Original Article

A streamlined method for the extraction of outer membrane vesicles (OMVs) from *Bordetella pertussis*

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ABSTRACT

Introduction: In spite of high vaccination coverage, whooping cough (pertussis) is still a worldwide health problem. The main reason for pertussis outbreak is waning immunity of safer acellular vaccines which have replaced the more reactogenic cellular vaccines. A new generation of pertussis vaccines that is potent and safe is desperately needed to control the disease. Previous studies have indicated that outer membrane vesicles (OMVs) obtained from *Bordetella pertussis* have desirable characteristics which make them a good candidate for application as pertussis vaccine. They contain surface immunogens in a native structure, are self-adjuvant and are easily uptaken by the antigen presenting cells. **Methods:** *B. pertussis* Tohama strain was cultured at 35°C in Stainer-Scholte broth. The OMVs were isolated by a new sequential ultracentrifugation method. The extracted OMVs were characterized by electron microscopy, SDS-PAGE and ELISA assays. **Results:** The existence of pertussis toxin, filamentous haemagglutinin and a 69-kDa antigen in *B. pertussis* OMVs was verified using an ELISA assay. Electron microscopy showed the size of these OMV's at 40-200 nm. The ELISA results indicated that the OMVs extracted using this protocol contain major immunogens. **Conclusion:** We report for the first time a simple protocol for the efficient extraction of *B. pertussis* OMVs. This protocol can be used in the process of making new generations of *B. pertussis* vaccines.

KEYWORDS: *Bordetella pertussis*, outer membrane vesicles, extraction, characterization, vaccine.

INTRODUCTION

Pertussis (also known as whooping cough) is a contagious respiratory illness that is particularly dangerous in infants. The causative agent of pertussis is the fastidious, Gram-negative bacterium *Bordetella pertussis* [1]. Pertussis is highly contagious and is preventable by vaccination. Despite massive vaccination programs over more than 60 years, this disease has remained a major global concern and one of the most life-threatening childhood diseases [2, 3]. The original sources of pertussis for infants are the family members [4-6]. The transmission is almost always directly from person to person [7]. The first generation of pertussis vaccines were inactivated and alum-adjuvanted which were developed in 1940s-50s with a good efficacy which are still in use in many countries [8, 9]. According to the WHO, from 1999 to 2014, pertussis vaccinations reduced infants death by around 100,000 cases [10]. There is, however, a growing concern about the safety of the whole-cell vaccination (wP) and its side-effects that has led to its replacement by the safe acellular vaccines (aP), containing immunogenic antigens of B. pertussis [11, 12].

The shift from more reactogenic wP to aP decreased vaccine side-effects, however, it led to several outbreaks and resurgence of pertussis [13, 14]. The causes of these outbreaks were low efficacy and short immunity duration induced by aP vaccines [15, 16]. Moreover, aP vaccines with alum as adjuvant induce mainly Th2 (humoral) response that contributes to reduced protection, while Th1/Th17 (cellular) response induced after wP priming leads to the lung clearance, long lasting immunity and protection [17, 18].

Outer membrane vesicles (OMVs) are spherical nanoparticles (10-300 nm in diameter) released from Gram-negative bacteria. These nano-spheres are principally composed of lipopolysaccharides (LPS), outer membrane proteins (OMPs) and periplasmic proteins [19-21]. OMVs have been identified as good vaccine candidates for bacterial infections [22]. The OMVs of B. pertussis contain several major immunogens that can be used as a new and potent acellular pertussis vaccine with

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low reactogenicity [23-26]. Here, a new extraction method of B. pertussis OMVs is introduced.

MATERIALS and METHODS

Strains and growth conditions

B. pertussis Tohama phase I strain was used in this study. A 10 µl aliquot of the stock bacterium was grown on Bordet-Gengou (BG) agar plate and incubated for 3-5 days at 35°C. A few small colonies were sub-cultured on Stainer-Scholte (SS) liquid medium for large-scale production of the cultures [27].

Isolation of OMVs

After approximately 48 h of incubation (decelerating growth phase), the culture sample was centrifuged at 10,000×g for 30 min at 4°C. The supernatant was discarded and the pellet was incubated at 56°C oven for 30 min, then washed twice with phosphate buffer saline (PBS) and centrifuged at 7,000×g for 10 min. The pellet was dissolved in sodium chloride buffer and homogenized by pipetting several times to make a uniform suspension. Then the suspension was incubated at room temperature for 45 min, after which 0.1 M Tris-HCl, pH: 8 and 10 mM EDTA was added (8 times weight of the suspension) and homogenized by shaking for 15 min. The suspension was then treated with 0.1 M Tris-HCl, pH: 7.5, 10 mM EDTA and 10% W/V sodium deoxycholate (up to 5% of the suspension) and mixed for 5 min, then centrifuged at 17,000×g for 1 h. The supernatant was separated carefully in a new tube and treated with 100 µl of 0.1 M Tris-HCl, pH: 7.5, 10 mM EDTA and 5% W/V sodium deoxycholate. Finally, the supernatant was pelleted at 60,000×g for 4 h at 4°C. The pellet was then dissolved in 5 ml of 3% sucrose and filtered through 0.2 µm filter (Milipore, Germany). The filtered sample containing OMVs was stored at 4°C.

Transmission electron microscopy (TEM)

The extracted OMVs were suspended in 0.1 M ammonium acetate (pH: 7). A 5-µl aliquot was drop-cast on a copper-coated grid. After evaporation of the liquid, the grid was stained with phosphotungstic acid and examined with a Zeiss EM10c TEM (Germany).

Protein assay

The concentration of total protein in OMVs was measured using the Bradford method with bovine serum albumin as standard [28].

Protein profile of OMVs

For the separation of proteins in OMVs, SDS-PAGE analysis was performed. The extracted OMVs (5 µg) were resuspended in laemmli sample buffer [29] and incubated at 95°C for 15 min. Electrophoresis was done at 22°C and constant voltage. After electrophoresis, the separated proteins were stained by Coomassie Blue R-250. OMVs from B. pertussis (Tahoma) were extracted 20 times with the method explained above. In all repeats, the size and SDS-PAGE profiles were determined.

ELISA

To evaluate the presence of specific pertussis antigens such as pertussis toxin (PT), filamentous haemagglutinin (FHA), and 69-kDa antigen, direct ELISA was carried out with standard antigens (NIBSC) and OMV- hyperimmune serum as antibody. First, hyperimmune serum with OMVs was produced as antigen and complete/incomplete Freund's adjuvant, according to the methods previously published [30]. Briefly, 40 µl of OMVs (50 µg total protein) was mixed with 40 µl of complete Freund's adjuvant and injected subcutaneously to 8 BALB/c mice at the time zero after homogenization. In addition, 2 booster immunizations were carried out at 2 and 4 weeks after the priming immunization with 40 µl incomplete Freund's adjuvant and 40 µl OMVs (50 µg total protein) per dose. After 14 days and bleeding from heart, serum samples were collected as hyperimmune serum. A checkerboard titration was carried out to determine the optimum antigen concentration. Antigen concentrations considered in the study included 150 ng/well for PT and FHA, and 40 ng/well for 69-kDa protein that were coated on 96-well ELISA plates, and incubated overnight at 4°C. The plate was then washed once with PBS and blocked with 150 µl/well of 3% skimmed milk (Sigma, Germany) and incubated for 24 h at 4°C. After thorough washing, 50 µl of hyperimmune serum (1:100 in 3% skimmed milk) was added and the mixture was incubated at 37°C for 2 h. As a negative control, few wells were coated with BSA (10 µg/ml). Each serum sample was tested in duplicate. The wells were washed 4 times and anti-mouse IgG HRP-conjugated (Sigma USA), diluted at 1:40,000, was added at 50 µLl/well and the samples were incubated at 37°C for 2 h. The wells were then washed four times to remove unbound antibodies. The plate was incubated with 100 µl of 3،3´, 5،5´-tettamethyl benzidine (TMB) at room temperature in dark. The reaction was stopped after 20 min by adding 25 µl stop solution (HCl 5.8%) and the absorbance was measured at 450 nm using an ELISA plate reader (STAT FAX, USA).

Specificity test

To perform the specificity test, the hyperimmune serum, normal mouse serum (NMS), diphtheria, tetanus serum (DT) and Pasteurella serum (PAS) were used. Three dilutions (1:100, 1:200, 1:400) were prepared from each sample. The serums were put in two separate plates and their optical density was measured. Only one dilution (1/100) was used for NMS.

RESULTS

OMVs isolation and characterization

The OMVs were extracted from B. pertussis Tohama cells according to the procedure described. For evaluation of the shapes and size, the extracted OMVs were examined with an electron microscope after negative phosphotungstate potassium staining (Fig. 1A). The size was determined to be 40-200 nm and topographical shapes were intact in 10 extraction examined via TEM. The protein profiles of extracted OMVs were studied by SDS-PAGE (Fig. 1B) and the results matched with that measured by the Bradford method [28] was 120 µg/ml.

ELISA

The assessment of antibody development pattern proved that all the extracted OMVs had specific pertussis antigens (PT, FHA, 69-kDa) as determined by ELISA (Fig. 2). The sera from the normal mouse serum (NMS) did not develop titer as confirmed by ELISA (Fig. 2). The results of specificity test are presented in the Table 1. The increase in the absorbance value was only observed in the pooled sample in all 3 dilutions whilst the other samples did not show any raise. The results of the specificity test thus showed that anti-PT, anti-FHA and anti-69-kDa were highly specific with no cross reaction.

Specificity test

The increase in the absorbance value was only observed in the hyperimmune serum sample in all 3 dilutions whilst other samples did not reveal any increase. Therefore, anti-PT, anti-FHA and anti-69-kDa were completely specific and there was no cross reaction with other antigens (Table1).

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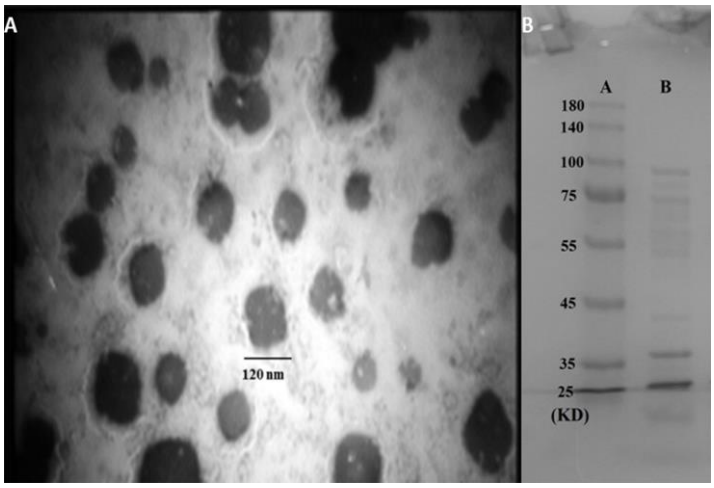


Fig. 1. Characterization of *Bordetella pertussis* Tohama strain phase I OMVs: (A), TEM image of negative stained OMVs extracted from *B. pertussis* Tohama. (B), SDS-PAGE 12.5% (w/v) of the extracted OMVs. Lane A shows the Mw marker and lane B shows the protein profile of the obtained OMVs.

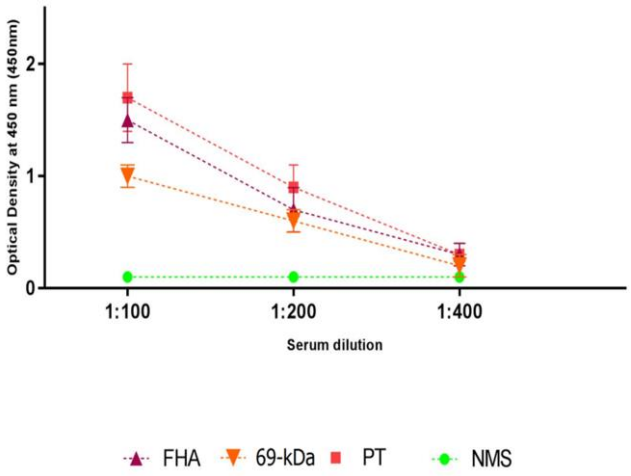


Fig. 2. Antibody levels in the hyperimmune serum and NMS against PT, FHA and 69-kDa

Table. 1. Optical density values of the hyperimmune serum, anti-DT and anti-PAS as antibodies and PT, FHA and 69-kDa as antigens in ELISA assay.

Sample name	Serum dilution	PT		FHA		69-kDa	
		A^{450} (plate1)	A^{450} (plate2)	A^{450} (plate1)	A^{450} (plate2)	A^{450} (plate1)	A^{450} (plate2)
Hyperimmune serum	1:100	1.852	1.801	1.636	1.702	1.091	1.126
	1:200	0.951	0.892	0.821	0.810	0.721	0.788
	1:400	0.562	0.502	0.522	0.501	0.412	0.458
DT	1:100	0.187	0.175	0.187	0.175	0.187	0.175
	1:200	0.144	0.151	0.144	0.151	0.144	0.151
	1:400	0.088	0.060	0.088	0.060	0.088	0.060
PAS	1:100	0.236	0.211	0.236	0.211	0.236	0.211
	1:200	0.221	0.289	0.221	0.289	0.221	0.289
	1:400	0.085	0.032	0.085	0.032	0.085	0.032
NMS	1:100	0.152	0.121	0.152	0.121	0.152	0.121

DISCUSSION

In this study we presented a novel procedure for the isolation of pertussis OMVs from Tohama strain. The extracted OMVs contain important immunogens (PT, FHA and 69-kDa) that could elicit host immune responses and could also be used to develop a new generation of efficient aP vaccine. The OMVs contain not only main immunogens but also LPS that can play the role of an adjuvant [31-33].

Our protocol of pertussis OMVs extraction includes a series of simple steps with high yields. In other studies, ultracentrifugation at 100,000×g has been used for the isolation of pertussis OMVs. However, an ultracentrifuge is not a commonly available item in many laboratories. Therefore, an alternative method with lower speed of centrifugation will be more useful. In this study we used 60,000×g for the isolation of OMVs. Unless otherwise specified by regulatory agencies, in aP vaccine production, there is no need for the purification of obtained OMVs and the isolated OMVs can be directly used as a vaccine candidate.

The extracted OMVs were nano-particle vesicles with the diameters ranging between 40-200 nm (Fig. 1A) that is consistent with other reports of OMVs extracted from other *B. pertussis* strains [24, 26, 34]. This size range also suggests that the nano-spheres can be readily uptaken by the antigen presenting cells [35]. The protein profiles of the extracted OMVs studied by SDS-PAGE showed several proteins that based on their molecular weights appear to be main immunogens, as confirmed by ELISA assay. At least 20 repetitions of OMVs extraction were performed. The similarity in morphology, size and presence of immunogens were observed in all cases. It is envisaged that the presented protocol may pave the way for an easier and more efficient extraction of OMVs for the future vaccine studies against pertussis.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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