Comparison of Two Different Methods in the Extraction of Outer Membrane Vesicles from the *Bordetella pertussis* as a Vaccine Candidate

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**ABSTRACT**

**Background & Objective:** Despite vaccination, pertussis is still a worldwide health problem. Outer membrane vesicles (OMVs) in gram-negative bacteria can stimulate the immune system due to several outer membrane proteins and are very good candidate in vaccine development, OMVs obtain from *Bordetella pertussis* contain several antigens, which are considered immunogenic that could make them a potential candidate for vaccine production. The aim of this study was to compare the current OMVs extraction method (with ultracentrifuge) and the modified extraction method (without ultracentrifuge) and to evaluate the physicochemical properties as well as the expression of their main virulence factors.

**Materials & Methods:** Vaccinal strain BP134 were grown on Bordet Gengo agar were inoculated in Modified Stainer-Scholte medium for mass cultivation. OMVs were prepared by using two different methods. The OMVs obtained were stained and examined with a transmission electron microscope. Protein contents were measured by the Bradford method and then the protein profile evaluated by SDS-PAGE. The presence of immunogenic antigens were detected by Western blotting.
**Results:** The size and shape of the OMVs obtained from the modified method without the use of ultracentrifuge were similar to the current method and with a size between 40 to 200 nm. The total protein yield of the OMV isolated using the current and modified method were 800 and 600 μg/ml respectively. Evaluating the protein profile of extracted OMVs, showed the presence of different proteins. Finally, the presence of PTX, PRN, and FHA in OMVs extracted from both methods was observed.

**Conclusions:** Comparison results of two OMV extraction methods showed that the obtained vesicles have a suitable and similar shape and size as well as the expression of three important pathogenic factors as immunogens. Despite the relatively low reduction of protein yield since the modified method does not require ultracentrifuge, this extraction method can be used as a suitable alternative for extracting the outer membrane vesicles from the *B. pertussis*, especially in developing countries. It should be noted that further experiments, including immunogenicity determination of OMVs, obtained as vaccine candidates in animal models are required.

**Keywords:** *B. pertussis*, outer membrane vesicle, virulence factors

**INTRODUCTION**

Pertussis or whooping cough is a highly contagious bacterial respiratory tract infection caused by *Bordetella pertussis* (Howard, 2016). Some of the major virulence factors which play important roles in its pathogenesis are pertussis toxin (PTX), pertactin (PRN), and filamentous hemagglutinin (FHA). The PTX induces protective immunity and plays an important role in the disease development (Mooi, 2010). The PRN is a surface protein that is involved in mediating adherence to the epithelium of the respiratory tract (Breakwell et al., 2016) and the FHA is also a major adhesion factor on the surface of *B. pertussis* (Zaretzky et al., 2002).

This respiratory disease was a major cause of infant mortality worldwide before the vaccine was introduced in the 1940s. Widespread vaccination with the first-generation of vaccines, whole cell pertussis (wP) vaccines that were consisted of detoxified killed whole bacteria significantly reduced morbidity attributed to the disease. However, in the 1970s, in some countries, concerns about the reactogenicity of wP vaccines led to rising rates of vaccine refusal and consequently to increase pertussis incidence (Edwards, 2005; Bolotin et al., 2015).
Since the 1980s, many countries replaced wP vaccines by less reactogenic acellular pertussis (aP) vaccines. Acellular vaccines are composed of pertussis toxin (PTX), as a major protective antigen and other surface proteins, as bivalent, trivalent and pentavalent pertussis vaccines. In general, five-component vaccines (pentavalent) including (pertussis toxin, filamentous hemagglutinin, pertactin, and fimbriae 2 and 3) are considered more effective than bivalent or trivalent vaccines (Gustafsson et al., 1996; Olin et al., 1997; Poolman and Hallander, 2007).

Unexpectedly, in recent years, despite the high vaccination rate, a large number of pertussis outbreaks were observed that not seen since the pre-vaccine days. Indeed, pertussis is now recognized as reemergence disease and still among the main cause of death in children worldwide (Tong et al., 2020; Mooi et al., 2009, 2014; Bart et al., 2010; Witt et al., 2013). This reemergence is due to short-term immunity induced by aP vaccines. Meanwhile, wP vaccines induce Th1/Th17 responses that lead to lung clearance and long-lasting immunity, aP vaccines mainly induce Th2 response. Due to the fact that *B. pertussis* is an intracellular bacterium, it needs to induce Th1 for lung clearance. Thus to overcome these problems a new generation of vaccines needs to be developed since outer membrane vesicles (OMVs) of *B. pertussis* contain phospholipids, lipooligosaccharides, nucleic acid, and several immunogenic antigens and also shown a basal level of protection against *B. pertussis* that also induces Th1 response, so it could be attractive alternatives over the currently available vaccines (Roberts et al., 2008; Gaillard et al., 2014).

The aim of this study was the extraction of outer membrane vesicles (OMVs) from *B. pertussis* using the modified procedure without using ultracentrifuge with very high speed in comparison with the current method using ultracentrifuge and evaluate the physicochemical properties and also the expression of its main virulence factors; PTX, PRN and FHA, of extracted OMVs as potential vaccine candidates.

**MATERIALS AND METHODS**

**Bacterial strains and cultures.** *B. pertussis* vaccinal strain BP134 was obtained from Razi Vaccine and Serum Research Institute (RVSRI). This strain was grown on Bordet-Gengou agar (BGA; Difco) supplemented with 10% defibrinated sheep blood at 37 °C for 72 h, then colonies were confirmed with biochemical tests and slide agglutination using *B. pertussis* antiserum (Difco) for the final approval. bacterial colonies were subcultured on the same medium for 48 h and then inoculated in liquid Modified Stainer-Scholte medium (MSS) with methyl-β-cyclodextrin at 200 rpm in a Beckman-Coulter shaker (BeckmanCoulter, Brea, CA).
until decelerating phases (optical densities, OD<sub>600</sub> between 0.7 and 1.0) were reached for large-scale production of cultures (Hozbor et al., 1999; Nikbin et al., 2013).

**Isolation of OMVs.** In the current method used in previous studies, sequential ultra-centrifugation with high speed above 100000 g has been used for isolation of OMV as described before (Hozbor et al., 1999; Roberts et al., 2008; Gaillard et al., 2014) but in this modify method we used simple steps with lower speed centrifuge as follows (according to the following)

600 ml of MSS broth was inoculated with 20 ml of a decelerating phase culture of *B. pertussis* for large scale production. After about 30h to reach the decelerating phase at 36 °C with aeration at 200 rpm (Beckman-Coulter, Brea, CA). The cultures were pelleted by centrifugation at 8000 g for 30 min at 4 °C and then the pellets were washed twice in phosphate-buffered saline (PBS) to eliminate cell debris. The pellet (1 g, wet weight) was subsequently resuspended in 7.5 ml of TE buffer (Tris-HCl, EDTA, pH 8.5) and homogenized completely to make a uniform suspension, next incubated at room temperature for 30 min. The suspension was sonicated for 10 min (MSE sonicator, 4-5 pm amplitude, 20 kHz on ice with intervals of 30 s) and centrifuged at 10000 g for 20 min at 4 °C. The pellets were washed with TE, centrifuged and the supernatants pelleted at 60000 g for 2 h at 4 °C. Subsequently, these resulting pellets were resuspended in 1 L PMTris, 10 mM EDTA, DOC (5 g/L) buffer, and mixed again several times by pipetting to make a homogenized suspension and incubated for 10 min and centrifuged for 2 h at 60000 g at 4 °C. Afterward, the supernatant was separated carefully in a new tube and treated with TE buffer and centrifuged again for 1 h at 60000 g at 4 °C. The pellets were dissolved in 5 ml of 3% sucrose and passed through 0.22 μm pore size filters (PVDF, syringe filters, Germany). The filtered sample containing the OMVs was inactivated by heating in a water-bath at 56 °C for 30 min. The suspensions were then spread on blood agar and Bordet-Gengou agar plates and incubated at 37 °C for 48 h to confirm the bacterial inactivation. Each extraction method repeated four times and in all repeats, the physiochemical properties of extracted OMVs by two methods such as shape, size, SDS-PAGE, Western profiles were determined (Hozbor et al., 1999; Roberts et al., 2008, Gaillard et al., 2014).

**Protein assay.** The amount of proteins in outer membrane vesicles were quantified by Bradford assay with bovine serum albumin (BSA-Sigma) as standard using Nanodrop (Thermo Scientific, Wilmington, DE, USA) (Hozbor et al., 1999; Gaillard et al., 2014).

**Transmission electron microscopy (TEM).** The OMVs preparations were suspended in 0.1 M ammonium acetate (pH 7.0) and a drop was placed on a grid coated with a carbon-reinforced formvar film. After 30 S evaporation, the excess fluid was removed by absorbing
with filter paper and the grids stained with 2% (w/v) phosphotungstic acid. The grids were examined on a Zeiss EM10C TEM (Germany) operating at an accelerating voltage of 100 kV (Hozbor et al., 1999; Roberts et al., 2008).

**SDS-PAGE and Western Blotting.** Protein profiles of the extracted OMVs were studied by SDS-PAGE followed by using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the separation of protein molecules based on their molecular weight. After electrophoresis, proteins were stained by 0.1% (w/v) Coomassie blue (DNA Biotech, IRAN) with gentle shaking for 1 h. The gels were then washed three times with methanol 40% (v/v) and acetic acid 10% (v/v) in double-distilled water (ddw) for 20 min. Washed solution was discharged and gels were fixed with a solution containing 2% phosphoric acid (w/v), 18% ethanol (v/v) and 15% ammonium sulfate (w/v) in ddW for 30 min with minor modifications (Roberts et al., 2008). Moreover, western immunoblotting analyses using specific antibodies against PTX, FHA, and PRN were employed to characterize and describe OMVs properties. While the proteins from the polyacrylamide gel were transferred to the polyvinylidene difluoride (PVDF) membrane which was blocked overnight with 5% skimmed milk in PBS. Membranes were then washed three times with PBS in 0.05% Tween 20 (PBST) next exposed to mouse monoclonal immune sera directed against the PTX, PRN and FHA (NIBSC No. 97/572, 97/558 and 97/564 respectively) for 1 h at 37 °C. Membranes were washed three times with PBST, followed by incubation with rabbit anti-sheep horseradish peroxidase-conjugated (HRP) antibody (PDZA Company, Iran) at a 1:1000 dilution, for 1 h at room temperature. Finally, after three times wash with PBST, the color reaction was ultimately generated in the presence of Metal Enhanced 3,3’-Diaminobenzidines (DAB) Substrate.

**RESULTS**

OMVs were isolated from *B. pertussis* vaccinal strain BP134 using the current method and modified method. Both obtained samples were negatively stained and examined with an electron microscope with a mean size of 70 nm and ranging from 40 to 200 nm (Figure 1). At least four independent extraction of OMVs with two methods and also characterization procedure were carried out in which similar morphology was observed in all cases and the OMVs size range was consistent from batch to batch and similar to previously described OMV preparations (Roberts et al., 2008).

The amounts of the membrane vesicle's protein were 800 and 600 μg/ml respectively.
In the present study, to further characterize the OMVs properties, including the virulence factors, the electrophoretic pattern was assessed by 12% SDS-PAGE. Bands similar to each other and to other studies, including 32, 69, 140-180 kDa were observed (Figure 2). Western blotting was performed using specific monoclonal antibodies against PTX, PRN and FHA, to confirm the presence of known pertussis immunogens in OMVs which finally confirmed and verified the presence of these important antigens in extracted OMVs by two methods as described by previous authors too (Hozbor et al., 1999).

DISCUSSION
The resurgence of pertussis was reported in several countries that shifted to aP vaccines from wP vaccines. Due to the side effects associated with whole-cell vaccines and also inadequacy to induce protective immunity had been led to the development of aP vaccines, but aP have some disadvantages, including low efficacy, waning the antibody after immunization, provided immunity against a limited number of antigens and also induces humoral response, according to these drawbacks many researches have focused on other parts of the bacterium. (Mooi et al., 2014; Sealey et al., 2016).

From the vaccine point of view, OMVs derived from *B. pertussis*, which contain main bacterial surface antigens has been shown to successfully exhibit a basal level of protection and can induce Th1, Th2, Th17 as similar as wP vaccines. Since the OMVs are spherical nanoparticles, it is expected to exert an improved uptake of the antigen by antigen-presenting cells than the bacterial whole cell (Gainlard et al., 2014). Another attractive feature of OMVs is, its advantages over the currently used aP vaccines since they are capable of conferring both long-lasting immunity and also protection against different strain genotypes that result in better protection. So, OMVs could be considered as good vaccine candidates against *B. pertussis* (Bottero et al., 2016).

In accordance, several researches revealed that since meningococcal OMVs express immunogenic antigens, it could be naturally taken advantage of important features required for a good vaccine. There are currently two licensed vaccines for serogroup B meningococcal disease based on OMVs. The efficacy and safety of these OMVs vaccines have been proved (de Kleijn et al., 2000; Nøkleby et al., 2007; Sandbu et al., 2007; Van de Waterbeemd et al., 2010). On the other hand, as the OMVs are stable even at room temperature and they don't require cold chain and buffer solution too, so these features consider the possibility of employing the OMVs as a good vaccine candidate for the prevention of diseases (Thornton et al., 2006).
Robert et al. (2008) Recently demonstrated that OMVs derived from *B. pertussis* can protect against intranasal pertussis challenge when administered by either intraperitoneal or intranasal route in a mouse model of infection (Roberts et al., 2008). Moreover, the isolation of OMVs has important advantages over purified proteins since OMVs extraction based on simple steps eliminates the need for costly prior purification of each antigen, which consist in the current acellular pertussis vaccines (Bottero et al., 2013).

This research is a comparison study between the current OMV extraction method and the modified method. At least five independent OMVs extractions were carried out by both methods where similar morphology and size ranging from 40 to 200 nm were observed in all. Analysis of OMVs isolated in this study demonstrated that they were numerous nanoparticle vesicles, which were fitted perfectly with *B. pertussis* OMVs used in other studies and also with each other (Hozbor et al., 1999; Roberts et al., 2008; Bottero et al., 2013; Hozbor, 2017). The main protective bacterial antigens in the development of effective pertussis vaccines are virulence factors such as filamentous hemagglutinin, fimbriae, and pertactin which allow *B. pertussis* to bind to the ciliated epithelial cells in the upper respiratory tract (Shrivastava and Miller, 2009; Howard, 2016). However, the presence of PTX, PRN, and FHA in the OMVs is very important (Smith et al., 2001; Pawloski et al., 2014). Our findings consequently showed the expression of PTX, PRN, and FHA in OMVs isolated from the vaccinal strain with the current method and modified method. As OMVs reported here contain several protective immunogens, it might be considered as a possible basic material for the development of *B. pertussis* vaccine.

Our results have revealed no noticeable differences in their size and shape as well as in their protein profile but modify product yield a little. It is worth noting that this method was modified from current previous method to avoid the need for ultracentrifuge with high speed which is an expensive and high technology and is not generally available in laboratories and many research centers, so this modify method can be a valuable alternative method, especially in developing countries.

It is obvious that further studies and challenges are necessary to determine the potential immunogenic effect of OMVs in formulation with different adjuvants and the probability of producing immune responses and the efficiency of the OMV as a vaccine candidate to improve the current *B. pertussis* vaccines.

**Ethics**
We hereby declare all ethical standards have been respected in preparation of the submitted article.

**Conflict of Interest**
The authors declare that they have no conflicts of interest regarding the publication of the study.

**References**


Figure 1. Negatively stained *Bordetella pertussis* OMVs examined with an electron microscope. (A) Shows the OMV obtained from *B. pertussis*, with the current method. (B) Shows the OMV obtained from *B. pertussis*, with the modified method. Vesicle sizes are ranging from 40 to 200 nm.

Figure 2. The SDS-PAGE Pattern of OMV Contains PTX1, Prn, FHA. Lane 1 shows the OMV obtained from *B. pertussis*, with the modified method and lane 2 shows the OMV obtained from the current protocol. M: Marker. Molecular weights are indicated at the right.
Figure 3. Western blot of OMVs using anti-PTX S1, anti-PRN and anti-HFA. Lane 1 shows the OMV obtained from *B pertussis* with the modified method and lane 2 shows the OMV obtained from the current protocol. Molecular weights are indicated at the right.