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Preparation of *Pseudomonas aeruginosa* alginate-flagellin immunoconjugate

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ABSTRACT

Mucoid strains of *Pseudomonas aeruginosa* are closely associated with chronic pulmonary infections. In this report we describe a straightforward approach to conjugate high molecular weight alginate to type b-flagellin (FLB) and investigation of its bioactivity. The conjugation process was performed by using ADH and EDAC. The endotoxin was eliminated from the candidate vaccine by LPS removal resin followed by LAL test. The bioconjugate molecules were verified by simultaneously determination of polysaccharide/protein content followed by gel filtration chromatography and FTIR spectroscopy. Groups of eight BALB/c mice were injected intranasally with 5 μg (per each nostril) of purified alginate, FLB and conjugated alginate-FLB with two week intervals. The functional activity of the vaccine was evaluated by ELISA and opsonophagocytosis tests. Vaccination with the alginate-FLB conjugate induced a significant ($P = 0.0033$) rise in alginate specific IgG in mice. At all dilution ranges, the opsonic activity of the conjugate vaccine antisera was significantly higher than alginate alone (61.9% vs. 17.3% at 1:4 dilution; $P = 0.0067$). The alginate-FLB conjugate could elicit high specific antibodies titer against alginate by improving its immunogenicity. In addition, the antisera raised against conjugate vaccine act as a suitable opsonin for phagocytosis of the mucoid strains of *P. aeruginosa*.

1. Introduction

As an opportunistic human pathogen, *Pseudomonas aeruginosa* (hereafter called *P. aeruginosa*) is seldom pathogenic to people with normal immune systems [1]. However, *P. aeruginosa* can cause serious and sometimes life-threatening diseases in immunocompromised hosts, such as HIV-infected patients, organ transplant patients, and patients and those with severe burn wounds, cystic fibrosis and individual with supportive therapy [2].

Natural and adaptive resistance to wide variety of antibiotics has made *P. aeruginosa* as one of greatest therapeutic challenges. This characteristic gives rise to the difficulties in treating of infections, resulting in a desire to pursue immunotherapeutic way to combat with this persistent pathogen [3]. One of the well-known adaptive resistance mechanisms to evade the host immune system is the ability of the bacterium to form biofilms [4]. The alginate-containing biofilm produced by the mucoid strains of *P. aeruginosa* can act as an obstacle to antibiotic penetration and so, inhibit phagocytosis of bacteria, whereas specific-antibody therapy can lead to enhance the bacterial clearance with increase the opsonophagocytosis of *P. aeruginosa* from the lung of infected mice

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[5]. Alginate-specific opsonic antibodies are also commonly found in the sera of cystic fibrosis (CF) patients without detectable *P. aeruginosa* infection [6]. The antigen-antibody reaction is then mediated by inflammation, leading to infiltration of neutrophils and other inflammatory cells around the respiratory tract. Alginate production by *P. aeruginosa* is a critical problem in CF patients whose dehydrated airway mucus layer of the lung provides a suitable environment for the bacterium [7].

Another important virulence factor that contributes in the initial stage of the pathogenesis of *P. aeruginosa* is flagellin. This antigen is a prominent surface-appendage that plays an essential role in the initial establishment of the infections [8]. Flagellin, the major structural subunit of flagella, is responsible for many functions, such as attachment of bacteria to host cells, motility, and activation of the inflammatory response via Toll-like receptor 5 (TLR5) [9,10]. The fact that flagellin is one of the first antigens exposed to dendritic cells (DCs) during initial stages of bacterial infection suggests that it could play an important role in generating protective immune response [11]. Immunization with recombinant flagellin-based vaccines induced potent B and T cell responses, including antibodies responses and specific CD^{8+} T cell [9,12].

Chemical conjugation of polysaccharide antigen to a carrier protein improves its restricted immunogenicity to efficient T-cell dependent immunity [13]. In a conjugate vaccine, antigen-presenting cells (APCs) such as B cells recognize the polysaccharide and take up the conjugate and present the processed protein to T cells via the MHC class II molecules [13]. Upon MHC class II-binding peptide recognized by the T-cell receptor, T cells become activated and secrete cytokines that induce B-cell maturation and antibody isotype switching to produce high specific antibodies against the polysaccharide antigen [14]. The main objectives of the present study were to prepare an alginate-flagellin conjugate vaccine using purified high molecular weight alginate and recombinant type b-flagellin (FLB) from *P. aeruginosa* and testing the immunogenicity of the conjugate to elicit serum IgG antibodies and evaluating the cross protection by opsonophagocytic activity.

2. Materials and methods

2.1. Bacterial strains

Two mucoid strains of *P. aeruginosa* were used in this study: *P. aeruginosa* 8821 M (kindly obtained from Dr. Ali Farjah, Department of Microbiology, Shahed University of Medical Sciences) and a mucoid clinical isolate, *P. aeruginosa* E6692, which was taken from CF patient.

2.2. Preparation of recombinant type b-flagellin

The FLB was prepared as previously described [15]. Briefly, *P. aeruginosa* FLB gene (*fliC*) was designed into pET28a vector and expressed in *E. coli*, BL21 (DE3) and finally purified as histidine-tagged protein in a bacterial expression system through nickel affinity chromatography.

2.3. Extraction and purification of alginate

Alginate was obtained from *P. aeruginosa* strain 8821 M as previously described with modifications [6]. The bacteria were grown for 72 h at 37 °C in Mian's broth medium (0.1 M Sodium D-gluconate, 0.1 M L-Glutamic acid, 16.8 mM Dipotassium phosphate, 7.5 mM Monosodium phosphate, 10 mM Magnesium sulfate, pH 7.2). After this time, the repeated centrifugation (45 min at 17700 × g at 4 °C) was used to pellet the bacterial cells, and the supernatant that contains alginate was collected, filtered and maintained for at least 4 h at 4 °C. The alginate was precipitated using dropwise addition of ice cold 80% ethanol (final concentration) at 4 °C. For better precipitation of alginate, the mixture was held at 4 °C for overnight. The precipitant was collected and washed two times with 80% ethanol and one time with 96% ethanol. The precipitated alginate was collected by centrifugation at 3000 × g for 14 min, dissolved in Phosphate-buffered saline (PBS) and then dialyzing against deionized water (dH_2O) for 48 h and finally lyophilized. The lyophilized alginate (2 mg/ml) was dissolved in PBS supplemented with 1 mM $CaCl_2$ and 10 mM $MgCl_2$. DNase I and RNase A, each at 100 μg/ml (Fermentas, USA), were used to remove any possible DNA and RNA contamination. Proteinase K was added (100 μg/ml, Fermentas) to the mixture for 4 h at 56 °C, and the mixture was heated for 30 min at 80 °C to inactivate and digest the enzymes. One volume of sample:phenol:chloroform (2:1:1) was added and then incubated for 30 min at 60 °C. After centrifugation (4000 × g for 20 min at 22 °C), the upper layer was collected and mixed with equal volume of chloroform. After 5 min incubation, the tubes were centrifuged (4000 × g for 45 min at 22 °C) and then dialyzing against dH_2O for 72 h and finally lyophilized. For isolation of polysaccharide with high molecular weight, the alginate was then dissolved at 5 mg/ml in PBS and passed through a XK 16 column (1.6 × 70 cm) packed with Sephacryl S-400 gel filtration column (GE Healthcare, Life Sciences, Swaziland). The uronic acid content from the eluted fractions was examined at 595 nm, and the positive fractions that eluted just past the void volume were collected. Eventually, the fractions containing high uronic acid were pooled, dialyzed against dH_2O, and lyophilized.

2.4. Biochemical analyses

The uronic acid content in the purified alginate and the conjugate was assayed by the carbazole-borate method using seaweed alginate (Sigma, St. Louis, MO, USA) as the internal standard [16]. The protein content of the FLB, alginate, and the conjugate were determined using the Bradford assay at 595 nm. The DNA content was assayed by determining the absorbance at 260 nm using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

2.5. Alginate-FLB conjugate synthesis

The FLB (10 mg) was dissolved in 2 ml normal saline and then 100 mg ADH (adipic acid dihydrazide, Sigma) as an efficient cross-linker was added. After dissolving, 20 mg EDAC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, Sigma) as a carboxyl activating agent wad added and the pH was maintained at 6 using 0.2 M HCl. The solution was slowly mixed for 4 h at room temperature (RT) with shaking and finally dialyzed against normal saline at 4 °C to get off the excess ADH. Alginate (5 mg) was dissolved in normal saline (3 ml) and then 25 mg EDAC wad added and the pH was immediately adjusted to 4.9 with 0.1 M HCl. The mixture was slowly stirred at RT for 15 min. For conjugation, the product of these two steps was mixed, and the pH was maintained at 6.5 with 0.2 M HCl. Finally, this mixture was incubated with gentle stirring at 4 °C for 4 h. After dialyzing against normal saline, the reaction mixture was subjected to gel filtration through a Sephacryl S-400 column. The void volume fractions that were positive for protein and uronic acids were determined, assumed to be polysaccharide-protein conjugates and were pooled, dialyzed, and lyophilized. Chemical characterization of the conjugate was confirmed using a Fourier transform infrared (FTIR) spectrophotometer (Bruker, Germany).

2.6. Endotoxin (LPS) removal

Endotoxins (or LPS, lipopolysaccaride) were removed from the conjugate using ε-poly-L-lysine-agarose (Pierce High Capacity Endotoxin Removal Spin Column, 0.5 ml, #88274; Thermo Fisher Scientific, Inc., USA). Endotoxin binding to the resin occurs at pH 6-8; therefore, the resin was equilibrated with an endotoxin-free buffer containing 10–50 mM Tris-HCl buffer containing 0.1–0.2 M NaCl (pH 7). The sample was added and incubated at 4–22 °C with gentle end-over-end mixing for one hour. The tube was centrifuged (500 × g for 1 min) and finally the sample was collected at the bottom of the column. The Limulus amebocyte lysate assay (Lonza, USA) was performed according to the manufacturer's directions to detect the presence of any remaining LPS (lipopolysaccaride or endotoxin) in the samples.

2.7. Immunogenicity studies

Six-eight weeks old female BALB/c mice (8/group) (purchased from Pasteur Institute of Iran, Karaj, Iran) were immunized intranasally four times with two week intervals with 10 μg of purified alginate, 10 μg of purified FLB, 10 μg of alginate in the conjugate vaccine (alginate-FLB), and PBS (as control group). The mice were bled prior to immunization and two weeks after each immunization, and the obtained serum samples were stored at −20 °C until use.

2.8. ELISA quantification assay

Serum samples from groups of immunized mice were analyzed for antigen-specific IgG by ELISA. ELISA plates were coated overnight at 4 °C with 100 μl/well of antigens (alginate and FLB) at 10 μg/ml, diluted in coating buffer (0.5 M Bicarbonate/carbonate buffer, pH 9.6). The plates were washed three times with PBS-T (PBS containing 0.05% Tween 20) at incubation intervals. PBS-T containing 2% skim milk was used as the blocking buffer for 1 h at 37 °C. The mice sera were diluted (1:50-1:6400) in PBS-T containing 0.5% BSA (Bovine serum albumin) and incubated for 90 min at 37 °C. After washing five times, HRP-conjugated anti-mouse IgG (Sigma, USA) diluted 1:10000 was used as secondary antibody for 90 min at 37 °C. After washing six times, the chromogenic substrate Tetramethylbenzidine (TMB, Sigma, USA) was used as a substrate to develop the reaction in the dark for 30 min. The reaction was stopped with 2 N H_2SO_4 and the results were read at an optical density of 450 nm (OD_{450}) by an ELISA reader (Awareness Stat Fax 2100, USA).

2.9. Opsonophagocytic killing assay

As previously described, the opsonic activity of the antibodies raised against conjugate vaccine was evaluated by opsonophagocytosis assay [9]. Briefly, 100 μl bacterial suspension (2×10^9 CFU/ml of *P. aeruginosa* 8821 M or the clinical isolate in 1% BSA) was incubated with an equal volume of heat-inactivated (at 56 °C for 30 min) pooled and diluted mouse sera (1:4 to 1:64) at 22 °C for 60 min and then washed twice with BSA (1% (w/v)) for elimination of excessive antibodies. 100 μl of mouse macrophages 2×10^7 CFUs/ml in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μl of 10% fresh infant rabbit serum, which served as a complement source, were added to the mixture and incubated in a shaker at 37 °C for 90 min. Control tubes were run with each assay by omitting antibody, macrophages, or complement. Finally, a 100 μl aliquot was removed, diluted in PBS, and plated for bacterial enumeration. The opsonic activity of the serum was calculated as following formula:

[1 - (CFU immune serum at 90 min/CFU of pre-immune serum at 90 min)] × 100

2.10. Statistical analysis

Statistical analysis was performed using the software GraphPad Prism version 6.0 for Windows, (GraphPad Software, San Diego, CA, USA). The data were analyzed using one-way analysis of variance (ANOVA). Antibody titers of groups of mice and opsonophagocytic killing activities were expressed as Means ± standard deviations (SD). The *P* values less than 0.05 was considered to be statistically significant.

3. Results

3.1. Purified alginate characterization

We performed enzymatic digestion to eliminate contamination of DNA, RNA, and protein. To select high molecular weight alginate, gel filtration chromatography was done using Sephacryl S-400, and fractions (10-26) containing high values of uronic acid that eluted near the void volume were collected (Fig. 1). The chemical compositions of the purified alginate indicated partial contamination (<2.6% protein, and <1.16% nucleic acid). No LPS was detected above the lower limit of the assay (<0.08 endotoxin unit/ml).

3.2. Characterization of alginate-FLB conjugate

The conjugate vaccine was verified by two methods: routine assay of polysaccharide/protein contents and FTIR spectrophotometry. In order to separate the conjugated molecules from un-conjugated, the conjugation product was applied into Sephacryl S-400 column for size exclusion chromatography. After fractionation, the optical density (OD) of all fractions was measured at 525 and 595 nm for uronic acid and protein contents, respectively. The first coupled peak that contained uronic acid (with high molecular weight) and protein was found near the void volume (Fig. 2). Therefore, these fractions were presumed to contain conjugated molecules, and were used as the alginate-FLB conjugate. A second peak was determined that presumably contained un-conjugated molecules and free proteins or conjugated molecules with low molecular weight polysaccharide. The alginate-FLB conjugate was

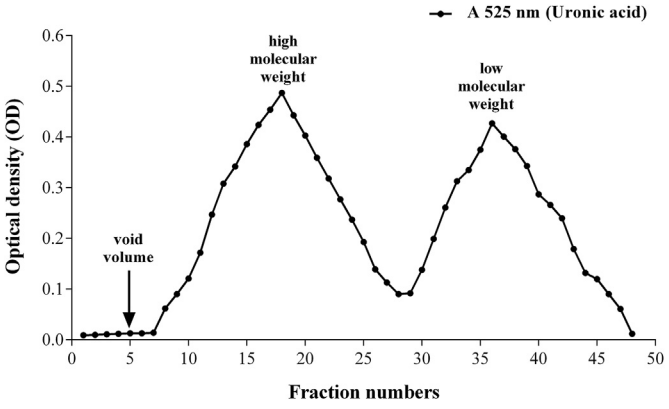

Fig. 1. Elution profile of the purified alginate through Sephacryl S-400. Fractions of 3 ml each were collected and analyzed for uronic acid content. Fractions 10-26 contained high values of uronic acid and were presumed to include high molecular weight alginate.

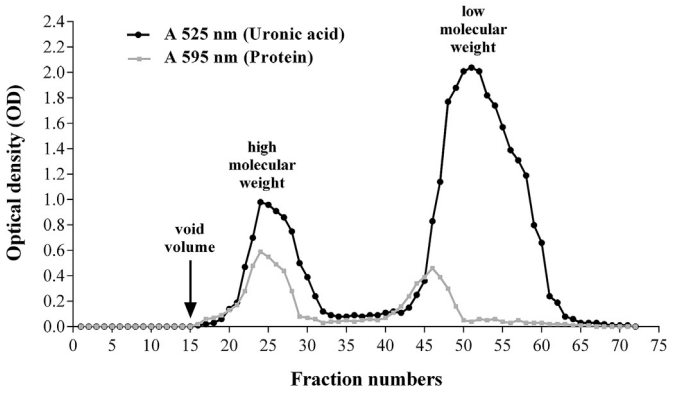

Fig. 2. Size exclusion chromatography of the alginate-FLB conjugate through Sephacryl S-400. The protein content of the fractions was measured using the Bradford assay at 595 nm, and uronic acid was measured using the carbazole-borate assay (525 nm). Fractions 22-30 contained protein and uronic acid components and were presumed to contain conjugate.

composed of 38% protein and 62% uronic acid by weight. The protein/polysaccharide ratio was 61.3%. The FTIR test was performed for structural analysis of the conjugate vaccine and conjugation bonds. FTIR test verified conjugation peaks for alginate and FLB. There was a peak at 1444-1557 $cm^{-1}$for amide N-H and another peak at 2830-2895 cm^{-1} for C-H alginate and flagellin (Fig. 3). These results indicated successful conjugation. This conjugate was not pyrogenic or toxic after intranasal immunization of mice.

3.3. Analysis of antibody responses

We assessed the ability of the alginate-FLB conjugate vaccine to elicit a humoral response in mice. Mice were immunized i.n. four times at two week intervals with 10 μg of alginate conjugated to type b-flagellin (alginate-FLB) or purified alginate or FLB. Mice were bled, and sera were evaluated by ELISA for IgG titers to alginate and FLB. As expected, vaccination with the alginate failed to induce high levels of the alginate antigen, whereas immunization with the alginate-FLB conjugate elicited a significant ($P = 0.0033$) rise in alginate specific IgG (see Fig. 4a), suggesting that the enhanced IgG response to alginate is a result of the covalent conjugation between FLB and alginate. We noted that the immune response to FLB elicited by both the FLB and alginate-FLB conjugate was high, even after 56 days post initiation of the immunization (Fig. 4b). Vaccination with the alginate-FLB conjugate extracted a significant ($P = 0.069$) increase in FLB specific IgG at 42 days after first immunization compared to FLB-immunized group.

3.4. Opsonophagocytic killing activity

The production of antigen-specific antibodies that can mediate bacterial uptake by phagocytes is correlated with the clearance of infection. By using antisera that taken from intranasal immunized mice, opsonic killing assay was performed *in vitro*. Pooled antisera from conjugate vaccine-immunized mice mediated significant killing at levels approaching 61.9% in a 1:4 dilution (see Fig. 5a), suggesting the conjugate vaccine induced a potent *P. aeruginosa* 8821M-specific antibody response. Killing was not observed in the group of mice that received non-immune mouse serum (NMS). As expected, there was a progressive decrease in the opsonic-killing activity with increased dilution of the antisera. At all dilution ranges, the opsonic activity of the conjugate vaccine group was significantly ($P = 0.0067$) higher when compared to FLB or alginate groups. Efficient killing of clinical isolate (53.8−5.8%) was also mediated by various dilutions of serum isolated from conjugate vaccine-immunized mice (Fig. 5b). The data also showed that the antibodies raised against purified alginate has same opsonic activity on both strains of *P. aeruginosa*, the data indicates the same compositions of the mucoid exopolysaccharide (alginate) in these strains.

4. Discussion

The goals of the present study were to conjugate the high

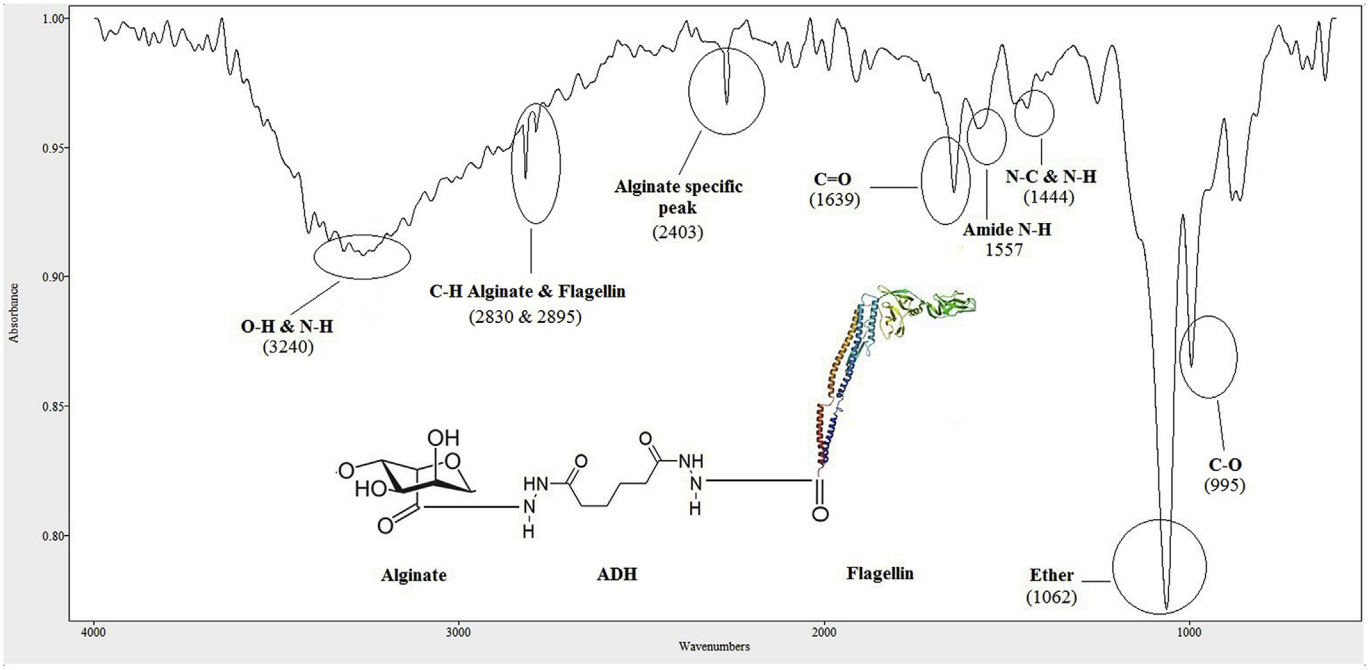

Fig. 3. FTIR-spectra of alginate-FLB conjugate.

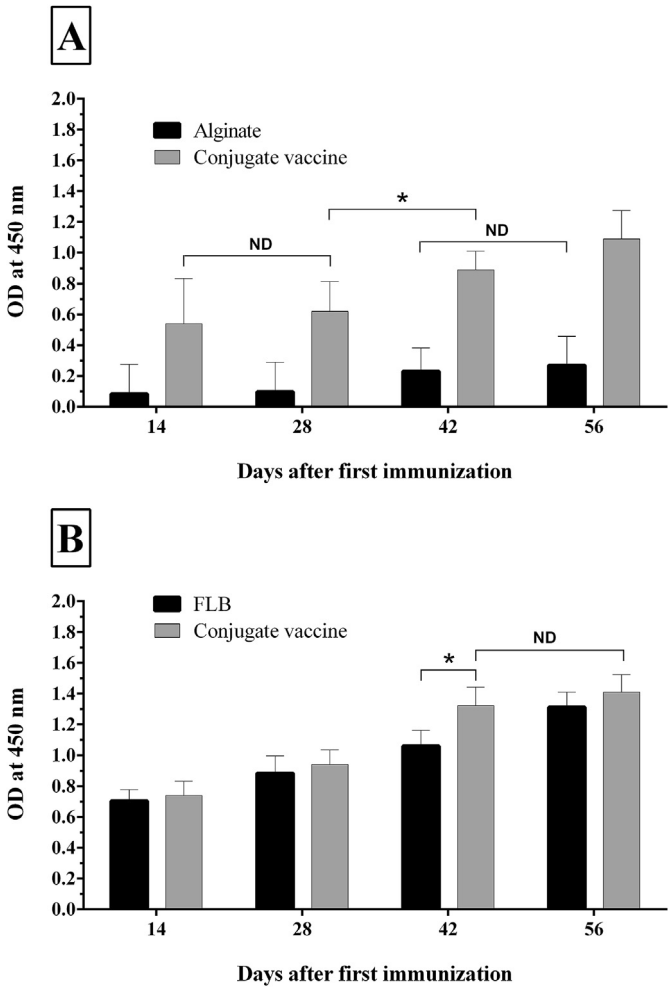

Fig. 4. Titers of IgG antibodies to alginate and FLB in the sera of mice immunized i.n. with 10 μg of alginate, FLB and alginate-FLB conjugate vaccine four times at two week intervals. (A) Titer to purified alginate. (B) Titer to purified FLB. Bars represent means of duplicate determinations in sera pooled from 6 animals, and error bars represent standard deviation (SD). The asterisks indicate the groups which were significantly different ($P < 0.05$) with each other, and the ND indicates not detectable differences between groups.

molecular weight alginate to FLB as possible candidate for a vaccine against *P. aeruginosa* pneumonia and also to determine the biological activities of the vaccine. It has been documented that the mucoadhesive property of alginate causes improvement in absorption and durability of vaccine in mucosal epithelium [17,18]. In our study, native high molecular weight alginate including β-D-mannuronic acid and α-L-guluronic acid was used. It is indicated that using native polymer of alginate (also named mucoid exopolysaccharide; MEP) by stabilizing its conformational epitopes could be vital in inducing opsonophagocytic antibodies, therefore it should be conserved in vaccine preparation [19–21]. Bacterial flagellin has been proposed to promote immunogenicity and as a choice for vaccine candidate as described by earlier studies [9,22]. In the present study, whole FLB was selected in conjugate vaccine design because that the protein was shown to have only one serotype, whereas, type a-flagellin is heterogeneous and composed of some serogroups [23]. Nevertheless, cross-protection between antibodies raised against type a and b-flagellin could be explained by moderately similarity and sequence arrangement in amino acid residues of the two types of flagellin [24]. It is indicated that routes of immunization could affect different the level of immune response [24]. In the present study, the mice immunization was performed via intranasal (i.n.) route. It has been well documented that i.n. delivery of vaccine has an appropriate influence on induction of mucosal and systemic immune reactions against respiratory pathogens [25–27]. In addition, easy accessibility to nasal cavity, needle-free injection and low antigenic dose are some other advantages of i.n. immunization [27].

The present study illustrated that the specific IgG titer to alginate taken from sera of mice immunized with alginate-FLB conjugate vaccine was significantly higher than the alginate alone, indicating conjugation of alginate-FLB leads to an increase in its immunogenity. Our finding was partially in agreement with that of Campodonico et al. [28] study where immunization of mice with the alginate-flagellin conjugate stimulated a higher level of specific IgG against alginate. However, in that study, the conjugate vaccine was composed of polymannuronic acid (PMA) and type a-flagellin (FLA). Another study showed that immunization of mice with MEP based conjugate using the outer membrane vesicle (OMV) of *Neisseria meningitidis* serogroup B as a carrier protein resulted in significantly higher titer of anti-alginate antibodies as compared to alginate alone [16]. It is concluded that this MEP-based conjugate vaccine could be potential protective against *P. aeruginosa* mucoid strains. It was also reported that the conjugation of MEP to KLH (keyhole limpet hemocyanin) as a carrier protein was significantly more potent in eliciting specific IgG against MEP compared with MEP alone [6]. However as one limitation, the carrier protein included in this conjugate vaccine has not been investigated yet in human trials. Cryz et al. structured a conjugate vaccine based on depolymerized alginate and exotoxin A from *P. aeruginosa*. They found that the conjugate can elicit anti-alginate and exotoxin A-neutralizing antibodies in mice [29]. As mentioned earlier, depolymerization of bacterial polysaccharide could destroy some its conformational epitopes [19–21,30].

We have found that immunization of mice with the conjugate vaccine containing alginate and FLB elicited increase in hormonal immune response to FLB in comparison to FLB alone. In the recent study, we showed that when flagellin is formulated in a HIV vaccine (in conjugated form), a significant increase in the total antibodies is observed in comparison with the control groups [31]. Our result was in contrast with the study conducted by Campodonico et al. in which immunization of mice with the PMA-FLA conjugate induced significantly lower level of specific IgG to flagellin when comparing to flagellin alone [28].

The present study illustrated that mice antisera to the conjugate vaccine showed more activity in opsonic killing of the bacterial cells as compared with antisera to FLB or alginate alone. The recent study identified that antisera raised against the PMA-FLA conjugate and un-conjugated PMA were similar in opsonic killing of *P. aeruginosa* mucoid strains; Of course, quantity of PMA-FLA conjugate was lower than un-conjugated PMA [28]. In the later study, specific antisera were obtained from rabbits, whereas in our study the antisera taken from mice, and also, serum dilutions for opsonic killing activity were so higher than the present study. It has been shown that un-conjugated PMA and other alginates from *P. aeruginosa* have high immunogenicity in rabbit [28,32]. One study showed that induction of opsonic antibodies was significantly higher in mice given MEP-conjugated vaccine in comparison to those given only MEP [6]. Our finding was partly in line with their result. However in contrast to our finding and that of Theilacker et al. [6], Garner et al. [19] found that immunization of mice with high dose of MEP alone induced opsonic antibodies. It was concluded that this variation between the results of MEP antigen might be attributed to the difference in their preparations and hereby in acetate proportion, Kd as well as small impurities [6,19]. In the recent study has been shown that at all dilution ranges, mice

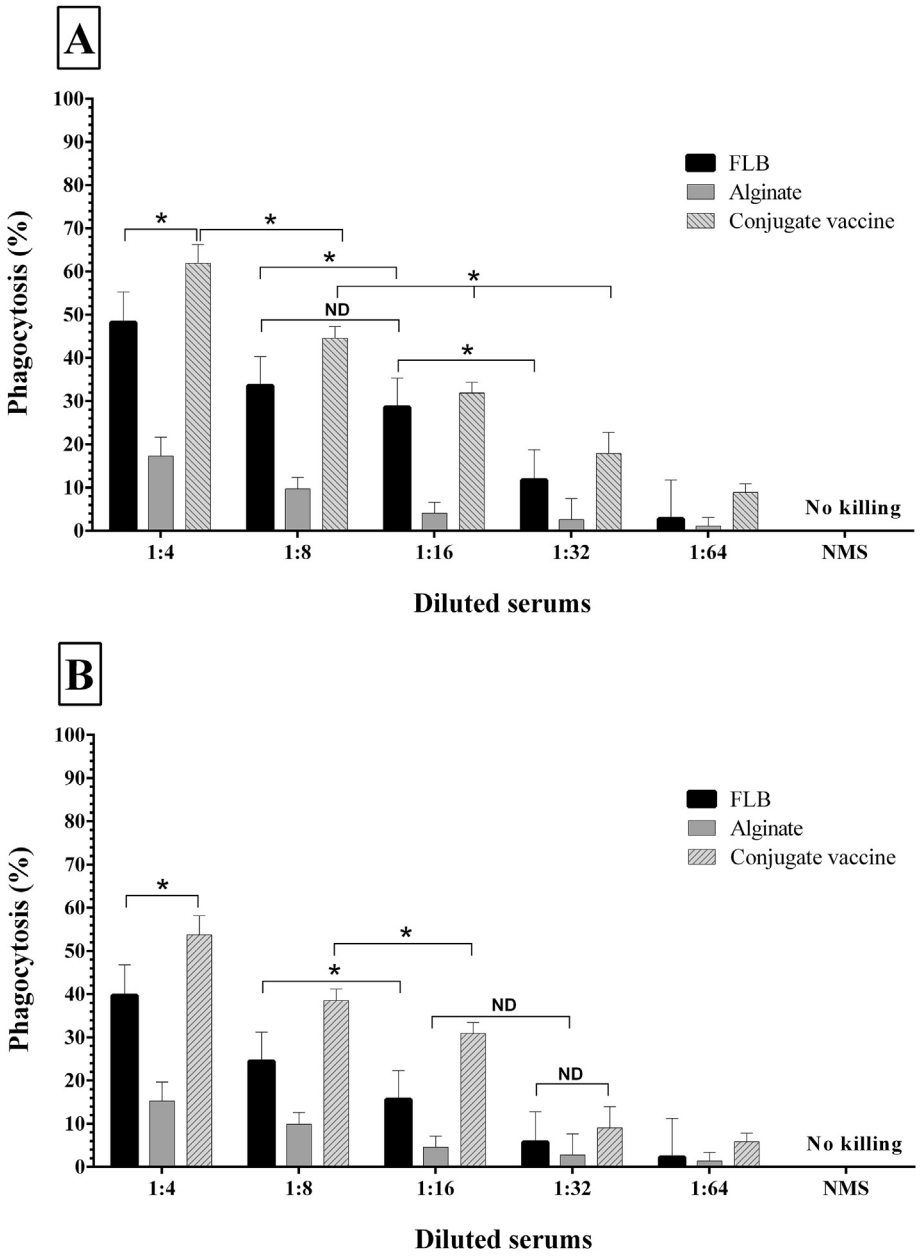

Fig. 5. The opsonic killing activity of different dilutions of specific antisera against *P. aeruginosa* strains 8821 M and E6692 (a clinical isolate). To do this test, all sera of each group were pooled together. The strains were incubated with different dilutions of specific antibodies and mouse macrophage in the presence of rabbit complement. Remarkable opsonic killing activity was observed when conjugate vaccine antiserum was treated with the 8821 M strain (A. Significant cross reaction was detected between the antiserum and E6692 strain (B). Bars represent means of duplicate determinations, and error bar indicate SD. Results were accepted to be significant at $P < 0.05$. The Asterisks represents the groups which were significantly different and the ND indicates not detectable differences between groups.

antisera raised to the conjugate vaccine (MEP-OMV) were stronger in opsonic killing of *P. aeruginosa* 8821 M and the clinical isolate as compared with alginate or the carrier protein alone. Also, the killing percent of the *P. aeruginosa* 8821 M was higher than that of the clinical isolate, it seems that standard strains like 8821 M to be less aggressive compared to clinical isolates [16].

Overall, we have concluded that preparation of conjugate vaccine containing native high molecular weight alginate and FLB from *P. aeruginosa* could elicit high specific IgG titer against alginate. Also, antisera raised against this conjugate vaccine indicate desirable influence on opsonophagocytic killing of the bacterial cells from mucoid standard strain as well as clinical isolate. According to these findings, the alginate-FLB conjugate vaccine might be suitable potential protective versus chronic lung infection resulting from mucoid *P. aeruginosa*. More research exploring the protective efficiency of the conjugate vaccine against mucoid and non-mucoid strains of *P. aeruginosa* as well as TRL5 signaling assay (as an index of innate immunity) would be welcomed.

Conflict of interest

The authors declare no conflict of interest associated with the present manuscript.

Authors' contribution

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