Original Article

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Immunogenicity Evaluation of PLGA Nanoparticles Contains Recombinant CfaB Protein from Enterotoxigenic *Escherichia Coli*

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ABSTRACT

BACKGROUND AND OBJECTIVE: Enterotoxigenic *Escherichia coli* (ETEC) are the most common causes of diarrhea among children. Colonization factors (CFs) are the major ETEC candidate vaccines. Considering the high antibiotics resistance reports, construction of effective immunogen against bacteria is major goal. Encapsulation of recombinant immunogen in nano carriers such as polymeric PLGA nanparticles, not only protects them from degradation in environmental elements but also provides the effective concentrations of immunogen in targets, hence increasing bioactivity. The aim of this study was to investigate the immunogenic properties of CfaB protein encapsulated in PLGA nanoparticles.

METHODS: In this research study, *cfaB* gene codon optimaztion was performed by OPTIMIZER software. Expression of recombinant protein in *E.coli* was induced with IPTG. The recombinant protein was purified using affinity chromatography and evaluated by Western Blotting analysis. PLGA nanoparticles containing recombinant protein were prepared by double emulsion method and their structures were characterized by SEM and DLS. 40 BALB/C mice in four group were immunized with nanoparticles and antibody titers were determined by ELISA. The efficiency of antibodies in preventing the attachment of ETEC bacteria to the Caco 2 cells was examined.

FINDINGS: The optimized gene had a Codon adaptation index (CAI) of 0.85. Expression of recombinant protein led to the production of CfaB with molecular weight of 18.9 kDa. Yield of protein was 5mg/L. Encapsulation efficiency of recombinant protein in PLGA nanoparticles was 85%. The average particle size was 170 nm. Immunization of mice induced serum antibody response. Immunized sera decreased %62.8 of ETEC adhesion to Caco2 cells.

CONCLUSION: Recombinant CfaB protein encapsulated in PLGA nanoparticles stimulate immunity against ETEC.

KEY WORDS: *PLGA, nanoparticle, ETEC, CfaB, Immunization*.

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Introduction

E. coli enterotoxigenic bacteria is one of the most important bacteria that causes digestive diseases and the prevalence of diarrhea due to it, is high especially in developing countries as well as in those who travel to these areas (1, 2). The bacteria is connected to the intestinal epithelium through its colonizing factors and colonization occurs at the level of the small intestine cells (3, 4). After bacterial binding and colonization, the bacterial enterotoxins affect the epithelial cells of that region (5, 6). One of the most important pathogensis factors in the enterotoxicogenic E. coli bacterium is the production of one or more of the colonization factors (CFs) and is considered as a target for the vaccine (7, 8). The CFA/I binding factor is the first CF and the most common colonization factor in ETEC strains, especially in indigenous South American regions and Asia (9, 10). The CfaB protein, as the main subunit of CFA / I, contains a signal peptide of 23 amino acids and an adult protein of 147 amino acids that plays a key role in the bacterial binding to the intestinal epithelial cells (1). Since some recombinant immunogens, including bacterial colonization factors, do not stimulate the immune system alone, so the use of adjuvants and carriers can improve immune function. The loading of recombinant immunogens including CfaB in nanoparticles, such as PLGA, has been considered as a mechanism for enhancing immunity (11, 12). Studies in the use of PLGA for the production of antibodies against CS3 and CS6 colonization factors have been successful (13, 14). Deng et al. examined the immunogenicity of LTB-ST protein in nanocapsule form (15). Nazarian and colleagues loaded fusion protein with binding and toxin factors in PLGA nanoparticles (16). Given that subunit proteins require the use of adjuvants, and considering the adjuvant function of carriers, the objective of this study is to evaluate the immunity against binding of ETEC bacteria to the CfaB protein in PLGA loaded in PLGA nanoparticles.

Methods

Optimization of cfaB gene: Optimization of rare codons, stability analysis of mRNA, and codon compatibility index (CAI) were performed by OPTIMIZER software and constructed by Shingene Company of China. The synthetic gene was cloned in the pET28a vector. For transformation of recombinant plasmid into susceptible cells used thermal shock method.

Expression and purification and confirmation of CFAB recombinant protein: expression of recombinant protein in host E.coliBL21-DE3 was induced under optimal conditions using IPTG (Fermentas) with a final concentration of 1 mM at 37 ° C and 5 hours. Extract of expression cells was investigated on SDS-PAGE gel. Purification of recombinant protein was carried out by affinity chromatography using denaturant method using 8-molar urea containing buffers with pH gradient in Ni-NTA (Qaigen) column. After removing urea from protein purified by urea gradient dialysis, protein concentration was measured by Bradford method.

Western blot protein confirmation: The resulting recombinant protein was evaluated using a Western Blot method using an anti-Histidine antibody conjugated to the HRP (Qaigen) enzyme. Protein was transferred to nitrocellulose paper and the protein-free regions were admixed with buffer containing 5% dry milk prepared in PBST (PBS buffer was 0.05% Tween 20). In the next step, anti-Histidine antibody was diluted 1: 10000 on an extra paper and placed at room temperature for 1 hour. Detection was performed with a rising solution containing diammonobenzidine (Sigma).

Loading of the recombinant protein in PLGA nanoparticles: For the production of nanoparticles, a dual emulsion solvent evaporation method was used (20, 22). 1 mg of protein was added to the 2 ml of aqueous phase in a solution of PLGA in methylene chloride and homogenized for 15 minutes. The water-oil emulsion was added to 30 ml of polyvinyl alcohol (2.5% w / v) and mixed by the homogenizer. 15 ml of water without ion was added to obtained double water emulsion in oil-water (W1 / O / W2), and was stirred for 1 hour. The nanoparticles were collected and rinsed with centrifuge 14000 rpm for 20 minutes. Polymer nanoparticles were used to study appearance and immunity.

Exponential Properties of Nanoparticles and Determination of the Efficiency of Loading of Protein: The apparent shape of nanoparticles prepared by scanning electron microscopy (SEM) of the LEO-1455VP model was investigated. To prepare the sample, the nanoparticles were dried on a foil and covered with gold. The imaging of nanoparticles was carried out at a voltage of 10 kV and a magnification of 10,000. The size and potential of Zeta particles were determined by dynamic light diffraction (DLS) method using the Zetasizer (Malvern) device. The loading efficiency was obtained by comparing the initial

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protein used and the amount of protein not absorbed in the polymer particles (16).

Experimental animal immunization and antibody titration by Eliza method: 40 female BALB / C mice with a mean weight of 25 g were divided into four groups of 10. 20 µg of the protein loaded in the nanoparticle was administered to each mouse in the first group and the protein with Freund's adjuvant was administered to the second group subcutaneously. The third and fourth groups were given Nanoparticles without antigene and PBS buffers, respectively. Injections were performed at intervals of two weeks and four times. To assess immunization, one week after the second, third and fourth injections, blood samples were taken from the eyes of immune and non-immune animals. Serums were isolated and used for later stages. The polyclonal antibody in the serum was evaluated by indirect ELISA method. Serial dilutions were prepared from 1.200 to 1.25600.

Examine the anti-coagulation and anti-inhibitory effects: Caco-2 trypsin cells were added to wells of culture plate containing microscopic slides and stored in the oven under conditions of 5% CO2 presure. A total of 107 isolated ETEC bacteria from the patient with separate CFA/I (17) were added to serum of the immune and non-immun mice and placed in incubator shaker for 30 minutes at room temperature. Sospanisson was added to the wells and placed an hour at room temperature on the shaker. The cells were stained with Giemsa and examined under a microscope. The percentage of inhibition of bacterial binding to the cell was calculated by comparing the binding of bacteria in the presence of immune serum and non-immune serum. Statistical analysis: To determine the normality of the data, the Kolmogroff-Irenozov test was used and repeated analysis of variance was used to determine the frequency of administration of the formulation. Duncan's test was used for comparisons between different groups and $p<0.05$ was considered significant.

Results

Optimization of cfaB gene: Codon optimization was performed on the CfaB protein encoding sequence to enhance the expression of recombinant protein. The codon adaptability index changed from 0.66 to 0.85 and the content of cytosine and guanine changed from 41 percent to 44.6 percent. The number of codons that have a good quality to increase protein expression changed from 44% to 74%. The minimum energy of mRNA for the structure before optimization was 10/117-kilocalories to 10/160 after optimization. The minimum energy of mRNA for the structure before optimization was 11/11-kilocalories reached to 160/160 after optimization.

Expression, purification and confirmation of recombinant protein: The synthetic genes in the expression vector pET28a were cloned with restriction enzymes EcoRI and HindIII (Fermentas). A 444-bp gene was confirmed by agarose gel (Fig. 1). Induction of protein expression was performed with IPTG. In the induced samples, the protein with a molecular weight of about 18.9 kDa was observed (Fig. 2). The recombinant protein had a high purity in the column output from the release buffer E (Fig. 2). The accuracy of produced recombinant protein was evaluated by western blotting was checked. The anti-histidine antibody detected and reacted with the six amino acids of the histidine recombinant amino acid sequence (Fig. 2).

Preparation of PLGA nanoparticles and estimation of protein loading efficiency: PLGA nanoparticles were prepared by dual emulsion method. The image of the electron microscope shows the smooth and spherical surface of the nanoparticles (Fig. 3). The results of DLS showed the preparation of PLGA nanoparticles with an average size of 170 nm (Fig. 3). The zeta potential of the sample was reached after loading of the recombinant protein to -5.87 (Fig. 3). The dispersion index of nanoparticles was 0.147. The protein loading efficiency in polymer nanoparticles was 85%.

Anti-CfaB antibody titration and the effect of antiserum on inhibition of bacterial binding: Animal immunization was performed by injecting protein loaded into nanoparticles and free protein with adjuvant. After the first, second and third injections, antibody titers were significantly increased in the antigen receiving groups, which was not seen in the control group (Figures 1 and 2). Antibody titers associated with adjuvant antigen (Fig. 1) showed a non-significant increase compared to the antibody titre of the antigen loaded in the nanoparticles (Fig. 2). There was no significant difference in the increase of antibody titres of fourth injection compared to the third injection (Figures 1 and 2). The analysis of variance of the antigen administration formulation and the frequency of administration with repeated measurements was performed and statistically was

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significant for the factors assessed in this study ($p < 0.05$). The prevention of ETEC binding to Caco-2 cells test was done. The results of counting the number of bundled bacteria showed that the antibody produced could reduce the binding of bacteria to Caco-2 cells. On average, the percentage of preventing bacterial binding to the antibody was 62.8%.

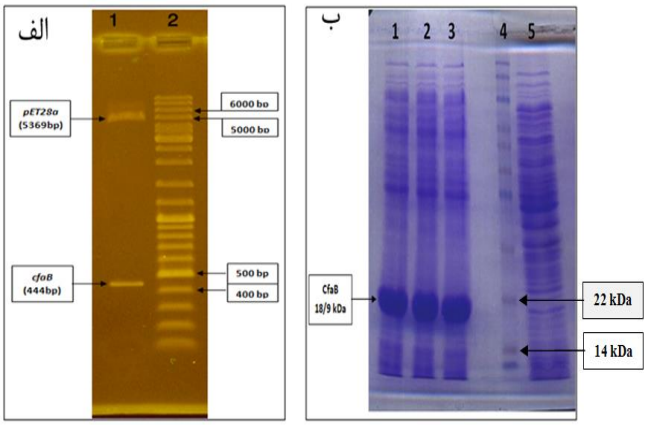

Figure1. (a) Confirmation of cloning of cfaB gene in pET28a plasmid by enzymatic digestion. Column 1: A recombinant plasmid was cut with restriction enzymes. Column 2: The DNA ladder mix size marker. (B) Expression of protein on SDS-PAGE gel 12%. Columns 1, 2, 3: IPTG-Induced Cell, Column 4: Vivantis PR0602 Protein Marker, Column 5: Induced Cells

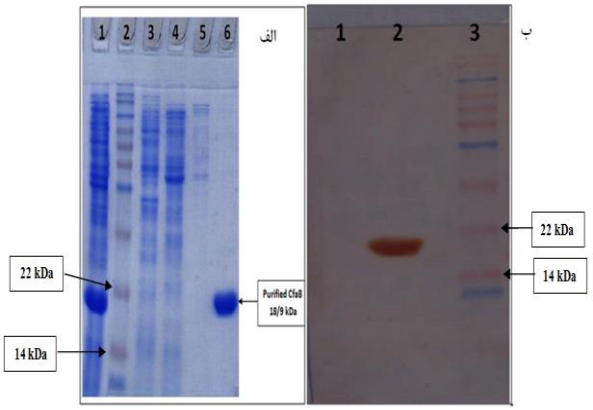

Figure2. (a) Purification of recombinant protein with NI-NTA affinity column. Column 1: Sample before column, Column 2: Vivantis PR0602 protein marker, Column 3, 4, 5: Output sample from the column after washing with buffer C, D and E, purification Protein with Imidazole 250mM. (B) Western blotting protein confirmation. Column 1: BSA Protein, Column 2: CfaB Protein, Column 3: Protein marker

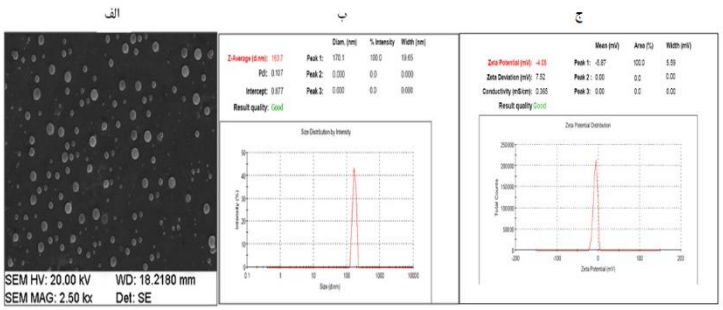

Figure3. (a): SEM image of the PLGA nanoparticles, (b): Evaluation of the distribution of the polymer particles produced size, (c): Zeta potential of the polymer nanoparticles having the protein

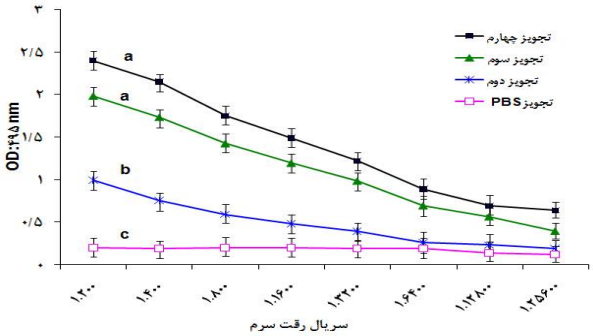

chart1. Antibody titre produced in mice after the administration of recombinant protein with adjuvant. Non-identical alphabets represent a significant difference

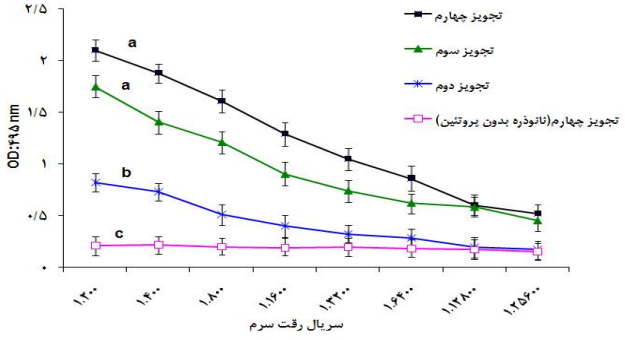

Chart2. Antibody titers produced in mice after administration of recombinant protein-containing

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nanoparticles. Non-identical alphabets represent a significant difference

Discussion

The results of the study showed that PLGA polymerase can be performed with gradual release as adjuvant and triggers an immune response against the fusion factor of ETEC. In this research, codon optimization was performed after sequencing. CAI increased to 0.87, and the absence of node structures or loops at the starting point of mRNA '5 caused the expression of the CFAB recombinant protein. Today, many studies have been carried out on the use of nanoparticles as carriers of immunogens. The PLGA polymer nanoparticles have been very much considered due to its good compatibility biodegradability, the possibility of targeted release and the protection of the compounds in the body (11, 18). Studies on the use of PLGA for the production of antibodies against ETC colonization factors CS3 and CS6 have been successful (14, 13). Deng et al. and Nazarian et al., in separate studies, investigated the protein fusion of ETEC as nanocapsules for immunization (15, 16). In various studies, PLGA polymer with ratios of 25: 75, 50, 50 and 75:25 is used from lactic acid to glycolic acid (19, 20). In this study, PLGA was used with 50:50 ratio to help appropriate elimination of polymer and to release sufficient amount of antigen to stimulate the immune system. In this study, polyvinyl alcohol was used as surfactant. Menon et al. showed that with increasing surfactant concentration up to 5%, smaller nanoparticles can be prepared (21). Since high concentrations of PVA surfactant can cause toxicity, in this study, instead of high concentrations, the concentration of 2.5% of the surfactant was used. The average particle size of the polymer produced in this study was 171 nm. The particle size containing the antigen is a determining factor in its absorption and bioavailability. Alexis et al showed that PLGA microparticles are not well absorbed in comparison with nanoparticles in the mouse body and do not stimulate the immune system properly (22). The results of this study also showed that PLGA nanoparticles can cause animal immunity in mice. In a similar study, Byrd et al. showed that reducing the size of the PLGA particles results in an appropriate immune response against the ETEC binding factors (14). The zeta potential of nanoparticles was also negative. The surface charge of particles is effective in removing them from the body. In a study by Alexis et al., Positive nanoparticles were eliminated more quickly than nanoparticles with negative superficial load (22). Accordingly, in the present study, PLGA was used to produce polymer nanoparticles that have a longer shelf life in the animal because of the negative zeta potential. Considering the immunogenicity results in this study it seems that PLGA polymer has been able to act as an adjuvant with the gradual release of antigen and its presentation to the immune system. In a similar study, Byrd et al. demonstrated the effect of the PLGA adjuvant on immunity against the CS3 factor of ETEC (13). However, Lapa et al. showed that nanoparticles containing CS6-binding factors did not show high immune responses and require the use of adjuvants (23). One of the reasons for this difference can be related to the difference in the nature of the antigen of various proteins. Antibody neutralization activity in the present study showed that the natural structure of the CFA / I colonization factor identified on the surface of the ETEC bacterium was detected by the antibody and prevented from binding to the cell receptor. This finding suggests that the structural sub-unit of the CFA / I colonization factor was loaded in PLGA nanoparticles without any changes in its epitopes. The results confirm further the key role of anti-CfaB antibodies in protecting against ETEC and preventing diarrhea (2, 3). The results of this study indicate that the recombinant CFAB protein loaded in PLGA polymer can be considered as an immunogen against infection and diarrhea caused by ETEC.

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