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Extraction and biological evaluation of *Mycobacterium bovis* extracellular vesicles as adjuvant and candidates for bovine tuberculosis vaccine

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ABSTRACT

Objectives: BCG vaccine is the only virtual vaccine that has significantly helped control tuberculosis for 80 years. Bacteria naturally release extracellular vesicles (EVs) in different environments during the growth process. The use of extracellular vesicles is an alternative way to transfer ligands that are detected by host cells. Vesicles range in size from 50 nm to 250 nm in diameter and contain phospholipids, proteins, and lipopolysaccharides, and can carry additional factors such as toxins, adhesive, or immune system compounds that are important in pathogens. Therefore, this study aimed to evaluate these compounds as adjuvants or candidates for the bovine tuberculosis vaccine.

Methods: In the present study, *Mycobacterium bovis* standard CRBIP7.121 was used. Extraction of membrane vesicles after mass culture was performed by a method based on ultracentrifugation and deoxycholate. After preparation and staining, the vesicles were examined by electron microscopy. Sample analysis was also performed by SDS-PAGE. The presence of LPS in the sample was measured by the LAL test. In addition, the harmlessness of bacterial EVs and the absence of any toxic agents in the sample were confirmed by pyrogenic tests in rabbits.

Results: The protein content of membrane vesicles is equal to 1.25 and 1.32 mg/ml. In SDS-page evaluation, bands of 35, 40, 50, and 70 kDa were observed and then membrane vesicles were observed and confirmed by electron microscopy. The amount of vesicle toxin contained by the LAL test was reported in the permissible range.

Conclusions: Discussion of the data obtained from the above research shows that at different stages of the purification process, EVs fully retained their spatial and natural form and lacked impurities. Therefore, due to the importance of external vesicles in developing immune responses, EVs extracted from *M. bovis* CRBIP7.121 can be considered a useful and effective immunogen against Mycobacterium infections.

Keywords: Extracellular vesicles, *Mycobacterium bovis* CRBIP7.121, adjuvant and vaccine

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Bovine tuberculosis is important in terms of public health in human societies. The ease and frequency of the spread of the causative agent of tuberculosis from animals to humans, especially in environments where bovine tuberculosis is not controlled, can make this disease an important common disease (Zoonosis). Tuberculosis infection in humans is caused by drinking contaminated milk in children, although transmission of the infection can also be by respiratory [1-3]. Bovine tuberculosis is caused by *Mycobacterium bovis* and is one of the most common diseases between humans and animals around the world. This bacillus infects domestic animals and wild mammals and makes them a reservoir for the disease, which makes it difficult to control the disease [4]. Vaccination is the best way to prevent the consequences of infectious diseases in humans and animals [5]. Common vaccines mainly include attenuated live pathogens and subunit vaccines such as inactivated bacterial toxins. Adjutants are chemical or biological compounds that cause nonspecific stimulation of the immune system against the antigens with which it is injected [5, 6]. *M. bovis* Calmett-Guerin (BCG) is the only virtual vaccine that has been instrumental in controlling tuberculosis for more than 80 years. The vaccine can provide about 80% immunity against Mycobacterium and Miliary TB in infants and young children, however, the protective effects of BCG against lung disease vary at different ages [7]. Bacteria naturally release extracellular vesicles (EVs) in different environments at different stages of growth. Vesicles range in size from 50 nm to 250 nm in diameter and contain phospholipids, proteins, and lipopolysaccharides (periplasmic compounds) and can carry additional factors such as toxins, adhesive, or immune stimulants that are important in patency [8]. EVs production has been recorded in various pathogenic and non-pathogenic species of Mycobacterium, indicating that the release of EVs is a conserved feature among Mycobacterium species. Proteomic analysis has shown extensively that only EVs from pathogenic species have the TLR2 agonist lipoprotein and have played an important role in stimulating the immune system [9]. The aim of this study was to purify the external vesicles of *M. bovis* to perform immunological evaluations of extractive vesicles.

METHODS

Bacterial Strain Culture

The standard culture of *M. bovis* was cultured in Lunstein-Johnson (LJ) medium with McFarland 1 turbidity for 3-4 weeks at 37°C to confirm Zill Nelson staining and microscopic observation and biochemical tests such as nitrate reduction, niacin and Catalase was used.

OMV Eextraction

Extraction of outer membrane vesicles was performed based on ultracentrifugation using solutions containing deoxycholate and sucrose gradient. In summary, after preparing a thick mass from the inactive *Mycobacterium bovis* cell body and preparing the cell mass with solutions containing Tris, EDTA, and deoxycholate, successive centrifuges were performed at high speed and finally, the precipitate obtained was dissolved in 3% sucrose. The resulting solution was sterilized using 0.2 filters.

Nanodrop

The standard concentration of the proteins of the extracted vesicles was measured using a nanodrape. The basis of this device is spectrophotometry.

SDS-PAGE

To determine the protein pattern in OMV and estimate their molecular weight at 30 µl of the purified sample was electrophoresed on a 12% gel and a protein marker was used to determine the protein weight (Fig. 1).

Electron Microscopy

Membrane vesicles were ultrasonically treated to disperse the vesicles followed by attaching to Formvar/carbon-coated nickel grids. Grids were washed with a 0.01 M PBS supplement, 0.1% gelatine (PBG), and 0.5% BSA. The vesicles on the grids were fixed with 1% glutaraldehyde in PBS at 4°C for 60 minutes and negatively stained with 1% potassium phosphotungstate pH 7.5. The grids were examined using a Zeiss EM10C transmission electron microscopy operated at 80 KV.

LAL Test

To measure the amount of LPS in the sample, Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit was used according to the manufacturer's protocol.

Pyrogen Test

The extracted external vesicles were tested on four healthy albino New Zealand rabbits. At the beginning of the study, the animal weight was limited between 1.8 and 3.8. Three rabbits were used for testing and one for control. The OMV was injected into rabbit peripheral ear veins at a dose proportional to the rabbit weights. The animals' rectal temperature was measured using a digital thermometer. If the rabbit did not showed a temperature rise of 0.6 C or greater or the sum of the three single temperature increases not exceeds of 1.4◦C, the test material has no toxicity.

Evaluation of the Level of total IgG Antibody against OMV in Mice Immunized by ELISA

After blood sampling and serum collection, the level of antibody against protein was evaluated by ELISA. Protein was prepared in PBS buffer at a concentration of 5 µg/100 µl. The mouse serum used was prepared in dilutions of 1: 250-1: 500-1: 1000 and 1: 2000. The mouse IgG concentration of 1.6000 was used and the samples were then read at 405nm wavelength.

RESULTS

Results of Electrophoresis of Membrane Vesicles

Electrophoretic motion analysis of proteins in the extracted vesicles of M. bovis showed protein bands in the 25, 40, and 50 kDa regions according to the markers.

Measurement of Protein using Laurie Test

Lowry method was used to estimate the concentration and quality of extracted membrane vesicles. The results of physicochemical analysis of vesicles extracted from M. bovis showed that the total protein

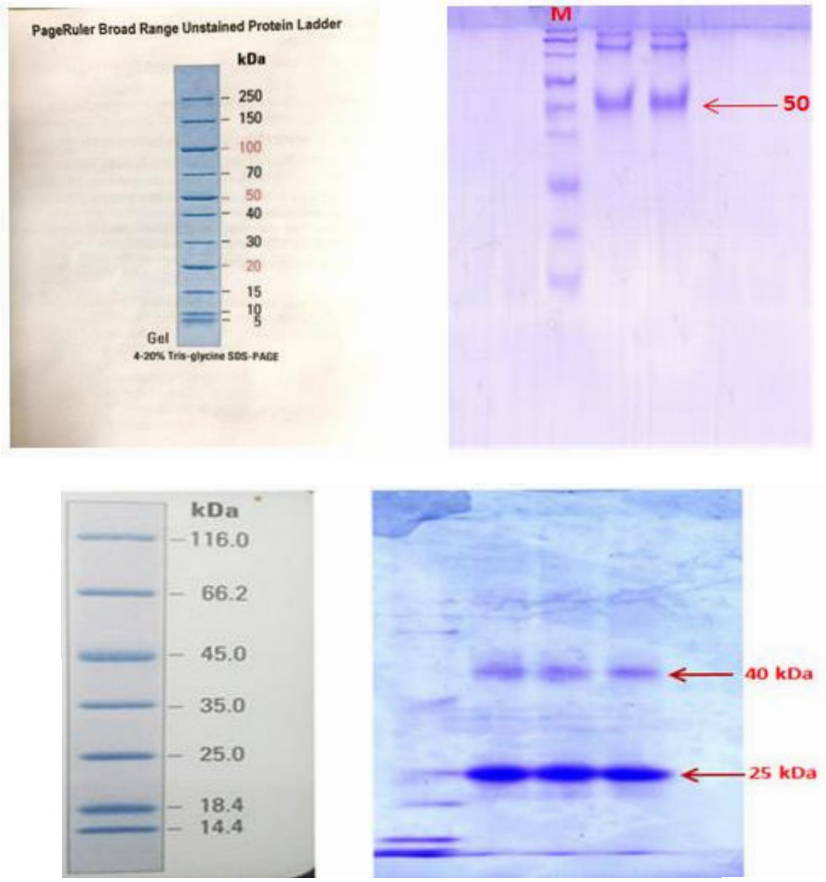

Fig. 1. Protein pattern of outer membrane vesicles by SDS_PAGE method

content of membrane vesicles was 1.32 mg/ml, which is an acceptable amount.

Electron Microscopy

The stability of the natural form of membrane vesicles at different stages of the purification process was investigated by electron microscopy. The spatial properties of the extracted membrane vesicles in negative contrast staining with the EM 900 electron microscope are shown below. As can be seen in the Fig. 2, the extracted vesicle is about 50-50 nm and retains its spatial properties at various stages of extraction and purification.

Determination of Endotoxin Levels in Membrane Vesicles by Chromogenic Limulus Amebocyte Lysate (LAL)

Results in this semi-quantitative test showed that the extracted vesicles were allowed to be used in the animal model within the safe limit (less than 300 IU).

Pyrogenic Test

After the test, no significant increase in temperature was observed in rabbits, indicating a lack of pyrogenicity.

DISCUSSION

The BCG vaccine was first introduced in 1921. This vaccine is by far the most widely used vaccine in the world. The current BCG vaccine, based on live attenuated bacteria from the *Mycobacterium bovis* strain that has been passed sequentially, is available and is used to protect infants and children against tuberculoses. However, this vaccine provides unreliable protection against pulmonary tuberculosis in the adult population. This vaccine is known to be the only effective live vaccine so that the killed BCG vaccine cannot provide protection in the animal sample. Also, *Mycobacterium tuberculosis* killed in humans provides poor protection and instability, and the level of immunity produced by the BCG vaccine varies from 0 to 80% [8, 9]. The BCG vaccine is largely safe, but in some cases has side effects such as abscesses at the injection site and local lesions such as osteoporosis [10, 11]. Another challenge in the development of bovine tuberculosis vaccines is the lack of knowledge of dominant immunogenic antigens. It has been suggested that the BCG vaccine used does not adequately express immunogenic antigens, and therefore mutant strains containing antigenic proteins are suggested for the development of improved BCG vaccines [12]. Recent studies have shown that infants with HIV are routinely vaccinated with the BCG vaccine at birth. Such infants and asymptomatic individuals, as well as those who have developed symptoms of AIDS, are at risk for developing BS [13]. The existence of such problems indicates the need to change the BCG vaccination strategy and produce a new generation of vaccines with lower risk and greater safety against subsequent consequences. Therefore, it seems that designing and introducing a new vaccine candidate can be considered a necessity. Various species to improve the BCG vaccine or to produce new vaccines are currently under investigation in many clinical trials. Vaccines currently on the production line are mainly based on protein subunits with adjuvants or viral vectors and some inactive bacteria from Mycobacterium strains [14]. A study of *M. tuberculosis* vesicles has shown

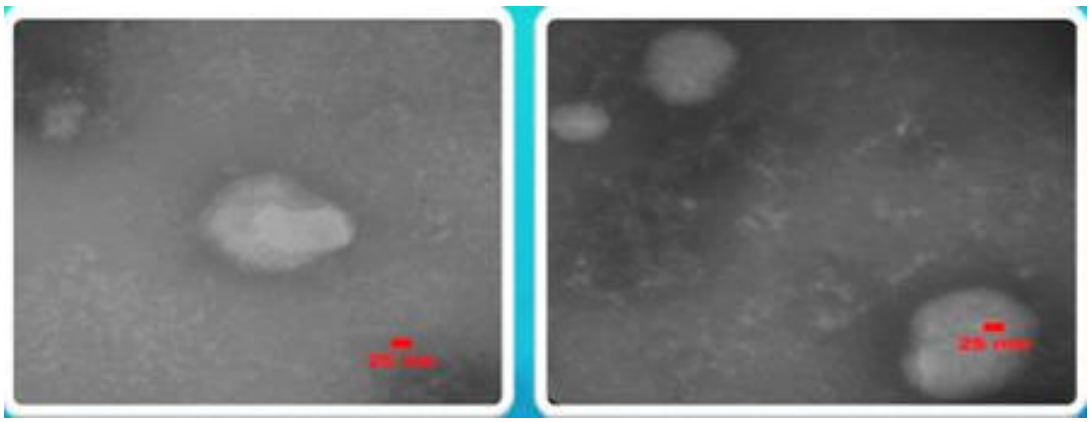

Fig. 2. Electron micrograph of outer membrane vesicles

that these vesicles contain TLR2 agonist lipoproteins so that macrophages infected with these vesicles are able to produce T2-dependent chemokines and T-dependent cytokines. It has also been shown that injecting extracellular vesicles into the lungs can stimulate an inflammatory response against them. The present study investigates the production of extracellular vesicles by the standard *Mycobacterium bovis* strain. In this study, the pattern of proteins extracted from this strain was protein bands in the 25, 40, and 50 kDa regions. Also, the extracted vesicle was 200-50 nm in size, and in terms of vesicle consistency, it maintained its spatial properties in different stages of extraction and purification. To study the function and structure of *Mycobacterium bovis* extracellular vesicles, no study has been done so far and all hypotheses are based on *M. tuberculosis* extracellular vesicles. The vesicles of this bacterium contain various compounds from the bacterial cell wall such as lipoarabinomannan and TLR2-stimulating lipoproteins, etc. In this bacterium, the VirR gene is responsible for the production of these vesicles, which play a very important role in controlling the stimulation of the immune system. Bacillus vesicles are also produced in large quantities under iron deficiency, which indicates the important role of these vesicles in iron deficiency conditions [15]. Due to its clinical use, meningococcal OMV vaccines, which have been used since 1980 and are an important tool in combating the spread of *Neisseria meningitidis* serogroup B, are one of the targets of vesicle extraction to produce vaccines. In fact, as of 2010, OMV was the only commercially approved vaccine against *N. meningitidis* reported [16]. According to these studies, it may be possible to evaluate the compounds in *M. bovis* vesicles and use them as alternative vaccine candidates. In addition to the vaccine role, the adjuvant role of these compounds is also considered. The goal of vaccination is to create a strong immune response to provide long-term protection against infection, and to achieve this goal, unlike attenuated live vaccines, the whole organism is usually killed or subunit vaccines need to add an adjuvant to be effective. [17]. Mycobacterium extracts have been widely used in vaccines as adjuvants. Among these cases is the complete adjuvant of Freund, which contains components of Mycobacterium. Mycobacterium cell wall contains a variety of antigens, including peptidoglycans, arabinoglycans, mycolic acids, proteins, phosphatidyl inositol, lipomannas, and arabinomans. These components stimulate dendritic cells via mannose and NOD2 receptors [18, 19]. In a study by Lee *et al*. [20], they showed that Mycobacterium vesicles contained cell wall compounds such as lipoproteins X, A, and G, and other compounds that appear to stimulate immune cells. Therefore, these compounds may be used as adjuvant [20]. Therefore, the vesicle extracted from *M. bovis* due to its preservation nature can be considered as a candidate for possible use for vaccines and adjuvant, which requires further research in this field.

Limitations

Further research of the Molecular pathobiology and immunological properties would lead to the development of better and safer vaccines.

CONCLUSION

The results indicate that these structures may be suitable candidates for vaccination and be considered as a new generation of vaccines against M. bovis infections, although further studies in this field are needed to evaluate antibody subclasses and responses.

Authors' Contribution

Study Conception: SS-y; Study Design: SS-Y; Supervision: ATM; Funding: AMS; Materials: AMS; Data Collection and/or Processing: ATM;, MS-S Statistical Analysis and/or Data Interpretation: MKSY, SDS; Literature Review: ATM; Manuscript Preparation: ATM and Critical Review: ATM, MK-G.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

The authors disclosed that they did not receive any grant during conduction or writing of this study.

Ethics Committee Approval

This article is not a study with human participants. There are no experiments on animals. This article does not contain any studies on human participants or animals performed by the author. There is no identifying

information of participants.

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