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Immunological Properties of Exotoxin A Toxoid — Detoxified Lipopolysaccharide — Gold Nanoparticles Conjugate Against *Pseudomonas aeruginosa* Infection

Faezeh Najafzadeh[1], Asghar Tanomand[2*], Azam Haddadi[1], Jafar Majidi[3]

[1]Department of Microbiology, Karaj Branch, Islamic Azad University, Karaj, [2]Departement of Microbiology, Maragheh University of Medical Sciences, Maragheh, [3]Department of Immunology and Immunology Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Background: *Pseudomonas aeruginosa* is eudomonas aeruginosa is an important opportunistic pathogen, especially in patients with compromised host defense.

Objective: To prepare the conjugate of detoxified lipopolysaccharide (D-LPS) and exotoxin A toxoid (T-ETA) from *P. aeruginosa* in gold nanoparticles (Au NPs) in a mice model.

Methods: LPS and ETA were purified from *P. aeruginosa* PAO1. D-LPS was conjugated with T-ETA via the amidation method. Au NPs were bound to D-LPS-T-ETA conjugate via electrostatic interaction. Mice were immunized with D-LPS, D-LPS-Au NPs, T-ETA, T-ETA-Au NPs, D-LPS-T-ETA, D-LPS-T-ETA-Au NPs, D-LPS-Au NPs+T-ETA-Au NPs, Au NPs, and phosphate-buffered saline (PBS), and specific IgG titers were determined by the ELISA and the whole-cell ELISA methods. Mice in the vaccinated and control groups were exposed to a $2\times LD_{50}$ of *P. aeruginosa* and mortality rates were recorded for one week.

Results: The results showed that vaccination by D-LPS, D-LPS-Au NPs, T-ETA, T-ETA-Au NPs, D-LPS-T-ETA, D-LPS-T-ETA-Au NPs and D-LPS-Au NPs+T-ETA-Au NPs induced specific IgG. Mice received the D-LPS-T-ETA-Au NPs conjugate showed significant protection against bacterial challenge.

Conclusion: These data indicate that D-LPS-T-ETA-Au NPs conjugate has a significant immunogenicity potential to be applied as a new vaccine against pseudomonas infections.

Keywords: Exotoxin A (ETA), Gold nanoparticles (Au NPs), Lipopolysaccharide (LPS), *Pseudomonas aeruginosa*, Vaccine candidate

*Corresponding author:
Asghar Tanomand,
Departement of Microbiology,
Maragheh University of Medical Sciences, P.O. Box: 551677-3111, Maragheh, Iran
Tel/Fax:+98 41 37256900
Email: tanomanda@tbzmed.ac.ir

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INTRODUCTION

P. aeruginosa is a Gram-negative opportunistic pathogen (1). Effective therapy and control of pseudomonal infections stay a continuous issue, mainly due to the natural resistance of *P. aeruginosa* and its notable capability to acquire resistance to many antimicrobial factors. As an alternative method to prevent pseudomonal infections in sensitive individuals, useful vaccines against *P. aeruginosa* have long been searched for (2).

ETA and LPS are the toxic factors of *P. aeruginosa*, which are strongly associated with the fatality of *P. aeruginosa* septicemia (3). Also, high levels of anti-LPS and anti-ETA antibodies have been related to higher survival rates following *P. aeruginosa* septicaemia (4).

LPS is the main surface antigen of *P. aeruginosa*, which is an important agent in pathogenesis, it also affects the host's responses to infection including the innate and the acquired ones (5, 6). LPS as a thymus-independent antigen induces an immune reaction without the interference of T lymphocytes. To overcome the problem, conjugation of polysaccharides to a carrier protein converts the thymus-independent polysaccharides antigen into a thymus-dependent antigen (7).

ETA is a single polypeptide chain compounded of three structural and functional domains: I is a receptor-binding domain, II is a transfer domain, and III is a toxic domain (8, 9). ETA conduces to the ADP-ribosylation of eukaryotic elongation factor 2 lead to prohibition of protein syntheses in the affected cell (10).

Researches about nanoparticles' application of vaccine delivery have shown a high capacity for rising vaccine efficiency, both by encapsulating antigens to modify their constancy or by increasing antigen presentation by arranging antigens onto the nanoparticle surface (11, 12).

In the research, we study the provision and assessment of detoxified lipopolysaccharide-exotoxin A toxoid-gold nanoparticles (D-LPS-T-ETA-Au NPs) conjugate as a vaccine to pseudomonal infections.

MATERIALS AND METHODS

Preparation of D-LPS and T-ETA

LPS from *P. aeruginosa* PAO1 was purified by using hot phenol procedure. The bacterial suspension was heated at 66 °C and then was combined with 90% phenol. This resulting combination was shaken at 66 °C, quickly cooled, and then centrifuged. Appeared aqueous layer was picked up, and then cold ethanol was combined and centrifuged. Finally, trichloroacetic acid (TCA) was mixed and centrifuged, and then dialyzed against deionized water; LPS was precipitated afterward. For the detoxification of LPS, the pellet containing LPS was solved in 0.2 N NaOH and stirred for 2 h at 100 °C. The sample was cooled and adjusted to pH 7. The next step was the dialysis, and D-LPS was precipitated (13, 14). ETA was extracted from the culture supernatant of *P. aeruginosa* PAO1. Strain PAO1 was cultured in trypticase soy broth (TSB) medium containing 1/20 volume of 1 M monosodium glutamate and 1% glycerol (v/v). Samples were shaken at 32 °C in an incubator shaker and then centrifuged. For the concentration of ETA, 0.3 M sodium citrate was added to a 1/10 volume of the supernatant fluid and then dialyzed against 0.01 M Tris buffer and finally centrifuged. The supernatant fluid contained ETA that was precipitated by adding ammonium sulfate. After the centrifugation, the precipitate was solved in 20 ml of 0.01 M Tris and then dialyzed. 10 ml of the sample was loaded into a column of DE-52 resin (25×2 ^{m}, Pharmacia), and elutes were collected in 50 ml fractions (15). To prepare the toxoid form of ETA, the purified ETA was incubated with PBS consisting of 0.01 M sodium phosphate, 0.15 M sodium chloride, and 4% formaldehyde at 37 °C for 4 consecutive days (16). Molecular weight analysis and purity of LPS and ETA were assessed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE). The endotoxin content of D-LPS and T-ETA was evaluated by Limulus amebocyte lysate (LAL) (13, 17, 18).

Preparation of D-LPS–T-ETA Conjugate

50 mg D-LPS in 5 ml of water (pH 10.5) was reacted with cyanogen bromide (0.1 g) and acetonitrile (0.5 ml). After that, it was combined with 5 ml of 0.5 M adipic acid dihydrazide (ADH) in 0.5 M NaHCO3 and its pH was continuously balanced to 8.5. Subsequently, the sample was incubated at 4 °C overnight and next dialyzed. ADH-derivatized D-LPS (20 mg) was mixed with the T-ETA (20 mg). After cooling, the pH was adjusted to 5.8. 0.1 N 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was mixed with this sample and rapidly cooled by shaking with ice and then dialyzed (13, 17, 18).

D-LPS–T-ETA conjugate was obtained by the fractionation of the purified sample by the utilization of CL-2B Sepharose column (1.5×90 cm). Void-volume fractions which were evaluated positive for both polysaccharide and protein were determined D-LPS–T-ETA and were pooled, dialyzed, and then were stored at -70 ºC (13, 17, 18). Zeta potential was used to specify the electrical properties of D-LPS–T-ETA (19). The amounts of protein in D-LPS–T-ETA were assessed according to the Bradford's method. The Phenol-sulfuric acid test was used to determine the amount of conjugate carbohydrate (9, 13, 17, 18).

Preparation of Au NPs Conjugates

Au NPs were provided by the reduction of chloroauric acid ($HAuCl_4$) with $NaBH_4$. Briefly, $NaBH_4$ was added to the $HAuCl_4$ solution and when the solution changed from colorless to dark pink, the stirring was stopped and the reaction mixture was kept overnight. The amine surface in dark pink solution was stirred for 12 h and the solution became colorless. HCL-KCl buffer was added to a gold surface. The enzyme solution was added to the buffer and the sample was stirred for 3 h and then eluted by HCL-KCl buffer (20, 21). The negatively charged T-ETA, D-LPS, and D-LPS-T-ETA are absorbed on the positively charged Au NPs by electrostatic interaction (22).

The Au NPs' size was acquired by the transmission electron microscopy (TEM) while Fourier transform infrared spectroscopy (FTIR) was carried out to assess different chemical groups (20, 23). Energy-dispersive X-ray spectrometry (EDS) was used to describe elemental combinations of samples (24).

Toxicity Test

For the toxicity assay of antigens in mice, 50, 100, 200, and 300 µg of each sample were subcutaneously injected into the groups which included 5 female BALB/c mice. Mice were monitored for 7 days post-challenge (13, 17).

Mice Immunization

Female BALB/c mice were bought from the Pasteur Institute of Iran. These animal procedures were performed with strict care, following ethical guidelines and international protocols (25). In addition, this research has the ethics code for work on animals: IR.IAU.K.REC.1396.036. The mice were distributed into 9 groups, every group consisting of 7 mice. The mice were injected subcutaneously with 20 µg of D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs, Au NPs , and 200 µl PBS on days 0 (by adding complete Freund's adjuvant), 14, 28 and 42 (by adding incomplete Freund's adjuvant). One week after the last injection, blood was obtained from the orbital sinus and gathered (17).

Enzyme-linked Immunosorbent Assay

Indirect enzyme-linked immunosorbent assays (ELISAs) were done as follows. Briefly, microplates were coated with D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs and Au NPs with a concentration of 5 µg/mL in 0.1 mol/L Na_2CO_3 buffer (100 µL per well) and

kept for 1 h at 37 °C. Blocking was performed with 2% BSA in PBS consisting of 0.05% Tween 20 (PBS-T) overnight at 4 °C. Among incubation phases, microplates were eluted 3 times with PBS-T. Serums diluted 1:100 in 1% BSA in PBS-T were kept for 1 h at 37 °C, then Horseradish Peroxidase-conjugated onto anti-mouse antibody (abcam) was added as the secondary antibody. The wells were coated with 3, 3′, 5, 5′ tetramethylbenzidine. In the last stage, 1 mol/L H_2SO_4 was added then absorbance at 450 nm was read (13, 26).

Whole-cell ELISAs were performed as previously described but in this method, microtiter plates were coated with *P. aeruginosa* strain PAO1 (13, 26).

Opsonophagocytosis Assay

P. aeruginosa strain PAO1 was cultured on Luria–Bertani broth and incubated overnight at 37 °C. The bacteria were eluted with PBS (pH 7.4), and suspended in PBS (10^6 bacteria/mL). Opsonophagocytosis assay was carried out by using 100 µL heat-inactivated mice sera collected from immunized and the control groups, 100 µL mouse macrophages (1×10^6 macrophages/ml), 100 µL infant rabbit serum, and 100 µL bacteria (10^6 CFU/mL). In the negative controls mice sera, macrophages, or complement was eliminated. Samples were incubated at 37 °C for 90 min and 10 µl of it was deleted, then cultured for bacterial counts. The percent kill was accounted by the following formula (9, 17, 27):

Percent kill=[1-(CFU of immune serum at 90 min/CFU of preimmune serum at 90 min)]×100

Protection Assay

P. aeruginosa was cultured in a TSB medium and incubated overnight at 37 °C. Cells were collected by centrifugation and eluted with PBS. The cell suspension was measured by the spread plate technique after a serial dilution in PBS. To determine the lethal dose 50 (LD_{50}) of bacterial infection, the mice were divided into 5 groups, each of which consisting of 6 mice. Each mouse received 0.1 ml of the bacterial suspension dilution (2.5×10^7, 5×10^7, 7.5×10^7, 1×10^8 and 12.5×10^8 CFU/ 0.1 ml per mouse) using the intraperitoneal route. The control group received PBS. The mice were monitored for ten days after infection, the fatality was checked, and afterward LD_{50} was calculated based on the Reed and Muench method (28). To protection assay in the immunized group mice, fourteen days after the last injection, the mice which received the D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs, Au NPs , and the control group were challenged intraperitoneally with 7.5×10^7 CFU of *P. aeruginosa* PAO1. Mice mortality was assessed daily for 10 days.

Statistical Analysis

For statistical analyzes in the study SPSS software (Version 22.0) was utilized. Standard deviation in the ELISA absorbance was compared by the T-student test. $P\leq0.05$ was considered significant. Survival percentage was used to survival assess (17).

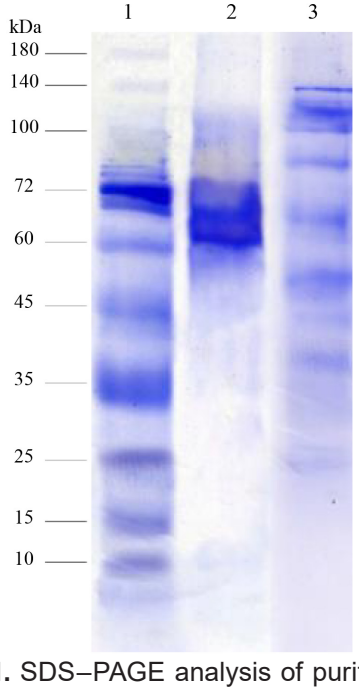

Figure 1. SDS–PAGE analysis of purified LPS and ETA of *P. aeruginosa*. Column: 1, standard protein size marker (SMOBio); 2, extracted ETA (66 KDa); 3, purified LPS. SDS-PAGE of the extracted LPS indicates a progressive ladder-like template of bands on the gel. The purified ETA has appeared as a protein with 66 KDa molecular weight in SDS-PAGE.

RESULTS

Preparation of D-LPS and T-ETA

SDS-PAGE analysis of *P. aeruginosa* LPS and ETA is shown in Figure 1. The result of the LAL method demonstrated that D-LPS and T-ETA were allowed for biological use. No pieces of evidence of toxicity were observed after the subcutaneous injection of the D-LPS and T-ETA to mice.

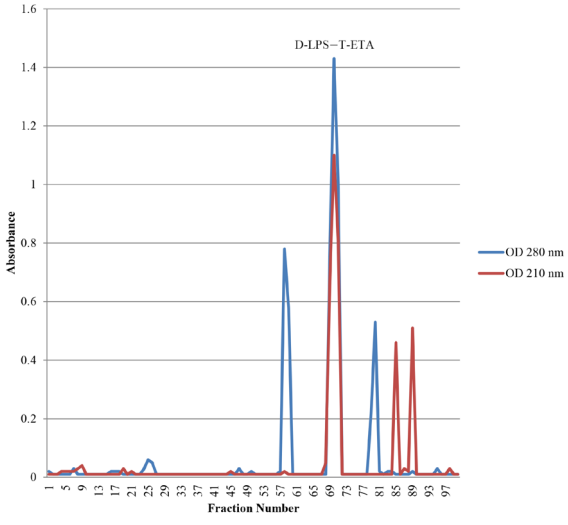

Figure 2. Gel filtration diagram of the D-LPS–T-ETA via a CL-2B Sepharose column. Fractions were assayed for LPS and ETA at 210 and 280 nm, respectively. Fractions 69 to 71 for protein and polysaccharides have become visible as a peak that shows the conjugation of D-LPS to T-ETA.

Preparation of D-LPS–T-ETA conjugate

According to Figure 2, fractions 69 to 71 for both LPS and ETA appeared as a peak that showed the conjugation of D-LPS to T-ETA. The zeta potential value of D-LPS–T-ETA was measured at 9.1 mV (Figure 3). The carbohydrate and protein amounts of D-LPS-T-ETA were 0.4 µg/ml and 0.5 mg/ml. D-LPS-T-ETA was non-toxic when injected with different doses. None of the tested mice died during the observation period.

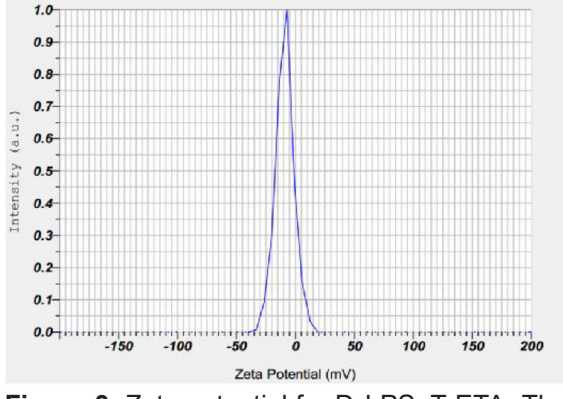

Figure 3. Zeta potential for D-LPS–T-ETA. The zeta potential value of D-LPS–T-ETA is 9.1 mV.

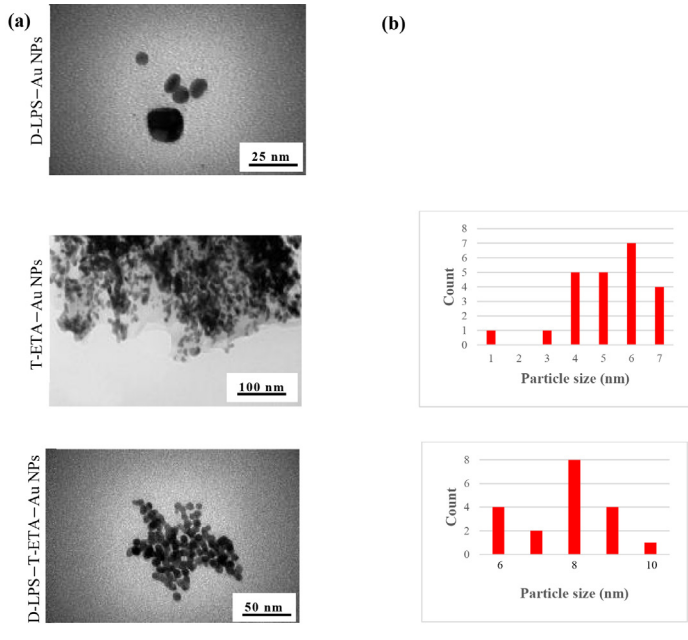

Figure 4. TEM images and size dispensation diagrams of Au NPs. TEM images of D-LPS–Au NPs, T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs (a). Scale bar: 25 nm in D-LPS–Au NPs, 100 nm in T-ETA–Au NPs, and 50 nm in D-LPS–T-ETA–Au NPs. Size distribution diagrams of T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs (b). In (a), the average size of Au NPs in D-LPS–Au NPs, T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs conjugates is 14 nm, 5 nm, and 8 nm, respectively.

Preparation of Au NPs Conjugates

The success of the conjugation based on the size and the charge of the nanoparticle was confirmed. The size of Au NPs was examined by TEM (Figure 4). The mean size of Au NPs in D-LPS–Au NPs, T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs conjugates was 14 nm, 5 nm, and 8 nm, respectively. The presence of antigenic functional groups in the structure of the NP and the formation of a P=O bond was confirmed with FTIR results and the corresponding courier form (Figure 5). Figures 5 (a) and (b) show FTIR spectra of D-LPS–Au NPs and T-ETA–Au NPs conjugates. In Figure 5 (a), peaks in the areas of 1120–1000 cm^{-1} and 1760-1710 cm^{-1} show the formation of P=O and C=O bonds, respectively, thus the areas indicate conjugation of Au NPs onto D-LPS. The FTIR spectrum extension of conjugate into areas 840-710 cm^{-1} and 1641 cm^{-1} is due to the presence of Au NPs (Figure 5 (a)). In Figure 5 (b), peak spreading in the FTIR spectrum of T-ETA–Au NPs in the 1280 cm^{-1} area represents the presence of P=O bond and these spectra supported conjugation of Au NPs onto T-ETA. Peaks in FTIR spectrum of T-ETA-Au NPs conjugate in regions 599 cm^{-1}, 1631 cm^{-1}, 2925 cm^{-1} and 3421 cm^{-1} are associated to Au NPs (Figure 5 (b)). Nanoconjugates were investigated using EDS and supported the existence of Au NPs (Figure 6). Au NPs conjugates were non-toxic when tested at various doses.

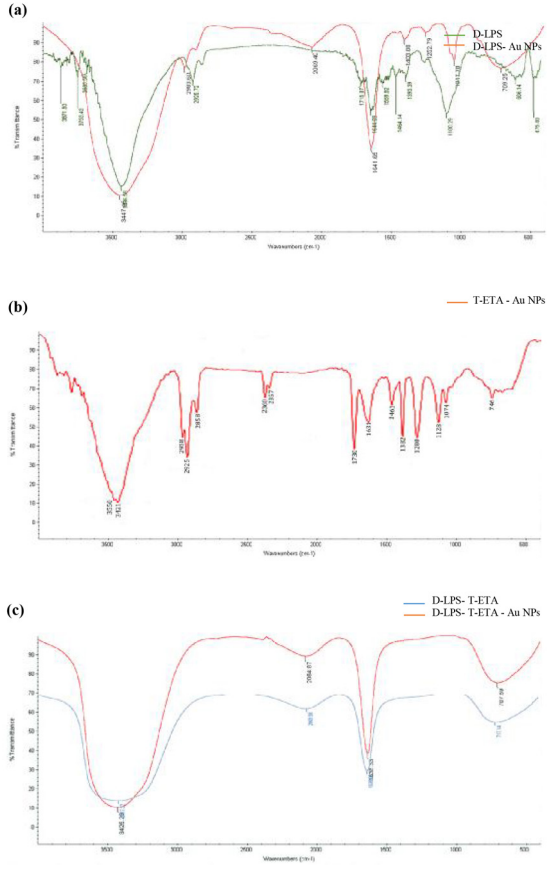

Figure 5. FTIR spectra of D-LPS–Au NPs, T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs. FTIR spectra of D-LPS–Au NPs (a), T-ETA–Au NPs (b), and D-LPS–T-ETA–Au NPs (c). In (a), peaks in regions of 1120–1000 cm^{-1} and 1760-1710 cm^{-1} are indicated the conjugation of Au NPs to the D-LPS. In (b), peaks in the 1280 cm^{-1} area in the FTIR spectrum of T-ETA–Au NPs are confirmed conjugation of Au NPs to the T-ETA.

ELISA

Antibody titers of vaccinated mice were assayed by the indirect ELISA. Table 1 shows the ELISA results in vaccinated and the control group mice. Antibody responses

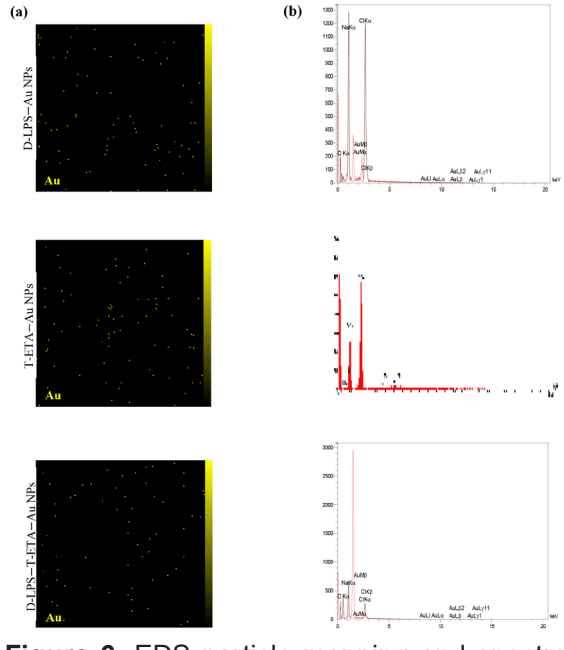

Figure 6. EDS particle mapping and spectra of Au NPs. EDS analysis results of D-LPS–Au NPs, T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs (a). EDS spectra of D-LPS–Au NPs, T-ETA–Au NPs, and D-LPS–T-ETA–Au NPs (b). In (a), EDS mapping analyses demonstrate that Au NPs were loaded as a homogeneous distribution in conjugates. In (b), the EDS spectra of conjugates indicated Au signals, an evidence of the attendance of elemental Au in samples.

Table 1. Responses of the antibody of mice which received the D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs, Au NPs and PBS at a dilution of 1:100 in sera obtained.

Immunogen	OD_{450} (Mean±SEM)	Standard deviation	P value	95% Confidence interval	
				Lower	Upper
D-LPS	1.646±0.14	0.37	≤0.05*	1.15	1.85
D-LPS–Au NPs	2.968±0.23	0.62	≤0.05*	2.24	3.40
T-ETA	2.103±0.14	0.37	≤0.05*	1.75	2.45
T-ETA–Au NPs	2.984±0.31	0.83	≤0.05*	2.22	3.75
D-LPS–T-ETA	2.334±0.12	0.33	≤0.05*	1.88	2.49
D-LPS–T-ETA–Au NPs	2.990±0.12	0.33	≤0.05*	2.54	3.14
D-LPS–Au NPs+T-ETA–Au NPs	2.902±0.29	0.76	≤0.05*	2.03	3.45
Au NPs	0.263±0.06	0.16	>0.05	0.17	0.41
PBS	0.145				

*Significant difference compared to control group (P≤0.05).

in vaccinated and the control group mice indicated that vaccination with D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, and D-LPS–Au NPs+T-ETA–Au NPs produced a significant amount of specific antibody. None of the Au NPs and PBS produced antibodies. D-LPS–T-ETA–Au NPs displayed antibody titers higher than others.

Table 2 shows the results of the whole-cell ELISAs. D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, and D-LPS–Au NPs+T-ETA–Au NPs were highly immunogenic (Table 2). The lowest antibody titers are seen in the Au NPs and the control groups. D-LPS–Au NPs+T-ETA–Au NPs displayed antibody titers higher than the others.

Opsonophagocytosis Assay

Opsonic killing levels measured against *P. aeruginosa* in sera of mice vaccinated are displayed in Figure 7. In opsonophagocytosis assay, antisera from mice which received the D-LPS–Au NPs, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs mediated phagocytic killing against *P. aeruginosa* PAO1 in contrast to the mice immunized with D-LPS, T-ETA , and Au NPs, which was less effective in mediating phagocytic killing. There were no opsonic antibodies in the control tubes.

Protection Assay

To determine the LD_{50} of bacterial infection, the mice were intraperitoneally vaccinated with 2.5×10^7, 5×10^7, 7.5×10^7,

Table 2. Antibody responses of mice which received the D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs, Au NPs , and PBS in whole-cell ELISAs at a dilution of 1:100 in sera obtained.

Immunogen	OD_{450} (Mean±SEM)	Standard deviation	P value	95% Confidence interval	
				Lower	Upper
D-LPS	1.86±0.18	0.48	≤0.05*	1.26	2.15
D-LPS–Au NPs	2.35±0.09	0.23	≤0.05*	1.98	2.42
T-ETA	1.9±0.1	0.26	≤0.05*	1.66	2.14
T-ETA–Au NPs	2.3±0.18	0.49	≤0.05*	1.85	2.77
D-LPS–T-ETA	2.42±0.14	0.37	≤0.05*	1.94	2.61
D-LPS–T-ETA–Au NPs	2.91±0.16	0.42	≤0.05*	2.38	3.15
D-LPS–Au NPs+T-ETA–Au NPs	2.92±0.13	0.36	≤0.05*	2.42	3.11
Au NPs	0.37±0.07	0.19	>0.05	0.2	0.55
PBS	0.14				

*Significant difference compared to the control group (P≤0.05).

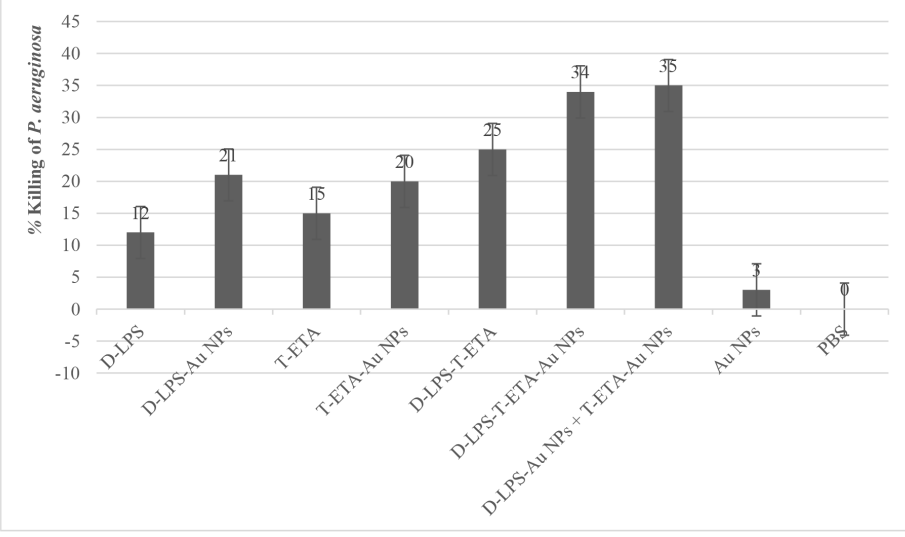

Figure 7. Phagocyte-dependent killing activity of the mice antibody to D-LPS, D-LPS–Au NPs, T-ETA, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs, Au NPs and PBS against *P. aeruginosa* PAO1. Vaccination with D-LPS–T-ETA–Au NPs, and D-LPS–Au NPs+T-ETA–Au NPs elicited a significant protective response against bacterial challenge compared to the other groups.

Table 3. Data of protection assay of vaccinated and the control group mice after bacterial challenge.

Immunogen	Frequency		Valid ratio		Live mice / total mice (survival percentage)
	Live mice	Killed mice	Live mice	Killed mice	
D-LPS	6	1	85.7	14.3	86
D-LPS–Au NPs	6	1	85.7	14.3	86
T-ETA	6	1	85.7	14.3	86
T-ETA–Au NPs	7	0	100	0	100
D-LPS–T-ETA	7	0	100	0	100
D-LPS–T-ETA–Au NPs	7	0	100	0	100
D-LPS–Au NPs+T-ETA–Au NPs	7	0	100	0	100
Au NPs	1	6	14.3	85.7	14
PBS	1	6	14.3	85.7	14

1×10^8, and 12.5×10^8 CFU of bacteria. The killed mice/injected mice per group were 0/6, 4/6, 6/6, 6/6, and 6/6, respectively. Thus, the dose which induced LD_{50} for *P. aeruginosa* in mice was 3.75×10^7 CFU, and 7.5×10^7 CFU was selected as $2\times LD_{50}$ for bacterial challenge assay in mice of vaccinated and the control group.

The data of the protection assay of vaccinated and the control group mice are displayed in Table 3. that The mice which received the T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs , and D-LPS–Au NPs+T-ETA–Au NPs conjugates indicated significant protection to challenge with 7.5×10^7 CFU of *P. aeruginosa* PAO1. The challenge dose killed 1/7 from mice which received the D-LPS, D-LPS–Au NPs, and T-ETA. There was no significant difference in the survival rates of group mice of Au NPs and the control.

DISCUSSION

Pseudomonas aeruginosa is still one of the

frequent causative agents of nosocomial illness with high fatality and incidence rates (5). The resistance of *P. aeruginosa* to various antibacterial agents and the emergence of antibiotic-resistant strains of this organism increased the demand for the production of a protective vaccine against this pathogen (18). In the study, we report the development of a conjugate vaccine consisting of both main virulence factors of *P. aeruginosa*: LPS and ETA onto Au NPs.

LPS is one of the important virulence factors in *P. aeruginosa* that affects both innate and acquired host immune responses (6). However, none of the LPS vaccines have been approved for clinical use due to their lipid's toxicity A (13). In the present research, the LPS from strain PAO1 was detoxified (14) in which the toxic lipid A moiety was cleaved. One of the problems in vaccine production with the polysaccharides is to develop a more effective anti-carbohydrate immune response. Conjugation of polysaccharides to proteins removes some of the limitations associated with these compounds (13, 14). Optimal protection against *P. aeruginosa* would be obtained by the use of a vaccine capable of eliciting both anti-LPS and anti-ETA antibodies (29). Therefore, in this study, we used ETA as a carrier protein as well as an immunogenic factor for conjugation to LPS as it was considerably more suitable for making this type of vaccine. ETA is the most toxic pathogenic factor of *P. aeruginosa*, which inhibits protein synthesis by host cells via ADP-ribosylation of elongation factor 2 (30). D-LPS was conjugated onto T-ETA with ADH as a spacer molecule and EDAC as a linker (13, 17, 18). Conjugates obtained by amidation prove to be highly stable (7).

After the fourth dose of the vaccination, the titers of IgG prepared from sera of mice which received the D-LPS–T-ETA indicated a significant rise compared to the D-LPS and T-ETA. Similar results have been found in *P. aeruginosa* conjugates of octavalent O-polysaccharide (OPS)–ETA (31), LPS–ETA (32), octavalent O-PS–ETA (4), and O polysaccharide–ETA (33). Meanwhile, immunizations with D-LPS–T-ETA conjugate showed significant protection against bacterial challenge, whereas there were partially effective survival rates of mice which received the D-LPS and T-ETA alone. The results were confirmed with the results of Abu-baker *et al*. (18). They demonstrated a high level of protection when the mice were vaccinated with O-polysaccharideрecombinant exoprotein A (O-PSrEPA) of *P. aeruginosa*.

Au NPs have been used as a carrier by conjugating the antigen from respiratory syncytial virus to its surface (12). The main notable traits of Au NPs as antigen carriers are: unlike other biological carriers, the Au NP carrier is inert; high densities of antigens can be presented on surfaces of Au NP; adjuvant activity of Au NPs are shown in peptide–Au NP conjugates; Au NPs can be produced in exact nanoparticle sizes on a large scale; they can be simply conjugated onto peptides, therefore it is a very suitable and effective method for making Au NP-based vaccines; and Au NP–Peptide conjugates have the capacity to lyophilize for making stable formulations. It has been suggested which Au NPs in the sizes of 8–17 nm induces an intense antibody immunity with a low cytotoxicity (34). So, we tried to synthesize Au NPs in this range by using the Natan method (35) and research their potential application as an antigen carrier for a conjugate vaccine of *P. aeruginosa*. Then we bound the made Au NPs to antigens and conjugate.

After the immunization of mice, total IgG titers prepared from the immunized mice sera with conjugate vaccines of Au NPs indicated a significant rise in comparison to non-conjugate vaccines of Au NPs. Similar protection has been obtained using *Yersinia pestis* F1-AuNP conjugate (36), and AuNP-FliC-LPS, AuNP-Hcp1-LPS , or AuNP-TetHc-LPS conjugates against *Burkholderia mallei* (11). We also found that the immunized mice sera with D-LPS–T-ETA–Au NPs conjugate showed a significant rise in total IgG titer compared to the other preparations. Thus,

D-LPS–T-ETA–Au NPs could be accepted as a capable vaccine candidate for eliciting both anti-LPS and anti-ETA antibodies. In the opsonophagocytic test, antisera of mice which received the D-LPS–Au NPs, T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs, D-LPS–Au NPs+T-ETA–Au NPs conjugates mediated phagocytic killing against *P. aeruginosa*, while antisera from mice which received the D-LPS and T-ETA alone were less efficient in mediating opsonic killing activity. These results corresponded with report a by Safari *et al*. (37) and indicated that the sera collected from mice that received the gold glyconanoparticles1 could be opsonized *Streptococcus pneumonia* type 14. In this research, the mice immunized with T-ETA–Au NPs, D-LPS–T-ETA, D-LPS–T-ETA–Au NPs , and D-LPS–Au NPs+T-ETA–Au NPs conjugates indicated a significant protection against challenge with 7.5×10^7 CFU of *P. aeruginosa* strain PAO1 while there was partially effective survival rates of the injected mice with D-LPS, D-LPS–Au NPs , and T-ETA. The results corresponded with the results of Gregory *et al*. (11) and demonstrated that the survival periods of the mice immunized with the AuNP-glycoconjugates lasted more than the mice vaccinated with LPS or AuNP-LPS.

In summary, this research indicated that a vaccine composed of LPS and ETA of *P. aeruginosa* and Au NPs appears to be promising for the development of a vaccine capable of evoking high levels of antibody to both ETA and LPS so that they exhibited the necessary functional attributes essential for the protection against infection.

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