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VETERINARY MICROBIOLOGY - SHORT COMMUNICATION

Identification of extracellular vesicles from J strain and wild isolate *of Mycoplasma hyopneumoniae*

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Abstract
Respiratory diseases constitute a major health challenge for the worldwide pork industry. Porcine enzootic pneumonia (PES) is caused by *Mycoplasma hyopneumoniae* (Mhyo). Mycoplasmas have the ability to produce extracellular vesicles (EVs), which can be useful for pathogenicity studies and as delivery systems for vaccines. The aim of this study was to demonstrate and compare, under laboratory conditions, EVs produced by Mhyo strain J and wild isolate in stressed and non-stressed in vitro conditions. Using differential centrifugation, density gradient ultracentrifugation, and transmission electron microscopy, the ability of Mhyo strains to produce EVs was demonstrated under favorable and unfavorable conditions.

Keywords Extracellular vesicle · Enzootic pneumonia · Autogenous vaccine

Introduction

In the last decade, extracellular vesicles (EVs) from cells, particularly those of pathogenic bacteria has become an explosion of interest [1]. EVs from prokaryotes, discovered in the 1960s, are secreted into the extracellular milieu; these are characterized by a lipid bilayer envelope, which may contain nucleic acid, proteins, lipids, metabolites, viruses, toxins, and contents reflecting the pathophysiological state of the host cells [1, 2]. Gram-negative EVs are referred to as outer membrane vesicles (OMVs) and display an LPS-containing outer membrane-derived

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envelope. Gram-positive compared to Gram-negative bacteria typically display lower levels of EVs [3].

In the absence of an outer membrane, *Mollicutes* class is phylogenetically related to Gram-positive lineages, suggesting common ancestry (*Clostridium* spp). These microorganisms are wall-less with a cholesterol-rich membrane, and due to the lack of a cell wall physical barrier, EV production is expected [4, 5]. *Mycoplasma hyopneumoniae* (Mhyo) is the main etiological agent of porcine enzootic pneumonia (PES), a disease that negatively impacts the porcine agronomy and is responsible for much of the cost related to antibiotics and vaccines in industrial pig farming [6]. Reports of EVs being released by other mycoplasmas species have been described [7]. In this work, we showed for the first time EVs from Mhyo strain J and wild isolate under stress conditions.

Materials and methods

Liquid culture medium

Friis medium was prepared as described by Cook [8]. Briefly, to make 500 ml of Friis medium, 1.5 g of brain heart infusion (BHI) (Difco), and 1.6 g PPLO (Difco) was dissolved in 365 ml water and sterilized by autoclaving. To this were added 18 ml of yeast extract (prepared from dried bakers'' yeast), 12.5-ml sterile solution A (160 g/l NaCl, 4 g/l; 8 g/l KCl, 2 g/l $MgSO_4 \cdot 7H_2O$, 2 g/l $MgCl_2 \cdot 6H_2O$, 3.7 g/l $CaCl_2 \cdot 2H_2O$), 12.5-ml sterile solution B (3.0 g/l $Na_2HPO_4 \cdot 12H_2O$, 1.2 g/l KH_2PO_4), 50-ml pig serum (Invitrogen) heat-treated at 56 °C for 20 min, 50-ml heat-treated horse serum (Invitrogen), 1-ml phenol red solution (0.6% in 0.1-M NaOH), azlocillin, and flucloxacillin (final concentration 50 µg/ml), and adjusted to pH 7.4 with 1.0-M NaOH.

Strains and culture

Strain J (NC_007295.1) and a wild isolate (SAMN11634267) were the strains used here; the wild strain was previously isolated by the group. Friis medium (1×) non-inoculated was used as the negative control. In the first experiment, Mhyo strain J and the wild isolate were inoculated in two tubes containing 100 mL of Friis medium and incubated at 37 °C until the reaching of the linear growth phase. In the second experiment, Mhyo strain J and the wild isolate were inoculated in a 10-mL Friis medium and 250-mM hydrogen peroxide, under the same conditions of the first experiment, during 4 consecutive days after reaching exponential growth phase.

Isolation of large EVs

The isolation protocol was adapted from Chutkan [9] and Chernov [5]. Briefly, cells were harvested by differential centrifugation (dC) washes and supernatant harvest at 500×g for 20 min (4 °C) followed by spinning at 3.000×g for 20 min (4 °C). Then, supernatants were filtered using 0.45-µm PVDF membranes. The filtrates were centrifuged again at 11.000×g for 20 min (4 °C). The pellets obtained after the last centrifugation were fixed as described.

Isolation of EVs by dGU

Initially, OptiPrep (60% stock) was added to the resuspended vesicles at a ratio of 1:3 (by volume) to adjust the vesicle preparation to 45% of OptiPrep (v/v). Then, 2 mL of vesicles was added to the bottom of a 12.5-mL ultraclear centrifuge tube. Carefully the tube was layered with 2 mL of each OptiPrep dilution in descending order on the top of the preceding layer from 45, 40, 35, 30 to 25%. The first ultracentrifugation was carried at 100,000×g in a Beckman Coulter ultracentrifuge (SW 40 Ti rotor) for 14 h at 4 °C. The second ultracentrifugation was diluted in 12 mL of PBS, and centrifuged at 250.000×g/3 h at 4 °C (SW 70 Ti rotor). Fractions of 1 mL were collected from the top of the tube to recover the fractions containing EVs. Each fraction was centrifuged again at 11.000×g for 20 min (4 °C) to constitute the pellets.

TEM

All pellets obtained in density gradient ultracentrifugation (dGU) were immediately fixed with a solution containing 2.5%-glutaraldehyde, 4%-formaldehyde, 0.1-M phosphate buffer (pH 7.2), and distilled water. After a minimum of 24 h, the pellets were washed with a phosphate buffer; postfixed in 1%-osmium tetroxide; dehydrated in an ascending series acetone (30 to 100%), and infiltrated by gradually replacing acetone with epoxy resin (Epon 812®). After replacement with the pure resin, the pellets were placed in molds and baked at 60 °C for 48 h for polymerization and preparation of the blocks. The blocks were cut in an ultramicrotome (Reichert Ultracut S, LEICA, Austria) to obtain ultrathin sections of approximately 70 nm in thickness, which were placed in a copper mesh (300 mesh). Grids were counterstained with 5% of uranyl acetate and lead citrate for negative staining and were observed under a TEM (Zeiss Transmission Electron Microscope, EM 109) [10].

Results and discussion

It was possible to note that early purification steps (dC process and 0.45-µm filtration) were enough to remove large particles and constituents of the medium from both experiments. These samples were not inoculated, and the serum used was not ultracentrifugated previously, suggesting that large vesicles from eukaryotes were not determinant for sample differentiation (Fig. 1a and b).

In the first experiment under favorable conditions, Mhyo strain J and the wild isolate were able to maintain EV production (Fig. 1c and d). For wild isolate, the production of extracellular vesicles can contribute to microbial survival or competition, and this is important for field isolates. Figure 1 e shows Mhyo strain J from the second experiment.

Mhyo strain J is considered type strain for *Mycoplasma hyopneumoniae* so this is in compliance with GCP (good laboratory practices).

In addition to comparing the occurrence of EVs between these different isolates (type strain and wild isolate),, the information obtained in the study is essential considering the antigenic potential of EVs, since many commercial vaccines against *Mycoplasma hyopneumoniae* has the J strain as master seed.

One of the major challenges today is to define methods that allow to differentiate between the distinct kinds of vesicles; invariably, EVs isolated by ultracentrifugation are likely to contain a mixed EV population of both. Current methods of isolation and purification include differential centrifugation, concentration, density gradient ultracentrifugation, affinity chromatography, or immunoaffinity method [11]. In this work, the protocol used was a filtration process based on differential centrifugation, which allows larger vesicles to be selected. However, it is important to observe that the EV size ranged from 200 to 500 nm, indicating that they were small to medium EVs.

It is categorically reported that the EV size range from 100 to 500 nm [4, 5], and that is why some authors use filter techniques (0.2 µm or 0.1 µm). However, this may result in the elimination of larger vesicles. Considering the exploratory work, we choose to filter at 0.45 µm to preserve larger EVs.

Bacteria under oxidative stress were separated only by dC, and although we did not quantify, we observed high production levels of EVs (Fig. 1f). Although images indicate larger cells do secrete vesicles, production rates for these were relatively low.

All the supernatants were used in the dGU. Figure 1g and h show EVs from Mhyo strain J under oxidative stress, purified by dC and dGU. Some researchers used a sample concentration before the ultracentrifugation process, so it was possible to carry out only dC and dGU. Figure 1g shows the fraction generated by dGU.

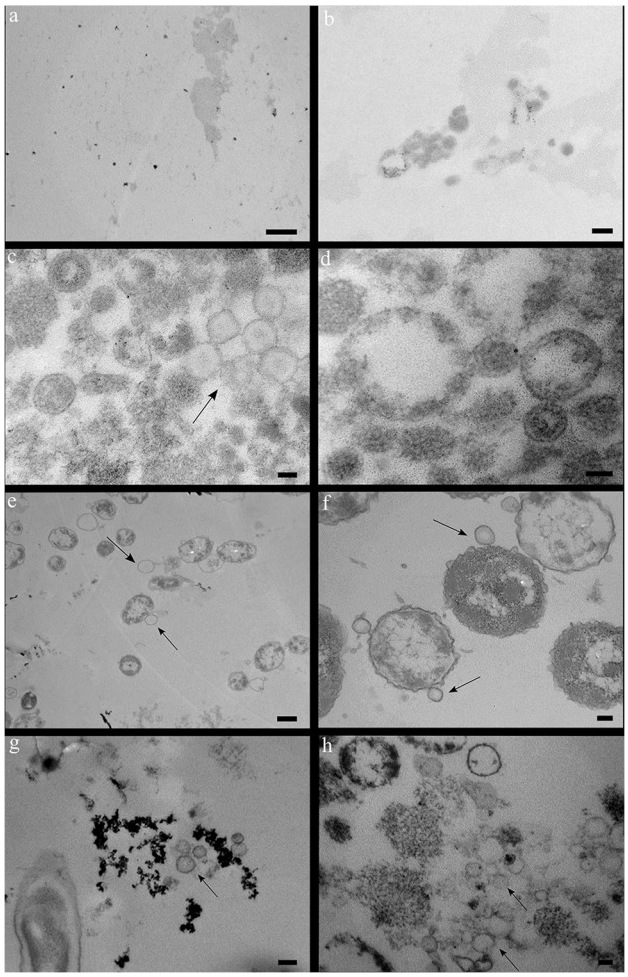

Fig. 1 **a**: EV purification technique, 10-mL Friis medium non-inoculated, after early purification steps. Bar 500 nm. **b**: control of EV purification technique, 100-mL Friis medium non-inoculated, after early purification steps. Bar 100 nm. **c**: 100-mL Friis medium inoculated with J strain under normal growth conditions. Bar 200 nm. **d**: 100-mL Friis medium inoculated with wild isolate under normal growth conditions. Bar 500 nm. **e**: 10-mL Friis medium inoculated with strain J under normal growth conditions. Bar 100 nm. **f**: 10-mL Friis medium inoculated with J strain under oxidative stress, separated only by differential centrifugation (dC). Bar 100 nm. **g**, **h**: 10-mL Friis medium inoculated with J strain under oxidative stress, purified by dC and differential gradient ultracentrifugation. The arrows highlight the EVs. Bar 100 nm

EV production has been widely demonstrated in bacteria due to a wide range of cell membranes, but not all processes are fully understood [1]. Due to their similar composition to the bacteria, the vesicles are often antigenic scaffolds of the bacterium itself, and may have potential uses as inducers of protection through vaccination. Immunogenicity, efficacy, and effectiveness of EV vaccines have generally been considered limited in terms of strain and type coverage

compared to conjugate vaccines [12]. Moreover, virulence factors found in EVs include so-called superantigens capable of activating a substantial portion of human T cell repertoire by microbe-host interactions [13]. Therefore, they are safer than OMV-based vaccines due the toxicity of LPS constituents [14].

We showed for the first time EV production by *Mycoplasma hyopenumoniae*. Future studies addressing how EVs are influenced by different environmental conditions would advance the current knowledge.

Conclusion

This study demonstrated for the first time that Mhyo strain J and a wild isolate have the ability of producing EVs. Mhyo EVs range in size from 100 to 500 nm. Also, we performed two independent experiments which indicate reproducibility.

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Declarations

Conflict of interest The authors declare no competing interests.

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References

1. Gill S, Catchpole R, Forterre P (2019) Extracellular membrane vesicles in the three domains of life and beyond. FEMS Microbiol Rev 43:273–303. https://doi.org/10.1093/femsre/fuy042
2. Kim KW (2018) Visualization of extracellular vesicles of prokaryotes and eukaryotic microbes. Appl Microsc 48:96–101. https://doi.org/10.9729/am.2018.48.4.96
3. Bielska E, Birch PRJ, Buck AH, et al (2019) Highlights of the mini-symposium on extracellular vesicles in inter-organismal communication, held in Munich, Germany, August 2018. J Extracell Vesicles 8:. https://doi.org/10.1080/20013078.2019.1590116
4. Gaurivaud P, Ganter S, Villard A et al (2018) Mycoplasmas are no exception to extracellular vesicles release: revisiting old concepts. PLoS ONE 13:14. https://doi.org/10.1371/journal.pone.0208160
5. Chernov VM, Mouzykantov AA, Baranova NB et al (2014) Extracellular membrane vesicles secreted by mycoplasma Acholeplasma laidlawii PG8 are enriched in virulence proteins. J Proteomics 110:117–128. https://doi.org/10.1016/j.jprot.2014.07.020
6. Health for Animals (2019) Roadmap to reducing the need for antibiotics
7. Théry C, Witwer KW, Aikawa E, et al (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles 7:. https://doi.org/10.1080/20013078.2018.1535750
8. Cook BS, Beddow JG, Manso-Silván L et al (2016) Selective medium for culture of Mycoplasma hyopneumoniae. Vet Microbiol 195:158–164. https://doi.org/10.1016/j.vetmic.2016.09.022
9. Chutkan H, MacDonald I, Manning A, Kuehn MJ (2013) Quantitative and qualitative preparations of bacterial outer membrane vesicles. In: Methods in molecular biology. pp 259–272
10. Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212. https://doi.org/10.1083/jcb.17.1.208
11. Ayers L, Pink R, Carter DRF, Nieuwland R (2019) Clinical requirements for extracellular vesicle assays. J Extracell Vesicles 8:. https://doi.org/10.1080/20013078.2019.1593755
12. Petousis-Harris H, Radcliff FJ (2019) Exploitation of Neisseria meningitidis Group B OMV vaccines against N. Gonorrhoeae to inform the development and deployment of effective gonorrhea vaccines. Front Immunol 10:1–11. https://doi.org/10.3389/fimmu.2019.00683
13. Liu Y, Defourny KAY, Smid EJ, Abee T (2018) Gram-positive bacterial extracellular vesicles and their impact on health and disease. Front Microbiol 9:1–8. https://doi.org/10.3389/fmicb.2018.01502
14. Acevedo R, Fernández S, Zayas C et al (2014) Bacterial outer membrane vesicles and vaccine applications. Front Immunol 5:1–6. https://doi.org/10.3389/fimmu.2014.00121