Iranian Journal of Virology 2021;15 (2): 9-14
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Original Article

Cloning and Poor Expression of S1 Gene of Infectious Bronchitis Virus (Genotype Variant 2) in pET28a

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Abstract

Background and Aims: Avian infectious bronchitis (IB) is an economically important, highly contagious, acute, upper-respiratory-tract disease of chickens and other bird species, caused by the avian coronavirus infectious bronchitis virus (IBV). The aim of this study was the expression of the S1 gene protein genotype variant-2 in pET28a. In vitro protein expression is an important method for obtaining many viral proteins to investigate their protective and biological properties. Since the S1 glycoprotein of the infectious bronchitis virus induces virus-neutralizing antibodies, it is an appropriate candidate for producing a recombinant vaccine against infectious bronchitis disease.

Materials and Methods: In this study, the S1 gene fragment of infectious bronchitis virus genotype IS/1494/06 was amplified using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and was inserted into the pTG19-T vector. The construct was subcloned into pET28a and inserted into E.coli BL21 competent cells.

Results: The insertion was proved by PCR analysis, and isolation of the S1 gene from the construct was carried out. Finally, it was sequenced. The SDS-PAGE assay showed that a protein about 60 kDa was expressed, but the western blot assay did not confirm the expression of the S1 protein.

Conclusion: S1 protein did not express well. To obtain properly expressed proteins, we suggest trying different expression vectors or choosing smaller important portions of the S1 gene.

Keywords: *Infectious bronchitis virus*; Cloning; *Escherichia coli*; Recombinant protein

Introduction

Infectious Bronchitis Virus is currently considered one of the most economically important viral pathogens in the poultry industry. Avian Infectious bronchitis virus (IBV) is a group 3 member of the genus Gammacoronavirus, from the Coronaviridae family and Nidovirales order [1].

It is a pleomorphic enveloped virus with club-shaped surface spikes on the surface of the virion, and its genome consists of a single-stranded positive-sense RNA approximately 27.6 kb, which encodes four structural proteins, including the spike glycoprotein (S) small membrane protein (E), membrane glycoprotein (M), and nucleocapsid protein (N) its 13 open reading frames are organized as UTR5′ –Pol-S-3a-3b-E-M-4b-4c-5a-5b-N-UTR3′[2].

IBV spike glycoprotein is a determinant of cell tropism [3], and it is posted transitionally cleaved into two glycoproteins, spike 1 and spike 2.

Following IBV first description by Schalk and Hawn in the USA in 1930[4], many serotypes or variants have been classified worldwide, against which little or no cross-protection exists [5, 6]. In Iran, IBV Mass type was first reported by Aghakhan et al. in 1994 [7].

Variant-2, 793/B, Mas IBV genotype, QX, IS/720 like, and IR-1 have been reported to be circulating in Iran in recent years [8, 9]. The prevalence of IS/1494-like IBV reached 70% between 2015 and 2017, becoming a dominant

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IBV genotype in Iran [10]. This study was conducted to provide a recombinant protein in the expression vector pET28a, which could be used to generate new vaccines.

Methods

Bacterial strain and virus: Escherichia coli stains Top 10 F' and BL21 (DE3) were obtained from Pasteur Institute and were cultured in Luria-Bertani agar. Virus genotype variant 2 of IBV was isolated from broiler flocks in Iran and ten times propagated in specific pathogen-free (SPF) eggs.

Viral RNA extraction: According to the manufacturer's protocol, RNA was extracted from allantoic fluid with the CinnaPure RNA Extraction Kit (SinaClon, Iran). The extracted RNA was stored at -70 °C.

Primer design: The primers were designed by primer 3 program (Forward: 5' GAATTCT TGGTGAAGTTCACTGTTTATAGTG 3' and Reverse: 5' CTTACGTCGTGTACGTGTAC 3') and were used for amplification of the full-length S1 glycoprotein gene.

Both primers were designed for two specific restriction endonuclease enzyme sites based on the expression vector cloning site.

cDNA synthesis: For cDNA synthesis, 1µL (0.2 µg) of random hexamer primer (SinaClon, Iran) was added to 10 µL of extracted RNA, and the mixture was incubated at 65°C for 5 minute and 5°C for 1 minute. 11 µL of a master mix containing 2.25 µL of DEPC-treated water (SinaClon, Iran), 2 µL of dNTP mix (SinaClon, Iran), 0.25 µL of RiboLock RNase Inhibitor (Thermo Fisher Scientific, USA), 0.5 µL of Revert Aid Reverse Transcriptase (Thermo Fisher Scientific, USA), and 4 µL of 5X RT reaction buffer were added to each tube, resulting in a final volume of 20 µL. The mixture was then incubated at 25 °C for 5 minutes, 42 °C for 1 hour, and 95 °C for 5 minutes. The cDNA was stored at -20 °C.

Amplification of the complete S1 gene: The RT-PCR was performed in Gradient Thermal Cycler with 3 µL of cDNA as the template in a total 20 µL volume of a reaction containing 1 µL of each primer and 2 µL DNase free water, and 13 µL of Taq DNA Polymerase Master Mix (Ampliqon, Denmark). The reaction was carried out at 95 °C for 4 min followed by 35 cycles of 95 °C for 1 min, 47 °C for 1min, and 72 °C for 1 min; the last step was performed at 72 °C for 7 min.

Insertion of complete S1 gene into the pTG10-T vector: The PCR product of the S1 gene was primarily cloned into cloning pTG19-T vector by TA cloning system according to the manufacturer's protocol of the kit (SinaClon, Iran) .

Transformation of E.coli Top 10F' cells and confirmation of insertion: The cloned pTG19-T vectors were transformed into Escherichia coli Top 10F' competent cells. The transformation was confirmed with PCR using forward and reverse primers of the S1 gene to test the insert's existence and approximate size.

S1 gene ligation to pET-28a expression vector and transformation of BL21 competent cells: The pTG19-T cloned vectors were extracted from positive E.coli Top 10F' cells using the AccuPrep® Plasmid Extraction Kit (Bioneer, South Korea) and digested with EcoRI (Fermentas) and HindIII (Fermentas) restriction enzymes. DNA fragments were separated with electrophoresis on 1% agarose gel.

The DNA band with an approximate size was excised from the gel and purified using the AccuPrep® Gel Purification Kit (Bioneer, South Korea). Subsequently, the expression vector pET28a was digested with the same restriction enzymes and purified with AccuPrep® PCR Purification Kit (Bioneer, South Korea). The concentration of purified inserted S1 gene and digested pET28a were measured by Eppendorf nanodrop.

After mixing in a 3:1 molecular ratio, the purified products were ligated by T4 DNA ligase (Fermentas) and subsequently transformed into competent E.coli BL21 cells. To confirm the integrity of the inserted S1 gene, we performed a direct colony PCR and extracted the recom-binant plasmid pET28a-S1; and it was digested with restriction enzymes, and results were observed 1% agarose gel.

SDS-PAGE analysis: The BL21 strain containing the recombinant plasmid was grown overnight with shaking at 37 °C. The overnight bacterial cultures were diluted in a scale of

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1:50 with fresh LB media and incubated at 37 °C with shaking to reach 0.5 optical density. After that, the production of S1 protein was induced with 1M IPTG and was incubated for a further 20- 22 hours. The cells were collected with centrifugation and examined by SDS-PAGE.

Western blot analysis: SDS-PAGE was used to separate expressed proteins. After that, they were transferred onto PVDF western blotting membrane at 15V for overnight. They were then blocked for 120 min with 4% skimmed milk at 37 °C and then washed with Tris-Buffered Saline (TBS) three times. After that, they were incubated with 570 µL Poly-His antibody conjugated with HRP (diluted 1:1000 in TBST containing ??% 17 mL skim milk) for 60 min at 37 °C. The membrane was washed with TBS three times, and then S1 Proteins were visualized with 3, 3'- Diaminobenzidine (DAB).

Results

The S1 gene of IBV genotype variant 2 was successfully amplified by RT-PCR using designed primers. Analysis of RT-PCR product on 1% agarose gel determined a sharp band with an approximate size of 1600-bp (Fig. 1).

Fig. 1. RT-PCR amplification of S1 gene. M: 1kb DNA marker, lane 1: negative control, lane 2: S1 gene positive sample.

Purified PCR products were then TA cloned into the pTG19-T vector, and the recombinant plasmids were amplified in competent E. coli strain Top10F'. Colony PCR was carried out on the recombinant clones and confirmed the existence and correct size of the inserted S1 gene (Fig. 2).

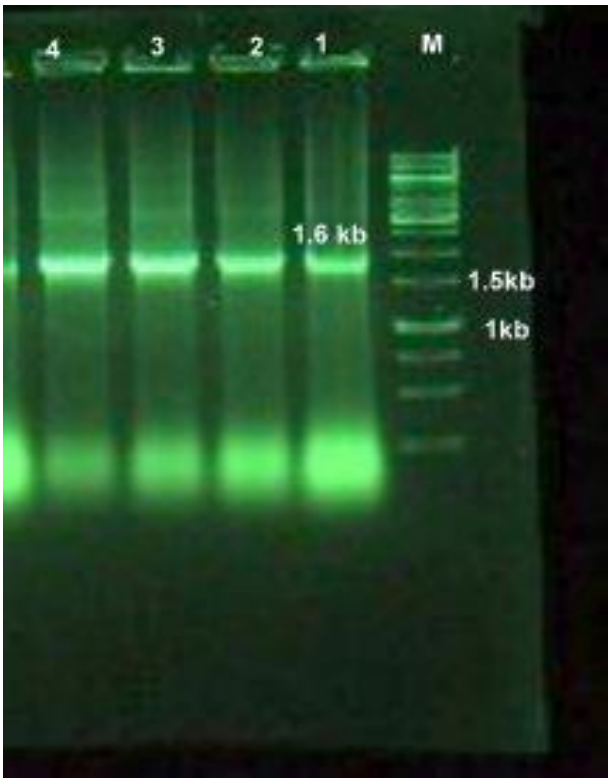

Fig. 2. Colony PCR of transformed S1 gene in pET28a vector with s1 and T7 primers. Lane M: marker 1kb, lanes 1, 2, 3, 4: amplified S1 gene.

The extracted recombinant constructs were digested with restriction enzymes EcoRI and HindIII. The digested product consists of two DNA fragments of about 3000 and 1600-bp relating to the linearized vector and the insert, respectively. The inserted S1 gene extracted from the agarose gel was subcloned into the pET28a expression vector. Recombinant plasmids were extracted from E.coli strain BL21. PCR amplification of recombinant plasmid with S1 primers and its digestion with EcoRI and HindIII were used to confirm the inserted S1 gene's size. Results acquired from digestion and colony PCR confirmed the cloning of the S1 gene (Fig. 2 and 3).

Recombinant expression vector pET28a-S1 was detected by adding 1M IPTG to collect recombinant S1 protein at different times ranging from 0, 4, 8, 12, and 22h. After IPTG inoculation, the results showed that a protein of 60 kDa was expressed 22h after addition to the culture (Fig. 4). But, the western blot technique

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did not confirm the expression of the S1 protein.

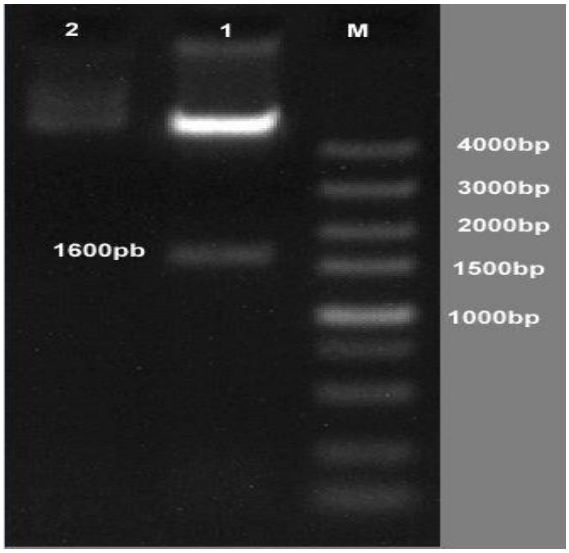

Fig. 3. Agarose gel electrophoresis of pET28a-S1. M (marker: 1 kb), Lane1: pET28a-S1 digested with EcoRI and HindIII, Lane 2: pET28a undigested.

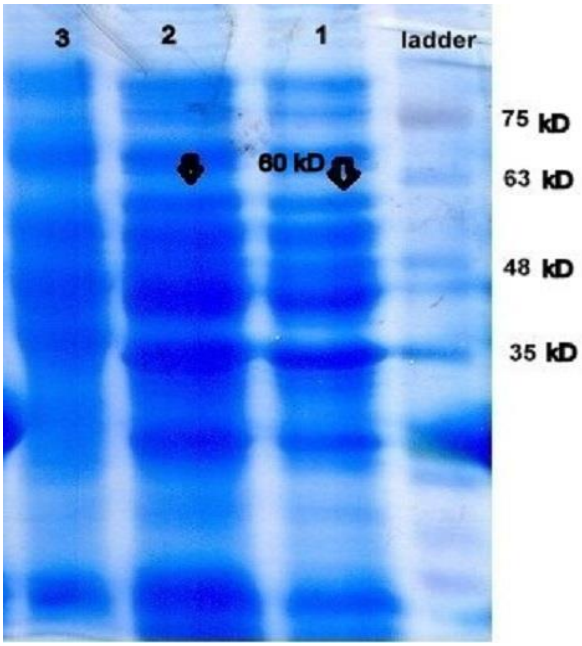

Fig. 4. SDS-PAGE gel results. Ladder (pre-stained protein ladder), lanes1, 2: samples collected at 22 h, lane 3: sample collected at 0 h.

Discussion

In this paper, we used an important genotype of IBV, variant2, recently isolated from broiler flocks in Iran [8].

For the first time, we amplified and cloned the full-length S1 gene of IBV genotype variant-2 to express S1 glyco-protein as a candidate of the subunit vaccine in the E. coli expression system. S1 glycoprotein in IBV induced serotype-specific and cross-reactive antibodies [11].

In the Middle East, variant-2 was first reported in Israel (unpublished data) and then isolated from other countries such as Libya, Egypt, Turkey, Iran, Iraq, Oman [8, 12-16]. Up to recent years, Varian-2 IBV has been limited to the Middle East countries, but lately, it was appeared in Europe [2]. Considering this issue, the specific and safe vaccine against genotype Variant-2 is necessary for prevention and control strategies.

In chickens primed with the attenuated H120 vaccine strain, the rS1 glycoprotein induced 83% protection of the kidney after two immunizations, and Haemagglutination-inhibition titers were also increased in chickens immunized with the rS1 glycoprotein after three immunizations, and significantly higher titers were detected after challenge [17].

protection of the recombinant baculovirus co-expressing S1 and N proteins was better than that of recombinant baculoviruses monoexpressing the S1 or N protein [18].

The complete sequence of the S1 gene (1617-bp) of infectious bronchitis virus serotype 793/B was successfully cloned in pTZ57 plasmid and transferred to E. coli- XL1 blue bacterium, and the construct was subcloned into pPICZB vectors transferred to P. past-oris Km71. P. pastoris expression system was used successfully for the produc-tion of recombinant heterologous proteins [19].

In 2018, Zeshan et al. clone and expression of truncated spike glycoprotein IBV in E. Coli using pET 32a (+) vector and suggest that it can be used as antigen to detect antibodies against IBV [20].

Ghani et al. [21] cloned and amplified the S1 gene of vaccine strain 4/91 of IBV and detected 33kD protein in nitrocellulose western blotting membrane for designation of ELISA detection kit. Additionally, Asadpour et al [22]. successfully obtained the recombinant S1 glycoprotein gene of IBV serotype 793/B in secretory Pichia pastoris for use in subunit vaccine.

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The single dose of a recombinant fowl adenovirus expressing the S1 gene from IBV was adequate to protect chickens [23].
Vaccination with S1 glycoprotein of M41 strain expressed in the E. coli system induced 40% protection, while vaccination with recombinant N protein provided no protection [24].
Chicken immunized orally and intra-muscularly with transgenic potato expressing S protein, generated the detectable levels of serum neutralizing antibodies and were properly protected against challenge against a virulent IBV strain [25].
Co-administration of the DNA vaccine containing chicken GM-CSF in an expressing plasmid, together with the pVAX-S1 plasmid, significantly enhanced the level of specific antibody compared to administration of the pVAX-S1plasmid alone [26].

Conclusion

Based on our results, the S1 glycoprotein was truncated after expression and did not express well. For obtaining the best results, the use of a column with a high capacity to elicit pure protein for carrying out in the western blotting technique is suggested. Additionally, we can use another type of pET expression vector and another expression system.

Acknowledgment

We Acknowledge the University of Tehran for providing the project grant.

Conflict of interest

The authors have no conflict of interest to declare.

Funding

This study was financially supported by University of Tehran.

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