

***E. coli* expression and immunological assessment of expressed recombinant Newcastle disease virus hemagglutinin-neuraminidase protein in chickens**

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Summary. – Every year, the poultry industry experiences significant economic losses due to epidemics of Newcastle disease virus (NDV). Developing new vaccines by identifying and using the immunogenic hemagglutinin-neuraminidase (HN) protein can protect the poultry industry. In the present study, the full-length HN protein was expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) cells, purified via affinity chromatography and detected via western blot analysis using His-specific antibodies. The purified HN protein was further evaluated in chickens to study the immune response against NDV. The successful production of HN-specific IgY proved the activity of the purified HN protein. IgY was present in the serum of immunized chickens. However, the immune response was higher in chickens immunized with purified HN protein along with complete and incomplete adjuvants than in chickens immunized with only the HN protein.

Keywords: protein; Newcastle disease virus; poultry; infectious diseases; vaccines

Introduction

The poultry industry is highly negatively affected by NDV, a member of the genus *Avulavirus* in the family *Paramyxoviridae*. NDV is an enveloped virus with a non-segmented, linear, negative-sense, single-stranded RNA genome (Shahid and Daniell, 2016; Sun *et al.*, 2017). The total NDV genome comprises nearly 15,186 nucleotides (Jin *et al.*, 2017) that encode 6 genes, including two transmembrane glycoproteins, a hemagglutinin-neuraminidase protein (HN) and a fusion protein (F). The NDV envelope includes two types of glycoproteins that are involved in viral infectivity and pathogenicity. Specifically, the F protein is associated with the pathogenicity of the virus.

The HN protein is a versatile molecule with distinctive actions i.e., fusion assistance, receptor binding and neuraminidase activity (NA). The two binding sites of the HN globular head (sites 1 and 2) are responsible for its neuraminidase and fusion activities, respectively. The stalk region promotes fusion by interacting with the F protein, which is responsible for virion penetration into the cell surface (Ewies *et al.*, 2017; Jin *et al.*, 2017; Dey *et al.*, 2019). The HN protein possesses neuraminidase activity; it binds sialic-acid-containing receptors (hemagglutination) on host cells. These receptors on the host cell cleave the sialic acid molecules and release the resultant viral particles. The HN protein also strongly promotes NDV virulence and tissue tropism. The high antigenic property and the ability to stimulate antibody production by inducing host's protective immune response make the HN protein a suitable target antigen for the production of recombinant vaccines against NDV (Habib *et al.*, 2015). Moreover, the length of the HN protein is very critical to its biological

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Abbreviations: ELISA(s) = enzyme-linked immunosorbent assay(s); HN = hemagglutinin-neuraminidase; NDV = Newcastle disease virus

activities, as a 571-aa HN protein has been reported only in highly virulent strains; a 577-aa HN protein exists both in avirulent and virulent strains, whereas a larger HN protein of 616 aa has been reported only in non-virulent strains (Römer-Oberdörfer *et al.*, 2003).

The poultry industry plays a vital role in strengthening the economy, especially in developing countries. In Pakistan, economic losses due to NDV have reached up to US \$106.5 million, or 10 billion Pakistan rupees (PKR), in the last decade (Grace, 2014). The morbidity and mortality rates are up to 100% for severe forms of the disease (Alexander *et al.*, 1997). Therefore, epidemiological studies have been conducted to control NDV outbreaks.

A number of studies have aimed to design NDV vaccines that are more efficient than conventional live attenuated and inactivated vaccines (Shahid and Daniell, 2016); these vaccines include DNA vaccines (Sawant *et al.*, 2011), reverse-genetics-generated recombinant vaccines (Zhao *et al.*, 2014; Wang *et al.*, 2015), subunit vaccines (Kumar *et al.*, 2011; Esaki *et al.*, 2013) and vector vaccines (Li *et al.*, 2014). Early diagnosis is critical to effectively control this epidemic disease. Hemagglutination inhibition (HI) tests and enzyme-linked immunosorbent assays (ELISAs) are effective for detecting anti-NDV antibodies. However, commercially available ELISA kits with pre-coated antigens are very expensive, particularly in developing countries. The use of purified recombinant NDV proteins, i.e., NP or HN, in ELISAs is considered an alternative solution to this problem. These proteins can be successfully expressed in *E. coli* and further used in indirect ELISAs for anti-NDV antibody quantification (Yusoff and Tan, 2001; Silva *et al.*, 2014).

In the present study, an attempt was made to characterize the HN protein under lab conditions and to further determine its efficacy in the production of antibodies against NDV in chickens.

Materials and Methods

Viral strain collection and propagation. The virulent NDV strain was obtained from the Veterinary Research Institute, Lahore, Pakistan (Acc. No. FJ430159.1). Viral propagation and dilution were conducted in a Biosafety Level III laboratory (CEMB, Lahore, Pakistan).

Amplification and sequence confirmation of the HN gene. RNA was isolated from the NDV strain using the TRIzol-based method. The RNA template, random hexamer primers and a Revert Aid first-strand cDNA synthesis kit (Thermo Scientific, K1622) were used for reverse transcription PCR (RT-PCR) according to the manufacturer's protocol. The NDV HN gene was amplified from cDNA with forward primer 5'-ATGGACAGCG CATGTAGCCAAGTT-3' and reverse primer 5'-TTAAACCCAC

CATCCTTGAG-3' under the following PCR conditions: initial denaturation at 95°C for 5 min; 35 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 15 min. The amplified fragment was visualized via 1% agarose gel electrophoresis. The amplified HN gene fragment was cloned into the pCR2.1 vector according to the manufacturer's protocol (Thermo Fisher TA cloning kit), and the sequence was confirmed by MacroGen Advanced Genomics, Korea.

Construction of *E. coli* expression vector and expression studies of recombinant HN. The pET30a expression vector, featuring a T7 promoter, N/C-terminal His-tag sequence, T7 terminator and Lac operator, was used, and the HN gene was ligated between the *EcoRI* and *HindIII* restriction sites. The HN-pET30a ligation product was transformed into TOP10 competent *E. coli* cells and spread on Luria Bertani plates containing kanamycin. Positive clones were screened via *EcoRI* and *HindIII* restriction digestion. BL21 (DE3) cells transformed with pET30a-HN were grown overnight at 37°C in LB media containing antibiotics. The primary culture was used to inoculate fresh media (1:10 v/v), which was then incubated for 3 h on a shaker. At an optical density of 0.6–0.8, expression of the recombinant protein was induced with 1 mM isopropyl B-D-1-thiogalactopyranoside (IPTG) and incubated at 30°C for 18 h. The cells were pelleted by centrifugation at 4,000 x g for 15 min at 4°C. Then, the cells were washed twice with 10 ml of 1x PBS and resuspended in lysis buffer (1x PBS, 500 mM NaCl, 1% Triton X-100, 6% glucose, 1 mM phenyl methane sulphonyl fluoride) for sonication. The sonication conditions were a continuous pulse at 20 kHz for 30 s on ice. To completely lyse the cells, the process was repeated 15–20 times for 60 s. The lysed cells were then centrifuged at 16,000 x g for 30 min, and the supernatant was stored at 4°C. The crude protein from *E. coli* extract was subjected to purification through affinity column chromatography in a Kelvinator chromatography refrigerator. Two column volumes of binding buffer containing 500 mM NaCl and 20 mM sodium phosphate buffer, pH 7.4, were used to equilibrate the Sepharose adsorbent. A linear flow rate of 150 cm/h was maintained to load the protein onto the column. The column was washed with 2 column volumes of wash buffer 1 (binding buffer and 20 mM imidazole, pH 7.4) and wash buffer 2 (binding buffer and 40 mM imidazole, pH 7.4) to successfully remove any unbound proteins and contaminating solutes. The recombinant HN protein was eluted from the column in a step-wise elution with five increasing concentrations of imidazole from 70 mM to 150 mM. Five 1-ml fractions from each elution concentration were collected, and the protein concentration was quantified using the Bradford assay. A total of 5 µg of protein was loaded into each well, and the protein purity was visualized via 12% dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) following the procedure of Laemmli (Laemmli, 1970). Coomassie brilliant blue (R 250; cat. No. 20278) staining was used to visualize the protein bands. For western blot, the protein was carefully transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Mil-

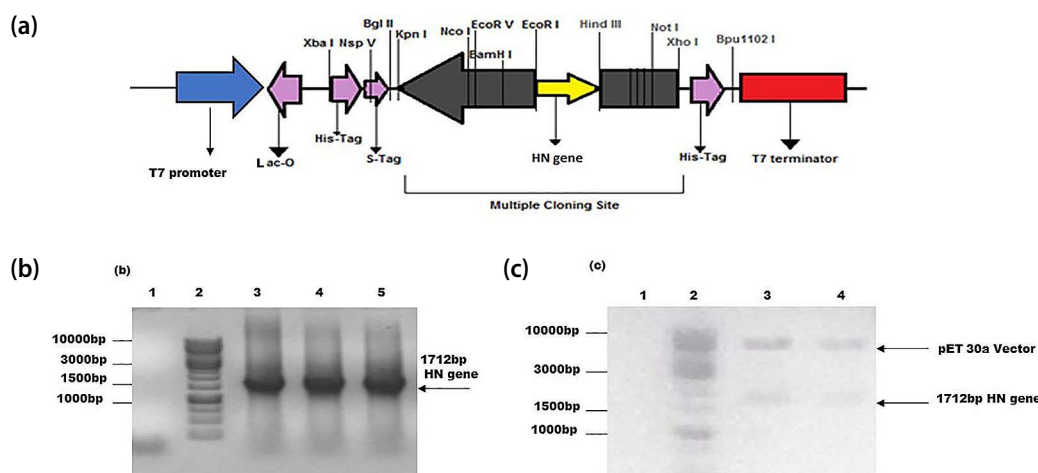


Fig. 1

Confirmation of the HN gene in the pET30a vector

(a) The presence of the HN gene in pET30a was confirmed via PCR. Lane 1 is a negative control (amplification without template); Lane 2 shows a 1-kb ladder, and lanes 3-5 show transformed clones. **(b)** The presence of the HN gene in the pET30a vector was confirmed via restriction digestion. Lane 2 shows a 1-kb ladder, and Lanes 3 and 4 show restriction-digested pET-HN clones.

lipore, USA) according to Chong *et al.* (2009). Recombinant HN protein was detected with rabbit polyclonal anti-His antibodies according to the manufacturer's guidelines (1:5,000 dilution; cat. No. sc-804; Santa Cruz). The 69-kDa recombinant HN protein was detected using alkaline phosphatase-conjugated 1-bromo-3-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate.

Immunogenicity assay. The levels of functional antibodies in the sera of immunized and control animals were assessed via ELISA as described previously (Berinstein *et al.*, 2005; Quan *et al.*, 2007; Silva *et al.*, 2014). Purified protein from BL21 (DE3) *E. coli* expressing the NDV HN protein was used as an antigen to promote the immune response. The immunogenicity assay was performed on ten-day-old specific pathogen-free (SPF) chickens purchased from hatcheries and reared in an animal facility. The chickens were separated into three groups, groups 1, 2 and 3, with 5 birds per group. The chickens in each group were allowed to feed ad libitum. Group 1 was used as a negative control. The chickens in group 2 were immunized with 20 µg of purified HN protein via subcutaneous injection at intervals of 0, 10, 20, 30, 40, 50 and 60 days. Group 3 was immunized with 20 µg of purified HN protein along with 0.2 mg/ml of complete Freund's adjuvant (CFA) and booster dose of incomplete Freund's adjuvant after 15 days interval by a subcutaneous injection. Blood was collected every ten days, and serum was isolated by incubating the samples at 25°C for 45 min, followed by centrifuging at 4°C (4,000 × g) for 15 min. The collected serum from each sample was evaluated for the presence of HN-specific IgY for up to 60 days using AP-conjugated goat anti-chicken IgY (ab 6877). To determine the IgY level in each serum sample, the absorbance values were recorded at 450 nm.

Statistical analysis. All data were analyzed using analysis of variance in a statistical analysis program (GraphPad Prism version 7) for Windows. To determine significant differences, the means of all treatments were compared using Tukey's multiple comparison test. Differences were considered significant when $P < 0.05$.

Results

Construction of an expression vector harboring the HN gene

The full-length 1712-bp HN gene was amplified from cDNA and confirmed through PCR (Fig. 1a). The specific 1712-bp HN fragment was similar to that of type III Mukteswar, a mesogenic NDV strain with accession number FJ430159.1. The resultant full-length HN gene was cloned into the pET30a protein expression vector. The full-length HN gene was confirmed to be in the pET30a protein expression vector through digestion, which generated a 1712-bp fragment (Fig. 1b).

Expression and purification of recombinant HN protein in *E. coli*

BL21 (DE3) *E. coli* cells were transformed with the recombinant pET30a vector containing the cloned HN gene. Upon induction with 1 mM IPTG, high protein expression

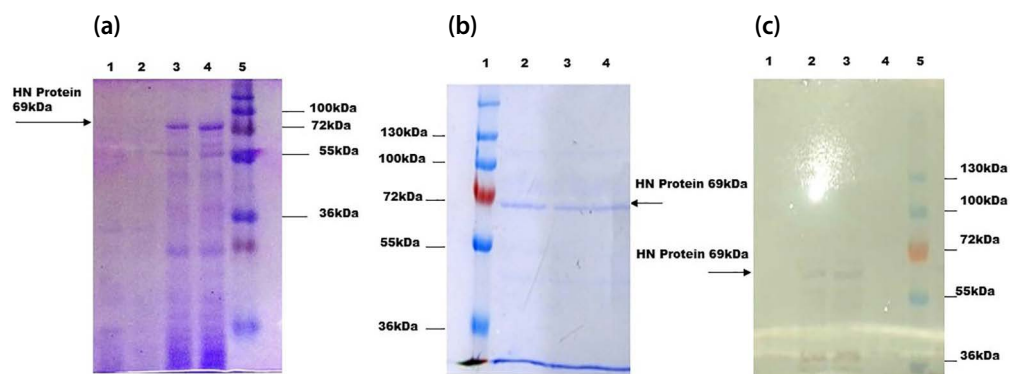


Fig. 2

Expression studies of the HN protein

(a) The expression of the HN protein was confirmed via SDS-PAGE. Lane 1 and 2 show a negative control (untransformed Rosetta cells), whereas Lanes 3 and 4 show Rosetta cells transformed with the HN gene, and Lane 5 contains a pre-stained protein marker (Fermentas)
 (b) The HN protein was purified via IMAC. Lane 1 is a pre-stained protein marker (Fermentas), and Lanes 2-4 show purified HN protein
 (c) The expression of the HN protein was confirmed via western blot analysis. Lane 1 shows a negative control (untransformed Rosetta cells); whereas Lanes 2-4 show Rosetta cells transformed with the HN gene, and Lane 5 contains a pre-stained protein marker (Fermentas).

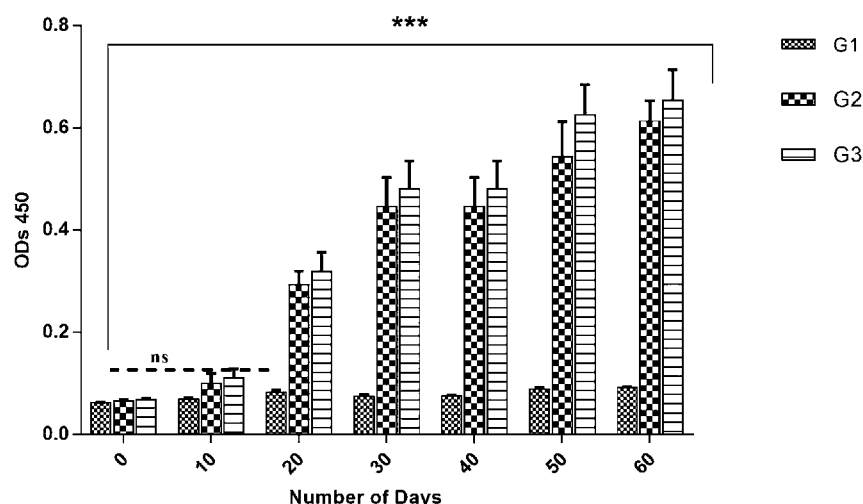


Fig. 3

Induction of the immune response against the HN protein

HN-specific serum IgY immune responses in chickens immunized with the HN protein (group 2) and chickens immunized with HN protein and boosted with complete and incomplete adjuvant (group 3) The chicken in group 3 showed a slightly greater humoral response with each booster than did chickens in group 2. No immunogenic response was observed in control group. Data shown are average \pm SD ($n = 5/\text{group}$). The significance of the data is determined by one-way ANOVA and $p < 0.001$ is indicated by "****" above bars, whereas "ns" represents non-significant.

was observed after 5-7 h at 25°C. SDS-PAGE and Coomassie blue staining of the obtained soluble fractions revealed a sharp band of 69 kDa (HN protein) (Fig. 2a). The expressed His-fused protein was purified via immobilized metal affinity chromatography (IMAC) with 100 mM imidazole, which resulted into a single 69-kDa band corresponding to the HN protein (Fig. 2b). The average yield of purified HN protein was quantified by referencing a known con-

centration of BSA (standard) (Bradford, 1976). The average HN protein concentration was 34.1 ng/ μl . The purified protein was electrophoretically transferred onto a PVDF membrane (Millipore, USA), and the 69-kDa HN protein was detected using polyclonal anti-His AP-conjugated antibodies (Santa Cruz, UK). Color development with BCIP/NBT substrate confirmed the presence of the 69-kDa HN protein (Fig. 2c).

Immunogenicity assay

The serum IgY level in immunized chickens was examined to determine the immune response after induction with purified HN protein. The antibody levels peaked 50–60 days post-vaccination. Chickens immunized with HN protein produced significantly higher antibody titers than did those in the negative control group. Chickens immunized with HN protein and boosted with complete and incomplete adjuvants showed slightly higher antibody titers against the HN protein than did those in group 2, which received only purified HN protein (Fig. 3).

Discussion

The control of NDV through vaccines is challenging due to various factors, such as genetic variation among endemic viral strains and mismatching of the NDV vaccine used in the field, causing incomplete protection against the disease and a failure of the vaccine to induce immunity against particular viral strains (Iram *et al.*, 2014; Shabbir *et al.*, 2016; Shahid *et al.*, 2017).

In this study, we described the immunogenic properties of recombinant NDV HN protein. The HN protein is the most important antigen that can induce a strong immune response. The HN protein is a capsid protein that attaches itself firmly to the viral membrane, thus making it very difficult to isolate and purify from infected cells (Shahid *et al.*, 2015). Immunization with recombinant HN protein has been attempted many times to study protective immune responses in chickens (Boursnell *et al.*, 1990; Edbauer *et al.*, 1990; Sun *et al.*, 2008; Su *et al.*, 2011). In the current study, an attempt was made to evaluate the immune response against recombinant HN in chickens at different time intervals; the stimulation of the immune response indicated that the recombinant HN protein had properly folded. The effectiveness of the recombinant HN protein obtained here still needs to be compared to the ND vaccines used in the field, which are produced in embryonated chicken eggs. We used only purified HN antigen at a concentration of 20 µg/dose in chickens. Our results indicated that 20 µg of HN protein was safe for the chickens and elicited a significant immune response that was stable for up to 50 days, but an immune response did not occur in the negative control group. The slightly enhanced immune responses in group 3 (chickens immunized with HN protein and boosted with complete and incomplete adjuvant) might be due to boosting with adjuvant (Shen *et al.*, 2013). The comparable immune responses between the two treatments [groups 2 (chickens immunized with HN protein) and group 3 (chickens immunized with HN protein and

boosted with complete and incomplete adjuvant] have practical significance because adjuvant use has not been endorsed for human vaccines (Aguilar-Yáñez *et al.*, 2010; Alving *et al.*, 2012).

Even if the recombinant HN obtained in this study produces immunity comparable to that of ND vaccines used in the field, it is more appealing, as the recombinant technology described here can produce at least 1000 more doses than can egg-based vaccines in a similar time interval. This technology can produce 2.2 million doses per day in a conventional 1,000 L bioreactor, which makes it better than conventional egg-based vaccines. Moreover, a bacterial-expression-based vaccine production system is a cost-effective solution for most endemic and pandemic veterinary diseases (Song *et al.*, 2008; Aguilar-Yáñez *et al.*, 2010).

Conclusion

We conclude that the recombinant HN protein produced in *E. coli* could potentially replace commercial egg-based vaccines. A number of potentially active recombinant vaccines against veterinary diseases have been produced in *E. coli* (Shen *et al.*, 2008; Chiu *et al.*, 2009). As an example, recombinant HA63–286 against avian influenza produced in *E. coli* was immunogenic, produced neutralizing antibodies and showed protection in a ferret model (Aguilar-Yáñez *et al.*, 2010). Similarly, a recombinant 60-kDa HA protein from H5N1 against avian influenza produced in *E. coli* yielded a protective immune response in mice (Shen *et al.*, 2008).

The recombinant HN protein obtained here has a potential for use as a diagnostic tool in laboratories to determine the efficiency of routinely used vaccines against NDV (Nallaiyan *et al.*, 2010). The current study shows that the NDV HN protein expressed in *E. coli* can be used as part of a generally low-cost and high-capacity platform for the poultry industry, which has low profit margins, during disease outbreaks. These attributes will also prove valuable in the development of more immunogenic vaccines for NDV by targeting more-immunogenic epitopes. Unfortunately, the laboratory facilities required for neutralization assays and for testing the ability of the vaccine to protect chickens from NDV infection are not available at our institution. Therefore, we were unable to perform these experiments. Collaborative work is in progress to evaluate the efficacy of the recombinant HN protein in protecting chickens against NDV infection.

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