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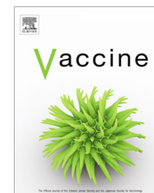

Immunogenicity of *Vibrio cholerae* outer membrane vesicles secreted at various environmental conditions

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ABSTRACT

Cholera is caused by toxigenic *Vibrio cholerae*. It is a significant health problem and an important cause of mortality of children in developing countries. Annually, about 5–7 million people are being infected worldwide, leading to death of 100,000 to 120,000. Immunization using the currently available cholera vaccines has been recommended by World Health Organization (WHO) in areas where cholera is endemic or at risk of outbreaks. Gram-negative bacteria secrete outer membrane vesicles (OMVs) that play important roles in virulence and host-pathogen interaction. The content of protein and lipid in OMVs are affected by purification methods and bacterial growth condition. OMVs released from *V. cholerae* are an appropriate candidate for vaccine development. The protection conferred by a new vaccine candidate prepared using different methods and in two different growth conditions with nanoparticles in an experimental model of cholera in mice was investigated. OMVs were encapsulated in chitosan-tripolyphosphate (TPP) nanoparticles prepared by an ionic gelation method and coated with Eudragit as an enteric polymer. OMVs loaded into nanoparticles (NP-OMVs) were homogeneous and spherical in shape, with a size of 417 nm. BALB/c mice (male, 20–24 g) were immunized via intraperitoneal (10 μg) or oral route (50 μg) with free or encapsulated OMVs. Seventy-eight days after first administration, serum of mice was infected with infection dose of *V. cholerae* ($\geq 10^7$ CFU). The new vaccine was able to protect fully against infection when it was administered via mucosa. By intraperitoneal route, the unpolymerized OMVs increased the protection against these bacteria.

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1. Introduction

Cholera is an acute diarrheal disease caused by *Vibrio cholerae* serogroups O1 and O139, and transmitted via fecal-oral route [1–3]. Pathogenesis is related to bacterial colonization in small intestine followed by secretion of a potent cholera toxin (CT) [4], causing watery diarrhea, hypotensive shock, and finally leading to death especially in children under 5 years of age [1,5]. Annual report of World Health Organization (WHO) for cholera is estimated to be 5–7 million cases, of which more than 100,000 lead to death [6]. Virulence factors such as CT and toxin-co-regulated pilus (TCP) common in O1 and O139 strains are being highly expressed in pathogenesis [1,7,8]. Serogroups without CT and TCP are nonpathogenic and named non-O1 and non-O139 (NOVC) [1]. Currently, three commercial oral vaccines are available against *Vibrio cholerae*. These vaccines were produced based on killed whole cells [1,9], killed whole cells along with recombinant B subunit of CT [10,11], and attenuated bacterial cells [12,13]. However, all commercially available vaccines beside their benefits have some disadvantages, including poor immunogenicity, the lowest efficacy in young children, limitation of administration to infants less than 6 months, and high reactogenicity, especially in parenteral cholera vaccine. Since, *V. cholerae* that is transmitted via fecal-oral route and naturally acts as a mucosal infection; therefore, the development of oral cholera vaccines is advisable [14]. Many virulence factors present in outer membrane (OM) of bacteria, are potentially considered to be candidate for vaccine development [15]. Outer membrane vesicles (OMVs) secreted from gram negative bacteria have been revealed to have role in protective transport of various factors such as toxins, DNA, OM proteins, and enzymes to infected tissue cells [15–19]. Growth conditions and purification methods affect the chemical composition of the OMVs as compared to native vesicles [20]. Various methods have been used for the purification of OMVs, such as mechanical shearing and the use of chemical agents like detergents. Application of detergents in OMVs purification has been commercialized due to efficient production and

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reduced toxicity of lipopolysaccharide (LPS) [21,22]. Dealing with protection against enteric pathogenic bacteria, oral immunization is an effective route which triggers both systemic and mucosal immune systems [23–25]. The harsh condition of the digestive tract is one of the obstacles facing the development of oral vaccine. The problem can be overcomed by proper targeting which can be achieved using methods which are based on time released approach, pH sensitive [26], and multiparticulate or nanoparticulate systems. The nano-micro particle-based system is the best approach for controlled and protective vaccine delivery in oral route [27]. Nano-micro particles are small, natural or synthetic polymers having particle sizes of 1–1000 μm [28]. A wide range of dedicated carriers are used for vaccine delivery in oral route. Nano-micro polymeric particles such as chitosan are an appropriate carrier for oral vaccine delivery [29,30]. Chitosan is safe and easily available, biodegradable with mucoadhesive properties [28,31,32]. However, chitosan partial release due to surface degradation in stomach condition is considered as a limitation [32] which can be overcomed by coating chitosan with pH dependent enteric polymers [33,34]. In this study, natural OMVs (nOMVs) and detergent treated OMVs (dOMVs) were purified from bacteria grown at two different temperatures of 37 (n) and 42 °C (s). Animals were orally and intraperitoneally administered with polymeric and non-polymeric OMVs and the immunogenicity of different OMVs were also evaluated.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Vibrio cholerae Inaba O1 ATCC 39315 is obtained from microbial collection center of Shahed University. Bacteria were grown in Luria-Bertani (LB) broth for 6 h at 37 and 42 °C with aeration of 150 rpm up to the late exponential phase.

2.2. Experimental animals

BALB/c mice were purchased from Pasture Institute of Iran. Mice were housed with food and water *ad libitum* and under caring staff in accordance with the standard rules of the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.3. Extraction and purification of LPS

LPS was extracted from *V. cholerae* O1 strain through the hot phenol-water method described previously [35] and analyzed on 16% tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using silver staining. In order to check any protein contamination in extracted LPS, Coomassie blue staining was done. LPS was quantified using the phenol-sulfuric acid method [36].

2.4. Extraction of dOMVs

dOMVs were prepared according to the method explained by Siadat et al. [37]. In brief, *Vibrio cholerae* O1 strain was grown in LB batch culture under controlled condition at two temperatures of 37 and 42 °C up to the late exponential phase. The bacteria were harvested and pellets were treated with 7.5 folds cells wet weight of 0.1 M Tris-10 mM EDTA buffer. Extraction of budding vesicles was performed using 1/20th volume of 0.1 M Tris-HCl (pH 8.6) buffer containing 10 mM EDTA and 0.5% (w/v) deoxycholate (Sigma-Aldrich, USA). Cells debris was removed by centrifugation at 18,000*g* for 90 min/4 °C. Vesicles in supernatant were concentrated by ultracentrifugation (Beckman SW32Ti rotor) at 125,000*g* for 2 h/4 °C. The pellet of OMVs was washed with 0.1 M Tris, 10 mM EDTA, and 0.5% (w/v) deoxycholate and then precipitated by centrifugation. The pellet of vesicles was suspended in 3% sucrose solution as a preservative and stored at 4 °C until it was used.

2.5. Extraction of nOMVs

The bacteria were cultured in 2 L of LB broth and incubated at 37 or 42 °C until it reached the late exponential phase. Bacterial cultures were centrifuged at 10,000*g* for 10 min/4 °C and supernatants were filtered using 0.45 μm pore-size Millipore filters. To confirm the complete removal of bacterial cells, 1 ml of each filtrate was plated on the LB agar plate and incubated for 24 h/37 °C. The filtrate was concentrated to 200 ml by freeze drying. The product was re-filtrated to remove any possible contamination. Finally, the OMVs were pelleted by ultracentrifugation at 140,000*g* for 4 h/4 °C. The OMVs were suspended in 1 ml of PBS and stored at −20 °C until it was used. Concentration of OMVs was estimated using the modified Lowry method.

2.6. Size determination of OMVs with transmission electron microscopy (TEM)

Samples were adsorbed on formvar carbon coated grids for 2 min and negatively stained with 2% uranyl acetate and then dried at room temperature. Grids were viewed in ziess-EM10C electron microscopy at 80-kV accelerating voltage. The different types of vesicles were analyzed for their homogenicity and the size of vesicles was estimated using program analysis (Soft imagine system, Switzerland).

2.7. OMVs encapsulation into chitosan-triphosphate (CS-TPP) nanoparticles

OMVs encapsulation into CS-TPP nanoparticles was done using ionic gelation method. CS (25 mg) (Sigma-Aldrich, USA) were added into 5 ml of 2% acetic acid with continuous stirring. The solution was sonicated (70 Hz, 0.5 cycle, 4 °C) to complete dissolving. TPP (Sigma-Aldrich, USA) solution at 2.5 mg ml^{-1} concentration was added dropwise into CS solution (pH 5.5), with continuous stirring and sonication. To prepare OMVs loaded nanoparticles, OMVs were added into CS solution before adding TPP. TPP was then added and the procedure was done as described for CS-TPP preparation.

2.8. Physicochemical properties of nanoparticles

Zeta potential and size of nanoparticles were measured using Zetasizer Nano-ZS on dynamic light scattering (DLS) techniques.

2.9. Entrapment efficiency

To estimate the entrapment efficiency, nanoparticles loaded with OMVs were centrifuged at 12,000*g* for 15 min/4 °C and concentration of free OMVs in supernatant was determined using Bradford method. Entrapment efficiency was calculated using the following equations:

$$\text{Loading Efficiency (LE)} = (\text{Total amount of OMVs} - \text{Free OMVs})/\text{Total amount of OMVs}$$

$$\text{Loading Capacity (LC)} = (\text{Total amount of OMVs} - \text{Free OMVs})/\text{Amount of CS}$$

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