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Production of specific IgY antibody to the recombinant FanC protein produced in *Escherichia coli*

Khadijeh Nasiri [1], Saeed Zibaee [2*], Mohammadreza Nassiri [3], Mojtaba Tahmoorespur [1], Alireza Haghparast [4]

[1] Department of Animal Science, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad , Iran
[2] Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran
[3] Department of Animal Science and Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran
[4] Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

Objective(s): *Enterotoxigenic Escherichia coli* (*ETEC*) strains are one of the primary causes of diarrhea in newborn calves and in humans, pigs, and sheep. IgY technology has been identified as a promising alternative to generating a mass amount of specific antibody for use in immunotherapy and immunodiagnostics. The purpose of this study was to produce specific antibody by egg yolk antibody (IgY) to recombinant FanC protein from *ETEC*.

Materials and Methods: FanC (K99) gene was amplified from *ETEC* by specific primers and polymerase chain reaction. The gene was cloned and subcloned into pTZ57R/T and pET32a (+) vectors, respectively. Recombinant vector was transferred into *E. coli BL21 CodonPlus (DE3)*. Protein expression was investigated by 1 mM IPTG induction. Hens were immunized by the purified recombinant FanC protein. The activity and specificity of the IgY antibody were detected by dot-blotting, Western blotting, and indirect ELISA.

Results: We obtained FanC specific IgYs by immunizing the hens with the recombinant FanC protein. The anti-FanC IgY showed binding specifically to the FanC protein of *ETEC*.

Conclusion: The results emphasize that specific IgY against the recombinant FanC protein could be recommended as a candidate for passive immunization against *ETEC* infection in animals and humans.

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Introduction

Enterotoxigenic Escherichia coli (*ETEC*) strains are the major cause of diarrhea (1). *ETEC* expressing K99 fimbriae is a bacterium that causes diarrhea in calves, lambs, piglets (2), and humans (3) resulting in mortality, morbidity, reduction of live weight, and huge economic losses (4). The K99 protein located at the surface of *ETEC* is a polymeric protein structure and has a diameter of 5 nm (2). K99 fragment encodes eight gene products named FanA to FanH, all of which are required for biosynthesis of K99 (5). The nucleotide sequence of the FanC gene comprises 159 amino acids which are proceeded by the signal sequence of 22 residues (6).

Chicken egg yolk (IgY) has been used widely for treatment and prevention of infections in humans and animals (7). It is used for passive protection against pathogen infections such as *Bovine rotavirus*, *ETEC, Bovine coronavirus*, *Salmonella*, *Staphylococcus* and *Pseudomonas* (8). Passive immunization using oral administration of specific antibodies such as IgY represents an effective strategy to prevent gastrointestinal infection in animals (9). The present study was carried out to characterize the specific IgY antibody produced by immunizing the hens to the recombinant FanC protein expressed in *E. coli.*

Materials and Methods

PCR amplification

A strain of Iranian *ETEC* was isolated from diarrhea samples of newborn calves that was positive for the K99 antigen. It was provided by Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran, and confirmed by biochemical and molecular tests. Genomic DNA of *ETEC* was extracted by using a DNA extraction kit (Bioneer, Korea). One pair of specific primer was designed and synthesized by Macrogen (South Korea) as follows: FanC-Forward: 5'-*CGCGGAT CCGC-G*GCGAATACAGGTACTATTAACTT-3' and FanC-

*Corresponding author: Saeed Zibaee. Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Mashhad, Iran. email: s.zibaee@mrazi.ac.ir

Reverse: 5'-*CCGCTCGAGCGG*CATATAAGTGACTAA-GAAGGATG-3'(492 bp). *Bam*HI and *Xho*I restriction sites were placed for gene transfer FanC to expression vector in forward and reverse primers, respectively (underlined). Polymerase chain reaction (PCR) was carried out in total volume of 25 µl containing 1.5 µl of $MgCl_2$ (50 mM), 2.5 µl of 10X PCR buffer, 2 µl of dNTPs (20 mM), 2 µl of mix primer (5 pmol/µl), 1 µl of DNA template (50 to 100 ng/µl) and 0.2 U/µl of *EXTaq* DNA polymerase, and 15.8 µl of deionized water. The PCR cycle conditions were an initial denaturation at 94 °C for 10 min, followed by 34 cycles of 94 °C for 30 sec, 48 °C for 30 sec, 72 °C for 30 sec, and final extension at 72 °C for 10 min. Amplified PCR fragment was separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, visualized under UV light and photographed with a UVidoc GEL Documentation System (UVitec, UK).

Cloning and sub-cloning the FanC (K99) gene

The purified PCR product by GeneJET Gel Extraction Kit (Fermentas) was ligated into pTZ57R/T cloning vector by T/A cloning. The recombinant vectors were transformed into competent *E. coli* DH5α. The bacterial clones harboring recombinant plasmid DNA were screened based on their ampicillin resistance. The recombinant vector was investigated by PCR reaction using FanC specific primers and M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGACC-3') primers. The PCR product was visualized by 1% agarose electrophoresis. The recombinant pTZ57R/T-FanC plasmid was purified by GeneJET Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instructions.

pET32a (+) vector was digested with *Bam*HI and *Xoh*I enzymes. Then the FanC fragment was ligated through T4 DNA ligase procedure in 16 hr at 16 ˚C into pET32a (+) vector. pET32a (+)-FanC plasmid was transformed into the *E. coli* strain *TOP10F'* cell and grown overnight at 37 ˚C on LB agar plates with ampicillin (100 µg/ml). The recombinant plasmids were screened by PCR colony and Miniprep plasmid was digested using *Bam*HI and *Xoh*I enzymes. The PCR colony was carried out with pET T7 primers and specific primers of the FanC gene. The recombinant plasmid was sequenced using T7 promoter (5'-TAATACGACTCACTATAGGG-3') and pET T7 terminator (5'- GCTAGTTATTGCTCAGCGG-3') primers by Macrogen (Korea) to confirm the constructed sequence.

Expression and protein purification of FaC gene

E. coli BL21 CondonPlus (DE3) harboring the FanC expression construct was grown in Luria broth (LB) culture supplemented with 100 µg/ml ampicillin and incubated overnight at 37 ˚C and 150 rpm. Fresh LB liquid (50 ml) containing 100 µg/ml ampicillin was incubated with 5 ml of preculture and was incubated at 37 ˚C and 150 rpm to reach OD_{600}:0.6. Then, the culture was induced with 1 mM IPTG and incubated at 37 ˚C with shaking at 150 rpm for 6 hr. Cells were harvested at different time points after induction. The FanC expression was evaluated on 12% SDS-PAGE and visualized using Coomassie-blue staining.

For purification, the pellet of the induced cells was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH=8), Lysozyme was added in concentration of 1 mg/ml. It was incubated on ice for 30 min. The cells were disrupted by sonication. Subsequently 1 ml of the 50% Ni-NTA (Qiagen, USA) slurry was added to 10 ml cleared lysate and was mixed by shaking at 4 ˚C for 60 min. The lysate-Ni-NTA mixture was load into a column with the bottom outlet capped. Then, the recombinant protein was washed once with 4 ml washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM Imidazole, pH=8). The 6Xhis-tagged recombinant protein was eluted 4 times with 0.5 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH=8) per column each time and was collected in 4 tubes. The concentration of the recombinant FanC protein was determined by Bradford assay (10). Before the injection of the recombinant FanC protein to the hens, the recombinant FanC protein was dialyzed to remove imidazole and other impurities.

Dot-blotting analysis of the recombinant FanC protein

The recombinant FanC protein (2 µg) was induced with 1 mM 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 (BSA) and was shaken for 30 min at room temperature, it was washed with PBST (PBS, 0.1% (v/v) Tween) for 2 min, then it was immersed in primary antibody (Anti-His tag rabbit) diluted 1:500 for 1 hr on a shaking incubator at room temperature. Afterward, the nitrocellulose membrane was washed 4 times for 5 min each time in PBST, it was incubated with a secondary antibody that was diluted 1:3000 for 1 hr on a shaking incubator at room temperature. The membrane was washed 4 times for 5 min each time in PBST. Color development was observed by addition of diaminobenzidine dissolved in PBS and H_2O_2.

Immunization

Immunization of the hens was performed by intramuscularly injecting with 200 µg of purified protein with an equal volume of Freund's complete adjuvant (Sigma, USA) for the first immunization. Freund's complete adjuvant without recombinant protein was injected to the control group. Two booster immunizations followed using incomplete Freund's adjuvant with two weeks interval. One week after the last of injection, the eggs were collected daily for eight weeks, marked and stored at 4 ˚C.

IgY antibody purification

IgY antibody was extracted and purified from egg yolk using polyethylene glycol 6000 (PEG 6000) powder (Merck, Germany) based on Polson's method (11). Egg yolk was separated from the egg. Phosphate–buffered saline (pH=7.6, twice the volume of yolk suspension) was added and mixed. The PEG 3.5% (w/v) was added and then shaken at room temperature for 10 min followed by centrifugation (13000 g, 20 min, 4 ˚C). The supernatant was collected and filtered through a Whatman filter paper to remove lipids. PEG 6000 was added to 8% (w/v) concentration, after gentle shaking at the room temperature for 10 min, again it was centrifuged (13000 g, 20 min, 4 ˚C). The precipitation was dissolved in 10 ml PBS and then 12% (w/v) PEG 6000 was added and centrifuged as described above. The pellet containing IgY was resuspended in 2 ml PBS and dialyzed against PBS overnight at 4 ˚C. Specific IgY anti-FanC content was measured by the Coomassie-blue method according to Bradford (10).

Dot-blotting and Western-blotting of anti -FanC IgY

2 µg of the recombinant protein was dot-blotted individually on two pieces of nitrocellulose membranes. After similar steps that were mentioned previously, each piece was immersed in primary antibody (hen anti-FanC IgY of the treatment group after injection 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 similar to steps mentioned previously.

The purified recombinant protein was separated using 12% SDS-PAGE and transferred onto nitrocellulose membrane. Sites were blocked with BSA 1.5% (w/v) and incubated at 4 ˚C overnight. The membrane was incubated with anti-FanC IgY antibody (1:1000) for 1 hr at room temperature. After washing, rabbit anti-chicken IgY H&L (HRP) diluted in PBS (1:2000) was added and incubated for 1 hr. The membrane was washed and the specific binding of IgY antibody to the FanC protein was investigated.

Indirect enzyme-linked immune sorbent assay (iELISA)

The specific activity of IgY antibody against the FanC protein was determined by iELISA. 100 µl of the recombinant protein was coated onto microtiter plates (65, 44, 33, and 26 ng) for 1 hr. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST) and nonspecific sites were blocked using blocking buffer (1.5% BSA in PBST) for 1 hr at 37 ˚C. After washing three times, 100 µl of diluted IgY antibody (1:2000) in PBST was added to the wells as the primary antibody and then incubated for 1 hr at 37 ˚C. Subsequently, the plate was washed again and incubated with 100 µl of labeled rabbit anti-chicken IgY H&L (HRP) at a diluted IgY antibody (1:10000) as the secondary antibody for 1 hr at 37 ˚C. The colorimetric detection was carried out using O-phenylenediamine (OPD, Sigma) as a chromogenic substrate of HRP and then wells were washed with PBST. The reaction was stopped by adding 50 µl/well 2N H_2SO_4. The absorbance of each well was measured at 492 nm.

Statistical analysis

The t-test was used to detect significant differences between means of ELISA absorbance for determination of specific IgY antibody. Also, a repeated measure ANOVA was used to compare the means of ELISA absorbances between weeks. *P*-value<0.05 was considered significant.

Results

PCR amplification, cloning and sub-cloning of the FanC gene

The FanC PCR product yielded a specific fragment 492 bp. The PCR amplified product was successfully cloned into pTZ57R/T after being extracted from the agarose gel. The results of PCR assay with specific primers, M13 primers (Figure 1a), and enzyme digestion showed that the target gene was correctly inserted into the recombinant pTZ57R/T plasmid (Figure 1b). A new sequence for the first time has been published on NCBI gene database under accession number KP05429.

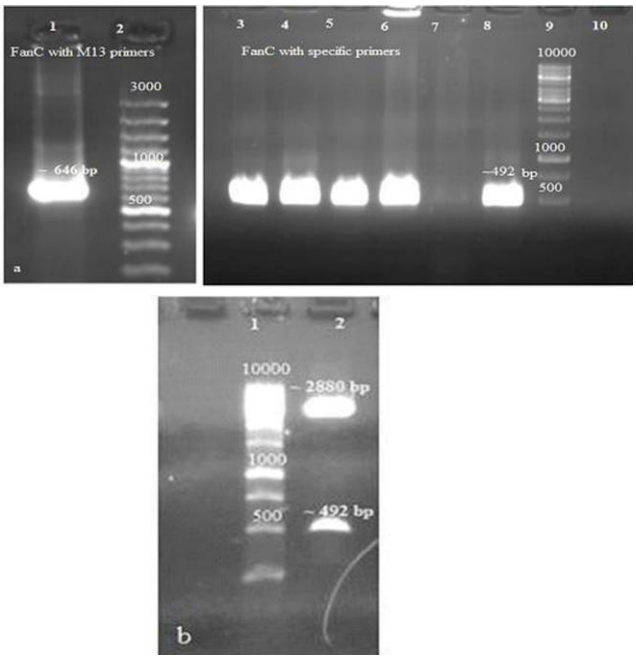

Figure 1. (a) Electrophoresis of colony PCR of the FanC gene with M13 and specific primers, (Lane 1: FanC gene, lane 2: DNA size marker, lane 3-8: FanC gene, lane 9: DNA size marker, lane 10: negative control). (b) digested pTZ57R/T-FanC plasmid, lane 1: DNA size marker and lane 2: digested pTZ57R/T-FanC)

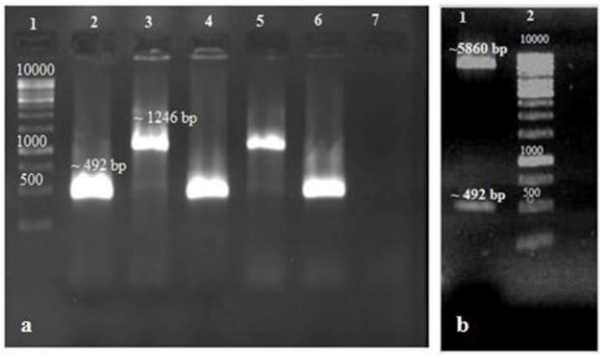

Figure 2. (a) Electrophoresis of colony PCR of the FanC gene with specific primers and pET T7 primers (lane 1: 1kb DNA size marker, lane 2 -6: FanC gene and lane 7: negative control). (b) Digestion of pET32a (+)-FanC (lane 1: digested pET32a(+)-FanC and lane 2: 1kb DNA size marker)

The results colonies of the recombinant pET32a (+)-FanC plasmid were confirmed by colony PCR with pET T7 primers (Figure 2a). The existence of FanC gene into pET32a (+) was verified by enzymatic digestion (Figure 2b).

Expression and purification of the recombinant FanC protein

The results of the FanC expression were shown on 12% SDS-PAGE electrophoresis by induction on 1 mM IPTG in 2, 4 and 6 hr after induction (Figure 3a). Figure 3 shows that the FanC antigen was highly expressed in *E. coli* BL21 CodonPlus. The recombinant FanC protein could be detected near 36 kDa (pET32a (+) +FanC). Also, Figure 3 shows proteins near 18.5 kDa that show pET32a (+) without a fragment of the FanC gene. pET32a (+) 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 increased the size of the expressed FanC protein near 18.5 KDa.

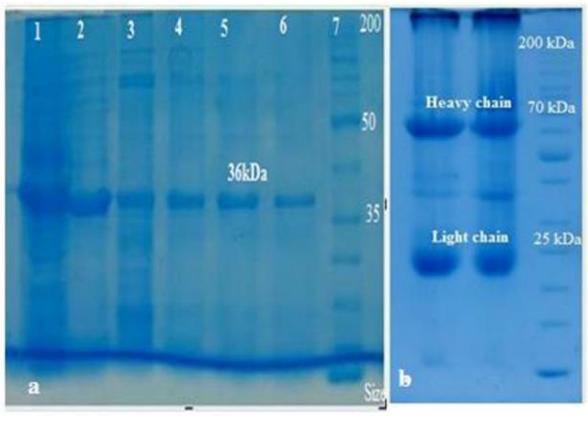

Figure 4. (a) 12% SDS-PAGE analysis of the protein purification. (Lane 1): the induced cell lysate; (lane 2): washed fraction; (lanes 3, 4, 5, and 6): fractions of the eluted proteins (E1, E2, E3, and E4), (lane 7): protein size marker. (b) SDS-PAGE analysis of the purified IgY antibody

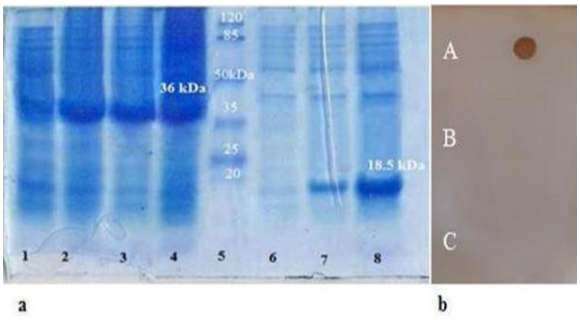

Figure 3. (a) The results of SDS-PAGE. (lane1: pellet uninduced by IPTG, lane 2, 3 and 4: pellets in 2, 4 and 6 hr after induction, respectively, lane 5: protein size marker, lane 6, 7 and 8: pET32a(+)/*E. coli* in 0 , 2, and 4 hr after induction.(b) Dot-blot analysis of the recombinant protein. Brown dot (A) (*E. coli*/ pET32a (+)/FanC) shows transformed bacteria induced by IPTG. (B)PBS and (C), *E. coli*/pET32a (+)/FanC before the induction shown as negative control

The result of dot-blot analysis confirmed the existence of the recombinant FanC protein (Figure 3b). The recombinant FanC protein was successfully purified using Ni-NTA agarose column (Figure 4a). The concentration of recombinant protein was calculated as 1.3 mg/ml.

Immunization and IgY purification

There was no decline in egg laying capacity after the immunization (*P*-value<0.05); the hens produced an average of one egg per day. The results showed that the combination antigen to Freund's adjuvant caused neither laying decrease nor adverse effects in the hens. IgY antibody was successfully extracted and purified from the egg yolk by PEG 6000. The purified IgY electrophoresed on SDS-PAGE using 12%, showed that IgY contained two major protein light (23 kDa) and heavy (68 kDa) chains (Figure 4b). In addition, some bands were presented around 40 kDa on the gel, probably due to the C-terminal fragment of the vitellogenin II precursor (12).

Dot blotting and western blotting analyses

Dot-blot analysis of the recombinant FanC protein showed that it could be recognized by the anti-FanC specific IgY in 1:2000 dilution of antibody. Western blot analysis indicated a 36 kDa protein band.

Indirect ELISA of anti-FanC IgY

The anti-FanC antibody activity was measured from ELISA absorbance data. ELISA assay (Figure 5a) indicated positive binding of IgY to FanC as expected. Also, Figure 5a shows that anti-FanC IgY (at 1:2000 dilution) was able to recognize the recombinant FanC protein in the treatment group (immunized by FanC) and there were significant differences between treatment and control groups (*P*-value<0.0001). These results indicated that FanC is highly immunogenic for chickens. The production of specific IgY from the first to eight weeks after the last

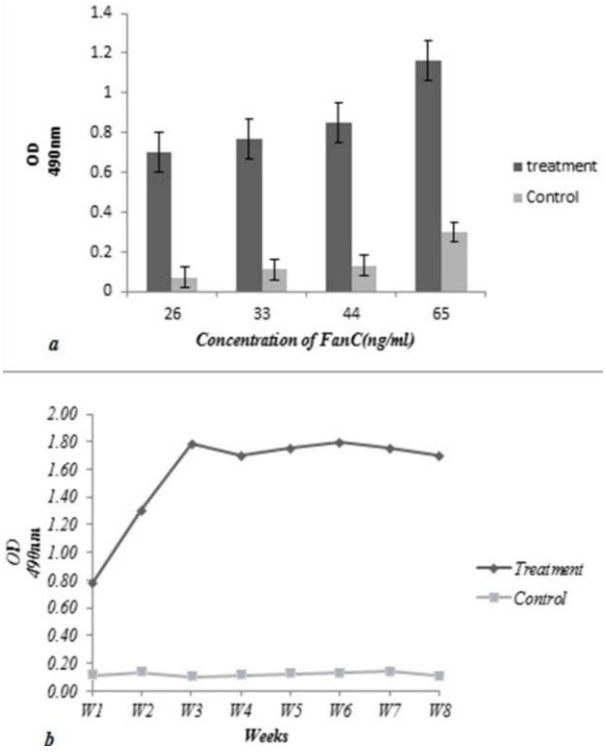

Figure 5. (a) Specific activity of anti-FanC IgY (at 1:2000 dilution) with the recombinant protein in treatment and control groups. (b) Changes in the anti-FanC antibody activity in the egg yolk after the last immunization (weeks)

immunization is shown in Figure 5b. The results indicated that high IgY level started to increase two weeks after the complete immunization (*P*-value<0.05). There was a significant increase of IgY production after the first week and the results showed specific antibody activity was increased (*P*-value<0.05). Also, significant differences were seen between the second week and other weeks. No significant difference was seen in specific IgY production from the third week to the eighth, indicating that the antibody level remained constant.

Discussion

Despite the improvement in herd management, nutrition, and facilities in animal husbandry, diarrhea in calves is still the main problem in this industry. Maternal antibodies (colostrum) protect the neonatal offspring against *ETEC* caused diarrhea in the first days of life (13). Moreover, milk antibodies drop rapidly in cattle that often develop diarrhea after neonatal calves become infected with *ETEC* (14). The development of vaccines or administration of milk supplements that make passive protection for the first several days of life induces a high protection against *ETEC* (15). IgY as an efficient biological agent has shown a significant efficacy in prevention of infectious diseases. The evidences of IgY efficacy reported in several studies. These studies are including specific egg yolk antibody against recombinant *cryptosporidium parvum* P23 protein (16), generation of egg yolk antibodies against *influenza* M2 protein (17), production of IgY against recombinant *porcine epidemic diarrhea virus* COE protein (18) and production of specific IgY against *helicobacter pylori* recombinant (19).

Researchers (20) indicated that passive protection of neonatal piglets achieved against K88, K99 and 987P fimbrial adhesions of *ETEC* with specific egg yolk immunoglobulin, and their results of scanning electron microscopy revealed resistance to bacterial adhesion in piglets with high-titer antibodies. In another study (21) IgY was applied for the protection of neonatal piglets from diarrhea caused by *E. coli* (*ETEC*). Malekan *et al* (22) produced anti-*E. coli k99* IgY and anti-*Salmonella* IgY by immunizing laying hens with inactivated *E. coli K99* and *Salmonella enteritidis* antigens. They indicated that immune eggs against *E. coli* k99 and *Salmonella* can be used to stimulate the immune system in newborn calves and protect them against the deleterious effects of these organisms. Hashish *et al* (13) constructed a multi-epitope antigen fusion (FanCSTa- E2) against *ETEC* and the homologous bovine viral diarrhea virus. They showed that this fusion antigen can potentially be developed as a vaccine for broad protection against bovine diarrhea.

In this study, FanC antigen of Iranian *ETEC* was isolated, it was cloned and subcloned into an expression vector and expressed in *E. coli* strain *BL21 (DE3);* the purified protein was injected to hens to produce anti-FanC IgY. We immunized the hens before the start of the laying period in order to avoid reduction in egg laying capacity by immunization because the adjuvant use in the immunization can directly decrease this capacity (23). Anti-FanC IgY antibody was produced by immunizing hens with the recombinant FanC protein, which can be used as a tool for the immune diagnosis. The results of this study are in agreement with the results of other studies that indicated immunized hens are an excellent alternative for production of polyclonal antibodies (24, 25). Also, the present results are the first molecular study on cloning and expression of FanC (K99) antigen of Iranian *ETEC* and they agree with results of the other studies that revealed *E. coli* expression system can be appropriate for expression at the high level of FanC antigen of *ETEC* (13, 26). The antigen FanC amount showed that it simulated the immunological response. It was reported the ideal does for simulating immune system ranges from 10 µg to 1 mg (27). In this study, FanC antigen concentration was within this ideal range. The antibody activity started to increase two weeks after intramuscular injection (*P*-value<0.05). Some studies indicated that anti-*E. coli* IgY activity increased two

weeks post immunization (*P*-value<0.05) but decreased 3~4 weeks later (21, 25 and 28); this phenomenon appeared in the fourth week in the present study, but it wasn't significant and started to reach a constant level. The iELISA results indicated that the recombinant FanC protein was recognized by anti-FanC IgY specifically.

Conclusion

The results of this study indicate that anti-FanC IgY antibody can be applied in immunotherapy for *ETEC* infection. Therefore, the anti-FanC IgY could be developed for diagnosis and treatment of *ETEC* infection. A further experiment with different titers of IgY and its preventive effects on the bacteria may give more practical information for an alternative application of IgY in the future as a supplement to passive immunity in newborn calves, sheep, and pigs against an economically important bacterial disease.

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Conflict of Interest

The authors declare no conflict of interest.

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