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ORIGINAL RESEARCH ARTICLE

Designing a Novel Recombinant HN Protein with Multi Neutralizing Antigenic Sites and Auto Tag Removal Ability Based on NDV-VIIj for Diagnosis and Vaccination Application

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Abstract Hemagglutinin–neuraminidase (HN) protein besides its mediation in viral pathogenesis, is composed of various antigenic sites which stimulate production of host's antibodies. Thus, application of this protein in serological tests and vaccination plays a major role in biosecurity and control programs. In the present study, we designed a recombinant HN protein containing different neutralizing antigenic sites with velogenic patterns, and sub-cloned it into pET-43.1a+ expression vector. The expression of NusA-HN recombinant protein was induced. Affinity chromatography protein purification using HisPur^{TM} Ni–NTA was then conducted. Moreover, we performed western-blot technique using HRP-conjugated Anti His-Tag. Results revealed that following induction of recombinant protein, two distinct bands of HN-61 kDa and NusA-63 kDa were purified and identified by western-blotting. We recommend further analysis should be carried out to determine the functional role of this recombinant protein in enzyme-linked immunosorbent assays for Newcastle disease diagnosis. This HN protein containing multi neutralizing antigenic sites might also be applicable in vaccination programs to increase vaccines potency.

Keywords Newcastle disease virus · Hemagglutinin–neuraminidase protein · Recombinant protein

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Introduction

The devastating infection leading to vast economic losses in avian industry is derived from a virus as a member of *Avulavirus* genus in *paramyxoviridae* family known as Newcastle disease virus (NDV) [1–3]. Clinical signs of the disease vary from mild symptoms to severe and fatal forms involving enteric, respiratory and nerve tracts in most species of wild and domestic birds [4]. The NDV virulence rate can be evaluated by intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI), and mean death time (MDT) in vivo tests, as well as molecular approaches focusing on Fusion protein cleavage site [2, 5–9].

The ND virus is comprising six proteins of Nucleoprotein (NP), Phosphoprotein (P), Matrix (M), Fusion (F), Hemagglutinin–Neuraminidase (HN), and RNA-dependent RNA-polymerase (L) encoded by the single stranded negative-sense RNA genome with 15 kb length [10, 11]. The HN protein as a surface glycoprotein on NDV envelope mediates viral pathogenicity and tropism by its attachment to sialic acid receptors following sialidase activity and releasing of progeny virions, besides virus-cell fusion facilitation [8, 12, 13]. The hemagglutinin–neuraminidase protein structure consists of a cytoplasmic domain, a transmembrane region, a stalk region and a globular head, of which the latter domain has identified to contain several distinct epitopes and a major linear antigenic site (345–353) based on monoclonal antibodies (MAbs) [14–17]. According to high antigenic properties of HN protein, it is characterized as the main factor for induction of host's protective immune response and stimulation of antibody production [14, 16, 17].

Diagnosis of ND viruses at early stages is critical to control programs. The haemagglutination inhibition (HI)

and enzyme-linked immunosorbent assays (ELISAs) with higher sensitivity than HI tests are broadly used to detect antibodies produced against viral infection for further strategies of efficient biosecurity programs and also evaluating the efficiency of applied vaccines and the antigenic response rates [17–21]. Furthermore, DNA vaccination of poultry against NDV infection which leads to induction of host's humoral and cell-mediated immune responses is also crucial for biosafety issues in avian industry [22, 23].

Overall, the hemagglutinin–neuraminidase protein is believed to play a significant role as the major target in immunological investigations [17]. In the present study, we designed a recombinant multi neutralizing antigenic sites HN protein with auto tag removal ability, while its cloning into pET43.1a+ expression vector, expression and affinity purification was conducted and analyzed.

Materials and Methods

Virus, Bacterial Strain and Plasmid

Behshahr isolate which we previously isolated from chicken in Behshahr city of Iran (2015) [24] was cultivated in the allantoic cavity of 9-day old SPF embryonated eggs following incubation at 37 °C.

The pET-43.1a+ expression vector bearing the T7 promoter, NusA sequence (for enhancement of protein solubility) and a N-terminal $(His)_6$ tag was selected for recombinant HN gene cloning. In addition, the competent *E. coli* BL21 (DE3) strain (*fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS, λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5)* was used as the host for expression. Bacterial cells were grown in Luria–Bertani (LB) medium containing ampicillin.

Cloning of Recombinant HN Gene

The extraction of total viral RNA of Behshahr isolate [using High Pure Viral RNA kit (Roche Diagnostic, Germany)], and amplification of HN gene fragment using specific forward and reverse primers were conducted in our previous work [25]. According to the sequenced (Macrogen Inc., South Korea) and characterized unique epitope pattern of HN gene related to the Behshahr isolate (HN coding sequence of Behshahr virus could be found under accession number of KU938925), we designed a recombinant construct with the backbone of Behshahr HN sequence containing a linear epitope pattern of HN related to majority of velogenic isolates in residues 343–355 (TCPDKQDYQIRMA) at the 5′-end of the construct instead of first 13 amino acids of HN (stalk region) related to the isolate under study. In addition, the *Eco*RI and *Xho*I recognition sites were added in 5′ and 3′ ends of the fragment respectively. Codons were then optimized using online codon optimization tool (Integrated DNA Technologies, Inc., USA) and recombinant HN fragment was sub-cloned into pET-43.1a+ expression vector.

Expression of Recombinant HN Protein

The transformation of pET-43.1a+/HN plasmids into *E. coli* BL21 (DE3) cells were performed using heatshock method [26] and grown in LB medium supplemented with ampicillin (50 µg/ml). Induction of recombinant HN protein expression inoculated into 2YT medium containing ampicillin (50 µg/ml) was then conducted (with OD 600 reached 0.8) using Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mmol/l). The expression cultures were incubated at 30 °C for 16 h. Verification of expressed recombinant protein was performed by SDS-PAGE using unstained protein molecular weight ladder (Thermo Fisher Scientific Inc., USA).

Purification of NusA-HN Protein and Western-Blot

The expression cell culture was centrifuged at $2700\times g$ at 4 °C for 10 min. Cells pellet was then resuspended in phosphate-buffered saline (PBS; 1X). Sonication of cells was performed for 5 cycles of 1 min bursts and 1 min intervals on ice. Afterwards, phenylmethylsulfonyl fluoride (PMSF; 0.1 mmol/l) was added to the lysate followed by centrifugation at $10{,}600\times g$ for 10 min. Here the supernatant contained the soluble recombinant protein.

The soluble His-tagged NusA-HN protein was purified with affinity chromatography method using HisPur™ Ni–NTA Resin following manufacturer's instructions (Thermo Fisher Scientific Inc., USA). And SDS-PAGE was conducted for analysis of the purified recombinant HN protein.

In order to confirm the presence of recombinant multi neutralizing epitopes HN protein, western-blot was carried out. The NusA-HN protein was blotted onto nitrocellulose membrane. Membrane was then incubated with HRP-conjugated Anti His-Tag, and the recombinant protein was detected by 3,3′-diaminobenzidine (DAB). To identify the recombinant protein size, a prestained protein molecular weight ladder (Vivantis Inc., Malaysia) was used.

Results and Discussion

Based on our recent findings, velogenic Behshahr isolate of NDV was clustered into novel sub-genotype VIIj in class II category [24], and analysis of hemagglutinin–neuraminidase coding sequence related to VIIj isolates revealed the unique epitope pattern in residues E347Q and

G362K located on its major linear antigenic site [25]. Thus recombinant HN gene containing VIIj backbone plus ^{343}TCPDKQDYQIRMA^{355} sequence instead of first 13 N-terminal amino acids (stalk region) with *Eco*RI and *Xho*I recognition sites was designed and successfully sub-cloned into pET-43.1a+ expression vector. The schematic figure of pET-43.1a+/HN was depicted in Fig. 1. Expression of recombinant HN protein was efficiently induced in *E. coli* BL21 (DE3) using IPTG (1 mmol/l) at 30 °C. Surprisingly, the NusA-HN protein expressed by pET-43.1a+/HN plasmid was observed as two distinct bands with approximate molecular weight of 61 and 63 kDa in SDS-PAGE instead of a 125 kDa of NusA-HN fusion protein. Figure 2 demonstrated the SDS-PAGE results of induced NusA-HN protein at different time courses. Furthermore, the recombinant NusA-HN protein expression in the supernatant of bacterial cell lysate showed its soluble nature due to binding of NusA sequence [17]. Based on presence of $(His)_6$ tag sequence on pET-43.1a+, the soluble NusA-HN protein was purified using HisPur^{TM} Ni–NTA Resin and fully eluted from affinity column by 250 mmol/l of imidazole. And recombinant protein concentration at A280 nm was measured, which showed the amount of 1.42 µg/µl. In addition, SDS-PAGE results confirmed the existence of two distinct bands for recombinant NusA-HN protein which was represented in Fig. 3. Results also revealed that the blotted proteins (using western-blot technique) were efficiently detected on nitrocellulose membrane using HRP-conjugated Anti His-Tag. Figure 4 indicated the western-blot results of NusA-HN protein.

The need to diagnose Newcastle disease (ND) at early stages is of great importance to ND biosecurity and control programs [20]. Among NDV proteins, hemagglutinin–neuraminidase protein plays a crucial role in stimulation of

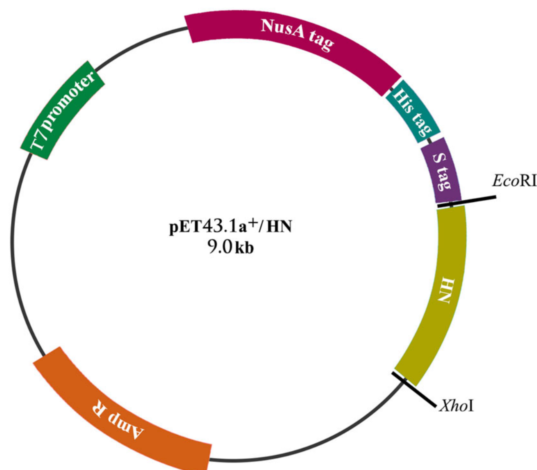

Fig. 1 The schematic figure illustrates pET-43.1a+/HN recombinant protein

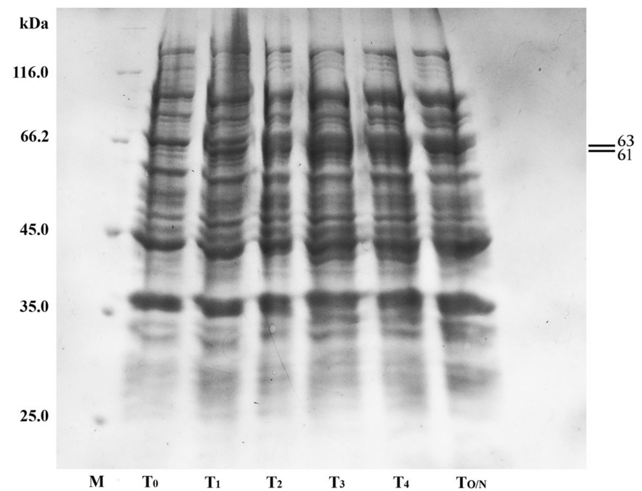

Fig. 2 Induction of expressed NusA-HN recombinant protein by IPTG (1 mmol/l) during 0, 1, 2, 3, 4 and O/N time courses. M is representative of unstained protein molecular weight marker. The induced recombinant proteins show two distict bands of 61 and 63 kDa

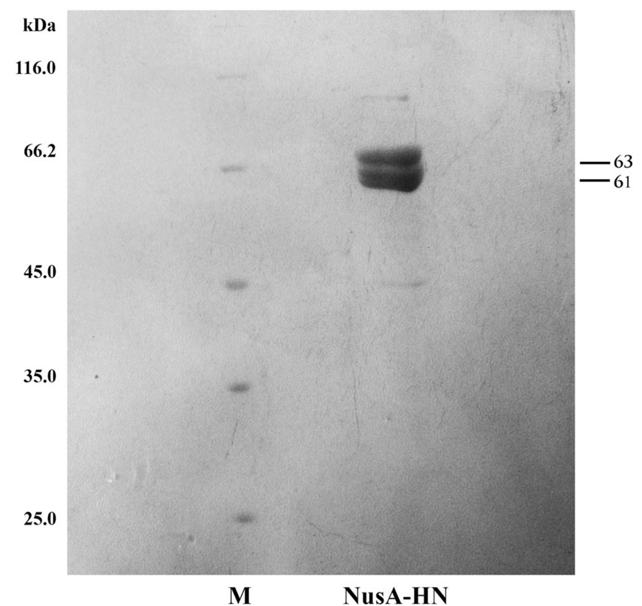

Fig. 3 Recombinant NusA-HN protein purification by affinity chromatography using HisPur^{TM} Ni–NTA Resin. Purified proteins demonstrate two bands (M_r of 61 and 63 kDa). M stands for marker of protein molecular weight

host's immunity and more specifically production of antibodies based on its various epitopes located on the globular head domain giving rise to its antigenic features [5, 16, 17]. Thus, HN protein acts as a significant target to serological tests for virus diagnosis [17]. Furthermore, according to our previous study, there exists major genetic variations in NDV genotype VII isolates and vaccine strains based on sialidase fragment of HN protein using restriction enzymes [27]. We also have characterized effective mutations in HN

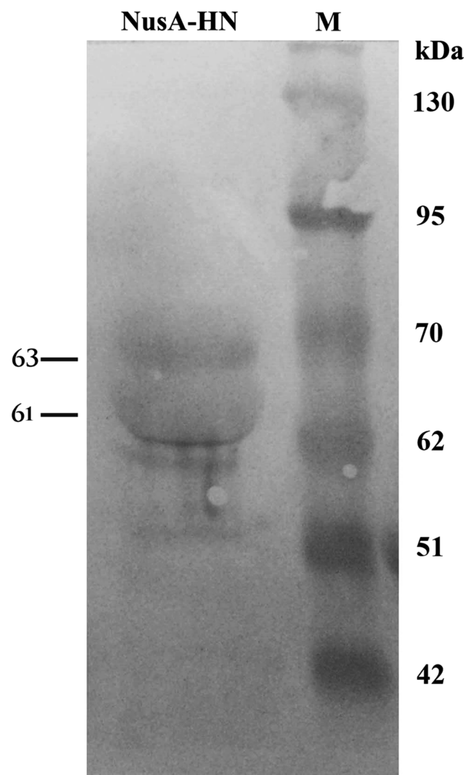

Fig. 4 Western-blot results of recombinant NusA-HN protein. The 61 and 63 kDa bands confirm the presence of purified recombinant protein using HRP-conjugated anti His-tag. Prestained protein molecular weight marker (M) is used in this technique

epitope pattern of Iranian VIIj isolates (velogenic) compared to vaccine strains recently, which may affect vaccination programs [25]. Hence, the goal of present study is expression of a novel recombinant HN protein containing genotype VII epitope patterns, in order to be used as an additive in poultry vaccination against ND to induce host's antibody response more effectively.

Here we have designed a specific HN construct which is comprised of overlapping conformational antigenic sites and the major neutralizing linear epitope (343–355) with its unique pattern related to velogenic VIIj sub-genotype ND viruses, in addition to the neutralizing antigenic site (343–355) sequence pattern of majority of velogenic NDV isolates. Another aim of this study is to apply the designed construct in immunodiagnostic approaches including HI, ELISA and virus-neutralization (VN) techniques which are based on detecting host cell antibodies and more importantly neutralizing antibodies stimulated by NDV antigenic sites [19–21]. Thus, we have sub-cloned the recombinant HN construct into pET-43.1a+ expression vector due to its NusA tag sequence which solubilize the fused protein.

The significant role of using serological assays (HI, ELISA, VN) is to track down produced antibodies against NDV which supports evaluation of antigenic responses. Moreover, these techniques help measuring vaccines efficiency and applicability for poultry industry [18]. Overall, HI test is thought not to have high sensitivity to neutralizing antibodies besides its false positive results. In addition, VN test using native NDV is a time consuming process [21]. Most ELISA assays have been reported to be based on applying purified virions, which is also a time consuming and expensive method. But recently recombinant proteins have been recommended for application in ELISA and also other serological assays [20]. Here, we propose that designing a virulent HN construct containing different virulent patterns of neutralizing epitopes (residues 343–355) besides its purity, help differentiating velogenic isolates of NDV from lentogenic isolates. Furthermore, another goal of this study is to increase the rate of concise and accurate diagnosis.

Following expression of recombinant NusA-HN protein, surprisingly two distinct bands of approximately 61 and 63 kDa have been detected instead of one with M_r of 125 kDa. Using autolytic site predictions, we have identified two potential residues, one just before (SAGKE$^{\downarrow}$-TAAA) and the other in the start (^{2}CPDKQ$^{\downarrow}$DYQI^{10}) of HN protein, either of which could be cleaved and resulted in production of two fragments. The purification of recombinant NusA-HN protein followed by western-blot technique have been also conducted for further serological assay applications. Affinity chromatography using His-Pur™ Ni–NTA Resin and HRP-conjugated Anti His-Tag treated western-blot have verified presence of the recombinant HN protein. We recommend further analysis should be conducted in order to define the functional role of the HN recombinant protein in ELISA assay.

According to the role of HN protein in production of neutralizing antibodies, this protein is regarded valuable in designing DNA vaccines [21]. In fact, purification of recombinant HN protein could be used for further application in vaccination. Here we suggest the current designed HN construct could be used as an additive in vaccination according to its different pattern neutralizing epitopes stimulating host's immune response. However, further investigations should be carried out to confirm this hypothesis.

To conclude, we have designed a HN recombinant protein containing a backbone of velogenic Behshahr isolate (VIIj sub-genotype) and an additional neutralizing epitope (343–355) of majority of velogenic NDV isolates. The construct has been sub-cloned into pET-43.1a+ expression vector following induction of NusA-HN protein expression. HisPur™ Ni–NTA Resin purification and western-blot techniques have been then performed. Application of this recombinant protein is suggested in ELISA assay for virus diagnosis. In addition, this novel recombinant HN protein might be useful in order to

improve vaccine potency. Further investigations should be performed to define the efficiency of the recombinant protein under study in control programs.

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Compliance with Ethical Standards

Conflict of interest The authors declare no competing interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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