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THE CYTOTOXICITY EFFECTS OF OUTER MEMBRANE VESICLES ISOLATED FROM HOSPITAL AND LABORATORY STRAINS OF PSEUDOMONAS AERUGINOSA ON HUMAN KERATINOCYTE CELL LINE

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ABSTRACT *Pseudomonas aeruginosa* is a leading cause of human morbidity and mortality, and it often targets epithelial surfaces. It procures pathogenicity by secreting outer membrane vesicles (OMVs) as a virulence factor. This study was aimed at determining the amount of OMVs of two strains of *Pseudomonas aeruginosa*, and evaluate the cytotoxicity of the isolated vesicles on human keratinocyte cell line (HaCaT). The results indicate that virulence factor of isolates from hospital strain was comparatively higher compared with the laboratory strain. This is consistent with earlier findings that the toxicity of hospital strain outweighed the laboratory strain. Laboratory strain that was treated with gentamicin showed a higher amount of OMVs compared with untreated strain. The antibiotic stimulated the bacteria to secrete more virulence factors seeking for protection. The number of bacteria (CFU) is inversely proportional to the release of OMVs. The toxic effect of outer membrane vesicles which acted as stress factor showed a loss of biomass in hospital strain and a moderate increase in the laboratory strain, and that supports earlier findings that hospital strains are more toxic. Cells biomass indicate the cells continue to grow rapidly even when they are treated with low concentrations of OMVs.

Keywords: Cytotoxicity, HaCaT cells, Keratinocytes, Outer-membrane Vesicles (OMVs), *Pseudomonas aeruginosa*

1. INTRODUCTION

Most bacteria produce extracellular and surface-associated components, such as membrane vesicles (MVs), extracellular DNA and moonlighting cytosolic proteins. Their biogenesis and export pathways are still not fully understood (Turnbull et al., 2016). Among gram negative bacteria, *Pseudomonas aeruginosa* is one of the most dangerous opportunistic pathogen that primarily infects immune compromised hosts, such as patients suffering from diabetes, cancer, AIDS, burns, and those suffering from chronic obstructive pulmonary disease and cystic fibrosis (Abdul Khalil et al., 2020; Krivonogova, Isaeva, Loretts, & Chentsova, 2019). *Pseudomonas aeruginosa* is now recognised as the most common cause of

disease in a variety of hosts (Shah et al., 2019). According to (Bjarnsholt et al., 2013; Sønderholm et al., 2017) that developed *P. aeruginosa* biofilms in vivo after interacting it with host cells showed bacterial cell agglomerates were embedded in the host material and were continuously exposed to the host's own nutrients flows, an entire range of the circulating communication cues, and to various host defence mechanisms. Most of the bacterial MVs were first found to be produced through controlled blebbing of the outer membrane of Gram-negative bacteria including *P. aeruginosa*. Therefore, they are often referred to as outer-membrane vesicles (OMVs) (Toyofuku, Nomura, & Eberl, 2019). The OMVs of gram negative bacteria are small membrane vesicles approximately 50-300 nm in size, and composed of lipopolysaccharides (LPS), various phospholipids, and protein. and in some, there are periplasmic components (Klimentová & Stulík, 2015). It was reported in (Kulp & Kuehn, 2010) that the increased release of OMVs is in response to external environmental factors, such as toxins, high temperature, contaminants, antibiotics and many other secondary metabolites, and also through the activation of the SOS response. Other studies suggested a positive correlation between stress conditions and the production and release of OMVs (Baumgarten et al., 2012; Sharma et al., 2008).

The OMVs are important virulent structures in *P. aeruginosa*, according to (MacDonald & Kuehn, 2012) who showed that the interaction of bacteria with host (specially human body) triggered release of OMVs, carrying many types of proteins and other adhesion molecules, such as aminopeptidase, on the bacterial cell surface. The *P. aeruginosa* OMVs act as bridges which accelerate the adhesion of cells with the host tissues (Wai et al., 2003) and help bacteria to invade the host. Many articles have been published regarding bacterial OMV release and the effects of stress factors (Baumgarten et al., 2012; Sharma et al., 2008), but only a few of them investigated the keratinocyte cell line (HaCaT) biomass and compared the actions of two different strains of bacteria. The current research is focused on isolating and quantifying the concentration of OMVs of two different strains of *P. aeruginosa*. Additionally, it investigates the cytotoxic effects of the isolated OMVs on human keratinocyte cell line (HaCaT) biomass.

2. MATERIAL AND METHODS

2.1. Microorganisms and preparation of inoculums

Two strains of *Pseudomonas aeruginosa* procured from a laboratory (NCIMB 10421) and a hospital (Ps3) were grown in plates of tryptone soy agar (TSA). They were incubated at 37°C for 24 hrs in aerobic conditions.

2.2. Isolation and quantification of OMVs

The outer membrane vesicles (OMVs) were isolated based on a well-established method first described by (Kadurugamuwa & Beveridge, 1999). In brief, each colony of *P.aeruginosa* was inoculated with 10 ml of tryptone soy broth (TSB) and incubated at 37°C overnight. The colony of *P. aeruginosa* (PS3) was inoculated with 300 ml of TSB and were placed in a shaker bed overnight at 37°C, 120 rpm. The culture was centrifuged at 10,000 x g (Beckman Coulter Avanti J-26 XPI centrifuge, F250 rotor) for 20 mins. The laboratory strain was inoculated with and without the presence of gentamicin (50µg/ml) in TSB. The culture was centrifuged and the supernatant was collected and filtered through 0.45µm pore, and the filtrate was ultra-centrifuged at 4°C for 2 hours to pellet out the outer membrane

vesicles. The precipitate was collected and ultra-centrifuged again at 4°C for 1.5 hrs. The supernatant was removed and the pellet obtained was re-suspended in different volumes for each strain in HEPES buffer PH 6.8, and the supernatant was filtered through 0.45 µm pore and stored at -20°C for further use.

2.3. Determination of bacterial count

Viable bacterial count was carried out before the isolation of OMV. 1ml of the bacterial culture was taken and diluted with the following concentrations: 10^2,10^4,10^6,10^8 and plated on TSB at 37°C overnight before the colonies were counted.

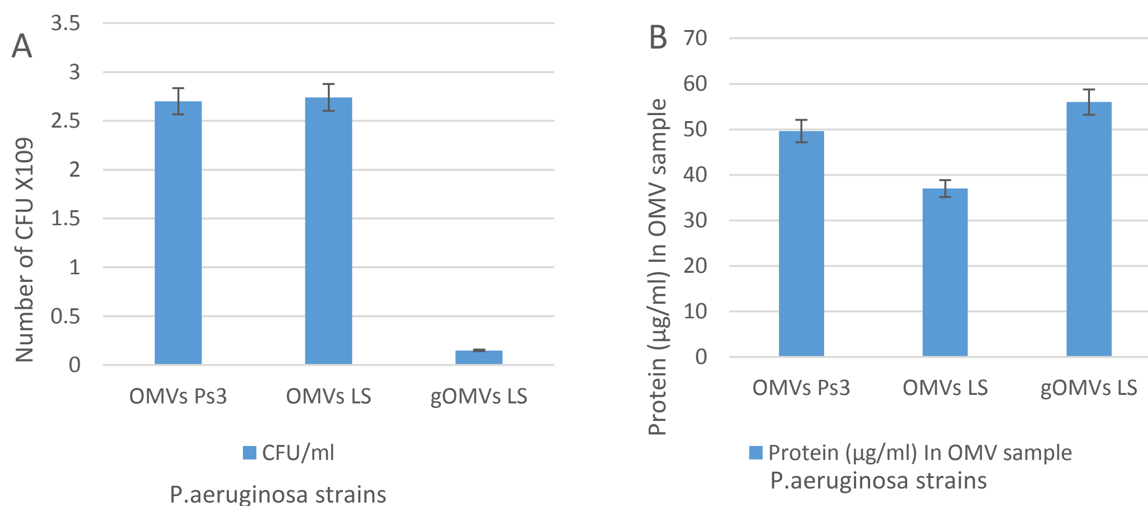

Figure 1. A. Bacterial count as Colony forming unit (CFU/ml). B. Protein concentration of OMVs purified from *P.aeruginosa* strains.

2.4. Concentration of OMVs

The different OMV concentrations were determined using Lowry assay. 40µL of all standard and OMVs protein samples were added into a 96 well plate; 200µL of Modified Lowry Reagent was added to each later using a multi-channel pipette. The contents of the wells were mixed immediately on a plate mixer for 30 seconds. The microplate was covered with sealing tape and incubated at room temperature (RT) for 10 minutes. 20µL of 1X Folin-Ciocalteu Reagent was added to each well and mixed immediately for 30 seconds. The microplate was covered and incubated for 30 minutes at room temperature. The average absorbance value at 750nm was measured using a plate reader.

2.5. Keratinocytes challenge with OMVs

Human keratinocyte cell line (HaCaT) was obtained from cell line services and grown in high concentration of glucose 4500mg/l in Dulbecco's modified Eagle's Medium (DMEM); the culture was supplemented with foetal bovine serum (FBS10%) and 2mM L-glutamine. The cell was incubated at 37°C with 100% constant humidity. The HaCaT cells were seeded into 6 well plates and incubated at 37°C until they became confluent. A week later the cells were challenged with 1ml of several concentrations of OMVs in tissue culture media for 4 hrs. The solution was later removed and 1 ml of tissue culture media was added to each well and incubated over night at 37°C.

2.6. Cytotoxicity procedure

Approximately 5000 cells were seeded onto 96 wells in tissue culture media and they were incubated overnight. The next day, the cells were challenged with 100µl of known concentration of OMV from either strain of *P. aeruginosa* or HEPES buffer as control for 4 hrs. The supernatant was later removed and the media was added to the cells and incubated for further 24 hrs. ELISA assay was carried out and pictures were taken for a visual assessment of cell viability.

3. RESULTS

3.1. Isolation and quantification of OMVs

The results are tabulated (Table 1) and they showed that protein concentration of OMVs for hospital strain (OMVs Ps3) and laboratory strain (OMVs LS) was 49.6µg per 1ml of bacteria and 37 µg per 1 ml of bacteria respectively. The laboratory strain with the presence of gentamicin (gOMVs LS) was 56 µg per 1ml of bacteria.

Table 1. Concentration of OMVs purified from *P.aeruginosa* strains, and CFU/ml of bacteria

Sample	CFU/ml	A750 with 40 µl of protein	Protein in OMV sample
OMVs Ps3	2.70×10^9	2.48	49.6 µg/ml
OMVs LS	2.74×10^9	3.70	37 µg/ml
gOMVs LS	0.15×10^9	2.80	56 µg/ml

3.2. Cytotoxicity assay

Picture (A) shows HaCaT cells without OMV challenge; picture (B) shows cells challenged with 14.28µg/ml of lab strain; picture (C) shows cell challenged with 71.4µg/ml of OMVs from the lab strain. Picture (D) shows cells exposed to 5.71 µg/ml of hospital strain OMV and picture (E) shows cells challenged with 28.55 µg/ml of the hospital strain. The enzyme-linked immunosorbent assay (ELISA) revealed that higher concentration of hospital strain OMV causes significant loss of cell viability.

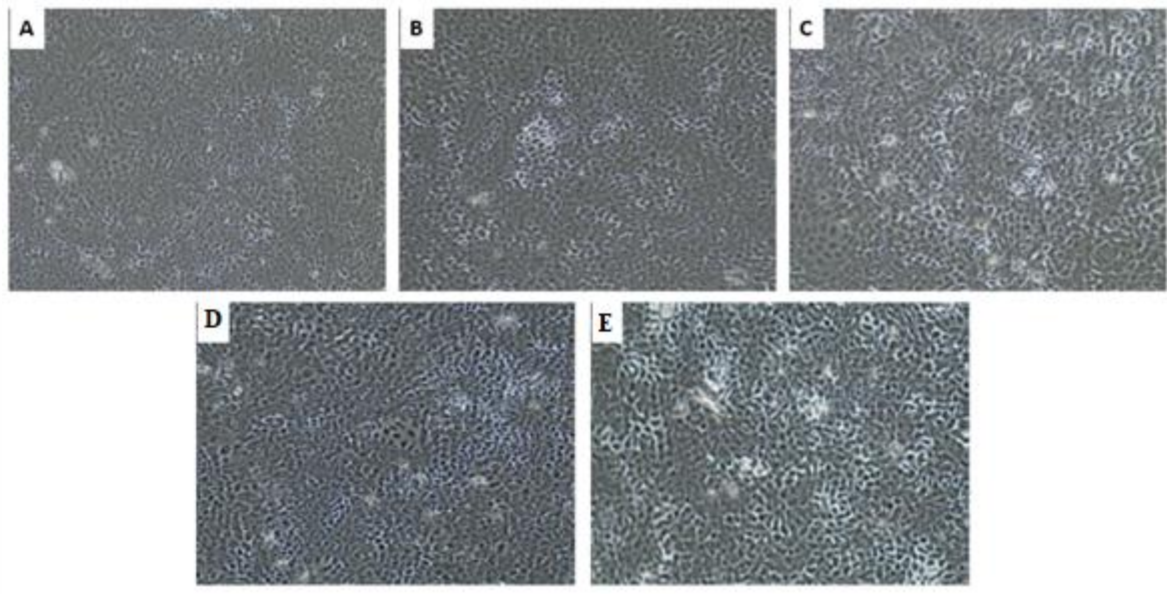

Figure 2. ELISA images showing cultured keratinocytes treated with OMV. (A) Normal HaCaT cells, (B to E) HaCaT cell challenged with different OMV concentrations

3.3. Cytotoxicity assay results

The biomass was calculated for each reading and its average plotted to measure the challenged cells. The biomass for the cells treated with 5.71µg/ml and 28.5µg/ml of hospital strain was about 105.055% and was 82.01211% respectively . Cells treated with 14.28µg/ml and 71.4µg/ml of lab strain was 130.6223% and 98.11628% respectively. The pie chart (Figure 3) shows the loss of cell biomass when treated with 28.5µg/ml OMV from the hospital strain.

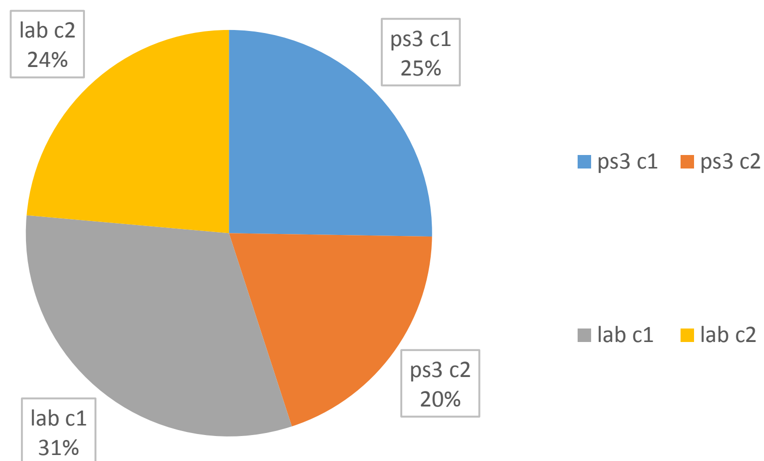

Figure 3. The average loss of cell biomass for HaCaT cells challenged with OMVs of hospital strain and laboratory strain in two different concentrations.

4. DISCUSSIONS

Outer membrane vesicles can potentially influence bacterial survival and pathogenesis. The results of the present study indicate vesicle yield in both hospital and laboratory strain in the presence of gentamicin antibiotic and this is measured by their protein concentration. In a similar study, (Metruccio, Evans, Gabriel, Kadurugamuwa, & Fleiszig, 2016) the authors used mucosal fluid as a stress factor to trigger OMV release from *P. aeruginosa*, and they concluded that the former greatly enhanced the latter compared with control strain that was treated with phosphate buffered saline. Other studies tested polymyxin and hydrogen peroxide as stress molecules resulting in similar increase in OMV release, which indicate that weakening the integrity of the bacterial cell wall stimulates OMV production. In contrast, tetracycline, a protein synthesis inhibitor targeting ribosome, had no effect on OMV production (MacDonald & Kuehn, 2013; Yahya, Alhawari, Amhimmid, AbuAeshah, & Saada, 2018; Yun et al., 2018). Viable bacterial count (VBC) was slightly different among the two strains and this might be due to the difference in OMV production. However, bacterial count (CFU) is inversely proportional with the amount of OMVs released. The VBC for hospital strain was slightly lower than the unchallenged laboratory strain. (Klimentová & Stulík, 2015) reported that production of OMVs depends on several stress factors, such as temperature, CFU of the bacteria, nutrition factors in the media, and other stress inducers. They found biosynthesis of cell wall inhibitor compounds, such as gentamycin and d-cycloserine, in addition to the OM targeted antimicrobial peptide polymyxin B, increased OMVs in *P. aeruginosa* (Klimentová & Stulík, 2015).

The present study found protein yield was higher for *P. aeruginosa* treated with gentamycin. Recent studies (Kameli, Beuken, Savelkoul, & Stassen, 2018; Perez-Cruz, Brianso, & Mercade, 2018) concluded that OMVs contribute to antibiotic resistance. The greater the OMV yield, the higher the resistance to antibiotics based on OprH protein in amino glycoside. The results of HaCaT cells biomass showed that the cells were not affected by the OMVs, where higher concentration of laboratory strain OMVs showed a very slight effect on cell viability a 71.4 µg/ml. The OMVs of 28.55 µg/ml from the hospital strain showed a lower biomass. Thus, these results support earlier findings that the hospital strain is more toxic compared with the laboratory one (Bushell, Tonner, Jabbari, Schmid, & Lund, 2019; Halstead et al., 2015). Multiple virulence factors of most gram negative bacteria capable of cytotoxic effects to human cells are associated with OMVs that include but not limited to, protease (elastase), alkaline phosphatase, phospholipase C, and Cif (Metruccio et al., 2016). However, current results indicate that HaCaT cells are not affected by low concentrations of OMVs for both laboratory and hospital strains respectively (14.28µg/ml, 5.71 µg/ml). Data showed that at low concentration of OMVs, the cells continued to grow rapidly leading to the increase of overall biomass compared with the control, and this was evident with the first concentration for the laboratory strain. These findings are consistent with that of (Al-Tameemi et al., 2014) who reported that stimulation of cells with low concentration of doxorubicin and 4-OH-CP led to an overall increase in HaCaT cells biomass. The results of the current study show that the most affected cells were the one exposed to the second concentration of hospital strain and the less affected cells were the first concentration of

OMVs for laboratory strain. Therefore, the OMVs for hospital strain are more toxigenic than the OMVs for laboratory strain as shown in the pictures (Kukavica-Ibrulj et al., 2007). The biomass and pictures indicate that even when treated with low concentration of OMVs, HaCaT cells grow rapidly.

5. CONCLUSION

The current study shows the highest OMVs were detected was from laboratory strain treated with gentamicin. It is believed the response to the antibiotic might have stimulated the bacteria to secrete more virulence factors seeking for protection. The OMVs in the isolated hospital strain was higher than the laboratory strain, which indicates it is more pathogenic. The toxicity of OMVs was determined by culture of HaCaT cells treated with two different concentrations for both hospital and laboratory strain. The most affected cells were the cells treated with 28.55 µg/ml concentration of OMV from hospital strain which showed maximum loss of biomass of the cells compared with other strains. The other concentrations showed increase of biomass of the HaCaT cells, meaning that the cells were not affected by OMVs. Even with low concentration of OMVs, the cells grow rapidly.

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