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ORIGINAL ARTICLE

Characterization of *Escherichia coli* outer membrane vesicles and the impact of pathogenic ones on NLR signaling pathways

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ABSTRACT

Background and Objectives: The secretion of outer membrane vesicles (OMVs) is a universal event among bacteria. In this study, we characterized OMVs from pathogenic and non-pathogenic strains of *Escherichia coli* and assessed the effect of pathogenic OMVs on NLR signaling pathways.
Materials and Methods: OMVs were extracted by differential centrifugation and characterized by scanning electron microscopy (SEM), SDS-PAGE, Limulus amebocyte lysate (LAL) test, and nucleic acid extraction. Then, the Caco-2 cells were treated with the pathogenic OMVs to evaluate their effect on NLR signaling pathways.
Results: SEM showed that pathogenic and non-pathogenic strains produced OMVs in the range of 9-72.9 and 45-270 nm, respectively. The SDS-PAGE revealed that both OMVs had protein bands ranging from 25 to 100 kDa. The LAL test displayed that the concentration of LPS was 2.368 and 0.055 EU/ml in pathogenic and non-pathogenic OMVs, respectively. The evaluation of nucleic acid contents showed no significant difference between both types of OMVs. The assessment of pathogenic OMVs' effect on NLR genes demonstrated that the expression level was changed in some genes.
Conclusion: The characterization of OMVs showed that both strains of *E. coli* secrete OMVs in different sizes and contents. Besides, it was revealed that OMVs can regulate gene expression.

Keywords: *Escherichia coli*; Outer membrane vesicles; NOD-like receptors; Signaling pathways

INTRODUCTION

All prokaryotic and eukaryotic cells can generate vesicles as an original mechanism for cell-free intercellular communications (1-3). Gram-negative bacteria-derived outer membrane vesicles (OMVs) are secreted by pathogenic and non-pathogenic strains during bacterial life cycle in vivo and in vitro (4). OMVs are nano-sized spherical phospholipids containing various cargo including DNA, RNA, proteins, glycolipids, and organic small molecules (5). Gram-negative bacteria have a complicated cell wall consisting of the inner membrane (IM), periplasmic space (peptidoglycan (PG) layer), and outer membrane (OM). Some specific proteins connect these three layers; Lpp- a lipoprotein (in *Escherichia coli*) binds OM to PG (covalent bonds), the Tol-Pal system binds IM to OM (non-covalent bonds), and OmpA-

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an OM protein binds OM to PG (non-covalent bonds) (6). Occasionally these connections are destroyed by repositioning or breaking which leads to OMV formation (7, 8). Meanwhile, the accumulation of curvature-inducing molecules such as lipopolysaccharide (LPS) and phospholipids in flimsy areas of OM (9) and the accumulation of PG fragments or misfolded proteins in the periplasmic space induce vesiculation (10). OMVs have several functions in a bacterial life cycle; OMVs serve as a secretion system to deliver their cargo (e.g. virulence factors and toxins) into the environment (e.g. host cells) (11). OMVs protect virulence factors from host cell enzymatic degradation allowing them to be transported safely and directly into host cells (12, 13). The cargo of OMVs contains immunomodulatory and antimicrobial components that will enable OMVs to help pathogenic bacteria capture their colonization niches, damage host cell functions, induce inflammatory responses, and adjust host defense (11, 14). OMVs enter the host cells through different mechanisms including i) lipid rafts, the most common OMVs entrance method into host cells (e.g., *Haemophilus influenzae* OMVs) (15), ii) dynamin-dependent, cholesterol-independent endocytosis (e.g., enterohemorrhagic *E. coli* OMVs) (16), and iii) cholesterol-independent, clathrin-mediated endocytosis (e.g., *Helicobacter pylori* OMVs) (17). OMVs' immunogenic cargos act as pathogen-associated molecular patterns (PAMPs) which can be sensed by pattern recognition receptors (PRRs, e.g., TLRs, NLRs, RLRs, ALRs) resulting in the induction of innate immunity (18, 19). NOD-like receptors (NLRs) are a family of cytosolic sensors, including 22 NLRs in humans, contributing to host innate immunity (20). NLRs recognize the host cells' molecules (e.g., ATPs, cholesterol crystals, uric acid, etc.) and microbial molecules (e. g. PG, flagellin, viral RNA, fungal hyphae, etc.) (21). Activated NLRs perform four different functions: inflammasome formation, signaling transduction, transcription activation, and autophagy (21). *E. coli*, a member of the Enterobacteriaceae family, has a broad range of strains from commensal inhabitants of the gastrointestinal tract to diverse harmful pathogens. In this study, we evaluated the physicochemical characteristics of OMVs extracted from pathogenic and non-pathogenic strains of *E. coli* and assessed the effect of pathogenic OMVs on the level of gene expression in the NLR signaling pathways. The value of this research lies in its potential to advance our knowledge of OMVs' cargo sorting, function, and their role in bacterial pathogenesis, offering new perspectives on how pathogenic *E. coli* strains interact with host immune systems and paving the way for vaccine technology and diagnostic methods.

MATERIALS AND METHODS

Bacterial strain. Standard strains of pathogenic (ATCC8739) and non-pathogenic (ATCC25922) *E. coli* were taken from the standard bacterial collection of the Pasteur Institute of Iran.

OMV preparation. Following the overnight incubation in LB broth media, OMVs were extracted from pathogenic and non-pathogenic strains of *E. coli*, as described in previous studies (Fig. 1) (22).

Evaluation of physicochemical properties of extracted OMVs:

Scanning electron microscopy (SEM). The morphology and size of extracted OMVs were detected by scanning electron microscopy (23). Briefly, after ultrasonic treatment, OMVs were affixed to Formvar/carbon-coated nickel grids. The grids were washed by a 0.01 M PBS supplemented with 0.5% bovine serum albumin (BSA-Sigma), and 0.1% gelatin (PBG-Sigma). Subsequently, the grids were fixed with 1% glutaraldehyde in PBS and negatively stained with potassium phosphotungstate (pH 6.0). The grids were examined by a field emission scanning electron microscope (FE-SEM) (HITACHI CS-4160).

Determination of OMVs lipopolysaccharide endotoxin (LPS) content. The content of the extracted OMVs LPS was measured using the Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific, USA). According to the manufacturer's instructions, the microplate was incubated at 37°C for 10 min. 50 µl of each sample and standards were dispensed into the microplate wells and the microplate was incubated at 37°C for 5 min. Then, 50 µl of LAL was added to each well and the microplate was incubated at 37°C for 10 min. 100 µl of substrate solution was poured into each well and the microplate was incubated at 37°C for 6 min. Finally, 50 µl of stop solution (25% acetic acid) was added to each well and the optical density (OD) was measured at 405-410 nm using a microplate reader.

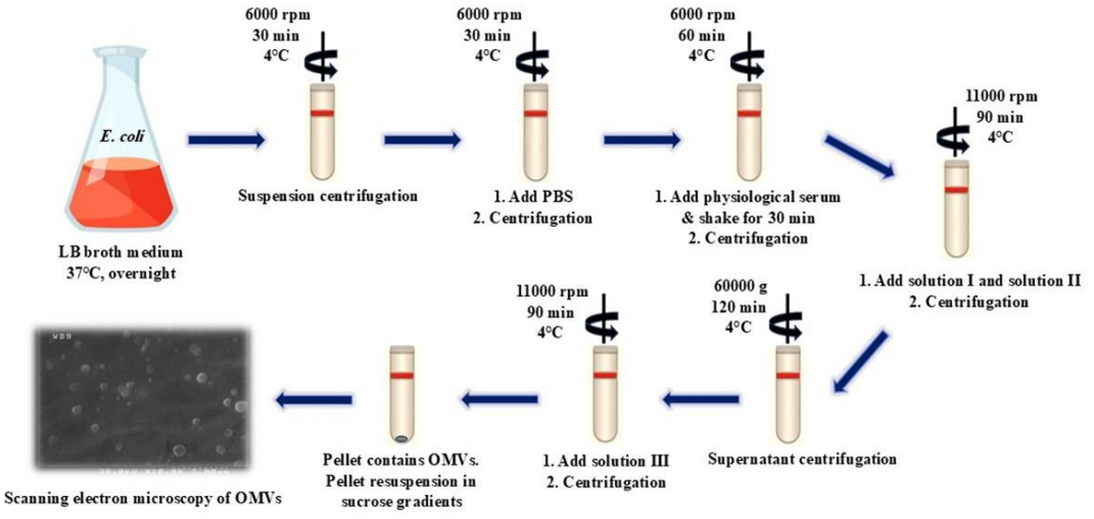

Fig. 1. Outer membrane vesicles (OMVs) extraction protocol.
Solution I (tris buffer + EDTA), solution II (tris buffer + EDTA + sodium deoxycholate), and solution III (tris buffer + sodium deoxycholate).

Determination of OMVs protein content and profiles. To determine the total amount of proteins, extracted OMVs were evaluated using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The protein profiles of extracted OMVs were visualized by SDS-PAGE on 12% gels using a mini-protein II electrophoresis apparatus (Bio-Rad, USA). The gel was stained by Coomassie brilliant blue.

Determination of OMVs nucleic acid content. To estimate the nucleic acid content of extracted OMVs, DNA and RNA were extracted using DNP TM Kit (SinaClon, Iran) and RNX- Plus (CinnaGen, Iran), respectively, according to the manufacturer's instructions.

Cell line and culture conditions. Caco-2 human colon adenocarcinoma cell line (ATCC HTB-37) was used to investigate how *E. coli* adheres to, invades, or affects intestinal epithelial cells (24). The Caco-2 cell line was taken from the Center for Genetic Reserves in Iran. The cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Gibco, Carlsbad, CA, USA), supplemented with 25 mM HEPES, 1% non-essential amino acids, 10% heat-inactivated fetal calf serum (FCS)(Gibco), penicillin G (100 U/mL), and streptomycin (100 µg/mL) (Gibco) at 37°C in a 5% CO_2 atmosphere.

Cell treatment with OMVs. Caco-2 cells were seeded at 3×10^5 cells/well in a six-well plate and experiments were performed when the cell count reached 10^6 cells. To evaluate OMVs' effect on Caco-2 cells, cells were treated with extracted OMVs (50 µg/ml) from a pathogenic strain of *E. coli* and incubated at 37°C in a 5% CO_2 atmosphere for 24 h for real-time PCR.

RNA extraction, cDNA synthesis, and real-time PCR. After 24 h, total RNA was extracted from the Caco-2 cells using the High Pure RNA Isolation Kit (Qiagen, Germany), following the manufacturer's instructions. The purity of RNA was assessed with a NanoDrop spectrophotometer and 1µg of the extracted RNA was used for cDNA synthesis, using the RT2 First Strand Kit (Qiagen). Real-time PCR, based on SYBR Green chemistry, was conducted with the NLR Signaling Pathways RT^2 Profiler PCR Array kit (Qiagen) according to the provided instructions. Data analysis was performed using a PCR array data analysis Web portal (SA Bioscience). The beta-actin (ACTB) gene was applied for normalization of the sample data. Also, the normalization of the housekeeping genes (β2 microglobulin (B2M), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1(HPRT1), and ribosomal protein lateral stalk subunit P0 (RPLP0)) was performed by calculating the cycle threshold (ΔΔCT) for each target gene in the plate. The RT^2

Profiler PCR array data analysis software calculates the fold change using the ΔΔCT method (25, 26). To ensure the accuracy of the test, reverse transcription control (RTC), positive PCR control (PPC), and human genomic DNA contamination control (HGDC) were included in the plate.

Statistical analyses. The real-time PCR data were statistically analyzed, using the Qiagen online analysis system, following the manufacturer's proposed guidelines.

RESULTS

Physicochemical properties of pathogenic and non-pathogenic *E. coli* OMVs. OMVs were extracted from the pathogenic and non-pathogenic strains of *E. coli* and visualized by SEM. SEM observations revealed that both strains produced OMVs in various sizes. The profile of OMVs protein was determined by SDS-PAGE. The LAL test displayed that the concentration of LPS was different between pathogenic and non-pathogenic OMVs. The evaluation of nucleic acid contents showed no significant difference between both types of OMVs. Table 1 and Fig. 2 summarize all the data.

The pathogenic OMVs internalized into the Caco-2 cells and regulated gene expression in NLR signaling pathways. To test if the OMVs' PAMPs are sensed by intracellular PRRs, NLRs gene expression was evaluated. As shown in Fig. 3, under the effect of OMVs, NLR genes were divided into three categories: up-regulated, down-regulated, and unchanged. Clustergram plot by mean log two-fold change of each gene in the Caco-2 cell line exposed to pathogenic OMVs was mapped using the online software of Qiagen. Up-regulated and down-regulated genes were detected in different signaling pathways (Table 2). Among up-regulated genes, the highest increase in gene expression level was shown in CASP1 (15.9-fold), NLRC5 (15.6-fold), CCL5 (13.6-fold), and IFNγ (10.5-fold) compared to untreated cells. Among down-regulated genes, the highest decrease in gene expression level was observed in PANX1 (-4.6-fold) and TXNIP (-3.2-fold) compared to untreated cells.

Table 1. Characterization of extracted outer membrane vesicles (OMVs) from pathogenic and non-pathogenic strains of *Escherichia coli.*

Strain	Pathogenic (ATCC8739)	Non-pathogenic (ATCC25922)
Size (nm)	9 - 72.9	45- 270
LPS content (EU/ml)	2.368	0.055
Protein content (mg/ml)	1	0.9
Protein size (kDa)	>25 -100	25 -100
DNA content (ng/µl)	20.5	48.8
RNA content (ng/µl)	1662.5	1565.7

DISCUSSION

Both pathogenic and non-pathogenic bacteria secrete OMVs in various sizes and contents (27). Bacteria exploit various approaches to interact with the host to fine-tune immunity and/or induce disease (28). Secretion of OMVs provides a pathway for bacteria to deliver their immunodominant cargo to the host cells which leads to induction of innate immune receptors, e. g. NLRs and TLRs (18, 19, 28). In the current study, we extracted OMVs from pathogenic and non-pathogenic strains of *E. coli* and evaluated their physicochemical characteristics (Table 1). In line with our previous study, electron microscopy images showed that pathogenic and non-pathogenic strains of *E. coli* can produce OMVs and pathogenic strain produce more and smaller ones (23). The size of the OMV is a critical factor for penetration into the host cell, so small-sized OMVs (< 120 nm) can easily internalize into cells and interact with PRRs (29-31). Our results showed that OMVs from pathogenic and non-pathogenic strains of *E. coli* had different protein profiles and sizes. Although OMVs were extracted from an equal volume of biomass, the pathogenic ones had more bands in close different sizes but the non-pathogenic ones had sharper bands. Fábrega et al. demonstrated that two non-pathogenic strains of *E. coli*, Nissle 1917 (EcN) (a probiotic strain) and ECOR12 (a commensal *E. coli* strain isolated from a healthy human stool sample) release OMVs ranging from 20-60 nm in diameter. This study showed that both strains had similar protein profiles and LPS amounts (32). In another study, it was shown that protein profiles of two pathogenic strains of *E. coli* including LB226692 (isolated during the 2011 outbreak from a patient with hemolytic uremic syndrome (HUS)) and C227-11Φcu (a *stx*$_{2a}$ -phage-cured derivative of the outbreak strain) were similar but not

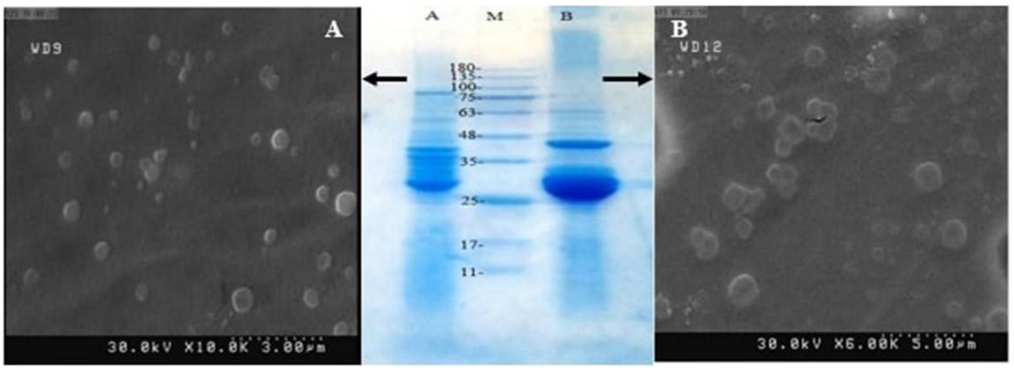

Fig. 2. Scanning electron microscopy observations and protein profiles of extracted outer membrane vesicles (OMVs) from pathogenic (A) and non-pathogenic (B) strains of *Escherichia coli*.

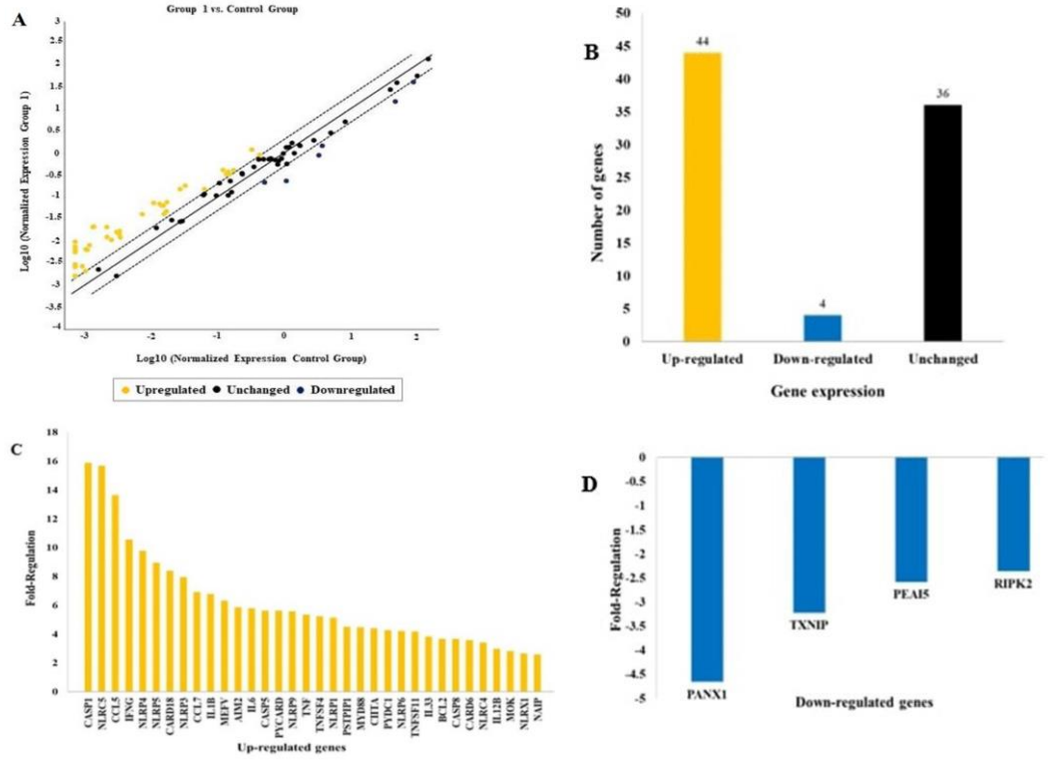

Fig. 3. Expression analysis of genes in NLR signaling pathways in Caco-2 cell line stimulated with pathogenic *Escherichia coli* outer membrane vesicles (OMVs). A) gene expression assay; B) gene expression chart; C) up-regulated genes; D) down-regulated genes. Data are presented as fold-change relative to housekeeping genes.

identical (33). The OMV protein cargo contributes to some biological processes such as outer membrane formation, pathogenesis, proper folding of a protein, siderophore transport, and the integration of protein into the membrane (34). We found that the concentration of LPS was much higher in pathogenic OMVs compared to non-pathogenic ones; this result was in line with our previous findings (23). OMV is a new vaccine candidate but native OMVs contain an excessive amount of endotoxic LPS, which triggers the secretion of proinflammatory cytokines in host cells (35). To achieve a safer and more applicable vaccine delivery system, OMVs have to endure some mutational inactivation, such as inactivation of the *msb*B gene encoding an acyltransferase, which plays a role in lipid A biosynthesis, to produce low toxic OMVs (36). The evaluation of OMVs for nucleic acid content demonstrated no significant difference between

Table 2. Pathogenic *Escherichia coli* outer membrane vesicles (OMVs) regulate gene expression in NLR signaling pathways.

Gene expression	Gene symbol	Signaling pathways
	AIM2	Inflammasomes: AIM2
	CASP1, CASP5	Inflammasomes: NLRP1
	PYCARD, NAIP	Inflammasomes: IPAF
Up-regulation	MYD88, IL-33, MOK, IL-1B, IL-12B, IFNγ	Inflammasomes: Downstream Signaling
	PSTPIP1, MEFV, PYDC1, TNFSF11, CARD18, TNF, BCL2, TNFSF4	Inflammasomes: Negative Regulation
	CCL5, CCL7, CASP8, CARD6, IL6	NOD-Like Receptors: Downstream Signaling
	NLRX1, NLRP1, NLRP3, NLRP4, NLRP5, NLRP6, NLRP9, CIITA, NLRC4, NLRC5	NOD-Like Receptors: Receptor
Down-regulation	PANX1, TXNIP, RIPK2	Inflammasomes: Downstream Signaling
	PEA15	NOD-Like Receptors: Downstream Signaling

pathogenic and non-pathogenic OMVs. Delivery of small signaling molecules and nucleic acid (DNA and RNA) by OMVs plays an important role in intra- and interspecies communication (37-39). In the next step in our study, Caco-2 cells were treated with the pathogenic OMVs and their effect on the level of gene expression was evaluated in NLR signaling pathways. OMVs act as a delivery system by presenting virulence and cytotoxic factors to the host cells; subsequently, they stimulate an immune response that leads to the production of cytokines, chemokines, and antimicrobial peptides (23, 40, 41). NLRs are intracellular innate immune receptors that can sense OMVs' immunodominant cargo (20). Following the treatment of Caco-2 cells with pathogenic OMVs, some genes in NLR signaling pathways were up-regulated; the highest increase in gene expression level was shown in CASP1(involving in inflammasome formation), NLRC5 (a NOD-Like Receptor), CCL5 (a NOD-Like Receptor), and IFNγ (involving in inflammasome formation) respectively. Besides, some genes were down-regulated; the highest decrease in gene expression level was observed in PANX1 and TXNIP (involving in inflammasome formation) (Table 2). Interestingly, several genes remained unchanged. These results demonstrate the regulatory effect of OMVs on various genes in host signaling pathways. Recognition of intracellular ligands by NLRs leads to the activation of nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK) signaling and finally the secretion of inflammatory cytokines (42). Previous studies have shown that cytoplasmic NOD host receptors NOD1 and NOD2 are activated by OMVs from pathogenic and non-pathogenic bacteria (*H. pylori*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Porphyromonas gingivalis*, and *E. coli* (ECOR12, Nissle 1917) (28). Despite these findings, NOD1 and NOD2 were not activated after OMV treatment in our study. In line with our results, OMVs from various bacterial species (*P. aeruginosa*, *Bordetella pertussis*, *E. coli* (K-12 BW25113), *N. gonorrhoeae*, and uropathogenic *E. coli*) activated NLRP3 and NLRC4 (28). It was shown that *Neisseria meningitidis* OMVs induce the production of CCL2, CCL3 and CCL5 (also known as RANTES), CXCL8, IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, and TNF43 in monocytes and macrophages (43). In accordance with this study, CCL5 was up-regulated in our study. Kim et al. found that intravenous injection of OMVs from *E. coli* induced a strong IFN-γ- and T cell-mediated anti-tumor response (44); in line with this study, IFN-γ was up-regulated in our study. Despite the extensive search, we did not find any studies examining the effects of OMVs on the specific genes we identified as up or down-regulated. Our research seems to be the

first to reveal how pathogenic *E. coli* OMVs modulate the expression of particular genes within the NLR signaling pathways. Our findings propose that OMVs from the pathogenic strains of *E. coli* (with exclusive content) can play vital roles in the simplification of infection and inflammation in the host.

CONCLUSION

In conclusion, our results demonstrated that pathogenic and non-pathogenic strains of *E. coli* secrete OMVs in different sizes and contents. Besides, it was revealed that pathogenic OMVs could regulate gene expression in NLR signaling pathways. To better understand the key role of outer membrane vesicles (OMVs) in host-microbe interactions, further investigation is needed into the sorting of OMV cargo and the regulation of signaling pathways by these vesicles. Future findings will facilitate the utilization of OMV for vaccine development and diagnostic methods (3).

Limitation. Due to limited financial resources, we only evaluated the effect of pathogenic OMVs on the level of gene expression in NLR signaling pathways. While the assessment of both pathogenic and non-pathogenic OMVs provides comparable and more comprehensive results.

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REFERENCES

1. Kuehn MJ, Kesty NC. Bacterial outer membrane vesicles and the host–pathogen interaction. *Genes Dev* 2005; 19: 2645-2655.
2. Mashburn-Warren LM, Whiteley M. Special delivery: vesicle trafficking in prokaryotes. *Mol Microbiol* 2006; 61: 839-846.
3. Mohammadzadeh R, Ghazvini K, Farsiani H, Soleimanpour S. *Mycobacterium tuberculosis* extracellular vesicles: exploitation for vaccine technology and diagnostic methods. *Crit Rev Microbiol* 2021; 47: 13-33.
4. Jan AT. Outer membrane vesicles (OMVs) of gram-negative bacteria: a perspective update. *Front Microbiol* 2017; 8: 1053.
5. Nagakubo T, Nomura N, Toyofuku M. Cracking open bacterial membrane vesicles. *Front Microbiol* 2020; 10: 3026.
6. Schwechheimer C, Sullivan CJ, Kuehn MJ. Envelope control of outer membrane vesicle production in Gram-negative bacteria. *Biochemistry* 2013; 52: 3031-3040.
7. Moon DC, Choi CH, Lee JH, Choi C-W, Kim H-Y, Park JS, et al. *Acinetobacter baumannii* outer membrane protein A modulates the biogenesis of outer membrane vesicles. *J Microbiol* 2012; 50: 155-160.
8. Schwechheimer C, Rodriguez DL, Kuehn MJ. NlpI-mediated modulation of outer membrane vesicle production through peptidoglycan dynamics in *Escherichia coli*. *Microbiologyopen* 2015; 4: 375-389.
9. Mashburn LM, Whiteley M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* 2005; 437: 422-425.
10. Schwechheimer C, Kulp A, Kuehn MJ. Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiol* 2014; 14: 324.
11. Amano A, Takeuchi H, Furuta N. Outer membrane vesicles function as offensive weapons in host–parasite interactions. *Microbes Infect* 2010; 12: 791-798.
12. Bomberger JM, MacEachran DP, Coutermarsh BA, Ye S, O'Toole GA, Stanton BA. Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog* 2009; 5(4): e1000382.
13. Jurkoshek KS, Wang Y, Athman JJ, Barton MR, Wearsch PA. Interspecies communication between pathogens and immune cells via bacterial membrane vesicles. *Front Cell Dev Biol* 2016; 4: 125.
14. Ellis TN, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol Mol Biol Rev* 2010; 74: 81-94.
15. Sharpe SW, Kuehn MJ, Mason KM. Elicitation of epithelial cell-derived immune effectors by outer membrane vesicles of nontypeable *Haemophilus influenzae*. *Infect Immun* 2011; 79: 4361-4369.
16. Bielaszewska M, Rüter C, Kunsmann L, Greune L, Bauwens A, Zhang W, et al. Enterohemorrhagic *Escherichia coli* hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis. *PLoS Pathog* 2013; 9(12): e1003797.
17. Parker H, Chitcholtan K, Hampton MB, Keenan JI. Uptake of *Helicobacter pylori* outer membrane vesicles by gastric epithelial cells. *Infect Immun* 2010; 78: 5054-5061.

18. Johnston EL, Kufer TA, Kaparakis-Liaskos M (2020). Immunodetection and pathogenesis mediated by bacterial membrane vesicles. Bacterial membrane vesicles. pp.159-188. https://link.springer.com/chapter/10.1007/978-3-030-36331-4_8
19. Kaparakis-Liaskos M, Ferrero RL. Immune modulation by bacterial outer membrane vesicles. *Nat Rev Immunol* 2015; 15: 375-387.
20. Mukherjee T, Hovingh ES, Foerster EG, Abdel-Nour M, Philpott DJ, Girardin SE. NOD1 and NOD2 in inflammation, immunity and disease. *Arch Biochem Biophys* 2019; 670: 69-81.
21. Motta V, Soares F, Sun T, Philpott DJ. NOD-like receptors: versatile cytosolic sentinels. *Physiol Rev* 2015; 95: 149-178.
22. Ahmadi Badi S, Moshiri A, Ettehad Marvasti F, Mojtahedzadeh M, Kazemi V, Siadat SD. Extraction and evaluation of outer membrane vesicles from two important gut microbiota members, Bacteroides fragilis and Bacteroides thetaiotaomicron. *Cell J* 2020; 22: 344-349.
23. Behrouzi A, Vaziri F, Riazi Rad F, Amanzadeh A, Fateh A, Moshiri A, et al. Comparative study of pathogenic and non-pathogenic *Escherichia coli* outer membrane vesicles and prediction of host-interactions with TLR signaling pathways. *BMC Res Notes* 2018; 11: 539.
24. Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol Toxicol* 2005; 21: 1-26.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408.
26. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008; 3: 1101-1108.
27. Deatherage BL, Lara JC, Bergsbaken T, Barrett SL, Lara S, Cookson BT. Biogenesis of bacterial membrane vesicles. *Mol Microbiol* 2009; 72: 1395-1407.
28. Johnston EL, Heras B, Kufer TA, Kaparakis-Liaskos M. Detection of bacterial membrane vesicles by NOD-like receptors. *Int J Mol Sci* 2021; 22: 1005.
29. Cecil JD, O'Brien-Simpson NM, Lenzo JC, Holden JA, Chen Y-Y, Singleton W, et al. Differential responses of pattern recognition receptors to outer membrane vesicles of three periodontal pathogens. *PLoS One* 2016; 11(4): e0151967.
30. Kaparakis M, Turnbull L, Carneiro L, Firth S, Coleman HA, Parkington HC, et al. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell Microbiol* 2010; 12: 372-385.
31. Patten DA, Hussein E, Davies SP, Humphreys PN, Collett A. Commensal-derived OMVs elicit a mild proinflammatory response in intestinal epithelial cells. *Microbiology (Reading)* 2017; 163: 702-711.
32. Fábrega MJ, Aguilera L, Giménez R, Varela E, Alexandra Canas M, Antolín M, et al. Activation of immune and defense responses in the intestinal mucosa by outer membrane vesicles of commensal and probiotic *Escherichia coli* strains. *Front Microbiol* 2016; 7: 705.
33. Kunsmann L, Rüter C, Bauwens A, Greune L, Glüder M, Kemper B, et al. Virulence from vesicles: Novel mechanisms of host cell injury by *Escherichia coli* O104: H4 outbreak strain. *Sci Rep* 2015; 5: 13252.
34. Lee J, Kim OY, Gho YS. Proteomic profiling of Gram-negative bacterial outer membrane vesicles: Current perspectives. Proteomics *Clin Appl* 2016; 10: 897-909.
35. Van Der Pol L, Stork M, van der Ley P. Outer membrane vesicles as platform vaccine technology. *Biotechnol J* 2015; 10: 1689-1706.
36. Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem* 2007; 76: 295-329.
37. Ahmadi Badi S, Bruno SP, Moshiri A, Tarashi S, Siadat SD, Masotti A. Small RNAs in outer membrane vesicles and their function in host-microbe interactions. *Front Microbiol* 2020; 11: 1209.
38. Dell'Annunziata F, Folliero V, Giugliano R, De Filippis A, Santarcangelo C, Izzo V, et al. Gene transfer potential of outer membrane vesicles of gram-negative bacteria. *Int J Mol Sci* 2021; 22: 5985.
39. Rodriguez BV, Kuehn MJ. *Staphylococcus aureus* secretes immunomodulatory RNA and DNA via membrane vesicles. *Sci Rep* 2020; 10: 18293.
40. Chatterjee D, Chaudhuri K. *Vibrio cholerae* O395 outer membrane vesicles modulate intestinal epithelial cells in a NOD1 protein-dependent manner and induce dendritic cell-mediated Th2/Th17 cell responses. *J Biol Chem* 2013; 288: 4299-4309.
41. Schertzer JW, Whiteley M. Bacterial outer membrane vesicles in trafficking, communication and the host-pathogen interaction. *J Mol Microbiol Biotechnol* 2013; 23: 118-130.
42. Franchi L, Warner N, Viani K, Nuñez G. Function of Nod-like receptors in microbial recognition and host defense. *Immunol Rev* 2009; 227: 106-128.
43. Tavano R, Franzoso S, Cecchini P, Cartocci E, Oriente F, Aricò B, et al. The membrane expression of *Neisseria meningitidis* adhesin A (NadA) increases the proimmune effects of MenB OMVs on human macrophages, compared with NadA–OMVs, without further stimulating their proinflammatory activity on circulating monocytes. *J Leukoc Biol* 2009; 86: 143-153.
44. Kim OY, Park HT, Dinh NTH, Choi SJ, Lee J, Kim JH, et al. Bacterial outer membrane vesicles suppress tumor by interferon-γ-mediated antitumor response. *Nat Commun* 2017; 8: 626.