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Oral immunization with a plant-derived chimeric protein in mice: Toward the development of a multipotent edible vaccine against *E. coli* O157: H7 and ETEC

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ABSTRACT

The most bacterial cause of infectious diseases associated with diarrhea are enterotoxigenic and enterohemorrhagic *Escherichia coli* (ETEC and EHEC, respectively). These strains use colonization factors for the attachment to the human intestinal mucosa, followed by enterotoxins production that could induce more host damage. The Heat-labile enterotoxin (LT) and colonization factors (CFs) are momentous factors for the pathogenesis of ETEC. Also, Intimin and Shiga like toxin (STX) are the main pathogenic factors expressed by EHEC. Because of mucosal surfaces are the major entry site for these pathogens, oral immunization with providing the protective secretary IgA antibody (sIgA) responses in the mucosa, could prevent the bacterial adherence to the intestine. In this study oral immunogenicity of a synthetic recombinant protein containing StxB, Intimin, CfaB and LtB (SICL) was investigated. For specific expression in canola seeds, the optimized gene was cloned in to plant expression vector containing the Fatty Acid Elongase (FAE) promoter. The evaluation of the expression level in canola seeds was approximately 0.4% of total soluble protein (TSP). Following to oral immunization of mice, serum IgG and fecal IgA antibody responses induced. Caco-2 cell binding assay with ETEC shows that the sera from immunized mice could neutralize the attachment properties of toxigenic *E. coli*. The reduction of bacterial shedding after the challenge of immunized mice with *E. coli* O157:H7 was significant. The sera from immunized mice in the rabbit ileal loop experiment exhibited a significant decrease in the fluid accumulation compared to the control. The results indicate efficacy of the recombinant chimeric protein SICL in transgenic canola seed as an effective immunogen, which elicits both systemic and mucosal immune responses as well as protection against EHEC and ETEC adherence and toxicity.

1. Introduction

Enteric infections is considered as the fourth main cause of death among children younger than 5 years old (Leitner et al., 2015; Troeger et al., 2017). Among the diarrhoeagenic *E. coli*, the enterotoxigenic and enterohemorrhagic *E. coli* (ETEC and EHEC) play a central role in human and domestic animal enteric infections (Dubreuil et al., 2016). Both pathogens attach to the mucosal surface through surface-associated structures and then inducing more damage by enterotoxins production. The main pathogenic factors of EHEC are Intimin for adherence of bacteria and Shiga like toxin (STX) (Mallick et al., 2012). Stx affects the endothelial cells in the glomerular capillaries and triggers a series of events that ultimately it causes hemolytic uremic syndrome (HUS) characterized by thrombocytopenia, hemolytic anemia, and renal failure which easily could cause death (Topal, 2010). The predominant agent in this group is O157: H7 which associated with HUS. The natural reservoir of the EHEC O157: H7 serotype is cattle, which could reserve the bacterium in its intestines. Outbreaks in humans are mostly linked to ingestion of food contaminated by bovine feces (Mohawk et al., 2010; Salehi et al., 2012). After the initial adherence of the bacteria to the epithelial cells, an adherence process is started which responsible for the attaching and effacing (A/E) lesions that disrupt the tight junctions and causes diarrhea (Topal, 2010). The virulence factors of ETEC are colonization factors (CFs) and a heat-

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labile toxin (LT) and/or a heat-stable toxin (ST) (Jafari et al., 2012). STX and LT belong to AB5 holotoxins family that consists of catalytic A subunit, associated with B subunit that binds to the cellular receptors (Tiwari et al., 2009). One of the most commonly antigenic types of CF is CFA/I that is composed of CfaB and CfaE proteins (Savar et al., 2014). It is possible to manage the infection more effectively by using toxin neutralizers and antibodies against pathogenic factors of these strains (Goldwater and Bettelheim, 2012). Hereupon the early immunization against ETEC and EHEC may be an effective preventive strategy.

Since the mucosal surfaces form the first defense barriers against infectious diseases entering the body, the production of vaccine subunit components in plant tissues and application as an edible vaccine is an impressible means of protection against a broad range of pathogens (Pelosi et al., 2012). In this study, we have targeted the surface structures of EHEC and ETEC in order to reduce the colonization activity and the enterotoxins of these two bacteria that prevent pathogenesis more effectively. Here the antigenic fragments of ETEC,i.e CfA/I and LtB and EHEC,i.e Intimin and StxB were selected for the chimera construction. In order to induce mucosal immunity, the transgenic canola as a delivery system was selected. Because of high protein content, nutritional value and several byproducts, canola seed has great potential for vaccine delivery systems. The water content of mature canola seeds is low that provides stability of expressed proteins (Barciszewski et al., 2000). In this study, we generated transgenic canola seeds and analyzed them as potential edible subunit vaccines to induce protective immunity against two important pathogenic bacteria, in mice model.

2. Materials and methods

2.1. Chimeric gene design

The chimeric construct (SICL) is containing 19–89 residues from StxB (S), 282 residues from the C-terminal of Intimin (I), 24–170 residues from CfaB (C) and 22–124 residues from LtB(L). The sequences were connected together by linkers containing EAAAK repeats so they can be well exposed to the immune system. To enhance the efficiency of protein production, the Kozak sequence (Kozak, 1989) and the sequence of the KDEL endoplasmic retention signal were added to the chimeric gene. The bioinformatics analysis was performed as described previously (Jeshvaghani et al., 2016). The multimeric gene was synthesized by the Biomatik company (Canada).

2.2. Construction of plant expression vectors

The chimeric codon optimized SICL gene was sub-cloned into the binary vector pBI1400 containing a canola seed-specific promoter called Fatty Acid Elongase (FAE) promoter and then was introduced to *Agrobacterium tumefaciens* cells (Zebarjadi, 2006). The confirmation of the accurate clones was done by PCR and restriction enzymes analysis (Sambrook and Russell, 2001).

2.3. Transformation of plant and tissue culture

The canola seeds (*Brassica napus L.*) cultivar; Hyola was taken from the Seed and Plant Improvement Research Institute of Iran. The sterile seeds were germinated on MS (Murashige and Skoog, 1962) basal medium with 30 g/l sucrose. Canola cotyledonary explants from 4 days old seedlings were infected with recombinant *A. tumefaciens*. After infection, the canola cotyledonary explants co-cultivated for 2 days at 22°C on MS medium contained 4.5 mg/l 6-BAP and 0.1 mg/l NAA in the dark condition. After two days the explants were placed on the selective medium (containing 200 mg/l cefotaxime and 8 mg/l kanamycin). The selected explants were transferred to root inducing MS and eventually, they were transferred to soil and grown under greenhouse condition for flowering and set seed (Kahrizi et al., 2007).

2.4. Molecular analysis of transgenic plants

DNA extraction from leaves of transgenic and non-transgenic plants was done by Dellaporta method (Dellaporta et al., 1983). The PCR analysis using the forward 5′ - GTTGAGAAGAACATTACCGTTACAGC -3′and the reverse 5′- TCAAAGTTCGTCCTTGTGATGATG -3′ primers, was applied to the determination of the *sicl* integration in transgenic plant genomic DNA. PCR was performed by a process of initial denaturing at 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 45 s, and a final extension step of 72 °C for 10 min. Total RNA was extracted using Plant Total RNA Kit (Roche, Germany) from seeds of transformed canola to determine the presence of *sicl* mRNA in the transformed canola seeds.

2.5. Preparation of specific antibody against recombinant SICL

Immunization of female BALB/c mice (Razi Vaccine and Serum Research Institute of Iran) was performed similar to the method described by Amani with 5 μg rSICL protein and PBS was used as a negative control (Amani et al., 2011). After the third injection, blood samples were obtained from the infraorbital region of immunized mice. The sera were placed at −70 °C for subsequent analyses.

2.6. Quantification of plant based rSICL by ELISA

The powder from grinding 200 mg of seed from each explant in liquid nitrogen was extracted with protein extraction buffer (200 mM Tris−HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA,14 mM 2-mercaptoethanol, 1 mM PMSF, 0.05% Tween-20) in presence of protease inhibitors cocktail (Roche, Germany). After centrifugation (5 min, 16,000 × g) by using the standard Bradford method (Kruger, 2002), the total soluble protein (TSP) content of each extract was determined. For quantification of rSICL, the ELISA assay with sera from immunized mice was used (Amani et al., 2011). An amplitude of 1 μg to 1 pg of rSICL, purified from *E. coli* BL21DE3 was applied as a standard curve. This analysis was repeated three times for each sample and the ANOVA test was performed using the SPSS software.

2.7. Oral immunization of mice

The mice immunization program was performed with three distinct mice groups. One mice group received 20 μg rSICL/dry weight canola in 500 μl extract orally for five doses. The second one immunized via the oral route (4 times) and receive a booster dose by subcutaneous injection(5 μg). The last group as the negative control was administered orally with a soluble protein extract made from nontransgenic canola seeds (Amani et al., 2011).

2.8. Antibody determination in serum and feces by ELISA

After last immunization for analysis of rSICL specific IgG and IgA antibodies, blood samples were taken from immunized mice. For detection of secretory IgA, the fecal pellets from groups of mice were homogenized in 500 μl PBS containing 0.2 mg/ml trypsin inhibitor, 0.05% sodium azide (w/v) and a cocktail of protease inhibitors (Roche) and stored overnight at 4 °C. After centrifugation (20,000 × g, 10 min, 4 °C), the supernatants were analyzed for sIgA (Amani et al., 2011).

2.9. Neutralization assay

The neutralization efficiency of immune serum on the toxicity of LT was evaluated by Rabbit ileal loop assay (De and Chatterje, 1953). ETEC bacteria (Bu-Ali Hospital, Tehran) at 1×10^{8} CFU/ml concentration were incubated (30 min,37 °C) with sera from different mice groups. After the animals sacrificing, the increase in the size of each ligated loop due to fluid secretion and the presence or absence of blood in the secreted fluid were investigated. Also, the index of enterotoxigenicity was calculated as the ratio of fluid accumulation versus loop length (g/cm). This work was conducted in agreement with the Animal Welfare Act at the Faculty of Veterinary Medicine, Tehran University. To evaluate of neutralizing effect on STX toxicity, the Vero cell cytotoxicity test was performed (Russo et al., 2014). The supernatant of an overnight culture of *E. coli* 0157: H7 (STX-2^+) (Bu-Ali Hospital, Tehran) was used as a source of STX-2 toxin. The 50% cytotoxic dose (CD50) of toxin was the dilution of this supernatant that killed 50% of the Vero cells as compared to the untreated control cells. A serial dilution of the immune serum was mixed with the 2,5 and 10 fold CD50 toxin, to specify the serum capacity for toxin neutralization.

2.10. Binding inhibition assay for ETEC

The binding inhibition assay with Caco-2 cells (Pasteur Institute of Iran, Tehran) was employed to characterize the neutralization effect of immunized serum on the attachment properties of toxigenic *E. coli* (Nazarian et al., 2014). The adhesion index was specified by estimation of the mean number of bacteria per cell in 100 examined Caco-2 cells. By comparing the adherence index in the presence and absence of the immune sera, the amount of inhibition was determined (Oliveira et al., 2007).

2.11. Bacterial challenge

The challenge with *E. coli* O157:H7 (10^{10} cells) via oral route was used to determine the rate of immunization effect by calculating the reduction of pathogenic *E. coli* O157:H7 shedding. Diurnal serial dilutions of the fecal pellet (0.1 g) were prepared in PBS. By plating these samples on to sorbitol MacConkey agar plates, the number of the viable *E. coli* O157: H7 was determined per gram of feces for 14 days after the challenge (Amani et al., 2011). Six days after the bacterial challenges, the mice were sacrificed and the macroscopic appearance of the colon and caecum was investigated(Amani et al., 2011).

2.12. Mouse toxicity model

The neutralizing effects of immune serum against STX were demonstrated by the mouse toxicity model (Sheoran et al., 2003). 4-week-old female BALB/c mice were injected with different amounts (50–500 µl) of Stx source and finally 130 µl of the culture supernatant calculated as lethal dose. The immunized and control mice groups were challenged with 130 µl of the bacterial culture supernatant as a source of STX-2 intraperitoneally. The survival rate of mice was evaluated daily for ten days.

3. Results

3.1. Design and cloning of the chimeric sicl gene

The synthetic gene consisting of StxB, Intimin, CfaB and LtB were selected. The Fatty Acid Elongase promoter was used as a canola seed-specific promoter (Zebarjadi, 2006). The synthetic construct was analyzed by bioinformatics software and codon optimized which described previously (Jeshvaghani et al., 2016).

3.2. Expression of the multimeric SICL protein in transgenic plants

Transgenic canola plants transformed with recombinant Agrobacterium harboring pBI1400-*sicl* plasmid were analyzed by PCR using specific primers to detect the presence of the chimeric *sicl* gene (Fig. 1a). Reverse transcriptase PCR (RT-PCR) analysis of *Brassica napus* L. seeds showed *sicl*-specific mRNAs at the expected size (Fig.1b). The positive plants with SICL-specific mRNA were selected for protein analysis. The level of expression for rSICL in canola seeds was estimated 0.4% of total soluble protein (TSP) (Fig. 2). The amount of multimeric recombinant SICL protein in canola seeds was calculated approximately 80 µg per gram of dry seed.

3.3. Humoral and mucosal immune responses in orally immunized mice

The survey of humoral and mucosal antibody responses was showed that the serum anti-SICL IgG titers were highest in prime-boost method (a single subcutaneous immunization and a set of 4 oral delivery) and reached the value of 2.23 (Fig. 3a). Also, there was a significant difference in the level of serum anti-SICL IgG in oral group in comparison with the control group ($p < 0.01$). The serum IgA titers from both groups were slightly high (1:128 serum dilution, $p < 0.01$) (Fig. 3b). The IgA concentration in feces sample from the prime-boost and orally administered groups were considerably high (Fig. 3c). The mice were administered the nontransgenic plant did not show any antibody response. These results demonstrated that a high anti-SICL IgG and IgA induction could be attained by oral administration.

3.4. Serum neutralization assay

The inhibition of fluid accumulation with anti-SICL antibody was further evaluated by rabbit ileal loop assay. The bloody enlarged loops as a result of secretion of fluids and blood were observed in both ETEC treated with the control serum and ETEC without any treatment. But in the case of PBS and ETEC treated with the mice immunized sera, the loops are morphologically natural in bright colors (Fig. 4a). The immunized mice sera exhibited a statistically significant decrease in the fluid accumulation compared to the control mice serum ($p < 0.001$) (Fig. 4b). The Vero cell cytotoxicity assay demonstrated that the CD50 of toxin was equivalent to a 10^{-2} dilution of the crude STX preparation. The serum of immunized and control mice groups were treated with 2, 5 and 10 CD50 of the crude STX-2 and the viability of Vero cells was measured 24 h after the exposure with serum-toxin solutions. The data indicated that the immunized sera could neutralized the toxicity of STX-2, whereas the sera of control group had no neutralizing activity ($p < 0.01$) (Fig. 5).

3.5. Binding inhibition of ETEC to Caco-2 cells

Caco-2 cell binding assay with ETEC (CFA/I positive strain) shows that the immunized sera could neutralize the attachment properties of toxigenic *E.coli*. The adhesion index (AI) for ETEC was 47.2 ± 9.5 bacteria per Caco-2 cell (in 100 microscopic field). In contrast, pretreatment of pathogenic ETEC cells with anti-SICL immune sera could decrease the Caco-2 cell binding capacity. In the case of immune sera treatment, the adhesion index reduced to 1.2 ± 0.6 bacteria per Caco-2 cell (Fig. 6). On the other hand, anti-SICL antibody would dramatically decrease the bacterial adhesion properties more than 97% ($p < 0.001$).

3.6. Challenging assay with pathogenic E. coli O157: H7

After the bacterial challenge assay with *E. coli* O157:H7 (ATCC:35218) a significant decrease in the bacterial shedding was seen in the immunized group ($p < 0.05$). The results show that there was no shedding in mice from both prime-boost and orally administrated group after 5 and 6 days post challenges, respectively. In control group, the bacterial shedding was detected to the end of the assay (14 days) (Fig.7). The histological examination from sacrificed mice at day 6 post-challenge showed that immunized mice had normal caecum and colon, without any mucosal thickening. However, non-immunized mice showed a thickened distal colon.

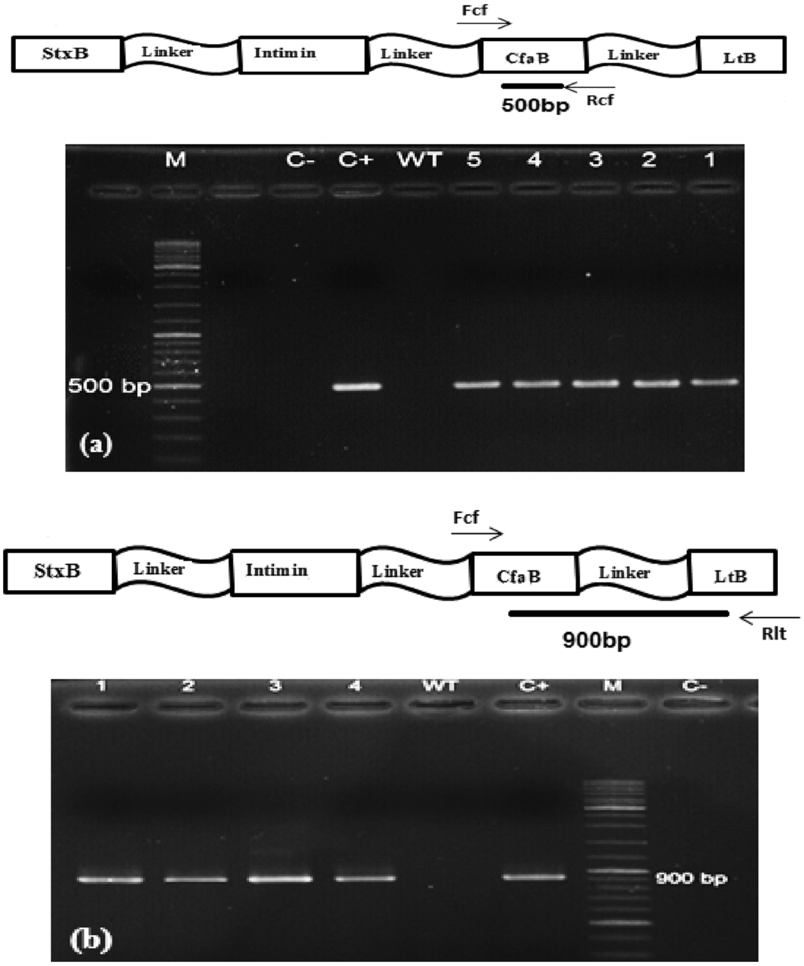

Fig. 1. a) PCR analysis of the *sicl* gene in transgenic plants with the specific primers. Lanes 1–5 transformed canola plants. b) Amplified SICL transcripts. Lanes 1–4, putative primary transformant Canola. Lane M, Ladder mix. C+, positive control using pBI-SICL. WT, wild-type plant (nontransformed canola) and C- (all of the components except the DNA template) as negative control.

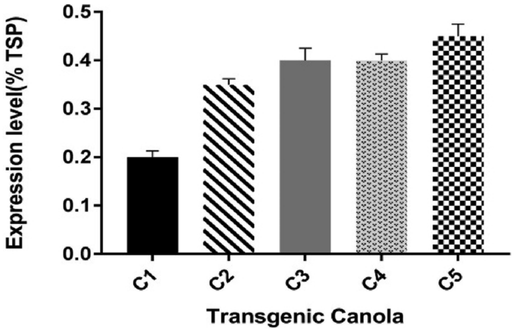

Fig. 2. Measurement of chimeric SICL protein in transgenic canola seeds by quantitative ELISA (C1-C5: different transgenic lines). The level of expression was estimated as 0.4% of TSP in canola seeds.

3.7. *In vivo* Neutralization assay

In order to characterize the relative strength of anti-SICL serum to neutralize *in vivo* STX activity, the mouse toxicity model was employed. All of the mice in control group died after 4 days, while in the immunized group, the survivance was calculated near 72% (Fig. 8). The prime-boost and orally administrated groups exhibited very similar effects on relative average survival and have no differ significantly.

4. Discussion

Infectious diseases are one of the momentous causes of deaths in developing countries, especially for children and enterotoxigenic and enterohemorrhagic *Escherichia coli* (ETEC and EHEC) are regarded as the important cause of bacterial diarrheal infections (Leitner et al., 2015; Nguyen and Sperandio, 2012). This is while vaccination are the most effective means for prevention, control and spread of such infections (Concha et al., 2017; Yusibov and Rabindran, 2008). So it is important to utilize new vaccine components or vaccination methods which it can overcome the limitations of the existing vaccines (Raj Kesar et al., 2012). The oral vaccine administration can induce mucosal immunity, produce antibody, immune response mediated by cell and thereby it could prevent colonization of infectious agents on mucosal surfaces (Laere et al., 2016; Pelosi et al., 2012). The immune response which is generated in one site, can transfer to other mucosal surfaces by movement of activated T-cells and cause immunity over a large surface area (Concha et al., 2017). Edible plants are capable of presenting the relevant immunogens for the antigen presenting cells, through the intestinal epithelium overlying gut-associated lymphoid tissue (GALT) such as Peyer's patches (PP) (Concha et al., 2017; Sharma and Sood, 2011). An appropriate plant for edible vaccine production should have several main properties such as accumulation of the protein in plentiful quantities, correct folding and appropriate post translational modifications of the protein similar to target host and finally the simplicity of the transformation procedure (Scissum et al., 2012). Plant seed tissue like canola seed with high protein content could be considered as an

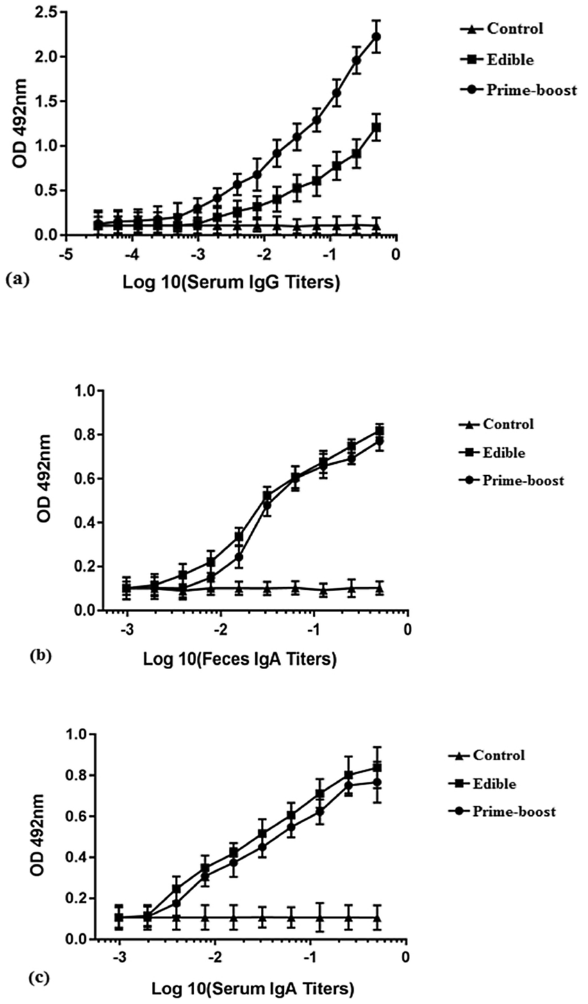

Fig. 3. Determination of SICL specific IgG and IgA by ELISA in edible, prime-boost and control groups (a) Anti-SICL IgG in serum samples from mice immunized via edible and prime-boost strategies. Anti-SICL IgA in serum (b) and fecal (c) from immunized and control mice groups. Control group was administered with a soluble protein extract made from nontransgenic canola seeds.

efficacious target for producing recombinant protein (Barciszewski et al., 2000; Tiwari et al., 2009). In this study, a chimeric recombinant protein containing StxB, Intimin, CfaB and LtB (SICL) was designed for immunological evaluation (Jeshvaghani et al., 2016). Previous studies demonstrated that the combination of antigens in a single recombinant protein could work more efficiently than the single one, especially if an bioadjuvant like LtB, was added to synthetic construct (Amani et al., 2011; Gao et al., 2009; Nazarian et al., 2014). The level of expression of SICL chimeric protein in transgenic canola seeds was 0.4% of total soluble proteins (TSP) under the control of seed preferred promoter, Fatty Acid Elongase (FAE). This promoter is highly active, embryo-specific and its activity is simultaneous with the period of main storage lipid accumulation, thus offering a competitive alternative to commonly used promoters (Rossak et al., 2001). The expression level of the SICL protein in transgenic canola seeds varied from 0.2% to 0.45% of TSP which is in the range expected for expression of a transgene in plants and consistent with other comparative studies (Conley et al., 2011; Rybicki, 2010; Tekoah et al., 2015). Accordingly, we can conclude that the utilization of the regulatory seed specific FAE promoter could express sufficient quantity of antigen which is required for immunization. The immunological analysis in mice revealed that all antigenic parts of this chimera have the property of stimulating the immune system. Furthermore, the fecal IgA level in both oral and in prime-boost was evaluated significantly (Fig. 3c). On the other hand, the plant-derived synthetic recombinant SICL polypeptide could evoke both humoral and mucosal immune response, effectively. These results were consistent with the findings of other research using recombinant proteins for immunogenicity in animal models (Bagheri et al., 2014; Kazemi et al., 2016; Martínez-González et al., 2011; Soh et al., 2015). In order to complete the previous research, in this study, the strategy of combining the antigens from two pathogenic bacteria and using different virulence factors was applied. So it is expected that this recombinant protein will be able to produce immunity responses against both of these strains. In fact, this new construct will be effective on the reduction of bacterial attachment and neutralization of their enterotoxins to prevent further damage. Therefore, if the antibody against the colonization factors as the first defensive line breaks down, can be expected that the second defensive line, the antibody against bacterial toxins, can neutralize the toxin. In order to evaluate the neutralization capacity of anti STXB antibody, the Vero cell neutralization assay was performed. There was no difference between the control group and mice which treated with semipurified STX toxin, whereas the immunized mice sera significantly showed toxin-neutralizing ability. This result suggests that the neutralizing epitopes of STXB in SICL molecule were suitably conserved and could stimulate the immune system (Boone et al., 2016; Kazemi et al., 2016; Liu et al., 2009). The effect of immune serum on the inhibition of fluid accumulation was evaluated by rabbit ileal loop assay. The finding of rabbit intestinal loops indicated that immune serum has the ability to neutralize toxin completely and block fluid accumulation (Alerasol et al., 2014; Bagheri et al., 2014; Maluta et al., 2014). We can conclude that, the enterotoxin-related parts in the chimeric recombinant structure are well exposed to the immune system, and the antibodies produced from those parts could be effectively neutralized the toxins from both strains. Previous research showed that the induction of the immune response in the animal model was stronger in ETEC with CFA/I compared to the other colonization factors (Byrd et al., 2003). For this reason, here we considered the CfaB as a main antigenic domain of CFA/I in the synthetic construct. The binding inhibition assay in the Caco-2 cell line showed that treatment of ETEC bacterial cells with the immune sera leading to a significant reduction in ETEC attachment to Caco-2 cells ($P < 0.001$) and the production of effective anti-CF antibodies in immunized mice sera was confirmed. It was already known that the Stxs can promote *E. coli* O157:H7 colonization (Lowe et al., 2009; Mohawk et al., 2010; Robinson et al., 2006; Yin et al., 2009). On the other hand, by neutralizing the STX activity, we could reduce the EHEC binding capacity more effectively. The shedding rate was consistent in the control group while the oral immunized group continued to the logarithmic decrease before one week (Fig. 7). In our previous work (Amani et al., 2011), the recombinant structure harboring the Intimin portion, and challenge the immunized mice with the *E. coli* O157:H7 showed that the shedding of bacteria on the eighth day reach to undetectable level. Hence, it can conclude that the presence of StxB in new chimeric protein (SICL) has been able to accelerate the reduction process up to two days compared to this work. The relative strength of anti-SICL to protect against EHEC was further evaluated by mouse toxicity model. All of the control group died within 4 days' post challenge with pathogenic EHEC, while in the immunized group, more than half of the mice survived after the bacterial challenge. Mohawk et al demonstrated that neutralizing antibodies to Stx could improve the survival of immunized group (Mohawk et al., 2010). As shown earlier in the Vero cells neutralization assay, the immunized mice sera have the ability to neutralize STX toxin and the viability of the cells is considerably higher than the control group. Therefore, anti-SICL antibody has improved the survival of immune mice by reducing the bacterial shedding of *E. coli* O157:H7 and neutralizing the STX effect.

In summary, we conclude that our synthetic construct and rout of immunization has ability to induce anti-toxin and anti-adhesion

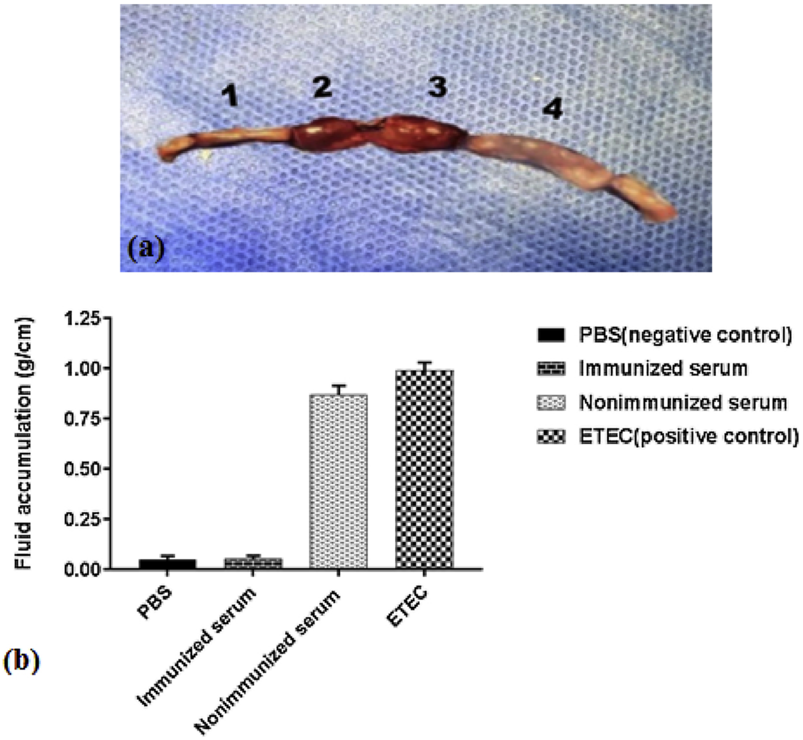

Fig. 4. a) Rabbit ligated ileal loops using the following samples: 1) PBS; 2) ETEC bacteria (positive control); 3) ETEC treated with the nonimmunized mice serum; 4) ETEC treated with the immunized mice sera. b) Estimation of the fluid accumulation ratio (g/cm) for each of samples. Differences between immunized and non-immunized groups were considered significant ($p < 0.001$).

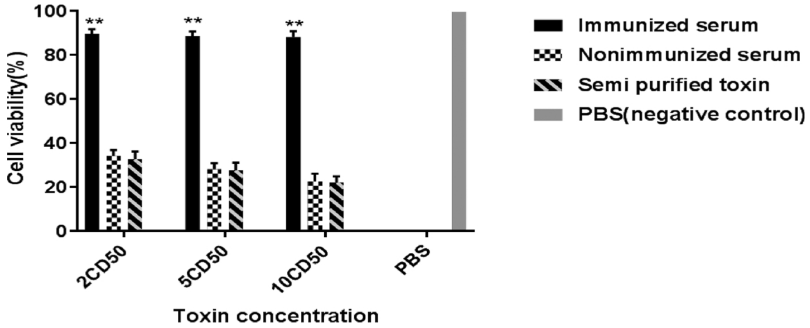

Fig. 5. The Vero cells neutralization assay. Cellular viability (%) of Vero cells was measured with different concentration of STX toxin. The sera of immunized mice neutralized the activity of STX-2 after 24 h of exposure ($p < 0.01$).

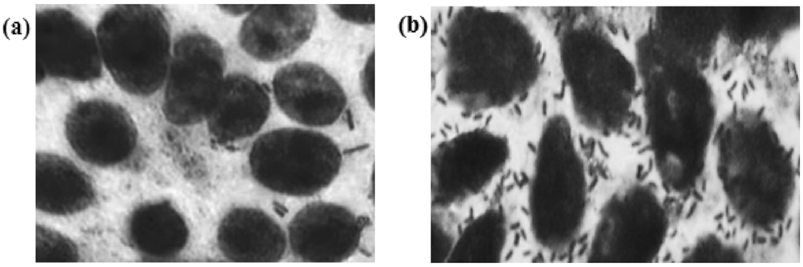

Fig. 6. Adherence of enterotoxigenic *Escherichia coli* (ETEC) strain to Caco-2 cells. The bacterial cells treated with immune serum(a) and non-immune serum (b), then incubated with Caco-2 cells.

antibodies and has the correct structure to elicit an effective humoral and mucosal immune response for protecting against EHEC and ETEC challenges. Therefore the application of transgenic plants harboring immunogenic moiety of antigens (SICL) could be considered as an efficient platform for production and delivery of vaccine candidate for *E. coli* O157:H7 and ETEC bacteria.

Conflict of interest declaration

No conflict of interest.

Acknowledgment

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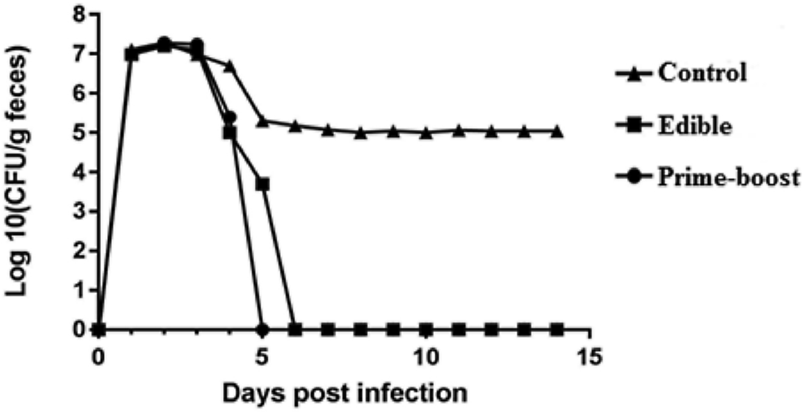

Fig. 7. Bacterial challenge assay with pathogenic *E. coli* O157: H7. Differences between immunized and non-immunized mice were considered significant ($p < 0.05$).

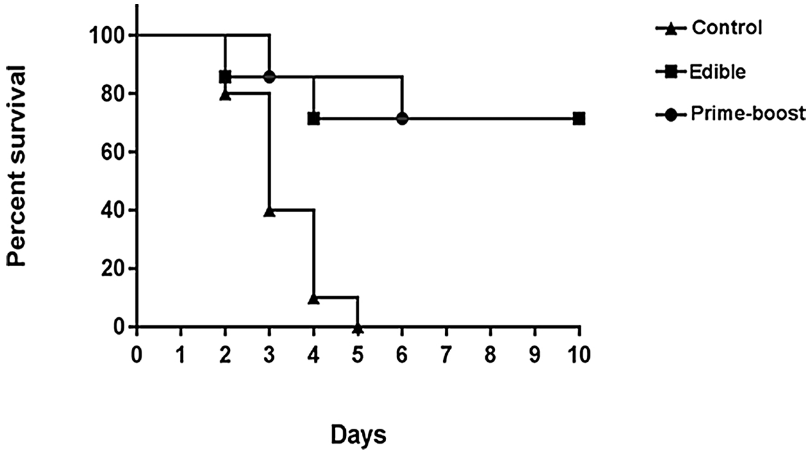

Fig. 8. The result of mouse toxicity assay. Mouse survival percentage by day post-EHEC infection. The anti-SICL considerably protected immunized mice as compared to control group.

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