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Expression of S1 protein from infectious bronchitis virus (IBV) serotype 793/B in *Pichia pastoris*

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ABSTRACT

Infectious bronchitis (IB) is a viral acute respiratory and contagious disease in chickens characterized by respiratory symptoms including nasal discharge, coughing, sneezing, and respiratory rales, and may be associated with the involvement of the reproductive system of females, digestion system and kidneys. This research was carried out to express recombinant S1 infectious bronchitis virus serotype 793/B. S1 gene complete (1617bp) was cloned in PTZ57 plasmid and transferred to *E. coli*- XL1 blue bacterium and next cloned to pPICZB vectors transferred to *P. pastoris* Km71. *P. pastoris* expression system is used successfully for the production of different recombinant heterologous proteins. Then *P. pastoris* has become an interesting and main alternative to bacterial expression system, for instance, *E. coli* especially when it needs typical eukaryotic post-translation modification or inclusive disulfide bridge, folding and glycosylation. In this study, the agent gene amplified successfully and it visualized validation and purity on agarose gel electrophoresis. Afterward, the products were ligated into PTZ57 cloning, pPICZB vectors and transfer to *E. coli* XL1 blue, *Pichia pastoris* competent cells. Next, by restriction digestion and PCR, the colonies were confirmed. The recombinant plasmid was confirmed by sequencing gene by using specific and universal M13 primers. Finally produced proteins were visualized by SDS PAGE gel that the recombinant protein was size 62 kDa.

Keywords: Expression, S1 protein, Infectious bronchitis virus (IBV), *Pichia pastoris*

1. INTRODUCTION

Infectious bronchitis (IB) is a viral acute respiratory and contagious disease in chickens characterized by respiratory symptoms including nasal discharge, coughing, sneezing, and respiratory rales, and may be associated with the involvement of the reproductive system of females, digestion system and kidneys (Cavanagh D, 2007; Bande F et al., 2017). Poultry infections have a negative effect on the trade of poultry and their products in developing and developed countries. Generally, the disease causes weight loss and reduction in feed efficiency of infected birds, resulting in a significant drop in egg production and quality among laying flocks. Hence, IB has limited the poultry industry, due to its worldwide dispersal and important economic losses. Vaccination is the most effective way for the prevention of such infectious disease, and the development of an effective vaccine is, even so, a priority.

The IB virus (IBV) is the member of the genus Coronavirus (Gammacoronavirus), family *Coronaviridae*. IBV was first detected in North American Dakota in 1930 (Bande F et al., 2016; OIE Terrestrial Manual., 2013). The virion is multiforme (circular, filamentous) with a capsid ranging from 80 to 220 nm diameters and its single-stranded RNA genome which is 27.6 to 31 kb in length. IBV genome encodes main proteins include: nucleoprotein (N. 50-60 kDa), and Spike protein (S. 180-220 kDa) It should be noted that virus incubation at 37 ° C causes loss of S1 subunit (Cavanagh and Naqi, 1983), membrane protein (M. 23-35 kDa), small membrane protein (E. 9-12 kDa), hemagglutinin-esterase (HE) (Maclachlan NJ and Dubove EJ, 2011; OIE Terrestrial Manual., 2013). S-glycoprotein provides the possibility of binding and fusion of the virus to the host cells at the onset of contamination. After binding of the HE to the host cell, S- glycoprotein is integrated at neutral pH with the membrane of the cell resulting in releasing the virus into a cell using the endosomal pathway. The genomic RNA is then transcribed by viral RNA polymerase, gene expression, and replication. Eventually, the virus goes into the plasmid membrane after vesicle sprouting and is released as exocytosis (Cavanagh D, 2007). Several strains of acute nephropathogenic viruses are associated with a potential death rate of over 30% in young birds. The most important serotype of IBV is 793/B, which was first detected in the United Kingdom in 1991 (Gough RE., 1992) and subsequently in other European countries (Cook et al., 1996). The presence of this serotype was confirmed by Momaize et al. and Abadi Shapouri in Iran in 2002 (Khalesi et al., 2017). Furthermore, initial reports showed that the Massachusetts (Mass) - like IBVs were the most common isolates in Iran. However next studies indicated that 4/91-like (also known as 793/B like IBV) was more prevalent than Mass-like serotypes among broiler chickens in Iran (Bande F et al., 2017). Glycoprotein S is a type 1 transmembrane protein containing 1162 amino acids in the IBV. It is highly glycosylated (21-35 N-glycosylation site), consisting homopolymers divided into two subunits S1 and S2 where contains 535 and 627 amino acids, respectively. Subunit S1 is a glycoprotein that plays an important role in binding to the host cell, diversity, and antibody neutralization of the virus. Changes in glycoprotein S1 are used to identify new genotypes as well as possibly anti-virus responses. Poultry IBV serotypes displayed approximately 20-25% of the difference in the S1 sequence (Bande F et al., 2017). It has been demonstrated that S1 protein was able to induce neutralizing antibody production, inhibiting coagglutination and neutralization. The 2nd end of the N-terminal and the C-terminal, as well as there, are identified 3 very variable regions (amino acids 52-90, 124-150, and 265-315). Previous studies have shown that the domain binding to the receptor (RBD), which usually consists of several hundred amino acids is located is S1 protein. The RBD can be

independently folded and is sufficient to connect to the cell receptor. Binding the IBV to the host cell sialic acid as a necessary stage for initiating infection is done by the S1 protein. It has been proven that the N-terminal portion of this protein (amino acids 19-272) is both necessary and sufficient for binding to the cells chicken respiratory tract (Nosrati M et al., 2013; Promkuntod N et al., 2014) regarding the production of a recombinant vaccine against IBV. A number of different expression systems such as insect cell, potato, and E. coli were used to express S1 (Li et al., 2010; Mei et al., 2006; Zhou et al., 2003; Zou et al., 2010). However, on the one hand, S1 is a hyperglycosylated protein. *E. coli* would not be a suitable host for expression of S1 due to lack of ability for post-translational modifications (PTMs). On the other hand, though eukaryotic expression systems like insect and potato are able to perform PMs, working with these hosts is difficult and large scale production of vaccines using this kind of systems would encounter with a number of limitations.

In this regard, *Pichia pastoris* (*P. pastoris*) a much more user-friendly expression host containing the PTM system seems to be a more effective choice in order to produce S1 glycoprotein. To date, no works have focused on the expression of the S1 glycoprotein in the yeast expression system *P. pastoris*. Within this purpose in mind, in the present study, the gene encoding S1 glycoprotein from IBV serotype 793/B was cloned into a non-secretory expression vector, pPICZB, and transformed and expressed *P. pastoris* cells regarding the production of a recombinant subunit vaccine against IB disease in poultry

2. MATERIALS AND METHODS

In this research was done on an infectious bronchitis virus (IR/773/2001/B/793) from Department of Avian Disease Research and Diagnosis, Razi Vaccine and Serum Research Institute (RVSRI).

Reverse Transcription (RT)

The infectious bronchitis virus of poultry was replicated in SPF eggs. Next RNA virus was extracted with RNA X kit (Sina Gene Company) according to the user manual and the RNA was stored at -70 ºC. cDNA synthesis of 20 µL was performed by RT Kit (General Company). As a result, the program was run at 55 ºC in 60 min. Next, is used as a template for S1 gene amplification.

PCR

The PCR step went through a primer pair available in the gene bank database (Accession number: HQ842715) the S1 gene was amplified by pair primer (table 1), which had EcoRI and Xba I cut enzyme position. The DNA of the gene was synthesized and multiplied using PCR kit (BIOfact kit) in thermal cycler (Applied Biosystem) fragment length of 1617bp. The PCR program Contains an initial denaturation step at 95ºC for5 min followed by 35 cycles of denaturation 95ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72 ºC for 2 min, next final extension at 72 ºC for 15 min.

Table 1 Primer Sequence with restriction enzyme

Gene	Sequence primer	Restriction enzyme	Size (bp)
S1	F:5' CG GAA TTC ATG TTG GGC AAA CCG CTT TTA CTA GTG R:5' GC TCT AGA CGT CTG GAG CGA CGT GTT CCG TTA G	EcoR I Xba I	1617

Cloning of S1 gene

After that amplicon was confirmed by gel electrophoresis, S1 gene was cloned in PTZ57 plasmid and transferred to *E. Coli*-XL1blue bacterium by T/A cloning kit (Ins T/A clone PCR product cloning kit, ferments). Then it cultured on LB medium containing antibiotics ampicillin (100 µg/ml), XGal and IPTG. White colonies containing plasmid were selected. The vector was extracted by a plasmid purification kit (Biofact Corporation) according to the user manual and digests with EcoRI and XbaI (Thermo Scientific) enzyme. These were confirmed the presence of S1 gene by PCR. Next, they were separated on 1% agarose gel. Following that the vector was sent to Macrogene Korea for sequencing using universal M13 forward and reverse primer. The PTZ57-S1 was digested in a large volume of the gene and S1 gene was purified by using pure PCR product purification kit (Biofact Corporation) then cloned using the TA cloning kit of the S1 gene into the original non secretive pPICZB vector of yeast Pichia pastoris according to user manual kit, which it was already enzymatically digested. The concentration of the vector and gene were measured by Thermos 1000 Nano drops. The new vector was transferred to X1blue bacteria and cultured on LB medium containing an antibiotic zeocine (25µg/ml). The plasmid pPICZB-S1 extraction is by purification plasmid kit (Biofact Corporation). It performed digestion and PCR again for

confirmation as before. As well as the pPICZB-S1 sent to sequence sent to Macrogene Korea for sequencing using universal AOX1 (forward and reverse primers) that it was verified insertion. After that the pPICZB-S1 replicated and extraction amount 10µg.

Expression and purification

For the expression, initial 10µg pPICZB were linear for transfection to the host Pichia pastoris (Km71) by SAC I (Thermo). Then Km71 yeast was grown in 5 ml of BMGY medium (Buffered glycerol-complex medium) temperature of 29 ° C shaker incubator. Next 100 micrograms of culture were transferred to 500 ml of the new medium and placed in a shaker incubator overnight next when in The wavelength of 600nm of yeast concentrations was up to 1/3 according to user manual Invitrogen yeast company (EasySelect ™ Pichia Expression Kit). The pPICZB-S1 linear amount of 10µg to 80 microliters of yeast is taken in Cuvette. The electroporation device was then placed on the Gene Pulser Xcell TM (BIO-RAD) previously set at 2000 volts and 4 milliseconds and triggered the start button, which switched on the 1977 voltage for 4 milliseconds. After that, add 1000 µl of sorbitol (1 M) and mix well. Transfer to 15 ml falcon sterilized and incubated for 1.5 hours in a 29°C incubator without shake. Then, 300 micrograms of cells were cultured in BMGY agar medium contain a zeocien antibiotic (100 µg/ml) next incubated at 29 ° C for 3 to 5 days. For increasing the copy number of multiple integrants in the yeast, these were cultured on BMGY agar medium containing concentrated antibiotics (1000 µg/ml). A colony was selected and after verifying the presence of the S1 gene by enzymatic digestion and PCR and observing them in an agarose gel 1%. First, a colony cultured at 5 ml of an antibiotic BMGY broth medium. After that, it was transferred to a new BMGY medium broth (500 ml) overnight was incubated at 29°C. After determining the concentration of the cell with spectrophotometry (Jenway England) at 600 nm and reaching the concentration between 2-7 cells under sterile conditions, 50 ml falcon at 3000g were precipitated for 5 minutes with a centrifuge (Sigma Germany), and for expression of the S1 protein was transferred to 100 ml of the expression BMMY (Buffered methanol- complexed medium) medium broth incubated at 29°C. Gene expression was induced for using pure methanol, which was inoculated daily by 500 microliters of alcohol per 100 ml of the medium. From day 0 to 72 hours, 2 ml of the cell was removed and after liquid centrifugation (Sigma), the liquid was discarded and the cell was stored in a -70 ° C. After completion of 96 hours, all cells were removed into 50 ml falcons that these Cells were centrifugation (3000g for 5 minutes). The *Pichia pastoris* were broken with acid-washed glass (0.5mm) and breaking buffer containing pmsf furthermore centrifuged at 3000g for 15 minutes, and the liquid was removed and stored in -70 ° C according to EasySelect ™ Pichia Expression Kit. The protein purification kit was purified according to the protein His tag Ni-IDA column (MN kit Ni-IDA 2000) and the SDS PAGE test was performed to determine the expression of the gene that was about 62 kDa.

Ethical considerations

Given that this study was not a human and animal study, it was not necessary to obtain a code of ethics from the university.

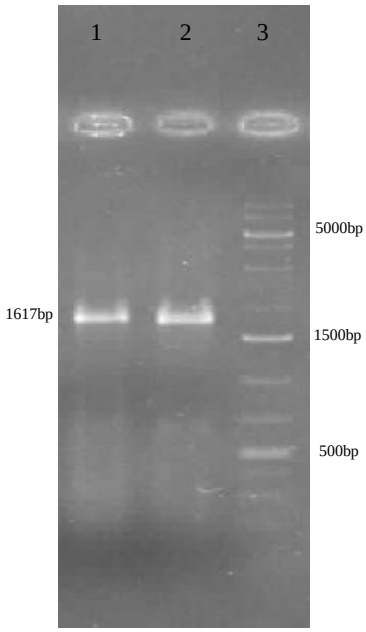

Figure 1 Result PCR product of S1 gene IBv793/B (1617 bp) lane1, 2 product and lane 3 ladder

3. RESULTS

In this study, the agent gene amplified successfully and it visualized validation and purity on agarose gel electrophoresis (figure 1). Afterward, the products were ligated into PTZ57 cloning, pPICZB vectors and transfer to *E. coli* Xl1blue, *Pichia pastoris* competent cells. Next, by restriction digestion (figure 2) and PCR, the colonies were confirmed. The recombinant plasmid was confirmed by sequencing gene by using specific and universal M13 primers. Finally produced proteins were visualized by SDS PAGE gel that the recombinant protein was size 62 kDa (figure 3).

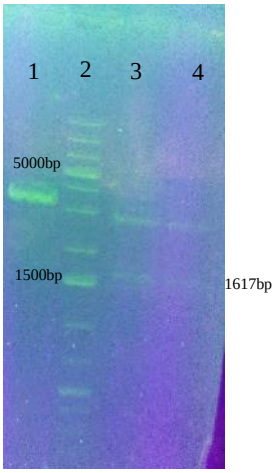

Figure 2 The result of the enzymatic digestion of Xba I and EcoR I, lane 1 vector non digest, lane2 ladder and lane 3, 4 vector digested

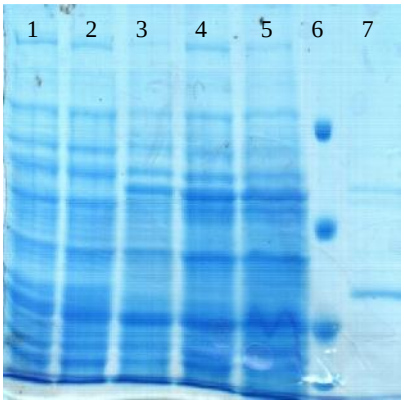

Figure 3 Results extract and purified protein SDS-PAGE gel lane 1-7, respectively 0, 24, 48, 72, 96, ladder and bond S1 gene 62KDa

4. DISCUSSION

This research was Succeeded to clone and expression recombinant S1-IBV serotype 793/B into *Pichia pastoris*. *P. pastoris* has become an interesting and main alternative to bacterial expression system, for instance, *E. coli* especially when it needs typical eukaryotic post-translation modification or inclusive disulfide bridge, folding and glycosylation and in previous studies, in 1998, Song and et al expressed the S1 gene (KM91 strain of IBV china) in Baculoviruses and said that this protein can induce a protective immune response as well as an antibody response (Song et al., 1998). In 2010, Zou and et al. expressed the S1 fragment in *E. coli* using a pET 32a (+) vector. Which used for ELISA test and the antibody titer or monoclonal antibody production against IBV was determined (Zou et al., 2010). Also in 2003 Zhou and et al. expressed S1 glycoprotein of IBV in transgenic potatoes which can be used as a source of the recombinant antigen for vaccine production (Zhou et al., 2003). In another study, Hosseinian and et al. expressed S1 glycoprotein gene of IBv isolated IRFIBV32 in *Lactococcus lactis*. They supposed that these recombinant bacteria can be a great candidate for the development of a new and less costly mucosal recombinant vaccine (Hosseinian et al., 2013). In addition, Babapoor, et al. in 2009, evaluated DNA vaccine with recombinant Spike gene (IBV M41) vaccine with interferon alpha as an

adjuvant. Their study showed that it was an effective vaccine against infectious bronchitis virus disease (Babapoor et al., 2009). Also, in 2018, Zeshan et al. clone and expression of truncated spike glycoprotein IBV in *E. Coli* using pET 32a (+) vector and suggest that can be used as antigen to detect antibodies against IBV (Zeshan et al., 2018). Yan and et al. in 2013 clone S1 gene in Plasmids pVAX1-16S1 with N and M gene as DNA vaccine that demonstrated that DNA vaccine targeting the S1, M, and N proteins of IBV can use against IBV (Yan et al., 2013). As well as Yuan et al. co-expressed S1 and N proteins in baculovirus that the bivalent subunit vaccine might be a strategy for the development of an IBV subunit vaccine (Yuan et al., 2018).

5. CONCLUSION

S1 protein is a suitable candidate for the vaccine based on recombinant viral proteins. Which would be safe to use and relatively simple to adapt to new variants. This present research, it was able to clone and express protein and it could be used as a candidate both for antigen against IBV 793/B and as antigen for making Elisa kit.

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Conflicts of interest

There are no conflicts of interest between the authors.

Financial resources

There are no financial resources.

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