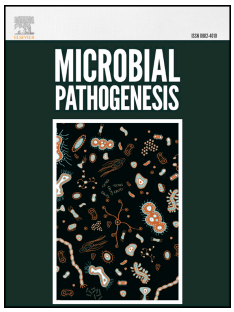
Accepted Manuscript



Immunization against *Vibrio cholerae*, ETEC, and EHEC with chitosan nanoparticle containing LSC chimeric protein

Bentol Hoda Ghaffari Marandi, Mohammad Reza Zolfaghari, Rouhollah Kazemi, Mohammad Javad Motamedi, Jafar Amani

PII: S0882-4010(19)30237-2

DOI: https://doi.org/10.1016/j.micpath.2019.103600

Article Number: 103600

Reference: YMPAT 103600

To appear in: *Microbial Pathogenesis*

Received Date: 6 February 2019

Revised Date: 12 June 2019

Accepted Date: 13 June 2019

Please cite this article as: Ghaffari Marandi BH, Zolfaghari MR, Kazemi R, Motamedi MJ, Amani J, Immunization against *Vibrio cholerae*, ETEC, and EHEC with chitosan nanoparticle containing LSC chimeric protein, *Microbial Pathogenesis* (2019), doi: https://doi.org/10.1016/j.micpath.2019.103600.

Immunization against *Vibrio cholerae*, ETEC, and EHEC with Chitosan Nanoparticle Containing LSC Chimeric Protein

Bentol Hoda Ghaffari Marandi[1], Mohammad Reza Zolfaghari[1], Rouhollah Kazemi[2], Mohammad Javad Motamedi[2], Jafar Amani[3*]

1. Department of Microbiology, Faculty of Basic Science, Qom Branch, Islamic Azad University, Qom, Iran.
2. Green Gene Company, Tehran, Iran.
3. Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran.

Corresponding author

Dr. Jafar Amani

Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Vanak Sq. Molasadra St. Tehran, Iran. P.O. Box 19395-5487.
Tel: +98 21 82482592 Fax: +98 21 88068924. Email: jafar.amani@gmail.com

Abstract

Introduction: Severe intestinal infections caused by *V. cholerae*, ETEC and EHEC have contributed to the mortality rate in developing countries. *Vibrio Cholera*, ETEC and EHEC bacterium with the production of CT, LT and Stx2 toxins respectively lead to severe watery and bloody diarrhea. This study aimed to investigate a trimeric vaccine candidate containing recombinant chimeric protein, encapsulate the protein in chitosan nanoparticles and assess its immunogenicity.

Methods: The LSC recombinant gene was used. It is composed of LTB (L), STXB (S) and CTXB (C) subunits respectively. The LSC recombinant protein was expressed and purified and confirmed by western blotting. The purified protein was encapsulated in chitosan nanoparticles, and its size was measured. BalB/c mice were immunized in four groups through oral and injection methods by LSC protein. The antibody titer was then evaluated by ELISA, and finally, the challenge test of the toxins from all three bacteria was done on the immunized mouse.

Results: After expression and purification LSC protein size of nanoparticles containing protein was measured at 104.6 nm. Nanoparticles were able to induce systemic and mucosal immune responses by generating a useful titer of IgG and IgA. The challenge results with LT, CT and Stx toxins showed that the LSC protein might partially neutralize the effect of toxins.

Conclusion: LSC chimeric protein with the simultaneous three essential antigens have a protective effect against the toxins produced by ETEC, EHEC and *Vibrio cholera* bacteria and it can be used in vaccines to prevent Diarrhea caused by these three bacteria.

Keywords: *Escherichia coli* O157:H7, Chitosan, Nanoparticle, Chimeric protein, EHEC, ETEC.

1. Introduction

Diarrhea is still a major cause of mortality in young children in developing countries with inadequate health facilities. Almost more than 600,000 people die of infectious diseases annually [1]. *V. cholerae*, enterotoxigenic *E. coli* (ETEC) and enterohemorrhagic *E. coli* (EHEC) are among the most important factors in the development of severe intestinal diseases.

Vibrio Cholera and ETEC are among the most important pathogens producing severe watery diarrhea in developing countries, which have been the second cause of mortality in infants[2, 3]. EHEC also causes many diseases, including bloody diarrhea and Hemolytic uremic syndrome (HUS), which is considered as a concern for public health globally [4]. The mechanism of action of these three microorganisms is very structurally and functionally similar, and all of them produce heterohexamer enterotoxin (AB5), resulting in diarrhea [5]. The ETEC is attached to the epithelial surface of the intestine by the colonization factor and produces LT toxin which is heat-sensitive, and its binding subunit is LTB [6]. The enterotoxin produced by *V. cholerae* is attached to the GM1 receptor at the epithelial surface and enters the host cell with endocytosis [7]. *E. coli* O157: H7, the most common EHEC serotype, has become a high-risk and pathogenic subtype of *E. coli* due to the production of Stx enterotoxin [4]. In recent years, the provision of effective subunit vaccine has been considered for the treatment and prevention of infections caused by these bacteria [8]. Hence, various antigens from these bacteria have been a candidate for vaccine production. Studies have shown that the LTB as an immunogen can produces weakened by attenuated effect against ETEC and it is an important candidate for vaccine production [9]. On the other hand, CTB which is non-toxic subunit of cholera toxin and has an adjuvant effect significant candidate for the vaccine against Vibrio Cholera is the. It also makes it possible that antigen attaches to the GM1 receptor that is present in many cells, such as neurons [10]. It should be noted that the combination of two subunits of CT-B and LT-B has synergistic effect and produce a more effective vaccine for diarrhea caused by *Vibrio cholera* and ETEC [11]. STXB is also an essential component in the development of the EHEC vaccine, as it can produce specific and neutralizing antibodies Stx toxin [12].

Diarrhea is the second leading cause of mortality worldwide and it is necessary to design and produce an effective vaccine for these diseases. Although weakened live vaccines produce strong cellular responses, they also present hazards, including uncontrolled disease and inflammation [13, 14]. Inactivated vaccines produce poorer immunity although they do not have these risks. Therefore, subunit vaccines that create more immunogenicity and lower risk have been considered [15]. Since orally-administered protein-based vaccines are confronted with the problem of enzymatic degradation, encapsulating of protein into the nanoparticles protect the protein and the antigen can be transported to Peyer's patches at the epithelial tissue [16, 17]. Chitosan (D-glucosamine and N-acetyl D-glucosamine) are the natural polymer can be

used for production of nanoparticle which has some advantages such as the ability to adhere to intestinal epithelium, biocompatibility, non-toxicity, and being antimicrobial [18-20]. Chitosan nanoparticles improve the permeability of the intestinal epithelium and induce more immune cellular and humoral responses by increasing the activity of white blood cells [21, 22]. This response is associated with increased fecal (SIgA) and serum (IgG, IgA) antibodies which is evaluated using the ELISA test [23]. Since oral vaccines affect mucosal tissue, they provide more protection by producing SIgA in the intestine and prevent bacterial binding to the intestinal epithelium. However, the titer level of this antibody is much lower than that of serum antibodies, but it plays an essential protective effect [23-25].

Given the fact that currently licensed vaccines against three crucial gastrointestinal pathogens such as *V. cholerae*, *S. Typhi* and rotavirus are available, but there is no vaccine against other intestinal pathogens, including ETEC, Shigella and EHEC [26, 27]. This study aimed to investigate the immunogenic effect of nanoparticles containing a recombinant chimeric protein that includes LT-B (L), STXB (S), and the CTXB (C) subunits, which correspond to three bacteria of ETEC, EHEC and *V. cholerae*, respectively. By encapsulating this protein into the chitosan nanoparticles, we expect the protein to be delivered to the proper site in the gastrointestinal tract without degradation and breakdown, and it induces more immunity at mucosal surfaces as a proper candidate for an oral vaccine.

2. Materials and methods

2.1, Materials

Chemical agents, kits and molecular markers from Merck, Sinagen, Qiagen, IPTG from Thermo, anti-IgG and IgA antibodies and Chitosan companies with an average molecular weight from Sigma-Aldrich were prepared.

2.2. Strains of bacteria

E.coli DH5α containing pET28-a-LSC plasmid was prepared from the National Institute of Genetic Engineering and Biotechnology (Iran). The LSC gene with accession no. JX866680 (GenBank) was used [5]. The size of the gene cloned in the pET28-a, is 960bp and it contains the subunits of lt-b, stxB, and ctxB. The product of this gene is the recombinant chimeric protein of LSC.

2.3. Transformation of pET28a-LSC into expression host

Plasmid extraction from *E.coli* DH5α contaning pET28a-lsc was performed by GeNet Bio kit. The extracted plasmid was transformed to *E. coli* Bl21 (DE3) and transformation was confirmed by Colony-PCR with T7 promoter primer (5' TAATACGACTCACTATAGGG3') and T7 terminator primer (5' GCTAGTTATTGCTCAGCGG3') which these primers were added 300 bp to the *lsc* gene.

2.4. Expression and purification of the LSC protein

E. coli BL21 (DE3) containing pET28a-LSC were cultured in LB growth medium with kanamycin (50 μg/ml) and the expression was induced by 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The expression was investigated at different time intervals (2 hours, 14 hours) at 37 ° C and the solubility of the protein was determined. The recombinant protein was purified with Ni-NTA column and the protein concentration was estimated by Bradford method. Finally, to remove urea and other salts, LSC protein was dialyzed against PBS buffer (pH of 2.7) [5, 28].

2.5. Immunoblotting of LSC protein

The purified protein was blotted on the PVDF membrane at 75 volts for 2 hours and was blocked by skim milk 5% , and then it was washed three times with TBS-T (TBS buffer plus the 0.05% Tween). HRP-conjugated anti-His tag antibody was added with 1: 2000 dilution in TBST and it was incubated with for 1 hour at 37 °C. After washing with TBS-T, the detection was carry out with 0.5 mM DAB (Sigma).

2.6. Preparation of nanoparticles containing LSC protein

The LSC protein was encapsulated in the chitosan nanoparticles by an ionic cross-linking method [29]. The antigen was added gradually into the chitosan solution (2 mg/ml) for 10 minutes. Then, the solution was completely mixed for 5 minutes, and the pH was adjusted to 5.5, and it was kept on the stirrer for 30 minutes. Then tripolyphosphate (TPP), an ion cross-linker, was added dropwise to a solution containing chitosan and an antigen. The sediment of the solution was collected by centrifugation at 13000 rpm (10 min). The supernatant solution was then isolated from the sediment containing encapsulated protein, and the percentage of antigen loading in the nanoparticles was calculated. The mean size of the nanoparticles was determined using the Malvern Particle Size Analyzer.

2.7. Immunogenicity in mice

BALB/c mice (6-7 weeks) were divided into four groups (oral, oral-injection, protein injection, and nanoparticle injection) and two control groups (oral and injection). In the oral group, the mice received four doses of nanoparticles containing the recombinant protein LSC (70μg) orally. For oral-injection mice, three doses of the nanoparticle of recombinant LSC protein (70μg) were administered orally, and one dose of LSC protein (15μg) was injected intraperitoneally. In the protein injection group, the recombinant (15μg) protein of LSC was injected into the mice in four doses (the first three doses were subcutaneous, and the last one was intraperitoneally). In the protein injection group the complete and incomplete Freund adjuvant was used in the first dose and later doses respectively. In the injection nanoparticle, the LSC protein encapsulated in nanoparticles (15μg) was injected in 4 doses (the first three doses were subcutaneous, and the last one was intraperitoneally). In the injection control and the oral

control group PBS injection and antigen-free chitosan were administered respectively. Administrations were done at intervals of two weeks. Then, blood sampling was done from the mice.

2.8. Titration of IgG and IgA antibody

Indirect ELISA was used to determine the fecal and serum antibody titers. Purified LSC was coated with coating buffer (64 mM Na2CO3, 136 mM NaHCO3, pH 9.8). All incubations were performed for 1 hour at 37 ° C. After washing with a PBS-T buffer; the blocking was performed using skim milk 5%. Serum serial dilution was prepared using PBS-T and was poured into the wells as a primary antibody. Washing was done, and the HRP-conjugated anti-mouse IgG was added. Then, the reaction was performed at room temperature with the addition of 0.5 mg/ml OPD (O-phenylene diamine dihydrochloride) substrate. The reaction was stopped with 2.5 M sulfuric acid and the plate was read at 492 nm. In the case of fecal IgA titer, 1.5 gram of fresh feces of mice was well mixed with PBS containing PMSF solution. Then, supernatant fluid was collected by centrifugation (13000 rpm, 15 minutes) and the serial dilution was prepared and added to the wells as a primary antibody.

2.9. Challenge assay

Two weeks after the immunization, three challenges were done for all three bacteria of EHEC, *V. cholerae* and ETEC. In the challenge for EHEC, 10^9 CFU *E. coli* O157:H7 which produced only Stx2 was administered orally to the mice, and then feces of mice were collected for two weeks. The feces were well combined with PBS and were cultured in Sorbitol-MacConkey agar, and the plate was incubated for 14 h at 37 ° C. Then, the colonies of *E. coli* O157: H7 were counted [30]. In the challenges for *V. cholerae* and ETEC (only LT producer), these bacteria were cultured in LB growth medium. Then, the supernatant solution was used as a toxin and injected into the mice peritoneally. Finally, their mortality rate was evaluated in the immunized and control mice for 14 days.

2.10. Statistical analysis

Data in each examination demonstrated of three independent experiments and illustrated as the mean ± standard deviation (SD).

3. Results

3.1. Expression and purification of LSC protein

Plasmid extraction from *E.coli* DH5α containing pET28a-LSC was performed and the plasmid was transformed to *E. coli* BL21DE3. The colony PCR was performed with T7 primer and the presence of LSC gene was confirmed by observing an approximate 1200 bp band on 1% agarose gel (Fig. 1A).

The recombinant LSC gene expression was confirmed by observing the 39 kDa band on the 12% SDS-PAGE gel (Fig. 1B). This result showed that the LSC protein was expressed mainly as an inclusion body. Purification was carried out under denaturation conditions with the Ni-NTA column, and the analysis of 12% SDS-PAGE indicated that the protein was present in the elution fraction (Fig. 1C). After dialysis the LSC recombinant protein was observed on the 12% SDS-PAGE gel. The expression of the recombinant LSC protein was confirmed by Western blotting (Fig. 1D).

3.2. Evaluation of chitosan nanoparticles containing LSC protein

The encapsulation and electron microscopy analysis of LSC protein in chitosan nanoparticles was accomplished by ion cross-linking method. The loading efficiency of this protein in chitosan nanoparticles was 88% and average size of nanoparticle was 10.46 nm with good quality (Fig. 2 A, B).

3.3. Determination of IgG and IgA antibody titer

The serum ELISA indicated a systemic response induction and increased serum IgG titer in all groups. In the LSC protein injected group indicated more raise of IgG antibodies than the nanoparticle injected group (Fig. 3A), followed by oral, and oral-injection groups respectively (Fig. 3B). The results showed that serum IgA titers of oral group were higher than the oral- injection and the fecal IgA titer (Fig. 3C).

3.4. Challenges in immunized mice

In a challenge with Ctx and LT, the mice were monitored for 14 days to determine their mortality. Six days after peritoneal injection of these two toxins, 100% of the control mice died. In a challenge with Ctx toxin in the oral-injection group, 66% of the mice survived until the end of day 14 and in the orally immunized group all of them died (Fig. 4A). In the group injected by nanoparticles, 33% of the mice survived, while 100% of the LSC protein injected group died (Fig. 4B). In a challenge with LT toxin, in the oral-injection group, 33% of mice survived until the end of the day 14 while the mice did not survive in other groups (Fig. 4C). Colony counting in the challenge with EHEC showed that the immunity generated against Stx could prevent bacterial binding to mice gut. Oral administration of these nanoparticles has had a more significant effect on preventing bacterial binding than other groups (Fig. 5).

4. Discussion

Severe intestinal infections caused by *V. cholerae*, ETEC, and EHEC, which lead to diarrhea, have accounted for a large proportion of the mortality rate in developing countries. Although diarrhea can be controlled by replacing fluids and electrolytes or by using antibiotics, due to resistance to antibiotics and antimicrobials, and the lack of healthy water sources, prevention, development and design of a vaccine can be considered as a more effective solution [3, 31, 32]. In recent years, attempts have been made to produce orally administered subunit vaccines and provide more protection in the intestine [33]. Toxins

from these bacteria (CT, LT, and Stx), have a key role in pathogenesis. These antigens are among the most important candidates for vaccine production and can induce more immune responses [34, 35]. A triple vaccine that can prevent these pathogens is desirable. In a study was indicated that using a chimeric protein composed of LTB and CTB could produce antibodies against both toxins [36]. Khalesi.et.al (2010), by injection of recombinant LTB protein, immunized the mice by neutralizing LTB [37]. In other study immunization of mice with Stx2 toxoid indicated that the anti-Stx2 antibody has protective effect and decrease colonization of EHEC in the intestine [38]. In this study, the recombinant LSC genes designed by Kazemi et al. (2016) were used which contain the subunit of ltb (l), stxb (s), and ctxb (c), respectively, belonging to the three bacteria of ETEC, EHEC, and *V. cholerae* [5]. In recent years, chitosan as a carrier in the medicinal field increases the antigen absorption by mucosal tissue through encapsulation of protein vaccines and induces more systemic and mucosal immunity [39]. Using of chitosan nanoparticles containing antigens from Vibrio *cholerae* [40] and EHEC [41] for immunization of mice showed appropriate immune response at the mucosal surfaces [40, 41]. In this study, chitosan nanoparticles containing LSC proteins were used to increase immunogenicity at mucosal surfaces and to deliver the vaccine, as a carrier, to the target cell in addition to protect the LSC protein from digestion and degradation. Mucosal vaccines are very appealing for children because they are administered without needle, pain, and fear. Also, these vaccines are preferred to traditional vaccine with advantages such as improvement of immunogenicity and ease of production [14, 23, 42]. The rate of protein loading in nanoparticles is also critical and shows the amount of vaccine in the nanoparticles that are supposed to reach the target cells. In this study, the percentage of loading of LSC protein was 88% which is appropriate compared with the research by Hosseini et al. (2018) (89.7%) and Noroozi et al. (2018) and Mirzaee Tabrizi et al. (2018) (90%) [43-45].

After immunization, the ELISA results showed raise of anti-LSC antibody in all immunized groups. The IgG titers in the mice immunized with recombinant LSC was higher than group immunized with nanoparticle containing LSC, followed by oral and oral-injection groups. The IgA antibody titer, which increases due to delivery of antigens at mucosal surfaces, was higher in the oral group than in the oral-injection group. The proper adhesion of nanoparticles to mucosal surfaces, as well as the reaction between positively charged nanoparticles and negatively charged mucosal surfaces, is an apparent reason for high levels of IgA titer in the oral group [30, 46]. Based on other studies the IgG for oral administration and IgA low in our research titration of oral administration and IgA in compare to other group was low but titration of IgG for injection groups was high [47, 48]. This data confirmed the results from Kazemi et al. [5]. Also in a study a chimeric protein containing CFA/I, CfaB, CS6, LTB, and STa was used to immunize mice and the challenge of this immunized mice with ETEC showed 30% protective

effect in oral-injection group [49]. Furthermore research on vaccination against EHEC and ETEC protected immunized mice after 6 days while in our research the immunized mice decreased shedding after 10 days[50]. In this study the oral-injection group which showed the highest raise of IgA and IgG could neutralize to some extent, the effect of both LT and CT toxins. The challenge of Stx toxin showed that the oral group receiving the four doses of LSC protein encapsulated in the chitosan nanoparticles had the highest inhibitory effect on the colonization of the EHEC bacterium in the intestine.These nanoparticles by increasing the absorption of antigens induced mucosal immunity and prevented the bacterial binding to these surfaces and reduced pathogenicity and led to a lower bacteria excretion of the feces in the mice. Similar result reported in other study which used Stx2 toxoid for immunization and Immunization study against chimeric antigen against EHEC and ETEC [38, 50].

In conclusion, the LSC chimeric protein encapsulated in the chitosan nanoparticles as a triple vaccine, while simultaneously providing three important molecules involved in the pathogenesis of *V. cholera*e, ETEC, and EHEC, can produce specific anti-LSC antibodies and thus has a proper protective effect against toxins of CT, LT, and Stx. Hence, its components can be considered as candidates for the design and development of a vaccine against intestinal diseases caused by these three bacteria.

Conflict of interest

The authors have no conflict of interest.

References:

[1] Zhang W, Sack DA. Current Progress in Developing Subunit Vaccines against Enterotoxigenic Escherichia coli-Associated Diarrhea. Clinical and vaccine immunology : CVI. 2015;22:983-91.

[2] Bhuiyan TR, Hoq MR, Nishat NS, Al Mahbuba D, Rashu R, Islam K, et al. Enumeration of Gut-Homing beta7-Positive, Pathogen-Specific Antibody-Secreting Cells in Whole Blood from Enterotoxigenic Escherichia coli- and Vibrio cholerae-Infected Patients, Determined Using an Enzyme-Linked Immunosorbent Spot Assay Technique. Clinical and vaccine immunology : CVI. 2016;23:27-36.

[3] Leitner DR, Lichtenegger S, Temel P, Zingl FG, Ratzberger D, Roier S, et al. A combined vaccine approach against Vibrio cholerae and ETEC based on outer membrane vesicles. Frontiers in microbiology. 2015;6:823.

[4] Eichhorn I, Heidemanns K, Semmler T, Kinnemann B, Mellmann A, Harmsen D, et al. Highly Virulent Non-O157 Enterohemorrhagic Escherichia coli (EHEC) Serotypes Reflect Similar Phylogenetic Lineages, Providing New Insights into the Evolution of EHEC. Applied and environmental microbiology. 2015;81:7041-7.

[5] Kazemi R, Akhavian A, Amani J, Salimian J, Motamedi MJ, Mousavi A, et al. Immunogenic properties of trivalent recombinant protein composed of B-subunits of LT, STX-2, and CT toxins. Microbes and infection. 2016;18:421-9.

[6] von Mentzer A, Connor TR, Wieler LH, Semmler T, Iguchi A, Thomson NR, et al. Identification of enterotoxigenic Escherichia coli (ETEC) clades with long-term global distribution. Nature genetics. 2014;46:1321-6.

[7] Heggelund JE, Burschowsky D, Bjornestad VA, Hodnik V, Anderluh G, Krengel U. High-Resolution Crystal Structures Elucidate the Molecular Basis of Cholera Blood Group Dependence. PLoS pathogens. 2016;12:e1005567.
[8] Larrie-Bagha SM, Rasooli I, Mousavi-Gargari SL, Rasooli Z, Nazarian S. Passive immunization by recombinant ferric enterobactin protein (FepA) from Escherichia coli O157. Iranian journal of microbiology. 2013;5:113-9.
[9] Bourgeois AL, Wierzba TF, Walker RI. Status of vaccine research and development for enterotoxigenic Escherichia coli. Vaccine. 2016;34:2880-6.
[10] Stratmann T. Cholera Toxin Subunit B as Adjuvant--An Accelerator in Protective Immunity and a Break in Autoimmunity. Vaccines. 2015;3:579-96.
[11] Karaman S, Cunnick J, Wang K. Expression of the cholera toxin B subunit (CT-B) in maize seeds and a combined mucosal treatment against cholera and traveler's diarrhea. Plant cell reports. 2012;31:527-37.
[12] Martorelli L, Garimano N, Fiorentino GA, Vilte DA, Garbaccio SG, Barth SA, et al. Efficacy of a recombinant Intimin, EspB and Shiga toxin 2B vaccine in calves experimentally challenged with Escherichia coli O157:H7. Vaccine. 2018;36:3949-59.
[13] Campos M, Godson DL. The effectiveness and limitations of immune memory: understanding protective immune responses. International journal for parasitology. 2003;33:655-61.
[14] Vela Ramirez JE, Sharpe LA, Peppas NA. Current state and challenges in developing oral vaccines. Advanced drug delivery reviews. 2017;114:116-31.
[15] Bagheri S, Mousavi Gargari SL, Rasooli I, Nazarian S, Alerasol M. A CssA, CssB and LTB chimeric protein induces protection against Enterotoxigenic Escherichia coli. The Brazilian journal of infectious diseases : an official publication of the Brazilian Society of Infectious Diseases. 2014;18:308-14.
[16] Morishita M, Peppas NA. Is the oral route possible for peptide and protein drug delivery? Drug discovery today. 2006;11:905-10.
[17] Bowman K, Leong KW. Chitosan nanoparticles for oral drug and gene delivery. International journal of nanomedicine. 2006;1:117-28.
[18] Madureira AR, Pereira A, Pintado M. Current state on the development of nanoparticles for use against bacterial gastrointestinal pathogens. Focus on chitosan nanoparticles loaded with phenolic compounds. Carbohydrate polymers. 2015;130:429-39.
[19] Cui H, Bai M, Rashed MM, Lin L. The antibacterial activity of clove oil/chitosan nanoparticles embedded gelatin nanofibers against Escherichia coli O157: H7 biofilms on cucumber. International journal of food microbiology. 2018;266:69-78.
[20] Cui H, Yuan L, Li W, Lin L. Edible film incorporated with chitosan and Artemisia annua oil nanoliposomes for inactivation of Escherichia coli O157: H7 on cherry tomato. International Journal of Food Science & Technology. 2017;52:687-98.
[21] Porporatto C, Bianco ID, Correa SG. Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration. Journal of leukocyte biology. 2005;78:62-9.
[22] Cui H, Yuan L, Lin L. Novel chitosan film embedded with liposome-encapsulated phage for biocontrol of Escherichia coli O157: H7 in beef. Carbohydrate polymers. 2017;177:156-64.
[23] Leach S. Approaches to Enhance and Evaluate the Immunogenicity of an Oral ETEC Vaccine2015.
[24] Nazarian S, Gargari SLM, Rasooli I, Hasannia S, Pirooznia N. A PLGA-encapsulated chimeric protein protects against adherence and toxicity of enterotoxigenic Escherichia coli. Microbiological research. 2014;169:205-12.
[25] Kim L, Martinez CJ, Hodgson KA, Trager GR, Brandl JR, Sandefer EP, et al. Systemic and mucosal immune responses following oral adenoviral delivery of influenza vaccine to the human intestine by radio controlled capsule. Scientific reports. 2016;6:37295.
[26] Czerkinsky C, Holmgren J. Vaccines against enteric infections for the developing world. Phil Trans R Soc B. 2015;370:20150142.
[27] Rojas-Lopez M, Monterio R, Pizza M, Desvaux M, Rosini R. Intestinal Pathogenic Escherichia coli: Insights for Vaccine Development. Frontiers in microbiology. 2018;9:440.
[28] Bollag DM, Edelstein SJ, Rozycki MD. Proteins methods1996.

[29] Ahmed TA, Aljaeid BM. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. Drug design, development and therapy. 2016;10:483.
[30] Khalouie F, Mousavi SL, Nazarian S, Amani J, Pourfarzam P. Immunogenic evaluation of chimeric recombinant protein against ETEC, EHEC and Shigella. Molecular biology research communications. 2017;6:101.
[31] Black RE. Epidemiology of diarrhoeal disease: implications for control by vaccines. Vaccine. 1993;11:100-6.
[32] Ochoa TJ, Ruiz J, Molina M, Del Valle LJ, Vargas M, Gil AI, et al. High frequency of antimicrobial drug resistance of diarrheagenic Escherichia coli in infants in Peru. The American journal of tropical medicine and hygiene. 2009;81:296-301.
[33] Kelly P. Infectious diarrhoea. Medicine. 2015;43:253-8.
[34] Glenn GM, Francis DH, Danielsen EM. Toxin-mediated effects on the innate mucosal defenses: implications for enteric vaccines. Infection and immunity. 2009;77:5206-15.
[35] Zhang X-h, He K-w, Zhang S-x, Lu W-c, Zhao P-d, Luan X-t, et al. Subcutaneous and intranasal immunization with Stx2B–Tir–Stx1B–Zot reduces colonization and shedding of Escherichia coli O157: H7 in mice. Vaccine. 2011;29:3923-9.
[36] Lebens M, Shahabi V, Bäckström M, Houze T, Lindblad N, Holmgren J. Synthesis of hybrid molecules between heat-labile enterotoxin and cholera toxin B subunits: potential for use in a broad-spectrum vaccine. Infection and immunity. 1996;64:2144-50.
[37] Khalesi R, Sh N, Ehsaei Z, Mansouri M, Amani J, Salimian J, et al. Optimization of gene expression and purification of enterotoxigenic Escherichia coli recombinant LTB protein and antibody production against it. Trauma Monthly. 2010;2010:141-7.
[38] Mohawk KL, Melton-Celsa AR, Robinson CM, O'Brien AD. Neutralizing antibodies to Shiga toxin type 2 (Stx2) reduce colonization of mice by Stx2-expressing Escherichia coli O157: H7. Vaccine. 2010;28:4777-85.
[39] van der Lubben IM, Verhoef JC, Borchard G, Junginger HE. Chitosan and its derivatives in mucosal drug and vaccine delivery. European Journal of Pharmaceutical Sciences. 2001;14:201-7.
[40] Adriani R, Gargari SLM, Nazarian S, Sarvary S, Noroozi N. Immunogenicity of Vibrio cholerae outer membrane vesicles secreted at various environmental conditions. Vaccine. 2018;36:322-30.
[41] Doavi T, Mousavi SL, Kamali M, Amani J, Ramandi MF. Chitosan-based intranasal vaccine against Escherichia coli O157: H7. Iranian biomedical journal. 2016;20:97.
[42] Shakya AK, Chowdhury MY, Tao W, Gill HS. Mucosal vaccine delivery: current state and a pediatric perspective. Journal of Controlled Release. 2016;240:394-413.
[43] Hosseini ZS, Amani J, Baghbani Arani F, Nazarian S, Motamedi MJ, Shafighian F. Immunogenicity of the nanovaccine containing intimin recombinant protein in the BALB/c mice. Clinical and experimental vaccine research. 2018;7:51-60.
[44] Noroozi N, Gargari SLM, Nazarian S, Sarvary S, Adriani RR. Immunogenicity of enterotoxigenic Escherichia coli outer membrane vesicles encapsulated in chitosan nanoparticles. Iranian journal of basic medical sciences. 2018;21:284.
[45] Tabrizi NM, Amani J, Ebrahimzadeh M, Nazarian S, Kazemi R, Almasian P. Preparation and evaluation of chitosan nanoparticles containing CtxB antigen against Vibrio cholera. Microbial pathogenesis. 2018;124:170-7.
[46] Elgadir MA, Uddin MS, Ferdosh S, Adam A, Chowdhury AJK, Sarker MZI. Impact of chitosan composites and chitosan nanoparticle composites on various drug delivery systems: A review. Journal of Food and Drug Analysis. 2015;23:619-29.
[47] Amani J, Mousavi SL, Rafati S, Salmanian AH. Immunogenicity of a plant-derived edible chimeric EspA, Intimin and Tir of Escherichia coli O157: H7 in mice. Plant science. 2011;180:620-7.
[48] Almasian P, Amani J, Arani FB, Nazarian S, Kazemi R, Tabrizi NM. Preparation of chitosan nanoparticle containing recombinant StxB antigen of EHEC and evaluation its immunogenicity in BALB/c mice. Iranian journal of microbiology. 2018;10:361.
[49] Zeinalzadeh N, Salmanian AH, Goujani G, Amani J, Ahangari G, Akhavian A, et al. A Chimeric protein of CFA/I, CS6 subunits and LTB/STa toxoid protects immunized mice against enterotoxigenic Escherichia coli. Microbiology and immunology. 2017;61:272-9.

[50] Shojaei Jeshvaghani F, Amani J, Kazemi R, Karimi Rahjerdi A, Jafari M, Abbasi S, et al. 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. Immunobiology. 2019;224:262-9.

Figure Legends:

Figure 1. Analyses of plasmid, expression, purification and western blot analysis of recombinant LSC protein. A) Agarose gel electrophoresis analysis of plasmid by PCR with T7 promoter and terminator primer. M, DNA ladder; 1, negative control; 2-4, PCR product of different colony of *E. coli* BL21 (DE3) containing recombinant lsc gene. B) Expression and purification of recombinant LSC analysis on the 12% SDS-PAGE. M, protein ladder; 1, before induction sample; 2-6, different colony of *E. coli* Bl21 (DE3) containing pET281-*lsc* induced by 1 mM IPTG. C) Analysis of purified LSC with Ni-NTA column; 1, bacterial lysate; 2, flow through; 3, wash; 4-8, elution D) Western blotting analysis with anti-his antibody, 1, Lysate of bacteria containing pET28a-LSC; 2, negative control, bacterial lysate before induction; M, protein ladder.

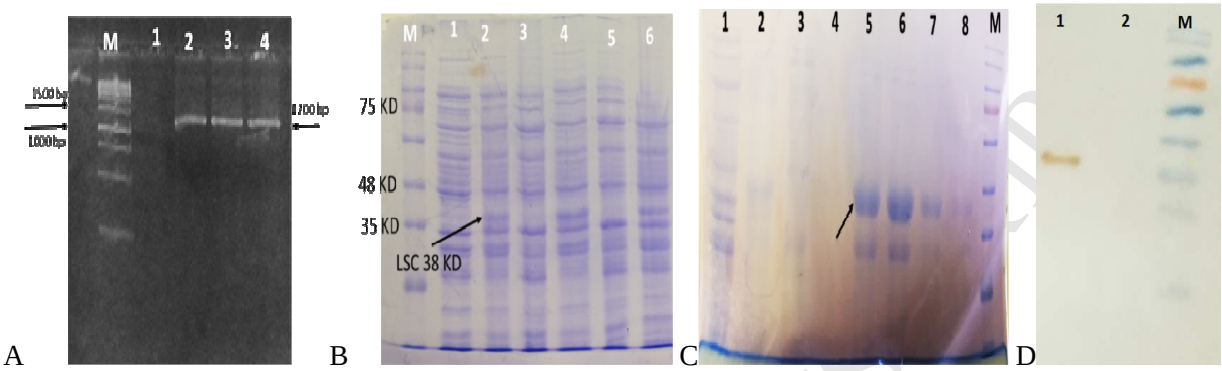
Figure 2. Zeta sizer A) and electron microscopy B) analysis of chitosan nanoparticle containing the purified LSC.

Figure 3. Titration of antibody in immunized mice. (A) Titration of IgG antibody in the groups were injected with recombinant LSC or chitosan nanoparticle containing LSC. (B) IgG titration in the groups were immunized orally with chitosan nanoparticle containing LSC and booster dose with inaction of LSC. (C) IgA titration in the serum and shedding of mice immunized orally and group with booster injection dose.

Figure 4. Challenge assay with toxin of vibrio cholerae and ETEC. (A) Survival of mice immunized orally with nanoparticle containing LSC after challenge with Ctx toxin. (B) Survival of injection group in challenge with Ctx toxin. (C) Survival of immunized mice in challenge with LT toxin.

Figure 5. Shedding colony count after challenge of immunized mice with 10^9 CFU of *E. coli* O157:H7.

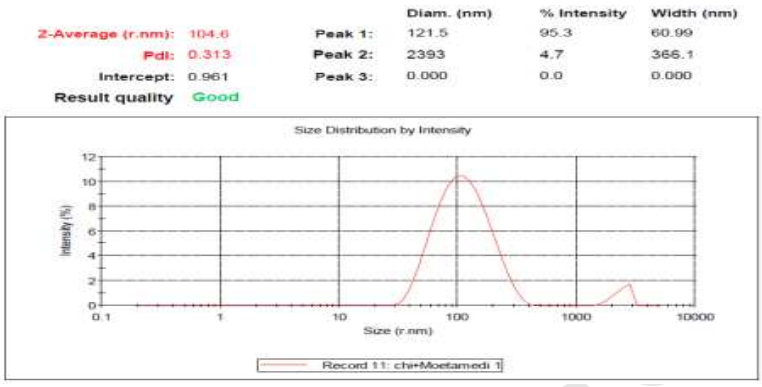
Figures1



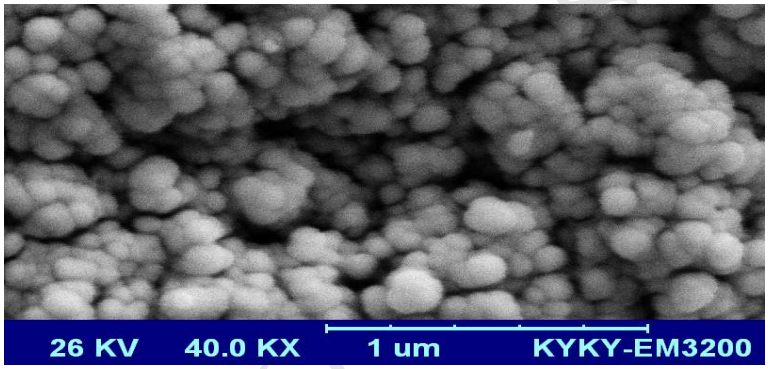
A B C D

Figures 2

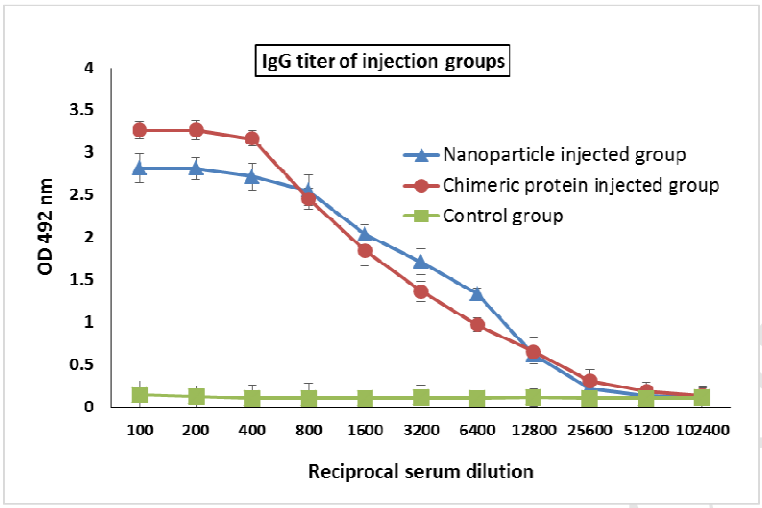
A


Z-Average (r.nm): 104.6
PdI: 0.313
Intercept: 0.961
Result quality Good
Diam. (nm) % Intensity Width (nm)
Peak 1: 121.5 95.3 60.99
Peak 2: 2393 4.7 366.1
Peak 3: 0.000 0.0 0.000
Size Distribution by Intensity
Intensity (%)
Size (r.nm)
Record 11: chi+Moetamedi 1

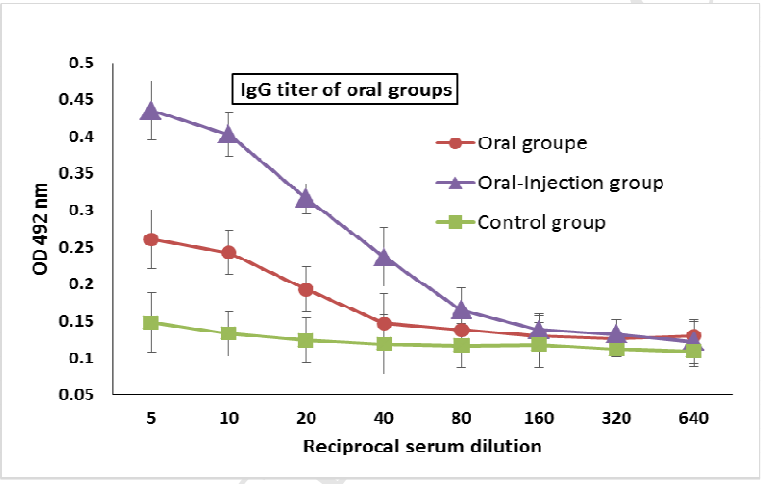
B


26 KV
40.0 KX
1 um
KYKY-EM3200

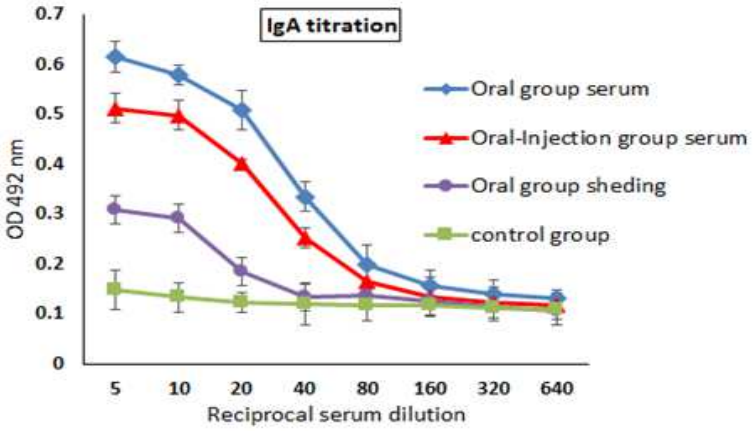
Figures 3



A

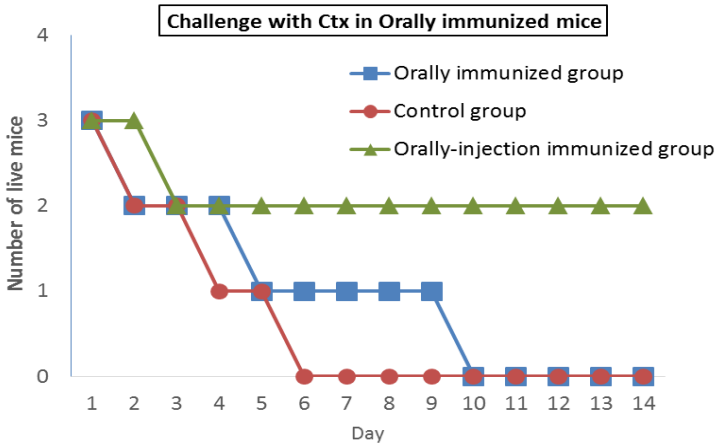


B

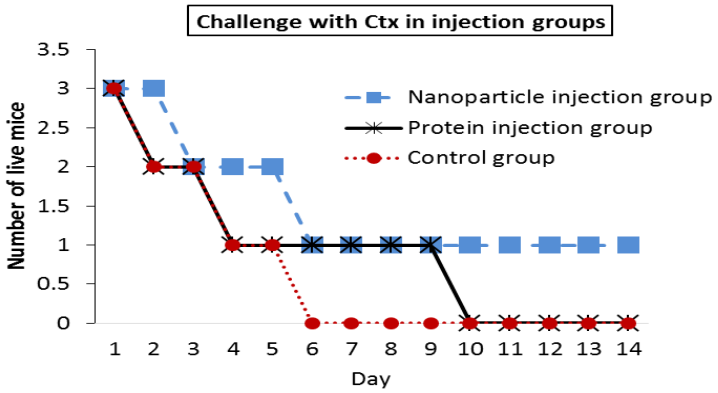


C

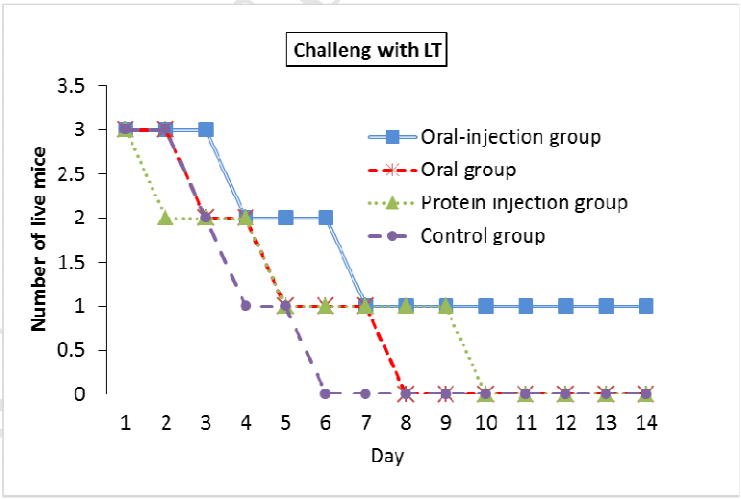
Figures 4



A

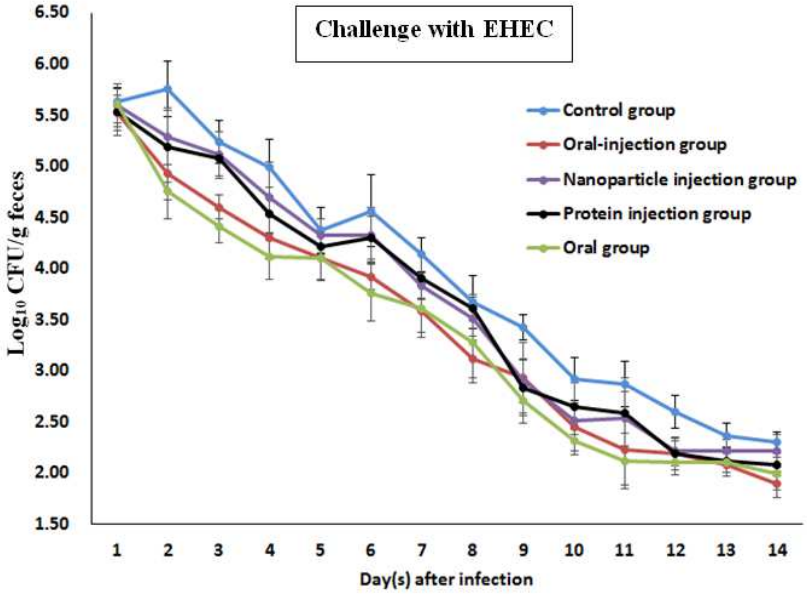


B



C

Figures 5



- *Vibrio cholerae*, ETEC, and EHEC are the most important bacteria causing diarrhea.
- This study investigated immunogenicity of a chitosan encapsulated chimeric LSC composed of LtB, StxB and CtxB subunits.
- LSC nanoparticles can be used in vaccines to prevent diarrhea caused by these three bacteria