Efficacy of recombinant S1 Protein expressed in *pichia pastoris* in chicken & using for detection titer antibodies against avian infectious bronchitis by ELISA

Abstract

Infectious bronchitis (IB) is an acute, extremely contagious upper respiratory disease in chickens. The objective of this study was expressed and using of recombinant S1 Protein serotype 793/B expressed in *pichia pastoris* in order to serodiagnosis against avian infectious bronchitis antibody. The complete S1 gene (1623bp) was cloned in PTZ57 plasmid and transferred to *E. coli* (Escherichia coli)-*XL1blue* bacterium. Next cloned to pPICZB vectors and transferred to *p. pastoris*(Km71H). Produced protein were visualized by SDS (sodium dodecyl sulfate) PAGE gel that was about with 62KDa. Finally, the S1 recombinant protein was used to coat 96 well plate to recognize antibodies in sera against live Infectious bronchitis virus (IBV 4/91). As a result, this recombinant S1 protein Antigen can be used in an enzyme-linked immunosorbent assay (ELISA) for recognizing antibody titer against IBV. This recombinant protein was evaluated by Elisa, using a panel of field sera for known IBV titer That in the commercial kits, the optical gain of positive control is 0/741 ± 0/007 and the negative control is 0/167 ± 0/002, next the average Optical Density (OD) in the three farms were 2.585, 2.001, 1.657, resulting to the S/P ratio of was 4.212, 2.886, 2.484, respectively. The results of our effort for positive control and the negative control are 0/9 ± 0/010, 0/067 ± 0/008 respectively. The mean Optical Density of sera in 3 Flocks were showed 3.390, 2.737, 2.921, and the ratio of S/P 4. 01, 3.242 and 3.46. These results showed that our outcomes are neared to Biochek kit. The results also showed that recombinant protein S1 was able to identify different kinds of infectious bronchitis virus serotypes in poultry. It can be used for commercial ELISA kit.

Keywords: IBV, ELISA, S1 glycoprotein, Cloning, Expression, Serodiagnosis.

Introduction

Infectious bronchitis (IB) is an acute, extremely contagious, and economically important respiratory disease in poultries. It is caused by the IB virus (IBV), which is a significant respiratory pathogen that causes considerable economic losses in the commercial poultry industry worldwide. First, IBV was reported in poultry flocks of Iran in 1994 by Aghakhan et al (1). The major clinical signs are included tracheal rales, sneezing and coughing. The IBV genome is a single-stranded, positive-sense RNA that is 27.6 kb in size. It is member of Gammacoronavirus, subfamily Coronavirinae, family Coronaviridae, Order Nidovirales (2,3,4,5). It encodes four major structural proteins, named, glycosylated spike protein (S), membrane protein (M), phosphorylated nucleoprotein (N), and envelope protein (E), and 15 nonstructural proteins (nsp2–nsp16). Generally, nonstructural proteins are present in infected cells but not in the virus, and they only play a role in the process of virus infection and replication (6).

In general, acute IBV infections can be diagnosed by detection of IBV virus (antigen) or the specific antibody response. The most common assays for routine virus detection are virus isolation (VI), immunofluorescence assay (IFA), immune peroxidase assay (IPA), polymerase chain reaction (PCR), and for antibody detection the haemagglutination inhibition (HI) test, agar gel precipitation test (AGPT), and enzyme linked immunosorbent assay (ELISA). The virus neutralization test (VNT) is rarely used for routine diagnosis because it is relatively expensive and laborious (7,8). All coronavirus S glycoproteins consist of four domains; a signal sequence that is cleaved during synthesis; the ectodomain, which is present on the outside of the virion particle; the transmembrane region responsible for anchoring the S protein into the lipid bilayer of the virion particle; and the cytoplasmic tail. The IBV S glycoprotein (1162 amino acids) is cleaved into two subunits, S1 (535 amino acids, 90-kDa) comprising the N-terminal subunit of the S protein and S2 (627 amino acids, 84-kDa) comprising the C terminal subunit of the S protein. The S1 subunit contains the receptor-binding activity of the S protein (9,10). This glycoprotein can be inducing neutralization of antibodies for protecting against virus. There are six antigenic sites on S1 protein that are induced of response immune (11).

The ELISA assay is a convenient method for monitoring of both the immune status and virus infection in chicken flocks. Several commercial ELISA kits for IBV specific antibodies detection

are already available. However, proliferation & purification of IBV is tiresome and expensive. Unlike full virions, recombinant proteins produced via gene engineering detect only specific antibodies to one certain protein and are safer, easier to produce and less expensive (12,13). Actually the S1 glycoprotein has potential as an antigen for developing an ELISA diagnosis instrument.

In this study, we keep complete S1 gen(1623bp) for expression in *pichia pastoris*. The polyclonal antibodies were produced in SPF chickens, and it used for making ELISA kit and used evaluate response system.

Materials and Methods

We got standard infectious bronchitis virus (IR/773/2001/B/793) from Razi Vaccine and Serum Research Institute (RVSRI).

1- Reverse Transcription (RT) and PCR (RT-PCR)

RNA virus was extracted with RNA X kit (Sina Gene Company) according to user manual and cDNA synthesis was performed by RT Kit (GeneAll Company). The program was run at 55 ºC in 60 min. Next it used as template for S1 gene amplification. The S1 gene was amplified pair of primer (table 1), which had EcoR I and Xba I cut enzyme positions. The DNA of the gene was synthesized and multiplied using PCR kit (BIOfact kit) in thermal cycler (Applyied Biosystem). The PCR program was done with initial denaturation step at 95ºC for 5 min next 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. The final fragment length was 1623 bp.

2- Cloning, Expression and purification 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, fermentase). Then the vector was extracted by a plasmid purification kit (Biofact Corporation) according to user manual. After that, S1 gene was cloned to the original non-secretive *pPICZB* vector (which it was already enzymatically digested by *EcoR I* & *Xba I*) of yeast Pichia pastoris by TA cloning kit according to user manual kit. The expression was initiated by 10µg *pPICZB* which was linear by SAC I(Thermo) for transfection to the host Pichia pastoris (Km71H). 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 it was stored in -70 ° C (according to EasySelect ™ *Pichia* Expression Kit). The protein purification was done by protein His Tag Ni-IDA column kit (MN kit Ni-IDA 2000) and the SDS PAGE test was performed to determine the expression of the 62 KDa. The purified protein concentration was determined by the Bradford method at 595 nm wavelength.

3- Western blot:

Recombinant S1 protein was separated by 12.5% SDS-PAGE. Next it was electrotransferred to nitrocellulose membrane overnight at 40 V by mini protein 3 (BIORAD) at 4˚c. The membrane was blocked in PBS-T buffer (contains BSA 2.5% & 0.1% Tween 20) by soaking in blotting buffer for 1 hour with shaking. Followed by incubating membrane in primary antibody(ab200537) anti-His tag (1:10000) for one hour then washing the blot three times with wash PBS-T buffer and incubated the blot in secondary antibody (goat anti rabbit ab205718) anti-His tag with horseradish peroxidase (1:10000) for one hour at room temperature. After that it was washed again with PBS-T buffer for removing the unbound antibodies and was developed by Add 10ul H2O2 (30%) to 10 ml of 0.05%DAB (3,3´-diaminodbenzidine) in PBS for 2-5 min.

4- Immunization and field serum:

For providing protective properties study of this recombinant antigen as a candidate vaccine, three group of chicken were selected. Live poultry avian infectious bronchitis 4/91 vaccine (Noblilis-Holland) was inoculated via eye drops in SPF chicken at the age two weeks (from Razi Institute). The recombinant protein was also injected intramuscularly with montanide oil adjuvant in the second group, and the third group was without injected antigens it was considered as negative control (10 chickens). After that field sera were collected from 94 layer hens (at the age 22 weeks) that their antibody titer was also determined with commercial Bio-check CK119 IBV ELISA kit according to the manufacturer's instructions.

5- ELISA assay:

In-house ELISA system was development briefly, Recombinant S1 protein as an antigen (approximately 50 ng/well) was coated to standard ELISA micro plates (Biomate plate 96 wells) using bicarbonate buffer at 0.1 M (pH: 9.6). The coated micro plates were incubated for 18 h at 4°C. This was continued by a triple washing treatment with 10mM PBS. Plates were coated with blocking through filling the wells with 150 µl of 2.5% BSA (Sigma-Germany). All steps were

carried out by incubating of plates at room temperature for one hour. The 10 mM PBS/Tween 20 and 1% BSA buffers were used to prepare 1/500 dilutions of serum samples. One hundred µl of treated serum was transferred to a fresh coated ELISA plate. This was followed by incubation at room temperature for 30 minutes when the plate was washed for 5 times with washing buffer. The next step was adding 100 µL of Horse Radish Peroxidase (HRP) conjugate (anti chicken, abcam-UK) diluted by 10mM PBS, pH 7.2) to the wells and incubation for 30 min at room temperature followed by a washing phase. Afterward, 100 µl of Tetra-Methyl Benzidine (TMB) substrate was added to wells and the plate held for 15 min at room temperature while keep avoids from light. This was followed by adding 100 µl of stop solution (HCl 1 N) to the wells. Finally, the whole process was read and recorded by an ELISA reader (Biotech-Model TS 800) at 450 nm wavelength.

Result

In this disquisition, the agent gene amplified and visualized on agarose gel electrophoresis (figure 1). Subsequently, the products were ligated into *pTZ57* cloning, *PpICZB* vectors (figure 5) that were confirmed by restriction Enzymes *EcoR* I & *Xba* I digestion (figure 2) and transferred to *E.Coli Xl1blue*,and finally *pichia pastoris* competent cells. The recombinant produced proteins were visualized by SDS PAGE gel and western blot. The recombinant protein's size was 62 KDa (figure 3,4). Then recombinant S1 protein was coated in 96 wells microplate. Henceforth, the plate run by positive and negative samples obtained from specific pathogen free chickens. After checkerboard with control positive & negative. We determined the appropriate spot for the amount of protein coated by the positive and negative control. As a result, the positive control level is Fixed on 0/9 at 450nm wavelength. This plate used for detection of sera field. We tested 94 sera from farms. In the commercial kits, the optical gain of positive control is 0/741 ± 0/007 and the negative control is 0/167 ± 0/002 next the average ratio OD in the three farms were 2.585, 2.001, 1.657, and the ratio of S/P was 4.212, 2.886, 2.484, respectively. The results of this experiment for positive and negative control were 0/9 ± 0/010, 0/067 ± 0/008, respectively. The mean Optical Density (OD) of sera in three Flocks were showed 3.390, 2.737, 2.921, and the ratio of S/P 4. 01, 3.242 and 3.46. These results showed that the outcomes of this study are very similar to Biochek kit (figure 6).

Discussion

This survey was Succeeded in cloning and expression of recombinant S1-IBV (1623 bp) serotype 793/B (IR/773/2001/B/793) in p.*pastoris*. The S1 glycoproteins observed at 62 KDa in SDS-PAGE and then was confirmed with a western blot. For in vivo evaluation of this recombinant antigen, SPF chickens were immunized with S1 glycoproteins. The expressed and purified recombinant antigen was also used to develop an in house ELISA plate to detect anti-IBV antibodies. The glycoprotein S1 is known for immunogenic properties. It is well known that the glycoprotein S1 subunit induces humoral protecting immunity (14). In previous studies, proteins have been expressed in the prokaryotic system, but Eukaryotic systems have the benefits such as packaging and glycosylation, which is not in the prokaryotic system or very low. Zehan & et al (2018) cloned & expressed the spike protein of IBV in E. coli which showed relatively acceptable immunogenicity in mice. They found that this protein can be used as antigen to detect antibodies against IBV (15). Hsun-Lin and et al (2012) they expressed S1 protein in prokaryotic system and applicate it for diagnosis of IBV antibodies (11). Wang (2002) was also used S1 spike polypeptide for ELISA. The results showed that S1 protein can be diagnosis antibodies against IBV (12). Song (1998) could clone and expressed S1 glycoprotein in Eukaryotic system by recombinant baculovirus. They indicated that S1 glycoprotein can induce immune response in chicken (16). Finger (2018) was used N protein IBV for making ELISA and western blot. He indicated that N protein can also be used for the immunodiagnosis of avian infectious bronchitis (3).

The major IBV immunogenic protein is S1 glycoprotein that could induce antibody response against IBV (17,18). The location of three overlapping antigenic sites on the IBV spike protein was similar to the location of antigenic sites on the spike protein of other coronaviruses (19). There are eight antigenic regions on the spike protein (S2, G-H) that six of them are on the S1(S1, A-F) (20,21). That main part of S1-f is conserved which is a proof for the cross-reactivity between the tested serotypes in antibodies based tests such as ELISA (12).

Generally, in chicken ELISA is used to diagnose disease and the antibodies titer of vaccinated chickens and for determining the next step of vaccination. Commercial kits use a logarithmic formula to determine the antibody titer, which is not possible manually. There is a parameter S/P($s/p = \frac{\text{OD sample} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$) that is based on positive and negative control, and

completely dependent on them. Based on the results obtained from the commercial kit, serum titers cannot be manipulated. Therefore, tried to obtain best controls point with the changes in the absorption rate positive and negative control, so that when the formula is used, the results of the manufactured kit are made close to the commercial titer. Also, there is a S/P ratio in the software Biocheck. This is ultimately used in logarithmic formulas. For this reason, understood that if the results of serum titers are closed to the results of the commercial kit, So the final logarithmic results are similar. Positive control was placed based on OD = 0.9 at 450nm wavelength. After several times the checkerboard tests are obtained by manipulating the positive control. As a result, the final outcomes will be close to the commercial kit. Although the purpose of this study was not to achieve to the commercial kits, but tried to work on this issue. This study showed that recombinant protein S1 was able to identify different kinds of infectious bronchitis virus serotypes in poultry. These results are similar to the previous studies. Such as Wang (12) and Lin (11) Which made the ELISA kit using recombinant protein. In this research, S1glycoprotein cloned, expressed and used it for diagnosis of antibodies titer against IBV. Then. It was tested with 94 serums from three farms chickens which showed a close relationship with the result of the commercial Biocheck kits.

Conclusions

The results showed that recombinant protein S1 was able to identify serotypes different kinds of infectious bronchitis virus have poultry. It can be used for making commercial ELISA kit.

Ethical: As per international standard guideline the ethical approval has been collected and preserved by the authors.

Consent; NA

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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	1623

Source: Ahmadi et al., 2019 [22]

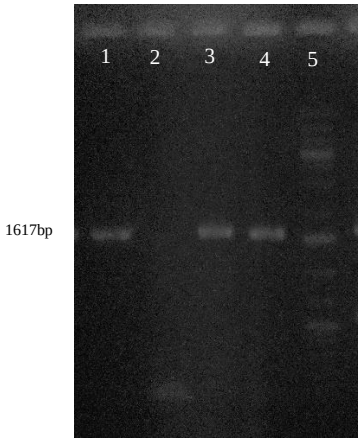

Figure1: Result PCR pro duct of S1 gene IBv793/B (1617 bp) lane1,3,4 product, 2 negative and lane 5 ladder

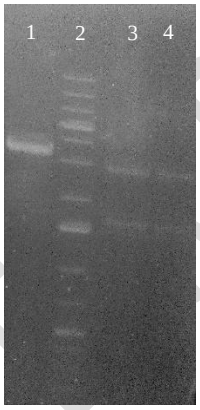

Figure 2: The result of the enzymatic digestion of Xba I and EcoR I, lane 1 vector non digest, lane 2 ladder and lane 3,4 vector digested

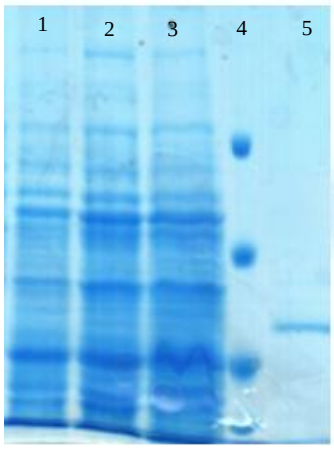

Figure 3: Results extract and purified protein SDS-PAGE gel lane 1-5, respectively 48, 72, 96, ladder and bond S1 gene 62KDa

Figure 4: Results western blot lane 1 ladder, 2 bond purified protein

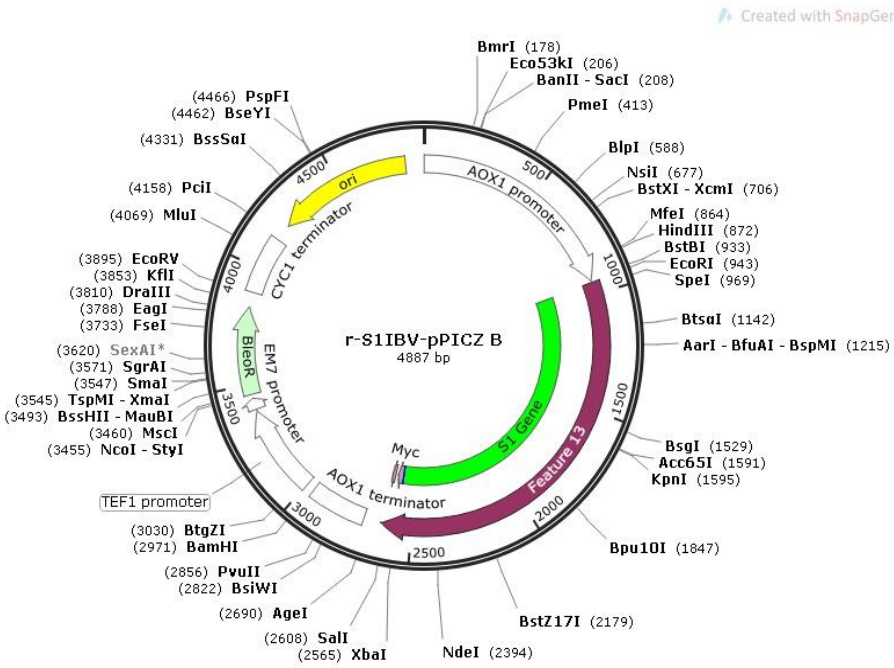

Figure 5: genetic map of plasmid pPICZB & S1793/B with 2 enzymatic cutting positions EcoR I & Xba I

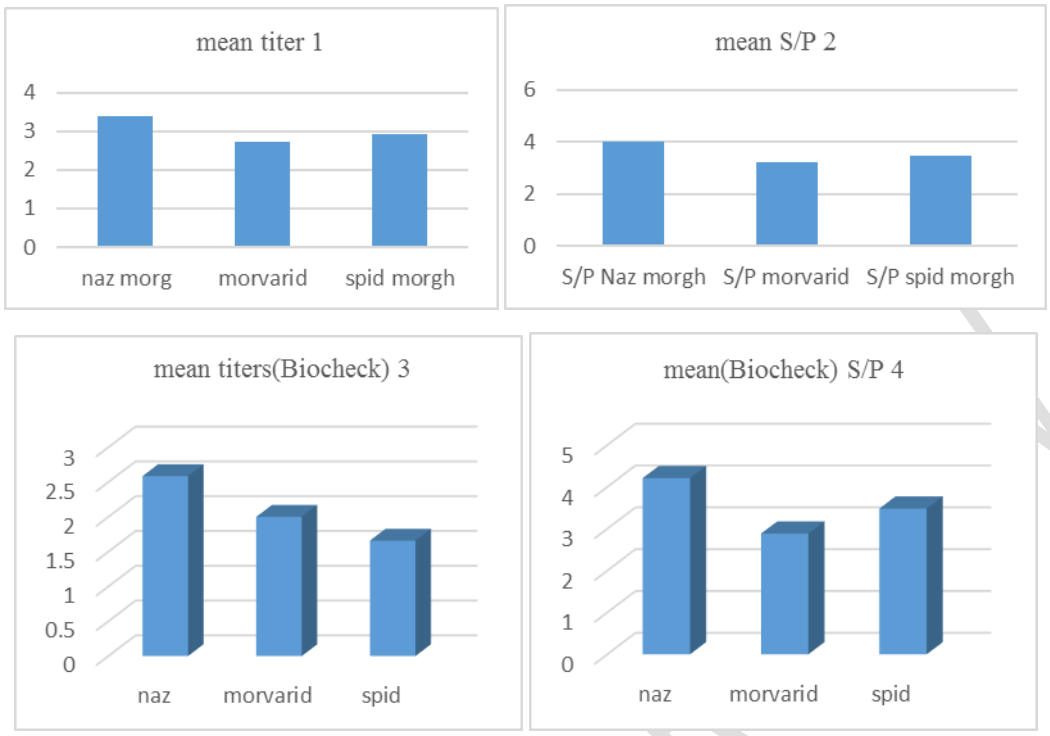

Figure 6: diagram 1 & 2 means of titers and S/P between farms, 3&4 means titers and S/P with Biocheck kit