Tropical Biomedicine 42(2): 213-219 (2025)
https://doi.org/10.47665/tb.42.2.014

RESEARCH ARTICLE

Discovery of alcohol dehydrogenase (ADH) as a potential vaccine target from *Mycolicibacterium smegmatis* extracellular vesicles via immunoproteomics

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ARTICLE HISTORY

Received: 16 December 2024
Revised: 27 March 2025
Accepted: 27 March 2025
Published: 30 June 2025

ABSTRACT

Mycobacterium tuberculosis (MTB), the causative agent of tuberculosis, releases extracellular vesicles (EVs) that impair macrophage functions and circulate bacterial components to modulate the host immune response. While EVs are increasingly investigated as new vaccines and biomarkers, studying MTB EVs is challenging due to the slow growth rate and pathogenic properties of MTB. *Mycolicibacterium smegmatis* (MSMEG), a non-pathogenic surrogate with a faster growth rate, offers a safer and more convenient option for laboratory studies due to its similarities to MTB. In this study, we explore the antigenic properties of MSMEG EVs to assess their potential use in developing safer tuberculosis vaccine strategies. Through an immunoproteomics approach that combines comprehensive protein separation by OFFGEL™ fractionation, Western blot analysis and mass spectrometry, we identified alcohol dehydrogenase (ADH) – a 46 kDa protein involved in mycobacterial cell wall synthesis – as an antigenic protein from MSMEG EVs. Our findings suggest that MSMEG EVs-derived ADH could improve tuberculosis vaccine formulations and potentially be used for coimmunization with the BCG vaccine, offering new and safer strategies to combat tuberculosis.

Keywords: extracellular vesicle; *Mycolicibacterium smegmatis*; tuberculosis; vaccine candidate; immunoproteomics.

INTRODUCTION

Tuberculosis (TB) remained one of the leading causes of death globally prior to the COVID-19 pandemic, with concerns escalating due to the rising prevalence of drug-resistant strains (Schirmer *et al*., 2022). The bacterium responsible for TB, *Mycobacterium tuberculosis* (MTB), releases extracellular vesicles (EVs) in various conditions, such as in culture, and within the lungs of infected mice and macrophages (Mehaffy *et al*., 2022). These nano-sized extracellular vesicles carry diverse biological materials such as proteins, lipids and glycolipids, which are critical to bacterial pathogenesis. EVs play a crucial role in delivering virulence factors, inducing cytotoxic and hemolytic activities, and modulating immune responses (Gupta & Rodriguez, 2019; Mehaffy *et al*., 2022). The surface components of EVs aid in targeting specific recipient cells and facilitating selective cargo delivery, while also serving as essential mediators of intercellular communication. Moreover, their excellent biocompatibility and ability to cross biological barriers, such as the blood-brain barrier, make EVs highly promising for various biomedical applications.

A key aspect of their potential lies in the presence of immunogenic proteins within EVs which stimulate host immune responses. This has sparked significant interest in their role in developing effective biomarkers and protective vaccines for TB. Recent research has shown that MTB EVs, being highly immunogenic without needing adjuvants, could boost the efficacy of the BCG vaccine (Mehaffy *et al*., 2022). Studies have also identified antigenic proteins in MTB and *M. bovis* EVs that appear in individuals infected with tuberculosis, providing valuable insights into immune response mechanisms and aiding in vaccine development strategies (Ziegenbalg *et al*., 2013; Schirmer *et al*., 2022).

Although studying MTB EVs is clinically significant, it is challenging due to safety concerns requiring biosafety level 3 containment. To circumvent these safety concerns, the fast-growing, non-pathogenic environmental mycobacterium, *Mycobacterium smegmatis* (MSMEG) – recently renamed *Mycolicibacterium smegmatis* in 2018 (Sparks *et al*., 2023), serves as an effective surrogate for MTB research. This alternative allows for studies to be conducted under less stringent safety requirements, providing a practical approach for exploring tuberculosis vaccine development (Kim *et al*., 2017; Kannan *et al*., 2020).

While research on MTB EVs is well documented (Rath *et al*., 2013; Prados-Rosales *et al*., 2014a, 2014b; Athman *et al*., 2017; Gupta *et al*., 2020; Schirmer *et al*., 2022), studies on MSMEG EVs remain comparatively scarce (Dahl, 2005; Prados-Rosales *et al*., 2011). Despite this, existing research underscore the promising

potential of MSMEG EVs as a protective vaccine. For example, Rodriguez *et al*. (2011) demonstrated that proteoliposomes derived from MSMEG exhibit cross-reactivity against MTB antigens in mice, marking this as a significant finding. Similarly, Prados-Rosales *et al*. (2011) showed that mice treated with MSMEG EVs had significantly lower bacterial counts of MTB H37Rv in their lungs compared to those treated with PBS. Conversely, unvaccinated mice treated with BCG EVs experienced a significant increase in MTB H37Rv bacterial counts in both the lungs and spleen after 23 days. These findings suggest that while BCG EVs promote bacterial dissemination, MSMEG EVs effectively suppress it, thereby providing a protective advantage to the host.

Despite these findings, there remains a lack of exploration into the specific antigenic proteins of this species. This gap highlights the necessity for further investigation to fully understand the potential benefits of MSMEG EV protein components. A promising approach to address this is through detailed protein studies using bottom-up proteomics. In this study, we utilized bottom up proteomics, incorporating advanced separation techniques such as OFFGEL™ electrophoresis, along with Western blotting and mass spectrometry. These methods allowed for comprehensive identification and accurate quantification of proteins based on the mass and sequence of the detected peptides, aiming to pinpoint antigenic proteins within MSMEG EVs. This work pioneers the exploration and identification of MSMEG EV proteins for their therapeutic applications in developing safer TB vaccine formulations that warrant further investigation.

MATERIALS AND METHODS

Cultivation of MSMEG for EVs production

A single colony of *M. smegmatis* ATCC 19420 was initially cultivated in 2 mL of Middlebrook M7H9 broth base supplemented with 10% ADC enrichment (BD Difco™) and 0.5% glycerol. The cultures were incubated at 37°C with agitation at 180 rpm until they reached an optical density (OD_{540}) of 0.8. The cultures were then plated onto Middlebrook M7H10 agar supplemented with 10% OADC enrichment (BD Difco™) and 0.5% glycerol. To prepare 1 L of low-iron medium, 5 g of KH_2PO_4, 5 g of L-asparagine, 2 g of dextrose, 0.5 µg of $ZnCl_2$, 0.04 g of $MgSO_4$ and 0.1 µg of $MnSO_4$ were dissolved in 1 L of deionized water, the pH was adjusted to 6.8 and the medium was filtered through a 0.22 µM filter before use, following the method described by Gupta and Rodriguez (2019). After three days of growth on the agar plates, a confluent bacterial layer was scraped off using a sterile cotton swab dabbed in the low-iron medium. This was used to prepare a 100 mL low-iron medium suspension adjusted to an OD_{540} of 1.0. The suspension was then diluted into 900 mL of fresh low-iron medium and incubated statically in 15 mL polypropylene centrifuge tubes at 37°C for ten days.

Extracellular vesicle isolation

To isolate EVs from the MSMEG culture, differential centrifugation method was performed following the method described by Gupta and Rodriguez (2019). The EVs were harvested from day-10 cultures due to a higher abundance of EVs based on the scanning electron microscope (SEM) images (Figure 1). Then, the cultures were centrifuged at 2850 x *g* for 10 minutes, followed by filtration through a 0.22 µM filter and concentration to approximately 300 mL using a 100 kDa centrifugal unit. After that, the concentrated supernatant was ultracentrifuged at 100,000 x *g* at 4°C for two hours. The membranous pellet containing MSMEG EVs was resuspended in sterile PBS, filtered through a 0.22 µM filter, and stored at –20°C until further use.

Verification of EVs and biofilm formation

To verify the presence of EVs from the MSMEG culture, the cell pellet obtained from the first centrifugation step was washed twice with PBS, dehydrated through graded alcohol concentrations (50, 75, 95, and 100%), and fixed using hexamethyldisilazane (HMDS) before being dried overnight, as described by Pardo *et al*. (2015). The samples were then imaged using an scanning electron microscope (SEM) (Regulus 8220) operated at 1 kV.

Validation of EVs morphology and size

To further validate the presence of EVs, their morphology and size were observed using a transmission electron microscope (TEM). For this, 50 µL of MSMEG EVs suspension was placed onto copper grids and stained with uranyl acetate following modified protocols by Théry *et al*. (2006). Imaging was conducted using a Zeiss EFTEM Libra 120. Additionally, nanoparticle tracking analysis (NTA) was performed using a Nanosight nanoparticle analyzer with NTA version 3.4 software to determine the size distribution of the EVs.

Protein separation by OFFGEL™ electrophoresis

To extract proteins from EVs, the membranous pellet suspensions of MSMEG EVs were homogenized via sonication for four 30-second cycles at a frequency of 1.5 Hz. After centrifugation, the supernatants were used to measure protein concentrations using the RC DC™ Protein Assay (Biorad, Hercules, California, USA). A 400 µL aliquot of MSMEG EV supernatant, adjusted to a protein concentration of 1 mg/mL, was then diluted in OFFGEL™ Protein solution. Subsequently, 150 µL of the diluted sample was loaded onto an Agilent 3100 OFFGEL™ Fractionator with a 12-well setup. Fractionation was performed at a maximum current of 50 µA, maximum power of 200 mW and voltages ranging from 200 to 1500 V overnight. The fractions were collected individually from 12 wells and analyzed by 10% SDS-PAGE with Coomassie staining.

Immunoreactivity evaluation via Western Blot

Following fractionation, the liquid samples were subjected to Western blot analysis to identify immunogenic proteins reactive to TB+ and TB- sera. The sera samples were provided by the Foundation for Innovative New Diagnostics, in collaboration with the Burnet Institute (Approval Number: 169/13). The immunoreactivities of the 12-fractionated proteins were tested using anti-human IgG antibody (Invitrogen, USA) on two groups of samples; TB+ (confirmed by positive smear and culture tests in patients) and TB- (initial provisional TB diagnosis, later confirmed negative by smear and culture tests in patients).

Electrophoresed gels were transferred onto nitrocellulose membranes, cut into strips and incubated for an hour in a buffer containing 5% skim milk in Tris-buffered saline (TBS). After three washes with TBS-T (0.05% Tween-20), the strips were incubated overnight at 4°C with pooled TB+ and TB- sera (1:250) as primary antibodies. Following another wash, the strips were probed with anti-human IgG HRP-conjugated secondary antibodies (1:6000, Invitrogen, USA) for one hour at room temperature. Visualization of immunogenic proteins was achieved using the SuperSignal™ West Pico chemiluminescence substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA.

In-gel tryptic digestion

The immunogenic bands identified on the Western blots were excised from the corresponding SDS-PAGE gel using a scalpel. The gel bands were transferred into 1.5 mL centrifuge tubes and destained twice at 37°C for 30 minutes using a solution containing 80 mg ammonium bicarbonate (AMBIC), 20 mL of acetonitrile (ACN) and 20 mL of ultrapure water. Following destaining, the gel pieces were reduced with 10 mM dithiothreitol in 100 mM AMBIC at 60°C for 30 minutes and alkylated with 50 mM iodoacetamide in AMBIC at room temperature for one hour in the dark. Further destaining was performed twice with the destaining solution, followed by dehydration with ACN. Sequencing-grade modified trypsin (12.5 ng/µL, Promega, USA) was added to the dehydrated gel

pieces, which were incubated overnight at 37°C with shaking at 300 rpm to facilitate protein digestion. The resulting peptide solution was collected, and the gel pieces were treated with 1% trifluoroacetic acid (TFA) for five minutes to further extract residual peptides. This process was repeated twice using 0.1% TFA. All peptide solutions were pooled and vacuum-dried before being sent for mass spectrometry analysis using the Agilent 6550 iFunnel QTOF LC/MS.

Data analysis

Peptide data were analyzed and matched against the Uniprot database using PEAKS (Bioinformatics Inc.) software. Proteins were considered significant if they had more than two unique peptides and a sequence coverage exceeding 16% (Kumarasamy *et al*., 2020).

RESULTS

Formation of MSMEG-EVs

MSMEG cultures were maintained in their stationary phase for two, five, and ten days. By the tenth day, scanning with SEM images revealed a marked increase in the abundance of extracellular vesicles (EVs), which were also integrated into the biofilm structures (Figure 1). This increase justified the selection of the tenth day for cell harvesting to maximize EV yield. Further observation under TEM following a negative staining process confirmed the characteristic cup-shaped morphology of the EVs, thereby validating the successful isolation of EVs (Figure 2A). Nanoparticle tracking analysis measured the size distribution of the EVs, which ranged from 22.5 - 217.5 nm as, with the majority observed at 43.3 +/- 0.9 nm (Figure 2B). The protein concentration of the EVs, determined by RC-DC protein assay, was 2.5 mg/mL from 1 L of culture. Further analysis combining ultracentrifugation and filtering confirmed the notable presence of vesicles, as evidenced by NTA and supported by TEM, which revealed vesicle sizes in the range of 50-200 nm.

OFFGEL™ fractionation and antigenicity evaluation of MSMEG biofilm EV proteins

OFFGEL™ fractions and the unfractionated crude EVs were run on 10% SDS-PAGE to compare their protein profile. The unfractionated crude EVs showed an abundance of unseparated proteins. In contrast, distinct proteins band were discernible in Fractions 2 through 9, whereas no proteins were found in Fractions 1 and 10-12, leading to their exclusion from further analysis (Figure 3). Additionally, Fraction 9 was also excluded due to its minimal volume. To further investigate, the protein reactivity within Fractions 2-8 were tested against two groups of sera; TB+ and TB-. Notably, Fraction 6 displayed positive reactivity to pooled positive serum samples, particularly around the ~30 and ~45 kDa sizes, as shown in Figure 4.

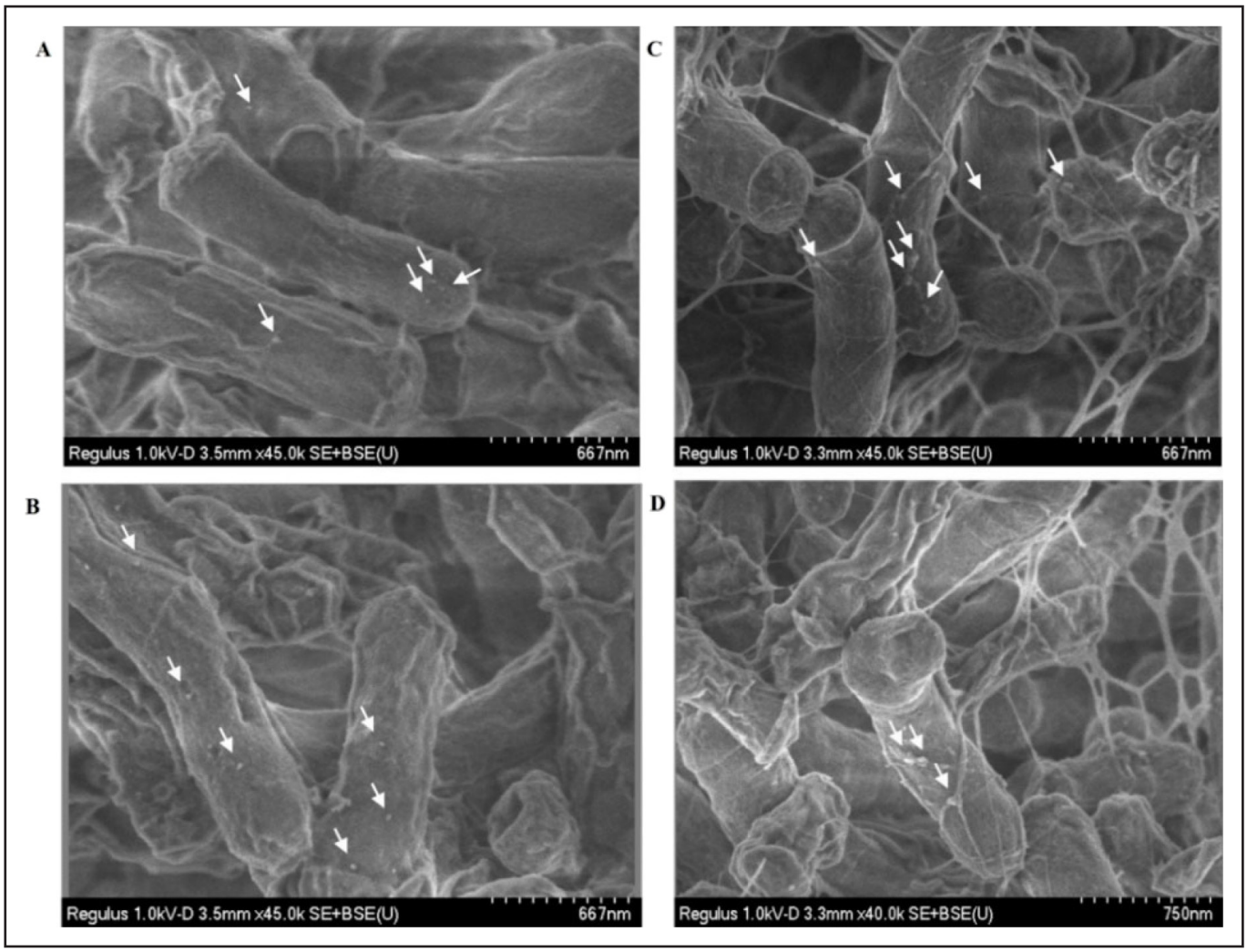

Figure 1. The observation of EVs in MSMEG cultures under SEM at 2 days (A), 5 days (B), and 10 days (C), respectively, with arrows indicating the EVs. A higher abundance of EVs was observed among biofilms on day-10 (C) (45K× magnification, scale bar 667nm). The EVs were integrated into the biofilm structure observed on day-10 (D) (40K× magnification, scale bar 750nm).

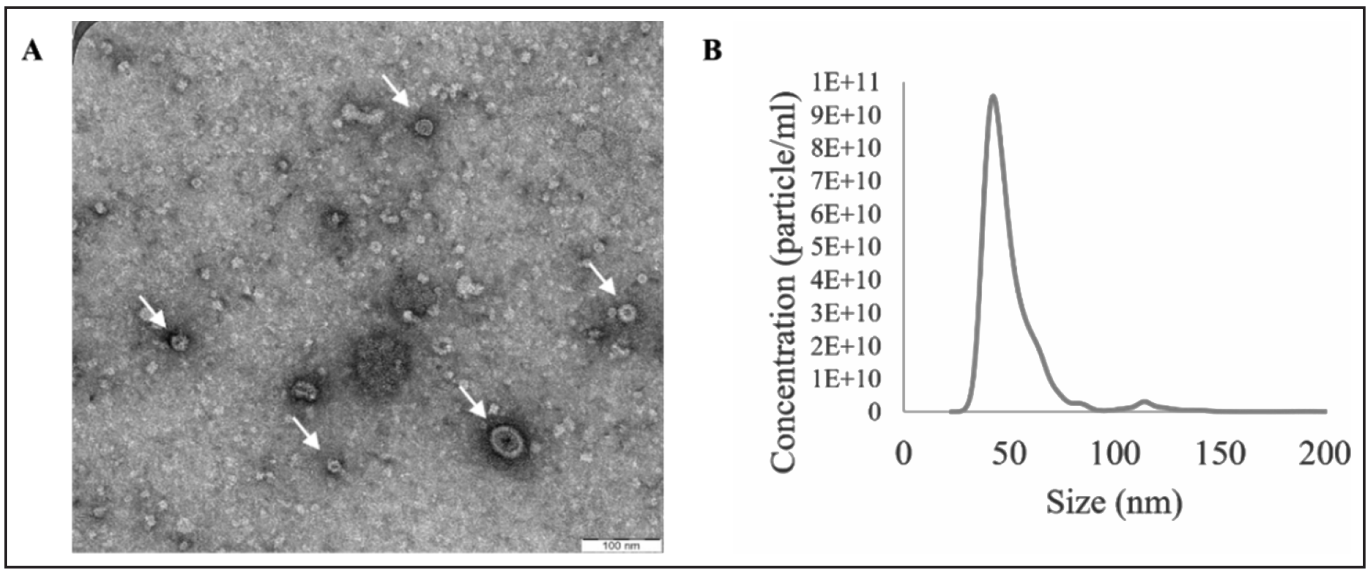

Figure 2. The cup-shaped morphology of the EVs, viewed under TEM, is indicated by arrows (A) (40K× magnification, scale bar 100 nm). The size distribution of the EVs as measured by NTA, revealed that most of the EVs were approximately 43.3 +/- 0.9 nm in size (B).

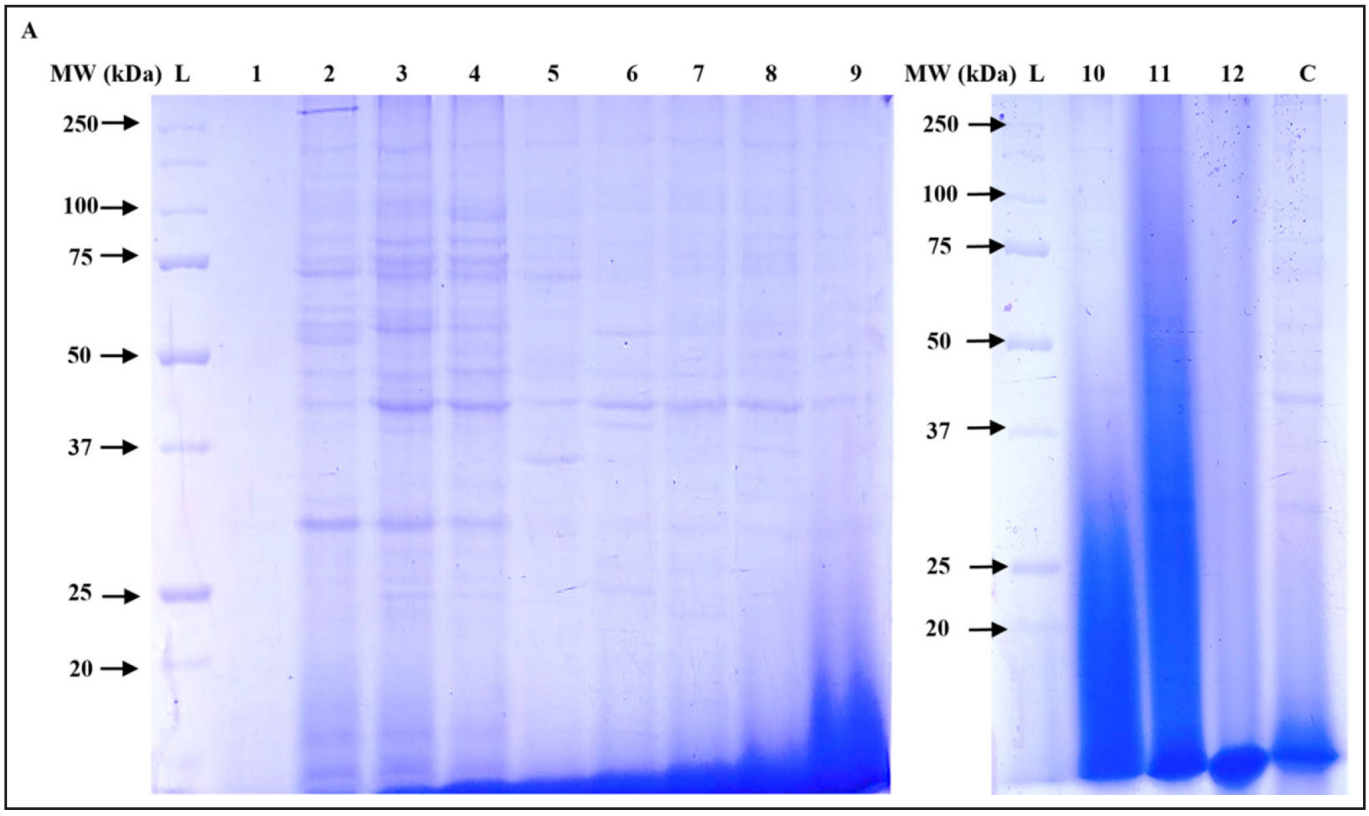

Figure 3. Protein profile of the MSMEG EVs across OFFGEL™ fractionated samples (1-12) and crude MSMEG EV protein (C) visualized with Coomassie staining. The analysis shows predominant protein bands ranging from 25 to 100 kDa, with notable bands observed between ~30 and ~100 kDa, indicating a diverse and heterogeneous expression pattern. L: Protein ladder.

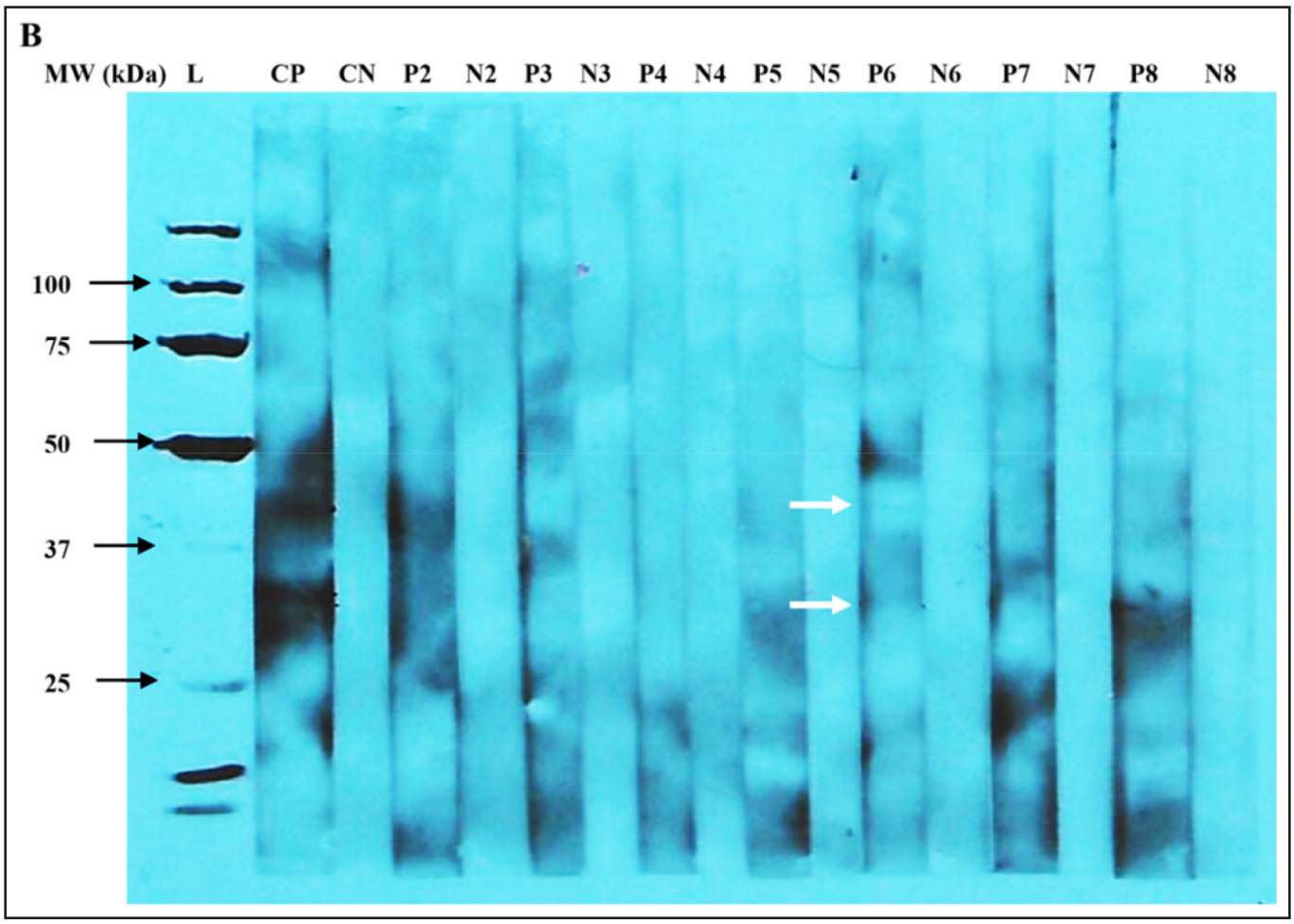

Figure 4. Identification of MSMEG EV immunogenic proteins by Western blot analysis using IgG antibodies against TB+ and TB- sera. White arrows showing specific protein bands in fraction 6, sized at ~30 kDa and ~45 kDa that reacted with pooled TB positive sera (P), but not with pooled TB negative sera (N). L: Protein marker, C: Unfractionated crude MSMEG EV protein, number 2-8: Fractions 2-8.

Table 1. Top Proteins Identified in reactive bands from Fraction 6 and Crude MSMEG EVs

	Accession Number	Name	Coverage	No of unique peptides	MW (kDa)
Crude EVs	A0A2U9PTN9	Glutamine synthetase	26	8	53.59
	A0A653F882	Homoserine O-acetyltransferase	30	8	38.4
	A0A653FNV5	Sulfate-binding lipoprotein	25	6	36.68
	A0A8B4R2H5	Glycosyl hydrolase glucoamylase	11	4	74.69
	I7GAD7	Iron-containing alcohol dehydrogenase	9	2	46.29
	Q939T2	Glutamine synthetase	11	3	53.59
	A0QQI7	O-succinylhomoserine sulfhydrylase	13	4	43.26
Fraction 6 (~30 kDa)	A0R5M3	Alcohol dehydrogenase iron-containing	15	5	46.29
	A0R4H0	29 kDa antigen Cfp29	11	2	28.73
Fraction 6 (~45)	I7GAD7	Iron-containing alcohol dehydrogenase	24	7	46.29

Protein identification of selected bands

Mass spectrometry analysis was conducted to further characterize the identity of the two reactive protein bands (30 kDa and 45 kDa) from Fraction 6 (P6) and crude MSMEG EVs (CP) (Figure 4). Table 1 lists the top proteins identified with over 9% coverage for each immunogenic band. Unfortunately, the 30 kDa protein did not match any significant identity. In contrast, the 45 kDa protein from Fraction 6 was confirmed as iron-containing ADH from *Mycolicibacterium smegmatis* (strain ATCC700084/mc (2)155) with UniProt accession number I7GAD7 and a sequence mass of 46 kDa. This protein exhibited 24% sequence coverage, with 7 matching peptides and 7 unique peptides. The molecular function of the protein was reported as oxidoreductase. Although iron-containing alcohol dehydrogenase was also identified in the crude EVs, it had higher coverage in Fraction 6 compared to the immunogenic bands from crude EVs.

DISCUSSION

MTB EV proteins have been prominently featured in numerous studies over the past decade as potential TB diagnostic markers and promising candidates for immunomodulation and vaccination (Prados-Rosales *et al*., 2011; Athman *et al*., 2015; Mehaffy *et al*., 2022; Schirmer *et al*., 2022). On the other hand, MSMEG, an environmental mycobacterium, has not been exclusively studied despite being less pathogenic than MTB. Given that MSMEG shares similarities with MTB and acts as a non-pathogenic surrogate with a faster growth rate, its cultivation in the laboratory offers a safer and more convenient research alternative. Recognizing its potential for safer vaccine production, this study pioneers the exploration and identification of MSMEG EV proteins for their therapeutic applications in immunomodulation and vaccination.

The limited research on MSMEG EVs highlights an over-reliance on studies focused on MTB-related findings. Culturing MSMEG requires iron-limited conditions that mimic the environment encountered by MTB in the host (Gupta & Rodriguez, 2019). These specific conditions have shown to enhance MSMEG EVs production compared to standard MTB cultivation media. In our study, we observed a high abundance of EVs produced by day 10 of cultivation, corroborating the findings for MTB by Prados-Rosales *et al*. (2014b). While MTB cultured in low-iron and high-iron produces EVs with similar proteins, the abundance and antigenicity of these proteins vary (Schirmer *et al*., 2022). Studies have reported that immunogenic proteins from MTB EVs cultured in low-iron conditions can distinguish between TB and non-TB individuals, as well as between active TB and latent TB infections (Schirmer *et al*., 2022). This suggests that EVs produced under iron limited conditions possessed proteins with significant potential for TB diagnosis and broader biomedical research. Consistent with these findings, our study identified several immunogenic proteins with significant therapeutic potential from EVs produced by MSMEG grown in low-iron minimal media. Among these, ADH was recognized as the most significant antigenic protein, detectable solely by TB+ sera.

This discovery was achieved through the integration of enhanced protein separation in bottom-up proteomics, which proved instrumental in the precise identification of target proteins. The OFFGEL™ technique is valued for its high efficiency in two-dimensional separation of complex protein samples via isoelectric focusing and molecular mass. This technique simplifies subsequent analysis as the fractionated proteins can be recovered in liquid form (Chenau *et al*., 2008). In our study, we achieved a clearer separation and observed a heterogeneous electrophoretic pattern across a pH range of 3-12, with distinct protein bands observed at ~30 and ~45 kDa. These observations underscore the utility of OFFGEL™ electrophoresis as an effective method for fractionating complex protein mixtures, serving as a critical preparatory step for downstream mass spectrometry analysis.

Analysis of these protein fractions revealed significant reactivity against TB+ serum samples in the 25–50 kDa sized range, specifically in the Fraction 6 with a pH range of 6–6.6. Further characterization via mass spectrometry identified iron-containing alcohol dehydrogenase (ADH) from *Mycolicibacterium smegmatis* as an immunogenic protein with 24% sequence coverage. However, although crude EVs before OFFGEL™ electrophoresis contained antigenic bands at 25–37kDa and 37–50kDa, in-gel digestion and characterization revealed many proteins within those bands, often with low sequence coverage. This suggests that OFFGEL™ electrophoresis is particularly effective in uniquely separating proteins from complex mixtures, which enhances the identification of antigenic proteins.

The 46 kDa protein of ADH with oxidoreductase function identified in this study was similar to the protein detected in both EVs and cellular components by Prados-Rosales *et al*. (2011) in their proteomic analysis. This finding suggests that cellular proteins can be selectively incorporated into EVs. Interestingly, while zinc-containing ADH was identified as a cellular protein in MSMEG, it was absent in MSMEG EVs, suggesting that protein packaging into EVs is selective, influenced by growth conditions and cellular needs. Unlike previous study that focused on the overall protein composition of MSMEG EVs and cellular components, our research uniquely identifies ADH as an antigenic protein, making it the first demonstration of its specific reactivity with TB+ sera. Supporting this, Lee *et al*. (2015) identified NADP-dependent ADH in MTB EVs, and Schirmer *et al*. (2022) confirmed the presence of both zinc-containing ADH and NADP-dependent ADH in EVs of MTB H37Rv grown under varying iron conditions, further validating the presence of ADH in EVs.

Beyond its role in alcohol metabolism, ADH is implicated in stress resistance, biofilm formation, and bacterial virulence (Larson *et al*., 2019). In *M. bovis* BCG, ADH is essential for synthesizing the mycobacterial cell envelope. Interestingly, MSMEG ADH shares 78% sequence similarities with ADH from *M. bovis* BCG and *M. tuberculosis* (76%), as well as with *M. avium* and *M. paratuberculosis* (76%), and *M. leprae* (75%) (Galamba *et al*., 2001). These findings highlight MSMEG EVs as a valuable model for studying the role of ADH in mycobacterial pathogenesis and its potential as a target for tuberculosis vaccine development.

Studies have also highlighted the involvement of ADH in various bacteria, including *Acinetobacter baumannii*, an opportunistic pathogen linked to increased nosocomial and community-acquired infections. Studies have notably shown that ADH modulates quorum sensing in *A. baumannii*, and that the presence of ethanol induces stress responses, leading to heightened virulence. These findings underscore the critical impact of ADH and environmental factors like ethanol on bacterial behavior and pathogenicity (Lin *et al*., 2021).

Our study lays the groundwork for future investigations into the potential of MSMEG-EVs as protective or diagnostic tools. By using the MSMEG model, we are able to examine the role of alcohol dehydrogenase (ADH) in a controlled setting, without the added complexities of pathogenic species. This approach offers foundational insights that can be applied to more intricate systems. However, it is crucial to recognize that the non-pathogenic nature of the MSMEG model may limit the direct applicability of our findings to pathogenic species. Additionally, the focus of this study on low-iron conditions and its limited sample size may constrain the generalizability of the results.

Future effort should focus on in vivo immunogenicity studies in mice and incorporating larger serum sample sizes to enhance the robustness of the findings. Concurrently, refining methods for isolating MSMEG EVs should remain a key area of focus. Moreover, given the lack of recent studies on the direct role of alcohol dehydrogenase (ADH) in tuberculosis, its potential involvement in MTB remains uncertain and calls for further exploration.

CONCLUSION

Through the precision of OFFGEL™ electrophoresis and immunoproteomics, we successfully identified ADH as an antigenic protein from MSMEG EVs. The ADH in MSMEG EVs exhibited antigenicity when reacted with TB-positive serum, highlighting its potential as a target for therapeutic applications. This finding suggests that, with further experimental validation, MSMEG-derived ADH could play a pivotal role in the development of novel protective vaccine formulations for tuberculosis. Specifically, the presence of ADH in MSMEG EVs suggests that these EVs could complement the existing BCG vaccine through co-immunization strategies, offering new avenues for combating this globally significant disease.

ACKNOWLEDGMENTS

We would like to thank Dr. Siti Nasuha Hamzah and IPPT Kepala Batas Oncology Unit for aiding in ultracentrifugation procedures. This research was funded by Ministry of Higher Education Malaysia Fundamental Research Grant Scheme (FRGS/1/2017/SKK11/USM/03/1) and FRGS/1/2018/SKK12/USM/02/1).

Conflict of Interests

The authors declare that they have no conflict of interest.

Ethics Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Alfred Ethics Committee (Approval Number: 169/13) and Universiti Sains Malaysia (USM) Human Research Ethics Committee (USM/JEPeM/PP/23010138).

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