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Original Article

The Design and Production of Engineered Sta Toxin as a Vaccine Candidate Against Enterotoxigenic *Escherichia coli*

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Abstract

Introduction: Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of watery diarrhea outbreak in children under five years of age and passengers in developing countries. The pathogenicity of ETEC is due to secretion of colonization factors (CFs) and two enterotoxins including heat-labile (LT) and heat stable (ST). Although diarrhea is considered as one of the causes of mortality in developing countries, no approved vaccine is available for the disease caused by ETEC. Accordingly, the objective of the present study was to design a vaccine candidate containing Sta toxin which accounts for 30%-75% of ETEC species.

Materials and Methods: A chimeric construct consisting of two Sta toxoid connected together by two linkers was designed. After expression and purification by Ni-NTA column, western blotting was performed to confirm the protein. The 2Sta protein was administered to BALB/c mice via injection and the serum and fecal antibodies titer was evaluated by the ELISA test.

Results: The recombinant 2Sta protein was expressed in insoluble form (inclusion body) and the 20 kDa band was observed on the SDS-PAGE 12%. The results of the ELISA test suggested that IgG and IgA antibodies had enhanced compared to the control group.

Conclusions: The Sta protein which was produced in most ETEC species can be induced in the immune system and raised the serum and fecal antibodies. Using this candidate subunit vaccine could actually protect bacterial infection.

Keywords: Heat Stable Enterotoxin, Enterotoxigenic *E. coli* (ETEC), Diarrhea, Sta Subunit

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Introduction

In spite of the global advances in the public health, diarrhea is still considered as one of the major life-threatening diseases for children in developing countries. Diarrhea which is caused by consuming contaminated food or water threatens the life of many people in the endemic regions.[1,2] Enterotoxigenic *Escherichia coli* (ETEC) is one of the major and important factors causing watery diarrhea in children aged under five years and passengers in developing countries annually (280-400 million and 100 million, respectively).[3,4] Diarrhea is recognized by symptoms such as pain in the abdominal region, headache, vomiting and fever. After five days, it improves without using any antibiotics or may result in death due to loss of excessive water.[5,6] The important factors in the pathogenicity of ETEC include colonization factors (CFs), heat-labile (LT) and heat-stable (ST) enterotoxins. This bacterium is first attached to the epithelial cells of the small intestine by the colonizing factors, and secretes ST and LT enterotoxins. With the secretion of the enterotoxins, the homeostasis or fluid balance in the epithelial cells of the intestine is disrupted, and leads to diarrhea.[7,8] Although the diarrhea caused by ETEC is one of the most important diseases affecting children in the endemic regions, there is no licensed vaccine available for it. Reports represented the reduction of the severity and infection rate in contaminated regions or increasing of immunogenicity in the endemic regions after infection with ETEC.[9,10] In recent years, the design and development of subunit vaccines such as LTB against ETEC have been considered as an effective strategy. One of the most important antigens of ETEC is LTB which are applied as a vaccine candidate because of their high immunogenicity.[11] Colonizing factors are other candidates for vaccine development and the production of antibodies against them can prevent ETEC infection or reduce the severity of the disease.[12] The Sta subunit, a small molecule with 19 amino acids, is non-immunogenic and if attached to a protein carrier it can cause immunogenicity. By binding to special receptors

of guanylate cyclase C, located on the surface membrane of intestinal epithelial cells, intracellular cGMP levels increase and will cause diarrhea. Between ETEC isolates, 49.4% were positive for the ST toxin, 25.4% were positive for the LT toxin only, and 25.2% were positive for both LT and ST. Producing anti-Sta antibodies is essential to induce immunity against ETEC infection which was applied in previous research.[13-16]

Since more than 66% of diarrheas caused by ETEC are due to ETEC strains that produce St toxin, using ST toxin in the development of candidate vaccines against ETEC is necessary.[17] Accordingly, the aim of the current study was to design and express a construct containing two Sta toxoid connected together by two linkers. The recombinant 2Sta protein was designed as a subunit vaccine against ETEC. The BALB/c mice were immunized by recombinant 2Sta and specific IgG and IgA antibodies against Sta toxin were measured.

Materials and Methods

Chemicals, kit, and molecular markers were purchased from Merck, Sinagen, and Qiagen companies; IPTG and kanamycin antibiotics were purchased from Thermo, and the Freund's complete and incomplete adjuvant as well as anti-IgG and anti-IgA mouse antibody were obtained from Sigma Aldrich.

Designing of the Chimeric 2Sta Gene

Sta toxin was used for designing 2Sta recombinant construct. The Sta amino acid sequence was retrieved from UniProt database. Since Sta toxin is a small molecule and cannot be expressed as a single immunogen protein, two Sta toxoid sequence connected together by two linkers (KKKKKKKKKKAEAAAKEAAAKAKKKKKKKKK). The chimeric protein was back translated and after on the gene was synthesized by Shinegene Company, China.

Expression and Purification of 2Sta Protein

The pET28a vector harboring 2*Sta* gene construct was transformed into *E. coli* BL21 (DE3) strain for the expression of the recombinant protein. The polymerase chain reaction (PCR) was carried out to confirm the 2*Sta* recombinant gene using universal T7 primers. Eventually, the PCR product was analyzed by 1% agarose gel and a 300 bp band observed using UV transilluminator. For protein expression, 500 µL of the overnight culture of the construct was inoculated to LB medium containing kanamycin (50 µg/mL), and incubated at 37°C until the optical density reached 0.7. The IPTG was added to the medium with the final concentration of 1 mM, and incubated for 5h at 37°C. For analysis of protein solubility, expression was performed in large-scale 50 mL cultureand bacterial precipitate dissolved in 5 mL of native buffer (50 mM NaH_2PO_4 and 300 mM NaCl, pH 8) and was mixthoroughly. Moreover, lysozyme (1 mg/mL) and sonication(6 x 10 s with 10 s pauses at 200–300 W) was applied toensure complete cell lysis. After centrifugation at 13000 gfor 10 minutes, supernatant containing soluble protein wascollected and the pellet resuspended in a denaturing buffer(100 mM NaH_2PO_4, 10mM Tris.Cl and 8 m urea, pH 8) andincubated in 37°C for 1 hour and the samples were analyzed on 12% SDS-PAGE gel. After cell disruption, the lysate was centrifuged (15 min, 10000 g, 4°C) and the supernatant was applied on a Nickel–nitrilotriacetic acid (Ni-NTA) affinity chromatography column (Qiagen) and the purification steps were performed according to the manufacturer instructions. The column was equilibrated with lysis buffer and the protein solution was loaded onto the column at a flow rate of 0.5 mL/min. The impurity was removed two times by washing the column with washing buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea pH 5.9). The protein was eluted with elution buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea) at pH 4.5. Protein concentration was determined by the Bradford method with BSA (bovine serum albumin) as a standard.

Confirming Sta Recombinant Proteins using Western Blotting

The recombinant protein was separated by 12% SDS-PAGE and electro transferred onto PVDF membrane (Roche). The membrane was blocked with 5% non-fat skim milk in TBS buffer (50 mM Tris-Cl, 150 mM NaCl, pH 7.5) containing 0.05% Tween 20 (37 °C, 2 hours). The membrane was incubated with HRP-conjugated mouse anti-poly His-tag antibody (1:2000 Roche). Finally, the membrane was soaked in 3, 3′-Diaminobenzidine tablet (DAB Reagents; Sigma) for signal development.[18]

Mice Immunization

To determine the immunogenicity of the recombinant Sta, 12 BALB/c mice (female, 6-7 weeks old, Pasteur Institute, Tehran, Iran) were randomly divided into two groups. Group I was inoculated with 100 µg of purified with complete Freund's complete adjuvant subcutaneously into mice. Group II received PBS as the negative control. Serum was prepared from the blood sample of each mice group (blood was transferred to vials and was allowed to clot for 30 minutes, then serum was collected by centrifugation) and frozen at -70 °C until use. The serum samples of each mice group were prepared for immunological analyses.

Determining the Titer of Serum IgG and IgA

The collected sera were subjected to ELISA-based antibody titer assays. The purified recombinant 2Sta (500 ng/well) were used to load MaxiSorb plates (Nunc, Denmark) with 100 µL bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) incubated for 2 hours at 37 °C. The wells were blocked for 2 hours at 37°C by the addition of 200 µL of 3% (w/v) non-fat skim milk in PBST (PBS containing 0.05% Tween-20) and washed three times with PBST. The wells were incubated with serially diluted serum from immunized mice in triplicate at 100 µL/well for 1 hour at 37°C. The bound antibodies were detected with HRP-conjugated goat anti-mouse IgG (Sigma) in a 1:5000 dilution for 1h and washed three times with PBST. The reaction was developed with O-phenylenediamine as a substrate (Sigma) for 15 minutes at room temperature in the dark. Sulfuric acid (2.5 M) was used to stop the reaction and the absorbance was measured at 492 nm in an ELISA reader.

Results

Confirmation of pET-28a Plasmid Containing Sta Gene

The results of plasmid extraction have been illustrated in Figure 1. To ensure the presence of 2*Sta* gene in pET28a plasmid, PCR was performed with T7 universal primers. The 2*Sta* fragment 600 bp was observed on 1% agarose gel.

Expression and Purification of 2Sta Protein

The expression of recombinant 2Sta protein with 6x His-tag (C-terminal) was analyzed on 12% SDS-PAGE (Figure 2). The recombinant protein produced as inclusion Bodies (IB) was subsequently solubilized using 8 M urea. Purification of the recombinant protein was performed by Ni-NTA affinity chromatography under denaturing conditions (Figure 3). Estimation of the purified recombinant protein by using the Bradford method indicated that the concentration of the 2Sta was 356 µg/mL.

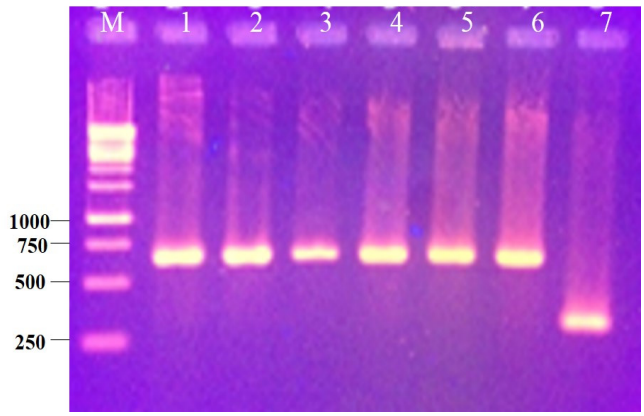

Figure 1. Electrophoresis of the PCR Products From 2*Sta* Gene (≈600bp) by Universal T7 Primers. Lane 1-6) positive samples; Lane 7) negative control (pET28a without insert); Lane M) 1 kb

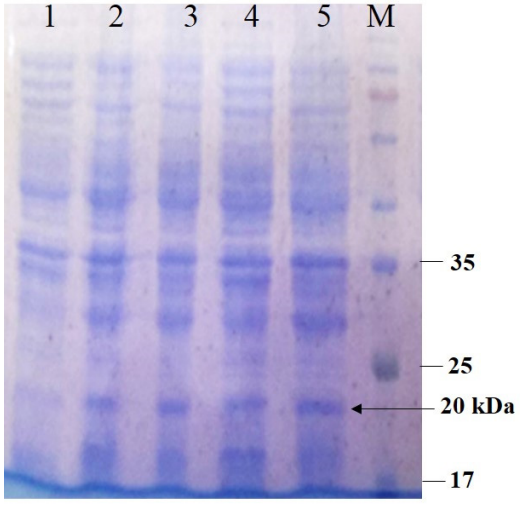

Figure 2. SDS-PAGE Analysis of Total Bacterial Proteins Containing pET28a-*2sta* (20 kDa) in Different Time Course. Lane1) before induction; Lane 2-5) 2, 4, 6, and 16 h after induction with IPTG, respectively; Lane M) protein molecular weight marker.

Western Blotting to Confirm the Expressed Protein of Sta

The authenticity of recombinant 2Sta (20 kDa) proteins were confirmed by anti-poly His-tag antibody. In contrast, no reactivity was observed in the negative control (Figure 4).

Determining the Titer of Serum IgG Antibodies

The results of serum ELISA in immunized mice indicated a systemic response induction and increased serum IgG titer against the recombinant Sta protein. Moreover, in the second administration, the serum IgG level in the injection group increased compared to the first administration and there was a significant difference (P<0.05) in the antibody titration (Figure 5). The ELISA results for the serum of mice immunized by 2Sta purified protein indicated that this protein caused induction of systemic response and elevation of serum IgG titer in the injection group. The level of this titer in the second blood sampling was far higher than that of the first bleeding compared to the control group (Figure 5).

Determining the Titer of Serum and Fecal IgA Antibodies

Titration of IgA was evaluated by ELISA methods. It showed that the serum and fecal IgA had increased (Figure 6).

Discussion

One of the infectious diseases which has developed serious and life-threatening problems in the public health in developing countries is watery diarrhea caused by Enterotoxigenic *E. coli* (ETEC).[19,20] The ETEC is one of the most prevalent pathogens isolated from the stool of patients in the hospitals of Tehran, Iran, along with *Shigella* which has caused the death of almost 500 000 children aged under five years in developing countries.[21,22] The treatment of diarrhea which is caused by ETEC is difficult because of its antibiotic resistance and also requires a strategy to reduce the incidence and severity of this infection. Since no approved vaccine is available against ETEC, in recent years, the development of

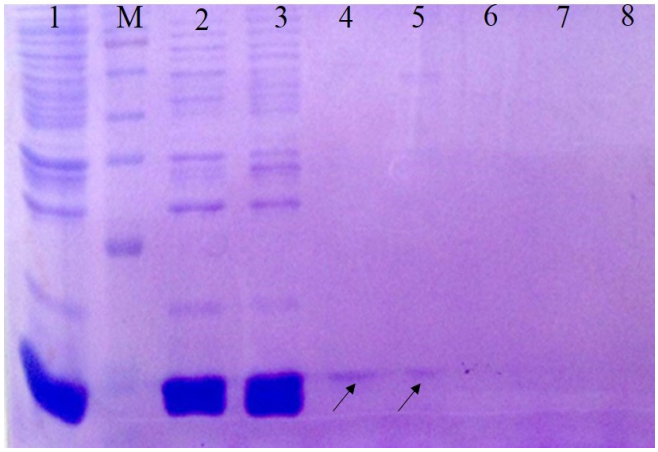

Figure 3. Purification of the Recombinant 2Sta Protein Under Denaturing Condition by Ni-NTA Column on SDS-PAGE 12%. Lane 1) lysate cell before column; Lane 2) flow through; Lane 3) washing column with wash buffer (pH 5.9); Lane 4 to 7) sample extracted fromthe column with elution buffer (pH 4.5); Lane 8) extracted with MES buffer, Lane M) protein weight marker.

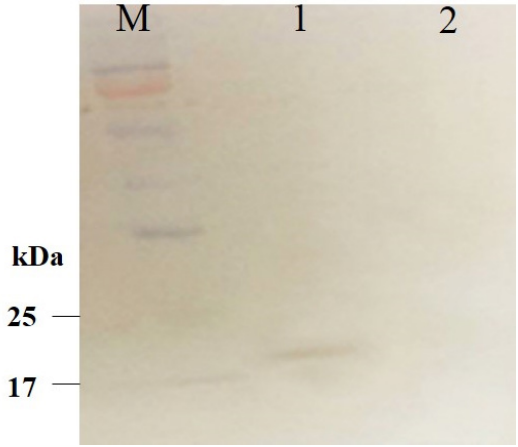

Figure 4. Analysis and Confirmation of 2Sta Purified Recombinant Proteins Using Western Blot. Lane 1) 2Sta purified protein samples with anti-His.tag antibody; Lane 2) negative control; Lane M) protein weight marker

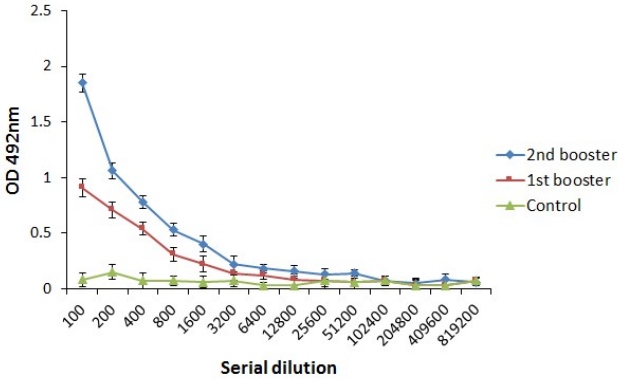

Figure 5. Estimation of Serum Antibody Response From Immunized Mice by Using Recombinant Purified 2Sta Proteins by ELISA. Control group was administered with PBS.

vaccines containing the ETEC antigens have been considered as an effective strategy for preventing infection and reducing mortality in developing countries.[23-25]

In many studies, Sta toxin was used for designing vaccines against ETEC because it produced 49.4% of ETEC species and by converting to the harmless toxoid; they achieved high levels of immunogenicity. The Sta toxin is a small and non-immunogenic molecule, which stimulated desirable immunogenicity only by chemically or genetically binding to a suitable carrier such as LtB, CtxB, or several copies of Sta sequence.[23,26-28] You et al intraperitoneally injected SLS protein consisting of subunit Sta, LtB, and Stb to mice and induced immunogenicity through producing antibodies against all of the three enterotoxins.[26] Zeinalzadeh et al, using chimeric protein consisting of CfaB, CFA/I, CS6, LtB, and Sta toxoid, achieved high immunity against ETEC in BALB/c mice.[27] In another study, Duan et al reported the successful expression of mutated *sta* along with Lt monomer and immunogenicity against both toxins in mice.[29] Owing to the importance of Sta subunit in the pathogenicity of ETEC, in the current study the recombinant construct which consists of two Sta sequences

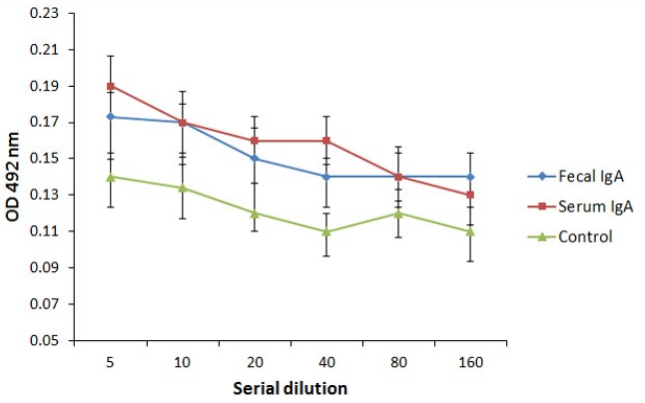

Figure 6. Evaluation of Fecal and Serum IgA Levels of Immunized Mice With Recombinant 2Sta Protein by ELISA. Control group was administered with PBS.

connected by two linkers was used.

Nandre et al found that the injection of a chimeric protein consisting of Sta and mutated LT antigens lead to inducing immunological response and enhancing the titration of IgG antibodies against both toxins in mice.[30] A similar study was carried out by Ruan et al in which a chimeric construct containing CFA/I/II/IV, Sta toxoid, dmLT could be elevated to the level of specific antibody against colonizing factors as well as both ETEC bacterial toxins.[31] In the present study, in order to achieve an acceptable immune response, three doses of the 2Sta recombinant protein were injected subcutaneously to the mice. Since the recombinant protein had not been administrated via mucosal route, the titration of serum and fecal IgA antibodies in injection group was raised slightly compared to the control group. On the other hand, ELISA results showed high levels of IgG antibody titration against the 2Sta recombinant protein in this group. These results were in line with the research performed by Nandre et al and Ruan et al in which Sta molecule has been able to enhance IgG antibody levels in the injection group by binding to proper carriers.[30,31] In this research, the maximum level of titer of antibody against Sta was observed in the second blood sampling which had received one more dose.

Conclusions

Acceding to the results, the use of 2Sta protein as a vaccine candidate has been able to stimulate the immune system and enhance the serum IgG antibody levels against Sta in mice. It was suggested that the chimeric protein would be able to cause a proper immunization in vaccinated people exposed to ETEC strain which only produce St Toxin. Furthermore, the use of its components could be considered in order to design a subunit and effective vaccine against ETEC.

Authors' Contributions

The authors contributed equally to this study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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