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Molecular Cloning and Expression of the Fusion (F) Gene from Newcastle Disease virus in *Escherichia coli*: A Platform for Further Studies

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ABSTRACT

Newcastle disease (ND) is a highly contagious viral disease affecting most avian species. The fusion protein in the ND virus serves as the target for immune response. The goal of this study was to develop the DNA vaccine using a fusion gene from the Newcastle virus. A new candidate DNA vaccine against Newcastle disease virus (NDV) has been developed. This innovative vaccine uses a fusion gene that encodes immunogenic proteins derived from NDV. The hypothesis behind this approach is that the fusion gene induces a strong immune response against the virus, potentially leading to long-term immunity in vaccinated individuals. Fusion gene RNA was extracted from the Newcastle virus and amplified by the reverse transcription-polymerase chain reaction (RT-PCR). Afterwards, it was sub-cloned in the pTG-19T vector, then into the expression vector pET43.1a E. coli BL21. Gene expression was induced by IPTG. The fusion protein was subjected to Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sequencing and PCR results confirmed the successful cloning of the fusion gene into the vector. Digestion results showed the target gene had been successfully inserted in the pET43.1a plasmid. SDS-PAGE revealed a protein band of about 54.7 kDa. Analysis of the constructs in E. coli cells demonstrated successful expression of gene inserts in vitro. Our results indicate that the fusion protein produced by pET43.1a in E. coli can be used as a DNA vaccine. However, a weak band of expressed protein was observed, indicating that the fusion protein produced by pET43.1a in E. coli was not highly efficient. This survey encourages further research to test the produced protein as a vaccine in vivo and in vitro.

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1. Introduction

Newcastle disease (ND) is one of the most important diseases in poultry farms all over the world (1). It causes harmful effects on domestic poultry production, and the range of the virus's infectivity varies from asymptomatic to quickly fatal (2, 3). Newcastle disease (ND) is a member of the *Paramyxoviridae* family in the genus *Avulavirus*. There is only one serotype of avian paramyxoviruses that causes Newcastle (ND) disease (APMV-1). APMV-1 is the serotype of Paramyxovirus that includes pathogenic NDV (4). NDV is a non-segmented, negative sense, and single-strand RNA (5). It is also classified into four pathotype categories based on clinical symptoms in infected chickens, including velogenic, mesogenic, lentogenic, and asymptomatic (6). The genome of NDV encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and RNA-dependent RNA polymerase (L) (3, 7, 8). Two surface glycoproteins, F and HN, are antigens and are present on the envelope (9). The protein can affect the binding of the virus to host cell membrane, and through several structural changes, lead to the movements of the virus particles into the cells, which is necessary for infection (8). Protein F1 is synthesized as an inactive protein, F0, which is cleaved into F1 and F2 polypeptides, activating the production of fusion protein. This cleavage is necessary for the virus to become infectious. Protein F is a key factor for disease and immunity. The amino acid sequence of F protein, along with host protein proteases, forms a molecular basis for the ND pathogenesis (10, 11).

Chick vaccination is carried out using inactive or live vaccines, which are temporary. In addition, these vaccines are expensive and time-consuming. Extensive and permanent immunity is produced by DNA vaccines. Importantly, DNA vaccines affect only humoral immunity. They has been used for various diseases. Studies have shown effective immunity of DNA vaccines against many infectious diseases, autoimmune diseases, as well as allergies and cancers (12). DNA vaccines have been studied for immunization against NDV strains. Therefore, the present study was conducted to determine the expression of the fusion gene in the expression vector pET43.1a. The goal of this study was to develop a DNA vaccine using a fusion gene from the Newcastle virus.

2. Materials and Methods

2.2. Virus and Viral RNA Isolation

The LaSota strain of the Newcastle disease virus was provided from Razi Vaccine and Serum Research Institute. To obtain the maximum titer of virus stocks, it was cultured in the allantoic fluid of 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs. After 72 hours of inoculation, viral RNA extraction from the allantoic fluid of the infected eggs was performed by using a QIAamp® Viral RNA kit (QIAGEN, Germany).

2.3. Primer Design

The primers were designed based on the fusion gene sequence in GenBank with accession number AY845400, which was related to the NDV LaSota strain. The F-specific primers 5'-ATGGGCTCCAAACCTTCTACC-3' and 5'-TTGTAGTGGCTCTCATCTGATC-3' were used to amplify the region of interest. The assessment of melting temperature and secondary structure of designed primers was conducted using primer3 software.

2.4. Amplification of the Viral Gene

Total RNA was extracted from the 140µl sample of allantoic fluid. The cDNA was produced according to the RevertAidTM first-strand cDNA synthesis kit protocol (Fermentas, Canada). Each RT-PCR reaction included hexamer primer, reverse primer, and 2µl of the cDNA (50ng cDNA). The reaction was carried out in a final volume of 25µl,containing 25mM10x PCR buffer, 2.5mM MgCl2, 0.2mM dNTP mix, 0.5U supperTaq, and each primer concentration 6.25mM, with following cycling conditions: 10min at 95ºC (hot start), followed by 40 cycles at 94ºC for 3min, 60ºC or 62ºC for 1min, 72ºC for 3 minutes ,and terminal extension for 20 minutes.

2.5. Enzyme Digestion and Sequencing

To confirm the specificity of the fusion gene and virus strain, the SacI enzyme was used. It cuts the fusion gene at position 600, which was compared theoretically in the GenBank database. The recombinant plasmids were extracted and subjected to sequencing to confirm that they aligned with a sequence of the F gene mRNA. Sequence findings were compared with GenBank, and ninety- eight percent alignment was achieved.

2.6. Gel Extraction

The RT-PCR product was extracted by using a Silica Bead DNA Gel Extraction kit (Fermentas, Canada). It was then purified to remove excess material in preparation for TA cloning.

2.7. Cloning Fusion Gene in pTG19

To determine the initial gene sequence and to create the sticky end in the fusion gene, the pTG19-T PCR cloning vector was employed.

2.8. Construction of Expression Plasmids

DNA Restriction Enzymes, including HindIII and KpnI, were used to Transfer a fragment of cloned DNA from pTG19 (Vivantis, Malaysia) to the pET43.1a (Invitrogen, USA) vector. These endonucleases cut both plasmid pET43.1a and fusion genes. Ligation of the Fusion gene with the vector was performed by ligase enzyme. The standard method to make the bacteria permeable to DNA involves treatment with calcium ions; This was followed by heat shock treatment at 42°C for 90 seconds to facilitate transformation. The plasmids contained AmpR as a selectable marker, providing resistance to ampicillin. Thus, the bacteria are grown on a medium containing the antibiotic only if the bacteria contain the plasmid. Transformed cells were cultured in Luria-Bertani (LB) medium containing ampicillin, and the target DNA was amplified.

Then, it was confirmed by colony PCR. Plasmid extraction and purification were carried out using the QIAprepMiniprep kit (QIAGEN, Germany). The plasmid DNA isolated from transformed cells was digested with HindIII and KpnI. The pattern of fragments on the gel electrophoresis indicates whether the plasmid contains the expected size insert. As the expression of the pET43.1a vector in the E. coli strainDH5α is low, DH5α was used for maintaining this vector. To express the fusion gene, pET43.1a was transformed into E. coli, BL21.

2.9. Preparing Total Protein and Expression Fusion Gene

The transformed bacteria were cultured in LB media containing ampicillin and incubated at 37°C. After the cell culture period, an OD600 of 0.4 to 0.6, Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce protein expression in E. coli. Following IPTG-induction, the bacterial cells were cultured at 28ºC and incubated under sufficiently aerated growth conditions.

To evaluate protein expression, bacterial suspensions were analyzed on 12% SDS-PAGE and stained by Coomassie Blue. As a negative control, the initial culture was grown without IPTG addition. The other samples were harvested at different hours, and each sample was subjected to Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3. Results

The Live LaSota virus was provided by Razi Vaccine and Serum Research Institute (Ahvaz, Iran). Each 1ml dose of the vaccine contains at least106.0 EID50 of LaSota strain NDV. However, this dose of vaccine showed no results. Therefore, the virus was cultured in the allantoic fluid to raise the titer. Amplification of 1662 bp fragment of fusion gene was performed with an annealing step at 61ºC. The size of the RT-PCR product was used for analysis, and agarose gel electrophoresis is shown in Figure 1. To confirm the specificity of fusion gene and virus strain, SacI enzyme was used, which cuts at position 600. The results were compared theoretically with GenBank database ,and sequencing confirmed this. For determining the initial gene sequence and for creating the sticky end in the fusion gene, the pTG19-T PCR cloning vector was used.

Sequencing analysis of the constructed plasmid confirmed that there were no amplification errors in the sequence of the cloned fusion gene. For expression of the fusion protein, a fusion gene was inserted between the KpnI and HindIII restriction sites of the pET43.1a plasmid. This resulted in a recombinant plasmid, pET43.1a, containing the interested fusion gene sequence. Following the transformation and plating of the bacterial cells on LB agar containing Ampicillin (50 mg/l), the transformed coloniesand the extracted recombinant pET43.a plasmids were PCR-positive against the fusion gene-specific primers. The presence of the fusion gene in the pET43.1a plasmid was confirmed by digestion with HindIII and KpnI restriction enzymes. Digestion results showed that the fusion gene had been inserted in thepET43.1a plasmid. To examine the expression of the fusion protein, a single colony of E. coli BL21 carrying the pET43.a-F plasmid was cultured and induced with IPTG at multiple times. Although SDS-PAGE analysis showed a protein band of about 54.7 kDa, a small band was detected in non-induced culture, as shown in Figure 2. After IPTG induction, expressed proteins were harvested at different times and compared by SDS-PAGE. However, increasing the duration of induction did not have a significant effect on protein production.

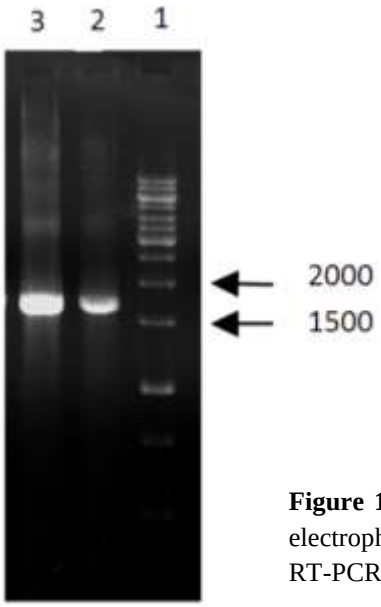

Figure 1. Electrophoresis of PCR product of the amplified fusion gene by RT-PCR on 1.5% agarose gel electrophoresis. Column 1 is 1kb marker. Columns 2 and 3 are PCR products. In this study, the length of the RT-PCR product was 1662 bp.

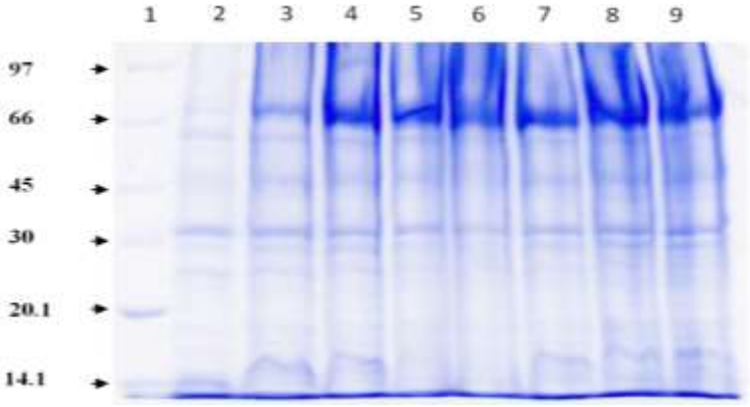

Figure 2. The protein pattern of recombinant bacteria carrying the pET43.1a plasmid was demonstrated by SDS-PAGE. Column 1(C-1) is the protein marker, C- 2 is the protein before induction, C- 3 is the protein after 2 hrs of induction, C- 4 is the protein after 4 hrs of induction, C- 5 is the protein after 6 hrs of induction, C- 6 is the protein after 8 hrs of induction, C-7 is the protein after 10 hrs of induction, C-8 is the protein after 12 hrs of inn, C- 9 is the protein after 14 hrs of induction.

4. Discussion

The genetic change of the virus can be a reason for viral variations (13).

Forty strains of NDV have been sequenced and are defined in three different lengths: 15.186 nt, 15.192 nt, and 15.198 nt (14-16). The NDV vaccine can play an important role in inducing mutations in the virus. Currently, commercial live vaccines are used to immunize chickens against Newcastle disease (13, 17). In addition, the DNA vaccine can provide sustained immunity that may help address this issue. Several studies have been conducted in this regard. It was shown that up to 40% protection against NDV could be achieved through a single vaccination with the linearized NDV F gene; however, no protection was observed with a plasmid expressing the F gene (18, 19). Also, up to 40% protection was reported by Loke et al. (20). Other studies demonstrated 73% protection in chickens against NDV using F and H proteins (1). Moreover, a high antibody response was detected in chickens vaccinated with the DNA plasmids (14). Both immune systems' cell-mediated immunity (CMI) and humoral response seem to play a major role in protecting chickens against NDV infection (21, 22). A recent study reported that the protective effect of the NDV DNA vaccine primarily involves humoral immunity rather than CMI (14).

The F gene is an important element in the infectivity of Newcastle Disease Virus (NDV). It encodes the fusion protein, which facilitates the entry of the virus into host cells. The F gene is highly immunogenic, making it a prime target for the immune system. This can result in a robust immunological response that helps avoid infection (23). A study showed that the fusion gene cloned into the PBI121 expression vector is expressed in the hairy roots of tobacco. Plant viruses can also be used as vectors although it is an effective way to produce recombinant protein, which is very time-consuming task. Additionally, the efficacy of viral vectors decreases when the size of the inserted gene exceeds a certain threshold (24, 25).

Consequently, we firstly inserted the fusion gene into the pTG19 plasmid and then into the pET43.1a vector. Recent research efforts have focused on increasing the amount of antigen produced from the DNA plasmids by changing the structure of the plasmid and using a stronger promoter (12, 26, 27). Therefore, we used a strong T7 promoter to improve fusion gene expression. Our findings offer significant benefits and advantages over conventional NDV vaccine production methods. First, using a DNA vaccine is a safer and more stable alternative to live viral vaccines, lowering the risk of accidental infection and reversion to virulence. Second, DNA vaccines can confer long-term protection, potentially eliminating the need for repeated booster vaccinations (28-30).

However, the results of this study showed that the fusion gene under the T7 promoter exhibited low expression levels. So, a weak band was observed on SDS-PAGE, indicating that the fusion protein produced by pET43.1a in E. coli was not very efficient. Because of the low expression under the T7 promoter, additional modifications to the expression system and in vivo testing are necessary to verify the vaccine's protective efficacy. This survey encourages additional research for in vivo and in vitro testing. Also, further experiments like Western blot analysis are needed to be done to confirm these findings. We selected the F genes from vaccine strains for our study owing to their safety profile. These vaccine strains have been thoroughly examined and shown to be attenuated, lowering the risk of illness in vaccinated animals. Future research might investigate the use of F genes from pathogenic viruses to potentially stimulate stronger immune response (25, 31).

The F gene is an important element in the infectivity of Newcastle Disease Virus (NDV), encoding the fusion protein for virus entry into host cells. The F gene's high immunogenicity makes it an ideal; target for the immune system. This can result in a robust immunological response, which may help avoid infection (32).

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Authors' Contribution

Study concept and design: S. P.
Acquisition of data: S. P.
Analysis and interpretation of data: S. P, H. G.
Drafting of the manuscript: S. P.
Critical revision of the manuscript for important intellectual content: H. G, MR. SAS, J F.
Statistical analysis: none.
Administrative, technical, and material support: H. G, MR. SAS, J. F.
Study supervision: H. G, MR. SAS, J. F.

Ethics

Not applicable.

Conflict of Interest

None declared.

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Data Availability

The data supporting the findings of this study are available upon request from the corresponding author.

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