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Original article

Production and characterization of egg yolk antibody (IgY) against recombinant VP8-S2 antigen

K. Nasiri[1], M.R. Nassiri[2], M. Tahmoorespur[3], A. Haghparast[4], S. Zibaee[5]

[1] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran
[2] Department of Animal Science and Institute of Biotechnology, Ferdowsi University of Mashhad. Iran
[3] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Iran
[4] Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Iran
[5] *Razi* Vaccine and Serum Research *Institute,* Mashhad, Iran

Abstract

Bovine Rotavirus and Bovine Coronavirus are the most important causes of diarrhea in newborn calves and in some other species such as pigs and sheep. VP8 subunit of rotavirus is the major determinant of the viral infectivity and neutralization. Spike glycoprotein of coronavirus is responsible for induction of neutralizing antibody response. Studies showed that immunoglobulin of egg yolk (IgY) from immunized hens has been identified to be a convenient source for specific antibodies for using in immunotherapy and immunodiagnostic to limit the infections.

In this study, chimeric VP8-S2 gene was designed using by computational techniques. The chimeric VP8-S2 gene was cloned and sub-cloned into pGH and pET32a (+) vectors. Then, recombinant pET32a-VP8-S2 vector was transferred into *E. coli* BL21 CodonPlus (DE3). The expressed protein was purified by Ni-NTA chromatography column. Hens were immunized with the purified VP8-S2 protein three times. IgY was purified from egg yolks using polyethylene glycol precipitation method. Activity and specificity of anti-VP8-S2 IgY were detected by dot-blotting, Western-blotting and indirect ELISA.

We obtained anti-VP8-S2 IgY by immunizing hens with the recombinant VP8-S2 protein. The anti-VP8-S2 IgY was showed to bind specifically to the chimeric VP8-S2 protein by dot-blotting, Western-blotting analyses and indirect ELISA. The result of this study indicated that such construction can be useful to investigate as candidates for development of detection methods for simultaneous diagnosis of both infections. Specific IgY against the recombinant VP8-S2 could be recommended as a candidate for passive immunization against bovine rotavirus and bovine coronavirus.

Key words: bovine rotavirus, bovine coronavirus, immunization, recombinant protein, IgY

Correspondence to: M.R. Nassiri, e-mail: nassiryr@um.ac.ir

Introduction

Rotavirus group A is one of the main causes of diarrhea and gastrointestinal disease in human infants, calves, sheep and swine (Kapikian et al. 1996). The BRV group A consists of 11 segments of double-stranded RNA surrounded by three concentric layers of protein (Kirkwood 1985). Rotavirus has six viral proteins which are called VP1, VP2, VP3, VP4, VP6 and VP7. Also, rotavirus has six nonstructural proteins (NSPs) that are only produced in cells infected by rotavirus, which are called NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6 (Kirkwood 1985).

The VP4 protein of rotavirus involved in some functions including hemagglutination, cell penetration, neutralization, attachment to cellular receptors and virulence (Jayaram et al. 2004). The VP4 protein is cleaved by trypsin to two subunits VP8 (28kDa N-terminal region) and VP5 (60kDa C-terminal region). The VP8 protein has the hemagglutination domain (Fiore et al. 1991).

Bovine coronavirus (BCV) is the primary cause of diarrhea in neonatal calves and winter dysentery in cattle worldwide (Saif et al. 1988). The BCV contains a large single and positive-stranded RNA genome. The BCV has proteins structural including nucleocapsid (N), tran-membrane (M), spike (S), small membrane (E), and hemagglutinin/esterase (HE) proteins (Abraham et al. 1990). The S protein is cleaved in position 768 to769 to two subunits S1 (N-terminal region) and S2 (C-terminal region) by trypsin (Abraham et al. 1990). The spike protein of coronavirus is involved in several important functions including induction of neutralizing antibody response and binding to susceptible cell (Takase-yoden et al. 1991)

Immunoglobulin Y (IgY) is an egg yolk antibody that has been used widely for treatment and prevention of infections in humans and animals (Kobayashi et al. 2004). IgY used for passive protection of the pathogen infections such as bovine and human rotavirus, *enterotoxigenic Escherichia coli*, bovine coronavirus, salmonella, staphylococcus and Pseudomonas (Mine and Kovacs-nolan 2002).

Because the VP8 and S2 proteins induce protective immune responses against BRV and BCV. In this study, specific IgY against the recombinant VP8-S2 protein was produced and anti-VP8-S2 IgY specificity was evaluated against the recombinant VP8-S2 antigen.

Materials and Methods

Bioinformatic tools for prediction antigenic regions

The nucleotide sequence of the VP8 (Fj598316) of G10P[11] genotype and S2 (NC_003045.1) gene were obtained from GenBank (http://www.ncbi.nih.gov/genbank/). For epitope prediction of the S2 protein, we chose rejoins of the S2 protein which was previously reported as conserved region. B and T-cell epitopes of the proteins encoded by these genes were predicted using different servers and software like as: ABCpred, BepiPred, BCPred, SVMTrip and LEPS for B-cell prediction and IEDB, SYFPEITH, PropredI and Propred for T-cell prediction. Secondary structure was predicted using SOPMA software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/ npsa-sopma.html). Tertiary structure was predicted by I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The epitopes of the VP8 and S2 proteins were screened for predicting their antigenicity using an online antigen prediction server VaxiJen v2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). Furthermore, enzymatic degradation sites, mass (Da) and pI were determined using the Protein Digest server (http://db.systemsbiology.net:8080/proteomicsToolkit/proteinDigest.html). The fragments fused together by linker $(G_4S)_3$. The chimeric VP8-S2 protein was codon- optimized for expression into *E. coli* by GENEray.

Cloning of chimeric VP8-S2 gene into bacterial expression vector

The pGH-VP8-S2 vector containing the gene of insert flanked by *Bam*HI and *Xoh*I restriction sites was constructed by GENEray. Then, pGH-VP8-S2 vector transformed into *E. coli* strain TOP10F'. It was cultured in LB medium which contained 100 µg/ml ampicillin. The pGH-VP8-S2 vector was digested by *Bam*HI and *Xoh*I enzymes. Then, VP8-S2 fragment ligated to pET32a(+) expression vector which digested by *the same restriction* enzymes by T4 DNA ligase. *E. coli* strain TOP10F' competent cells was used for transformation with pET32a-VP8-S2 plasmid. The transformed bacteria were grown on LB agar plates with ampicillin (100 µg/ml) overnight at 37°C. The suspected colony was further analyzed by PCR colony and restriction enzyme digestion. The correctness of the VP8-S2 sequence was confirmed by DNA sequencing using pET T7 promoter and terminator primers.

Expression of the recombinant VP8-S2 protein

E. coli BL21 CondonPlus (DE3) was transformed with pET32a-VP8-S2 plasmid and grown in Luria broth (LB) culture supplemented with 100 µg/ml am-

picillin on shaking incubator overnight at 37°C in 150 rpm. Fresh LB liquid (50 ml) containing 100 µg/ml ampicillin was inoculated by 5 ml of pre-culture and it was incubated at 37°C at 150 rpm to reach OD600:0.6. Expression of the VP8-S2 protein was induced by IPTG to a final concentration of 1mM and incubated for two hours.

Protein solubility determination and protein purification

For solubility determination, cells were harvested by centrifugation at 8000 rpm at 4°C for 10 minutes. The cells were resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, pH=8). Lysozyme was added in concentration of 1 mg/ml. The cells were disrupted by sonication. Supernatant and pellet were investigated to find whether the supernatant contained the soluble VP8-S2 or insoluble VP8-S2 was present in the pellet. The recombinant VP8-S2 protein was purified based on manufacturer's instructions on Qiagen nickel-nitrilotriacetic acid agarose Column (Qiagen, Hilden, Germany). In order to solubilize inclusion bodies pellet, the pellet was resuspended in buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH=8) for 60 minutes on ice, lysate was centrifuged at 10000 rpm for 20 minutes in room temperature. Subsequently 1ml of the 50% Ni-NTA slurry was added to 4 ml lysate and was mixed by shaking for 60 minutes. The lysate-Ni-NTA mixture was loaded into a column with the bottom outlet capped. The recombinant protein was washed once with 4 ml of buffer C (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH=6.3), it was eluted four times with 0.5 ml of buffer D (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH=5.9) and followed four times with 0.5 ml of buffer E (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8M Urea, pH=4.5) per column. Protein concentration was quantified by Bradford assay (Bradford 1976). Before injection of the recombinant VP8-S2 protein to hens, it was dialyzed to remove Imidazole and other impurities.

Dot-blotting analysis of the recombinant VP8-S2 protein

The recombinant VP8-S2 protein (2 µg) induced by IPTG, the extract of the transformed bacteria uninduced by IPTG and PBS were dot-blotted on nitrocellulose membrane. The membrane was immersed in 1% bovine serum albumin and was shaken in the incubator for 30 minutes at room temperature, and it was washed with PBST (PBS, 0.1% (v/v) Tween) for 2 min, and then it was immersed in primary antibody (anti His-tag rabbit) diluted 1:500 for 1 hour on shaking incubator at room temperature. After that, the nitrocellulose membrane was washed four times for 5 minutes each time in PBST, it was incubated with secondary antibody (anti-rabbit IgG conjugated to HRP) that was diluted 1:3000 for 1 hour on shaking incubator at room temperature. The membrane was washed four times for 5 minutes each time in PBST. Color development was observed by adding diaminobenzidine dissolved in PBS and H_2O_2.

Immunization of hens

Sixteen-week-old laying hens (Mashhad, Iran) were kept in individual cages with food and water ad *libitum*. Immunization of hens was performed by intramuscularly injecting with 200 µg of purified protein with an equal volume of Freund's complete adjuvant into two sides of chest area (Sigma, USA) for the first immunization. Freund's complete adjuvant without the recombinant protein was injected to the control group. Two booster immunizations followed up using incomplete Freund's adjuvant in two weeks interval. One week after the last injection, the eggs were collected daily for 8 weeks, marked and stored at 4°C.

Purification of IgY

Purification of IgY was carried out by polyethylene glycol precipitation method using PEG 6000 powder (Merck, Germany) based method of Polson (Polson et al. 1980).

Dot-blotting and Western-blotting of anti-VP8-S2 IgY

For dot-blotting analysis, 2 µg of the recombinant VP8-S2 protein was dot-blotted individually on two pieces of nitrocellulose membranes. After similar steps as were mentioned previously, each of pieces was immersed in primary antibody (hen anti-VP8-S2 IgY of the treatment group and hen IgY of the control group) diluted 1:2000 and followed with secondary antibody [rabbit anti-chicken IgY H&L (HRP)] (Abcam, USA) diluted 1:2000. Other steps were carried similarly to steps mentioned previously.

For Western-blot, the purified VP8-S2 recombinant protein was separated using 12% SDS-PAGE and transferred onto nitrocellulose membrane. The nonspecific binding sites were blocked with BSA 1.5% (w/v) and incubated at 4°C overnight. The membrane

Table 1. Final epitopes after filtration and antigenicity ability of predicted epitopes.

Antigen	Number	Final epitops after Filtration	VaxiJen Score
VP8	1	$_{8}$QLLYNSYSVDLSDEITNIGAEK$_{29}$	0.72
VP8	2	$_{180}$ADTQGDLRVGTYSNPVPNAVV$_{200}$	0.57
VP8	3	$_{223}$GLPAMQTTTYVTPISYAIR$_{242}$	0.92
S2	4	$_{975}$ATSASLFPPWSAAAGVPFYLNVQYR$_{999}$	0.92
S2	5	$_{1187}$SGYFVNVNNTW$_{1198}$	0.84

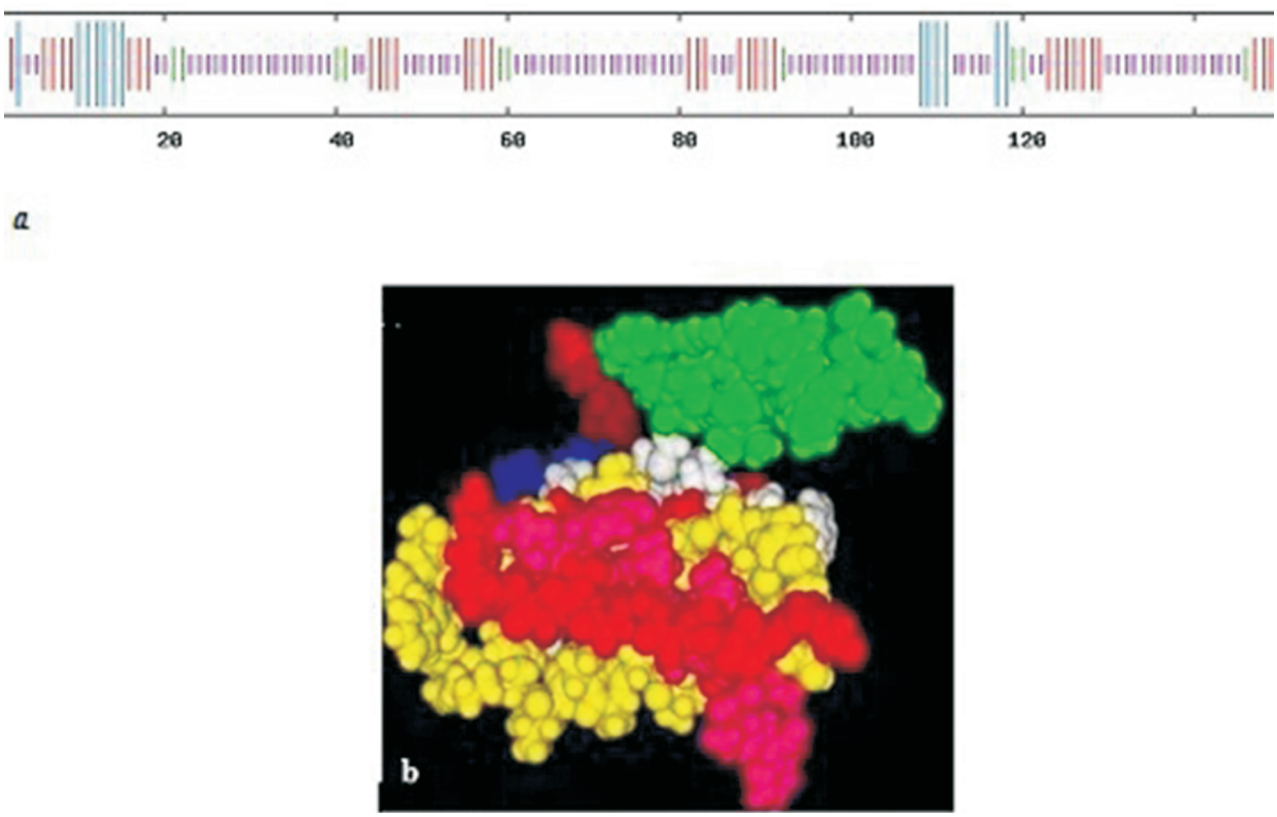

Fig. 1. (a) Secondary structure prediction results. Blue: α helix; green: β turn; red: extended strand; and Purple: random coil. (b)Tertiary structure prediction results of the VP8-S2 protein.

incubated with anti-VP8-S2 IgY antibody (1:2000) for 1 hour at room temperature. After washing three times in PBST, rabbit anti-chicken IgY H&L (HRP) diluted in PBS (1:2000) was added and incubated for one hour at room temperature. The membrane was washed three times with PBST and the specific binding of IgY antibody to the VP8-S2 protein was detected.

Indirect Enzyme-linked immune sorbent assay

Specific activity of IgY antibody against the VP8-S2 protein was determined by iELISA. 100 µl of the recombinant VP8-S2 protein were coated onto microtiter plates (60, 43 and 30 ng/ml) for 1 hour. After washing three times with PBS containing 0.05% (v/v) Tween 20(PBST) and blocking nonspecific sites with blocking buffer (1.5% BSA in PBST) at 37°C for 1 hour. Then, 100 µL of a diluted IgY antibody (1:3000) in PBST was added to the wells as the primary antibody and incubated at 37°C for 1 hour. Subsequently, the plate was washed again and incubated with 100 µL of labeled rabbit anti-chicken IgY H&L (HRP) at a dilution of 1:10000 as the secondary antibody for 1 hour at 37°C. The colorimetric detection was carried out using O-phenylenediamine (OPD, Sigma) as a chromogenic substrate of HRP. The reaction was stopped by adding 50 µl of 3 mol/l H_2SO_4 to the wells.

Statistical analysis

A T-test was used to detect significant differences between means of ELISA absorbance for determination of specific IgY antibody. Also, repeated measures ANOVA was performed for mean comparisons of ELISA absorbances between weeks. The p value < 0.05 was considered significant.

Results

Epitope prediction of the VP8 and S2 proteins

The B-cell and T-cell epitope predictions were successfully performed using different online software. Five epitopes were chosen as final epitopes (Table 1). The chimeric VP8-S2 protein was identified as an antigen by the VaxiJen 2.0 server (threshold 0.5) with a final score of 0.59. The antigenicity of the final predicted epitopes shows in Table 1. The results of VaxiJen 2.0 analysis indicated that five predicted epitopes of the VP8-S2 protein were identified as antigen. To assess the antigenic features of the VP8-S2 protein, we predicted secondary structure using SOPMA server. A greater proportion of extended strands and random coils present in the structure of VP8-S2 protein with an increased likelihood of the protein forming an antigenic epitope (Fig. 1). The results revealed that the proportion of random coils, β turns, α helices and extended strands (β folds) accounted for 58.60, 13, 14 and 38% of the secondary structure, respectively. The 3D structure of the chimeric VP8-S2 protein show in Fig. 1.

Protein expression and purification

The recombinant pGH-VP8-S2 vector was successfully transformed into TOP10F'. The digestion results of the recombinant vector are shown in Fig 2. The results of the colonies of recombinant pET32a(+)-VP8-S2 plasmid were investigated by the PCR colony using pET T7 primers (Fig. 3a). Also, the existence of the VP8-S2 fragment in pET32a(+) vector was confirmed by enzymatic digestion (Fig. 3b).

The recombinant VP8-S2 protein was successfully expressed by 1mM IPTG in 2 hours post induction (Fig. 4a). A protein 33 kDa (pET3a (18.5kDa) + VP8-S2 (14.45 kDa)) can be detected in Coomassie blue staining. This vector is able to express a fusion protein with a 6-histidine tag at thrombin site and a T7 tag at the N-terminus. These additional amino acids increase the size of expressed protein to near 18.5 kDa. Expression analysis indicated that the recombinant VP8-S2 protein could be highly expressed in *E. coli* cells. The result of dot-blot analysis confirmed the existence of recombinant VP8-S2 protein by anti-His tag. The analysis of the supernatant and pellet of *E. coli* BL21CodonPlus (DE3) cells revealed that the expressed VP8-S2 protein was found in pellet. The recombinant VP8-S2 protein was successfully purified using Ni-NTA agarose Column (Fig. 4b). The concentration of the recombinant VP8-S2 protein was calculated as 3 mg/ml.

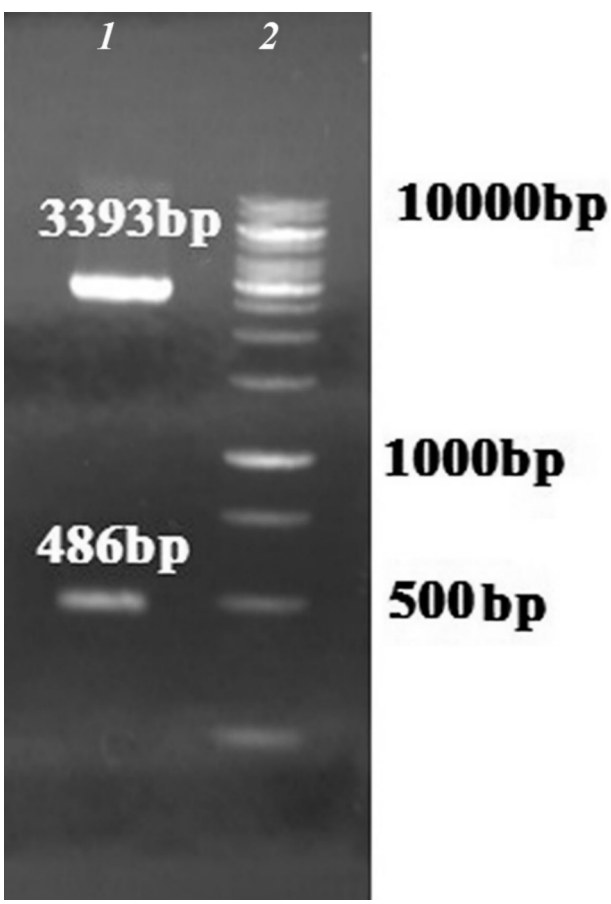

Fig. 2. Digestion of the recombinant pGH-VP8-S2 plasmid (lane 1: digested recombinant plasmid 2: DNA size marker).

Immunization and purification of IgY

There was no decrease in egg laying capacity after the immunization ($p<0.05$). The results showed that the combination of antigen with Freund's adjuvant generated neither laying decrease nor had adverse effects in hens. The purified IgY was electrophoresed on SDS-PAGE using 12% polyacrylamide gel. It showed that IgY contained two major proteins 23kDa (light chain) and 68 kDa (heavy chain) (Fig 5a).

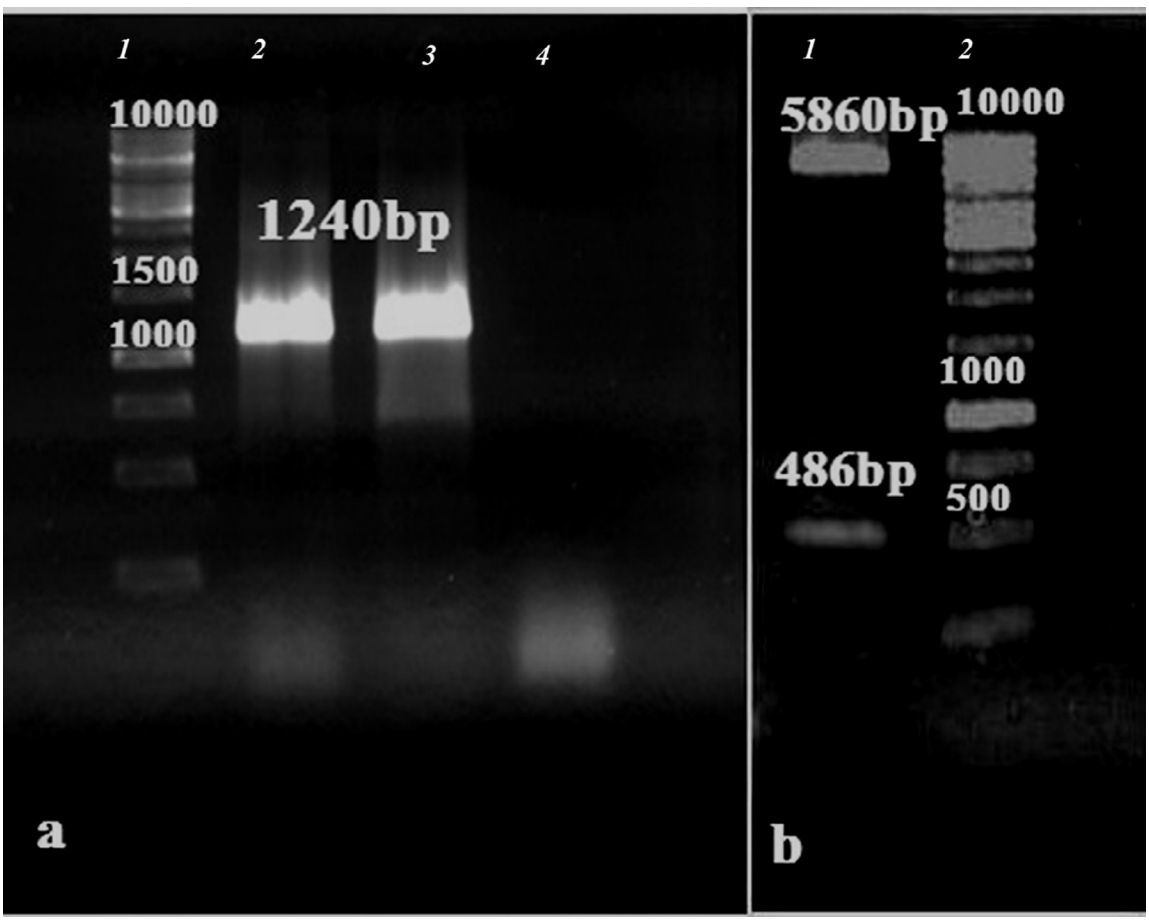

Fig. 3. (a) Electrophoresis of the PCR colony by pET T7 primers (lane 1: DNA size marker, lane 2 and 3: the VP8-S2 gene and lane 4: negative control). (b) Digestion of the recombinant pET32a(+)-VP8-S2 plasmid (lane 1: digested pET32a(+)-VP8-S2 plasmid and lane 2: 1kb DNA size marker).

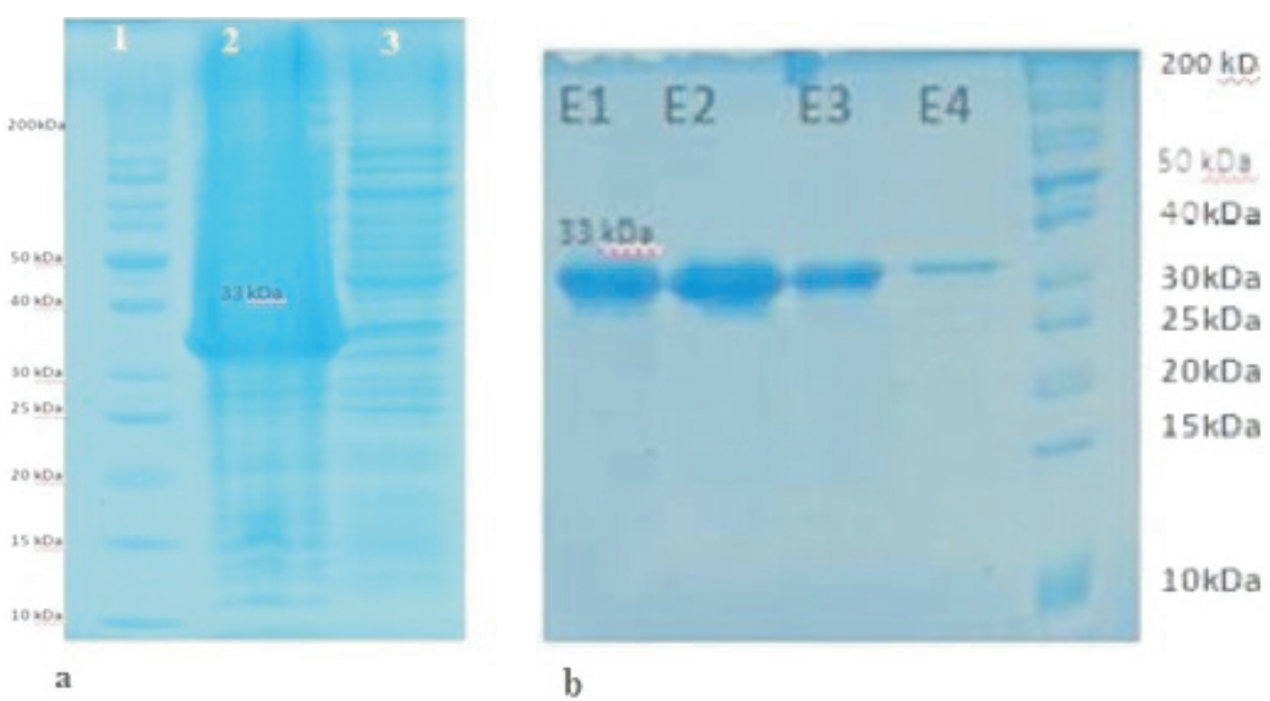

Fig. 4. (a) SDS-PAGE of the recombinant protein (lane1: pellet uninduced by IPTG, lane 2: pellet inducted by IPTG and lane 3: protein size marker 200kDa). (b) SDS-PAGE analysis of the purification steps: (Lane 1): protein size marker, (Lane 2, 3, 4 and 5): four fractions of the eluted proteins.

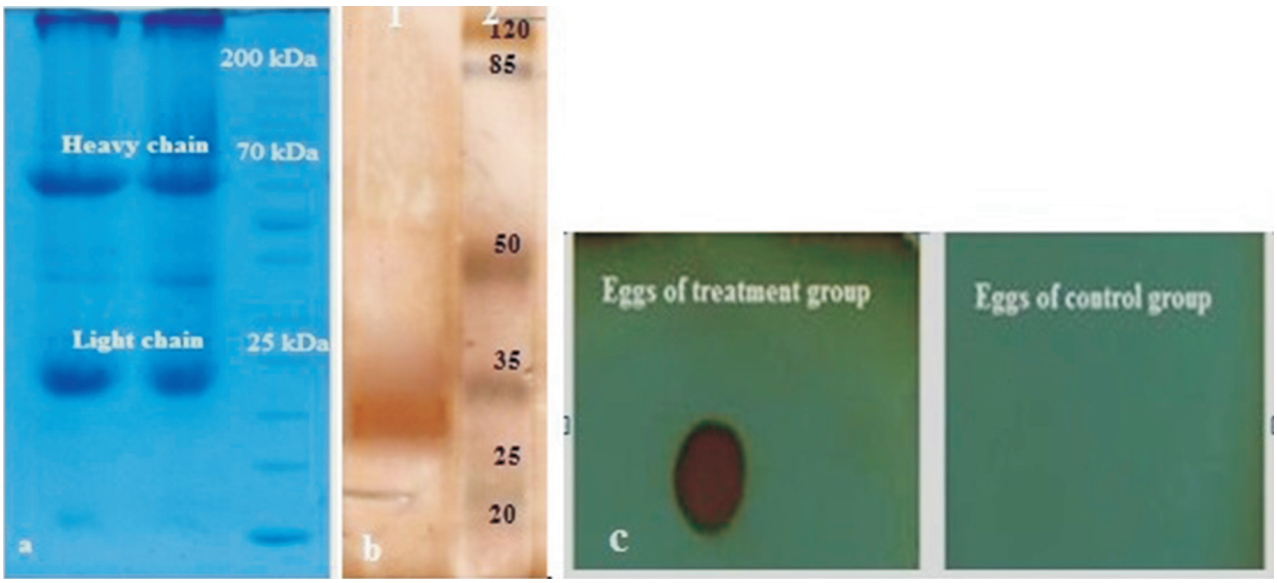

Fig. 5. (a) SDS-PAGE of the purified IgY. (b) Western blotting of the recombinant protein. Lane 1: the recombinant VP8-S2 antigen and Lane 2: protein size marker. (c) Dot blotting analysis of the treatment (immunized by VP8-S2 antigen) and control groups.

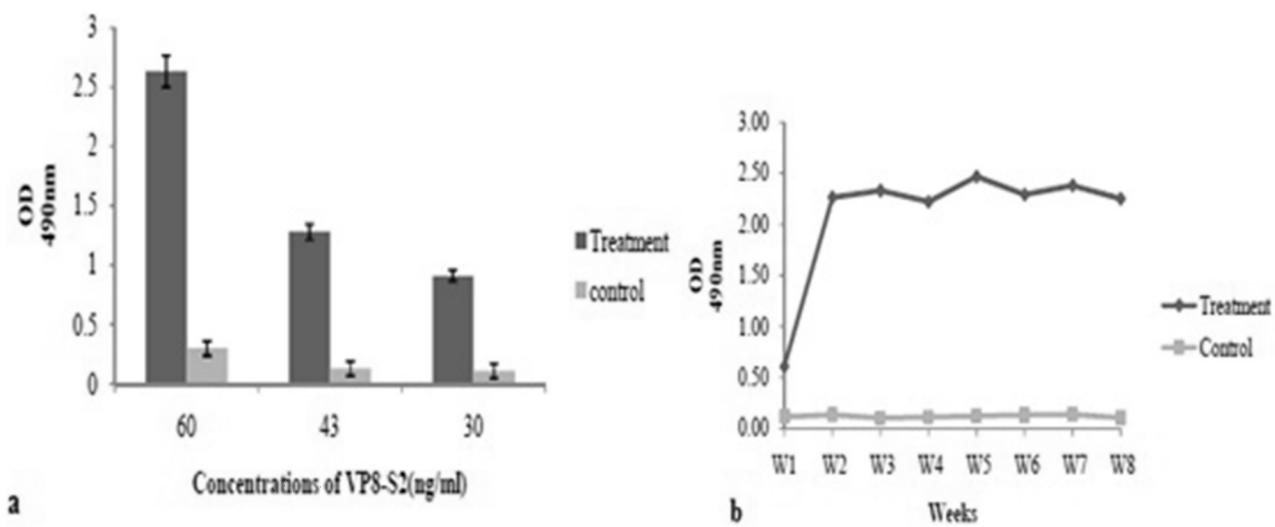

Fig. 6. (a) Specific activity of anti-VP8-S2 IgY (at 1:3000 dilution) with the recombinant protein in treatment and control groups. (b) Changes in the anti-VP8-S2 antibody activity after the last immunization.

Western blotting and dot blotting analyses

Western blot analysis indicated a 33kDa protein band that showed the presence of anti-VP8-S2 specific IgY in purified IgY of egg yolks collected from hens immunized with the recombinant VP8-S2 protein (Fig 5b). Dot-blot analysis of the recombinant VP8-S2 protein showed that it could be recognized by the anti-VP8-S2 IgY in 1:2000 dilution of antibody (Fig. 5c).

Specific activity of IgY antibody against VP8-S2 protein by Indirect ELISA

The anti-VP8-S2 IgY activity was measured as ELISA absorbance. ELISA assay (Fig 6a) indicated positive binding of IgY to VP8-S2 protein. Also, Fig 6a shows that the recombinant VP8-S2 protein in treatment group were recognized by anti-VP8-S2 IgY ($p<0.0001$).The results indicated that VP8-S2 antigen is highly immunogenic to hens. Anti-VP8-S2 IgY produced from the first to eight weeks after the last

immunization is shown in Fig 6b. The results indicated that immunoglobulin production was started to increase one week after the last immunization. Significant differences were shown between the first week and other weeks ($p<0.05$). There was a significant increase of the specific IgY activity in the second week after the last immunization ($p<0.05$). Also, significant differences were shown in the specific IgY activity between the second and third weeks, fourth and fifth weeks and fourth and sixth week ($p<0.05$). No significant difference was observed in the specific IgY activity in the last weeks of egg collection indicating that the antibody level remained constant.

Discussion

Maternal antibodies (colostrum) protect the neonatal offspring against rotavirus and coronavirus diarrhea for the first several days of life. However, milk antibodies drop rapidly in cattle and develops neonatal diarrhea often after calves become infected with rotavirus and coronavirus (Acres and Babiuk 1978). The development of vaccines or administration of milk supplements that make passive protection for the first several days of life induces high protection against bovine rotavirus and coronavirus (Parreño et al. 2010). Previous studies have indicated that treatment with colostrum, monoclonal and polyclonal antibodies provided protection against bovine rotavirus and bovine coronavirus in calves and human (Fernandez et al. 1998, Vega et al. 2011). Vega et al. (2011) reported that supplementing newborn calves; diets for the first 14 days of the life with egg yolk, represents a promising strategy to prevent bovine rotavirus diarrhea. Ikemori et al. (1997) indicated that the orally administered egg yolk of immunized hens with bovine coronavirus and colostrums powders protected against bovine coronavirus which induced diarrhea in neonatal calves.

The main strategy in this study was to design a chimeric gene (named VP8-S2) carrying various epitopes of the bovine rotavirus and bovine coronavirus proteins for co-expression VP8-S2 proteins. We selected well-known online epitope prediction servers and a multi-method analysis approach to enhance the accuracy of epitopes prediction. The chimeric VP8-S2 antigen was expressed at high levels. We immunized the hens before beginning of the laying period to avoid of reduction in egg laying capacity by immunization. Anti-VP8-S2 IgY antibody was produced by immunizing hens with the recombinant VP8-S2 protein. The results of this study were in agreement with the results of other studies that indicated that immunizied hens are excellent alternative to produce polyclonal antibodies (Liou et al. 2011, Han et al. 2012). Also, the present results are the first molecular study on design and construction of chimeric VP8-S2 antigen, cloning and co-expression of the VP8 and S2 antigenic regions. It is in agreement with results of the other studies that showed *E. coli* expression system as appropriate for expression at high levels of the VP8 gene of the bovine rotavirus (Yoo et al. 1997, Favacho et al. 2006). The finding of this study was in agreement with the other findings that reported the VP8 antigen have been expressed as fusion protein in insoluble form (Larralde and Gorziglia 1992, Favacho et al. 2006). The results were that anti-VP8-S2 IgY was able to recognize the VP8-S2 antigen specifically.

The results indicated that the anti-VP8-S2 IgY antibody may be applied into the immunotherapy for the bovine rotavirus and bovine coronavirus infections. Therefore, the anti-VP8-S2 IgY may be useful in the diagnosis and treatment of bovine rotavirus and coronavirus infections, but the efficiency should be more investigated.

Acknowledgments

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