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Original Article

Construction of Recombinant Bacmid DNA Encoding Newcastle Disease Virus (NDV) Fusion Protein Gene

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Abstract

Background and Aims: Newcastle disease virus (NDV) is one of the major pathogen in poultry. Vaccination is intended to control the disease as an effective solution; nevertheless this virus is a growing threat to the poultry industry. F gene open reading frame (ORF) from NDV is 1650 bp, encoding a protein of 553 amino acids that can induce protective immunity alone. The F glycoprotein on the surface of NDV is important for virus infectivity and pathogenicity. Towards protection goal, the full-length of F gene was isolated using specific primers and cloned into the baculovirus derived bacmid shuttle vector to produce recombinant F-protein in insect cells.

Materials and Methods: F gene ORF from avirulent strain La Sota of NDV was amplified by RT-PCR to produce F cDNA. The amplicon was cloned firstly into the T/A cloning vector and then subcloned into the pFastBac Dual donor plasmid through *NcoI/KpnI* sites. After the verification of cloning process by PCR and enzymatic digestion analysis, the accuracy of F gene ORF in the T/A cloning vector was confirmed by sequencing. Finally, F-containing recombinant bacmid was subsequently generated and confirmed by PCR using F specific primers and M_{13} universal primers.

Results: Results showed that a recombinant baculovirus containing a correct and in framework sequence of Newcastle F gene under the control of p_{10} promoter was constructed.

Conclusion: The above mentioned F-containing recombinant baculovirus, in addition to its independent application, can be used with other individual recombinant baculoviruses expressing NH and NP genes to produce Newcastle VLPs in insect cell line.

Keywords: Newcastle disease; F protein; Baculovirus; Bacmid shuttle vector

Introduction

Newcastle disease virus (NDV) is one of the major pathogens in poultry. Vaccination is intended to control the disease as an effective solution, nevertheless this virus is a growing threat to the poultry industry (1). NDV belongs to the *Avulavirus* genus within the *Paramyxoviridae* family of negative-stranded RNA viruses. The viral genome encodes six proteins from the 5' terminus to the 3' terminus: RNA-directed RNA polymerase (L gene), hemagglutinin-neuraminidase (HN gene), fusion (F gene), matrix (M gene), phosphoprotein (P gene), and nucleocapsid (NP gene) protein (2). Both HN and F glycoproteins on the surface of NDV are important for virus infectivity and pathogenicity, and either of these proteins can induce protective immunity (3-9). The F protein is responsible for membrane fusion

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required in the first stage of infection (10). Furthermore, if inserted into the host cell it can mediate fusion between the plasma membranes of the two cells. The molecular basis of NDV pathogenicity depends on the F protein cleavage site amino acid sequence (11). The F protein is synthesized as an inactive precursor F_0 (M_r 68,000) which is proteolytically cleaved into two disulfide-linked subunits F_1 and F_2 that is necessary for the infectivity of paramyxoviruses (Le *et al* 1988). F gene open reading frame (ORF) from NDV is 1650bp encoding a protein of 553 amino acids. NDV causes a disease that varies in clinical severity and transmissibility depending on the pathotype involved. NDV strains can be categorised into three groups based on their virulence, as lentogenic (nonvirulent), mesogenic (intermediately virulent) and velogenic (highly virulent). Lentogenic strains, especially in adult chickens may cause minimal or no clinical sign. However, the disease produced by mesogenic strains may cause mortality that can reach 25% and that by velogenic strains may be reached up to100% (12). NDV F protein can be effectively stimulating the immune system (13). Protein expressed, is enough to induce immune response alone, and vaccine derived from it can be used for immunization (13). As well, changes in the structure of the F protein were created by genetic engineering techniques that would optimize the immune response (13). The recombinant baculovirus and insect cell expression system provides high levels of recombinant proteins that undergo post-translational modifications like glycosylation (14-15). Therefore, application of such system allows large quantity production of a desirable protein, in the native conformation as a vaccine. In this study, F gene was isolated and amplified from NDV and then a clone of F-containing recombinant bacmid was constructed. This construct can be used to produce VLPs of different NDV genotypes, because F sequence has been conserved evolutionarily in all NDVs, recorded during the last decades (16). F-containing recombinant bacmid also can be used to produce large scale of fusion protein, suitable for vaccine production.

Methods

RNA Extraction and cDNA synthesis

Viral RNA was extracted from 200 µl of harvested cell culture supernatant using a Roche commercial kit (Germany) according to the manufacturer's instructions. The cDNA synthesis was performed using ThermoScript™ RT-PCR System (Invitrogen-USA) according to the manufacturer's instructions. Towards this goal, specific primers (at the concentration of 10µM or 10pmol/µl for each primer), dNTPs (10mM for each base), special buffers of enzymatic reactions and a RNAase inhibitor were used. It is to be noted that in all stages, the RNAase/DNase free micro-tubes (Extra gene, USA) were used.

RT-PCR

Specific primers (forward and reverse) were designed by CLC Main Workbench 4.5 (QIAGEN Co.) to amplify the complete F gene ORF of NDV (1650 bp). 5µl of cDNA was added to a 20µl total volume of PCR mixture containing 10pmol of each forward primer 5' GGT-ACC-TCA-TGT-TCT-TGT-AGT-G 3' and reverse primer 5' CCA-TGG-ATG-GGC-TCC-AAA-CCT 3' (underlined nucleotides correspond to *NcoI* and *KpnI* sites respectively), 10 mM Tris-Cl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 10 mM dNTPs and high fidelity PCR Enzyme Mix (Genetbio-Korea). Amplification reactions were performed in a thermocycler (Biorad, USA) under the following profile: 5 min at 95°C followed by 30 cycles at 95°C for 30 sec, 55°C for 30sec and 72°C for 80 sec, with a final extension step at 72°C for 7 min. PCR products were analyzed on 1% (w/v) electrophoresis agarose gel stained with safe view (Kiagene, IRI) upon preparation (17-18).

Cloning of the F gene

The RT-PCR product was extracted from the low melting agarose gel by using a DNA extraction kit (Vivantis-Korea) and subsequently cloned into the T/A cloning vector and subcloned into the pFastBac Dual

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donor plasmid (Invitrogen-USA) through *NcoI/KpnI* sites. The F-containing recombinant pFastBac Dual donor vector was confirmed using PCR and enzymatic digestion analysis. Subsequently it was subjected to perform automatic one-directed sequencing using forward primer, described previously for F gene amplification.

Generation of a recombinant bacmid

The F-containing recombinant pFastBac Dual donor plasmid was transferred into the *E. coli* $DH_{10}Bac$ competent cells for site-specific transposition of the F DNA from the transposing vector to a bacmid DNA through *lacZ* gene disruption. The transformed cells were cultured on a LB agar plate containing kanamycin (50µg/ml), gentamicin (7µg/ml), tetracycline (10µg/ml), X-gal (100µg/ml) and isopropylthio-β-galactoside (IPTG, 40µg/ml) and incubated at 37ºC for 16h. The high-molecular-weight bacmid DNA was isolated from the overnight cultures by alkaline lysis purification method, according to the instructions supplied by the manufacturer (Invitrogen-USA). Successful transposition was verified by PCR analysis using both M_{13}/pUC and F-specific primers.

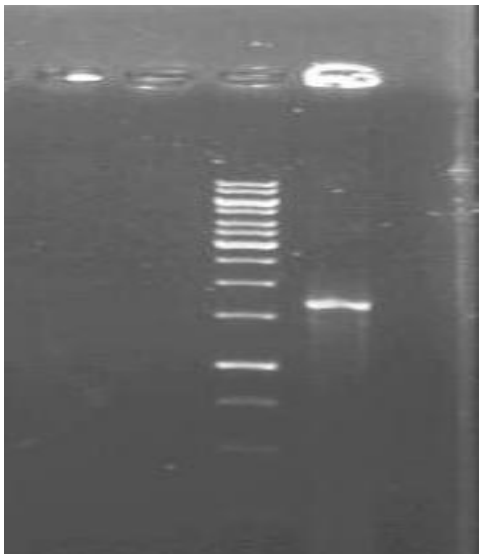


Fig. 1. Electrophoresis gel analysis of F gene (1650 bp) RT-PCR products, Lane 1: negative control (blank) Lane 2: 1 Kb DNA size marker (Fermetas). Lane 3: demonstrating a thick bond of 1650bp amplicon.

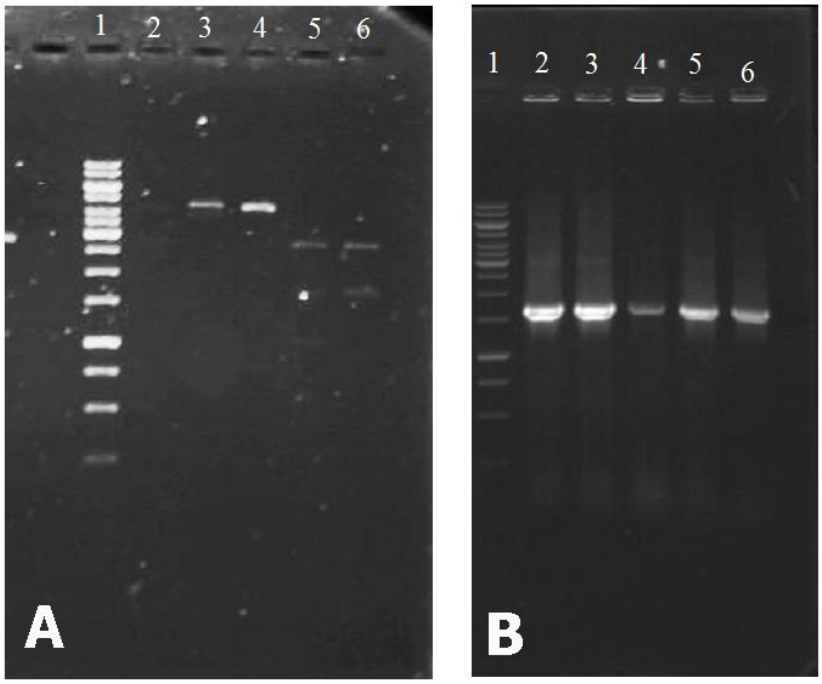


Fig. 2. A: F gene PCR with specific primer, five white colonies was selected randomly. Lane 1: 1 Kb DNA size marker (Fermetas). Lanes 2-6: F-gene fragment (1662 bp). **B :** Enzymatic digestion map of the new F-containing recombinant T/A cloning vector. Lane 1: 1 Kb DNA size marker (Fermetas). Lanes 2-4: single digested revealed expected fragment (4368 bp), Lanes 5 and 6: *NcoI/KpnI* double digestion on the F-containing recombinant T/A cloning vector (clone), revealed expected 2727 bp and 1662 bp fragments.

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Results

The full-length sequence of F gene (1650 bp) was amplified by RT-PCR using the designed specific primers (Fig. 1). The fragment produced, was cloned into the T/A cloning vector after extraction from agarose gel and confirmed using PCR (Fig 2a) and enzymatic digestion analysis (Fig 2b). The accuracy of the F gene ORF in the T/A cloning vector was confirmed by sequencing. Analysis of sequencing was accomplished by Chromas software (version 1.45-Australia). Subsequently, F fragment was Subcloned into the pFastBac Dual donor plasmid, as described previously in the methods section. The recombinant vector was confirmed by PCR and subsequently by enzymatic digestion. (Results not presented).

Construction of the recombinant bacmid DNA

The F gene was subcloned into the pFastBac Dual donor plasmid and verified by endonuclease digestion in comparison with non-recombinant pFastBac Dual, as the control. Following the transformation and plating of the cells on LB agar, the transposed colonies were visible as large white colonies among the blue ones harboring the unaltered bacmids. The selected white colonies were restreaked onto a LB agar plate to ensure if they have true white phenotype. Since verification of the high molecular weight recombinant bacmid DNA is not convenient by digestion, PCR was performed using both M_{13}/pUC and F-specific primers to ensure that proper transposition of the target gene has been doen in the recombinant bacmid. The bacmid DNA contains M_{13} forward and reverse priming sites, flanking the Tn_7 mini-att site within the LacZ α-complementation region. A panel of PCR was done using M_{13}/pUC universal primers, F gene specific forward and M_{13} reverse primers, F gene specific reverse and M_{13} forward primers, and finally F gene specific primers, respectively. Amplification of the non-recombinant bacmid using M_{13}/pUC primers generated a 303bp band (Fig. 3).

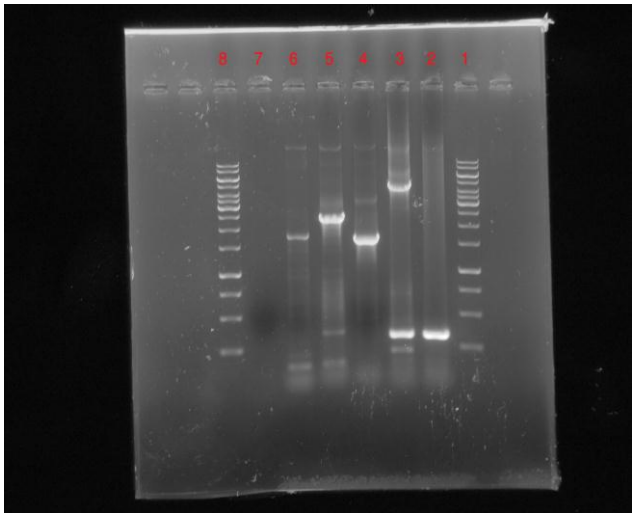


Fig. 3. The panel of PCR, done to confirm F-containing recombinant Bacmid :
Lane 1 and 8: 1Kb DNA size marker (Fermentas).
Lane 2: PCR product using M_{13} universal primers (~ 300 bp) produced by the nonrecombinant bacmid extracted from a blue colony as a negative control.
Lane 3: PCR product using M_{13} specific primers (4222 bp) produced by the recombinant bacmid extracted from a white colony.
Lane 4: PCR product using F gene forward and M_{13} reverse primers (~1500 bp) produced by the recombinant bacmid extracted from the same white colony.
Lane 5: PCR product using M13 forward and F reverse primers (~ 2500 bp) produced by the recombinant bacmid extracted from the same white colony.
Lane 6: PCR product using F gene specific primers (1662 bp) produced by the recombinant bacmid extracted from the same white colony.

Discussion

Expression of eukaryotic genes using baculovirus expression vectors takes advantages of their protein synthesis machinery and facilitates proper folding and

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post-translational modifications including glycosylation, acetylation, olygomerization and proteolysis. In addition, the insect cell cytoplasmic environment provides appropriate disulfide bridge assembly (19-20). These features are important in the case of NDV F-protein, as its proper structure is critical for its function as a nexus that gathers ribonucleoprotein complex and outer envelope-bound HN and F proteins together for packaging in the host cell (21-22). Production of Influenza VLPs was demonstrated with different expression systems including recombinant DNA expression vectors (23), recombinant vaccinia viruses (21), and DNA plasmid transfection with T7 RNA polymerase-expressing vaccinia virus (22). However, recombinant baculovirus/insect cell expression system yields high levels of recombinant proteins and is more important. VLPs formed in such systems have shown high immunogenicity both in the raise of neutralizing antibodies and the induction of cellular immune responses. Several research groups have reported the expression of NDV F-gene in baculovirus-infected insect cells. Most of them planned to produce various Influenza VLPs of different genotypes and apply them in vaccine research studies (24-27). Gomez-Puertas and colleagues' studies have clearly demonstrated the significance of F protein in assembly and the process of NDV VLP formation (22, 28). Therefore, in the most of NDV VLP studies, F protein has been considered as an essential component (19, 23, 25-26). It has been also demonstrated that VLPs lacking HN have been formed and released from the cells expressing F through recombinant DNA or virus infection (21). In other words, only the expression of F protein is sufficient to induce the particle formation.

Conclusions

In this study, a recombinant baculovirus containing a correct and in framework sequence of Newcastle F gene under the control of p_{10} promoter was constructed. To produce NDV VLPs as the ultimate goal, we choose the Bac-to-Bac expression system (Invitrogen life technologies) because apart from the advantages mentioned above, it is a valid and multipurpose system for the production of recombinant proteins. In ongoing study, the recombinant baculovirus expressing F gene (F recombinant bacmid) constructed here, will transfect into the cultured Sf_9 (Spodoptera frugiperda) insect cell line to produce F protein. Recombinant baculoviruses expressing F gene can be used with the other individual recombinant baculoviruses expressing HN gene to produce NDV VLPs. Moreover, the purified F protein expressed in this system maybe applicable in several vaccine research projects

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