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Extraction of Extracellular Membrane Vesicles (EMV) from *Streptococcus pneumoniae* using Ultracentrifugation, Ultrafiltration and Iodixanol Gradient Fractionation

Nurul Fathiyah Zaipul Anuar[1], Mohd Nasir Mohd Desa[2], Jamal Hussaini[3,4], Eng Hwa Wong[5] , Vanitha Mariappan[6], Kumutha Malar Vellasamy[7], and Navindra Kumari P[1,3*]

[1]Institute of Medical Molecular Biotechnology (IMMB), Faculty of Medicine, Universiti Teknologi MARA (UiTM) Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia

[2]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia

[3]Department of Medical Microbiology and Parasitology, Faculty of Medicine, Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, Sungai Buloh, Selangor, Malaysia

[4]Institute of Pathology, Laboratory and Forensic Medicine (I-PPerForM), Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, Sungai Buloh, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia

[5]School of Medicine, Taylor's University Lakeside Campus, Subang Jaya, Selangor, Malaysia

[6]Center for Toxicology and Health Risk Studies, Faculty of Health Sciences, Universiti Kebangsaan Malaysia (UKM), Jalan Raja Muda Abdul Aziz, 50300, Kuala Lumpur, Malaysia

[7]Department of Medical Microbiology, Faculty of Medicine, Universiti Malaya, 50603 Kuala Lumpur, Malaysia

*Corresponding author: navindra@uitm.edu.my

Abstract

Extracellular membrane vesicles (EMVs) are membranous structures that are excreted by gram-positive bacteria. These vesicles are involved in a multitude of biological functions, essential for adaptability to the environment, cellular component exchange, antigen and virulence factor distribution, and infection transmission. Recently, bacterial EMVs have gained attention due to their potential as highly effective vaccine targets. However, extraction of EMVs from bacterial cells has been difficult, especially among gram-positive organisms. This study aimed to optimize a method to extract EMVs from *Streptococcus pneumoniae* which can be used as a potential vaccine candidate. The EMVs of *S. pneumoniae* was extracted from its common serotypes (6A, 14, 19A, 19F, and 23F) using ultracentrifugation, ultrafiltration, and iodixanol gradient fractionation. The extracted EMVs were validated by viewing their morphology using a transmission electron microscope (TEM). The six *S. pneumoniae* serotypes used were found to release extracellular vesicles, albeit in different numbers and sizes (22 nm - 250 nm). They are believed to contain various biologically active proteins required for bacterial nutrient acquisition, biofilm formation, and pathogenesis. The success of extracting EMVs from *S. pneumoniae* using a modified method has paved a path to study better drug targets for *S. pneumoniae* since bacterial EMVs are non-viable components of the bacteria that act as an antigen, hence able to induce host immune response. This suggests EMVs as potential vaccine candidates for this bacterium.

Keywords: *Streptococcus pneumoniae*, extracellular membrane vesicle, ultracentrifugation

Introduction

Streptococcus pneumoniae causes invasive and non-invasive diseases such as pneumonia, sepsis, meningitis, and otitis

media, especially in young children and the elderly (Henriques-Normark & Tuomanen, 2013; Scelfo et al., 2021). One of the characteristics that contribute to the virulence factor of *S. pneumoniae* is the polysaccharide capsule that allows the bacterium to avoid the host immune system (Brooks and Mias, 2018; Paton and Trappetti, 2019), and this is used for the epidemiological classification of strains into serotypes and serogroups (Yu et al., 2019). About 98 different capsular pneumococcal serotypes have been reported among 46 serogroups, based on the biochemical structures of the capsular polysaccharide (Geno et al., 2015). The distribution of pneumococcal serotypes varies geographically, and some serotypes are specifically correlated with disease in children or adults (Grant et al., 2023). A few pneumococcal vaccines have been developed based on different polysaccharide capsule serotypes. The first pneumococcal conjugate vaccine (PCV), approved in 2000, provides coverage for seven serotypes (PCV7: 14, 6B, 19F, 23F, 4, 9V and 18C) (Hicks et al., 2007), followed by PCV10, (provides protection against serotypes 1, 5 and 7F in addition to serotypes included in PCV7) in 2009 (Esposito and Principi, 2015) and PCV13 (provides protection against serotypes 3, 6A and 19A in addition to serotypes included in PCV10) in 2010 (Geno et al., 2015). The pneumococcal polysaccharide vaccine (PPSV23) is less immunogenic than PCV, especially in infants and 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). In order to combat pneumococcal disease, a more universal and non-serotype-based candidate is essential, hence the extracellular membrane vesicles (EMVs) were explored. EMVs are membranous structures excreted by gram-negative and gram-positive bacteria. These vesicles are involved in a multitude of biological functions, essential for adaptability to the environment, cellular component exchange, antigen and virulence factor distribution, and infection transmission. Recently, bacterial EMVs have gained attention due to their potential as highly effective vaccine targets. In a similar context, the outer membrane vesicles (OMVs) released by gram-negative bacteria, which developed from the outer membrane of gram-negative bacteria under the control of membrane blebbing, have been the main subject of studies on bacterial EMVs thus far (Brown et al., 2015; Toyofuku et al., 2019). While gram-negative bacteria are well-known for their extracellular vesicle (EV) production, gram-positive bacteria have been largely ignored because of the assumption that their thick peptidoglycan cell wall prevents the release of EMV due to physical barriers (Brown et al., 2015; Toyofuku et al., 2019). Research on the biogenesis of EMVs in gram-positive bacteria has been sparked by the discovery of physiologically active EMVs from these bacteria in recent years (Furi et al., 2017). However, due to technical limitations, it is still difficult to observe and quantify EMV formation in gram-positive bacteria (Toyofuku et al., 2019). Besides that, extracting EMVs from gram-positive bacterial cells has been challenging (Konoshenko et al., 2018; Northrop-Albrecht et al., 2022).

In this study, we optimized a method to extract EMVs from *S. pneumoniae* which can be used as a potential vaccine candidate. The EMVs of *S. pneumoniae* were extracted from common serotypes (6A, 14, 19A, 19F, and 23F) using a combination of three methods (ultracentrifugation, ultrafiltration and iodixanol gradient fractionation). The extracted EMVs was validated by viewing its morphology by transmission electron microscope (TEM).

Material and Methods

Bacterial Strains and Growth Conditions

The six *S. pneumoniae* strains of serotypes 6A, 14, 19A, 19F, and 23F were used in this study. The bacteria were cultured onto blood agar and incubated at 37°C in the presence of 5% CO_2 for 16 to 24 hours. The colonies were then stored in brain heart infusion (BHI) broth supplemented with 20% glycerol and kept at -80°C until further use. For the purpose of EMV

extraction, the strains were grown in 1L BHI broth at 37°C supplemented with 5% CO_2 until an OD_{600nm} 1.0 was reached.

Extraction and Purification of EMVs

The OD_{600nm} 1.0 culture were pelleted at 17,000 X *g*, at 4°C for 30 mins. Then, the supernatants were filtered using vacuum filtration (PES with 0.22 µm pore size) (JetBiofil, China). Following that, the supernatant was concentrated and ultrafiltrated at 5000 X *g*, 4°C, 30 mins using Amicon® Ultra-15 Centrifugal Filter Devices (Merck, USA). Subsequently, the concentrated supernatant was transferred into 13.2 mL, Open-Top Thinwall Ultra-Clear Tube, 14 x 89 mm (Beckman, USA) and ultracentrifugation was done using Beckman Optima XL-100 K Ultracentrifuge. These tubes were centrifuged at 120,000 X *g* for 2 hrs at 4°C to cause sedimentation of the vesicles and the supernatant was discarded. Next, the pellet which contained vesicles were washed and resuspended in phosphate-buffered saline (PBS). Crude vesicle preparations were further purified by density gradient centrifugation using OptiPrep density gradient medium (Sigma, USA). Briefly, the density gradient was prepared by layers of different concentration (5%, 30% and 50%) of OptiPrep density gradient medium (Sigma, USA). Next the crude vesicle of EMVs was added on the top of the layer and the gradients were centrifuged at 155,000 X *g* for 3 hrs at 4°C, and the first 3 mL on top (containing the EMVs) was collected and stored at -80°C.

Observation of EMVs under Transmission Electron Microscope (TEM)

A 2% aqueous solution of uranyl acetate (staining reagent) was prepared. A drop of sample (suspended liquid) was placed onto a copper grid coated with carbon film (400 mesh). The sample was let to deposit 5 mins and then excess sample was removed with filter paper. Small drop of uranyl acetate was applied on the carbon coated grid for 10 sec and washed quickly with a small drop of distilled water. Next, excess liquid was removed using filter paper and the copper grid was let to air dry at room temperature before viewing by TEM instrument (Thermo Scientific Talos L120C) operated at 120 kV.

Results

The findings showed that the EMVs from all six serotypes were successfully recovered by employing three combinations of procedures (ultracentrifugation, ultrafiltration, and iodixanol gradient fractionation). These methods were used in conjunction with one another. The extracted EMVs had their morphology examined with a TEM in order to verify their authenticity. The six serotypes that were used in this study all showed evidence of releasing EVs, albeit in a variety of quantities and sizes. Figure 1 demonstrates that the diameter of the EVs ranges from 20 to 250 nm.

Discussion

Bacterial extracellular vesicles (BEVs), can be isolated through the use of a variety of different methods, including differential centrifugation or ultracentrifugation, microfiltration, sucrose or iodixanol gradient centrifugation, antibody-coated magnetic beads, microfluidic / lab on a chip (LOC) device, and size-exclusion chromatography (SEC) (Theel & Schwaminger, 2022; Sun & Sethu, 2017). Although differential centrifugation is the primary and most popular method for isolating and purifying EVs, different users' methods might result in inconsistent EV recovery. This is primarily due to variations in the viscosity of the biofluid that is being used. For instance, microfiltration techniques can be employed to skip the initial centrifugation stage, which can both shorten the isolation process and increase the quality of the EVs that are isolated. This can have a positive impact on both aspects. In addition, additional purification procedures can be conducted after the stage of final ultracentrifugation, such as sucrose or iodixanol gradient centrifugation, to produce a collection of EVs with a higher purity that is

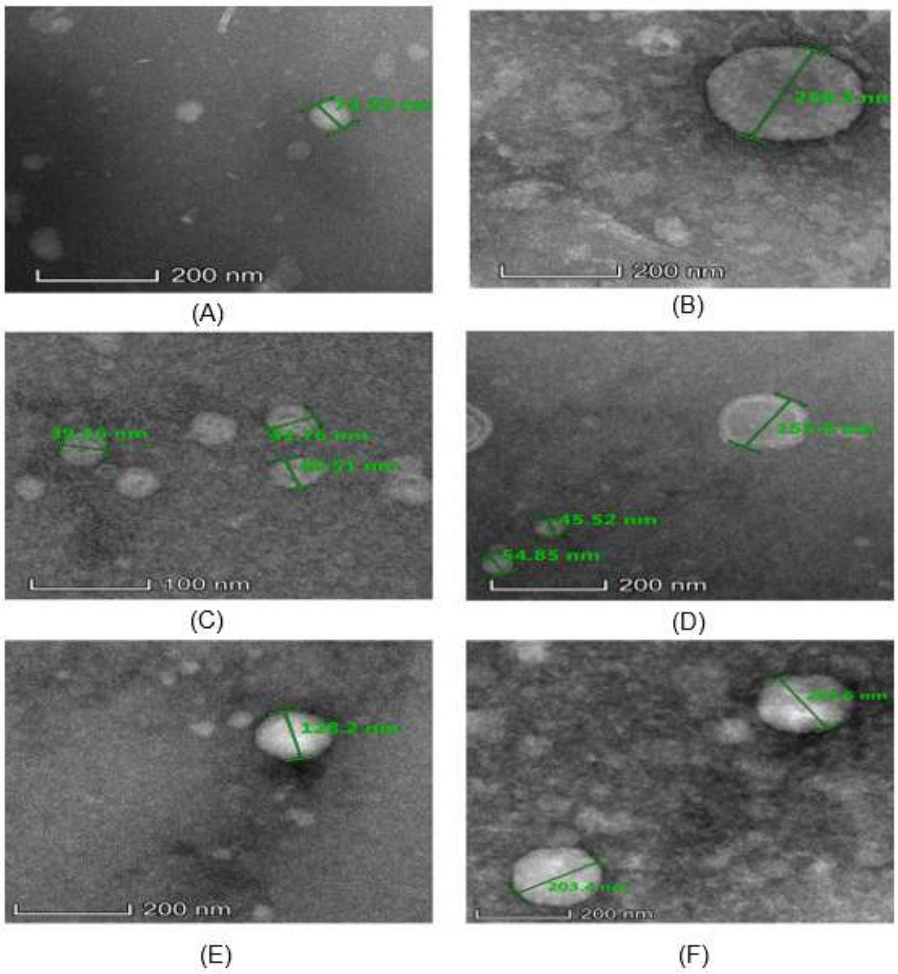

Figure 1: Extracellular membrane vesicles (EMVs) from different serotypes of *Streptococcus pneumoniae* under transmission electron microscope (TEM). (A) ATCC 49619 (serotype 19F); (B) Sample T39 (Serotype 19F); (C) Sample T19 (Serotype 14); (D) Sample T43 (Serotype 6A); (E) Sample T28 (Serotype 19A); (F) Sample T20 (Serotype 23F).

free of EV-associated proteins and nucleic acid. These additional purification steps can be undertaken in order to obtain the desired result. In this study, we used three combinations methods which are ultracentrifugation, ultrafiltration, and iodixanol gradient fractionation. Firstly, for the ultracentrifugation phase, the process can be performed in several different ways, but it usually involves two stages of centrifugation: initially, a low-speed spin that eliminates dead cells and bulky apoptotic debris; and second, a higher speed spin, which can range anywhere from 1,000 *g* to 20,000 *g* (depending on the availability in the laboratory), to eliminate larger vesicles and debris (Momen-Heravi et al., 2013). According to Konoshenko et al. (2018), filtration is a relatively new technology for isolating EVs, and it has the potential to perform successfully when paired with other procedures such as differential centrifugation protocols and ultracentrifugation protocols.

Aside from that, this process can be done in any laboratory because it only needs a centrifuge machine running at its normal speed. Besides, ultrafiltration is ideally suited for concentrating EVs and is often used as the primary EV isolation/concentration strategy (Liangsupree et al., 2021). When using differential centrifugation to isolate EVs, the co-precipitation of protein aggregates, apoptotic bodies, or nucleosome fragments may reduce the sample purity and correct protein binding (Furi et al., 2017). One possible solution to these issues is to use a sucrose or iodixanol gradient centrifuge, which sorts vesicles according to their respective flotation densities. Iodixanol (OptiprepTM) gradients have distinct benefits over sucrose gradients. OptiprepTM density gradient centrifugation is capable of separating EVs with a higher level of selectivity when compared to sucrose density gradient centrifugation and SEC (Pavani et al., 2020). In addition to this, iodixanol gradient solutions can be readily made by diluting OptiPrepTM with the homogenization medium. In contrast, accurate preparation and measurement of sucrose can be fairly difficult. Images obtained using TEM can demonstrate the differences between sucrose gradient centrifugation and conventional deferential centrifugation with regard to the amount of protein aggregation and the degree of sample purity. The findings of this study showed that the EMVs from these serotypes were successfully recovered by employing three combinations of procedures (ultracentrifugation, ultrafiltration, and iodixanol gradient fractionation). The extracted EMVs had their morphology examined with a TEM in order to verify their authenticity. All six serotypes used in this study showed evidence of releasing extracellular vesicles, albeit in a variety of quantities and sizes (22 nm -250 nm).

Conclusion

The success of extracting EMVs from *S. pneumoniae* using the modified method has now opened a path to study better drug targets for *S. pneumoniae* since bacterial EMVs are non-viable component of the bacteria that acts as an antigen, hence able to induce host immune response. This suggests it to be a potential vaccine candidate for this bacterium. However, further studies are warranted to elucidate the role of EMV as a vaccine candidate.

Conflict of Interest

The authors declare no conflict of interest.

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