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IMMUNOGENIC PROPERTIES OF RECOMBINANT ECTODOMAIN OF NEWCASTLE DISEASE VIRUS HEMAGGLUTININ-NEURAMINIDASE PROTEIN EXPRESSED IN *ESCHERICHIA COLI*

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Summary. – Hemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) plays a vital role in the viral infectivity, host immunity, and disease diagnosis. A portion of the HN gene encoding the ectodomain (nt 142–1739) was cloned and expressed in *Escherichia coli* yielding an insoluble HN protein and a soluble NusA-HN protein containing N-utilization substance A (NusA) fusion component. Both recombinant proteins were purified and used for immunization of chickens. The recombinant HN protein induced higher antibody titers as compared to the recombinant NusA-HN protein. These antibodies were able to react in immunoblot analysis with the corresponding recombinant proteins as well as with the HN protein of NDV.

Keywords: Newcastle disease virus; hemagglutinin-neuraminidase; cloning; expression; immunogenicity

Introduction

The HN protein is one of the two surface glycoproteins of NDV (Yusoff and Tan, 2001). It is a multifunctional protein and plays a key role in the infection. HN protein recognizes sialic acid on the receptor of host cells, what leads to the virus attachment. Also, it contains neuraminidase activity that is involved in hydrolysis of progeny's sialic acid to prevent self-agglutination. Finally, the HN protein together with the fusion protein supports a viral penetration process and determines tropism and virulence of the virus (Huang *et al*., 2004). The HN is highly antigenic and together with the fusion protein stimulates the production of protective antibodies (Yusoff and Tan, 2001). Studies using monoclonal antibodies revealed a presence of various epitopes within the HN protein (Long *et al*., 1986). The hemagglutination-inhibition test based on the HN protein is widely employed in the diagnosis of NDV infection (Alexander, 1989).

The HN protein has become a dominant target in many immunological studies. Advances in recombinant DNA technology led to the intensive studies of HN protein using various expression systems. The HN gene was cloned into Vaccinia virus (Nishino *et al*., 1991), Fowlpox virus (Boursnell *et al*., 1990; Edbauer *et al*., 1990; Iritani *et al*., 1991), retrovirus (Morrison *et al*., 1990; Cosset *et al*., 1991), herpesvirus of turkey (Heckert *et al*., 1996), baculovirus (Nagy *et al*., 1991; Niikura *et al*., 1991; Ong *et al*., 1999), Cytomegalovirus (Loke *et al*., 2005), and plant (Berinstein *et al*., 2005) recombinant vectors.

However, there is a relatively limited knowledge about the immunological properties of the recombinant HN pro-

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Abbreviations: $(His)_6$ = hexa-histidine; HN = hemagglutinin-neuraminidase; IMAC = immobilized metal affinity chromatography; NDV = Newcastle disease virus; NusA = N-utilization substance A

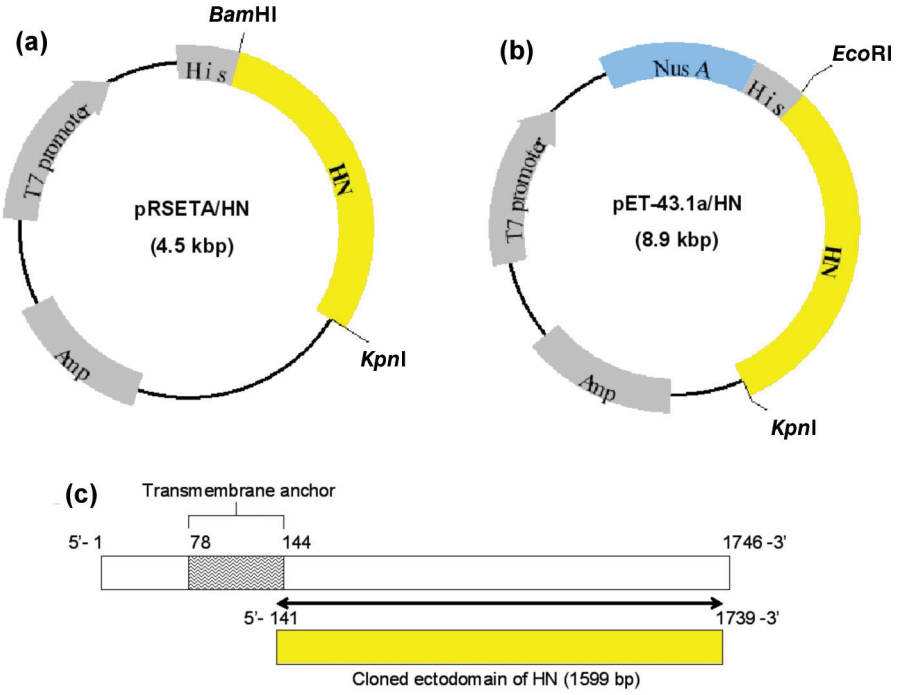

Fig. 1

Recombinant plasmid constructs

pRSET/HN (a), pET-43.1a/HN (b), and the HN gene (c).

tein produced in *Escherichia coli*. The rapid growth of this bacterium provided in simple and inexpensive culture media has made an advantage over eukaryotic systems. Numerous viral proteins used in immunological studies were produced in *E. coli* as hemagglutinin of influenza virus (Davis *et al*., 1981), glycoprotein of Rabies virus (Yelverton *et al*., 1983), gC glycoprotein of Varicella-zoster virus (Ellis *et al*., 1985), gC-II glycoprotein of Human cytomegalovirus (Kari *et al*., 1994), Pseudorabies virus glycoprotein gE (Ro *et al*., 1995), and glycoproteins (gC, gE, and gp60) of Infectious laryngotracheitis virus (Chang *et al*., 2002).

To investigate the immunogenicity of the recombinant HN protein in chickens, we cloned and expressed the HN gene using the *E. coli* expression system. We produced the insoluble and soluble forms of the recombinant HN proteins through the pRSET and pET-43.1a vectors and the purified recombinant proteins were used to immunize chickens. We examined the production of antibodies against the recombinant HN proteins using ELISA and immunoblot analysis. We found that the recombinant HN proteins triggered significant antibody titers with reactivity toward the authentic and recombinant HN proteins.

Materials and Methods

Bacterial strains and plasmids. E. coli TOP10 [F$^-$ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG; Invitrogen] was used as a general host for plasmid propagation. *E. coli* BL21-SI [F$^-$ *omp*T *Ion hsd*SB(rB$^-$mB$^-$) *gal dcm end*A1 *proUp*::T7 RNAP::*mal*Q-*lacZ* Tet^s; Gibco] is a salt-inducible host for expression of the pRSETA/HN' plasmid construct. *E. coli* Origami B(DE3) [F$^-$ ompT *hsd*SB(rB$^-$mB$^-$) gal dcm lacY1 ahpC gor522::Tn10 (TcR) trxB::kan (DE3); Novagen] was employed to harbor the pET-43.1a/HN' expression construct. The pPICZαA (Invitrogen) and the pCITE2a (Novagen) are plasmid yeast and *in vitro* expression vector, respectively. The pRSETA vector (Invitrogen) harbors the T7 promoter and an N-terminal hexa-histidine $(His)_6$ detection/affinity tag. The pET-43.1a vector (Novagen) carries the T7 promoter, N-terminal $(His)_6$ tag, and NusA solubility carrier. *E. coli* cells were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) with the exception that *E. coli* BL21-SI was cultured in NaCl-free LB medium.

Cloning of HN gene. Oligonucleotides 5'-GAA**GAATTC**AT GGGAGTTAGCACACCAA-3' (forward) and 5'-GATA**GGTAC** CTGACTCAACCGGCCAGATCT-3' (reverse) were designed based on the nucleotide sequence of NDV strain AF2240 (Tan *et al*., 1995). The underlined nucleotides represent the cutting sites of

*Eco*RI and *Kpn*I, respectively. The HN gene (nt 142–1739; Fig. 1) was amplified from the viral genome using RT-PCR. The reaction conditions were 48°C/45 mins, 94°C/2 mins, 30 cycles of 94°C/30 secs, 55°C/1 min, and 68°C/2 mins, and 68°C/7 mins. The HN gene was cloned into pPICZαA and pCITE2a plasmids and inserted between the *Bam*HI-*Kpn*I restriction sites of pRSETA vector to produce the pRSETA/HN' plasmid (Fig. 1). Furthermore, the HN gene was subcloned into the *Eco*RI-*Kpn*I restriction sites of pET-43.1a vector to generate the pET-43.1a/HN'plasmid (Fig. 1).

Expression of recombinant HN proteins. The pRSETA/HN' and pET43.1a/HN' plasmids were used to transform *E. coli* BL21-SI and Origami B(DE3), respectively, by the heat shock method (Sambrook *et al*., 1989). Expression of the recombinant HN proteins was induced when cell density measured by A600 reached 0.6–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mmol/l) and NaCl (0.3 mol/l) were added as inducers for *E. coli* Origami B(DE3) and BL21-SI, respectively. The expression cultures were incubated at 30°C for 3 hrs for BL21-SI, and at 37°C for 6 hrs for Origami B(DE3).

Purification of recombinant HN proteins. The expression cultures were harvested and resuspended in 1/25 volume of lysis buffer (20 mmol/l Na_3PO_4, 0.1 mol/l NaCl, pH 7.4). Cells were lysed by lysozyme (1 mg/g of cells) at 4°C for 1 hr followed by sonication at power output of 40 W (Braun) for 10 cycles of 30 secs. The lysate was spun at 2,500 x g for 5 mins to remove cell debris followed by centrifugation at 20,000 x g at 4°C for 20 mins. The supernatant contained soluble proteins, while the insoluble proteins remained in the pellet.

The soluble $(His)_6$-tagged NusA-HN protein was precipitated with ammonium sulfate at 30% saturation. The pellet was dialyzed twice against phosphate buffer (20 mmol/l NaH_2PO_4/Na_2HPO_4, 0.1 mol/l NaCl, pH 7.6) at 4°C for 36 hrs prior to purification with immobilized metal affinity chromatography (IMAC) using ProBond™ resin (Invitrogen). The bound recombinant protein was eluted from the column with PBS (4.3 mmol/l Na_2HPO_4, 1.4 mmol/l KH_2PO_4, 137 mmol/l NaCl, 2.7 mmol/l KCl, pH 7.4) containing imidazole (0.1 mol/l).

The inclusion bodies containing the recombinant HN protein were extracted with increasing concentrations 0.1, 0.2, 0.5, and 1% of Triton X-100 made in PBS. The inclusion bodies were washed twice in PBS (5 ml) to remove excess of the detergent and recovered by centrifugation at 20,000 x g at 4°C for 15 mins.

Total protein assay. The amount of NusA-HN protein was measured by the Bradford assay (Bradford, 1976). The insoluble recombinant HN protein was analyzed with SDS-PAGE. The protein bands were stained with Coomassie Brilliant Blue and analyzed in the BioRad imaging system using the Quantity One software (Version 4.2.2). Bovine serum albumin was used as a standard protein.

Immunization of chickens. Groups of five 2-week-old specific pathogen-free chickens were immunized with the purified recombinant HN and NusA-HN proteins. The chickens were inoculated subcutaneously with 50 μg of recombinant protein in 100 μl of PBS, emulsified with the same volume of Freund's complete adjuvant. Two weeks later, booster injections were given using the same amount of protein in Freund's incomplete adjuvant. The chickens were bled from the wing veins at day 0, 14, and 28 of the immunization procedure. Control groups of chicken were inoculated with PBS and NusA protein.

ELISA. Antibody titers in the serum samples were evaluated by the Newcastle disease antibody test kit (IDEXX). All serum samples were analyzed in duplicates. The positive and negative control sera were provided by the kit.

Immunoblot analysis. The recombinant HN, NusA-HN proteins and NDV (0.1–0.5 μg) were analyzed by SDS-PAGE and blotted onto nitrocellulose membranes. Skim milk (5% in PBS) was used as a blocking agent. The blot was probed with chicken serum samples (e.g. antibodies against NDV, recombinant HN and NusA-HN proteins) at 1:500–1:1,000 dilutions in PBS at room temperature for 1 hr. The blot was washed three times in PBS containing Tween 20 (0.01%) followed by incubation with goat anti-chicken IgG conjugated to alkaline phosphatase (1:5,000 dilution in PBS) for 1 hr at room temperature. Color development was achieved by adding nitroblue tetrazolium (66 μl) and bromochloroindolyl phosphate (33 μl) in the alkaline phosphatase buffer (100 mmol/l Tris, 100 mmol/l NaCl, 5 mmol/l $MgCl_2$, pH 9.5).

Results and Discussion

Cloning, expression, and purification of recombinant HN proteins

The gene portion (nt 141–1739) encoding the ectodomain (aa 47–580) of the HN protein was amplified and cloned (Fig. 1). The recombinant HN protein was expressed in *E. coli* BL21-SI harboring the pRSETA/HN. The HN protein was observed as a band with M_r of 65 K in SDS-PAGE (Fig. 2).

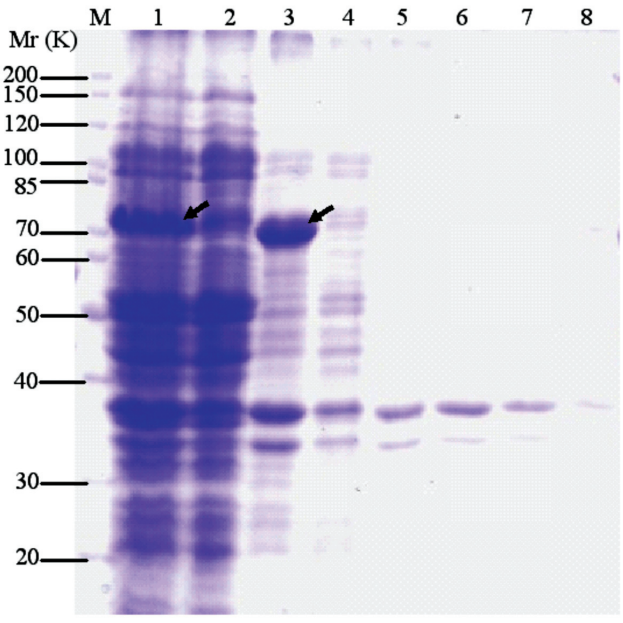

Fig. 2

SDS-PAGE of different fractions obtained during purification of recombinant HN protein

Protein size markers (M), total bacterial lysate (1), soluble protein fraction (2), insoluble protein fraction (3), proteins extracted with 0.1, 0.2, 0.5, and 1% Triton X-100 (4, 5, 6, 7, respectively). The arrows indicate the HN protein.

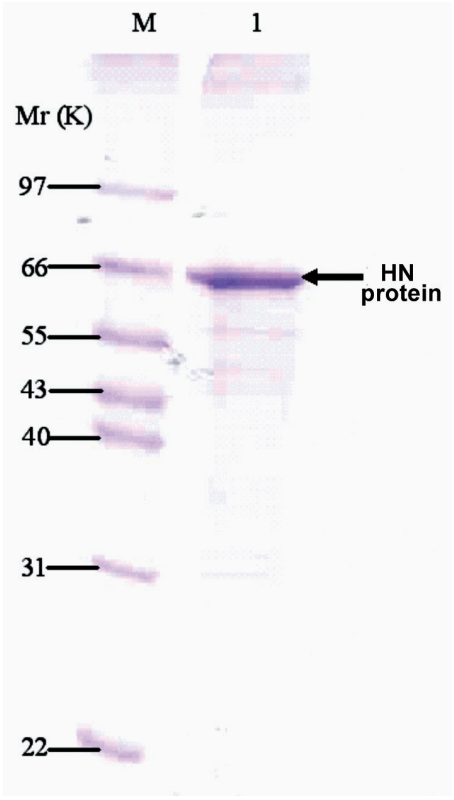

Fig. 3

SDS-PAGE of purified recombinant HN protein (1)

Protein size markers (M).

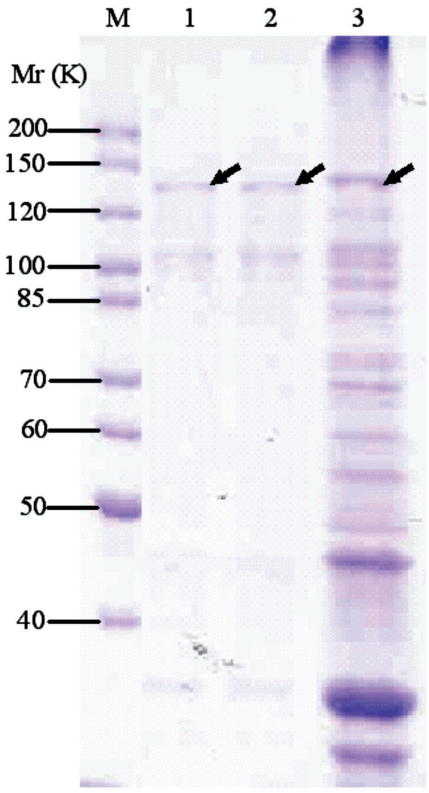

Fig. 4

SDS-PAGE of NusA-HN protein (1, 2) purified by affinity chromatography from bacterial lysate (3)

The arrows indicate the NusA-HN protein. Protein size markers (M).

It was extensively produced at the expression level of 6.7% of the total host cell protein. The recombinant HN protein was found in the pellet of the bacterial cell lysate (Fig. 2) indicating that HN protein was expressed in insoluble aggregates or deposits known as inclusion bodies (Marston, 1986). Formation of inclusion bodies is a common obstacle in preserving the native structure and biological function of the recombinant protein. However, these insoluble protein aggregates have an advantage in the purification process, since they can be easily isolated and enriched with centrifugation and in addition, they are protected from a proteolysis (Rudolph and Lilie, 1996; Jonasson *et al*., 2002). However, the inclusion bodies containing recombinant protein are often contaminated with other bacterial membrane proteins such as OmpF, OmpC, and OmpA (Rinas and Bailey, 1992). Therefore, we extracted the preparation of insoluble HN protein with solutions of Triton X-100 in different concentrations, what successfully removed most of the bacterial proteins (Fig. 3).

E. coli Origami B(DE3) cells transformed with the pET-43.1a/HN plasmids expressed the NusA-HN fusion protein. The NusA-HN fusion protein (M_r = 125 K) was produced at the level of 4.2% of the total cell protein (Fig. 4). Unlike the recombinant HN protein, the NusA-HN fusion protein was expressed in bacterial host as a soluble protein. The pET-43.1a vector contains an NusA gene at the 5'-end of its multiple cloning sites. The product of the gene, NusA protein has been identified as the highly soluble protein with a potential to improve solubility of a protein fused to its C-terminus (Davis *et al*., 1999). Fusion of the HN polypeptide to the C-terminus of NusA protein led to the expression of NusA-HN protein that was present in the soluble fraction of the bacterial cell lysate. The soluble NusA-HN fusion protein contained a $(His)_6$ tag that interacted with a bivalent ions such as Ni^{2+}. Unlike the insoluble recombinant HN protein, which was contaminated with relatively low number of the host proteins, the crude preparation of NusA-HN protein contained a quantity of bacterial cytoplasmic proteins. Hence, a differential precipitation with ammonium sulfate was carried out to fractionate this mixture. The procedure was useful for removal of a portion of the soluble protein contaminants that might bind non-specifically to the Ni^{2+}

resin and decrease the efficiency of IMAC purification process. The NusA-HN protein was efficiently eluted from the column by 0.1 mol/l of imidazole. However, multiple bands were detected below the band of full-length NusA-HN protein suggesting that the expressed protein was partially degraded (Fig. 4). A degradation of the expressed protein might be attributed to the absence of glycosylation due to the protein expression in a prokaryotic system. It is known that the oligosaccharide chains protect the native glycoproteins against proteolysis (Olden *et al*., 1982; Rudd *et al*., 1994).

Immunogenicity of recombinant HN proteins

The IDEXX Newcastle disease antibody test kit quoted that the antibody titers beyond 396 (or log >2.60) indicated an exposure to NDV or a vaccination of the tested birds. The ELISA results showed that all chickens immunized with the recombinant HN and NusA-HN proteins showed positive antibody titers (Fig. 5). The control birds injected with PBS or the NusA protein gave insignificant antibody titers (Fig. 5). None of the pre-immunization serum samples tested positive for the anti-NDV antibodies. It was observed that the insoluble recombinant HN protein elicited a more rapid and stronger immune response than the soluble NusA-HN protein. Chickens immunized with the recombinant HN protein showed significant antibody titers 2 weeks

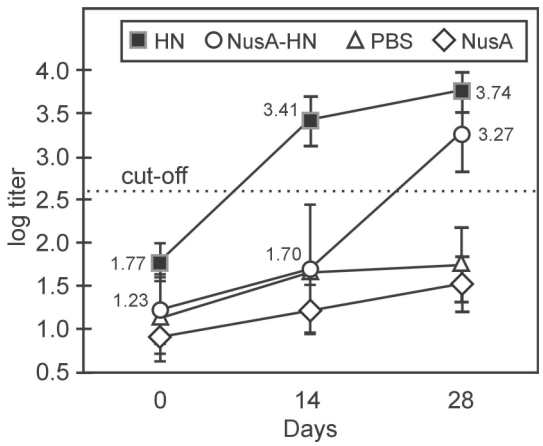

Fig. 5

Serum antibody titers in chickens immunized with recombinant HN and NusA-HN proteins

PBS and NusA were used for preparation of control antisera. x-axis: days of immunization; ---------- cut off line

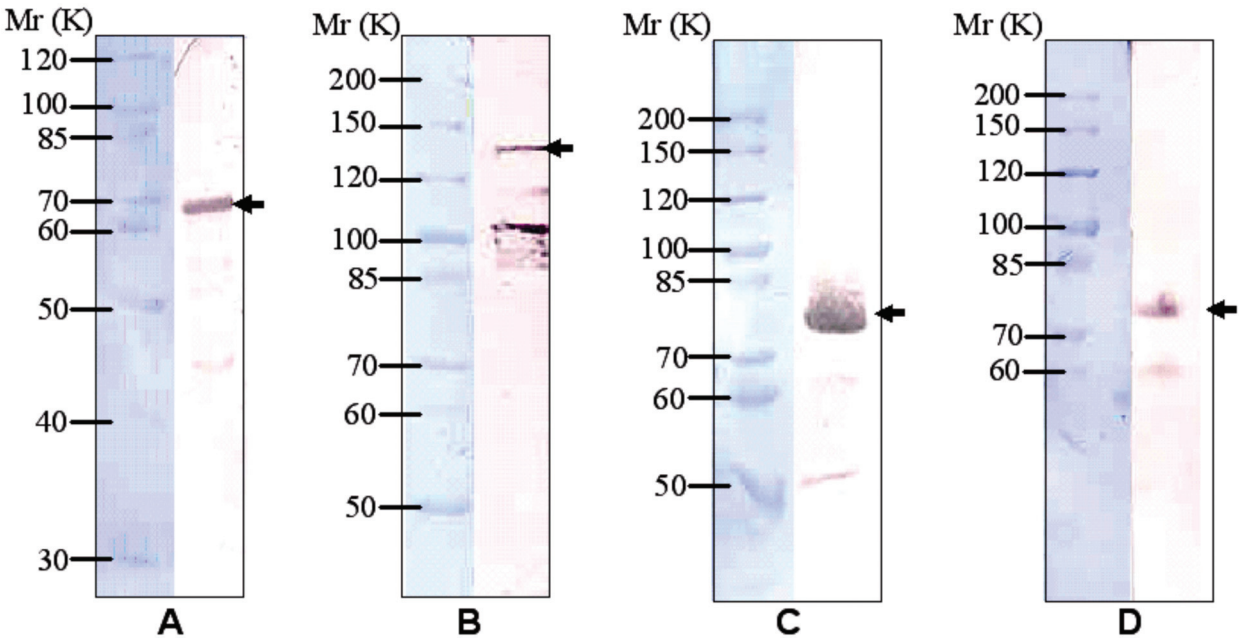

Fig. 6

Immunoblot analysis

The blotted antigens represent recombinant HN protein (A), recombinant NusA-HN protein (B), and purified NDV (C, D). Sera of chickens immunized with NDV (A, B), recombinant HN protein (C), and recombinant NusA-HN protein (D).

after the primary injection. The antibody titers had doubled even after the first injection of the recombinant HN protein (log = 1.77–3.41). After a booster injection the titers went up steadily to log = 3.74. In contrast, chickens injected with the soluble NusA-HN protein did not yield positive titers until the booster injection was given. The antibody titers increased slightly from log = 1.23–1.70 after the first injection of the NusA-HN protein. After the second injection, the titers increased nearly 2-fold to a significant level of log = 3.27. However, the soluble antigen could have influenced the immune response of the chickens. The insoluble recombinant HN protein in inclusion bodies was in the state of protein aggregates that could increase the likelihood of effective T-cell epitope response and engage the antigen-processing cells (Hanly *et al*., 1995).

Immunoblot analysis of recombinant HN proteins

ELISA represents a quantitative analysis of antibodies against both continuous and discontinuous epitopes on the antigen. On the other hand, immunoblot analysis involves mainly reaction of continuous epitopes and corresponding antibodies. The NDV, HN and NusA-HN proteins were denatured by SDS and 2-mercaptoethanol, separated in SDS-PAGE, and electroblotted. The results demonstrated positive reactions between all examined chicken sera raised against NDV, HN and NusA-HN proteins and the corresponding blotted proteins. The antisera against the recombinant HN and the NusA-HN proteins detected the authentic HN glycoprotein of the NDV (Fig. 6). Conversely, the anti-NDV serum reacted with both recombinant HN and the NusA-HN proteins (Fig. 6). Several studies defined the nature of the epitopes present on the blotted HN protein. Most of them were continuous epitopes or epitopes able to regenerate by refolding after denaturation in SDS-PAGE (Russell *et al*., 1983; Long *et al*., 1986; Chambers *et al*., 1988; Yusoff *et al*., 1988). Furthermore, *E. coli* cells are devoid of any glycosylation machinery and are unable to add oligosaccharide chain to the recombinant HN and NusA-HN proteins. Therefore, binding of the antibodies to the recombinant proteins involved epitopes devoid of carbohydrates.

The detection of the authentic SDS-denatured NDV HN glycoprotein with the antibodies against the recombinant HN and NusA-HN proteins revealed the potential application of these antibodies as probes for the viral glycoprotein in immunoblot analysis. In addition, reactivity of the anti-NDV serum toward the denatured recombinant HN and NusA-HN proteins in immunoblotting opens the possible use of these recombinant proteins as antigens for diagnosis of Newcastle disease in poultry. The recombinant HN and NusA-HN proteins produced in *E. coli* may be used to substitute the conventional use of whole NDV as an antigen with advantages in terms of safety, cost, and technical convenience of production.

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