

Synthesis, characterization and immunological properties of *Escherichia coli* O157:H7 lipopolysaccharide- diphtheria toxoid conjugate vaccine

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ABSTRACT

Background and Objective: *Escherichia coli* O157:H7, an emerging pathogen, causes severe enteritis and the extraintestinal complication of hemolytic-uremic syndrome. The goal of this study was to evaluate the conjugate of *E. coli* O157: H7 lipopolysaccharide (LPS) with diphtheria toxoid (DT) as a candidate vaccine in mice model.

Material and Methods: LPS from *E. coli* O157:H7 was extracted by hot phenol method and then detoxified. Purified LPS was coupled to DT with adipic acid dihydrazide (ADH) as a spacer and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as a linker. The coupling molar ratio of LPS to DT was 3:1. Clinical evaluation of *E. coli* O157:H7 LPS-DT conjugate was also performed.

Results: The conjugate was devoid of endotoxin activity and indicated 0.125 U/ml of D-LPS. Mice immunization with D-LPS DT conjugate elicited fourfold higher IgG antibody in comparison to D-LPS. Also, in vivo protection of mice with conjugate provided high protection against the LD₅₀ of *E. coli* O157:H7, which indicated a good correlation with the IgG titer.

Conclusion: Our results showed that the suggested vaccine composed of *E. coli* O157:H7 LPS and DT had a significant potential to protect against *E. coli* infections.

Keywords: *Escherichia coli* O157:H7, Conjugate vaccine, Lipopolysaccharide (LPS), Diphtheria toxoid (DT).

INTRODUCTION

Escherichia coli O157:H7 is an emerging infectious agent that causes a spectrum of diseases including

severe hemorrhagic enteritis, hemolytic-uremic syndrome (HUS), diarrhea and dysentery (1). Although, it has been nearly 30 years since *E. coli* O157:H7 was discovered as an important pathogen and despite the increase in the rate of severe disease associated with the serotype, no effective treatment yet exists. Treatment of infection is difficult because antibiotics do not change the course of the enteritis caused by *E. coli* O157:H7 and may increase the incidence of HUS (2). Currently, infections are not treated with antibiotics as it has been shown to increase HUS

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development, as well as lead to the increased release of shiga-like toxins. A variety of treatment and prevention strategies to protect against *E. coli* O157:H7 are in development; those include toxin receptor analogs, passive antibody therapy, and vaccines (3, 4). There is increasing evidence that serum antibodies to the surface polysaccharides such as lipopolysaccharide (LPS) of *E. coli* O157:H7 may confer protective immunity to this enteric pathogen (5). O-specific polysaccharide (O-SP) conjugate vaccines for *E. coli* O157:H7 infections have been designed to elicit anti-LPS serum immunoglobulin G (IgG) (6). A phase 1 clinical trial of *E. coli* O157:H7 O-SP coupled to *Pseudomonas aeruginosa* recombinant exoprotein A (rEPA) showed the statistically significant increases in levels of anti-LPS IgG (7).

The conjugation of poly-or oligosaccharide to highly immunogenic protein carrier has the advantage of stimulating the T cell dependent responses that protect infants and young children (8). In this regard, a conjugate vaccine based on the LPS and carrier proteins such as *P. aeruginosa* rEPA (7) and nontoxic B subunit of Stx1 (Stx1B) (8) has been reported for *E. coli* O157:H7. The aim of this study was to prepare and investigate the immunological properties of a conjugate vaccine composed of *E. coli* O157:H7 LPS and diphtheria toxoid (DT) as a carrier. The immunogenicity and bactericidal activity of the conjugate was examined as well.

MATERIALS AND METHODS

Materials. *E. coli* O157:H7 was provided by the Biological Research Center, Islamic Azad University, Zanjan Branch, Iran. It was cultured in nutrient broth medium in shaker incubator at 37°C for 72 h (9). DT was obtained as a gift from the Research and Production Complex of Pasteur Institute of Iran, Karaj, Iran.

LPS purification. LPS from *E. coli* O157:H7 was purified by hot phenol method with several modifications. The bacterial suspension was heated at 66°C for 20 min and 90% phenol was added. The resulting mixture was stirred at 66°C for 30 min, rapidly cooled by stirring on ice and then centrifuged at 4000 g at 4°C for 45 min. The aqueous phase (phenol layer) was collected, 95% cold ethanol was added and centrifuged at 4000 g at 4°C

for 45 min. 10% TCA was added, and stirred at 4°C for 30 min and centrifuged as before. The phenol layer was dialyzed extensively against water at 4°C for 24 h with three changes of water. LPS was precipitated with 100% cold ethanol, lyophilized and stored at 4°C. Purified LPS was analyzed by sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE). The protein content was measured according to the method of Bradford using BSA as a standard. The nucleic acid content was also estimated by the UV absorbance at 260 nm (10, 11).

Detoxification and analysis of LPS. LPS was detoxified by the alkaline method. LPS pellet was dissolved in 0.2 N NaOH and heated at 100°C for 2 h. The mixture was adjusted to pH 7.0 with 1 N HCl, and dialyzed extensively against water at 4°C for 2 days. Detoxified LPS (D-LPS) was mixed with cold ethanol, placed overnight at 4°C and centrifuged at 4000 g at 4°C for 45 min (12). The endotoxin level in D-LPS was assayed by the *Limulus polyphemus* amoebocyte lysate (LAL) test. Pyrogenicity test was also performed in three rabbits. Rectal temperatures were measured with indwelling rectal thermostats and recorded ever 15 min for 1 h before injection and ever 15 min for 3 h after injection (13).

Conjugation of D-LPS with DT. D-LPS was covalently coupled to DT as a carrier protein via amidation method with adipic acid dihydrazide (ADH) as a spacer molecule and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as a linker. NaCNBH₄ (0.1 g) and acetonitrile (0.5 ml) were added to D-LPS and the reaction mixture was stirred at 4°C. After 10 min, an equal volume of 0.5 N ADH in 0.5 N NaHCO₃ was added, and the pH was adjusted to 8.5. The reaction was carried out at 4°C for 18 h and then dialyzed against water at 4°C for 24 h with three changes of water. After that, 4 ml DT containing 10 mg/ml protein and 0.1 N EDAC was added to the conjugation reaction, and then stirred on ice for 4 h. The reaction mixture was dialyzed against distilled water at 4°C for 2 days with six changes of the outer fluid. The D-LPS DT conjugate product was purified by gel filtration using CL-2B Sepharose column (1.5 90 cm). Fractions with the maximum absorbance for both protein and polysaccharide were pooled, dialyzed and then lyophilized under aseptic conditions (14).

Analyze of D-LPS DT conjugate. The protein content of D-LPS DT conjugate was estimated according to the method of Bradford using BSA as a standard. Carbohydrate content of conjugate was also measured by the phenol-sulfuric acid method with glucose as a standard (15). Pyrogenicity test for conjugate was performed as described above. The lethal effect of D-LPS–DT conjugate vaccine was evaluated in five mice. One human dose of conjugate (10 µg/ml) was administered intraperitoneally to each mice. Mice were monitored for weight change and mortality for 7 days post-challenge (16).

Immunization. The immunogenicity of the conjugate vaccine was evaluated in female BALB/c mice. 60 female BALB/c mice aged 6-8 weeks were immunized intraperitoneally in four groups of fifteen on days 0, 14 and 28 with either 10 µg of D-LPS, DT, D-LPS DT conjugate. Normal saline was also used as control treatment. Antibody response level against D-LPS, DT, and D-LPS DT were measured by enzyme linked immunosorbent assay (ELISA) method. DT sample solution (2 µg/ml in 0.05 M carbonate buffer, pH 9.6) was coated into plate and incubated at 4°C for overnight. After washing, blocking buffer was added and incubated at room temperature for 1 h. Mouse sera were diluted in PBS (1:10) and incubated at room temperature for 2 h. Horseradish peroxidase conjugated goat anti-mouse IgG (diluted 1:3000 in 1% BSA-PBST) was added, and incubated at room temperature for 1 h. PBS containing 0.05% Tween 20 (PBST) was used for washing (3X) between steps. O-Phenylenediamine dihydrochloride and H₂O₂ were used as substrate. The reaction was stopped by addition of 50 µl H₂SO₄ and the absorbance at 450 nm was measured (4, 17).

Vaccination of mice with D-LPS–DT conjugate. The lethal dose (LD) of *E. coli* O157:H7 strain was measured in order to estimate the infecting inocula for in vivo protection test. The bacterial cell was cultivated in nutrient broth medium for 10 h with shaking. The pelleted cells were resuspended in sterile PBS and measured by spectrophotometer at 600 nm. Colony forming units (CFU) were estimated using spread plate method after a serial dilution of bacterial suspension (10⁸–10¹⁰ CFU/ml PBS), then three female mice groups (six mice per group, weighing 25 ± 2 g each) were challenged i.p. with the prepared bacterial suspension (1 ml). Control group received ster-

ile saline. The mice were daily monitored for mortality for 7 days. For in vivo protection study, three inbred BALB/c mice groups (five mice each group, 6–8 weeks old) were selected. Groups A and B were immunized (on days 0, 7 and 14) with 5 mg D-LPS DT conjugate and D-LPS, respectively. Group C contained unimmunized control mice. Ten days after the last injection, mice were challenged i.p. with 3 × 10⁸ CFU (4 × LD₅₀) of the homologous strain of *E. coli* O157:H7 suspended in sterile PBS, pH 7.4. The mice were daily monitored for mortality for 7 days (18).

Statistical analysis. Antibodies sera titers are expressed as a geometric mean. Comparison of geometric means was performed with the One-Way ANOVA test (tukey) by P<0.01 (16).

RESULTS

Characterization of *E. coli* O157:H7 LPS. The results of SDS-PAGE electrophoresis showed the purified *E. coli* O157:H7 LPS as a single band (Fig. 1). The obtained LPS contained 1 mg/ml protein, 1 µg/ml nucleic acid and 0.125 EU/ml, which was acceptable for immunization. The rabbit thermal induction test showed no excess in body temperature.

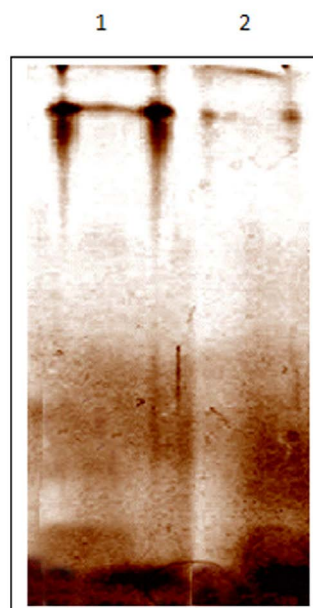


Fig. 1. Silver nitrate-stained SDS-PAGE of *E. coli* O157:H7 LPS. Lane 1: 10 µg of extracted LPS. Lane 2: 5 µg of extracted LPS.

Characterization of the conjugate vaccine. As seen in Fig. 2, positive fractions (fractions 72 to 74) for polysaccharide and protein appeared as a single peak, indicating the D-LPS conjugation to DT. The molar ratio of LPS to protein in was calculated to be 3:1. The protein and carbohydrate contents of the purified D-LPS DT were 1 mg/ml, and 0.4 mg/ml, respectively.

Pyrogenicity, toxicity and sterility of D-LPS DT conjugate. D-LPS DT conjugate was non-pyrogenic and evoked $<0.5^{\circ}\text{C}$ increase in body temperature of each rabbit after 24 h. There were no overt signs of toxicity after intraperitoneal administration of the conjugate vaccine to mice. No evidence of microbial

growth was found. Sterility test also showed that the resulting conjugate was sterile.

Immunogenicity studies. Table 1 shows anti-LPS antibody titers in the immunized mice. As observed, two weeks after the first injection, there was no significant difference between the antibody levels in groups that were immunized by D-LPS and D-LPS DT ($P<0.01$). Additionally, neither DT, nor saline elicited antibodies. However, D-LPS DT conjugate elicited high levels of IgG and IgM LPS antibodies following the second injection and booster responses after the third injection ($P<0.01$). The control groups indicated the lowest titers of LPS antibodies.

Fig. 2. Gel filtration profile of D-LPS DT conjugate through a CL-2B Sepharose column.

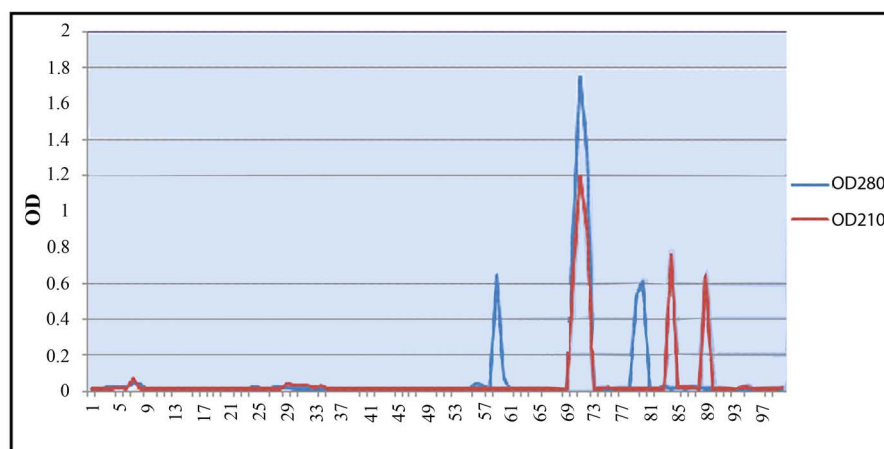


Table 1. Antibody response of mice immunized with D-LPS DT, D-LPS, DT and normal saline as control group.

Immunogen	IgM			IgG		
	Day 14	Day 28	Day 42	Day 14	Day 28	Day 42
D-LPS DT	110.0	270.0	838.8	392.0*	1220*	1899*
D-LPS	110.0*	200.0*	235.6*	100.8*	418*	431*
DT	0	0	0	0	0	0
Negative Control	0	0	0	0	0	0

* The mean difference is significant at the 0.01 level.

Protective capacity of the conjugate vaccine. The dose that induced 50% mortality (LD_{50}) for *E. coli* O157:H7 in mice was 3.4×10^8 CFU/g body weight. In vivo protective capacity of the conjugate was performed by challenging the immunized mice with ten times of the determined LD_{50} . This challenge dose killed 0/5 of mice immunized with conjugate and 1/5 of mice immunized with D-LPS alone. No protection was observed in the control group.

DISCUSSION

Polysaccharides of bacteria are a source of the most highly conserved protective epitopes. One of the challenges in vaccine development based on the polysaccharides is to develop a better anti-carbohydrate immune response. Conjugation of polysaccharides to proteins has provided a solution to some of the limitations found for these compounds (16). The covalent binding of carbohydrate to antigenic proteins offers an attractive method for increasing the immunogenicity and also stimulating a longer-lived IgG response (19). Conjugate vaccines are composed of carrier proteins being covalently linked to the purified polysaccharides. The carrier proteins that have already been used to vaccinate humans are including tetanus toxoid (TT), mutant DT CRM197, and bovine serum albumin (BSA). Since infants receive diphtheria and tetanus toxoids in their DT-TT-pertussis (DTP) vaccinations, conjugation of these proteins with LPS avoids introducing the new protein antigens in the immunization (20).

Treatment of *E. coli* O157:H7 infection with antibiotics does not change the course of enteritis and may increase the incidence of HUS. For this reason, efforts for treatment of *E. coli* O157:H7 infections have been directed toward prevention. There is increasing evidence that serum antibodies to the surface polysaccharides such as LPS of *E. coli* O157:H7, *Salmonella typhimurium*, *Vibrio cholera* and *Shigella*, confer protective immunity to these enteric pathogens. In this regard, conjugate vaccines based on the O-specific polysaccharide (O-SP) of *E. coli* O157:H7 and carriers including BAS and B subunit of Shiga toxin 1 (Stx1) has also been reported (7, 8). In this work, we used DT as a carrier protein to construct a conjugate vaccine because DT is readily available and a part of the pediatric immunizations, which is done within the frame of the Expanded Programme of Im-

munization of the WHO and Unicef. The DT has been shown to enhance immunogenicity of polysaccharide vaccines when used as the carrier protein in conjugate vaccine (21). The D-LPS was conjugated to DT via the amidation method. The spacing and density of the saccharide are likely to have major impacts on the ability of conjugate to induce an immune response. The conjugation molar ratio of LPS to DT was 3:1. After second and third doses of vaccination, total IgG titers prepared from the immunized mice sera with D-LPS DT showed significant rise in comparison to D-LPS. In fact, in the absence of DT, there was no significant enhancement of immunogenicity with D-LPS. Effective protection of DT has been observed using LPS-DT conjugate derived from *Leptospira interrogans* (19). Meanwhile, in vivo protection of mice with the conjugate indicated its protective ability. This result was in a good correlation with the elicited IgG titer in the serum. Similar results have been found in conjugates of *E. coli* O157:H7 O-SP- with *P. aeruginosa* recombinant exoprotein A (rEPA) (7), and nontoxic B subunit of Stx1 (Stx1B) (8). Therefore, it can be interpreted that conjugation of *E. coli* O157 LPS may be considered for making hyperimmune therapeutic sera. Collectively, conjugation of LPS from *E. coli* O157:H7 to DT appears to be promising for production of an LPS-based conjugate vaccine. Our results showed that the constructed LPS-DT conjugate vaccine had considerable potential to protect against *E. coli* infections.

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