Immunogenicity comparison of conjugate vaccines composed of alginate and lipopolysaccharide of *Pseudomonas aeruginosa* bound to diphtheria toxoid

Faezeh Najafzadeh1, Ghazaleh Jaberi2, Reza Shapouri1, Mehdi Rahnema3, Ashraf Karimlinik2, Anvarsadat Kianmehr4*

1Department of Microbiology, Zanjan Branch, Islamic Azad University, Zanjan, Iran.
2Department of Microbiology, Science and Research Branch, Islamic Azad University, Kerman, Iran.
3Biologic Research Center, Zanjan Branch, Islamic Azad University, Zanjan, Iran.
4Medical Biotechnology Department, School of Advanced Medical Sciences, Tabriz University
Of Medical Sciences, Tabriz, Iran

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**ABSTRACT**

**Background and Objectives:** Treatment of *Pseudomonas aeruginosa* infections is greatly hampered by innate and acquired antibiotic resistance. The goal of this study was to compare the immunogenicity of conjugates of *P. aeruginosa* depolymerized alginate-diphtheria toxoid (D-ALGDT) and *P. aeruginosa* detoxified lipopolysaccharide-diphtheria toxoid (D-LPSDT) in mouse model.

**Materials and Methods:** Alginate and LPS were purified from *P. aeruginosa* strain PAO1. The resulting depolymerized alginate (D-ALG) and detoxified LPS (D-LPS) were covalently coupled to diphtheria toxoid (DT) as a carrier protein with adipic acid dihydrazide (ADH) as a spacer molecule and carbodiimide as a linker. Sterility, safety and pyrogenicity tests were performed. 30 mice in two groups were immunized intraperitoneally on days 0, 14 and 28 with 10 µg of D-ALGDT and D-LPSDT. Conjugates specific antibody levels were also determined by enzyme-linked immunosorbent assay (ELISA).

**Results:** The conjugates were non-toxic and non-pyrogenic. Conjugates of D-ALGDT and D-LPSDT were shown to be safe and to elicit total IgG, IgM, IgA, IgG1, IgG2a, IgG2b and IgG3 antibodies in mice. ELISA results indicated that antibodies titer of D-ALGDT was more than D-LPSDT.

**Conclusion:** Immunization with D-ALGDT showed significant increase in all types of antibodies titers in versus D-LPSDT, suggesting D-ALGDT as a vaccine candidate against *P. aeruginosa* infections.

**Keywords:** *Pseudomonas aeruginosa*, lipopolysaccharide (LPS), alginate (ALG), conjugate vaccine, diphtheria toxoid (DT)

**INTRODUCTION**

*Pseudomonas aeruginosa*, a ubiquitous environmental Gram-negative microorganism, is one of the most important opportunist bacteria in hospital-acquired infections and causes a wide variety of serious infections in individuals with thermal burn, mechanical extensive trauma, cancer, cystic fibrosis and surgical site infections (1-3). Despite considerable advances in antimicrobial therapy, effective treatment
and control of *P. aeruginosa* infections remains a persistent problem, primarily because of the natural resistance of the organism and its remarkable ability to acquire resistance to multiple antimicrobial agents by various mechanisms. As an alternative strategy to prevent *P. aeruginosa* infections in susceptible populations, effective immunotherapies or vaccines against *P. aeruginosa* have long been sought for (4).

Several *P. aeruginosa* antigens are used for vaccine development including LPS alone, polysaccharides alginate, extracellular proteins, exotoxin A, and whole killed cell (5). Alginate and LPS are the major surface components and the immunity confers protection (6, 7).

Alginate (ALG) is a mucoid exopolysaccharide produced by *P. aeruginosa*. Alginate, like LPS, functions as an adhesin, anchoring *P. aeruginosa* to the colonized respiratory epithelium (7). Because of the association between mucoid *P. aeruginosa* and the pathogenesis of these infections in cystic fibrosis patients, interest was generated in using *Pseudomonas* alginate as immunogen to prevent and treat *P. aeruginosa* infections in this patient population (8). Immunization with alginate antigen gives rise to antibodies that have opsonic activity and lead to clearance of mucoid *P. aeruginosa* from the respiratory tract in mice and rats (9). Active immunization against alginate is in the process of being optimized, particularly through conjugation with exotoxin A, in order to compensate for the variable efficacy of antibodies produced in response to alginate (7). LPS is the major surface antigen of *P. aeruginosa* playing an important role in the interaction with the host immune system, as well as it is responsible for determining the various serotypes of *P. aeruginosa* (10).

This immunogenicity makes them obvious targets for immunotherapy. However, the active immunization elicited by O-antigen based vaccines is lacking in protectiveness. To circumvent this problem, multiple serotype conjugates can be further conjugated with another target such as exotoxin A (7). LPS based conjugate vaccine has been evaluated by many investigators. In general, the conjugate vaccine appears to be recommended for immunization over other immunogens because of its safety and its potential to elicit high quantities of protective antibodies against O-PS antigen, which confer protective immunity against the pathogen (10). In this study, we compared immunogenicity of conjugates composed of D-ALG-DT and D-LPS-DT as *P. aeruginosa* vaccine candidates.

## MATERIALS AND METHODS

### Strain and growth conditions.

*P. aeruginosa* strain PAO1 was obtained from the Biologic Research Center, Zanjan Branch, Islamic Azad University, Zanjan, Iran. It was grown on Mueller-Hinton agar at 37°C for 24 h (11).

### Purification of alginate.

The mucoid bacteria were cultured on selective medium containing glycerol, dextrose, L-glutamine, Na₂HPO₄, K₂HPO₄ and MgSO₄.7H₂O and incubated at 37°C for 72 h. The bacterial cells were removed by centrifugation at 4000 × g at 4°C for 30 min. Alginate was extracted by repeated ethanol precipitation, dialysis and enzymatic digestion. Crude alginate was precipitated from the supernatant by the addition of cold absolute ethanol to a final concentration of 80% (v/v). The precipitate was collected by centrifugation at 4000 g for 30 min. Crude alginate was re-dissolved at 2 mg/ml in PBS, pH 7.5, supplemented with 0.5% sodium dodecyl sulfate (SDS) and 10 mM CaCl₂. Proteinase K was added (100 μg/ml) for 2 h with incubation at 56°C and then kept at 4°C overnight. DNase and RNase were added (each at 100 μg/ml) for 3 h with incubation at 37°C and centrifuged. Alginate was precipitated by the addition of cold absolute ethanol to a final concentration of 80% (v/v). Following centrifugation, the pellet was collected, sterile filtered, and freeze-dried (9, 12).

### Purification of LPS.

After the growth of bacteria, *P. aeruginosa* colonies were cultured on nutrient broth in shaker incubator at 37°C for 72 h (13). The biomass was centrifuged at 4000 × g at 4°C for 30 min, sedimented bacteria were collected and used for LPS extraction and purification (14). Extraction of LPS was carried by optimized hot phenol method. The bacterial suspension (20 g cellular wet weight in 68 ml distilled water) was heated at 66°C for 20 min, mixed with 90% ethanol and placed at 4°C overnight. DNase and RNase were added (each at 100 μg/ml) for 3 h with incubation at 37°C and centrifuged. Alginate was precipitated by the addition of cold absolute ethanol to a final concentration of 80% (v/v). Following centrifugation, the pellet was collected, sterile filtered, and freeze-dried (9, 12).
After centrifugation, trichloroacetic acid (TCA) (1 g per 10 ml of solution) was added and stirred at 4°C for 30 min. Following centrifugation, the supernatant was dialyzed against distilled water at 4°C until the phenol was completely eliminated and LPS was concentrated by alcohol precipitation. The pellet was then collected by centrifugation at 4000 × g for 45 min, and lyophilized (15-17).

Analysis of alginate and LPS. LPS was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver nitrate staining (18). The protein content of alginate and LPS was measured by the method of Bradford, using bovine serum albumin (BSA) as a standard (19). The nucleic acid content of alginate and LPS was measured by the UV absorbance at 260 nm (12).

Depolymerization of alginate and detoxification of LPS. Alginate was depolymerized by controlled heating in dilute acid. Alginate was depolymerized in 1% (vol/vol) acetic acid and heated at 121°C for 15 min. After cooling, the solution was extensively dialyzed against distilled water at 4°C for 1 day with three changes of distilled water and lyophilized (20). LPS was detoxified by the method of alkaline. Pellet of LPS was dissolved in NaOH to a final concentration of 0.2 N and the mixture was heated at 100°C for 2 h. After cooling on ice, the mixture was adjusted to pH 7 with 1 M HCl. LPS was dialyzed against distilled water at 4°C for 2 days with six changes of the outer fluid and centrifuged at low speed to remove a small amount of precipitate. The conjugate was passed through a Sepharose CL-2B column (1.5 by 90 cm) in 0.2 M NaCl, the void volume peak was collected. Similarly, D-LPS-DT conjugate prepared with ADH as the spacer and EDAC as the linker. The conjugates (D-ALG-DT and D-LPS-DT) were stored at 4°C (23, 25, 26).

Analyses of D-ALG-DT and D-LPS-DT. The protein content of D-ALG-DT and D-LPS-DT was measured by the method of Bradford, using BSA as a standard. The carbohydrate content of conjugates was determined by the method of phenol-sulfuric acid, using glucose as a standard (19). Pyrogenicity testing was performed in rabbits as previously described. The ability of the toxic effect of D-ALG-DT and D-LPS-DT for mice (5 per group) was evaluated by the intraperitoneal injection of graded doses of conjugates. Each mouse received 10 µg/ml from conjugate. Mice were observed daily for 7 days post-injection (9). Sterility testing was performed (27).

Immunization. For evaluation of immunogenicity, 6-8 weeks old female BALB/c mice from the Pasteur Institute of Iran, Karaj, Iran (15 per group) were injected intraperitoneally three times at 2-week intervals with 10 µg of immunogens of the D-ALG-DT or the D-LPS-DT. A control group received one
injection of 10 µg of normal saline. Five mice were randomly chosen from each group and exsanguinated 2 weeks after each injection, and sera were pooled, sterile filtered and stored at -20°C (28, 29).

**ELISA test.** Conjugates specific antibody levels in sera were determined using an ELISA for total IgG by indirect ELISA and IgM, IgA, IgG1, IgG2a, IgG2b and IgG3 by antigen mediated ELISA. Each well of the plates was coated with 100 µl of either D-ALG-DT or D-LPS-DT at a concentration of 2 µg/ml in 0.05 M carbonate buffer, pH 9.6 and kept overnight at 4°C. Plates were washed three times with wash buffer (PBS containing 0.05% Tween 20). After a washing, plates were blocked with blocking buffer for 1 h at room temperature. Plates were washed three times with wash buffer. Sera samples were diluted in PBS (1:10), assayed in triplicate and incubated for 2 h at room temperature. Plates were washed three times with wash buffer. Goat anti-mouse antibody was diluted in PBS (1:1000), added, incubated for 1 h at room temperature and plates were washed three times with wash buffer. Goat anti-mouse antibody was diluted in PBS (1:1000), added, incubated for 1 h at room temperature and plates were washed three times with wash buffer (this step was only in antigen mediated ELISA). Horseradish peroxidase conjugated to goat anti-mouse IgG was diluted in PBS (1:3000), added. After 1 h at room temperature, plates were washed. O-Phenylenediamine dihydrochloride and H2 O2 were added as substrate. After 15 min of incubation in the dark, the reaction was stopped with 50 µl H2 SO4 and the absorbance was measured at 450 nm (9, 18, 27-31).

**Statistical analysis.** Comparison of geometric means was performed with the One-Way ANOVA test (Tukey’s test). Values of P<0.01 were considered to be significant. The Statistical Analysis System was used for all data analysis. Statistics were performed with SPSS version 16.

**RESULTS**

**Characterization of D-ALG and D-LPS.** Fig. 1 shows the electrophoretic patterns of the LPS bands of *P. aeruginosa* on an SDS-PAGE. Various characteristics of D-ALG and D-LPS were also shown in Table 1. As can be found from the Table 1, the endotoxin activity assay showed 0.125 EU/ml of D-ALG and D-LPS that were acceptable to use for immunization. D-ALG and D-LPS were non-pyrogenic when tested at a dose of 50 µg/kg and evoked <0.5°C increase in temperature after 24 h.

**Characterization of D-ALG-DT and D-LPS-DT.** D-ALG-DT and D-LPS-DT were isolated by gel filtration, monitoring the elution profile by the UV absorbance at both 210 nm (for the presence of ALG and LPS) and 280 nm (for the presence of DT) and refractive index. Fractions with the maximum absorbance for both 210 and 280 nm (Fig. 2, fractions 72 to 77 and Fig. 3, fractions 72 to 74) were indicated the formation of D-ALG-DT and D-LPS-DT conjugate molecules. Various characteristics of conjugate vaccines are shown in Table 2. The

| Table 1. Characteristics of D-ALG and D-LPS |
|-----------------------------------------------|
| Composition | Protein (mg/g) | Nucleic acid (µg/g) | Endotoxicity (EU/ml) | Pyrogenicity (µg/kg) |
|-------------|----------------|---------------------|---------------------|---------------------|
| D-ALG       | 1.5             | 1.4                 | 0.125               | 50                  |
| D-LPS       | 1               | 1.1                 | 0.125               | 50                  |

* When administered intravenously to rabbits, 50 µg of D-ALG and D-LPS per kg body weight evoked <0.5°C increase in temperature.

| Table 2. Characteristics of D-ALG-DT and D-LPS-DT Conjugates |
|---------------------------------------------------------------|
| Composition | Protein (mg/g) | Carbohydrate (mg/g) | Pyrogenicity (µg/kg) | Toxicity (µg/ml) |
|-------------|----------------|---------------------|---------------------|------------------|
| D-ALG-DT    | 1.2             | 0.5                 | 50                  | 10               |
| D-LPS-DT    | 1               | 0.4                 | 50                  | 10               |

* When administered intravenously to rabbits, 50 µg of vaccines per kg body weight evoked <0.5°C increase in temperature.
# When administered intraperitoneally to mice, 10 µg/ml of vaccines were not observed decrease in weight and mortality.
conjugates were non-pyrogenic when tested at a dose of 50 μg/kg and evoked <0.5°C increase in temperature after 24 h. The conjugates were non-toxic upon intraperitoneal administration to mice. No overt signs of illness and decrease in weight were observed and all mice survived. Mediums were observed after incubation at 37°C for 24 to 48 h. No signs of microorganism growth were observed. Sterility testing showed that the resulting conjugates were sterile. The above results, demonstrating the safety and stability of the conjugate vaccines, led us to evaluate its acceptability and immunogenicity in animals.

**Comparison of Immunogenicity of D-ALG-DT and D-LPS-DT conjugates.** The immunogenicity of the D-LPS-DT and D-ALG-DT conjugate vaccines were analyzed by immunization in mice. Figs. 4, 5 and 6 shows antibody titers in the immunized mice. As shown in these figures, D-ALG-DT displayed higher titers in the IgG and IgM antibodies than D-LPS-DT (P<0.01). The control groups also indicated the lowest antibody titers.

**DISCUSSION**

*P. aeruginosa* has been considered to be a difficult target for antimicrobial chemotherapy (33). Different approaches have been tested to protect patients with *P. aeruginosa* infections including passive immunization with monoclonal and polyclonal antibody. In this respect, the conjugate vaccine composed of bacterial antigens and carrier protein is a well-established method because of its safety and its potential to elicit high quantities of protective antibodies (9). Anti-LPS antibody has been shown to be highly protective against *P. aeruginosa* infections (6). In this study, the LPS and ALG from *P. aeruginosa* PAO-1 was investigated for the conjugate vaccine preparation. Conjugation of bacterial antigens to carrier proteins has been applied to increase their immunogenicity and create effective vaccines (7). Some proteins such as tetanus toxoid (TT) and bovine serum albumin (BSA) have been used as carrier proteins to conjugation with O-polysaccharide (O-PS) from *P. aeruginosa* (18). However, in the case of ALG, because of its large molecular mass, conjugating it to carrier proteins to produce immunogenic vaccines has proven to be difficult. Here, we used DT as the carrier because...
DT is readily available and a part of the pediatric immunizations, which is done within the frame of the Expanded Programme of Immunization of the WHO and UNICEF (17). The DT has been shown to enhance immunogenicity of vaccines when used as the carrier in conjugate vaccine. The detoxified D-LPS and D-ALG were conjugated to DT via the amidation method using EDAC as a linker and ADH as a spacer molecule. The molar ratio of LPS and ALG to DT conjugation was 3:1. Total IgG titers prepared from the immunized mice sera with D-ALG-DT vaccine showed significant rise in comparison to D-LPS-DT. Similar finding has been observed using D-ALG with exotoxin A conjugate derived from \textit{P. aeruginosa} (9). Briefly, these results showed that the conjugate vaccine based on LPS from \textit{P. aeruginosa} and diphtheria toxoid raised LPS antibodies. Briefly, our results showed that the conjugate vaccine based on D-ALG from \textit{P. aeruginosa} and DT raised more antibodies than the D-LPS-DT. In conclusion, our data indicated that the D-ALG-DT can be used as a potential vaccine candidate against \textit{P. aeruginosa} infections.

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