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Original Article

Co-production of hemagglutinin H9N2 influenza virus and fusion protein Newcastle virus in insect cell using baculovirus expression system

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Abstract

Influenza and Newcastle disease are the most important poultry diseases that cause high annual damage to poultry farms worldwide. Newcastle virus fusion (F) gene and Influenza Virus Hemagglutinin (HA) gene are capable of encoding F and HA proteins that are the main factors in creating immunity, so this study aimed to clone and express these genes in Spodoptera frugiperda (Sf9) cells using baculovirus expression system. After isolating the Newcastle and Influenza virus genome, the HA gene of influenza virus and the F gene of Newcastle virus were amplified by reverse transcriptase PCR and specific primers and then cloned into pFastBacTM Dual plasmid. A recombinant sucker with these genes was produced in the DH10Bac host cell. By transfecting Sf9 cells with recombinant bacmid, expression was assessed by SDS-PAGE, western blotting, and Bradford methods. Cloning of genes into the bacmid was successful. By transfecting the recombinant bacmid into Spodoptera frugiperda cells, 218 µg/ml of the recombinant protein was obtained in the supernatant. In addition, the presence of protein was confirmed by western blotting. The PCR products of HA and F genes showed one band of 1.7 kb size using specific primers. The pFastHA1 vector was about 7 kb in size. Two bands of about 7 kb and 1.7 kb were created by ligation of the F gene and pFastHA1 vector based on enzymatic digestion, indicating the correct ligation of F gene under the P10 promoter. This is the first report on the cloning and Co-expression of two HA and F genes using baculovirus expression system and can be a candidate for dual influenza and Newcastle vaccine. Mixtures of these recombinant proteins can be used as vaccine candidates against both avian influenza and Newcastle disease.

Keywords: pFastBacTM, Spodoptera frugiperda, PCR, Cloning, Expression.

1. Introduction

Influenza and Newcastle disease are poultry's most economically important viral pathogens worldwide [1]. Avian influenza is one of poultry's most contagious viral respiratory diseases that is highly spreading. A virus belonging to the orthomyxovirus family causes it, and all types of it in poultry belong to influenza virus type A [2]. The main proteins of influenza virus coatings are hemagglutinin (HA) and neuraminidase (NA), which are involved in the entry and spread of the virus [3]. These surface glycoproteins are the main components of the antigenic structure of the virus and the essential component of influenza vaccines [4].

Hemagglutinin with a molecular weight of about 75-70 kDa is the predominant membrane glycoprotein. Hemagglutinin is the most abundant surface protein of the outer membrane of the virus, triggering a high immunogenicity, HA protein which is a surface glycoprotein is produced as inactive (HA0). Then it is proteolytically cleaved by trypsin-like proteases into HA1 and HA2. HA consists of two subunits large (HA1) and small (HA2). Almost all virus antigen sites are located on the HA1 domain of this glycoprotein and can stimulate the production of an immune response equal to the complete protein of the virus [5].

Newcastle virus belongs to the avian paramyxovirus Type 1 [6]. Newcastle contains a single-strand negative-sense RNA genome encoding 6 major structural and non-structural proteins, nucleocapsid, phosphoprotein, matrix, fusion, hemagglutinin neuraminidase, and RNA-dependent RNA polymerase [7].

F protein is a glycoprotein involved in infectivity and pathogenesis, resulting in stimulation of the immune system [8]. F protein is a membrane fusion protein and it appears that the placement of this part of the protein in the target cell membrane initiates fusion [9]. Unfortunately, in Iranian vaccines, the main immunogenic factors including HA and F, are largely lost due to the use of old production methods, especially inactivation stages. Therefore, the immunogenicity of these vaccines is not appropriate. Therefore, both the immunogenicity of these vaccines will be increased and the possibility of recombination will be reduced to zero if the main immunogenic agents of these

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viruses can be used purely as vaccines [10].

Therefore, this study aimed to clone and express the HA and F genes of influenza H9N2 and Newcastle disease viruses in Sf9 cells using baculovirus expression system simultaneously, which might be used as a dual vaccine against these diseases.

This is the first report on the cloning and Co-expression of two HA and F genes using baculovirus expression system and can be a candidate for dual influenza and Newcastle vaccine.

2. Materials and Methods

2.1. RNA extraction

To extract virus RNA, inactivated H9N2 strain of influenza and inactivated Newcastle vaccine were prepared from the Razi Vaccine and Serum Institute (Iran). RNA extraction was performed by RNA extraction kit (Sinaclone; Iran) using the standard protocol of the kit.

2.2. cDNA synthesis

Each of the specific primers of the H9N2 influenza virus HA gene and Newcastle virus F gene was designed using sequences in NCBI gene bank (accession number for HA gene is AJ404626.1and accession number for F gene is ACW19917.1) and bioinformatics software. *Nco*I and *Kpn*I sites were designed at the beginning and end of the F gene, respectively, and *BamH*I and *Xba*I sites were also designed at the beginning and end of the HA gene, respectively (Table 1).

Real-time reverse transcription-polymerase chain reaction (rRT-PCR) reaction was performed for cDNA synthesis using ThermoScript ™ RT-PCR System (Invitrogen ™, USA) and Reverse primers. The temperature program consisted of 60 minutes at 42 ℃ for reverse transcription, 5 minutes at 37 ℃ for completing the synthesis period, 5 minutes at 70℃, and finally 15 minutes at 4℃.

The Polymerase chain reaction was performed to amplify F and HA genes using Exprime master mix (2X) and specific primers. The reaction mixture consisted of 12.5 µl of master mix, 1 µl (10 picomoles) of each of the forward and reverse primers, 1 µl of the synthesized cDNA (approximately 100 ng/µl) and a final volume of 25 µl. The amplification was performed by the following program: initial denaturation at 95 ℃ for 3 minutes, followed by35 cycles consisting of denaturation at 95 ℃ for 30 s, annealing at 49 ℃ for 30 s, extension at 72 C for 1 min and a 5 min final extension step at 72 ℃. The reaction product was cloned into the TA cloning vector pTZ57R by the standard method of the kit and then transferred to the *E. coli* competent cell by heat shock method [11].

2.3. Preparation of F and HA genes and pFastBac™ Dual vector

The fragments were generated by restriction enzymes digestion as follows: *Bam*HI and *Xba*I for the F gene and two enzymes *Nco*I and *Kpn*I (ThermoFisher, USA) for the HA gene at 37 ° C for 3 hours. F and HA genes were ligated into the pFastBac™ Dual vector in a final volume of 10 µl. Initially, the ligation reaction mixture consisted of 1.5 µl of pFastBac™ Dual vector, 3 µl of HA gene, 3 µl of F gene, 1 µl of T4 DNA ligase (Thermofischer USA), 1 µl of 10 X ligation buffer and 1 µl of polyethylene glycol. The reaction mixture was placed at 22 ° C for 60 minutes. 5 µl of the ligation reaction was transformed in the *E. coli* DH5α by standard heat shock method [11]. Recombinant colonies were cultured on the LB agar medium containing ampicillin. The accuracy of cloning was confirmed by colony PCR and enzymatic digestion.

2.4. Recombinant bacmid construction

Recombinant plasmid pFastHA-F was extracted using the purification kit (Pouya Gene Azma, Iran) according to the standard protocol of the kit. Recombinant plasmid pFastHA-F was transferred to *E. coli* DH10Bac competent cell using the standard heat shock method [10]. Recombinant DH10Bac competent cells were cultured in LB agar medium containing kanamycin, tetracycline and gentamicin (at concentrations of 50, 10, and 7 g/ml, respectively). Large bacmid DNA size (about 135 kb) leads to difficulty in enzymatic digestion, Therefore, a PCR reaction was used to confirm the recombinant bacmid. Bacmid has M13 primer sequences on both sides of the transposon site in the laczα region, so M13-specific primers were used for performing PCR (Table 2). The reaction mixture consisted of 2.5 µl Buffer (10X), 0.5 µl dNTP Mix (10 mM each), 1 µl of each primer, 1 µl of DreamTaq DNA Polymerase, 5 µl of recombinant plasmid in a final volume of 25 µl. The amplification was performed by the following program: one cycle at 94 ℃ for 4 minutes, followed by 94℃ for 45 s, 55℃ for 45 s, 35 cycles at 72 ℃ for 5 minutes and one cycle at 72℃ for 7 minutes.

2.5. Expression of recombinant proteins in Sf9 cells

Sf9 cell was used to express the recombinant protein. After preparing Sf9 insect cells from Cell Bank of Pasteur Institute of Iran, 800 ×106 young cells in logarithmic phase containing Grace's Supplemented Insect Medium)10% FBS, and 1% of penicillin and streptomycin antibiotics (were cultured at 37 ° C. Recombinant Bac-PPRHF complex was formed by combining 8 µl of cellfectin and 1 µg of DNA. The mixture was incubated at room tempe-

Table 1. Primers for amplifying H and F genes.

Name	Primer
HA	F: GGATCCATGGAAACAATATCACTAATAACTATAC R: AAGCTTTTATATACAAATGTTGCATCTGCAAGATC
F	F: CCATGGATGGGCTCCAAACCTTC R: GGTACCTCATGTTCTTGTAGTGGC

Table 2. M13 specific primers.

Name	Primer
M13	F: CCCAGTCAC GAC GTT GTA AAA CG R: AGCGGA TAA CAA TTT CAC ACA GG

rature for 30 minutes. The transfection mixture was added dropwise to the cells and the cells were kept at 27 ° C for 5 h. After removing the transfection mixture, 2 ml of complete culture medium containing 10% FBS was added to the cells and the cells were incubated at 27 ° C. After the onset of cytopathic effects on the third day after infection, the supernatant was removed, and the solution was then centrifuged at 2000 g for 4 minutes at 4 ° C to remove cell debris. Regarding the presence of peptide signal at the beginning of both genes and expectation of secretion of recombinant protein into culture medium, or production of VLP, the supernatant was examined for the presence of protein by standard SDS-PAGE method [12].

2.6. Western blotting

The recombinant protein was transferred from the SDS-PAGE gel to the nitrocellulose membrane by the standard semi-dry method. Conjugation was performed by adding 15 ml of PBS buffer containing 7.5 µl of Alkaline phosphatase (AP)-conjugated secondary antibody and stirring gently for one hour at laboratory temperature. The membrane was transferred to 15 ml PBS buffer containing 45 NBT and 35 µl X-Phosphate and placed in a dark place. Bradford standard method was used to evaluate the amount of recombinant protein produced.

3. Results

The accuracy of HA gene cloning in pFastBac™ Dual vector was performed using colony PCR (Figure 1) and enzymatic digestion (Figures 2 and 3). The results showed the accuracy of HA gene ligation with pFastBac™ Dual vector under polyhedrin promoter.

Examination of the gene on the recombinant bacmid using PCR and specific primers of HA and F genes, as well as M13 primer, where the recombinant bacmid revealed both HA and F genes (Figures 4 and 5).

After transfection of recombinant cells into Sf9 insect cells, examination of cell growth and comparison with non-transfected cells showed that cell division stopped and the cells enlarged and formed CPE in Sf9 cells infected with the virus (Figure 6).

Examination of the supernatant of Sf9 cells transfected with the recombinant virus carrying both genes by SDS-PAGE showed a band of about 62 kDa. Because the molecular weight of both proteins is about 61 kDa, it is

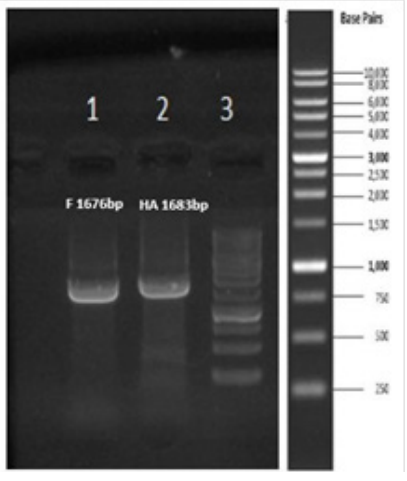

Fig. 1. Electrophoresis of PCR product on agarose gel. Lane1: F gene PCR product, Lane2: HA gene PCR product, Lane3: 1kb ladder.

Fig. 2. Electrophoresis of pFastHA1 vector digested with *Bam*HI and *Xba*I enzymes. Lane1: 1kb plus ladder, Lane2: Vector digested with two enzymes. Two lanes belonging to vector (5 kb) and the pFastHA1 vector was about 7 kb in size. Two bands of about 7 kb and 1.7 kb were created by ligation of the F gene and pFastHA1 vector based on enzymatic digestion, indicating the correct ligation of the F gene under the P10 promoter.

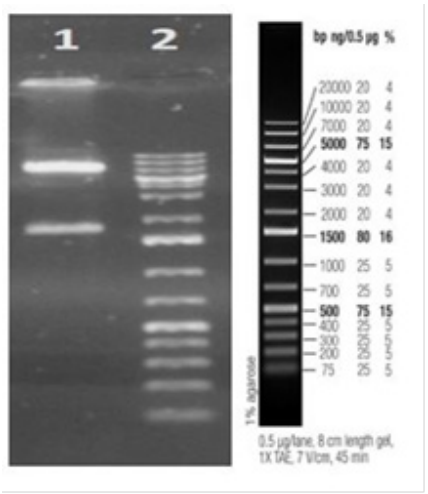

Fig. 3. Electrophoresis of pFastHAF vector digestion using *Nco*I and *Kpn*I enzymes. Lane 1: Vector carrying HA and F genes after enzymatic digestion. As it is known, two bands are formed, the band of about 1.7 kb belongs to F gene and the band of about 7 kb belongs to the vector-carrying HA gene. Lane 2: 1kb ladder.

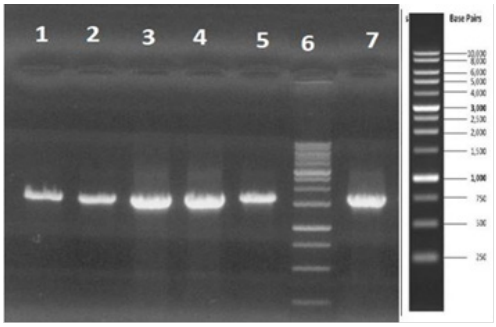

Fig. 4. Electrophoresis of PCR product on recombinant bacmids with specific primers of HA and F genes. Lanes 1 to 3: PCR product of HAF bacmids with specific primer of HA gene with 1.7 kb size. Lanes 4, 5 and 7: PCR products of HAF bacmids using F gene specific primers with 1.7 kb size. Lane 6: 1kb ladder.

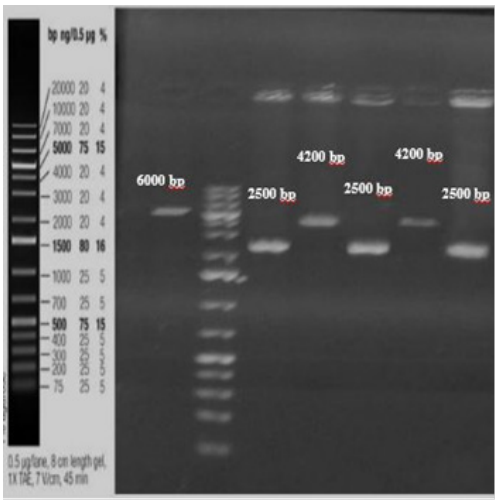

Fig. 5. Gel electrophoresis of recombinant bacmid product. Lane 1: DH10Bac including both genes. Lane 2: (1+) kb Ladder. Lane 3: Negative control without gene of interest. Lane 4: DH10Bac carrying HA gene. Lane 5: Negative control without HA gene. Lane 6: DH-10Bac including F gene. Lane 7: Negative control without F gene.

not possible to separate them on SDS-PAGE gel, so it was expected to see a band of about 60 to 65 kDa (Figure 7).

As shown in Figure 7, only a band was observed in the supernatant. However, the expression band of both genes is stronger than the expression band of the expressed protein of one gene, and the band of HA was stronger than the band of the F protein. Bacmid containing F and HA genes were also used as controls in SDS PAGE. The amount of recombinant protein in the supernatant was determined at 241 µg/ml by the Bradford standard method through a vector carrying two genes.

In Western blotting, the binding of F and HA antisera to the extracted protein resulted in a band of about 60 kDa in the supernatant solution (Figure 8). In the supernatant sample of cells transfected with recombinant bacmid, it is completely identified with polyclonal antibody containing antibodies against F and HA protein. While in the sample of the supernatant of cells transfected with bacmids without the gene, no band appeared.

4. Discussion

Newcastle disease and the flu annually cause great damage to the poultry industry worldwide; But despite huge research investments, the disease has not yet been controlled [13]. However, many vaccines against these viruses have been developed to date [14]. However, they do reappear once a year and spread so rapidly that they cause a widespread epidemic of these viruses [15], causing great damage to the poultry industry [16]. In recent years, the prevalence of H9N2 viruses has increased in several countries. The increase also includes countries where the virus was first detected [17].

Hemagglutinin is the most abundant surface protein in the viral capsid, which is the main target for immunity [18]. Recently, mRNA-based vaccines have been developed to control infectious diseases, and studies have shown that these vaccines have the potential to develop strong humoral cells as well as cellular immune mediators that demonstrate their ability to fully protect against lethal challenges [19]. Attempts have been made to use F and Hemagglutinin-neuraminidase (HN) proteins in recombi-

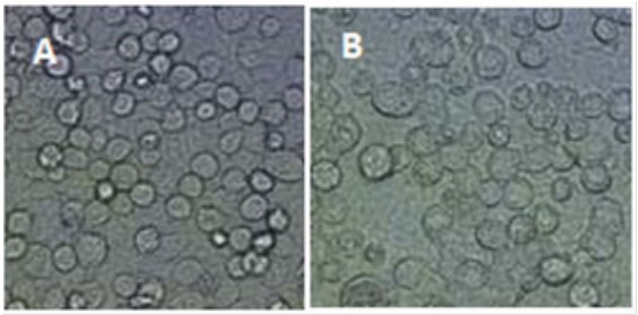

Fig. 6. Microscopic image of uninfected (A) and infected (B) Sf9 cells. In Sf9 cells infected with the recombinant virus, cell division stopped and the cells became larger.

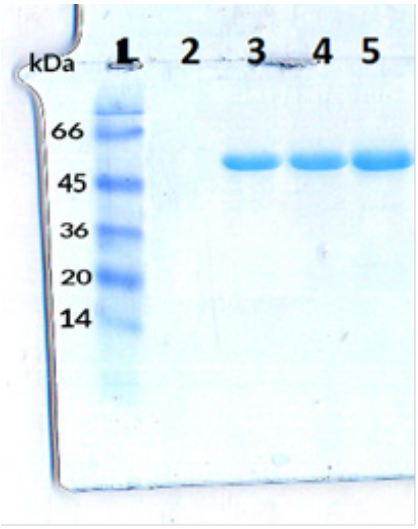

Fig. 7. Evaluation of recombinant proteins by SDS-PAGE. Lane1: Protein Ladder, Lane2: Supernatant of cells transfected with bacmid without gene, Lane3: Supernatant of cells transfected with bacmid carrying F gene, Lane4: Supernatant of cells transfected with bacmid carrying HA gene, Lane5: Supernatant of cells transfected with bacmid carrying HA and F genes.

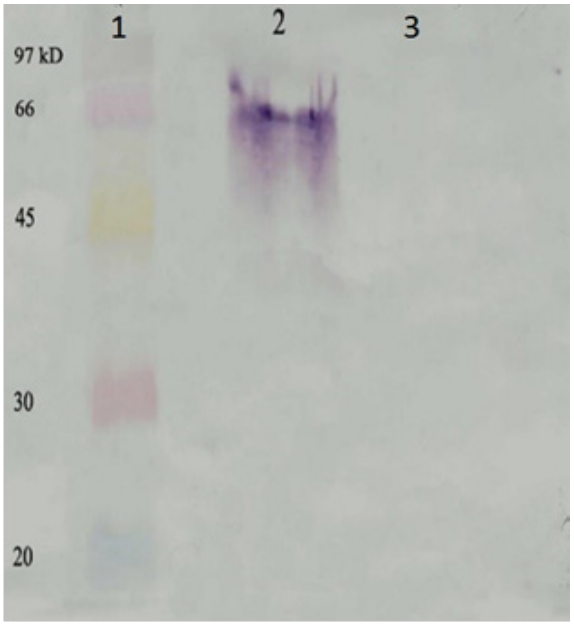

Fig. 8. Lane1: supernatant of cells transfected with bacmids without genes, lane2: supernatants of cells transfected with recombinant bacmids, Lane3: protein marker.

nant subunit vaccines against Newcastle disease. Recombinant vaccines containing F and HN subunits have been produced in baculovirus vectors [20].

Attempts have been made to use F and HN proteins in recombinant subunit vaccines against Newcastle disease. Recombinant vaccines containing F and HN subunits have been produced in baculovirus vectors [20].

In 2006, a study converted the HPAI pathotype to the attenuated LPAI pathotype by inserting the outer part of the hemagglutinin-neuraminidase (HN) gene of Newcastle disease virus into the influenza virus H5 gene fragment, by which vaccinated chickens were protected by stimulating humoral immunity against both Newcastle disease and influenza via intra-embryo [21]. The "primary amplification strategy" has also been used in DNA-based inactivated trivalent (TIV) vaccines for *Hemophilus influenza*, resulting in a large number of neutralizing antibodies and a cross-immune response [22].

Recently, mRNA-based vaccines have been developed to control infectious diseases, and such studies have shown that these vaccines have the potential to develop strong humoral cells and cellular immune mediators that demonstrate their ability to fully protect against lethal challenges [23].

Evaluation of influenza virus strains showed that the HA2 region of the hemagglutinin is significantly more conserved than the HA1 segment [24]. A study on the construction of Newcastle virus vaccine in baculovirus vector found that high-titer neutralizing antibodies elevated IL-4, IFN-γ, and IL-2 levels, indicating that baculovirus vaccine has both the advantages of recombinant viral vector-based vaccines and recombinant DNA vaccines. These vaccines effectively deliver extracellular antigens to avian immune cells, which increases humoral cell production, stimulating the cellular immune response [25].

HA and F genes were selected to make a subunit vaccine against influenza and Newcastle disease in the present study. The genes were first cloned into the pFastBac™ Dual vector. Three recombinant vectors carrying the F and HA genes were cloned on the bacmid (modified Baculovirus genome) and the recombinant bacmid was transfected into the Sf9 cell. This study is the first to simultaneously clone two HA and F genes in the baculovirus expression system. This system has many vital activities of eukaryotic cells such as protein processing and transport. The protein produced retains its nature. The formation of disulfide bonds, oligomerization, glycosylation, phosphorylation and folding is done correctly on them and the splicing of the intron is done naturally and the resulting protein is both structurally and functionally similar to the natural protein.

There was no report on the expression of HA and F genes in cloned hosts. Because HA and F genes were cloned under the control of polyhedrin promoter and P10 promoter, the expression level was found to be 241 g/ml. In the supernatant of cells transfected with HAF bacmid, the presence of both recombinant HA and F proteins was confirmed by Southern blotting.

5. Conclusion

In the present study, HA and F genes were cloned separately and together on the pFastBac™ Dual plasmid vector. After transfer to the bacmid and transfection into Sf9 cells, proper expression of genes was obtained in the supernatant of Sf9 cells by 3 passages. In the supernatant of cells transfected with HAF bacmid, the presence of both recombinant HA and F proteins was confirmed by Southern blotting. Mixtures of these recombinant proteins can be used as vaccine candidates against both avian influenza and Newcastle disease.

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Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
No humans or animals were used in the present research.

Informed consent
The authors declare not used any patients in this research.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Mohaddeseh Moheb Shahedin: data collections and lab work, Majid Moghbeli: designing the study and writing the manuscript, Mohammad Kargar: data analysis, Mohsen Forouzanfar helped with data analysis and writing manuscript.

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Non.

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