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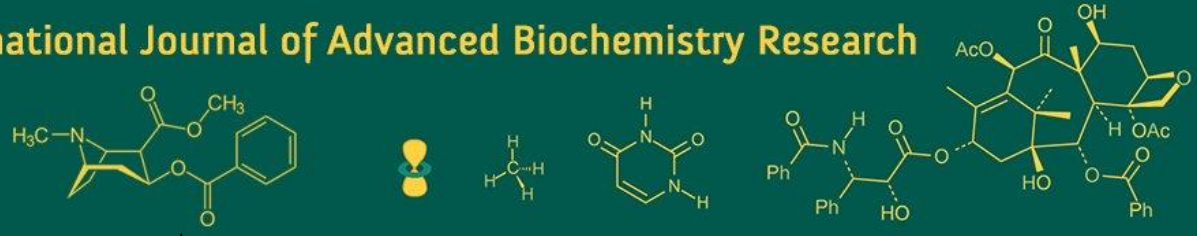

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Structural characterization and proteomic analysis of outer membrane vesicles from *Pasteurella multocida* strain p52

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Abstract
Outer membrane vesicles (OMVs) are bacterial-origin natural nanoparticles potentially emerging as vaccine adjuvants for different Nano-vaccine formulations. They are the spherical derivative product of pathogenic or nonpathogenic bacteria including both gram negative and gram positive. Some archaea and eukaryotes like fungi also generate OMVs which may possess inherent immune-stimulatory properties and can act as delivery candidates for different cargo proteins. In this study, we report the production and characterization of bacterial OMVs collected from vaccine strain P52 of *P. multocida* and discuss their role as adjuvants in vaccine development. Brain heart infusion broth (BHI) with yeast extract was used to grow *Pasteurella multocida.* Using ultracentrifugation procedure OMVs were isolated and characterized by different protocols viz Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), Zeta potential, SDS-PAGE, and LC-MS/MS. The collected OMVs with a protein concentration of 8.7 mg/ml were spherical, bilayer with size of 75 to 300 nm in diameter. Proteomic analysis revealed the presence of a group of proteins like the major outer membrane protein and OMP A, which may cause the production of robust immune responses. The findings related to OMV of P. *multocida* provide foresight in the field of adjuvants and novel vaccine development.

Keywords: Structural characterization, proteomic analysis, outer membrane vesicles, *Pasteurella multocida*

Introduction
India is predominantly an agriculture-based country. Indian economy mostly relies on agriculture where livestock plays an important role The fastest-growing livestock sector of India contributes 6% to the nation's gross domestic product. But this agriculture industry is always threatened by acute, infectious, or contagious deadly diseases which may create abrupt economic fall.

Pasteurella multocida, a Gram-negative bipolar bacterium, having zoonotic importance is responsible for several economically important diseases hemorrhagic septicemia (HS) also called shipping fever, endemic animal pneumonia, porcine atrophic rhinitis, and avian cholera. High morbidity and mortality of *Pasteurella multocida* can be prevented with effective disease control measures such as vaccination. Infected cattle may die suddenly without showing any significant clinical signs. In sub-acute cases, within five days of their incubation period animals may exhibit clinical signs and symptoms such as high rise of fever, respiratory discomfort, edema in the neck or brisket region, recumbency, and finally death. In India out of five capsular types (A, B, D, E, and F) serotypes and 16 somatic types of *P. multocida*, the B:2 serotype is the predominant cause strain for HS in cattle. Effective vaccination with killed alum-precipitated or oil-adjuvant vaccines is the ultimate key to preventing the disease in endemic regions. Despite effective protection, they have a limited period of immunogenicity usually from 6 to 9 months. Reactogenicity and reversion of vaccine to virulence are other drawbacks of these vaccines. Therefore, it is essential to produce safe, stable, novel, and economical vaccines which can provide long-lasting immunity [1, 2].

In recent years OMVs have emerged as a fascinating field of study because of their varied roles and potential applications. These vesicles are nano-sized, spherical in structures, and

natural by-products of both Gram-negative and positive bacteria formed in the time of the growth cycle. OMVs recapitulate the bacterial parent cell's content and represent a potent tool for virulence, intercellular signaling, and adaptation to environmental challenges. Mostly composed of proteins, double-layer lipids, nucleic acids, and other biomolecules hence they can play a vital role in host-pathogen interaction, bacterial pathogenesis, and their community operations. These Nano-sized vesicles act as a carriers for different cargo molecules such as growth factors, enzymes, toxins, and different cell signaling compounds for both bacterial and host cells, revising host physiology and immune response system. Furthermore, OMVs represent parent bacterial genetic makeup and are involved in horizontal gene transfer aiding in the spread of antibiotic resistance and other resilient qualities [3]. They have also emerged as a promising tool in biotechnology and biomedical research as potential vaccines, drug delivery systems, immune potentiators, and diagnostic bio-indicators owing to their natural immunogenicity, biocompatibility, and ability to target specific cells or tissues [4]. By leveraging the uniqueness of OMVs, different novel innovative therapeutic strategies are developing against infectious diseases, cancer, and other health challenges. In this study, we have characterized the OMVs isolated from *P. multocida vaccine stain p52* using different techniques, including TEM, DLS, Zeta potential analysis, SDS-PAGE, and LC-MS/MS. The current investigation may encourage the development of OMV-derived vaccines for HS and potential adjuvants for different bacterial or viral vaccine preparations.

Materials and Methods

Bacterial culture and OMV isolation

The bacterial OMV was isolated as described previously [5]. Briefly, the *P. multocida* B:2 vaccine strain (P52), maintained at IVRI, Bangalore, was grown aerobically in BHI broth at 37 °C for 18 hours with shaking at 120 rpm. BHI broth with bacterial cells was centrifuged at 6,500 rpm for 20 minutes at 4 °C to obtain cell-free supernatant. The collected supernatant was then filtered through 0.45 µm and subsequently 0.2 µm pore size filters to ensure the complete removal of any remaining bacterial cells. The BHI broth filtrate was further subjected to ultracentrifugation at 27,000 rpm for 5 hours at 4 °C. Finally, the OMVs were collected as pellets at the bottom of the ultracentrifuge tube, eluted in 1 ml of PBS (pH 7.4), and stored at -20 °C for future use. The protein concentration of the OMV preparation was estimated using nanodrop spectrophotometry.

TEM, DLS, and Zeta Potential

OMV samples were diluted 1:5000 in PBS and subjected to size determination and zeta potential estimation using a ZetaPALS instrument (Brookhaven). For TEM analysis, the diluted OMV samples were deposited on 200 mesh copper grids and stained with 2% uranyl acetate. Air-dried copper grids were then analyzed using a "Hitachi, HT7700" Transmission Electron Microscope.

SDS-PAGE and LC-MS/MS

The protein profile of OMVs was analyzed in 12% acrylamide gel by SDS-PAGE as per the method of Laemmli (1970) [6]. Prominent bands were cut from the gel digested with trypsin and submitted for LC-MS/MS analysis at IISc, Bengaluru.

Results and Discussion

The recent research study of OMVs has depicted an image of potential vaccine candidates and an immune booster also called an adjuvant, due to their Nano dimensional morphology and their capacity to trigger both antibody-mediated and cell-mediated immune responses. Nanoscale OMVs are naturally released during the growth period by Gram-negative bacteria. Their biochemical makeup is mainly attributed to lipopolysaccharides, proteins, and nucleic acids, playing a key role in immune modulation [7]. The current study is mainly aimed at the structure characterization of the OMVs extracted from the Indian vaccine strain P52 of *P. multocida*. Due to its excellent antigenicity and immunogenicity, it can be extensively utilized as a vaccine or an adjuvant.

The BHI bacterial broth from an overnight-grown culture was made sterile using a 0.22µm filter and ultra-centrifuged to produce an OMV with a protein yield of 8.7 mg/mL. This yield is significantly higher compared to previous reports, which recorded yields of 6.2 mg/mL [8], 0.11 mg/mL [9], and 0.12 mg/mL [10]. The variation in protein yield can be attributed to differences in parameters chosen for the growth of the bacterial strain used and the purification protocols [11]. TEM pictures signified OMVs as a spherical double-layer membrane structure diameter ranging from 25-200 nm (Fig 1) and approximately 75 nm recorded from DLS (Fig 2). The analyzed zeta value was -2.63 mv (Fig 3) which is within the range for OMV reported from other Gram-negative bacteria -22±1.05 [12] but some other studies reported zeta potentials of -28.86 mv [13], and -16 mv [8] for *P. multocida*. This variation may be due to the growth environment, any stress factors involved, isolation procedures, storing conditions, and the type of instruments used for analysis having different calibration and specificity. The ultra-purified OMV in the SDS-PAGE displayed two distinct bands between the 25-35 kDa range and the other two bands in the 63-75 kDa range. The clear band at 35 kDa is the indicative of omp A protein of P. *multocida* [14]. Some faint bands were also seen from 110 kDa to 25 kDa (Fig. 4). Similar results were also observed by other workers (5,8,9). The LC-MS/MS analysis (Table 1) of SDS-PAGE gel identified six proteins which are Major Outer Membrane Protein, Outer Membrane Protein A, D-galactose-binding periplasmic Protein, Ribose Import Binding Protein (RbsB), Ferric Binding Protein A (FbpA) and an Uncharacterized Protein. Similar reports were found for OMVs of *Escherichia coli* [11] and *Neisseria meningitidis* [15]. Furthermore, Sun's research work highlighted the presence of an extensive variety of proteins in the OMVs of P. multocida, corroborating the diversity of protein composition recognized in the study [13]. The strong immunogenicity is likely due to this diversity of protein composition of OMVs, positing them as strong immune stimulators and a promising candidate for vaccine development.

The data generated in this study will be useful for the potency assessment of the OMVs from *P. multocida* as an immunogenic and immune stimulator or adjuvant. Depending on the observed data exploration of new aspects of the research area has been started in our laboratories. Different *in-vitro* as well as in-vivo testing systems are

employed to explore the OMVs' efficacy to produce strong immune responses and help to intensify the effect of vaccine particles as does adjuvant [16]. Further studies are warranted to elucidate the mechanisms by which these vesicles enhance immune responses and to optimize their use in vaccines and therapeutic applications. This research could be a passage for the evolution of novel vaccines for *P. multocida* and potentially pathogenic other bacteria, harnessing the unique properties of OMVs to enhance vaccine efficacy and safety.

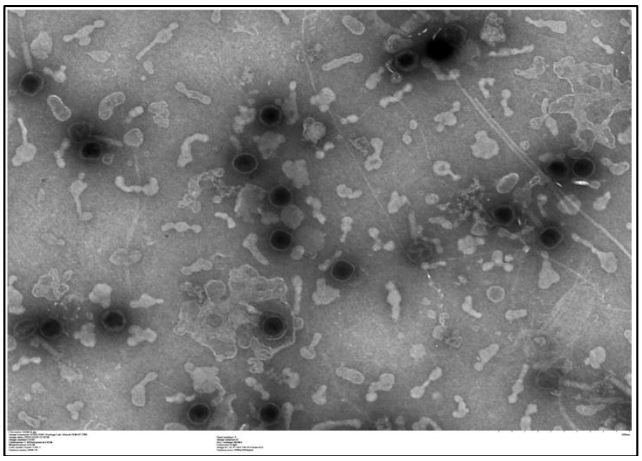

Fig 1: Transmission electron micrograph of OMVs from *P. Multocida*

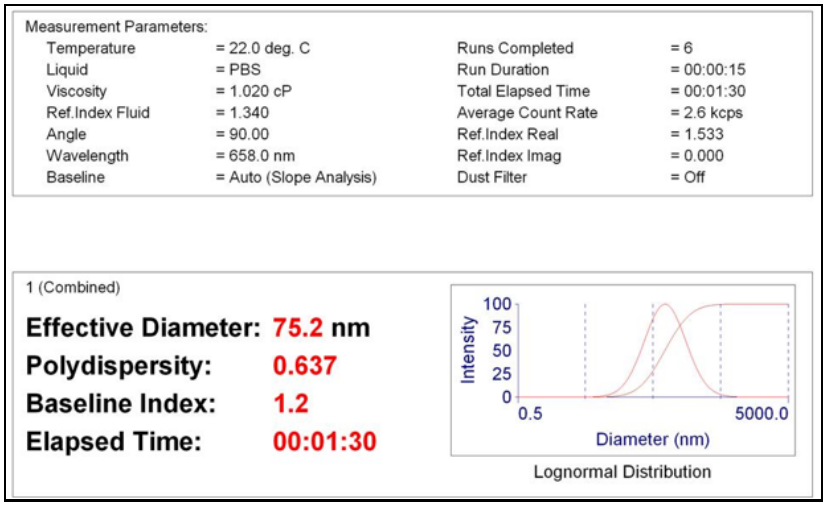

Fig 2: DLS analysis of the OMVs from *P. multocida*

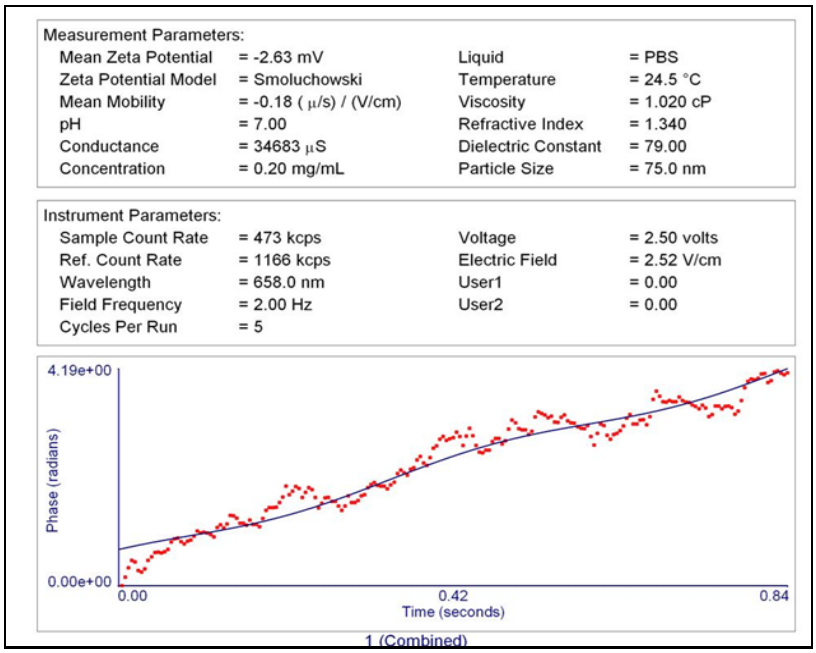

Fig 3: Zetapotential analysis of the OMVs from *P. multocida*

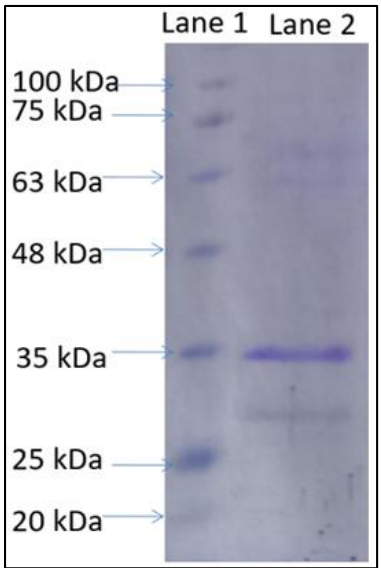

Fig 4: SDS–PAGE analysis of OMVs from *P. Multocida*, with lane 1 showing a 10-245-kDa protein ladder, lane 2 representing *P. multocida* OMV.

Conclusion

This research highlights the potential of Outer Membrane Vesicles (OMVs) from the Indian vaccine strain P52 of *Pasteurella multocida* as promising vaccine candidates and immune boosters. The study demonstrated a significant protein yield of 8.7 mg/mL and characterized OMVs with a spherical double-layer membrane structure, with a diameter between 25-200 nm. Proteomic analysis revealed a diverse protein composition, including Major Outer Membrane Protein and Outer Membrane Protein A, enhancing their immunogenic potential. These findings underscore the suitability of OMVs as both immunogens and adjuvants. Future research will focus on validating their efficacy in vaccine development and therapeutic applications, paving the way for novel approaches in combating bacterial infections.

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