rnasin ribonuclease inhibitor (Promega, USA) and 1X reaction buffer [50 mM Tris-HCl, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM Dithiothreitol (DTT)], The mixture was incubated at 42°C for 90 min and then heated at 94°C for 5 min. Two primers, NCP sense (5'-AAG CCT TCT GCC AAC ATG TC-3') and NCP antisense (5'-TTT GTC CA**T CA**A TAC CCC CA -3') were designed and used to amplify the NCP gene. The antisense primer contains a stop codon (TCA, in bold) that was incorporated into the PCR product. A PCR was carried out in a 50 μl mixture containing 3 U *Taq* DNA polymerase (Promega, USA), 3 mM $MgCl_2$ and 1X reaction buffer [5 mM Tris-HCl (pH 8.0), 10 mM NaCl, 0.01 mM EDTA, 0.1 mM DTT, 5% glycerol, 0.1% Triton X-100], 0.3 mM of each dNTP (Promega, USA) and 5 μl of cDNA template synthesized above. The PCR mixture was subjected to a 35-cycle PCR profile of 94°C/30 sec, 53°C/90 sec, 72°C/1 min, followed by a final extension step of 72°C/5 min.

Cloning and sequencing

The purified PCR product was ligated into a pGEM-T easy vector (Promega, USA). The ligation mixture was then transformed into *E. coli* JM109 (Promega, USA). A positive clone was isolated and plasmid extracted with a DNA purification kit (Bioneer, Korea). The 1.48 kb *Eco*RI fragment containing the NCP gene was ligated into the *Eco*RI site of a pET28b vector (Invitrogen, USA). The ligation mixture was transformed into *E. coli* BL21 (Promega, USA). Three positive clones were selected and the complete sequence of the NCP gene was obtained by reading both strands of the insert DNA.

Expression, purification and analysis of recombinant NCP

The verified clone was cultured in 1 l of LB medium containing 25 μg/ml kanamycin at 37°C with vigorous shaking. When the absorbance of the cultures at 600 nm reached 0.6, NCP was induced by adding 1 mM isopropylthio-β-galactoside (IPTG) and incubating for another 3 h. Cells were harvested and broken by sonication. The cell lysate was centrifuged at 10,000 × *g* at 4°C for 30 min and the supernatant was collected by filtering through a 0.45 μm filter. The filtered lysate from 1 l of culture was applied to a 5-ml Ni-NTA column at flow rate of 1 ml/min. To assess the optimal concentration of imidazole for elution, the column was loaded with elution buffer varying in the concentration of imidazole (0 to 250 mM). After optimization, the column was washed with lysis buffer and wash buffer [50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole (pH. 8.0)] until the baseline absorbance at 280 nm was stable. After that, the protein was eluted with five column volumes of elution buffer [50 mM NaH_2PO_4,

300 mM NaCl, 50 mM imidazole (pH. 8.0)]. The purified protein was then dialyzed in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4).

The expressed NCP was analyzed by SDS-PAGE (10% polyacrylamide) [4] and confirmed by Western immunoblot [12]. After electrophoresis, proteins were transferred onto nitrocellulose membrane (Sigma, USA) and blocked with 3% bovine serum albumin (BSA) in PBS. Proteins were detected using chicken anti-NDV La Sota antibody and horseradish peroxidase-labeled goat anti-chicken antibody (Sigma, USA).

ELISA

Total of 43 sera were used for validity of NCP based ELISA. All sera were tested by HI test and identified positive and negative. The 96-well microtiter plates were coated with purified NCP or whole inactivated NDV in carbonate coating buffer (0.5 M sodium carbonate, pH 9.6) and incubated overnight at 4°C. Following three washes with PBS-0.05% Tween 20, the plates were blocked with 1% BSA. Chicken sera against NDV were serially diluted in PBS and dispensed into each well and incubated for 30 min. Plates were washed as above and 100 μl of goat anti-chicken (1 : 3,000) antibodies conjugated with horseradish peroxidase were added to each well. Following incubation at 37°C for 30 min, the plates were washed before loading azinodiethylbenzthiazolin sulfonate (ABTS) solution [3.5 mM citric acid, 293 mM glycine, 0.5 mM 2,2'-ABTS, 0.015% H_2O_2, pH 4.0]. Bound antibody was quantitated by measuring absorbance at 410 nm with an automated plate reader (Biotek, USA).

Results

RT-PCR and sequence determination of the cloned NCP gene

Amplification of the NCP gene by RT-PCR produced DNA fragments of the expected size, 1,492 bp (Fig. 1). These fragments were cloned into a pET28a plasmid and completely sequenced. The cloned NCP gene showed 99% homology with the NDV La Sota sequence in the GenBank database (Accession No. AF077761). Conceptual translation of the open reading frame of the cloned NCP gene revealed a polypeptide of 513 residues, including vector-derived amino acids, with a calculated molecular weight (MW) of 55.7 kDa.

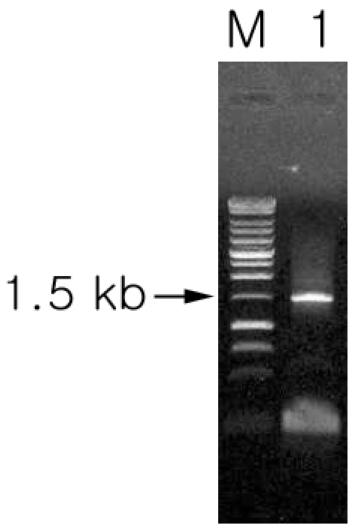

Fig. 1. Agarose gel (1.2%) electrophoresis of Reverse transcriptase-polymerase chain reaction (RT-PCR) products of the coding region of the nucleocapsid protein (NCP) gene. Lanes: M, DNA molecular weight marker of 1-kb DNA ladder; 1, RT-PCR product of the NCP coding region (1,492 bp). The arrow indicates the size marker.

Expression and purification of recombinant NCP in *E. coli*

Transformed *E. coli* cells were cultured in 1 mM IPTG to induce expression of recombinant NCP. After harvesting the cells, they were lysed and the recombinant protein was purified using a Ni-NTA column. The size of the expressed NCP, as determined by SDS-PAGE, was 55 kDa, consistent with the calculated MW of 55.7 kDa (Fig. 2A, lanes 5-8). The expressed protein was also detected on a Western blot probed with chicken antiserum against La Sota NDV, confirming its viral identity (Fig. 2B). No specific bands were observed in extracts of cells transformed with pET28b vector only (data not shown).

Twenty milliliters of sonicated lysate from a 1-l culture were loaded onto a 5 ml Ni-NTA column. The elution step was optimized by varying the concentration of imidazole from 0 to 250 mM in the elution buffer. On a gel stained with Coomassie blue, the expected band was observed when ≥ 50 mM imidazole was used (Fig. 2A, lanes 5-8). However, in a Western blot analysis, detection of the same molecular-weight protein was achieved at ≥ 25 mM imidazole. To minimize the loss of the expressed NCP during purification and to prevent its contamination by other proteins, 50 mM imidazole was used for further purification. A lower

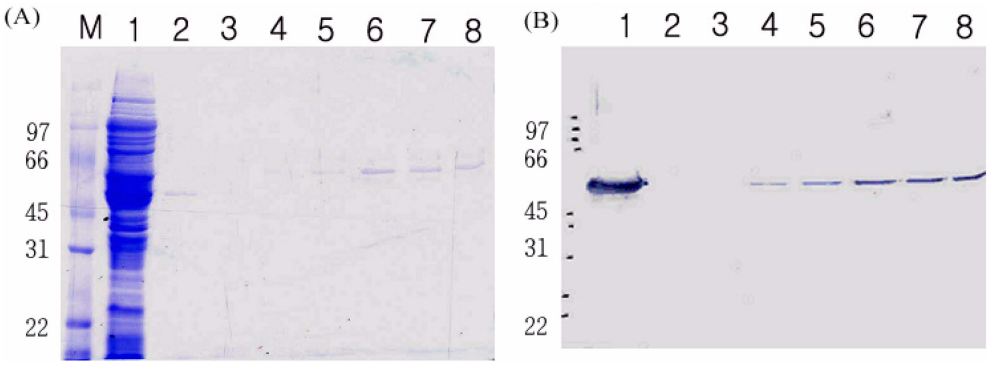

Fig. 2. Analysis of expressed NCP according to the imidazole concentration in elution buffer. (A) Coomassie blue stained gel after SDS-PAGE. (B) Western blot analysis with antibody to La Sota Newcastle disease virus. Lanes: M, molecular weight marker in kDa; 1-8, eluate by concentration of imidazole in elution buffer, 0, 5, 10, 25, 50, 100, 150, and 250 mM, respectively. The numbers at the left indicate molecular weights.

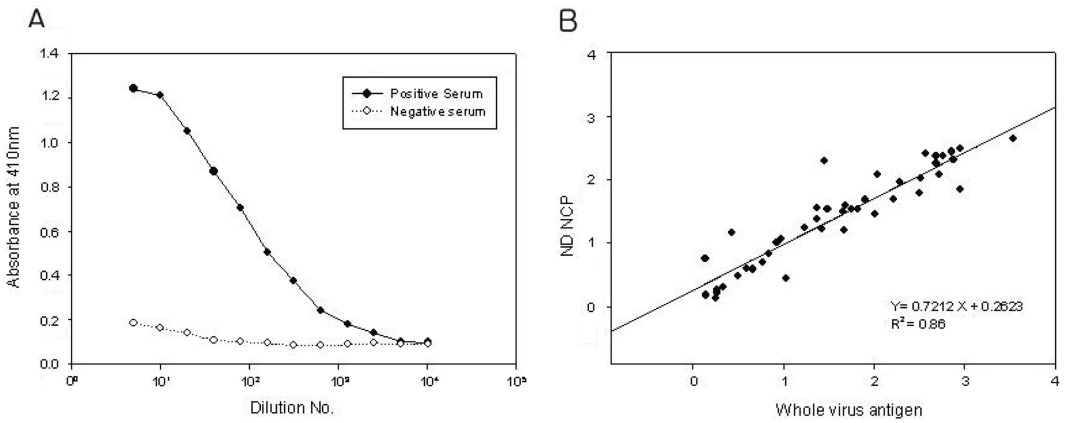

Fig. 3. Reactivity of the NCP-coated enzyme-linked immunosorbent assay (ELISA) plates versus inactivated whole virus antigen-based ELISA plates. (A) To determine the sensitivity and specificity of NCP-coated plates, a two-fold dilution series of positive and negative serum was tested. At the 80-fold dilution points, the P/N ratio for the NCP coated plates was 8.02. (B) Comparison of recombinant NCP- versus whole Newcastle disease virus- based ELISAs. Sera were examined in duplicate. The correlation coefficient is +0.972 for the two antigens.

molecular-weight band visible on the Coomassie blue stained gel (Fig. 2A, lane 2) did not appear on the Western blot (Fig. 2).

From a 1-l culture of transformed *E. coli* BL21, 1.7 mg of NDV recombinant NCP was purified, representing 1.5% of the total protein in the lysate (Table 1). The final concentration of purified NCP was 0.33 mg/ml, as assayed by the Lowry method [5].

Table 1. Purification efficiency from 1 l culture of transformed *E. coli*

	Volume (ml)	Protein conc. (mg/ml)	Protein (mg)	Percentage
Lysate	10	5.44	108.8	100.0
Elute	25	0.33	1.7	1.5

Comparison of recombinant NCP with inactivated whole NDV as ELISA antigens

NCP-coated ELISA plates showed positive reactions against ND positive chicken sera by HI test. For example, at the 80-fold dilution point, where the absorbance value for negative serum was below the negative threshold (average absorbance of negative serum ± 3 SD), the positive/negative ratio was 8.02 (Fig. 3A). This result demonstrated that NCP-coated ELISA plates are both sensitive and specific for detection of antibodies against NDV. To validate the method, a total of 43 sera were examined in duplicate

to compare inactivated whole NDV antigen- and NCP-based ELISAs (Fig. 3B). The correlation coefficient ($r = +0.972$) for these two methods was highly significant ($p < 0.0001$).

Discussion

The transmission of avian viruses both between bird populations and from birds to non-avian species, including humans, is a major health concern. Tracking such infections requires rapid and sensitive diagnostic methods, such as the ELISA. Here, recombinant NCP expressed in *E. coli* was evaluated as a potential antigen for use in an ELISA intended to identify birds exposed to NDV. Two previous studies used NCP in cell lysates from a baculovirus expression system as the coating antigen for such a diagnostic ELISA. Despite the use of non-purified NCP, those assays gave results comparable to a commercial ELISA kit and consistent with HI test data [3, 6]. However, recombinant protein production in baculovirus expression systems is generally limited by high cost. The present study found NCP expressed in *E. coli* to be an excellent antigen for use in a diagnostic ELISA, as shown here applied to chicken sera.

Among the structural proteins encoded by NDV, NCP has been the main focus of research because of its highly immunogenic properties. Moreover, the NCP gene of NDV is highly conserved between lentogenic (low-virulence), mesogenic (moderate-virulence), and velogenic (high-virulence) strains [7]. For example, Yusoff and Tan (2001) found 91 to 98% identity between the predicted amino acid sequences of NCP from five different strains of NDV [15]. Therefore, the NCP ELISA based on the La Sota strain should be effective for identifying all NDV strains.

In initial tests, bacterially expressed NCP showed a strong reaction against a known NDV positive serum. To further examine the ability of recombinant NCP from *E. coli* to substitute for whole inactivated virion as an ELISA coating antigen, ELISA plates were coated with purified NCP. All sera identified as being positive by whole virus coated ELISA also showed a strong reaction against purified NCP; the negative serum showed a very weak, background-level reaction. Thus, the present study demonstrated that an ELISA based on recombinant NCP has both the sensitivity and specificity required for detecting antibody against NDV. Finally, results from ELISAs using NCP-coated plates versus whole NDV-coated plates were compared and found to be highly correlated ($r = +0.927$, $p < 0.0001$). This suggests that NCP produced in *E. coli* is antigenically similar to NDV NCP, and thus should provide a reliable source of antigen for NDV ELISA. Moreover, the NCP-based ELISA described here should, in principle, be able to differentiate between birds exposed to natural NDV, whole NDV vaccine, or vaccine consisting only of HN or F protein, since anti-NCP antibody would be present only in birds exposed to whole NDV vaccine and natural NDV infection [6, 8].

The NCP ELISA has not yet been compared with a commercially available NDV ELISA kit (IDEXX ELISA). However, the NCP ELISA is expected to show similar performance characteristics because the IDEXX ELISA is very similar to the whole NDV ELISA used in the present study. Further studies comparing NCP-based and IDEXX ELISAs using a large panel of NDV positive and negative sera are planned.

In conclusion, expressing NCP in *E. coli* is a safe and reliable method of producing antigen suitable for use in a sensitive and specific ELISA for identifying birds exposed to NDV.

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