Prevention of EHEC infection by chitosan nano-structure coupled with synthetic recombinant antigen

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ABSTRACT

One of highly effective methods for prevention and control of Entrohemorragic Esherichia coli (EHEC) infections is to use vaccination against extremely immunogenic part of attachment factors. In this study rEIT (EspA, Intimin, Tir) was produced in bacteria and then encapsulated with chitosan nanoparticle as a candidate nano-vaccine.

A chimeric trivalent recombinant protein which was previously found to provide reasonable immunogenicity against E.coli O157:H7 was used as a base. Mice immunized orally with chitosan based nanoparticle containing rEIT antigen. The rEIT-specific immune responses (IgG and IgA) were measured by indirect ELISA. In challenging tests different groups of immunized mice were infected orally with E.coli O157:H7.

The results showed that the recombinant nanovaccine candidate could induce the strong humoral and mucosal immune responses and protect the mice from live EHEC O157:H7 challenge. Higher titers of serum anti rEIT IgG were achieved after the last immunization in all of the groups. Comparison of the amount of IgA titers in serum and feces showed higher values for the latter. In vitro study of binding inhibition assay on Caco-2 cell monolayers by pre-incubated antisera with EHEC bacteria, showed that immunized mice antibody could reduce adhesion properties of E. coli O157:H7. In a challenging study with EHEC bacteria, reduction in number of colonies was observed in all of the immunized groups for over two weeks.

Results from the present study prove that nanovaccine candidate with rEIT can reduce signs and symptoms of EHEC infections. This novel approach can be a new strategy for inducing immunity against E. coli O157:H7. This study suggests the use of oral –injection combined vaccination routes comparing to other methods available in order to achieve higher humoral and mucosal immunogenicity levels.

1. Introduction

The Entrohemorrhagic E. coli (EHEC) O157: H7 strain is one of the major pathogens that causes hemorrhagic colitis and hemolytic-uremic syndrome in humans. The main reservoir of this bacteria is in the intestines of livestock, which can cause severe human infections through contaminated food and water (Stevens et al., 2002). The prevalence of acute diarrhea caused by Escherichia coli strains is generally very high in the world considered as a major causes of mortality, among children, in the areas having a lower levels of health (Pop et al., 2014). This bacterium causes about 22% of severe intestinal and hemorrhagic infection cases worldwide, and may create severe impairment in renal function as well. Since the treatment by antibiotics may eliminate the natural flora of the body and change the population of microorganisms (Ferens and Hovde, 2011), therefore it is not considered effective enough and thus vaccination and other health raising methods can play a complimentary role.

The recombinant vaccines are a rather new approach to control

disease (Ellis and Brodeur, 2012). With the advent of genetic engineering it is no longer necessary to use the whole body of the pathogen as a vaccine, instead, specific genes and expressions are used to produce these recombinant vaccines(Garcia-Angulo et al., 2013).. Accordingly, researchers should identify the most immunogenic component of microorganisms following to highly specialized procedures. These are usually membrane proteins (Karmali, 1989). Bacterial binding to the gastrointestinal tract controlled by the pathogenicity islands. These islands are extra-chromosomal genetic materials which contain Attachment/Effacing genes and the type III and IV secretion system. The type III secretion system is involved in secretion of various proteins including Tir, EspA, EspB, and EspD (Cid et al., 2001). The Intimin, Tir, and EspA are three important binding proteins to the intestinal epithelial cells. By selecting and assembling the effective parts of these proteins, they can be used as a vaccine candidate to enhance immunization levels (Amani et al., 2009).

EspA which is encoded by *espa* gene could provide a filamentary structure in the host cell. This apparatus provides a bridge for transferring other factors involved in bacterial implantation. The Tir protein is transmitted through the TTSS (the type III secretion system) to the host cell and, after processing on the surface of the host cell, is ready to receive its own ligand, *i.e.* Intimin (Fan et al., 2012). Intimin is a membrane protein encoded by the *eae* gene and is one of the most important factors for the binding of bacteria to the host cell.

Nowadays, studies have focused on methods for more effective immunization against these three antigens. Previous report showed that with the preparation of the trivalent gene construct, the mice immunogenicity will be higher than each of proteins separately (Amani et al., 2010). To further improve immunogenicity several studies have also been conducted to use dedicated carriers and digestive enzymes inhibitors including colloidal carriers (Hajizade et al., 2015). Chitosan with properties such as biodegradability and biocompatibility can effectively adhere to mucosal tissues and prevent destruction of antigens by proteolytic enzymes. (Sekhon and Saluja, 2011) (Metcalfe and Fahmy, 2012; Vinsova and Vavrikova, 2011; Xu and Du, 2003).

Researchers have reported some antibacterial effect of nanoparticles on *E.coli* enhance infection control (Mantravadi, 2017; Shrivastava et al., 2007). Even if these protective responses do not have the ability to prevent infection, they can delay the onset of bacteria and provide an appropriate opportunity for the antibiotics treatment(Qi et al., 2004). Among the various methods for nanoparticle formulation, ionic gelation is less toxic than other methods (Zhao et al., 2014). Some advantages of this method was ascribed to its condition achieved without applying harmful organic solvent, strong agitation or heat, which negatively affect sensitive proteins(Mohammadpour Dounighi et al., 2012).

In this study, after expression and purification the rEIT trivalent recombinant protein (Amani, Salmanian, Rafati and Mousavi, 2010) was nanoparticulated with chitosan by an ionic gelation process. Subsequently, this nanovaccine candidate was prescribed as Oral, Oral-Injection and Injection only, to BALB/c mice and their immunogenicity were measured.

2. Materials and methods

2.1. Expression and purification of recombinant synthetic protein

EHEC serotype O157:H7 (strain ATCC 35218) and the constructed rEIT in pET28a plasmid provided by the NIGEB laboratory. Recombinant EIT is a construction containing 120 amino acid of EspA, lacking 36 amino acids from N-terminal of the protein, 282 amino acids from the C-terminal of Intimin, and Tir 103, residues 258–361 which interact with Intimin (Amani et al., 2009; Amani et al., 2010).Expression of the synthetic *eit* gene was performed in *E.coli* BL21(DE3) with the $6 \times$ His tag in the N- terminal in Luria-Bertani (LB) broth at 37 °C.The media were supplemented with kanamycin (40 μg/ml). The

rEIT protein was purified with Ni-NAT (Qiagen, Germany) resin under native condition,and verified on 12% SDS-PAGE.

2.2. Western blotting analysis

Purified rEIT antigen from 12% SDS-PAGE was transferred to PVDF membrane using transfer buffer. The chimeric recombinant EIT was incubated with HRP conjugated anti- His-tag (1:2000; sigma) in PBS/T (PBS contain 0.05% Tween 20), *via* gentle shaking for 1 h at 37 °C. Detection finally was carried out using HRP staining solution (DAB). Chromogenic reaction was stopped by rinsing the membrane with distilled water. Also to verify the reactivity of immune sera with rEIT, this antigen was blotted and incubated using antisera from the mice immunized with rEIT.

2.3. Preparation of chitosan solution

Chitosan nanoparticles were prepared by ionic cross linking of chitosan solution with TPP anions. The 500 μg of rEIT antigen was mixed with 7.5 ml of solution of chitosan (2 mg/ml, pH 5.5) in 2% of acetic acid during 10 min. The solution was mixed with drop wise addition of sodium tripolyphosphate (1 mg/ml) under magnetic stirring at room temperature for 1 h. The resulting mixture was centrifuged at 1300 rpm for 20 min at 4 °C. The supernatant was subjected for encapsulation efficacy. The pellet was collected and was kept at 4 °C for further characterization.

2.4. Determination of the size and the surface charge of particles

Particle size and zeta potential of nanoparticles with and without rEIT were measured by dynamic light scattering and Laser Doppler Electrophoresis using Zetasizer (Nano-ZS, Malvern, UK) at wavelength of 633 nm at 25 °C.

2.5. Encapsulation efficiency

To check the encapsulation efficiency, the free rEIT that was present in the supernatant after centrifugation was measured in spectrophotometer at 595 nm *via* Bradford protein assay (Kruger, 2002). The encapsulation efficiency was calculated using the formula:

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Total rEIT} - \text{rEIT in supernatant}}{\text{Total rEIT}} \times 100$$

2.6. In vitro antigen release

The nanoparticled recombinant EIT with chitosan in PBS buffer was centrifuged at 13000 rpm for 20 min at 4 °C and was placed in shaker incubator at 37 °C. Then in times period of 0, 1, 3, 12, 24, 48, 72, 96 and 120 h the amount of antigen released was determined by Bradford protein assay.

2.7. Animal immunization

25 Female- five- week old BALB/c mice (Pasteur Institute of Iran) were divided into three Tests (T1, T2, T3) and two control groups (C1, C2). Each mouse in the T1 group was immunized directly by oral gavage administration of 100 μg chitosan nanoparticled rEIT protein at two weeks intervals for four times. The second group (T2) was immunized by oral gavage same amount for three times and the intraperitoneal injection of 15 μg pure rEIT for last period. The mice of T3 group was injected subcutaneously with 15 μg recombinant EIT protein with complete Freund's adjuvant (CFA) for first time and with the same amount antigens using incomplete Freund adjuvant (IFA) for the second and third time. Last injection was performed without adjuvant. C1 group was injected intraperitoneally with PBS and C2 group was

Table 1
Immunization administered to the groups of mice.

Experimental group	Route of administration	Administrated formulation	Immunized Schedule		Adjuvant	Atigen dose (μg/mice)
			Step	Day		
T1	Oral gavage	Nanoparticled rEIT	1st	0	–	100
			2nd	14	–	100
			3rd	28	–	100
			4th	42	–	100
T2	Oral gavage	Nanoparticled rEIT	1st	0	–	100
			2nd	14	–	100
			3rd	28	–	100
	Intrapretoneal	Pure rEIT	4th	42	–	15
T3	Subcutaneous	Pure rEIT	1st	0	CFA	15
			2nd	14	IFA	15
			3rd	28	IFA	15
			4th	42	–	15
C1	Subcutaneous	PBS	1st	0	–	–
			2nd	14	–	–
			3rd	28	–	–
			4th	42	–	–
C2	Oral gavage	Chitosan Without rEIT	1st	0	–	–
			2nd	14	–	–
			3rd	28	–	–
			4th	42	–	–

administrated orally gavage with only chitosan without antigen. The description of groups is shown in Table 1. Pooled blood samples from each groups of mice were collected retro-orbitally after the third and fourth immunization. In order to recover antibodies from feces, 1 g of pooled samples collected from each groups were mixed thoroughly with 500 μl of PBS containing 0.05% (*w/v*) sodium azide and 10 μl/mg protease inhibitor cocktail (Roche, Switzerland). The supernatant of the fecal after centrifugation (4 °C 6000 rpm 10 min), and sera samples were stored at -70 °C for further analyses. Animal study approval is available under ref. number IR.PNU.REC.1397.39 on www.ethics. research.ac.ir.

2.8. Determination of serum IgG and fecal IgA antibody responses to rEIT

Antibody specific responses were determined by an indirect Enzyme-Linked Immunosorbent assay (ELISA). 96-well plates (Nunc) were coated with 5 μg of purified rEIT protein diluted in coating buffer (64 mM Na_2CO_3 136 mM $NaHCO_3$, pH 9.8) at 4 °C, overnight. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS/T) and the non-specific sites were blocked with 3% skimmed milk in PBS/T. For determination of the relative IgG and IgA titers, sera samples were serially diluted in PBS/T from 1:100 to 1:400,000 for IgG and from 1:5 to 1:10,000 for IgA whereas fecal samples. The diluted samples were added to ELISA plates and incubated at 37 °C for 1 h. The plates were washed three times with PBS/T and HRP rabbit anti-mouse IgG (1/2000 in PBS/T) (Sigma) or - IgA (1/10000 in PBS/T) (Sigma) were added to the ELISA plates as a- secondary antibody. Plates were incubated (30 min at 37 °C) and washed three times in PBS/T. The wells added with 100 μl of citrate buffer containing 0.06% (*W/V*) of O-Phenylenediamine Dihydrochloride (OPD)(Sigma) and 0.06% (*V/V*) hydrogen peroxide were incubated at room temperature for 15 min. The reaction was stopped with 100 μl 0f 2 M H_2SO_4 and the OD $_{492}$ was read on micro plate ELISA reader (Garni DA-3200, Iran).

2.9. Challenging the immunized mice

To analyze the effect of antibodies on bacterial growth, two weeks after the last immunization, the mice were orally challenged with a lethal dose of *E.coli* O157:H7. All mice groups were pretreated with streptomycin sulfate (5 mg/ml) for reduction of gut bacterial flora two days before. The fasted antibiotic-treated mice while a night was gavaged with 10^{10} CFU of *E. coli* O157:H7.The mice fecal samples were

collected daily for two weeks. 0.1 g of fecal samples were suspended in 1 ml LB broth and incubated at room temp for 3–4 h, then vortexed for more homogenization, followed by plating onto Sorbitol Mac Conkey agar plates containing Cefixime and Tellurite. After incubation (37 °C overnight) the *E.coli* O157:H7 colonies were counted.

2.10. Binding inhibition assay for bacterial adhesion effect

Caco-2 cells (Pasteur Institute of Iran, Tehran) were grown to confluence in a culture flask containing DMEM medium and 10% FBS trypsinized and distributed onto a sterile round cover slip placed on the bottom of a 6-well cell culture plate (Nunk) and incubated in 5% CO_2 at 37 °C for 48 h. An overnight culture of *E.coli* O157:H7 grown to exponential growth phase was washed three times with PBS (pH 7.5) and adjusted to an OD: 0.5 at 600 nm (~10^8 bacteria). 300 μl of the bacterial suspension was pretreated with 150 μl of immunized T1, T2 and T3 mice antisera. The Caco-2 cell line treated with unimmunized mice antisera were used as negative control. The bacteria/serum mixture was added to cells and incubated for 1 h at room temperature. After three times washing with PBS, the Caco-2 cells were fixed (1 ml of 100% Methanol for 5 min) and stained with Giemsa staining solution (SIGMA) for 5 min and then destained with PBS. The cover slips containing the Caco-2 cells bound with *E. coli* O 157:H7 were observed under the optical microscope at 1000× magnification. The number of bacteria that adhered to each cell was counted and determined the percentage of positive cells by examining of 100 Caco-2 cells on each cover slip.

2.11. Statistical analysis

The data were representative of three separate experiments and described the mean ± standard deviation. Student *t*-test was performed to analyze the antibody data of the mice groups. Analysis for the significance differences of EHEC attachment inhibition on Caco-2 cells was also tested using Bonferroni multiple comparison test. In all experiments the $p < .05$ was considered statistically significant.

3. Results

3.1. Expression and purification of recombinant protein

The chimeric gene consisting of 120 amino acids from C-terminal of EspA, 282 amino acids from Tir-binding carboxy terminal of Intimin
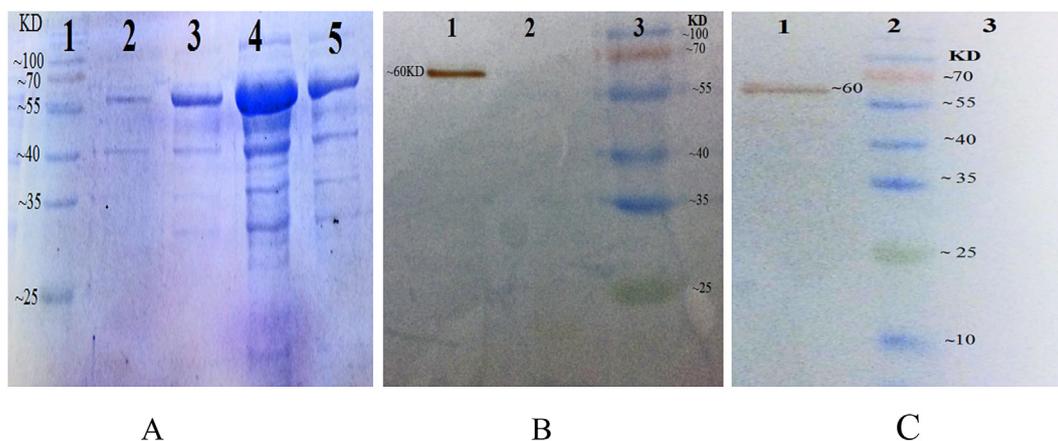
Fig. 1. Purification of recombinant EIT and western blotting analysis. A) Purified 6 × -His-tagged protein after elution with 250 mM imidazole. Lane 1, Protein weight marker, Lane 2–5 purified protein after elution. B) Western blot analysis of rEIT using anti 6 × -His-tag antibodies (~60 KD). Lane 1, rEIT. Lane 2, total protein of *E. coli* BL21DE3/pET28a without *eit* gene after induction as control. Lane 3 protein molecular weight marker. C) Western blot carried out with mice serum EIT antibody, Lane1, rEIT antigen. Lane2, protein marker. Lane3, control.

and residues 258–361 of Tir fragment interacting with Intimin were selected (Amani et al., 2010).

The synthetic *eit* gene was expressed in *E. coli* (BL21 DE3), with the N-terminal His-tag. Purification of the rEIT were carried out under native condition and 12% SDS-PAGE analysis showed the presence of near 61 KD of chimeric antigen as a major band in all the eluted fractions (Fig. 1A). The expression of recombinant EIT protein confirmed with Western blotting (Fig. 1B). To verify the reactivity of the immune sera with antigen, the recombinant EIT were blotted and incubated with antisera of immunized mice (Fig. 1C).

3.2. Measurement of particle size and zeta potential of nanoparticles with rEIT

The particle size and Zeta potential of nanoparticles with and without rEIT are shown in Figs. 2 (A, B). The average size of chitosan particles along with antigen was about 116.6 nm with a polydispersity index of about 0.384 and without antigen were 104.6 nm with an index of 0.31 respectively.

Surface charge of chitosan nanoparticles with and without rEIT was measured and the values of + 19.3 mV and + 11.4 mV were obtained respectively indicating that chitosan nanoparticles possessed positive surface charges with a good mobility (Fig. 2.C) Fig. 5 shows that the higher titres of serum anti rEIT IgG could be achieved after the fourth immunisation in all of the groups.

3.3. Encapsulation efficiency of rEIT in chitosan and in vitro antigen release

The encapsulation efficiency was calculated and shows that 91% of the rEIT antigen loaded into chitosan nanoparticles. The *in vitro* antigen release was studied. Fig. 3 displayed a graph showing 76% of rEIT release of chitosan nanoparticles during 120 h.

3.4. Determination of antibody responses to rEIT

Titration of serum samples from immunized mice of T1, T2 and T3 groups compared to controls groups C1 and C2 showed a significant EIT-specific IgG and IgA antibody responses. The titers of anti-EIT-specific IgG antibody were clearly detectable even at 1:400000 dilutions (Fig. 4A). The titers of EIT-specific IgA in serum and fecal of three immunized mice groups were compared with each other (Fig. 4 B, C). The Fig. 5 was shown that the higher titers of serum anti rEIT IgG could be achieved after the fourth immunization in all of the groups.

3.5. Animal challenge with E. coli O157:H7

In order to determine that the rEIT-specific antibodies in immunized mice sera could prevent the *E. coli* O157:H7 shedding in feces, three immunized (T1, T2, T3) and two control groups (C1, C2) were infected orally with 10^{10} CFU of *E. coli* O157:H7. Shedding of mice was detected in feces of each groups. Result showed that all unimmunized control mice groups indicated high levels of bacterial shedding in their feces during two weeks sampling period. All immunized mice exhibited a statistically significant decrease during colonization of bacteria compared to the mice control groups ($p < .05$). There was no significant difference in the duration of shedding between three groups (Fig. 6).

3.6. Bacterial adhesion and growth inhibitory effect

Colony count of Caco-2 cell monolayers revealed that *E. coli* O157:H7 cells pretreated with serum from the immunized and non-immunized mice has shown different adhesion percentage (Table 2). In non-immunized collections almost all the Caco-2 cells (up to 90%) were observed to bind one or more bacteria with a mean number around 15 bacteria per cell. But in immunized samples showed lower percentage (< 50%).That means the percentage of *E. coli* O157:H7 cells treated with different groups of immunized mice sera was significantly lower than that treated with non-immunized mice sera ($p < .05$). All experiments were carried out in duplicate on separate time (Fig. 7).

4. Discussion

Diarrhea is the major cause of morbidity and mortality in infants and young children. (Organization, W.H., 2012). It is estimated that between 4 and 6 million children die from diarrhea every year. The study of *E. coli*: O157: H7, the most important serotype of the Enterohemorrhagic *E. coli*, has a great importance because of severe digestive pathogenesis, including hemorrhagic colitis, and renal failure development (Wales et al., 2005) (Blecher et al., 2011).

In recent years, studies have been conducted to improve vaccine efficacy in order to provide better immunization(Nascimento and Leite, 2012). To be used as more effective vaccines, the proteins need adjuvants which increase the immunity response. In this regard, the new field in the nanobiotechnology is development of the vaccines based on nanocarriers. In many studies, the positive properties of chitosan have been confirmed in terms of its harmlessness, mucosal adhesion, biodegradability, nontoxicity, small size and low cost (Kumar et al., 2004; Ma et al., 2016) (Van der Lubben et al., 2001).

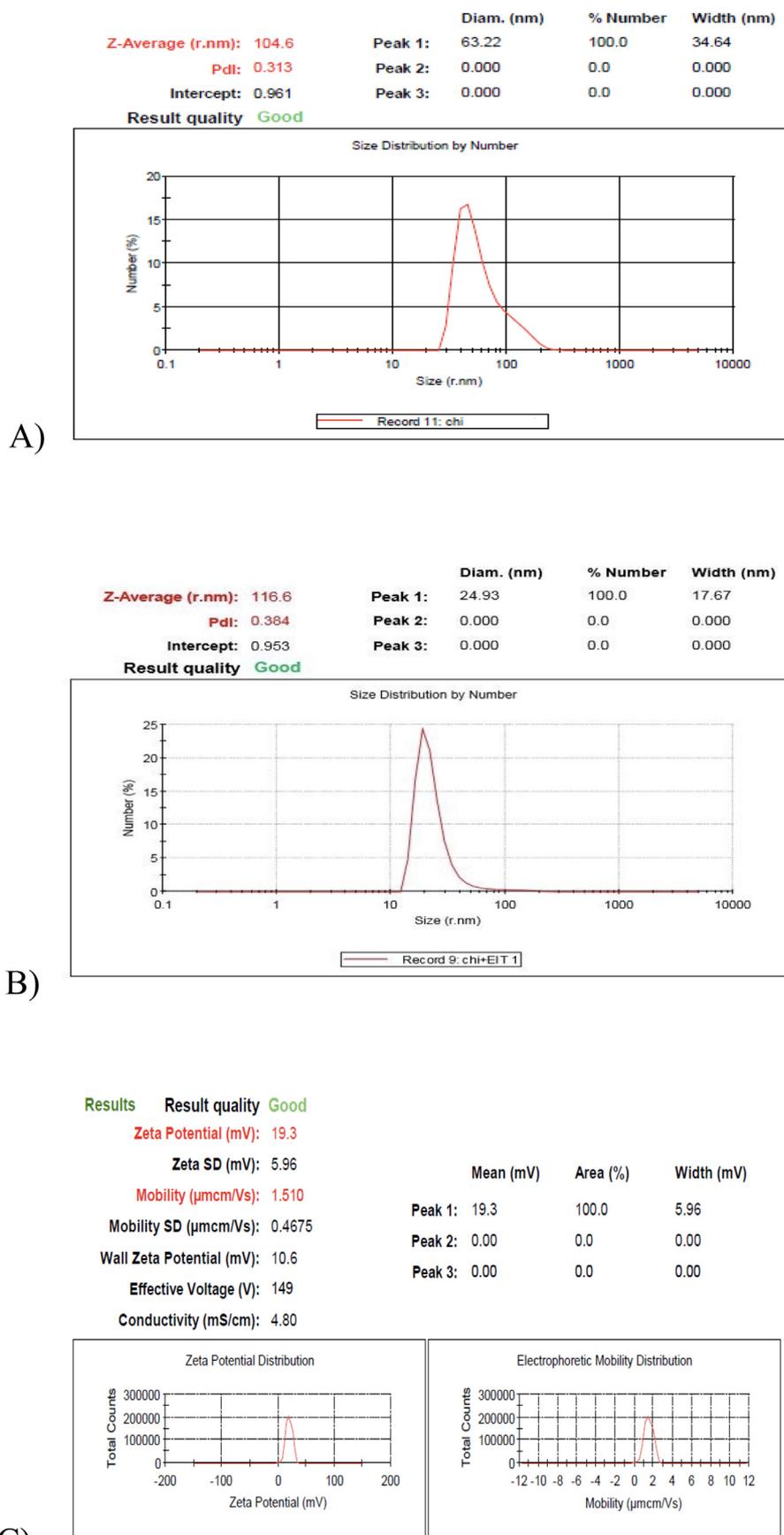Mucosal vaccines have low proteolytic enzymatic activity thus

Fig. 2. Size distribution, Zeta potential and Mobility of chitosan nanoparticles. A) Chitosan nanoparticles without rEIT, B) Chitosan nanoparticles containing rEIT and C) Chitosan nanoparticles containing rEIT measured by MALVERN Zetasizer,
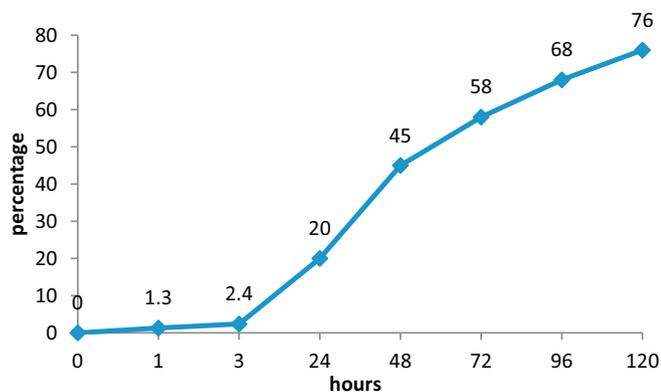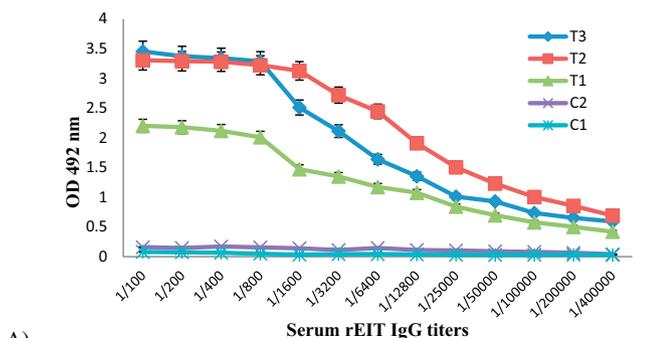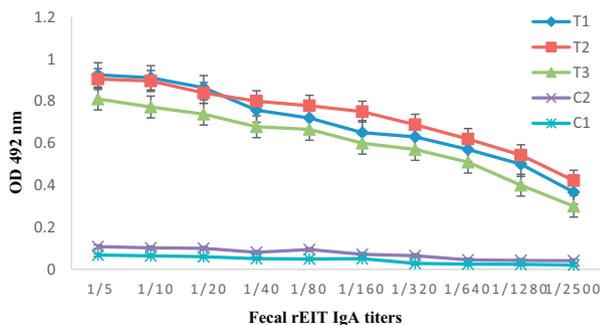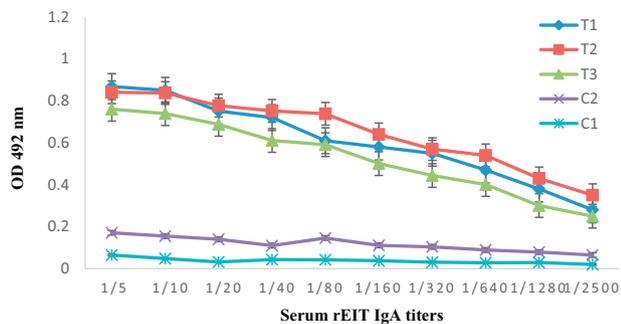
Fig. 3. Graph of *in vitro* antigen release of nanoparticled rEIT based on chitosan.

Fig. 5. Comparison of serum rEIT-IgG titers (1:100) after third (S1) and fourth (S2) immunization in mice groups. T1: Oral nanovaccine; T2: Combination (oral-injection) strategies; T3: Parenteral recombinant vaccine.
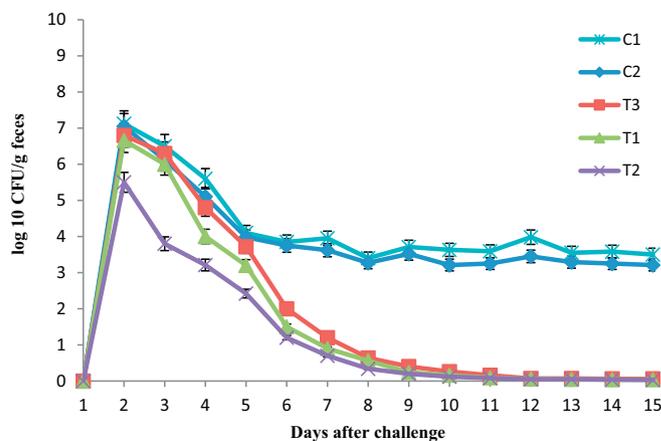
Fig. 6. *E. coli* O 157:H7 shedding following intramuscular administration in mice. Test groups (T1, T2, T3) were orally fed with 10^{10} *E. coli* O157:H7 and shedding was monitored daily in feces of each group for two weeks. Differences were considered significant whenever $p < .05$. T1: Oral nanovaccine; T2: Combination strategies; T3: Parenteral recombinant vaccine; C1: Control-PBS; C2: Control with chitosan.

Table 2

Inhibition of *E. coli* O157:H7 binding to Caco-2 cells.

Test Sera	Positive Caco-2 cells(%)[a]	Mean number adhesion[b]
Non -immune mice (C1) A	98	15.1 ± 0.02
Non- Immune mice (C2) B	93	14.8 ± 0.08
Immune mice (T1) C	47	2.9 ± 0.05
Immune mice (T2) D	33	2 ± 0.09
Immune mice (T3) E	32	1.9 ± 0.02

T1: Oral nanovaccine; T2: Combination strategies; T3: Parenteral recombinant rEIT; C1: Control-PBS; C2: Control with chitosan.

[a] The percentage of Caco-2 cells with at least one adhering *E. coli* O157:H7 bacterial cell.

[b] The mean number of adherent bacteria cells per Caco-2 cell. A, B, C, D, E above images of test and control groups.

Fig. 4. Graph of serum IgG and IgA titers of rEIT. A) Graph of serum IgG, serum B) Graph of fecal IgA titers C) using indirect ELISA method after 42 days. T1: the test group of mice was immunized with gavage by rEIT nanoparticle on chitosan, T2) The mice group using gavage and intrapretoneal vaccination, T3: the test group of mice used only subcutaneous injection with rEIT adjuvanted by Ferund's adjuvant. C1: control group subcutaneously immunized only with PBS, C2: Control group immunized with orally gavage with chitosan without antigen.
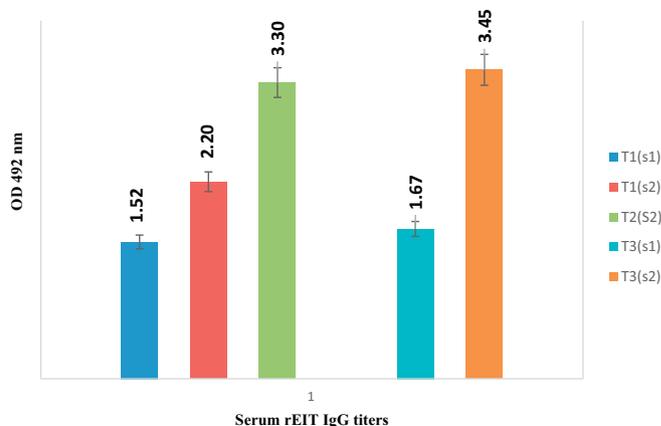
provides more antigenic constancy and need lower doses of antigens (Chen et al., 2011; Giudice and Campbell, 2006). Chitosan is a desirable candidate due to its non-toxic properties and the ability to easily take form while preparing (Thanou et al., 2001).

In this research, the preparation and purification of the nanovaccine candidate was performed. This immunogen consists of a chimeric antigen construct (EIT) (Amani et al., 2009) from three important
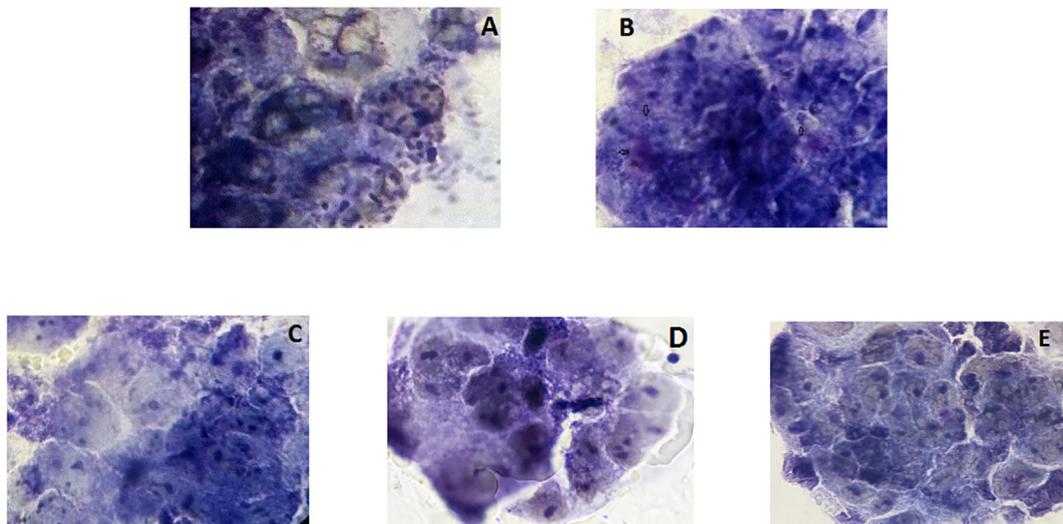
Fig. 7. Light micrographs of Giemsa-stained Caco-2 cells with adhesion of *E. coli* O157:H7; Before incubation with Caco-2 cells, the bacterial cells were pretreated with: A) Negative control, unimmunized sera (C1), B) Negative control, non-immunized sera (C2); C, D, E) Test groups: immunized mice antisera (T1, T2, T3).

binding proteins of the *E. coli* O157: H7 (EspA, Intimin and Tir) loaded on chitosan, then its immunization capability was evaluated in the animal model (mice). Recent studies suggest that evaluation of pure three-part chimeric construct in vaccinated mice (Amani et al., 2011) and rEIT nanovaccine candidate in nasal electrospray manner (Doavi et al., 2016) provide valuable results and favorable immunization.

In this study, the size of nanoparticles containing the rEIT antigen was found to be very favorable in the range of 116.6 nm (mean) in comparison to 104.6 nm obtained from chitosan nanoparticle without antigen as a control. Most researchers have come up with a single consensus on the desired size (100 to 500 nm), which is influenced by the nature of the polymer (Amidi et al., 2010). The small size of the nanoparticles and the positive zeta potential at 19.3 mV in comparison to 11.8 mV from chitosan without antigens indicate their better adhesion to the mucosal cells of the intestine consistent with other examines (Bhattarai et al., 2010). These results show that encapsulation of rEIT antigen with chitosan nanoparticles can provide easy delivering and slow releasing of antigens (Nandedkar, 2009).

In the present study, the release rate of recombinant antigens from chitosan was investigated. It took at least four days to gradually release 76% of the protein from chitosan polymer nanoparticles in PBS buffer. This experiment was performed to indicate that quantized release of rEIT has taken place during the assay, further steps may be carried out by taking proper buffer for stomach and intestine conditions and pH value changes into consideration. It is concluded that the body will find enough time to make more effective immunogenic response against pathogens.

The immunogenicity analysis (Fig. 4.A) showed a rise in IgG antibody titers in the injection group than the oral and oral-injection groups compared to the control groups. Fecal IgA antibody titration (Fig.4.B) was increased in oral and oral-injection groups compared to the injection only group. These data indicated that due to first colonization of *E. coli* O157:H7 through the mucosa of gastrointestinal tract, oral-injection routes of mucosal vaccination were induced a more effectual immune response, since general vaccination dose not usually increase these antibody levels enough. Fig. 4.C indicate similar results dispite titers of IgA antibody being lower than fecal. By administration of three oral dose of nanoparticled rEIT and one dose of injection both specific IgG and IgA in appropriate level were detected. Raising rEIT-specific IgG titers in serum of all groups after last vaccination showed a more proper stimulation of immune system in mice (Fig. 5). Identification of particular antibodies in the mice serum which were produced against the EIT recombinant protein through the western blotting procedure

proves the correctness of approvals taken in this research, However it is not yet clear against which part of the EIT this antibody was produced. It is probable this combination can be the cause of these antibodies production against one or several epitopes of the mentioned antigen. The presented data are in accordance with those obtained previously by other researchers in mice with non nanopaticulated rEIT subcutaneously and with intranasal route of vaccination by nanoparticulated rEIT and also in cattle with intramuscular route by rEIT without the usage of nanoparticle for immunization (Amani et al., 2010; Doavi et al., 2016; McNeilly et al., 2010).

In a challenging study with different groups of immunized mice *versus* control groups by feeding EHEC bacteria the colonies of this bacterium were counted after their fecal extract culture on the specific Mac Conkey Sorbitol Agar medium. The reduction in the number of colonies over two weeks was more in the oral and oral-injection groups compared to the injection group. Giemsa-stained Caco-2 cells monolayers of the neutralization test indicated lower adhesion of *E. coli* O157:H7 bacteria to Caco-2 cells pretreated by immune sera (Fig. 7). Results of microscopic slides (Table 2) showed that the serum of immunized mice with injection group prevents 67% of binding of *E. coli*: O157: H7 to cells also for oral and oral -Injection groups, these values were 53%, 68% respectively. These data were in concurrence with the values obtained previously in mice when the subcutaneous and intranasal routes were used for immunization with rEIT (Amani et al., 2010; Doavi et al., 2016).Given that the bacteria enters the human digestive system through contaminated food, the prevention of the bacterial binding to Caco-2 cells and reduction of bacteria in the vaccinated animal feces (mice) believed to be due to some efficacy of nanovaccine candidates. Also, for obtaining the appropriate titration of IgG antibodies and acceptable titers of the serum and fecal IgA in the animal models (mice), it was decided to follow the T2 group as a model *i.e.* with combination of oral-injection routs of vaccination.

5. Conclusion

It can be concluded that the use of the rEIT nanovaccine candidate is very beneficial for preventing the disease caused by this bacteria, but the balance between benefit and risk must be calculated before clinical examination. Painless, effective and safe needle-free routes are some of the advantages of this method. A significant result of challenges of inhibition binding assay on the human epithelial cells and reduction of EHEC bacteria shedding of immunized mice groups by gavage with *E. coli* O157:H7 showed a clear and effective immunogenicity against this

bacteria.

Conflict of interest

The authors have no conflict of interest.

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