ISOLATION AND PURIFICATION OF LEPTOSPIRAL EXTRACELLULAR VESICLES

Eakalak Phanchamnan[1,3], Kanitha Patarakul[2,3]

[1]Medical Microbiology, interdisciplinary program, Graduate School, Chulalongkorn University

[2]Department of Microbiology, Faculty of Medicine, Chulalongkorn University

[3]Center of Excellence in Vaccine Research and Development (Chula Vaccine Research Center, Chula VRC), Chulalongkorn University

---

ABSTRACT

Several pathogenic gram-negative bacteria including *Leptospira* produce extracellular vesicles (ECVs) possibly derived from bacterial outer membrane. ECVs harbor multiple active molecules that serve many functions such as secretion system, communication tool, and vaccine candidates. The previous report demonstrated that the chemically induced leptospiral ECV (iECV)-based vaccines conferred protective immunity against lethal infection in the animal model. Therefore, leptospiral ECVs may contain potential vaccine antigens. However, the natural leptospiral ECV (nECV) has not been characterized. In this study, we isolated and purified both nECV and iECV from *Leptospira interrogans* serovar Pomona by filtration and ultracentrifugation and then purified by density sucrose gradient. Our results indicated that the nECV and iECV were mainly separated into 50% sucrose fraction. Moreover, the immunoblotting analysis revealed that LipL32, known leptospiral outer membrane lipoprotein, was present in both nECV and iECV. This study should lead to further investigation in the role of ECVs in pathogenesis and identification of new vaccine candidates for leptospirosis.

Keywords: Extracellular vesicles, *Leptospira*, Isolation and Purification

1. Introduction

Bacterial extracellular vesicles (ECVs) are spherical membrane-bound particles of 10-300 nm in diameter. These vesicles are commonly produced by gram-negative bacteria and not only found in pathogenic but also saprophytic species, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Helicobacter pylori* and *Leptospira interrogans* (Cañas et al., 2016; Haake & Matsunaga, 2002; Keenan et al., 2000; MacDonald & Kuehn, 2013; Mondal et al., 2016). Natural ECVs may be pinched off at the outer membrane leaflet and simultaneously harbor multiple molecules including nucleic acid (DNA and/or RNA), proteins, lipopolysaccharide, and metabolites, that serve potential functions such as secretion system, communication tool, and vaccine candidates (Jan, 2017). For instance, Shiga toxin-containing ECVs of pathogenic *E. coli* induced target cell deaths in colon epithelial cell line (Bielaszewska et al., 2017). Moreover, ECVs derived from *Neisseria meningitidis* showed the protective effect and promising vaccine candidates (Acevedo et al., 2017). Besides natural ECV, chemically

induced ECVs (iECVs) have been studied (Haake & Matsunaga, 2002; Williams et al., 2007). The chemically induced ECVs are produced by treating intact cells with chemical reagents in order to break down and subsequently reform of debris membrane to become vesicles (Klimentova & Stulik, 2015; Kunjantarachot, 2014). Like natural ECVs, chemically induced ECVs are spherical nanosized particles which harbor multiple active molecules (Williams et al., 2007). Furthermore, the iECVs have been demonstrated as vaccine candidates in several infectious diseases (Acevedo et al., 2017; van der Pol, Stork, & van der Ley, 2015).

Pathogenic *Leptospira* spp. are causative agents of leptospirosis, a globally widespread zoonosis with public health concern which causes 1 million leptospirosis cases and approximately 60,000 deaths annually (Jimenez, Marroquin, Richards, & Amin, 2018). Endemic areas are mostly in tropical and subtropical countries. The transmission of leptospirosis is commonly through direct or indirect contact with the reservoirs including rodents, pigs, dogs, or contaminated water and soil with urine, mucous or blood of the infected host. Leptospires enter host via broken skin or mucous membrane and subsequently penetrate though endothelial layer of blood vessel by using endoflagella and then reach target organs such as kidney, liver, lung, brain, and heart (Lambert et al., 2012). Human leptospirosis typically encompasses two phases of clinical manifestations. The symptoms in the first acute phase are non-specific such as headache, fever, loss of appetite, vomiting, myalgia, and nausea. Clinical manifestations in the second phase or immunologic phase are related to target organ involvements including pulmonary hemorrhagic syndrome, myocarditis, meningitis, hepatic failure, and renal failure that may lead to death (Adler & de la Peña Moctezuma, 2010).

Recently, the chemically induced leptospiral ECVs (iECVs), which were produced by using citrate buffer pH 3.0 to induce cell lysis and reforming of vesicles, were demonstrated as vaccine candidates and conferred 100% survival in hamster model with significantly decrease bacterial load in target organs (Kunjantarachot, 2014). Moreover, proteomic analysis revealed that iECVs contained outer membrane proteins (OMPs) including OmpL1, LipL32, LipL36, and LipL46 (Haake & Matsunaga, 2002). However, the efficient and reproducible methods for leptospiral nECVs isolation and purification have not been verified. In this study we aim to evaluate the methods to isolate and purify both nECVs and iECVs from pathogenic *Leptospira*.

2. Objectives of the study

To isolate and purify extracellular vesicles from pathogenic *Leptospira interrogans* serovar Pomona.

3. Materials and methods

Bacterial cultivation. *L. interrogans* serovar Pomona were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% bovine serum albumin (BSA) at 28-30°C until the log phase (approximately 2-5x10^{8} cells/ml) was reached.

Isolation and purification of leptospiral ECVs. To remove intact cells, leptospiral cultures at optimal concentration were centrifuged at 12,000xg at 25°C for 40 min. The supernatant were collected and filtered through 0.45 µm and 0.22 µm pore size filter membranes, respectively. The filtered supernatants were subjected to ultracentrifugation at 50,000xg for 1 h and subsequently 100,000xg, 4°C for 3 h using ultra-clear polycarbonate ultracentrifuge tube (Backman Coulter, USA), Ti 45 type rotor (Backman Coulter, USA). The pellets were collected and resuspended in EMJH media without albumin to obtain natural ECVs (nECV).

To produce leptospiral iECVs after centrifugation of leptospiral cultures at 12,000xg for 25°C for 40 min to remove intact cells as mentioned above, 0.1 M citrate buffer pH 3.0 was added to the cell pellet on ice and then shaked vigorously for 3 h with vortexing for 2 min at 20-min intervals. The suspension was centrifuged at 10,000xg for 10 min and the pellet was resuspended in the final volume of 20 ml 0.1 M citrate buffer pH 3.0 then passed through a pressure homogenizer at 12,000 psi then ultracentrifuged at 100,000xg, 4°C for 1 h. The pellet was resuspended in 1.6 ml at the final volume of 0.1 M citrate buffer pH 3.0 and subsequently centrifuged at 200xg for 5 min. The supernatant contained chemically induced ECVs (iECVs).

The natural and chemically induced ECVs were further purified by density sucrose gradient at 10%-60% (wt/vol) of sucrose concentrations then ultracentrifugation at 77,000xg for 16-18 h, 4°C using polypropylene centrifuge tube (Backman Coulter, USA) and SW 41 swing rotor (Backman Coulter, USA).

Transmission electron miscopy (TEM). To observe the morphology and size of ECVs, the purified ECVs were adsorbed to polyvinyl formal-carbon-coated grids (EMS) for 10 min, and negatively stained with 4% (w/v) uranyl acetate for 5 min. the grids were washed twice with sterile water and examined using a transmission electron microscope (S-4800,Hitachi) at the Petroleum and Petrochemical College, Chulalongkorn University.

Gel electrophoresis and immunoblotting. To confirm the ECVs were produced from *L. interrogans* serovar Pomona, the ECVs were solubilized in sample buffer composed of 20 mM sodium phosphate, 2% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, 0.2 M dithiothreitol (DTT), and 2% glycerol and separated on 15% SDS-polyacrylamide gel (SDS-PAGE). The gel was stained with Coomassie Brilliant Blue R250 or transferred to 0.45 µm pore size nitrocellulose membrane (Millipore) for immunoblotting. Subsequently, the membrane was blocked with 1% BSA in 0.05% tween20-PBS for 1 h at room temperature, incubated for 1 h with anti-LipL32 diluted 1:5,000 in 0.05% tween20-PBS, probed with mouse anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase diluted 1:10,000 in 0.05% tween20-PBS. The antigen-antibody binding was detected by chemiluminescent system (BioRad).

4. Results

The culture supernatant of leptospiral cultivation was filtered and ultracentrifuged and then the pellet was used to isolate nECVs. Treatment of *L. interrogans* serovar Pomona with citrate buffer pH 3.0 and passed through

pressure homogenizer to break cells and then outer compartment reformed to vesicles as iECVs. Both nECVs and iECVs were purified by density sucrose gradient (Fig 1A). The result showed that both nECVs and iECVs were separated mainly into 50% w/v sucrose fractions (Fig 1B, 1C).

TEM with negative staining showed *L. interrogans* serovar Pomona producing ECV (fig 2A). Moreover, the nECVs and iECVs were spherical vesicles in nanosize and were absent of contaminated cell debris compared to crude nECVs obtained from ultracentrifugation at 100,000xg (fig 2B, 2C, 2D). Moreover, the size of iECVs was approximately 10-50 nm in diameter which was smaller than that of nECVs (approximately 50 to 100 nm in diameter) and a number of iECVs were higher than nECVs (Fig 2B, 2C).

To confirm that nECVs and iECVs were produced from outer membrane of *L. interrogans*, nECVs and iECVs were separated on 15% polyacrylamide gel and immunoblotting against anti-LipL32 antibody, the result showed that LipL32 was detected in both nECVs and iECVs (Fig 3).

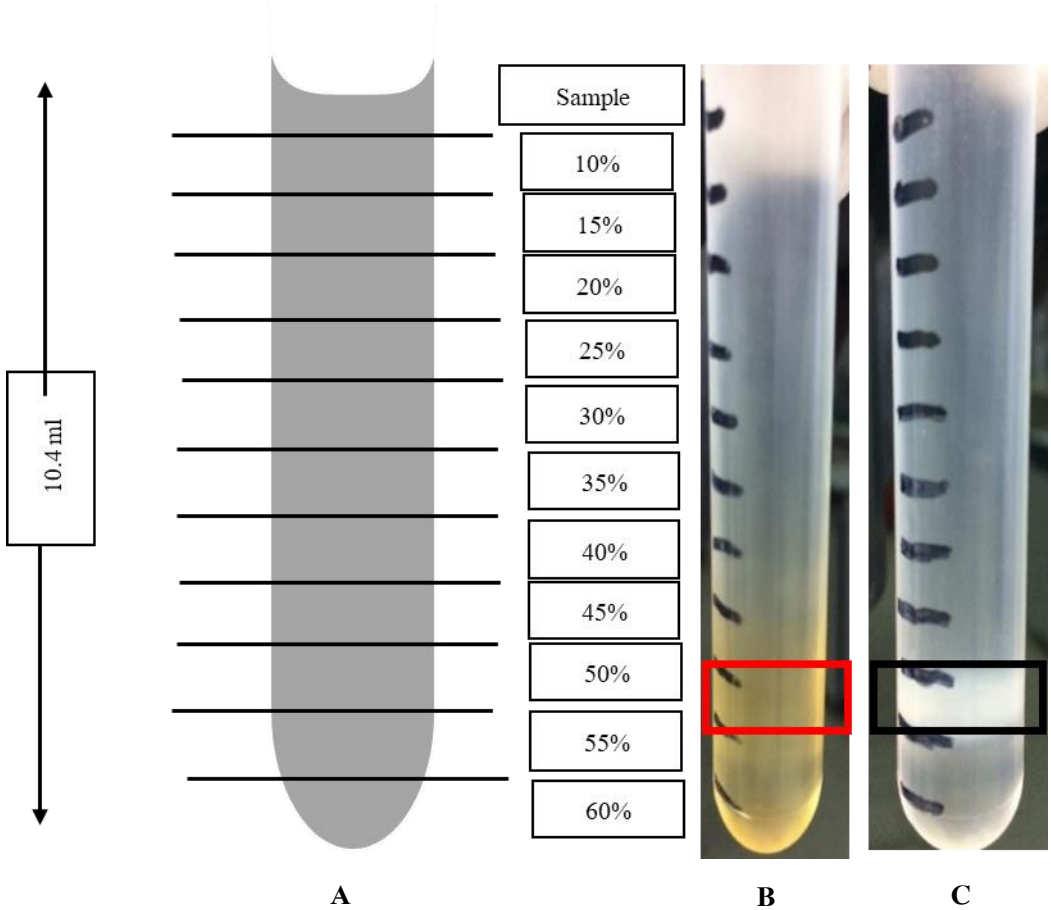

Figure 1. Purification of leptospiral ECVs by density sucrose gradient at 10% - 60% w/v sucrose. A, a diagram of the centrifuge tube and percentage of concentration of sucrose in each fraction. B, nECVs represent in red box (the yellow ring at approximately 50% w/v sucrose fraction). C, iECVs represent in black box (the white ring at 50 % w/v sucrose fraction).

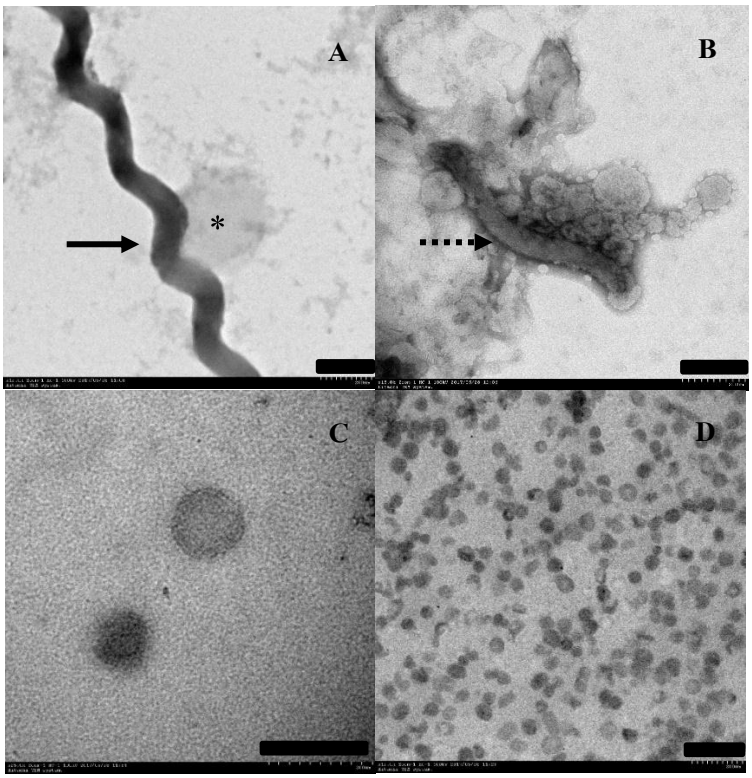

Figure 2. Transmission electron micrograph. A, *Leptospira interrogans* serovar Pomona intact cell, solid arrow indicates the intact cell, star indicates the ECV. B, natural leptospiral extracellular vesicles (nECVs) isolated by ultracentrifugation at 100,000xg, 4°C for 3 h, broken arrow indicates the debris cell. C, natural extracellular vesicles (nECVs) purified by density sucrose gradient. D, chemically induced leptospiral extracellular vesicles (iECVs) purified by density sucrose gradient. Black bar represents 200 nm.

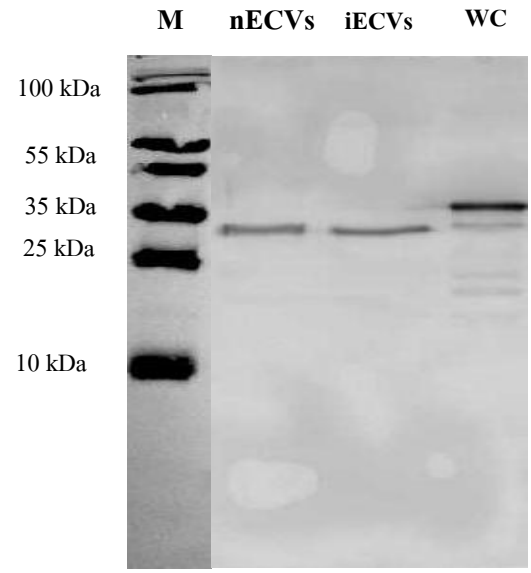

Figure 3. Detection of LipL32 in leptospiral ECVs. nECVs and iECVs were subjected for immunoblotting against anti-LipL32 antibody. M, marker. nECVs, natural extracellular vesicles. iECVs, chemically-induced extracellular vesicles. WC, whole cell lysate.

5. Discussion

Bacterial extracellular vesicles are pinched off outer membrane leaflet of bacteria and therefore simultaneously harbor multiple active molecules, which serve as secretion system, communication tool, and vaccine candidates. Furthermore, ECV derived from pathogenic bacteria showed a role in pathogenesis. In order to characterize ECV, purified ECVs are required. To decrease contaminants including secreted proteins and debris cells, density sucrose gradient is commonly used for purification. In this study, both nECVs and iECVs were sedemented in 50% w/v sucrose or 1.64 g/ml, which differed from the density of sucrose gradient at 1.19 g/ml used to purify alkaline plasmolysis buffer treated leptospiral ECVs of similar size from *L. kirschneri* strain RM52. This may be because of buoyant density of the ECVs (Klimentova & Stulik, 2015).

Currently available killed-whole cell vaccines, bacterins, confer protective immunity against leptospirosis, but there are diversed side effect and serovar-specific (Wang, Jin, & Węgrzyn, 2007). Lipopolysaccharide (LPS) was found to be immunodominant epitopes that stimulated host immune responses and therefore provide short-term immunity against leptospirosis (Sonrier et al., 2000). For these reasons, protein antigen-based vaccines have been evaluated for subunit vaccines, especially outer membrane proteins (Wang et al., 2007). Bacterial extracellular vesicles harbor immunogenic molecules including outer membrane protein, LPS, flagellin, and nucleic acid (Marini et al., 2017; Roberts et al., 2008). These molecules have been considered as pathogen-associated molecular patterns (PAMPs) that are recognized by pathogen recognition receptors (PRRs) on host immune cells in order to stimulate innate immune response and subsequently promote adaptive immunity against particular pathogens (Marini et al.,

2017). The nECV and iECV contained LipL32 (Fig 3). Similar evidences were reported (Haake & Matsunaga, 2002; Kunjantarachot, 2014). The LipL32 is an outer membrane protein of pathogenic *Leptospira* that previously demonstrated as a vaccine candidate (Dellagostin et al., 2011). Furthermore, immunohistochemical examination of the infected tissues revealed that ECVs were observed suggesting that leptospiral ECV may play a role in pathogenesis and protective immunity (Monahan, Callanan, & Nally, 2008).

In summary, our results provide isolation and purification methods of leptospiral ECVs in order to decrease contaminant for further analysis. Outer membrane proteins of leptospiral ECVs may play a role in pathogenesis and could be used as subunit vaccine candidates in the future.

6. Conclusion

Pathogenic *Leptospira* spontaneously produced natural ECVs (nECVs) during routine culture in EMJH medium. The chemically induced ECVs (iECVs) were prepared by treatment of intact leptospiral cells with 0.1 M citrate buffer pH 3. The nECVs and iECVs were separated into 50% sucrose fraction in purification step by density sucrose gradient. The nECVs and iECVs consist of outer membrane proteins including LipL32. Further characterization of ECVs are required to study their role in pathogenesis and potential subunit vaccine candidates.

Acknowledgements

I would like to acknowledge The National Research Council of Thailand and the Higher Education Research Promotion and the National Research University Project of Thailand, Office of the Higher Education Commission and Chulalongkorn Academic Advancement into Its 2nd Century Project.

References

Acevedo, R., Zayas, C., Norheim, G., Fernandez, S., Cedre, B., Aranguren, Y., . . . Garcia, L. (2017). Outer membrane vesicles extracted from *Neisseria meningitidis* serogroup X for prevention of meningococcal disease in Africa. *Pharmacol Res, 121*, 194-201. doi: 10.1016/j.phrs.2017.04.030

Adler, B., & de la Peña Moctezuma, A. (2010). Leptospira and leptospirosis. *Vet Microbiol, 140*(3), 287-296. doi: https://doi.org/10.1016/j.vetmic.2009.03.012

Bielaszewska, M., Ruter, C., Bauwens, A., Greune, L., Jarosch, K. A., Steil, D., . . . Karch, H. (2017). Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: Intracellular delivery, trafficking and mechanisms of cell injury. *PLoS Pathog, 13*(2), e1006159. doi: 10.1371/journal.ppat.1006159

Cañas, M.-A., Giménez, R., Fábrega, M.-J., Toloza, L., Baldomà, L., & Badia, J. (2016). Outer Membrane Vesicles from the Probiotic *Escherichia coli* Nissle 1917 and the Commensal ECOR12 Enter Intestinal Epithelial Cells via Clathrin-Dependent Endocytosis and Elicit Differential Effects on DNA Damage. *PLoS One, 11*(8), e0160374. doi: 10.1371/journal.pone.0160374

Dellagostin, O. A., Grassmann, A. A., Hartwig, D. D., Félix, S. R., da Silva, É. F., & McBride, A. J. A. (2011). Recombinant vaccines against Leptospirosis. *Human Vaccines, 7*(11), 1215-1224. doi: 10.4161/hv.7.11.17944

Haake, D. A., & Matsunaga, J. (2002). Characterization of the leptospiral outer membrane and description of three novel leptospiral membrane proteins. *Infect Immun, 70*(9), 4936-4945.

Jan, A. T. (2017). Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. *Front Microbiol, 8*, 1053. doi: 10.3389/fmicb.2017.01053

Jimenez, J. I. S., Marroquin, J. L. H., Richards, G. A., & Amin, P. (2018). Leptospirosis: Report from the task force on tropical diseases by the World Federation of Societies of Intensive and Critical Care Medicine. *J Crit Care, 43*, 361-365. doi: 10.1016/j.jcrc.2017.11.005

Keenan, J., Day, T., Neal, S., Cook, B., Perez-Perez, G., Allardyce, R., & Bagshaw, P. (2000). A role for the bacterial outer membrane in the pathogenesis of *Helicobacter pylori* infection. *FEMS Microbiol Lett, 182*(2), 259-264. doi: 10.1111/j.1574-6968.2000.tb08905.x

Klimentova, J., & Stulik, J. (2015). Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiol Res, 170*, 1-9. doi: 10.1016/j.micres.2014.09.006

Kunjantarachot, A. (2014). *Immunogenicity of Leptospira interrogans Outer Membrane Vesicles in a Hamster Model* (Vol. 05).

Lambert, A., Picardeau, M., Haake, D. A., Sermswan, R. W., Srikram, A., Adler, B., & Murray, G. A. (2012). FlaA proteins in *Leptospira interrogans* are essential for motility and virulence but are not required for formation of the flagellum sheath. *Infect Immun, 80*(6), 2019-2025. doi: 10.1128/IAI.00131-12

MacDonald, I. A., & Kuehn, M. J. (2013). Stress-Induced Outer Membrane Vesicle Production by Pseudomonas aeruginosa. *J Bacteriol, 195*(13), 2971-2981. doi: 10.1128/JB.02267-12

Marini, A., Rossi, O., Aruta, M. G., Micoli, F., Rondini, S., Guadagnuolo, S., . . . Koeberling, O. (2017). Contribution of factor H-Binding protein sequence to the cross-reactivity of meningococcal native outer membrane vesicle vaccines with over-expressed fHbp variant group 1. *PLoS One, 12*(7), e0181508. doi: 10.1371/journal.pone.0181508

Monahan, A. M., Callanan, J. J., & Nally, J. E. (2008). Proteomic analysis of *Leptospira interrogans* shed in urine of chronically infected hosts. *Infect Immun, 76*(11), 4952-4958. doi: 10.1128/IAI.00511-08

Mondal, A., Tapader, R., Chatterjee, N. S., Ghosh, A., Sinha, R., Koley, H., . . . Pal, A. (2016). Cytotoxic and Inflammatory Responses Induced by Outer Membrane Vesicle-Associated Biologically Active Proteases from *Vibrio cholerae*. *Infect Immun, 84*(5), 1478-1490. doi: 10.1128/IAI.01365-15

Roberts, R., Moreno, G., Bottero, D., Gaillard, M. E., Fingermann, M., Graieb, A., . . . Hozbor, D. (2008). Outer membrane vesicles as acellular vaccine against pertussis. *Vaccine, 26*(36), 4639-4646. doi: 10.1016/j.vaccine.2008.07.004

Sonrier, C., Branger, C., Michel, V., Ruvoën-Clouet, N., Ganière, J. P., & André-Fontaine, G. (2000). Evidence of cross-protection within *Leptospira interrogans* in an experimental model. *Vaccine, 19*(1), 86-94. doi: https://doi.org/10.1016/S0264-410X(00)00129-8

van der Pol, L., Stork, M., & van der Ley, P. (2015). Outer membrane vesicles as platform vaccine technology. *Biotechnol J, 10*(11), 1689-1706. doi: 10.1002/biot.201400395

Wang, Z., Jin, L., & Węgrzyn, A. (2007). Leptospirosis vaccines. *Microbial Cell Factories, 6*, 39-39. doi: 10.1186/1475-2859-6-39

Williams, J. N., Skipp, P. J., Humphries, H. E., Christodoulides, M., O'Connor, C. D., & Heckels, J. E. (2007). Proteomic analysis of outer membranes and vesicles from wild-type serogroup B *Neisseria meningitidis* and a lipopolysaccharide-deficient mutant. *Infect Immun, 75*(3), 1364-1372. doi: 10.1128/IAI.01424-06