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Full Length Research Paper

Molecular cloning of S1 glycoprotein gene of infectious bronchitis virus (IBV) serotype 793/B in secretory *Pichia pastoris* vector

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***In vitro* protein expression is an important method of obtaining large amounts of viral proteins to investigate their biological properties. The S1 glycoprotein of infectious bronchitis virus, due to its effective immune-dominant role is an appropriate candidate for production of recombinant vaccine against infectious bronchitis disease. In this study, the S1 gene fragment of infectious bronchitis virus strain793/B was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR) and purified. It was then cloned into pPICZαA a secretory expression vector of *Pichia pastoris*. The insertion was proved by PCR analysis and isolation of gene from construct by restriction enzymes and finally, it was sequenced. After the expression of S1 gene in *P. pastoris* expression system, it was found that it could be used in the production of recombinant vaccines against infectious bronchitis disease.**

Key words: Infectious bronchitis, S1 glycoprotein, cloning, *Pichia pastoris*.

INTRODUCTION

Infectious bronchitis virus (IBV), one of the prototype of the family, Coronaviridae, is the causative agent of an acute, highly contagious respiratory, renal and urogenital disease characterized by high mortality rates in affected flocks and affecting both production and egg quality.

The IBV genome encodes three major structural proteins; the nucleo - capsid, membrane and spikes(S) proteins. The S protein is cleaved to the N-terminal S1 and C-terminal S2 glyco-proteins during post-translational process. The S1 glycoprotein is responsible for attachment to the host cellular membrane and induces virus-neutralizing and hemagglutination–inhibiting antibodies (Cavanagh et al., 1986; Moore et al., 1997; King and Cavanagh, 1991).

Although the S1 subunit is highly variable (Cavanagh et al., 1988, 1992), previous studies demonstrated that it is an excellent candidate for developing subunit vaccines against infectious bronchitis (IB). The corresponding monoclonal antibodies (m Abs) were main tools for identifying functional epitopes and for developing the diagnostic reagents (Wang et al., 2002; Johnson et al., 2003; Kapczynski et al., 2003; Zhou et al., 2003).

The commercial live and inactivated vaccines that have been used to control IBV-associated disease in chickens (Cavanagh et al., 1997) have some disadvantages. Inactivated vaccines are safe but are costly and less effective than live attenuated vaccines, while attenuated vaccines maybe associated with the emergence of variant strains of the virus (Wang et al., 1993; Moore et al., 1997; Zhou et al., 2002). The live vaccine strains are also spreading in the field (Meulemans et al., 2001). So we need vaccines with higher efficacy and fewer side effects.

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Abbreviations: IBV, Infectious bronchitis virus; **IB,** infectious bronchitis; **RT-PCR,** transcriptase-polymerase chain reaction; **ELISA,** enzyme-linked immunosorbent assay.

Table 1. List of PCR primers.

S/N	Primer	Sequence(5' to 3')
1	For-S1	GCCGGAATTCATGTTGGGCAAACCGCT
2	Rev-S1	GGCGCGGCCGCACGTCTAGAGCGACGTGTTCC

In previous studies, some expression systems, such as insect cells, *Pichia pastoris* and potato were used to express S1 (Dai et al., 2003; Huang et al., 2003; Zhou et al., 2003, 2004). In this study, we cloned the S1 glycoprotein gene of infectious bronchitis virus serotype 793/B in pPICZαA a secretory expression vector of *P. pastoris* (Invitrogen) to contribute in developing IB subunit vaccine.

MATERIALS AND METHODS

Virus and viral RNA purification

The IBV was inoculated in 11 days-old specific-pathogen-free chicken embryonated eggs at 37°C for 48 h. Then the allantoic fluid was harvested from eggs and clarified by low-speed centrifugation.

Viral RNA was extracted from EM-confirmed virus- positive allantoic fluid samples, using high pure viral nucleic acid purification kit (Roche), according to the manufacture instruction and the RNA was stored at -70°C. So it was applied as a template for S1 gene amplification.

Reverse transcriptase-polymerase chain reaction (RT- PCR) amplification of S1 gene

The oligonucleotides primers were used in RT-PCR designed according to the S1 sequence available in the gene bank database (Accession number: AF093793). Also multiple cloning site of pPICZαA a secretory plasmid of *P. pastoris* expression system (Invitrogen) was applied. The sequence of RT- PCR primers is shown in Table 1. RT- PCR was performed by using one step RT-PCR kit (one step- titan kit, Roche) with a thermal cycling program of 45°C for 45 min, to prepare the cDNA and followed by 94°C for 3 min, 35 amplification cycle of 94°C for 1 min, 59°C for 2 min and 68°C for 2 min. The final elongation step was 10 min at 68°C.

Cloning of S1 gene into pPICZα A

The PCR- product of S1 gene was first cloned in pTZ57R vector of T/A cloning system based on the manufacture protocol of the kit (Ins T/A clone TM PCR product cloning kit , fermentas) then white bacterial colonies were selected and plasmids were extracted using high pure plasmid isolation kit (QIA Gene) according to the manufacturer and digested with *NOT I* (MBI fermentas) and ECOR I (MBI fermentas). These were confirmed with PCR using forward and reverse primers of S1 gene to test the existence and approximate size of the insert (S1 gene), and it was sent to MWG, Germany for DNA sequencing using universal M13 forward and reverse primers. The construct, pTZ57R- S1, was then digested by *Not I* and ECOR I restriction enzymes and DNA fragments were separated on 1% agarose gel. The DNA band with an approximate size was excised from the gel and purified by using high pure PCR product purification kit (Roche) according to the manufacture protocol. Subsequently, the secretory expression vector pPICZαA was digested with the same two restriction enzymes and purified as described above. The concentrations of purified product were measured by Ependorf nanodrop. After mixing in a 3:1 molecular ratio, the purified products were ligated by T4 DNA ligase at 16°C overnight and then transformed into *Escherichia coli* Top10F competent cells. Transformants selected on low-salt Luria-Bertani (LB) plates containing 25 µg/ml zeocin by digestion of purified plasmids and direct colony PCR.

In order to confirm the integrity of the insert (S1 gene) and that no errors have taken place at the ligation sites, the construct pPICZαA-S1 extracted from bacterial colonies, was sent to MWG, Germany for DNA sequencing by vector specific primers (AOX 1 forward and reverse primers) and the sequence of inserts were verified. The sequence data obtained were compared with sequence of the S1 gene of IBV strain 793/B (accession number: AF093793).

RESULTS

The S1 gene of infectious bronchitis virus strain 793/B was successfully amplified in RT- PCR with designed primers. Analysis of PCR product on agarose gel revealed a sharp band with an approximate size of 1.7 kb (Figure 1). Purified PCR product was then ligated into T/A cloning vector pTZ57R/T and the recombinant plasmids were amplified in competent *E. coli* strain Top 10F. The sequences of inserts were verified by sequencing. Then purified recombinant plasmids were digested with restriction enzymes EcoRI and NotI. As demonstrated in the Figure 2, digestion produced two DNA fragments of about 3000 and 1700 bp corresponding to linearized vector and insert.

The insert purified from agarose gel was subsequently cloned into the pPICZαA expression vector of *P. pastoris* (Figure 3). PCR amplification of recombinant pPICZαA with S1 primers and its digestion with EcoRI and NotI confirmed the correct size of the insert (Figure 4). To confirm the identity of the insert, sequence of S1 gene inserted in pPICZαA was determined by sequencing using vector specific primers and when compared with the original sequence of S1 gene of IBV strain 793/B, did not show significant differences between them.

DISCUSSION

In this study, we amplified and cloned the S1 glycoprotein gene of the strain 793/B of IBV, in order to express S1 glycoprotein as a candidate of subunit vaccine in *P. pastoris* expression system. This is the first report of cloning of the S1 glycoprotein gene of this IBV strain that is regularly used in vaccines in Iran. However, vaccination with live attenuated and inactivated IBV is performed in Iran

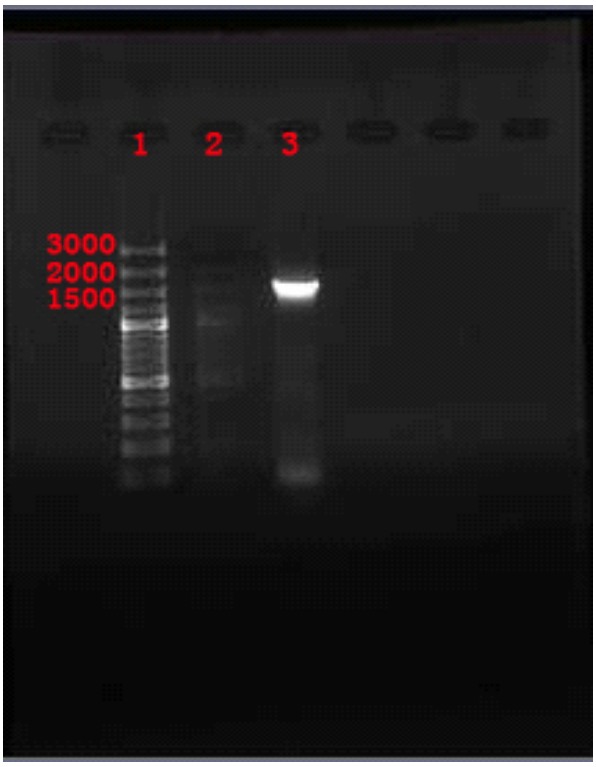

Figure 1. PCR product of amplified S1 gene. Lane 1: Ladder (100 bp); lane 2: negative control; lane 3: amplified S1 gene (about 1700 bp).

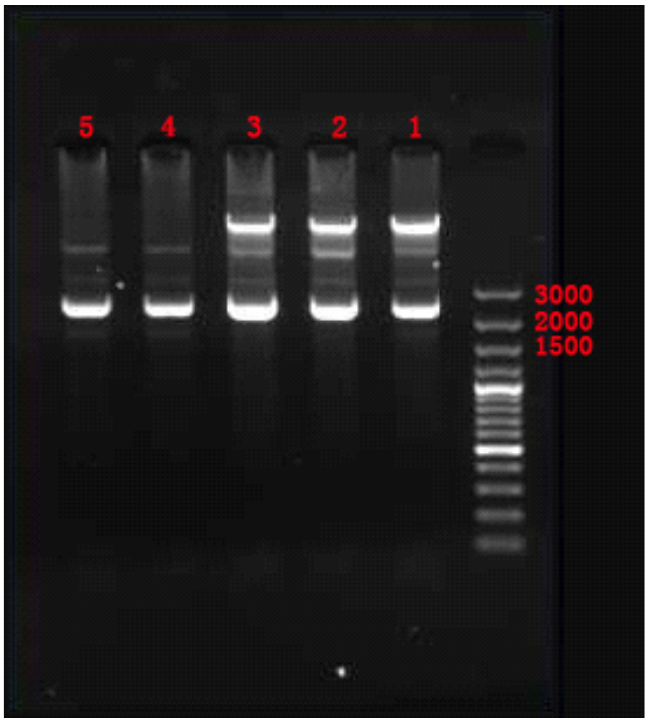

Figure 3. Construct (S1-pPICZαA) agarose gel electrophoresis. Ladder (100 bp); lane 1 - 5 construct (S1-pPICZαA).

Figure 2. Agarose gel electrophoresis of construct (S1-pTZ57R) digested by EcoRI and NotI. Lane1: Ladder (100 bp); lane 2: digested construct.

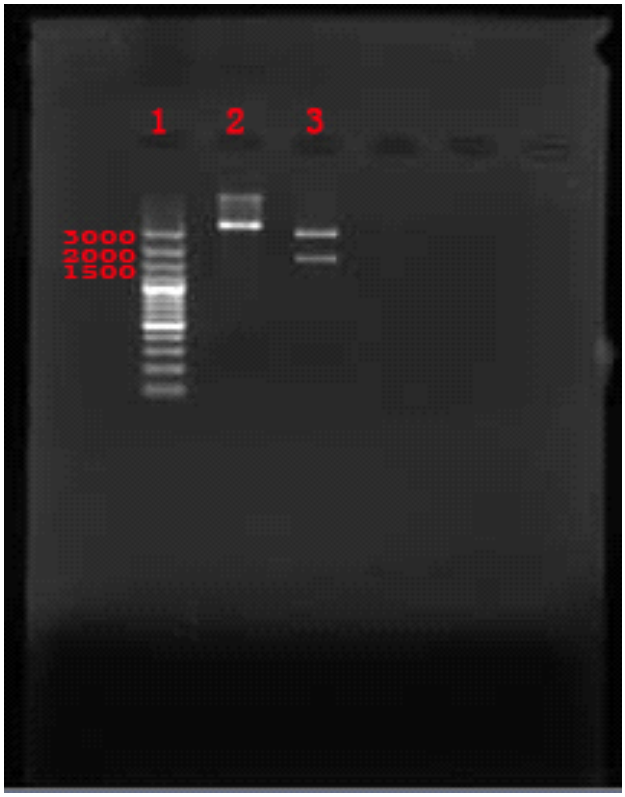

Figure 4. Agarose gel electrophoresis of construct S1-pPICZαA digested by EcoRI and NotI. Lane 1: ladder (100 bp); lane 2: undigested construct; lane 3: digested construct.

to protect broiler chicken flocks against infectious bronchitis; the disease has remained as a serious problem and causes significant financial losses. In fact, in all over the world, chicken infectious bronchitis has a significant economic impact in poultry industry.

Although other virus polypeptides play a role in protection and recovery from infection in the chicken, the S1 glycoprotein is an excellent candidate for the development of the novel IBV vaccines. The S1 protein is a structural glycoprotein and the glycosylation modification is important for its activity. *P. pastoris* is a eukaryotic expression system with high efficiency and proteins expressed in this system can get appropriate post–translational modifications, including glycolsylation. Therefore, the *P. pastoris* system is more suitable for the expression of the S1 protein *in vitro* than *E. coli* (Grinner and Tschopp, 1989; Kukuru Zinska et al., 1987; Romanos et al., 1992). In a previous report, mice which were vaccinated with the S1 glycoprotein of IBV, expressed in recombinant vaccinia virus, produced antibodies that recognized the S antigen in an enzyme-linked immunosorbent assay (ELISA) and neutralized IBV infectivity (Tomley et al., 1987).

Song et al. (1998) have shown that recombinant S1 glycoprotein expressed in baculovirus induced protective immunity against a challenge with virulent IBV in chickens. Additionally, Johnson et al. (2003) reported that the S1 protein of IBV expressed with fowl adenovirus was able to induced protective immunity against virulent challenge in chickens. In this study, we amplified S1 glycoprotein gene coding region of IBV strain 793/B genome and cloned it in pPICZαA vector of *P. pastoris* and sequenced after cloning. The result indicates the accuracy of joining site and we expect the construct pPICZαA-S1 to be used in the production of S1 protein of IBV in the yeast system.

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