Research Article

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

Abstract

Newcastle disease (ND), is a highly contagious viral disease, affecting most of the 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, amplified by the reverse transcription-polymerase chain reaction (RT-PCR). After that, it was sub-cloned in the pTG-19T vector and then in 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 findings confirmed the cloning of the fusion gene into the vector. Digestion results showed the target gene had been inserted in the pET43.1a plasmid, successfully. SDS-PAGE revealed a protein band of about 54.7 kDa. Analysis of the constructs in E.coli cells revealed the successful expression of gene inserts in vitro. Our results show 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 found and the fusion protein produced by pET43.1a in E. coli was not so efficient. This survey encourages researchers to do more studies for testing the produced protein as a vaccine in vivo and in vitro.

Keywords: DNA vaccine, NDV, cloning, *E. coli*, Fusion gene, gene expression.

1. INTRODUCTION

Newcastle disease (ND) is one of the most important diseases in poultry farms all over the world (1, 29). It causes harmful effects in domestic poultry production and the range of the virus infecting is 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 cause Newcastle (ND) disease (APMV-1). APMV-1 is the Paramyxovirus serotype that includes pathogenic NDV (4, 30). NDV is a non-segmented negative sense and single-strand RNA (5, 32). It is also classified into four pathotype categories based

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٣٧ on clinical symptoms in infected chickens, including velogenic, mesogenic,
٣٨ lentogenic, and asymptomatic (6). The genome of NDV encodes six structural
٣٩ proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion
٤٠ protein (F), hemagglutinin-neuraminidase protein (HN), and RNA-dependent RNA
٤١ polymerase (L) (7, 8, 3, 28). Two surface glycoproteins, F and HN are antigens and
٤٢ are presented on envelope (9). The protein can affect the binding of the membrane
٤٣ of the virus and, with several structural changes, leads to movements of the virus
٤٤ particles into the cells that are necessary for infection (8). Protein F1 is synthesized
٤٥ as an inactive protein F0 and cleaved into F1 and F2 polypeptides, which activates
٤٦ the production of fusion protein. This virus cleavage is necessary for becoming
٤٧ infectious. Protein F is a key factor for disease and immunity. The amino acid
٤٨ sequence of F protein along with host protein proteases is a molecular basis for the
٤٩ ND pathogenesis (10, 11).

٥٠ Chick vaccination is carried out using inactive or live vaccines which are
٥١ temporary. In addition, these vaccines are expensive and time-consuming.
٥٢ Extensive and permanent immunity is produced by DNA vaccines. Importantly,
٥٣ DNA vaccines affect only on humoral immunity. It has been used for various
٥٤ diseases. Studies have shown effective immunity of DNA vaccine against many
٥٥ infections and autoimmune diseases, as well as allergies and cancers (12).

٥٦ DNA vaccine has been studied for immunization against NDV strains. Therefore,
٥٧ the present study was conducted to determine the expression of the fusion gene in
٥٨ the expression pET43.1a vector (27). The goal of this study was to develop a DNA
٥٩ vaccine using a fusion gene from the Newcastle virus.

٦٠ ## 2. MATERIAL AND METHOD

٦١ ### 2.2. Virus and viral RNA isolation

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

٦٨ ### 2.3. Primer Design

٦٩ The primers were designed based on the fusion gene sequence in GenBank with
٧٠ accession number AY845400 which was related to the NDV LaSota strain. The F-
٧١ specific primers 5'-ATGGGCTCCAAACCTTCTACC-3' and 5'-

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٧٢ TTGTAGTGGCTCTCATCTGATC-3' were used to amplify the region of interest.
٧٣ The assessment of melting temperature and secondary structure of designed
٧٤ primers was conducted using primer3software.

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٧٦ 2.4. Amplification of the viral gene

٧٧ Total RNA was extracted from the 140µl sample allantoic fluid. The cDNA was
٧٨ produced according to the revertAidTM first-strand cDNA synthesis kit protocol
٧٩ (Fermentas, Canada). Each reaction of RT-PCR included hexamer primer, reverse
٨٠ primer, and 2µl of the cDNA (50ng cDNA). The reaction was carried out in a final
٨١ volume of 25µl containing 25mM10x PCR buffer, 2.5mM MgCl2, 0.2mM dNTP
٨٢ mix, 0.5U supperTaq, and each primer concentration 6.25mM, with following
٨٣ cycling conditions: 10min at 95ºC (hot start), following by 40 cycles at 94ºC for
٨٤ 3min, 60ºC or 62ºC for 1min, 72ºC for 3 min and terminal extension for 20min.

٨٥ 2.5. Enzyme Digestion and Sequencing

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

٩٢ 2.6. Gel Extraction

٩٣ The RT-PCR product was extracted by using a Silica Bead DNA Gel Extraction kit
٩٤ (Fermentas, Canada). Then, it was purified to clean up the extra material to do TA
٩٥ cloning.

٩٦ 2.7. Cloning fusion gene in pTG19

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

٩٩ 2.8 Construction of expression plasmids

١٠٠ DNA Restriction Enzymes, including HindIII and KpnI, were used for
١٠١ Transferring a fragment of cloned DNA from pTG19 (Vivantis, Malaysia) to the
١٠٢ pET43.1a (Invitrogen, USA) vector. These endonucleases cut both plasmid
١٠٣ pET43.1a and fusion genes. Ligation of the Fusion gene with the vector was
١٠٤ performed by ligase enzyme. The standard method for making the bacteria

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١٠٥ permeable to DNA involves treatment with calcium ions. Then, treatment with
١٠٦ Ca+2 was followed by heat shock treatment at 42oC for the process of
١٠٧ transformation for 90 sec. plasmids also contained AmpR as selectable markers.
١٠٨ So, resistance to ampicillin was provided. Thus, the bacteria are grown on a
١٠٩ medium containing the antibiotic only if the bacteria contain the plasmid.
١١٠ Transformed cells were grown in Luria-Bertani (LB) medium containing
١١١ ampicillin and the target DNA was amplified. Then, it was confirmed by clony
١١٢ PCR.

١١٣ Plasmid extraction and purification were performed by the QIAprepMiniprep kit
١١٤ (QIAGEN, Germany). Plasmid DNA isolated from transformed cells was digested
١١٥ by HindIII and KpnI. The pattern of the fragments on the gel electrophoresis can
١١٦ be indicated if the plasmid contains the expected size insert. As the expression of
١١٧ the pET43.1a vector in the E. coli strain, DH5α is low; so, for maintaining this
١١٨ vector, DH5α was used. Thus, to express the fusion gene, pET43.1a was
١١٩ transformed into E. coli, BL21.

١٢٠ 2.9. Preparing total protein and expression fusion gene

١٢١ The transformed bacteria were cultured in LB media containing ampicillin and
١٢٢ then incubated at 37°C. After the cell culture period, an OD600 of 0.4 to 0.6,
١٢٣ Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of
١٢٤ 1 mM for expression protein in E. coli. After IPTG-induction, the bacterial cells
١٢٥ were cultured at 28ºC and incubated under sufficient aerated growth conditions. To
١٢٦ evaluate the expression protein, bacterial suspensions were analyzed on 12% SDS-
١٢٧ PAGE and stained by Coomassie Blue. As a negative control, the initial culture
١٢٨ was cultured without IPTG addition. The others were harvested at different hours.
١٢٩ Then, each sample was subjected to Sodium dodecyl-sulfate polyacrylamide gel
١٣٠ electrophoresis (SDS-PAGE).

١٣١ 3. Result

١٣٢ The Live LaSota virus was provided by the Razi Research Vaccine and Serum
١٣٣ Institute (Ahvaz, Iran). Each 1ml dose of the vaccine contains at least106.0 EID50
١٣٤ of LaSota strain NDV. However, this dose of vaccine showed no result. Therefore,
١٣٥ the virus was cultured in the allantoic fluid to raise the titer. Amplification of 1662
١٣٦ bp fragment of fusion gene was performed with an annealing step at 61ºC. The size
١٣٧ of the RT-PCR product was used for the analysis of RT-PCR products. Agarose
١٣٨ gel electrophoresis is shown in Figure 1. To confirm the specification of fusion
١٣٩ gene and virus strain, SacI enzyme was used and it cuts at position 600. Then, it
١٤٠ was compared theoretically with GenBank. Also, the result of sequencing
١٤١ confirmed that. For determining the initial gene sequence and also for creating the

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sticky end in the fusion gene, the pTG19-T PCR cloning vector was used. Sequencing analysis of the constructed plasmid confirmed that there was no amplification error in the sequence of the cloned fusion gene. To the 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 that contained the interested fusion gene sequence. Following the transformation and plating of the bacterial cells on LB agar containing Ampicillin (50 mg/l), the transformed colonies, and 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 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 multiple times. Although SDS-PAGE analysis showed a protein band of about 54.7 kDa, a small band detected in non-induced culture, as it was shown in Figure 2. After IPTG induction, expressed proteins were harvested at different times and compared by SDS-PAGE. However, it was found that increasing in time of induction did not affect protein production.

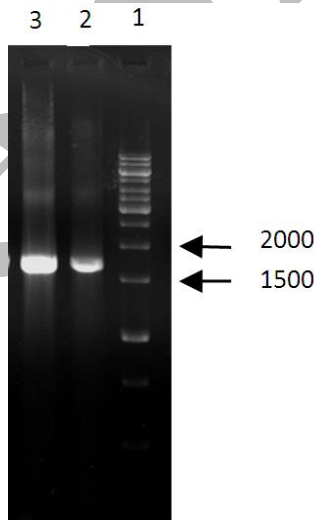

Figure 1. Electrophoresis of PCR product of the amplified fusion gene by RT-PCR on 1.5% agarose gel electrophoresis. Column 1 is 1kb marker. Columns 2 and 3 are PCR products. In this study, the length of the RT-PCR product was 1662 bp.

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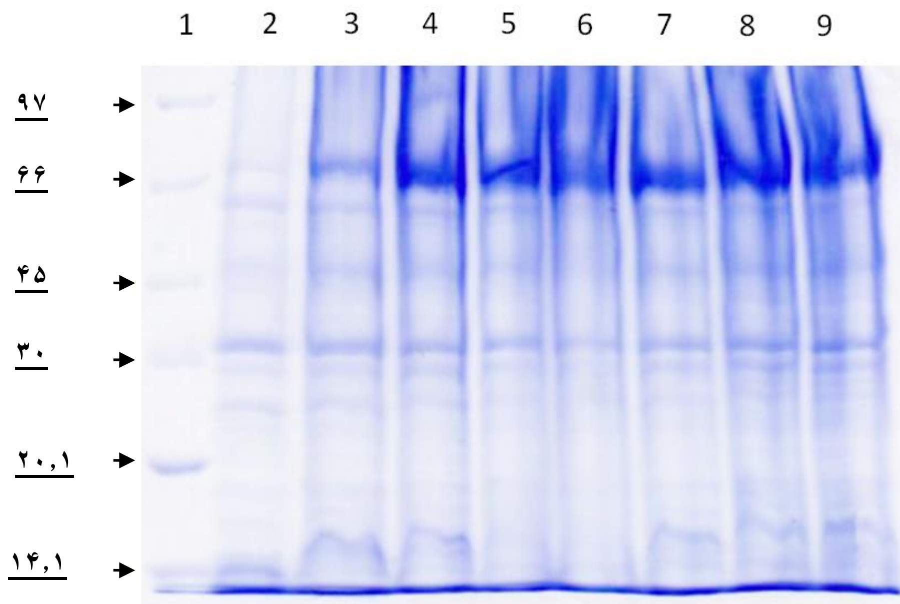

Figure. 2. The protein pattern of recombinant bacteria carrying the pET43.1a plasmid was demonstrated by SDS-PAGE. Column 1(C-1) is the protein marker, C- 2 is the protein before induction, C- 3 is the protein after 2 hrs of induction, C- 4 is the protein after 4 hrs of induction, C- 5 is the protein 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 changes (13). Forty strains of NDV have been sequenced that are defined in three different lengths 15.186 nt, 15.192 nt, and 15.198 nt (14, 15, 16). The NDV vaccine can play an important role in making changes to the virus. Now commercial live vaccines are used to immune chickens from Newcastle disease (17, 13). In addition, the DNA vaccine can provide a sustained immunity that can solve this problem. Studies have been conducted in this regard. It was shown that up to 40% protection against NDV through a single vaccination with the linearized NDV F gene; however, no protection with a plasmid expressing the F gene was obtained (18, 19). Also, up to 40% protection was reported by Loke et al. (20). Studies showed that there is 73% protection by F and H proteins in chickens against NDV (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 is on 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 allows the virus to enter host cells. The F gene is highly immunogenic, therefore it is a target for the immune system. This can result in a robust immunological response, which can help avoid infection (23). A study showed that the fusion gene cloned in 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. However, the production of viral vectors is a very time-consuming task. Another disadvantage of viral vectors is that the length of insertion genes larger than the threshold size will reduce the efficacy of the vector (24, 31). So, we inserted the fusion gene first into the pTG19 plasmid and then into the pET43.1a vector. New research efforts aimed at increasing the amount of antigen produced from the DNA plasmids. This can be achieved by changing the structure of the plasmid and using a stronger promoter (25, 12, 26). Therefore, we used a strong T7 promoter to improve fusion gene expression. Our findings provide significant benefits over conventional NDV vaccine production strategies. To begin, using a DNA vaccine is a safer and more stable alternative to live viral vaccines, lowering the possibility of accidental infection and reversion to virulence. Second, DNA vaccines can confer long-term protection, potentially removing the need for repeated booster immunizations. The results of the present study showed that the fusion gene under the T7 promoter has a low expression level. So, a weak band appeared by SDS-PAGE and it was found that the fusion protein produced by pET43.1a in E. coli was not very efficient. because of the results showed a low level of expression under the T7 promoter, additional modification of the expression system and in vivo testing are required to verify the vaccine's protective effectiveness. This survey encourages researchers to do more studies for testing the vaccine in vivo and in vitro. Also, more tests like western blot need to be done to confirm these findings. We picked the F genes of vaccination strains for our study owing to their safety profile. 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 a higher immune response. The F gene is an important element in the infectivity of Newcastle Disease Virus (NDV). It encodes the fusion protein, which allows the virus to enter host cells. The F gene is highly immunogenic; therefore, it is a target for the immune system. This can result in a robust immunological response, which can help avoid infection.

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Conflict of Interest. None declared.

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Authors' contribution

Sahere Parvas developed the original idea and the protocol, abstracted and analyzed data, wrote the manuscript, and is guarantor. Interpretation of findings by Sahereh parvas, Hamid Galehdari done. Hamid Galehdari, Masoud Reza Seyfi Abad Shapouri and Jamal Fayazi contributed to the development of the protocol, abstracted data, and prepared the manuscript.

1- Study concept and design: Sahere Parvas

2- Acquisition of data: Sahere Parvas

3- Analysis and interpretation of data:Sahere Parvas, Hamid Galehdari

4- Drafting of the manuscript: Sahere Parvas

5- Critical revision of the manuscript for important intellectual content: Hamid Galehdari, Masoud Reza Seyfi Abad Shapouri and Jamal Fayazi

6- Statistical analysis: none

7- Administrative, technical, and material support: Hamid Galehdari, Masoud Reza Seyfi Abad Shapouri and Jamal Fayazi

8- Study supervision: Hamid Galehdari, Masoud Reza Seyfi Abad Shapouri and Jamal Fayazi**Ethics**

Ethics

Not applicable.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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