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Full Length Research Paper

Expression of enterotoxigenic *Escherichia coli* colonization factor I major subunit (CfaB) and evaluation of its immunogenicity

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Enterotoxigenic *Escherichia coli* (ETEC) is the most significant agent leading to childhood diarrhea and death in developing countries. This bacterium is the cause of 380 thousand deaths in children under five years of age. Due to its prevalence as well as difficulties in its treatment, designing effective vaccines against ETEC is a goal of the World Health Organization (WHO). The Colonization factor B (CfaB) as major subunit of fimberiae has a critical role in bacterial attachment to small intestine epithelium. Hence, the molecule alone or together with other candidate molecules has been considered in vaccine design. In this work, we produced recombinant CfaB in *E. coli* with the aim of studying its immunogenicity as a component of vaccine. The cfaB gene was cloned into pET28a and regarding the presence of rare codons in cfaB gene, it was not expressed. Therefore, an optimized gene with codon preferences was synthesized and cloned into pET28a vector and subsequently expressed. The recombinant protein was purified with Ni-NTA column and used as an antigen for mice immunization and in ELISA test. Microplate agglutination inhibition test was utilized to show antibody blocking activity. In conclusion, codon optimization is a useful approach for obtaining large quantities of a desired protein. Relying on agglutination inhibition experiment, anti-CfaB serum was able to block the binding of colonization factor antigen I (CFA/I) fimbriated ETEC to erythrocytes.

Key words: Enterotoxigenic *Escherichia coli* (ETEC), colonization factor antigen I, expression, agglutination inhibition.

INTRODUCTION

Enteric pathogens constitute a serious pediatric threat particularly in less developed countries (Walker, 2005). Enterotoxigenic *Escherichia coli* (ETEC) is among the most frequent bacterial causes of diarrhea (Qadri et al., 2005) and infects adults from industrialized countries visiting these regions, thus it is known as travelers' disease (Stauffer et al., 2002). This organism accounts for over 200 million episodes of illness and annually 380,000 deaths in children (Svennerholm and Tobias, 2008; Walker et al., 2007).

Two important virulence characteristics of ETEC differentiate these bacteria from other diarrheagenic *E. coli*: first, adherence to epithelial cells of the small intestine; and second, production of enterotoxins that act locally on enterocyte and cause diarrhea (Shaheen et al., 2009). Adherence and colonization in host cell via filamentous surface structures known as colonization factors (CFs) is a crucial step in ETEC pathogenesis (Anantha et al., 2004). More than 25 human ETEC colonization factors have been identified so far (Fleckenstein et al., 2010); however, up to 70% of strains

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express colonization factor antigen I (CFA/I), CFA/II and CFA/IV (Qadri et al., 2000; Shaheen et al., 2003; Turner et al., 2006).

The epidemiological studies have reported that CFA/I producing strains are widely distributed in endemic areas such as South America, Asia and Africa (Alves et al., 2000; Shaheen et al., 2004). CFA/I molecule consist of a polymerized stalk of thousand copies of the major subunit CfaB and tip localized minor subunit (CfaE) (Li et al., 2009; Mu et al., 2008). CfaB as the major subunit is a carbohydrate binding protein which specifically interacts with a number of glycosphingolipids and glycoproteins in human small intestinal epithelium (Jansson et al., 2006). Also, binding of CfaB protein to erythrocyte carbohydrate receptors causes mannose-resistant hemagglutination of human type A erythrocytes (Evans et al., 1977; Sedlock et al., 1981).

Humoral immunity to CFs and toxins protects against ETEC infection (Turner et al., 2006). Because of high immunogenicity of CFA/I and its important role in pathogenesis, it's essential proteinaceous subunits are central to ETEC vaccines currently under development (Cassels et al., 1992) (Svennerholm and Tobias, 2008) (Shaheen et al., 2009). Regarding the widespread prevalence and high mortality rate of ETEC, the World Health Organization (WHO) plans much research on vaccine design against ETEC (Walker et al., 2007).

In the present study, we intend to isolate and express the CfaB gene from a local isolate of ETEC. The comparison is made between the expression of the native and codon-optimized sequences in the prokaryotic systems. The purification of recombinant CfaB, its immunogenicity and effectiveness of antibody against rCfaB in inhibition of ETEC binding to its receptor are investigated as well.

MATERIALS AND METHODS

Amplification and cloning of cfaB gene

An ETEC bacterium isolate was taken from Reference laboratory of Bu Ali hospital, Tehran, Iran. Amplification of cfaB encoding gene (cfaB) derived from genomic DNA of ETEC bacterium isolate was carried out with two primers (forward 5′TGTGCAGTGAGTGCTAAGCTTGTAGAG3′ and reverse 5′AATACTCGAGTCAGGATCCCAAAGTC3′) with *Hind*III and *Xho*I restriction sites (underlined) in a high fidelity PCR system (Fermentas, Lithuania). These PCR primers were designed based on cfaB gene (Accession number: M55661). PCR conditions were as follows: 95°C for 5 min, 31 cycles at 94°C for 15 s, 58°C for 30 s and 68°C for 4 min, with final extension of 7 min at 72°C. The amplified fragments were run on 1% agarose gel with 100bp DNA ladder and confirmed by restriction digestion with *Eco*RI. Polymerase chain reaction (PCR) fragment was ligated into pTZ57R (Fermentas, Lithuania) and the ligated DNA was then transformed into *E. coli* DH5α (Invitrogen, Canada). The recombinant colonies were screened with LacZ-based blue/white selection and restriction digestion. After that, cfaB gene was subcloned in pET28a expression vector. The construct was subsequently transformed into *E. coli* BL21 (DE3) pLysS cells (Stratagene, USA). The accuracy of cloning was determined by PCR with specific primers, restriction enzyme digestion and sequencing system. All of the procedure was carried out according to the standard protocol (Sambrook and Russell, 2001).

Analysis of *in vitro* expression of pET28a-native cfaB

The expression circumstances were evaluated under various conditions such as inducer (isopropyl-b-D-thiogalactopyranoside, IPTG) concentration (0.25, 0.5, 0.75, 1 and 1.5 mM), incubation times (1, 2, 3, 4 and 5 h) and incubation temperatures (25, 35 and 37°C).

Also, the expression plasmid (pET28a-cfaB) was transformed into Rosetta cells and expression was induced as previously described (Shaheen, 2009). Afterwards, the induced cells were harvested by centrifugation (5000 rpm, 10 min) and lyzed by sonication on ice (45 s, in 75% maximum outputs). Protein concentration was measured using the Bradford assay and subjected to electrophoresis in SDS polyacrylamide gel (Bollag, 1996).

Construction of the synthetic codon-optimized cfaB gene

The construct of the codon-optimized cfaB gene was designed by using optimum geneTM algorithms and the most frequently used codons for *E. coli*. This construct was synthesized by ShineGene Bio-Technologies, Inc. (Shanghai, China), cloned into pET28a vector and transformed into BL21 (DE3) pLysS. The cfaB optimized sequence was submitted to GenBank (GeneBank. GU355642).

Expression and purification of CfaB

The synthetic cfaB gene-pET28a was expressed by promoter induction via adding IPTG (final concentration 1mM) and incubation at 37°C for 3 h. Harvested cell pellets were re-suspended in 1:4 (w/v) buffer A (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4) and were sonicated on ice as aforementioned. The soluble protein extracts were then loaded on a 12% SDS-polyacrylamide gel. Purification of 6x His-tagged proteins was performed by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen, Canada) under non-denaturing condition. Recombinant protein was eluted by 50 mM NaH_2PO_4, pH 8.0, containing 300 mM NaCl and a linear gradient of 20 to 250 mM imidazole. Fractions containing the expected protein were determined by SDS-PAGE (Bollag, 1996).

Confirmation of rCfaB by Immunoblot analysis

The expression of the recombinant protein was confirmed by Western blotting using monoclonal anti-His tag (Roche, Germany) and polyclonal anti-CfaB (provided by Dr. Salmanian, NIGEB, Iran). Subsequent to electrophoresis, the protein band was blotted to nitrocellulose membrane using Bio-Rad Protean II system (USA) and transfer buffer (39 mM glycin, 48 mM Tris-Base, 0.037% SDS and 20% methanol). Non-specific binding was blocked by incubating the membrane in blocking buffer of 3% skim milk in PBST (phosphate-buffered saline, pH 7.2, % 0.05 Tween 20) for 2 h at room temperature. The membrane was washed three times with PBST at each step. The membrane was separately incubated with anti-His tag and anti-CfaB (1/8000 and 1/1000 dilution, respectively, with gentle shaking for 1 h at RT) and then incubated with a 1/1000 dilution of anti-mouse IgG peroxidase conjugate

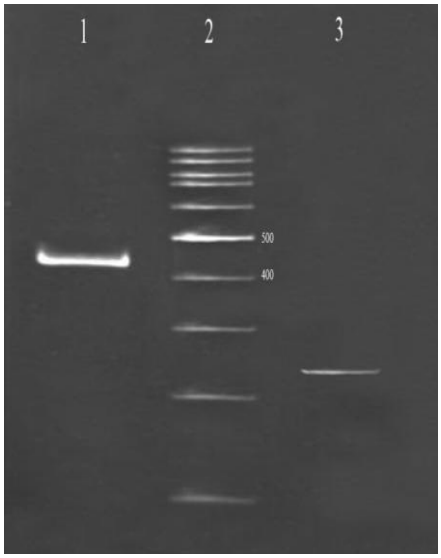


Figure 1. PCR product of cfaB gene, lane1: amplified product cfaB gene (444 bp), lane 2: 100 bp DNA ladder (fermentas), lane 3: digestion of PCR product with *Eco*RI (digested fragment~228 bp).

(Sigma, Germany). The specific band was developed with 3, 3′-diaminobenzidine (DAB) (Sigma, Germany) in buffer (10 mM Tris, pH 7.5, 10 µl H_2O_2).

Immunization procedure and ELISA

Six-week-old female BALB/C mice were immunized with recombinant CfaB protein. Briefly, each mouse was injected s.c. with 20 µg recombinant CfaB protein with complete ferund's adjuvant and then three booster doses with incomplete ferund's adjuvant every two weeks.

After bleeding and collection of mouse antiserum, CfaB specific ELISA was performed with serum samples using purified recombinant CfaB. Each well of Maxisorp plate (Nunc, Denmark) was coated with 1 µg (100 µl) of CfaB in carbonate- bicarbonate buffer (pH 9.6) at 37°C for 1 h. The plate was washed three times with PBST at each step. After 1 h of blocking at 37°C with 1% (w/v) skim milk in PBST to prevent nonspecific binding, the plate was incubated with serially diluted sera for 1 h at 37°C. Anti-mouse IgG peroxidase conjugated (Sigma, Germany) (100 µl, 1:10,000) was added to the plate and incubated for 1 h at 37°C. Finally, the plate was developed with 100 µl per well of OPD (Sigma, Germany) substrates in citrate-phosphate buffer (pH 5) for 20 min at room temperature in darkness. After stopping the reaction with sulfuric acid (2.5 M), the plate was read at 492 nm with an ELISA reader (Dynex, USA).

Purification of antibody

Anti-CfaB IgG polyclonal antibodies were purified from immunized mouse sera by protein G affinity column chromatography. Sera from different non immunized mouse were treated in an identical manner. After washing, the matrix-bound proteins were eluted and separated by SDS–PAGE (10%) and used for subsequent immunoblotting to verify specific reaction to CfaB (Data not shown).

Hemagglutination-inhibition assays for determining anti-CfaB antibody capacity

The ETEC strains attach to intestinal epithelial cells by CFA/I pili and also mediate agglutination of human type A erythrocytes. Hemagglutination-inhibition is a useful technique to examine anti-CfaB antibody function. To this end, ETEC strain (ATCC: 35401) was obtained from Department of Foodborne and Diarrheal Disease, Shaheed Beheshti University, Tehran and were grown overnight at 37°C on CFA agar (1% casamino acids, 0.15% yeast extract, 2% agar, and 0.0005% MnCl2, 0.005% MgSO4.7H2O) and suspended in PBS (pH 7.4) to reach OD_{600} 2. A 9.0 ml of human blood type A was mixed with 1.0 ml of 3.8% citric acid in distilled water. Blood was diluted 1:4 with PBS, pH 7.2 and 1:4 with 1% mannose in PBS (on ice and at room temperature, in different bacterial densities). Subsequently, 10 µl of anti-CfaB antibody was preincubated with 50 µl of bacterial suspensions for 20 min at room temperature and then mixed with RBC. Non-immunized mice anti-sera were used as negative controls (Evans, 1991).

RESULTS

Cloning and expression of native cfaB gene

The native form of CfaB gene without promoter and signal peptide sequences encodes a mature protein of 147 amino acid residues with a molecular weight of 16.2 kDa. This gene without promoter and signal peptide was amplified from the genomic DNA by PCR with high fidelity enzyme and its size on 1% agarose gel was 444 bp. After digestion of PCR product by restriction enzyme (EcoRI), two fragments (216 and 228 bp) that were of almost the same size were produced (Figure 1). Digested PCR product was subcloned into pET28a expression vector and transformed into *E. coli* BL21 (DE3) pLysS. To further confirm the presence of cfaB gene, colonies were analyzed by colony PCR, *Xho*I/*Hind*III excision and *Eco*RI digestion (Figure 2). The sequencing result was confirmed by its comparison with databases using Basic Local Alignment Search Tool (BLAST) software.

Induction of recombinant protein expression was investigated at different temperatures, induction times, IPTG concentrations and different strain of host such as Rosseta. Unexpectedly, a desired protein band was not seen in SDS-PAGE.

Expression and purification of the optimized cfaB gene

The native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. In order to improve the expression levels, the cfaB gene were designed with the most frequently codon usage of *E. coli*. Additionally, to

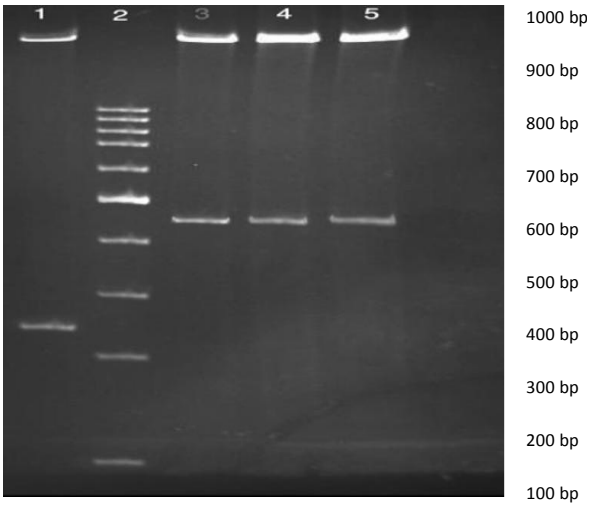
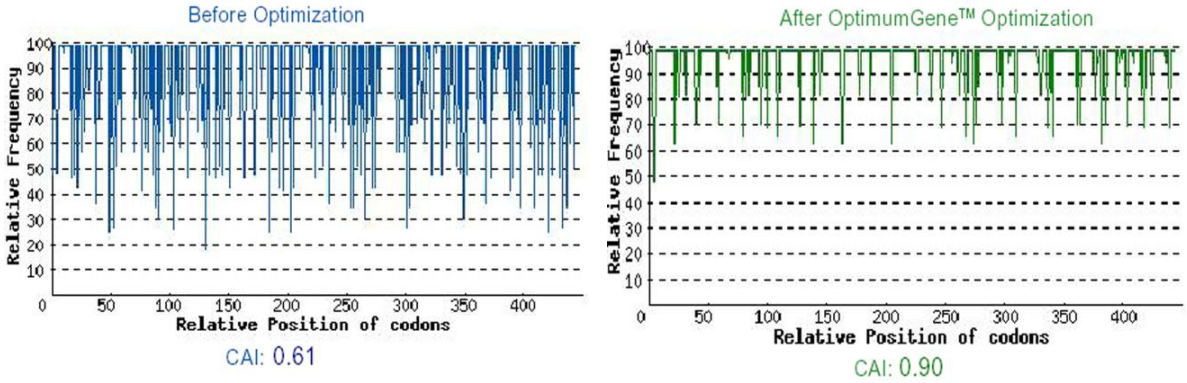


Figure 2. Analysis of recombinant vector with restriction enzyme digestion, lane 1: pET28a-*cfaB* single digestion with *Eco*RI enzyme (~228 bp insert), lane 2: 100 bp DNA ladder (fermentas), lane 3 to 5: pET28a-*cfaB* doubles digestion with *Xho*I and *Hind*III enzymes (444 bp insert).



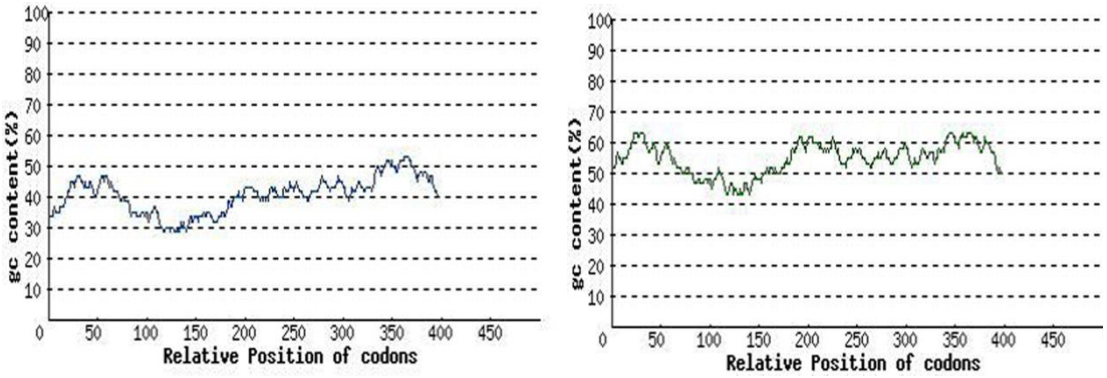
Before optimization

After optimization

Figure 3. Codon Adaptation Index (CAI): The distribution of codons usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.9 is regarded as very good, in terms of high gene expression level. CAI was upgraded from 0.61 to 0.90.

increase RNA stability, the stem-loop structures which impact ribosomal binding were deleted. Codon adaptation index (CAI) is an important factor for estimation of gene expression scale. Furthermore, based on OptimumGene™ algorithm, CAI was upgraded from 0.61 to 0.90 (Figure 3). Besides, GC content was optimized to prolong the half-life of the mRNA. So, GC content average after optimization increased from 54.4 to 77.54 (Figure 4). Meanwhile, the percentage of high frequency codons (<90%) increased to 70% after optimization (Figure 5). Finally, the optimized sequence of cfaB gene was submitted to GenBank (GeneBank. GU355642).

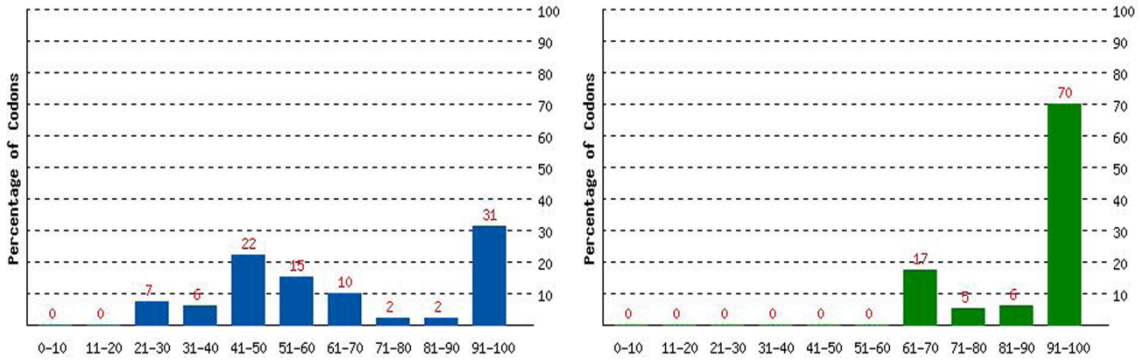
The optimized gene which was expressed under T7 promoter control was seen on SDS-PAGE to be approximately 20 kDa (Figure 6). Based on the N-terminal His tag, the highly soluble recombinant CfaB was purified by Ni-NTA affinity chromatography (Figure 6). Western blotting results showed that anti-CfaB and anti-His tag antibody detected recombinant CfaB protein (Figure 7).



Before optimization

After optimization

Figure 4. GC Content Adjustment: The ideal percentage range of GC content is between 30 to 70%. GC content average after optimization increased from 54.4 to 77.54.



Before optimization

After optimization

Figure 5. Frequency of Optimal Codons (FOP): The percentage distribution of codons in computed codon quality groups. The value of 100 is set for the codons with the highest usage frequency for a given amino acid in the desired expression organism. The percentage of high frequency codons (<90%) increased to 70% after optimization.

Immunization of mice and examination of the capacity of anti-CfaB antibody

This purified protein (CfaB) was used for the immunization of mice. There was a significant difference ($P < 0.001$) between the pre-immunization and post-immunization titers of serum antibodies in all immunized mice. Pre-immunization titers never exceeded 1:15 for IgG, while anti-CfaB antibody titer reached amounts up to 1:256000 (Figure 8).

The effectiveness of anti-CfaB antibodies to prevent of ETEC binding to erythrocyte was examined by hemagglutination-inhibition assay. Based upon this experiment, the anti-CfaB antibodies (1:20 dilution) prevented the CFA/I-mediated binding of ETEC to red blood cell receptors.

DISCUSSION

After natural ETEC infection, the immune system especially in mucosal site responses to colonization factor antigens and these immune responses may have an essential role in illness limitation and natural immunity (Svennerholm and Tobias, 2008; Rudin and Svennerholm, 1994). Therefore, colonization factor antigens have been taken into consideration in vaccine development against this organism (Svennerholm and Tobias, 2008; Walker et al., 2007). Among ETEC colonization factors, CFA/I has been presented in majority of ETEC strains in endemic countries (Turner et al., 2006; Alves et al., 2000; Shaheen et al., 2004) and immunity to this antigen could widely protect against ETEC diarrhea (Svennerholm and Tobias, 2008). CfaB

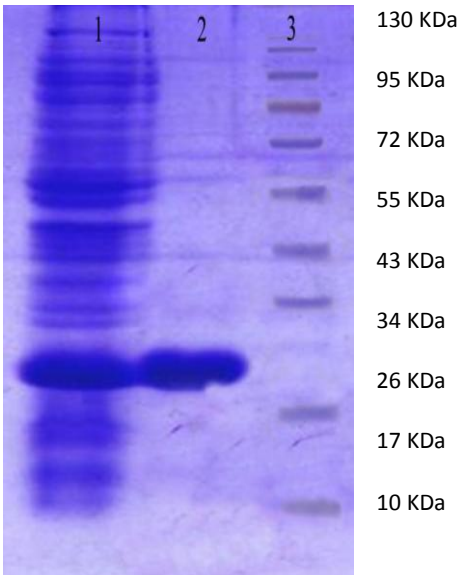


Figure 6. Analysis of recombinant CFaB expression, purification on 12% SDS-PAGE, lane1: induced *E. coli* BL21DE3/pET28a-codon optimized cfaB gene with IPTG, lane 2: Purified CFaB protein under non-denaturation condition (20 kDa), Lane 3: protein weight marker (Fermentas, #SM0671).

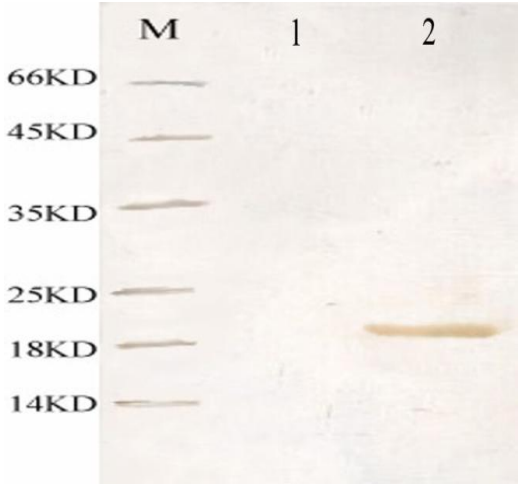


Figure 7. Western blot analysis of CFaB using anti His-tag antibody and anti-CfaB antibody, M: protein molecular weight marker (Fermentas, #SM0431), Lane 1: non-induced clone (control), Lane 2: recombinant CfaB (Only reaction of CfaB protein with anti-CfaB antibody was shown).

as a major subunit of CFA/I fimbriae specifically binds to carbohydrate receptors on enterocytes (Jansson et al., 2006). The antibody against this molecule can inhibit

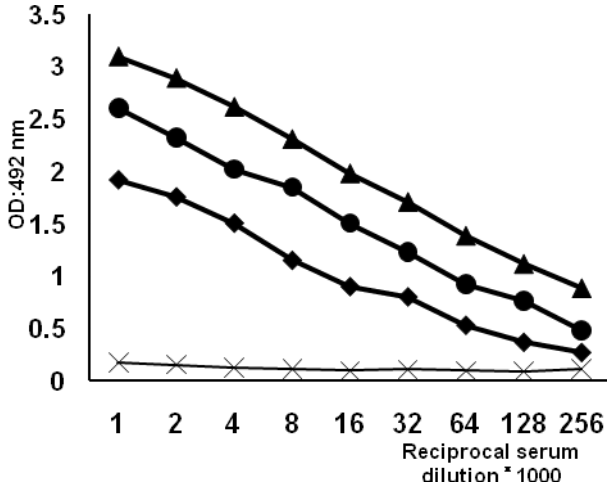


Figure 8. Titers of anti CfaB-antibodies after immunization of mice with 4 doses were determined by ELISA on mice sera. The figure shows the mean OD of ELISA wells in repeated experiments. (__▲__After 4th injection, __●__After 3rd injection, __♦__After 2nd injection, __×__ Controls).

ETEC colonization; consequently, this molecule is introduced as an appropriate candidate vaccine.

In the present study, two strategies were proposed for recombinant CfaB production, and we compared the expression of native and codon-optimized cfaB genes. In the first strategy, native cfaB gene was amplified by high fidelity enzyme PCR mix (unique blend of *Taq* polymerase and a DNA polymerase with proofreading activity). After subcloning and induction of cfaB expression in pET28 expression vector, the SDS-PAGE analysis clearly demonstrated that native cfaB gene was not expressed in this expression vector (pET28a).

On the other hand, researchers have previously reported the successful expression of this native gene (Favre et al., 2006; Li et al., 2009; Tobias et al., 2010). Favre et al. (2006) produced recombinant colonization factors in CF medium. It was postulated that, salt and minerals in this medium as regulator of gene transcription can produce secondary metabolites in this strain and cause optimum expression. However, applying these changes had no effect on recombinant protein expression.

Although we used *E. coli* BL21 (DE3) host strain for its benefits such as proteases deficiency, this protein was not expressed. The native gene employs tandem rare codons that can reduce the efficiency of translation or even disengage the translational machinery. As Rosetta™ host strain supplies extra copies of tRNA genes to enhance the expression of proteins that contain codons rarely used in *E. coli,* we transformed the recombinant construct to Rosetta™ host strain. Despite

the changes made in circumstances, protein expression was not observed on gel.

In 2004, Anantha et al. (2004) cloned cfaB gene (residues 24 up to 170) in pMAL-p2 vector and this protein was expressed as a fusion construct (CfaB-MBP) under the tac promoter in BL21 (DE3) host. A strategy to increase protein expression is to fuse the genes to the highly expressed sequence. It appeared to be a successful strategy for CfaB expression (Sorensen and Mortensen, 2005). Also, Li et al. (2009) linked cfaB to cfaE by a linker (Asp-Asn-Lys-Glu) and expressed in pET24a vector. Structure and promoter of this vector is very similar to pET28a, except that the distance between promoter and multiple cloning sites in pET 24a is very short due to the lack of ribosome binding site and His tag. These differences make this vector appropriate for transcription but not for expression, and thus regulatory elements must be added to the sequence for gene translation and expression. Maybe insertion of regulatory elements or codon optimization not been mentioned in that paper.

In *E. coli*, degree of codon bias is highly related to level of gene expression. Optimal codons help to achieve faster translation rates and high accuracy (Sharp and Li, 1986). The cfaB gene has an AT-rich sequence and consists of rare codons. Hence, optimization of cfaB codon increases the production of recombinant protein. The cfaB sequence was optimized by using Optimum Gene^{TM} algorithm. These changes increased the GC content, codon adaptation index (CAI) and mRNA stability. CAI explains the compatibility of codon usage between the native gene sequence and that of the expression host (Sharp and Li, 1987). Following this strategy, we achieved higher level of expression for CfaB protein.

The soluble expressed CfaB protein containing the 6xHis tag was purified with Ni-NTA column and confirmed with anti-His tag and anti-CfaB antibodies in immunoblotting. This recombinant protein was utilized to immunize the mice. The capacity of anti-CfaB antibody was investigated by hemagglutination-inhibition assay. The result showed that these antibodies are functionally potent and are able to prevent CfaB-receptor interaction. This result is in accordance with Svenerholm's study (Rudin and Svennerholm, 1994).

In conclusion, CfaB protein as an immunogen can be a major vaccine component against ETEC infection. In addition, codon optimization and expression in different strain of *E. coli* hosts provide a valuable technique for the high level production of recombinant proteins.

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