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Comparison of Outer Membrane Vesicles of Three Different Isolates from *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* (PA) is an opportunistic mucosal human pathogen responsible for a wide range of acute and chronic infections. PA releases outer membrane vesicles (OMVs) in all situations and environments. OMVs are bilayered proteolipids ranging in diameter from 50 to 250 nm. Recent studies have demonstrated that OMVs are related to PA pathogenesis. According to strain-dependent components of OMV, in this study, we aimed at identifying significant physicochemical differences among OMVs from lab strain ATCC 17933, an antibiotic-susceptible and an antibiotic-resistant PA clinical strains. **Methods:** OMVs of the three strains were purified using differential centrifugation with deoxycholate and EDTA. Chemical analyses were assessed using nano-drop, SDS-PAGE and the limulus amebocyte lysate (LAL) test. Moreover, electron microscopy was performed to verify the stability and totality of the extracted OMVs. **Results:** The nanodrop method and the LAL test showed that total protein and endotoxin concentrations were significantly different among all the 3 mentioned strains. In addition, the quality control of OMVs illustrated that the lab and the antibiotic-susceptible strains were approximately similar in terms of the vesicle yield and size; however they differed in protein contents. Moreover, OMVs generated from the resistant strain had a higher density, smaller size and sharper protein bands as observed by electron microscopy and SDS-PAGE, respectively. Endotoxins measurement were 2.8, 2.9 and 3 EU/ml for OMVs from the lab, the antibiotic-susceptible and the resistant strains, respectively. **Conclusion:** The results of the current study demonstrated that OMVs of the resistant PA strain may produce vesicles with a particular composition. This characterization profile provides a basis for future studies to elucidate immune responses to OMVs from PA and developing vaccines against Pseudomonal infections as a common nosocomial infection with extremely high resistance to antibiotics.

INTRODUCTION

Pseudomonas aeruginosa (PA) is a common Gram-negative opportunistic pathogen that causes nosocomial pneumonia, acute or chronic infections in the lung, blood stream, urinary tract, and surgical or burn wounds [1]. Patients with cystic fibrosis and hospitalized patients are associated with fatal PA infections due to their weakened immune system [2]. PA releases outer membrane vesicles (OMVs) like other Gram-negative bacteria [3], which are bilayered spherical proteolipids with an average diameter of 20–200 nm. OMVs contain lipopolysaccharide (LPS), glycerophospholipids, proteins of the outer membrane, DNA and RNA [4]. Moreover, phospholipase C, alkaline phosphatase, proelastase, hemolysin [5], murein hydrolases [6], antibiotic resistance [7] and quorum sensing molecules have been reported as PA OMVs contents. OMVs play a significant role in bacterial pathogenesis [8]; therefore, previous studies have paid attention to assay the possibility to use them for the vaccine development. According to the results of these studies, vesicles of Gram-negative bacteria such as *Neisseria meningitides* [9], *Acinetobacter baumannii* [10], *Helicobacter pylori* [11] and *Klebsiella pneumonia* [12] are capable of producing immunity against the infections in animal models. Based on previous studies which have shown the activation of alveolar epithelial cells and induction of interleukin 8 (IL-8) release *in vitro* by PA OMVs [2, 13] as well as the results of our previous study with attention to strong induction of toll like receptor (TLR) pathways (especially intracellular TLRs and inflammatory cytokines [14]), it appears that PA OMVs are capable of inducing an appropriate immune

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response in epithelial cells. It should also be noted that developing new and alternative cure strategies are desperately needed due to the presence of multiple-drug resistant and even pan-drug resistant PA isolates which make it increasingly difficult to treat the related infections [15]. Thus, in the current study, physicochemical properties of PA OMVs of different strains were compared. This could be an important step forward in their application as new biotechnological tools in areas such as vaccine manufacturing, adjuvants and drug delivery.

MATERIALS and METHODS

Bacterial Strains: A laboratory PA strain (ATCC 17933) was obtained from the Pasture Institute of Iran. Moreover, two clinical isolates termed as antibiotic-susceptible and antibiotic (*i.e.* Amikacin, Gentamicin, Ciprofloxacin, Ceftazidime, Cefepime, Imipenem, Ceftriaxone, and Cotrimoxazole)-resistant, were obtained from eye and sputum samples, respectively (Loghman Hospital, Tehran, Iran).

OMV Isolation: OMVs released by the PA strains were isolated by the protocols described previously [2]. Briefly, 500 ml of the strains were grown (at 37°C) overnight. Isolation of OMVs was performed by Tris-HCl, EDTA and 100 g/L deoxycholate and consecutive centrifugation at 20,000 x g for 30 min. Finally, OMVs were pelleted using ultracentrifugation at 125,000 x g for 2 h. The pellets were suspended in sucrose (% 3) and stored at -80˚C before use.

OMV Physicochemical Analyses: The amounts of the purified proteins were measured using spectrophotometry using a NanoDrop instrument (Thermo scientific, USA). The protein contents of the OMVs were assessed using 12% SDS-PAGE and staining was done by Coomassie blue [2].

Scanning Electron Microscopy (SEM): The integrity, morphology and size of the negatively stained OMVs were performed using scanning electron microscopy (SEM). In brief, we placed the vesicles on carbon-coated nickel grids and fixed them using glutaraldehyde (2% in PBS). Then, potassium phosphotangstate was used for negative staining and visualized using a field emission scanning electron microscope (SEM S4160, Hitachi, Japan) at 30 Kv.

Endotoxin Analysis by Limulus Amebocyte Lysate (LAL) Method: The Lipopolysaccharides (LPS) contents of the OMV preparations were assayed according to a Limulus assay using pierce LAL chromogenic endotoxin quantitation kit (Thermo Scientific, USA). The microplate reader (BioTek, USA) was used for reading the plates.

RESULTS

Purification and Physicochemical Comparison of OMVs Derived from Different PA Strains: The integrity of the vesicles was confirmed by images obtained after the SEM examination. Although they all had almost closed vesicular forms, the OMVs of the lab and the antibiotic-susceptible strains were similar in size and density (Figs. 1B, C) while they had different protein compositions (Fig. 1A). Specifically, OMV from both the antibiotic-susceptible and the antibiotic-resistant strains had similar protein compositions with only delicate differences in their band intensities (Fig. 1A). The molecular weights of the PA-derived OMVs ranged along the 11-160 kDa marker and bands corresponding to ~57 kDa could be detected in OMVs of the antibiotic-susceptible and the antibiotic-resistant samples but not in the laboratory strain sample (Fig. 1A, blue arrow). The resistant strain had dense OMVs with the smallest size among the tested OMVs (Fig. 1D) while it had approximately sharper bands with a distinct pattern on SDS–PAGE, compared to the other strains (Fig. 1A). The measured concentrations of the OMVs proteins indicated 0.731, 2.823 and 3.191 mg/ml of protein content for the laboratory, the antibiotic-susceptible and the antibiotic-resistant strains, respectively. Moreover, 2.8, 2.9 and 3 EU/ml were obtained as the LPS amounts in OMVs from the laboratory, the antibiotic-susceptible and the antibiotic-resistant strains, respectively.

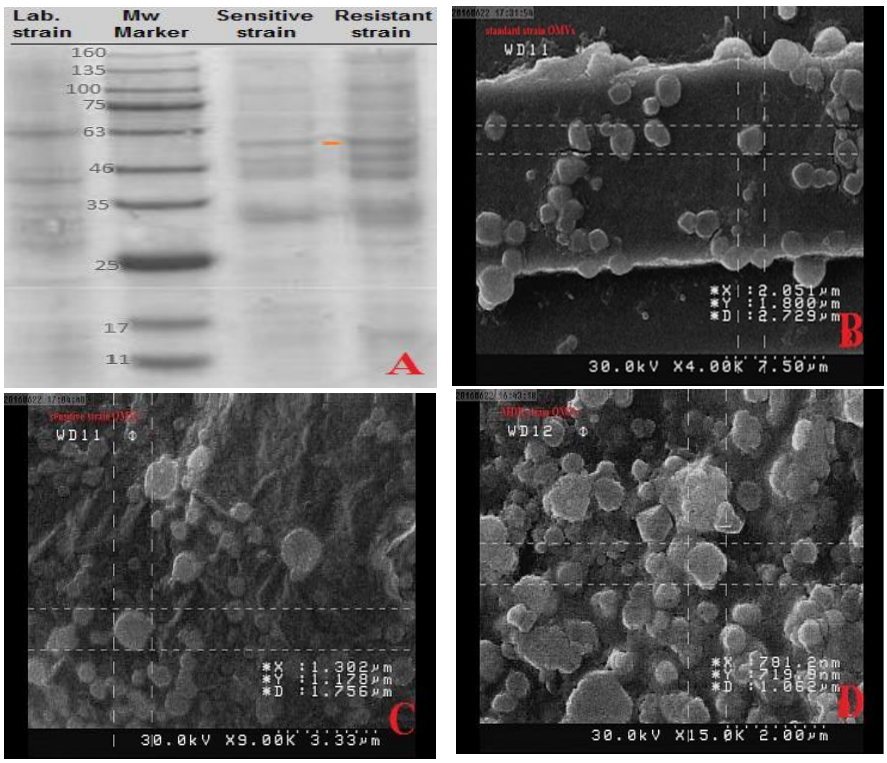

Fig. 1. (A) The SDS-PAGE of the purified OMVs from three different PA strains; A ~57 kDa band could be detected in vesicles from the antibiotic-susceptible (sensitive) and the antibiotic-resistant strains. **(B, C, and D)** SEM images of the purified OMVs of the laboratory, the antibiotic-susceptible and the antibiotic-resistant strains, respectively.

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DISCUSSION

In our previous study, we showed that intestinal epithelial cells exposed to OMVs of different PA strains would lead to strain-specific induction in the expression profiles of TLRs, signaling pathway-related genes and different secretions levels of pro-inflammatory cytokines and chemokines [14]. Our data demonstrated that the vesicle sizes, their contents and their amount are strain-dependent. Here, to isolate the OMVs of the tested PA, we performed the ultracentrifugation method, which is the most common method for OMV isolation. We also applied SEM for the confirmation of the spatial shape and sizes of the PA-derived vesicles which indicated that the sizes were within ~ 30-250 nm range. Further, the PA resistant strain showed dense OMVs with variable sizes which were more spherical and smaller than the vesicles from the other tested strains (Figs. 1B, C, D). Strain-dependent differences in the vesicle yield, morphology, size, and protein compositions were expected, as reported by previous studies [16, 17, 18], which may be due to the composition of the vesicles [19]. In addition, our data were consistent with previous results, demonstrating that pathogenic bacteria produce more vesicles with smaller sizes than their non-pathogenic counterparts [20, 21] . The molecular weights of the tested OMVs ranged from 11 to 160 kDa and were highly consistent with previous studies [22, 23]. Bands corresponding to the molecular weight of ~57 kDa were visible in the susceptible and the resistant vesicles which could be the protein identified as an extracellular aminopeptidase determined PaAP [16, 24]; although its proper identification require further studies. PaAP has been specified recently as a zinc-dependent leucine aminopeptidase that is partly controlled by the las quorum-sensing system [2]. The average size of the purified vesicles was consistent with vesicle sizes reported for PA and other bacteria [25]. We observed that vesicles from the resistant strain were smaller and more spherical with more protein and LPS content. As stated by Bauman and Kuehn, the protein and LPS ratios can affect the size and shape of the OMVs [2].

In conclusion, our results demonstrated that the conformation and components of PA-derived OMVs were strain dependent. However, the resistant strain produced vesicles with a particular composition. Identification of active components of PA secreting OMVs and its potential therapeutic use remain to be investigated. Infections caused by PA are increasingly difficult to be cured due to the emergence of multiple-drug resistant isolates [26]. Moreover, further researches are necessary to elucidate the safety, efficacy, practicality and mechanisms of action of PA OMVs in therapeutic and preventive clinical practices, especially as vaccine delivery vehicles.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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