Iranian Journal of Immunology (IJI)
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ORIGINAL ARTICLE

Immunogenic Evaluation of Bivalent Vaccine Candidate against *Enterohemorrhagic* and *Enterotoxigenic Escherichia coli*

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ABSTRACT

Background: Caused by bacterial, viral, and parasitic pathogens, diarrhea is the second leading cause of death among children under five. Two strains of *E. coli*, namely *Enterotoxigenic*, ETEC and *Enterohemorrhagic* EHEC are the most important causes of this disease in developing countries. EHEC is a major causative agent of bloody diarrhea and hemorrhagic uremic syndrome, while ETEC is the most important cause of diarrhea in neonates and travelers. **Objectives:** To evaluate the immunologic properties of a subunit vaccine candidate comprising the main immunogenic epitopes from these two bacterial strains. **Methods:** The construct comprised of LTB and CfaB antigens from ETEC, and Intimin and Stx2B antigens from EHEC, was designed, analyzed and synthesized using bioinformatics methods. The chimeric gene was sub-cloned in the expression vector and expressed in *E. coli* host. The purified chimera protein was injected subcutaneously into the experimental animals. The production of specific antibodies was confirmed by immunological methods, and the protection capacity was evaluated by the challenge of immunized mice with the pathogenic bacteria. **Results:** Chimeric recombinant protein was able to increase IgG titer. Neutralization assay indicated that the antibodies generated against LtB moiety were able to neutralize ETEC toxin. In animal challenge study, all non-immune mice died within 3 days after the injection of toxin, but all immunized mice survived from Stx toxin. **Conclusions:** The immunity to both ETEC and EHEC bacteria is significant, and this structure can be considered as a candidate for vaccine production against these bacterial strains.

Received: 2018-11-12, Revised: 2019-06-24, Accepted: 2019-07-17.
Citation: Karimi Rahjerdi A, Jafari M, Motamedi MJ, Amani J, Hatef Salmanian A. Immunogenic Evaluation of Bivalent Vaccine Candidate against Enterohemorrhagic and Enterotoxigenic *Escherichia coli*. *Iran J Immunol.* 2019; 16(3):200-211. doi: 10.22034/iji.2019.80271.

Keywords: EHEC, ETEC, Recombinant Vaccine

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INTRODUCTION

Diarrhea is a common disease around the world which not only threaten the health of humans but also influenced significantly on society and the economy (1). In April 2013, WHO, along with UNICEF, implemented a comprehensive global action plan for the prevention and control of the two common diseases (pneumonia and diarrhea) in childhood (2,3). *E.coli* diarrheagenic strains are classified in at least 6 separate groups based on the mechanism of disease development and pathogenicity (4,5). *E. coli* O157H:7 is one of the most important bacteria that causes bloody diarrhea and fever, which in the absence of treatment, sometimes leads to hemolytic uremic syndrome (HUS) syndrome (6). Vaccines made against EHEC contained one of its important antigens either the recombinant subunits or the killed bacteria, which could not significantly prevent colonization of the bacteria (7). Proteins that play a major role in binding to the intestinal epithelial surface are including Intimin, Tir, and EspA. Given that these proteins play an essential role in the bacterial binding and colonization, preventing their function could prevent the onset of the disease. According to studies, these proteins are believed to be suitable candidates for the production of immunogenic agents against the bacterium. The EspA protein, which is a component of type III secretion system, formed a channel at the bacterial surfaces and transferred the Tir protein to the host cell. This protein can act as a receptor for Intimin, a membrane protein which localizes in the surface of the bacterium (8). Regarding the importance of this molecular interaction for preventing the bacterial binding to the host cell surface, it could efficiently inhibit bacterial pathogenicity in the early stages. Furthermore the Shiga-like toxin (Stx) plays an important role in the pathogenesis of *enterohemorrhagic E. coli* strains (EHEC) and leads to hemorrhagic colitis, damage to the central nervous system and hemolytic uremic syndrome (HUS) (9). The Stx toxin consists of two subunits, StxA and StxB, which together create a pentameric donut structure and are responsible for connecting the toxin to the receptors (10). Stx specific receptor is a neutralized sphingolipid called globotriaosylceramide (Gb3), which is highly expressed at some eukaryotic cell surfaces, such as endothelial cells of the kidneys and arteries (11). ETEC is a major cause of diarrhea in travelers (12,13). The pathogenic strains of ETEC are different in humans and animals and it can be entered into the small intestine through contaminated water and food (14). More than 22 colonization factors mainly Fimbriae (Pilli) or fibrous proteins are known in human ETEC strains (15,16). *In vivo* and *in vitro* studies have shown that the bacteria with different types of colonization factors can cause diarrhea, while their isogenic mutants that deplete these factors are not capable of causing diarrhea (17). Among all discovered colonization factors, CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS14, CS17, and CS21 are more commonly reported in ETEC diarrhea strains (18). CFA/I is a fimbrial colonization factor consisting of about 1000 structural subunits of CfaB, and at least one CfaE as end connectors. The CfaB is a 147 amino acid protein and binds to specific receptors on the surface of the intestinal epithelial cells. Structural studies have shown that the last 36 N-terminal residues of the CfaB protein, in spite of having conserved sequence among Class 5 Fimbrial members), is not a good antigen candidate (19). It is due to the fact that upon the assembly of the CFA/I structure, this domain is buried in the internal part of Fimbria and is not actually exposed to the immune system (20). ETEC can produce two toxins, a heat-labile enterotoxin (LT) which is structurally and functionally similar to *Vibrio cholera* toxin, along with a heat-stable enterotoxin (ST). LT toxin belongs to AB5 toxin family and consists of 2 subunits A and B. The single unit A is responsible for the catalytic

activity of the toxin and the subunit B, through the formation of a homopentamer, recognizes its receptors on the intestinal cells. Heat-stable enterotoxin (ST), which is produced by a human strain of the ETEC, is made of a 72 amino acid precursor which becomes mature with 19 amino acids after the maturation process. ST toxin is very weak in terms of immunogenicity and has a specific receptor on the surface of the intestinal epithelial cells (21). So far, many types of researches and studies have been conducted to control the infection of ETEC and EHEC using subunit vaccines; to this end, the candidate immunogenic agents have been used either in combination or after genetic binding. However, there has been no report for the genetically binding of more than three immunogenic agents for human ETEC and EHEC. In the present study, we are going to achieve a more effective and integrated immunogenic agent through combining an increased number of these factors which have been chosen more accurately, while in the immunization and protection studies.

MATERIALS AND METHODS

Sequence Analysis and Construct Design. The sequences encoding for four proteins, consist of CfaB, LtB, Intimin, and Stx2B ,namely SICL, were connected and linkers were placed among them and bioinformatics analysis was performed as described previously (22). The synthetic genes were provided by Biomatik Company from Canada.

Preparation of Chimeric Gene and Confirmation. The synthetic gene was cloned into the pUC57 plasmid with blunt ends and delivered in a lyophilized state. Samples were dissolved in 50 µl of distilled water and 5 µl was transferred to the DH5α strain of the *E. coli* competent cells. After overnight incubation at 37°C, colonies containing the recombinant plasmid appear on ampicillin (80 µg/ml) LB media. After ensuring the presence of the plasmid in bacteria (enzymatic digestion) and gene confirmation by the sequencing method, the glycerol stokes were prepared and stored at -70°C for future application.

Cloning and Expression of Recombinant Chimeric SICL Gene. The synthetic chimeric construct was subcloned to pET28a expression vector. For this purpose chimeric gene and pET28 vector were digested with *EcoRI* and *HindIII* restriction enzymes for 5 hours at 37°C, and then purified by purification kit (iNtRON, Korea). Ligation reaction between the insert and the linear vector was performed using T4 ligase for 10 hours at 12°C. The ligation product was transformed into competent *E. coli* BL21 DE3 and confirm by PCR and restriction enzyme analysis. Corrected colonies were cultured in LB broth containing 50 µg/ml kanamycin at 37°C, when the culture was reached to OD_{600nm}: 0.6, the bacteria induced by 1mM IPTG and incubated for further 5 h at 37°C. Each sample was evaluated on 10% SDS-PAGE. For optimization of expression the chimeric gene, factors such as IPTG concentration and incubation temperature were analyzed.

Solubility Evaluation and Purification of SICL Protein. To determine the solubility of the recombinant protein, expression was performed in 50 ml culture medium after precipitation the cells by centrifugation (13000 rpm, 4°C). The liquid phase containing soluble proteins was collected. The cell pellet was dissolved in a denaturant buffer (100mM NaH_2PO_4, 10mM Tris HCl, and 8M Urea). Both samples were analyzed by 12% SDS-PAGE. For purification the supernatant was transferred to Ni-NTA column. Washing and elution were performed according to the standard methods and the samples from the column were analyzed on a 10% SDS-PAGE. In order to remove the urea from

the protein solution, an equivalent volume of denaturing buffer containing glycerol stabilizer (100 mM Glycine, 100 mM NaCl, 100 mM Tris pH 8.5, 2-mercaptoethanol 140 mM, 2.5% Glycerol) was mixed with protein solution and then dialyzed against PBS solution for 12 hours. The protein was then stored at -70°C.

Western Blotting Analysis. Induced and un-induced samples were separated by electrophoresis on 12% SDS-PAGE and then transferred to PVDF membranes and floated in blocking buffer (3% Skim milk). The membrane was incubated with anti His-tag antibody (1/1500 dilution) conjugated with HRP with shaking for one hour at R/T. Membranes were subsequently washed three times with PBST buffer. Finally DAB solution was used to stain the bands. The reaction was terminated by the addition of distilled water upon appearance of the bands.

Immunization of BALB/c Mice with SICL Protein. A total of 20 BALB/c female mice at the age of 6-7 weeks were divided into test and control groups. In the first injection, 10 µg of antigen were subcutaneously injected with complete Freund's adjuvant. The second and third injections were performed with the incomplete Freund's adjuvant. The control group received PBS buffer with complete and incomplete adjuvant. Blood samples were taken from mice one week after each injection.

Antibody Titration by ELISA Method. ELISA was used to measure the antibody titration. One microgram of recombinant antigen was coated at the bottom of each well (Nunc Denmark) with 100 µl of coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6, 4°C, O/N). The plate was blocked with 100 µl of 3% (w/v) nonfat milk in PBST for 1h at 37°C. The plate was incubated with 1/100 to 1/204800 dilutions of the serum from immunized mice for 45 min at 37°C. Diluted (1/5000) HRP goat anti-mouse IgG (Sigma) were added to the each well (45 min at 37°C). After each step plate was washed with PBST (PBS with 0.05% (v/v) Tween 20). The substrate (OPD, Sigma) was added and the reaction was stopped with 100 µl of 2 M H_2SO_4 and the OD_{492} was read on a microplate reader (Bio-Rad).

***In vitro* Neutralization of Toxin with the Immunized Mice Serum.** The neutralization capacity of immunized sera was assayed by Hela cell toxicity test. In brief the CD_{50} of Stx2 on the Hela cells was measured (24). Serial dilutions of Stx2 toxin were prepared in EMEM medium and added to Hela cells (16000 cells/well) in a 96-well plate. After 18 to 20 hours of incubation (37°C and 5% CO_2) the culture medium containing dead cells were discarded and the remaining live cells were fixed with 2% formalin and staining with 0.13% crystal violet for 20 min. Ethanol (50% V/V) was added to each well and the OD_{595nm} was measured. To check the neutralization of Stx based on above procedure, the 50% cytotoxic dose (CD50) of toxin was defined as the dilution that killed 50% of the Hela cells, as compared to the untreated control cells. To investigate the effect of neutralizing serum of immune mice on LT toxin (23) a CHO elongation test was used and cell deformation was investigated (24).

Immunized Mice Challenge Analyses. 100, 150 and 200 µL of supernatant from *E. coli* O157:H7 medium (25,26) were used as a source for Stx2 toxin and was injected to peritoneal of animal model. The amount of 150 µL of crud toxin results in the death of 100% of mice at most after 3 days. To evaluate the protective effect in immunized mice, two weeks after the last dose of recombinant protein, 150 µL of Stx2 toxin was injected intraperitoneally. The mortality rate was investigated for up to 10 days after injections.

Statistical Analysis. The data were presented of three experiments separately. SPSS 15.0 was used for all statistical analysis. Student t-test was performed to analyze the antibody response in immunized and non-immunized animal groups. A value of $p<0.05$ was

considered statistically significant. Mice survival differences were studied by one-sided Fisher's exact test.

RESULTS

Construction of synthetic *SICL* gene and confirmation by enzyme digestion.
Based on our previous study (22), the molecular weight of construct in the recombinant cloning plasmid was calculated near 4700 bp which was analyzed on 1% agarose gel. The 1998 bp synthetic fragment was digested by *HindIII/EcoRI* restriction enzymes and subcloned to pET28a (Figure 1a). The recombinant expression plasmids were analyzed by with *HindIII* and *EcoRI* restriction enzyme digestion (Figure 1b).

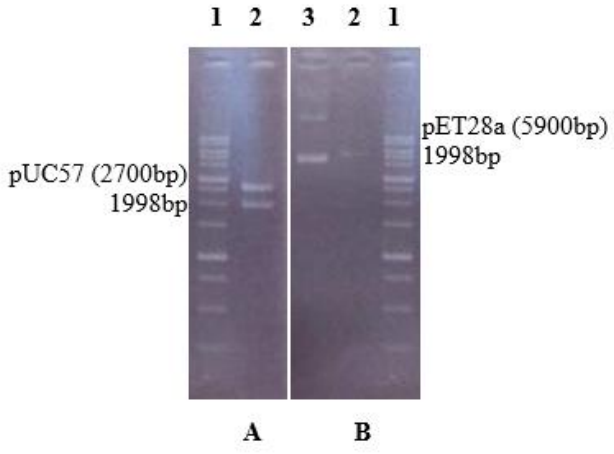

Figure 1. Agarose 1% gel electrophoresis of enzymatic digestion of pUC57 and pET28a containing SICL gene (1998bp) with *EcoRI* and *HindIII*. A) Lane1, DNA ladder. lane 2, pUC57 vector containing *SICL* gene (1998bp) digested with *Eco*RI and *Hin*dIII. B) Lane1, DNA ladder. Lane 2,3, pET28a vector containing SICL gene (1998bp) digested with *EcoRI* and *HindIII*.

Expression of recombinant SICL gene.
After induction of authentic expression clones with IPTG, the expressed protein was separated using denatured and native condition which was examined using 10% SDS-PAGE. As it has been shown in Figure 2a, the molecular weight of recombinant protein was near 70 kDa.

Optimization of recombinant SICL gene expression.
In order to optimize the expression condition, the different factors such as initial cell density for induction, IPTG concentration, incubation temperature and time of induction were optimized. The optimized expression condition was found at induction at OD_{600}=0.5 with 1 mM IPTG and 16 hours of induction at 18°C (Figure 2 b,c).

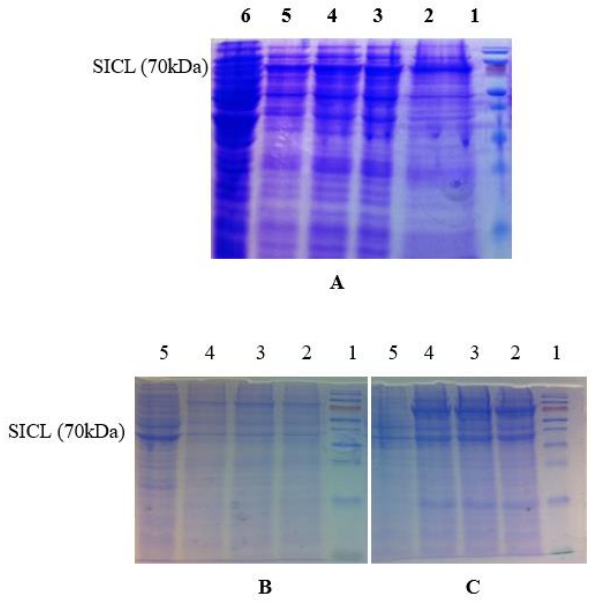

Figure 2. Expression and optimization of *E.coli* BL21 DE3 containing pET28a-SICL on the SDS-PAGE 10%. a) Lanes 1, protein ladder. Lane 2,3,4,5, expression of recombinant SICL induced by IPTG. Lanes 6, uninduced *sicl* as control. b) Lanes 1, protein ladder. Lane 2,3,4, expression of recombinant SICL induced by concentration 0.25, 0.5, 1 mM IPTG. Lanes 5, uninduced *sicl* as control at 37°C, c) at 18°C.

Recombinant protein purification and western blot analysis.

The chimeric SICL protein was purified under denaturant condition. The purified protein was analyzed on 10% SDS-PAGE (Figure 3a). The concentration of SICL recombinant protein was calculated near 400 µg/ml. The western blotting with anti His-tag was used to confirm the integrity of recombinant protein (Figure 3b).

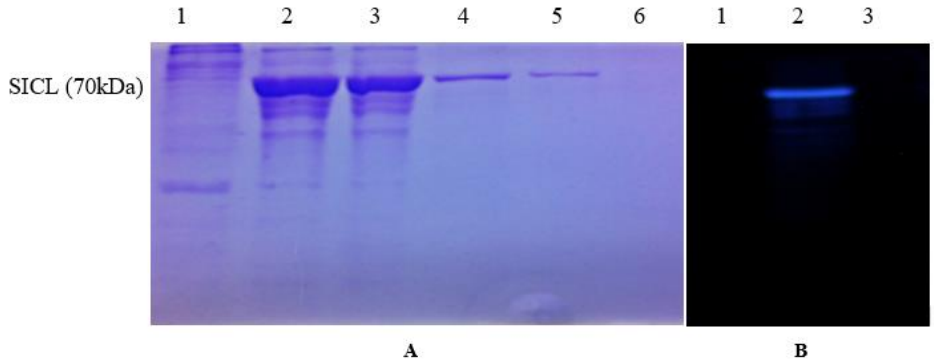

Figure 3. Purification and western blot analysis of recombinant SICL. A) Purification of recombinant SICL protein with His-tag. Lane 1, protein ladder. Lane 2, flow. Lane 3, wash column with 20 mM imidazole, Lanes 4-6, purified protein with elution buffer containing 250 mM imidazole. B) Lanes 1, protein ladder. Lanes 2, recombinant SICL protein, Lanes 3, uninduced SICL as control.

Quantification of antibody titration.

IgG antibody titers against SICL were evaluated in the serum of immunized mice. Compared to the control group, a significant increase in the antibody titer was observed following each injection (Figure 4).

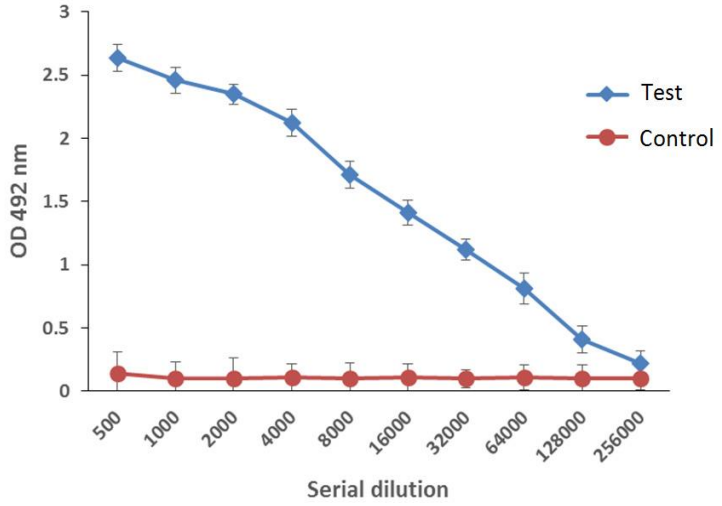

Figure 4. Titration of serum IgG after subcutaneous immunization which was determined by ELISA method. Serum from no immunized mice was used as control.

***In vitro* neutralization assay for Lt toxin.**

The result from CHO cells assay showed that antibodies generated against recombinant LtB was able to neutralize the secreted Lt toxin, effectively (Figure 5).

Stx2 toxin protection and animal challenge.

The animal challenge was done by LD_{50} of Stx toxin two weeks after the last dose of recombinant SICL protein injection. All non-immune mice died at most 3 days after the injection of toxin. In the immunized group mice however, 100% survived after toxin injection.

DISCUSSION

Enterohemorrhagic E. coli (EHEC) and *Enterotoxigenic E. coli* (ETEC) are endemic pathogens in developing countries. ETEC are the main reason of diarrhea in travelers to these countries and EHEC are the main factor of large outbreaks in the world, specifically in developed countries (27). Until now, many approaches have offered to control EHEC and ETEC infection but vaccination are still best way (28,29). Many studies worked on vaccination against EHEC. In a study the fusion protein containing the A1 subunit of Stx2 toxin and the N-terminus of EspA was assayed for its immunogenicity. The Immunized mice with EspA-Stx2A1 shows the high titers of IgG antibodies and over >95% mice survived in challenge with Stx2 toxin (28). Another research focused on two types of Shiga-toxins, Stx1/Stx2, and a shortened intimin protein which was increased IgG

antibody titers in mice. The immunized mice orally challenged with EHEC O157:H7 and demonstrated 100% survival rate (30).

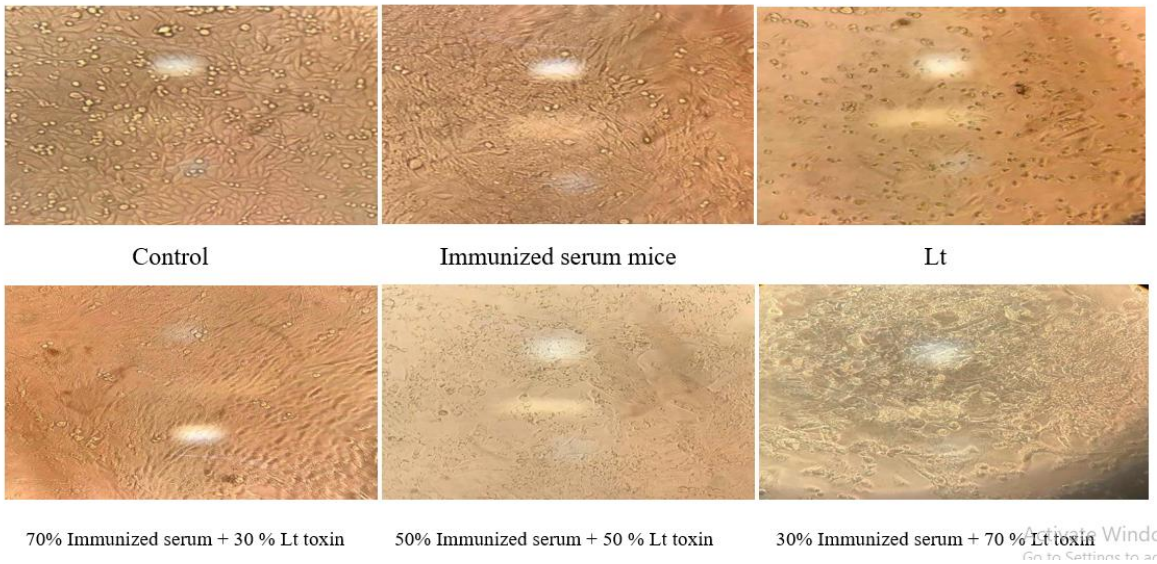

Figure 5. Neutralization effect of mice immunized sera on the elongation of CHO cells. Mice immunized sera with SICL blocked the effect of Lt toxin, whereas sera of control mice showed no inhibitory effect on elongation of CHO cells.

Immunization with Stx and intimin fusion protein produced antibodies against both antigens (31) and mice immunized with this chimeric protein challenged with a lysed EHEC showed more than 90% survival. Specific peptide which was designed by Wan and coworker could create protection against EHEC. The C terminal region of intimin which related to A/E lesions protected immunized mice against *E. coli* O157:H7 challenges (32). Amani J. and his colleague evaluated chimeric construct contain truncated Intimin, EspA and Tir protein, namely EIT, in a synthetic construct. The challenge of immunized mice with *E.coli* O157:H7 shows %100 surveillance (33,34). In another study which conducted in Iran the research results show that the production of immunogenic parts of the EspA (E), Intimin (I), and Tir (T) proteins in unique structure, its expression in transgenic canola plants and finally the antigen administration through oral delivery can induce the humoral and mucosal immunity in mice model (35). The major and main virulence factors in ETEC are colonization factor A (CFA), heat-labile (LT) and heat-stable (ST) toxins, which are responsible for water and electrolyte discharge during infection (36). For example the transcutaneous injection used to immunize human volunteers with LT toxin raised anti-LT IgG and IgA but did not prevent the illness after challenge (37). The ST subunit A (STa) is known to be low immunogenic property although its immunogenicity can accelerate by conjugated with stronger antigens, like LT or other adhesion factor, and reducing toxicity by mutation in major essential amino acids. Mice immunized with a STaP13F-LTR192G toxoid fusion protein produced specific IgG antibodies for LT and STa proteins in serum and feces and IgA in feces (38). Another research is the MEV or Etvax vaccine which contain cocktail of four inactivated bacteria which expressing separately different CFs, in which CS6 is expressed

in a K12 strain and CFA/I, CS3, CS5 in an ETEC O78 toxin-negative strain. This four inactivated bacteria by formalin were applied in clinical phase I studies (39). This studies was carried out based on our previous research (22). The main research was production of a multivalent construct composed of immunogenic proteins of EHEC and ETEC. These factors (CfaB, Intimin, Stx and LtB), including adhesins and toxin, are the most important pathogenesis factor in these bacteria and it is believed that production antibody against these factors can provide protection. It is expected that by expressing the immunogenic regions of these bacteria in the *E.coli* and presenting them to the immune system of animal model can make a significant immune responses against two important bacterial based diarrhea diseases. In the first step optimize expression of recombinant chimeric SICL was carried out and the results showed that two main modifications conditions such as decreasing the temperature in culture media and concentration of IPTG can significantly increase the solubility of recombinant protein (Figure 2). Recombinant SICL protein was purified by metal based Ni-NTA column and confirmed by western blotting analysis (Figure 3). The ELISA results using serum of immunized mice showed that after each injection, the amount of IgG antibody in mice was increased which representing the efficacy of immune system to induce the B cell molecules which lead to increase the titer of the IgG and IgA antibodies (Figure 4). Neutralization assay by immunized mice sera was showed that the toxin could not activity against immunized sera (Figure 5), this result confirmed the result from Cheng *et al.* which the anti-EspA-Stx2A1 serum was able to neutralize the action for Stx2 on the *in vitro* assays on HeLa cells (28). Furthermore this data compatible with result from Lu Liu *et al.* study (40). The survival rates for challenging the immunized mice with Stx 100%, it showed that the construct can protect mice against toxin, the data similar to result of Xue Qin Ran, Cai kun *et al.*, and Xiang Gao *et al.* (29-31). Based on the results, SICL chimeric protein could provide an effective immune response and effective protection against toxins produced either by ETEC and EHEC furthermore could be used as a vaccine candidate to prevent diarrhea. On the other hand, the SICL multi-component could be used as an effective part of a vaccine candidate to prevent bacterial diarrhea originated by ETEC and EHEC.

ACKNOWLEDGEMENTS

This research is a part of the PhD thesis of the Biotechnology that approved by Department of Plant Biotechnology with project code NIGEB III-450, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.

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