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MOLECULAR CHARACTERIZATION AND IMMUNOSTIMULATORY PROPERTIES OF EXTRACELLULAR MEMBRANE VESICLES (EMVS) OF *Streptococcus pneumoniae*

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ABSTRACT

Streptococcus pneumoniae causes invasive and non-invasive diseases such as pneumonia, sepsis, meningitis, and otitis media, especially in young children and the elderly. There are over 103 serotypes of *S. pneumoniae*, but current vaccines protect against only 24 serotypes. Therefore, universal vaccine candidates are necessary for alternative prevention. Recent research has revealed that gram-positive bacteria like *S. pneumoniae* can produce extracellular membrane vesicles (EMVs). The role of EMVs as carriers for bacterial proteins is well established, but their impact on cellular processes and immune system interactions remains poorly understood. This study investigate the molecular characterization and immunostimulatory properties of EMVs from different serotypes of *S. pneumoniae*. First, *S. pneumoniae* isolates were isolated from invasive and non-invasive sites. After that, these isolates were identified using gram stain, catalase test, optochin test and bile solubility test. Then, the serotypes of *S. pneumoniae* isolates were determined using multiplex PCR. In this study, the EMVs were extracted using ultracentrifugation, ultrafiltration, and iodixanol gradient fractionation methods. Subsequently, the EMVs were observed under the transmission electron microscope and the proteins in the EMVs of *S. pneumoniae* isolates were identified using liquid chromatography-mass spectrometry (LC-MS). VaxiJen v.2.0 was used to evaluate the protein antigenicity and the protein subcellular localization was determined using PSORTb v3.0.2. For cytotoxicity assessment, the A549 lung epithelial cells were treated with EMVs at concentrations of 25, 50, and 100 µg/mL. Lastly, the immune response in the A549 lung epithelial cell line upon treatment of the EMVs from various serotypes of *S. pneumoniae* (6A, 14, 19A, 19F, 23F) were elucidated using RT^2 Profiler™ PCR Array Human Innate and Adaptive Immune Response. These five serotypes were shown to release extracellular vesicles, albeit in varying sizes (22 nm -250 nm). The LC-MS/MS analysis of *S. pneumoniae* EMVs identified 61 proteins, including 12 highly antigenic candidates such as PspA, PavB, HtrA, and ZmpB, which are known virulence factors involved in immune evasion and bacterial pathogenesis. The presence of membrane, cell wall, and extracellular proteins suggests that EMVs could serve as antigen delivery vehicles, potentially eliciting protective immune responses. For the MTT assay, according to the ISO 10993-5:2009 cytotoxicity classification, EMVs at concentrations of 25 µg/mL and 50 µg/mL exhibit weak cytotoxicity (viability above 60%). At 100 µg/mL, EMVs show moderate cytotoxicity, with the lowest viability recorded at 64%. These results suggest that EMVs are not highly cytotoxic to A549 lung epithelial cells, as viability remains above 40% in all tested conditions From the PCR array analysis, this study demonstrates that EMVs derived from various serotypes of *S. pneumoniae* can induce both innate and adaptive immune responses in the A549 lung epithelial cell line. The activation of innate immune pathways not only initiates immediate defense mechanisms but also primes the adaptive immune system for a more tailored and sustained response. The release pattern of cytokines from the treated A549 lung epithelial cell line was altered by EMVs, suggesting a potential immune system interaction. These findings suggest that EMVs from *S. pneumoniae* have potential as a basis for novel vaccine strategies. However, further *in vivo* model is required to validate their immunogenicity and efficacy.

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CHAPTER 1
INTRODUCTION

1.1 Research Background

Streptococcus pneumoniae (pneumococcus) causes invasive and non-invasive diseases such as pneumonia, sepsis, meningitis, and otitis media, especially in young children and the elderly (Lister et al., 2021; Ezhumalai et al., 2020; Jefferies et al., 2014; Johnson et al., 2010). One of the characteristics that attributes to the virulence of *S. pneumoniae* is the polysaccharide capsule that allows the bacterium to avoid the host immune system (Nelson et al., 2007), and this is used for the epidemiological classification of strains into serotypes and serogroups (Geno et al., 2015). There are about 103 different capsular pneumococcal serotypes that have been reported based on the capsular polysaccharide variations. The distribution of pneumococcal serotypes varies geographically, and some serotypes are specifically correlated with disease in either children or adults (Hausdorff et al., 2000). A few pneumococcal vaccines have been developed based on differences in their polysaccharide capsule composition. The first Pneumococcal Conjugate Vaccine (PCV), approved in 2000, covered seven serotypes (PCV7: 14, 6B, 19F, 23F, 4, 9V and 18C) (Hicks et al., 2007), followed by PCV10 (includes serotypes covered in PCV7 and serotypes 1, 5 and 7F) in 2009 (Esposito and Principi, 2015) and PCV13 (includes serotypes covered in PCV10 and serotypes 3, 6A and 19A) in 2010 (Geno et al., 2015). Other vaccines have been developed, such as the 15 serotype Vaxneuvance (manufactured by Merck Sharp & Dohme Corporation) and the 20 serotype Apexxnar (manufactured by Pfizer, Inc.). Both of these vaccines have recently been approved for use by the European Medicines Agency and the Food and Drug Administration for implementation in those aged above 6 weeks and 18 years, respectively (Centers for Disease Control and Prevention, 2023; European Medicines Agency, 2023). The pneumococcal polysaccharide serotype vaccine (PPSV23) was introduced in 1983 but is less immunogenic than conjugate vaccines especially in infants although it targets 23 different capsular polysaccharides (1, 2, 3, 4, 5, 6B, 7F, 8, 9 N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C,19A, 19F, 20, 22F, 23F and 33F) (Diao et al., 2016).