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EXPRESSION OF HAEMAGGLUTININ-NEURAMINIDASE OF NEWCASTLE VIRUS GENOTYPE VIII IN INSECT CELL SF9

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ABSTRACT

The hemagglutinin-neuraminidase (HN) protein is the surface glycoprotein of Newcastle disease virus (NDV) which is involved in the process of virus entry into host cells. HN exhibits the capability to induce agglutination of chicken red blood cells, thereby contributing to the viral infection of the host. As a consequence, HN is a potential antigen for the production of subunit vaccines for protecting poultry from NDV infections. In this study, the *hn* gene coding for the HN antigen of NDV genotype VIII was artificially synthesized, ligated into donor vector pFastBacHT A and integrated into the baculovirus genome for the purpose of HN expression in *Spodoptera frugiperda* 9 (Sf9) cells. The recombinant HN protein was successfully expressed in Sf9 cells in the SF900 III medium. The expressed recombinant HN protein, with a size of approximately 63 kDa, exhibited hemagglutinin activity (HA) of 4log2 when interacting with chicken erythrocyte fluid. The findings of this study lay the foundation for subsequent research endeavors aimed at the development of Newcastle disease virus (NDV) vaccines tailored for poultry in Vietnam.

Keywords: Hemagglutinin-neuraminidase, HN, Newcastle disease virus, NDV, genotype VIII.

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INTRODUCTION

Newcastle disease virus (NDV) is an avian *paramyxovirus* in the genus *Avulavirus* of the Paramyxoviridae family (Amarasinghe et al., 2019). This viral pathogen is known to cause Newcastle disease in poultry in Vietnam annually. The hemagglutinin-neuraminidase (HN) protein is a surface glycoprotein, which involved in the process of entering host cells. HN mediates the binding of the virus to sialic acid-containing receptor molecules on the host cell surface, which promotes fusion activity by activating the F protein, and it functions as a neuraminidase in the process of virus budding through its receptor-destroying activity (Bose et al., 2011; Yuan et al., 2011).

Hemagglutinin-neuraminidase plays an important role in the host's immune response, therefore a lot of efforts have been made to produce recombinant HN for vaccine production purpose. Niikura (1991) has created a recombinant baculovirus containing the cDNA encoding HN of the Newcastle disease virus. The *Spodoptera frugiperda* cells infected with this recombinant virus produced large amounts of recombinant HN (rHN) and the recombinant HN protein has been used as a subunit vaccine against Newcastle disease (Nagy et al., 1991). Ong and colleagues have successfully expressed recombinant HN in *S. frugiperda* cells. Their study showed that the recombinant HN exhibits haemagglutination and neuraminidase activities (Ong et al., 2000). In another study, Choi and colleagues expressed recombinant HN in Sf9 cells, the rHN protein expressed from infected cells was used as an antigen in a hemagglutination inhibition (HI) test for the detection and titration of NDV-specific antibodies present in chicken sera. The rHN antigen produced a high HA titer (2^{13} per 25 µL), similar to the NDV antigen produced using chicken eggs, and it remained stable without significant loss of HA activity for at least 12 weeks at 4 ^{o}C (Choi et al., 2013).

Newcastle disease virus genotype VIII was isolated in South Africa and South Asia in the 1960s, 1980s, and 1990s. The genotype also was first isolated in Western China in the 1980s (Liang et al., 2002), in Iran in 2013 (Sabouri et al., 2018). Recently, several highly virulent NDV strains were detected from vaccinated fighting chickens in Guangxi, China that caused 100% morbidity and mortality of the challenged birds. Analysis of their complete genomes based on the sequences of NDV showed that these strains belonged to NDV genotype VIII (Wei et al., 2019). The above studies show that the re-emergence and the risk of highly virulent NDV strains of genotype VIII can cause a pandemic in poultry. In this study, we present the results of the expression of the *hn* gene from NDV genotype VIII in Sf9 cells with the ultimate aim for vaccine development protecting poultry from NDV infections.

MATERIALS AND METHODS

Materials

Gene, cell line, vector: The haemagglutinin-neuraminidase (*hn*) gene was artificially synthesized based on the gene sequence encoding for HN from strain Q4 (gene accession: FJ751919.1) (Cao et al., 2013). *S. frugiperda* 9 (Sf9) cells and baculovirus donor vector pFastBacHT-A were obtained from Invitrogen (USA).

Bacteria: Escherichia coli DH10B for cloning, DH10Bac for *hn* insertion in baculovirus were obtained from Invitrogen (USA).

Medium: Medium for *E. coli:* LB (1% tryptone, 0.5% yeast extract, 1% NaCl), kanamycin (50 µg/mL), gentamicin (7 µg/mL), tetracycline (10 µg/mL), Bluo-gal (100 µg/mL) and IPTG (40 µg/mL) were added to the medium as needed. Medium for Sf9 cell: SF900 III, supplemented Grace's Insect Medium (SGIM), Unsupplemented Grace's Insect Medium (UGIM), FBS were obtained from Invitrogen (USA).

Methods

Artificial synthesis of hn gene: The sequence of *hn* gene (1,716 bp) from NDV genotype VIII strain Q4 (Accession No: FJ751919.1) was analyzed using Graphical Codon Usage Analyser and was optimized by

replacing the codons predicted to be less frequently used in the Sf9 cell expression system. The *hn* gene was artificially synthesized by GeneScript (USA), then inserted into the pFastBacHT-A vector through the BamHI and NotI enzyme recognition sites.

Manipulating DNA methods: Plasmids were transformed into *E. coli* by electroporation. The plasmid carrying the gene of interest was then extracted from recombinant *E. coli* and analyzed as described by Sambrook (1989).

Creating Bacmid: The bacmid was prepared following the instructions of the Bac-to-Bac Baculovirus Expression System (Invitrogen). In brief, 1 µL of pFastBac-HN (100 ng/µL) was transformed into DH10Bac competent cells (Invitrogen) by heat shock. The transformed cells were spread on LB medium supplemented with kanamycin (50 µg/mL), gentamicin (7 µg/mL), tetracycline (10 µg/mL), Bluo-gal (100 µg/mL) and IPTG (40 µg/mL) then incubated at 37 ^{o}C for 2–3 days. The white colonies were selected for isolation of Bacmid DNA by Bacmid HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific), analyzed for the presence of Bacmid-HN by PCR using specific primers of *hn* (forward primer: 5'-atgggtgcctcaaccccatcc-3', reverse primer: 5'-ttacacacggtcgtccttcag-3').

Transfection Bacmid into Sf9 cells: The transfection was carried out according to instructions in the manual of Bac-to-Bac Baculovirus Expression System: Sf9 cells were cultured in Supplemented Grace's Insect Medium (SGIM) with 10% of FBS at log phase 1.5 × 10^6 cells/mL. The cells were collected then transferred to a 6-well cell culture plate containing 2 mL of UGIM, seeding concentration of 8 × 10^5 cells/mL, the mixture were left for 15 minutes in the incubator before adding mixture 1 (8 µL of Cellfectin® II with 100 µL of UGIM) and mixture 2 (100 µL of UGIM supplemented with 1 µL of Bacmid-HN DNA). The mixture was incubated for 15–30 minutes before adding to each well of the cell culture plates. The cell culture plates were incubated at 27 ^{o}C for 3–5 hours then removed the trasfection solution and replaced with 2 ml of SGIM medium. The plates were cultured for 72 hours at 27 ^{o}C until the baculovirus infected cell signalling were observed.

Expression of the HN protein: Sf9 cells were grown in 5 ml of Sf900 III medium until cell density reached 2 × 10^6 cells/mL. The cells were harvested then seeded at 6 × 10^5 cells/mL into 5 mL of Sf900 III medium in a 25 cm^2 tissue culture flask, incubated for 30 minutes at room temperature then added 100 µL of the recombinant-virus infected cell lysates from the second generation (P2). The cells were cultured at 27 ^{o}C for 5 days to allow recombinant baculovirus growth and expression of rHN.

Collection of recombinant HN: After expression, the cells were collected into a fancol tube and centrifuged at 3,000 rpm for 5 minutes to collect cells. The cells were resuspended in 5 mL 0.01M PBS buffer, lysed by gentle sonication and centrifuged at 8,000 rpm for 20 minutes to remove cell debris. The clear fluid containing recombinant protein was collected and stored at -20 ^{o}C for the next experiment.

Haemagglutination (HA) assay: HA assay was performed on 1% chicken erythrocytes in V-bottomed microwell plates according to OIE standard diagnostic testing guidelines (Afonso, 2012).

Western Blotting: The cell lysate containing rHN was incubated at 95 ^{o}C for 5 minutes in a sample buffer before electrophoresis in 12% (w/v) SDS-PAGE according to the method of Laemmli (1970) & Wisdom (1994). The gel was blotted onto the PVDF membrane, blocked with skim milk solution then incubated with NDV chicken polyclonal antibody (1:200), alkaline phosphatase-conjugated goat anti-chicken antibody. The rHN band was detected using BCIP/NBT reagent (Roche) according to the manufacturer's instructions.

RESULTS

Construction of donor vector pFastBac-HN

For the purpose of expressing the HN protein of NDV genotype VIII in SF9 insect cells, the gene sequence encoding HN of strain Q4 (gene accession: FJ751919.1) was used (Cao, 2013). The *hn* gene (1,716 bp) was designed from ATG encoding the starting methionine to the stop codon of the gene. The codon-optimized gene sequence for protein expression in insect cells was artificially synthesized (Fig. 1) by GeneScript company (USA).

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atggatagagccgtcaacagagtggtgctggagaacgaagagagggaggctaagaacaca
 M  D  R  A  V  N  R  V  V  L  E  N  E  E  R  E  A  K  N  T
tggagattggtttccgtattgcggtgctgttcctgatcgtgaccactctggccatctca
 W  R  L  V  F  R  I  A  V  L  F  L  I  V  T  T  L  A  I  S
gctgccgctctgatctacagcatgggtgcctcaaccccatccaacctggctggaatccct
 A  A  A  L  I  Y  S  M  G  A  S  T  P  S  N  L  A  G  I  P
actgccatcagcaaggctgaggaccgcatcacctctctgctgtccagcaaccaggacgtg
 T  A  I  S  K  A  E  D  R  I  T  S  L  L  S  S  N  Q  D  V
gtggaccgtatctacaagcaggtggccctggaatcacctctggctctgctgaacaccgag
 V  D  R  I  Y  K  Q  V  A  L  E  S  P  L  A  L  L  N  T  E
tccatcatcatgaacgccatcacttctctgtcataccagatcaacggcgccgctaacaac
 S  I  I  M  N  A  I  T  S  L  S  Y  Q  I  N  G  A  A  N  N
tcaggttgcggcgctccagtccacgaccctgactacatcggtggcatcggaaaggaactg
 S  G  C  G  A  P  V  H  D  P  D  Y  I  G  G  I  G  K  E  L
atcgtggacgacgtcagcgacgtgacctctttctacccctcagcctaccaggagcacctg
 I  V  D  D  V  S  D  V  T  S  F  Y  P  S  A  Y  Q  E  H  L
aacttcatcccagctcctaccactggttccggttgcactcgcatcccttccttcgacatg
 N  F  I  P  A  P  T  T  G  S  G  C  T  R  I  P  S  F  D  M
agcgccacccactactgctacactcacaacgtgatcctgtcaggatgccgcgaccactcc
 S  A  T  H  Y  C  Y  T  H  N  V  I  L  S  G  C  R  D  H  S
cacagctaccagtacctcgccctgggagtgctgaggacctccgctactggtagagtgttc
 H  S  Y  Q  Y  L  A  L  G  V  L  R  T  S  A  T  G  R  V  F
ttctccaccctgaggtcaatcaacctggacgacactcagaacagaaagtcttgctcagtg
 F  S  T  L  R  S  I  N  L  D  D  T  Q  N  R  K  S  C  S  V
tccgctacccctctgggctgcgacatgctgtgcttcaaggtcaccgagactgaggaagag
 S  A  T  P  L  G  C  D  M  L  C  F  K  V  T  E  T  E  E  E
gactacaagtccgtcactcctactagcatggtgcacggccgcctgggattcgacggacag
 D  Y  K  S  V  T  P  T  S  M  V  H  G  R  L  G  F  D  G  Q
taccacgaagaggacctggacatcactgtcctgttcaaggactgggtggccaactacccc
 Y  H  E  E  D  L  D  I  T  V  L  F  K  D  W  V  A  N  Y  P
ggtgtcggaggtggctccttcatcgacgaccgtgtctggttcccagtgtacggaggtctg
 G  V  G  G  G  S  F  I  D  D  R  V  W  F  P  V  Y  G  G  L
gacccctaacagcccctctgacaccgctcaggaaggccgctacgtcatctacaagcgttac
 D  P  N  S  P  S  D  T  A  Q  E  G  R  Y  V  I  Y  K  R  Y
aacaacacttgccctgacgagcaggactaccagatcaggatggccaagtcctcctacaag
 N  N  T  C  P  D  E  Q  D  Y  Q  I  R  M  A  K  S  S  Y  K
cctcgccgtttcggcggaaagagagtgcagcaggctatcctgtccatcaaggtgtccacc
 P  R  R  F  G  G  K  R  V  Q  Q  A  I  L  S  I  K  V  S  T
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 S  L  G  E  D  P  V  L  T  V  P  P  N  T  V  T  L  M  G  A
gagggaagggtcctgactgtgggtactagccacttcctgtaccagagaggctccagctac
 E  G  R  V  L  T  V  G  T  S  H  F  L  Y  Q  R  G  S  S  Y
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ccatacaccttcaacgctttcactcgccccggttccgtgccttgccacgccagcgctagg
 P  Y  T  F  N  A  F  T  R  P  G  S  V  P  C  H  A  S  A  R
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 C  P  N  S  C  I  T  G  V  Y  T  D  P  Y  P  L  I  F  H  R
aaccacaccctgcgtggtgtgttcggcactatgctggacgacgaacaggcccgcctgaac
 N  H  T  L  R  G  V  F  G  T  M  L  D  D  E  Q  A  R  L  N
cctgtcagcgctgtgttcgacaacatctcccgctcccgcatgacccgcgtgtcctcctcc
 P  V  S  A  V  F  D  N  I  S  R  S  R  M  T  R  V  S  S  S
tccaccaaggctgcttacaccacttctacctgcttcaaggtggtcaagactaacaaggtc
 S  T  K  A  A  Y  T  T  S  T  C  F  K  V  V  K  T  N  K  V
tactgcctgtctatcgctgagatcagcaacaccctgttcggagagttccgtattgtgcca
 Y  C  L  S  I  A  E  I  S  N  T  L  F  G  E  F  R  I  V  P
ttgttggttgagattctgaaggacgaccgtgtgtaa
 L  L  V  E  I  L  K  D  D  R  V  -
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Figure 1. The nucleotide sequence of the artificially synthesized *hn* gene and the deduced amino acid sequence. The ORF is 1,716 bp, encoding a protein of 571 amino acids. The start codon is atg, and the stop codon is taa

The artificially synthesized *hn* was then inserted into the donor vector pFastBacHT-A through the *Bam*HI and *Not*I sites to create a recombinant plasmid (pFastBacHT-hn), allowing the *hn* gene to be expressed under the control of the polyhedrin promoter available in the vector. The *hn* and pFastBacHT-A were treated with *Bam*HI and *Not*I for 3 hours, purified with QIAquick PCR Purification Kit (Qiagen) and ligated with T4 ligase (Invitrogen). The ligation mixture was transformed into *E. coli* DH10B by the electroporation method. Transformants carrying the plasmid were cultured on LB ampicillin (100 µg/mL) selective medium. The plasmids were cut with restriction enzymes *Bam*HI and *Not*I before separated on agarose gel electrophoresion to check the inserted gene.

The electrophoretic diagram showed the appearance of DNA bands of pFastBacHT-A (5 kb) and *hn* gene (1.7 kb) after the recombinant vector were digested with *Bam*HI and *Not*I (Fig. 2). This proves that the *hn* gene has been inserted into donor vector pFastBacHT-A. The donor vector carrying the *hn* gene is named pFastBac-HN.

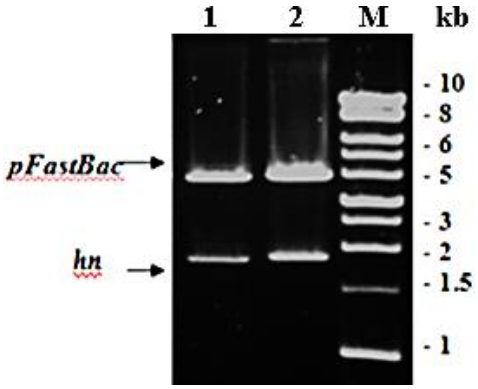

Figure 2. Analysis of the digested products of thepFastBac-HN with *Bam*HI and *Not*I on agarose gel by electrophoresis. 1 and 2: pFastBac-HN; M: DNA marker (Invitrogen)

Creation of Bacmid-HN

Bacmid-HN was created by transforming pFastBac-HN into *E. coli* DH10Bac cells carrying Baculovirus by electroporation method. Once pFastBac-HN was transformed into DH10Bac cells, the pFastBac-HN was integrated into the Baculovirus genome, resulting in Bacmid-HN. The *E. coli* cells carrying Bacmid-HN were selected on LB plate agar medium supplemented with kanamycin, gentamicin, tetracycline, Bluo-gal and IPTG. To screen for Bacmids carrying the *hn* gene, the white colonies were collected and DNA Bacmid was isolated using the Bacmid HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific). Bacmids were tested for the presence of *hn* by PCR with specific primers of the *hn* gene. The PCR results showed that both tested Bacmids carried the *hn* gene while the negative control sample (Bacmid without inserted gene) had no DNA band (Fig. 3). This proves that Bacmid-HN was successfully created.

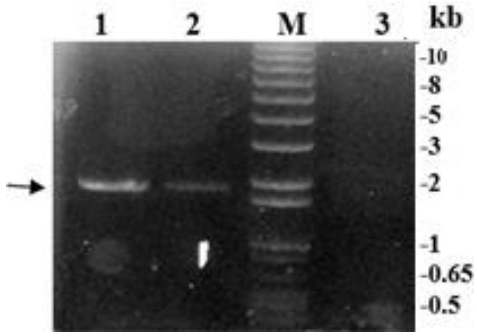

Figure 3. Detection of *hn* in Bacmid-HN by PCR using *hn*-specific primers. 1–2: Bacmid-HN; 3: nagative control; M: DNA marker 1 kb (Invitrogen). The *hn* was indicated in arrow

Generation of recombinant Baculovirus (rBac-HN)

To create a recombinant baculovirus expressing HN, Bacmid-HN DNA was purified using the Bacmid HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific) and then transfected into Sf9 cells as instructed by the manufacture "Bac-to-Bac Baculovirus Expression System" (Thermo Fisher Scientific). After 4 days post-transfection in an incubator at 27 ^{o}C, it was possible to observe on the inverted microscope that the infected Sf9 cells had died and sloughed off in

clusters while the negative control sample (uninfected cells) grew normally (Fig. 4). This proves that Bacmid-HN was transfected into Sf9 cells and the recombinant baculovirus was developed. The recombinant baculovirus was named rBac-HN.

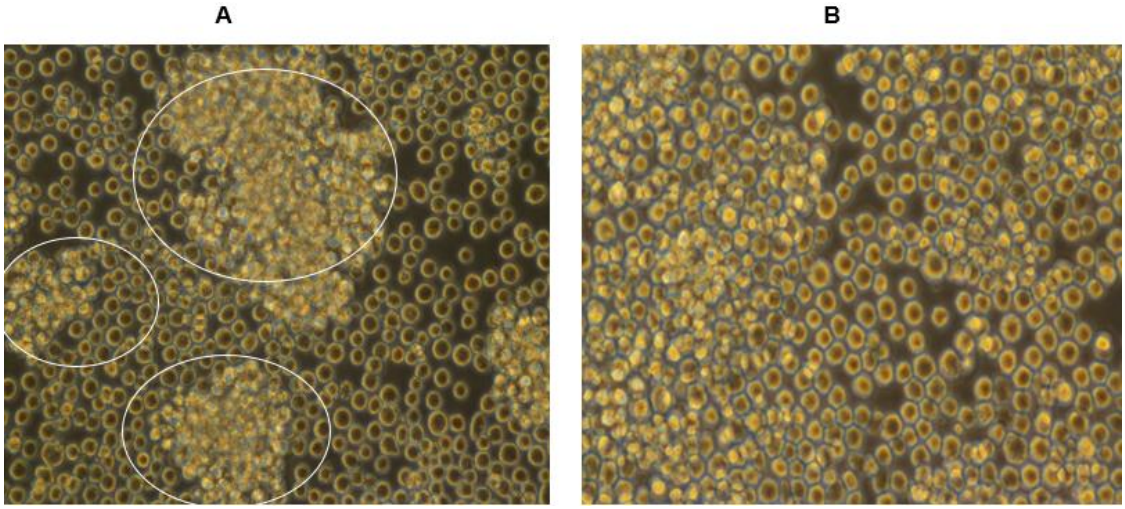

Figure 4. Sf9 cells after 4 days post-transfection with Bacmid–HN (A) and negative control (no transfection) (B) on an inverted microscope (Olympus). The dead cells are marked in circles

Expression of recombinant protein rHN in Sf9 cells

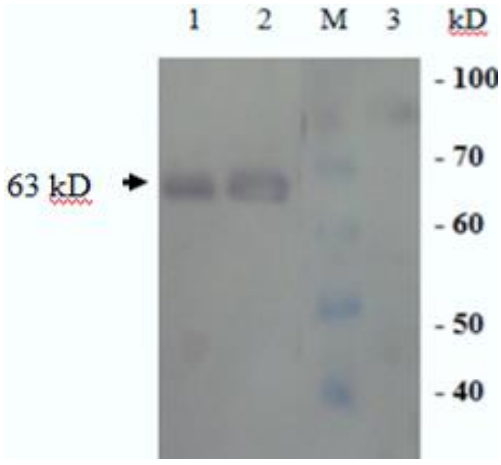

Figure 5. Western Blot analysis of various virus-infected cell **lysates**. 1–2: rBac-HN infected Sf9 cells; 3: uninfected Sf9 cells; M: protein maker

The recombinant protein rHN was expressed in a 25 cm^2 tissue culture flask as described in the method section. After 5 days of post-infection, the Sf9 cells were collected by centrifugation and lysed by ultrasonic to obtain total protein. Western blot results showed that an HN band of about 63 kDa was observed in the lysate of infected-cells while the control (uninfected cells) did not show any protein band (Fig. 5). Evaluation of the haemagglutination (HA) activity of rHN showed that the HA titer of rHN was 2^{-4} (4log2), while it was not detected in uninfected Sf9 cells (Fig. 6). This result proves that HN was expressed in Sf9 cells transfected with recombinant baculovirus rBac-HN.

The hemagglutinin-neuraminidase is a surface glycoprotein involved in the process of entering host cells. HN serves as a crucial antigen for the development of vaccines intended for poultry. Numerous scientists have undertaken the expression of the HN protein for vaccine production. Ong and colleagues have effectively expressed HN derived from a virulent NDV strain isolated in Malaysia within Spodoptera frugiperda (Sf9) cells. The recombinant HN was observed on the surface of the host cells, exhibiting both haemagglutinin (HA) and neuraminidase

(HN) activities. Western blot analysis revealed two distinct protein bands, approximately ~63 kDa (corresponding to non-glycosylated HN) and ~75 kDa (corresponding to glycosylated HN) (Ong et al., 2000).

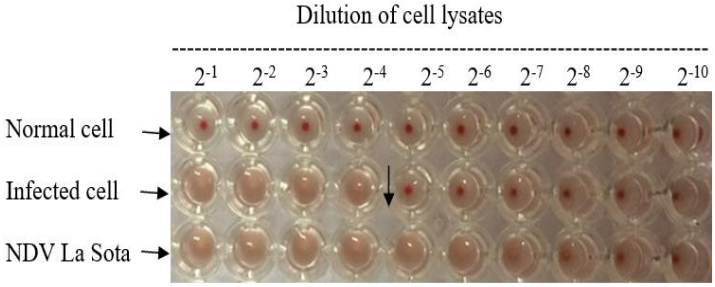

Figure 6. Hemagglutination titer of cell lysates from virus-infected cells. Normal cell: Sf9; infected cell: rBac-HN infected Sf9 cell; NDV La Sota: NDV La Sota solution (HA of 8log2). The HA titer was determined as the reciprocal of the highest dilution with HA activity (down arrow)

Choi (2012) expressed the gene encoding HN from the La Sota virus strain in Spodoptera frugiperda (Sf9) cells. The recombinant HN (rHN) antigen exhibited hemagglutination (HA) titers comparable to NDV antigen produced using chicken eggs and demonstrated stability without significant loss of HA activity for at least 12 weeks at 4 ^{o}C (Choi et al., 2012). In our study, the size of the rHN protein was approximately 63 kDa, consistent with the HN expression results reported by Ong et al. (2000), confirming that they are non-glycosylated proteins. The hemagglutinin activity of rHN showed a titer of 4log2, indicating its potential application for vaccine production against NDV.

CONCLUSION

The HN gene of the NDV genotype VIII strain was effectively expressed in the baculovirus-Sf9 expression system using the SF9000 III medium. The resulting recombinant HN (rHN) protein has a molecular weight of approximately 63 kDa, confirming its non-glycosylated nature. Furthermore, the hemagglutinin activity of rHN reached a titer of 4log2, demonstrating its significant potential for applications in vaccine production or related studies against NDV.

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