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Klebsiella pneumoniae derived outer membrane vesicles mediated bacterial virulence, antibiotic resistance, host immune responses and clinical applications

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ABSTRACT

Klebsiella pneumoniae is a gram-negative pathogen that can cause multiple diseases including sepsis, urinary tract infections, and pneumonia. The escalating detections of hypervirulent and antibiotic-resistant isolates are giving rise to growing public concerns. Outer membrane vesicles (OMVs) are spherical vesicles containing bioactive substances including lipopolysaccharides, peptidoglycans, periplasmic and cytoplasmic proteins, and nucleic acids. Emerging studies have reported various roles of OMVs in bacterial virulence, antibiotic resistance, stress adaptation, and host interactions, whereas knowledge on their roles in *K. pneumoniae* is currently unclear. In this review, we summarized recent progress on the biogenesis, components, and biological function of *K. pneumoniae* OMVs, the impact and action mechanism in virulence, antibiotic resistance, and host immune response. We also deliberated on the potential of *K. pneumoniae* OMVs in vaccine development, as diagnostic biomarkers, and as drug nanocarriers. In conclusion, *K. pneumoniae* OMVs hold great promise in the prevention and control of infectious diseases, which merits further investigation.

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Outer membrane vesicles; *Klebsiella pneumoniae*; virulence; host immune responses; clinical application

Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is a commensal and opportunistic pathogen that can cause multiple diseases including sepsis, urinary tract infections, liver abscesses, and pneumonia, especially in people with a compromised immune system [1,2]. The emergence of hypervirulent and antibiotic-resistant *K. pneumoniae* is a cause of increased concern for this pathogen, especially the infections caused by ST11 hypervirulent carbapenem-resistant *K. pneumoniae* (hvCRKP) strains [3–5]. The World Health Organization (WHO) has published its surveillance report on antimicrobial-resistant carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains, which have emerged in all WHO regions and been listed as the priority pathogen with urgent need of antibacterial drug developments [6]. According to the data of WHO GLASS in 2021, CRKP resistance rates of meropenem and imipenem were 12.34% and 10.63%, respectively, and the rate varied in different regions and populations [7]. The data of China Antimicrobial Surveillance Network (CHINET) in 2023 indicated that the resistant rate of *K. pneumoniae* to meropenem has dramatically increased from 2.9% in 2005 to 30.0% in 2023 [8]. Limited treatments for CRKP made related infection a public threat.

Transport and accumulation of membranous vesicles are essential for intercellular communication; numerous bacteria secrete membrane vesicles under physiological conditions. Bacterial extracellular vesicles (BEVs) or outer membrane vesicles (OMVs) are spherical vesicles secreted by bacteria, which are 20–400 nm in diameter, and contain a variety of bioactive substances including lipopolysaccharides, peptidoglycans, periplasmic and cytoplasmic proteins, toxins, and nucleic acids [9]. Both Gram-negative and Gram-positive bacteria secrete vesicles; the one produced by Gram-negative bacteria was often called OMV, whereas since Gram-positive bacteria do not contain an outer membrane and the vesicles they produced were called BEVs [10]. The diameter of OMVs ranged from 20 to 250 nm [11]. BEVs play important roles in bacterial virulence, pathogenesis, antibiotic resistance, stress adaptation, horizontal gene transfer, transport of cell metabolites, and bacterial interactions within bacterial communities and with host [12–16]. OMV is also being investigated as a potential vaccine ingredient due to its immunogenicity

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[17,18]. In addition, since OMV is widely present in body fluids (such as the cerebrospinal fluid, blood, and stool) after infection [19], it may serve as a potential biomarker for the clinical identification of bacterial infection. There are growing interests on the research of *K. pneumoniae* OMVs in recent years, indicating import[1] roles in host interaction and potential clinical application (Figure 1).

In this review, we summarized recent progress on the biogenesis, components, and biological function of *K. pneumoniae* OMVs and the impact and action mechanism of *K. pneumoniae* OMVs in antibiotic resistance, virulence, and host immune response during infection. We also discussed the potential application of *K. pneumoniae* OMVs in the vaccine development, as diagnostic biomarkers and as drug carriers.

Biogenesis and components of OMVs

OMVs were found in the culture medium of *Escherichia coli* early in the 1960s, and the bacteria adapted to the environment by releasing vesicles, which can transmit extracellular membrane proteins, virulence factors, and antigens to the host cells [20,21]. OMVs are secretory vesicles produced by bacteria in a programmed manner, similar to exosomes or microvesicles in eukaryotes, which can be generated by membrane budding and extruding spherical membrane particles outward. There are two main routes for Gram-negative bacteria to form vesicles: blebbing of the bacterial outer membrane bubbles, thus forming outer membrane vesicles, or producing inner and outer membrane vesicles or explosive outer membrane vesicles through explosive cell lysis [19]. The detailed mechanisms of OMV synthesis were summarized as the following four models [22]: (1) reduced cross-linking between the outer membrane and the peptidoglycan layer; (2) accumulation of certain envelope proteins, peptidoglycan, and membrane lipids, which exert pressure on the membrane and result in membrane budding and OMV production; (3) accumulation of lipopolysaccharides (LPS) and phospholipids, leading

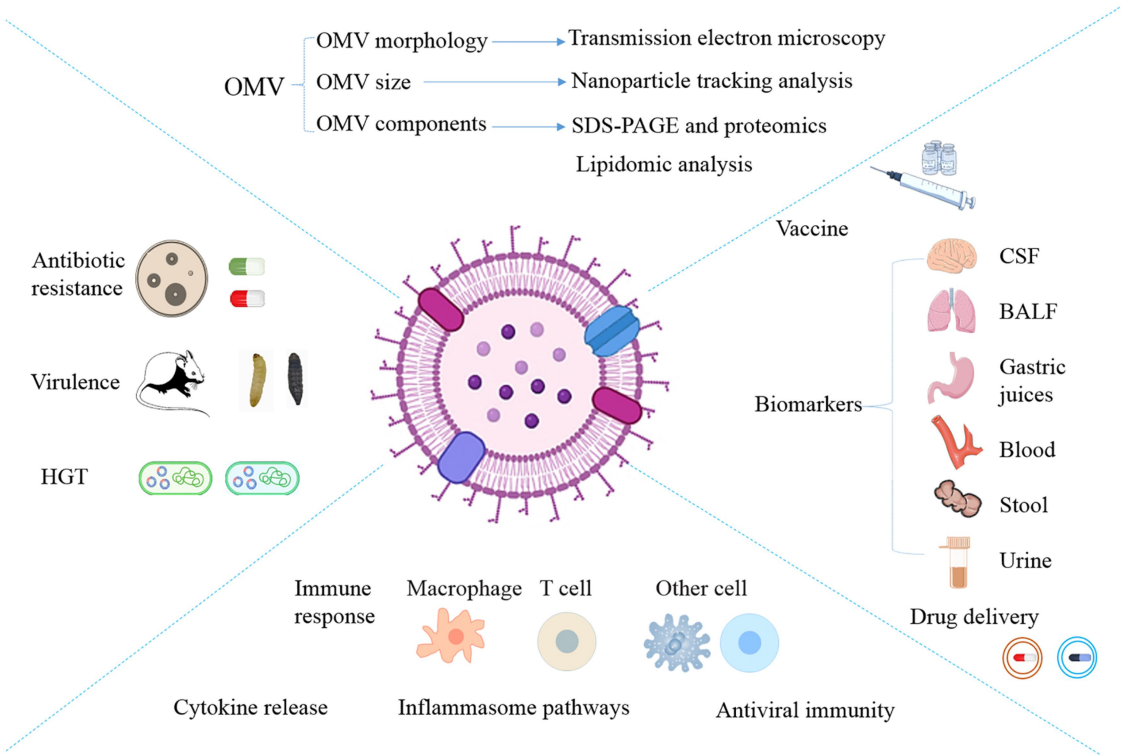

Figure 1. Characterization, biological functions, action mechanisms, and potential application of *K. pneumoniae* OMVs. The identification of OMVs mainly includes the morphology, size, and composition of OMVs. OMVs play roles in bacterial antibiotic resistance, virulence, pathogenesis, horizontal gene transfer, and host immune responses via different cells. OMVs are present in various body fluids after infection; hence, it may serve as a potential biomarker for the clinical identification of bacterial infection. OMVs are also utilized in the vaccine development and drug delivery to combat related infectious diseases. Drawn by BioRender and modified by Microsoft PowerPoint.

[1]import should be important.

to OMV biogenesis; and (4) extracellular stimulation to promote the formation of local curvature of the bacterial outer membrane (Figure 2). OMVs can be generated at any stage of bacterial growth; however, the bacterial growth conditions, namely, on the solid plate, in liquid media, and in the biofilm state, exerted significant effects on their production or composition. In addition, the bacterial external environment such as temperature, nutrient depletion, and antibiotics can cause increased OMV production and changed the composition of biochemical macromolecules in the OMVs [23]. Iron-limited media can result in HvKP releasing more OMVs than classic *K. pneumoniae* [24]. The deletion of the *wbbO* gene involved in the O antigen modification of LPS could change the composition of *K. pneumoniae* OMVs by decreasing the amounts of outer membrane proteins and increasing the expression of cytoplasmic proteins [25]. The treatment of meropenem, amikacin, polymyxin B, and a combination of these agents could change the lipidome or protein patterns of *K. pneumoniae* OMVs [26–28]. Further studies are necessary to reveal the genetic regulation of OMV biogenesis and release.

OMVs are secreted from the bacterial surface and located in the cell-free supernatant of the bacterial culture. Commonly employed methods for the extraction and purification of BEVs include ultracentrifugation, density gradient centrifugation, ultrafiltration, size exclusion chromatography, tangential flow filtration, polyethylene glycol precipitation, immunomagnetic bead separation, microfluidics, and aptamer-based magnetic separation [29]. Among them, ultracentrifugation and density gradient centrifugation are the most prevalent methods, known as the "gold standard" for OMV separation [30,31]. The combination of density gradient centrifugation and ultracentrifugation method is commonly utilized to attain high-purity OMVs through prolonged centrifugation and the addition of a specific separation medium, such as sucrose and iodixanol, by exploiting the disparities in size, molecular weight, and density between OMVs and other biological molecules [9]. Due to the high separation purity of this method, it can mitigate the damage inflicted by centrifugal force on OMVs and is applicable for functional studies, biomarker detection, and content analysis of OMVs [32]. For large volume samples such as bacterial culture supernatants,

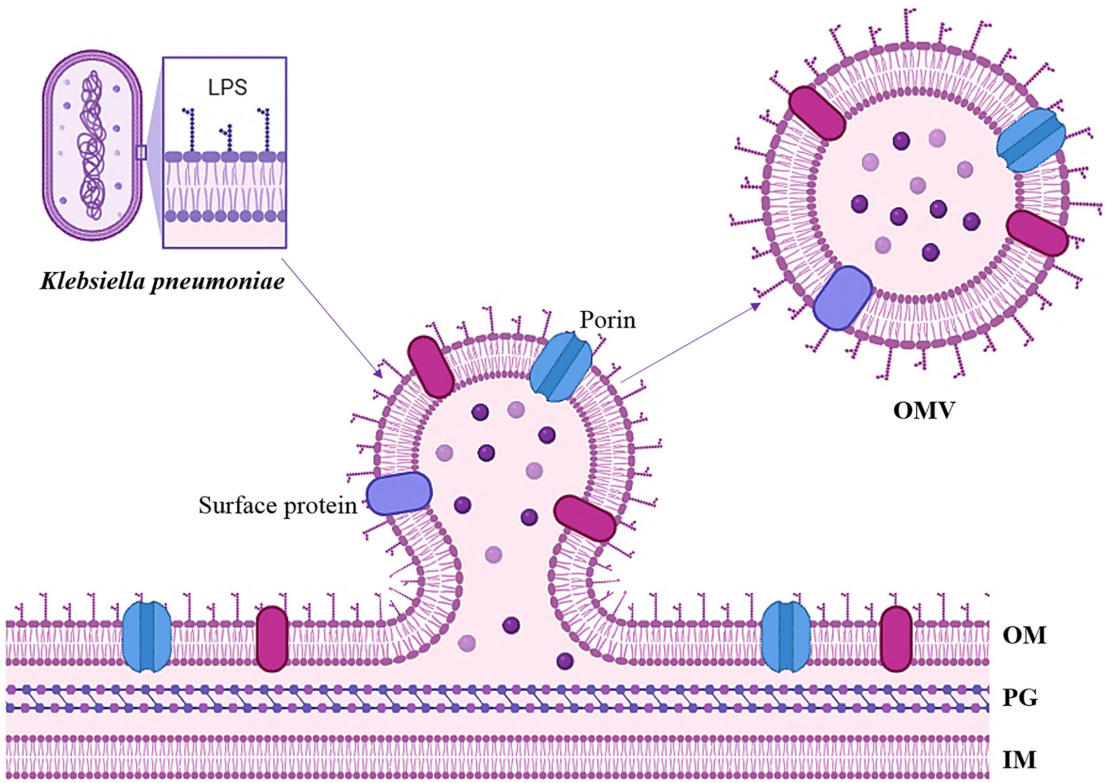

Figure 2. Schematic figure of OMV formation by outer membrane blebbing in *Klebsiella pneumoniae*. Abbreviations: OM, out membrane; PG, peptidoglycan; IM, inner membrance. Drawn by BioRender and modified by Microsoft PowerPoint.

ultracentrifugation and density gradient centrifugation can achieve relatively high extraction efficiency at a lower cost.

Different separation methods have their own advantages and disadvantages; therefore, choosing the appropriate separation and purification method for studying OMVs is very important.[2]

Once OMVs are purified, their quantification and quality determination constitute vital steps for further application. The identification of OMVs includes their morphology, size, and composition [33]. Transmission electron microscopy (TEM) is considered as the "gold standard" for identifying the OMV morphology. TEM can provide high-resolution images of OMVs, which can then be analysed using image processing software to quantify vesicle numbers per unit area. Nanoparticle tracking analysis (NTA), the most prevalently utilized and well-established method, has been extensively employed for characterizing the size and distribution of BEVs [34]. NTA is a method that utilizes light scattering to track individual particles in solution, allowing for the determination of both the particle size and the concentration of OMVs. This technique can be particularly useful for analysing heterogeneous populations of OMVs. Nevertheless, NTA itself has stringent requirements for temperature, concentration, and calibration during detection and exhibits poor specificity, being unable to discriminate OMVs, cell debris, and potential contaminants in the solution. Dynamic light scattering (DLS) is a technique that is typically employed to measure the size distribution of extracellular vesicles in liquid suspension [9,34]. DLS is easy to operate, sample preparation is straightforward, and the detection sensitivity is elevated, enabling rapid measurement of particles as small as 1 nm. However, DLS is prone to the physical and chemical properties of the particles, such as colour, charge, and magnetism, and is highly susceptible to dust and impurities. Hence, it is merely suitable for the size measurement of relatively high concentration and well-defined nanoparticles, and is unable to measure the concentration of particles in the sample, with low specificity. Tunable Resistive Pulse Sensing (TRPS) measures variations in electrical current as particles traverse a nanopore, enabling the sizing and enumeration of individual vesicles based on their volume and surface charge [35]. Flow cytometry can be utilized to analyse OMVs by marking them with fluorescent dyes or antibodies specific to surface markers, allowing for quantitative assessment based on fluorescence intensity [35]. As for the components of OMVs, lipids can be identified by liquid chromatograph–mass spectrometry, and protein components are usually analysed by SDS-PAGE and proteomics [33,36]. It is important to note that each method has its own set of properties regarding accuracy, precision, sample preparation requirements, and potential interference from contaminants, and thus careful selection should be made according to specific research needs. Therefore, different methods can usually be combined to characterize the features of OMVs.

K. pneumoniae-derived OMVs can be generated under both normal and stressed conditions containing proteins with diverse biological functions. Lee et al. investigated the production of OMVs by *K. pneumoniae* ATCC 13883 cultured in LB broth and showed that *K. pneumoniae* produced spherical bilayer OMVs (with diameters ranging from 20 to 200 nm), as indicated by transmission electron microscopy analysis [37]. Proteome analysis of *K. pneumoniae*-derived OMVs identified 159 proteins from various bacterial locations, including extracellular space, outer membrane, periplasmic space, inner membrane, and the cytoplasm. The most abundant proteins in the OMVs included the outer membrane protein X, murein lipoprotein, phage shock protein, protein YgdR, and 30S ribosomal protein S20. Lan et al. reported that there is no difference in the production of OMVs between HvKP and classic *K. pneumoniae* (cKP) in iron-sufficient medium, whereas HvKP released more OMVs than cKP in iron-deficient medium, indicating the roles of OMVs in the adaptive response of HvKP to iron limitation [24]. Proteome analysis revealed that OMVs' proteins participated in 11 biological processes, including the cellular process, cellular component biogenesis, localization, biological regulation, reproductive process, biological adhesion, metabolic process, multi-organism process, stress response, developmental process, and carbon utilization. Proteome analysis of OMVs identified a variety of proteins involved in iron acquisition, binding, and transportation. The compositions of both the outer membrane and OMVs of *K. pneumoniae* 43816 were affected by the deletion of the *wbbO* gene, which encodes the galactosyltransferase involved in the O antigen modification of LPS [25]. OMVs from the wild type strain were enriched in outer membrane proteins, whereas loss of O antigen resulted in significantly decreased amounts of outer membrane proteins and enriched cytoplasmic proteins

[2]The sentence should be included in the previous paragraph, right after the "at a lower cost".

in Δ*wbbO* OMVs. Proteins enriched in OMVs included porins (such as OmpA, OmpK17, OmpK35, and OmpK36), murein lipoprotein, SlyB, Tsx, and YfaZ. The OMVs of Δ*wbbO* reduced the content of outer membrane proteins and increased proteins involved in post-translational modification, protein turnover, and chaperones.

Virulence and resistance-related proteins or genes were also detected in OMVs of *K. pneumoniae* strains, indicating their roles in bacterial virulence, antibiotic resistance, and other stress response processes. Jasim et al. performed lipidomic analysis and demonstrated that the lipid composition of OMVs from *K. pneumoniae* strains was mainly composed of glycerophospholipids (~35%), fatty acids (~33%), and sphingolipids (~20%), with a lower proportion of glycerolipids (~4%), sterol lipids (~3%), and prenol lipids (~4%) [38]. The global lipidome of OMVs changed significantly between the polymyxin-susceptible and polymyxin-resistant *K. pneumoniae* strains. Polymyxin B treatment reduced the glycerophospholipid, fatty acid, lysoglycerophosphate, and sphingolipid content in the OMVs isolated from the polymyxin-susceptible strains. Conversely, the OMVs isolated from the polymyxin-resistant strains were richer in these lipid species whether under polymyxin treatment or not. Wang et al. identified virulence and resistance genes in the OMVs of *K. pneumoniae* strains [39]. Several virulence genes, including *rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg344*, were detected in the OMVs of the hypervirulent *K. pneumoniae* (HvKP) strain hvK2115. Meanwhile, resistance genes *bla*KPC-2, *bla*CTX-M-1, and *mph(A)* were detected in the OMVs of the CRKP strain CRK3022. Li et al. also detected carbapenemase genes and virulence genes in the OMVs of a CR-HvKP strain (NUHL30457) [40].

Antibiotic treatments altered the content composition of *K. pneumoniae*-derived in a strain phenotype-dependent manner. Different effects on the protein composition were reported for the OMVs of the polymyxin-susceptible and resistant strains [26]. Comparative proteomics of OMVs from paired polymyxin-susceptible and an extremely resistant *K. pneumoniae* strains revealed the existence of proteins involved in outer membrane assembly (lipopolysaccharide, O-antigen, and peptidoglycan biosynthesis), cationic antimicrobial peptide resistance, β-lactam resistance, and quorum sensing. Polymyxin B treatment reduced the expression of OMV proteins related to adhesion, virulence, and the cell envelope stress responses in the polymyxin-susceptible strain [26]. In the polymyxin-resistant strain, proteins involved in LPS biosynthesis, RNA degradation, and nucleotide excision repair significantly increased under polymyxin B treatment. Fan et al. revealed a heterogeneous protein pattern for the OMVs purified from CRKP strains cultivated with or without meropenem induction [27]. The OMVs' proteome profile analysis indicated that differentially expressed proteins were involved in bacterial virulence, pathogenicity, drug resistance, stress response, bacterial survival, and cellular metabolism. The resistance-associated proteins included penicillin-binding protein 1B, EmrA, $Na^{(+)}$/anti-drug transporter, arnT, arnA, arnC, *bla*KPC, *bla*SHV, SHV-112, macB, outer membrane channel protein, and outer membrane protein OmpK36. In addition, the meropenem treatment could significantly increase the contents of proteins and DNA packed in the OMVs of CRKP [41]. Lucena et al. reported changed protein cargo of OMVs isolated from extensively drug-resistant (XDR) *K. pneumoniae* under the treatment of subinhibitory concentrations of meropenem, amikacin, polymyxin B, and a combination of these agents [28]. Different antibiotic treatments altered the OMV proteome profile by changing proteins involved in genetic information processing, energy metabolism, cell envelope formation, environmental adaption, and antibiotic resistance. These suggests that OMVs may be associated with pathogenicity, survival, stress response, and resistance dissemination.

In summary, OMVs of *K. pneumoniae* strains contained proteins, DNA, and lipids; the composition of OMVs varied depending on the growth conditions, the strain phenotype, and antibiotic induction. The detection of virulence factors, antibiotic resistant determinants, and stress response proteins indicated multiple functions of OMVs in *K. pneumoniae*. Currently, a certain degree of comprehension has been achieved regarding the composition and structure of *K. pneumoniae* OMVs. However, the disparities between different strains and under diverse growth conditions still require further investigation.

Biological function of OMVs

OMVs mediated interactions with host cells

Since OMVs encompass numerous pathogenic proteins derived from their parental bacterium, they can initiate and modulate pro-inflammatory responses within the host, which are largely dependent on their uptake and entry into host cells. Phagocytosis constitutes the principal pathway employed by the phagocytic cells (neutrophils, macrophages, dendritic cells) of the immune system for the internalization of BEVs [42]. OMVs enter non-phagocytic epithelial cells through mechanisms including lipid-raft-dependent, or lipid-raft-

independent mechanisms, in addition to the requirement for endocytosis or macropinocytosis [43]. The contribution of the size of *Helicobacter pylori* OMVs in determining the mode of OMV entry into non-phagocytic epithelial cells and affecting the composition of OMVs was reported [44]. *H. pylori* OMVs with heterogeneous sizes entered epithelial cells through macropinocytosis and clathrin-dependent and caveolin-dependent endocytosis. Smaller OMVs ranging from 20 to 100 nm in size preferentially entered host cells via caveolin-mediated endocytosis, whereas larger OMVs ranging between 90 and 450 nm in size entered host epithelial cells via macropinocytosis and endocytosis. It was also reported that OMVs with heterogeneous sizes had different toxin distributions [45]. The size-dependent uptake mechanisms can influence the processing and presentation of antigens within these cells, thereby shaping the subsequent immune response and triggering different signalling pathways. A previous study identified that lipid vesicles with a diameter smaller than 100 nm elicited higher levels of NF-κB activity compared to larger OMVs, suggesting that these smaller OMVs could be more efficient at entering host epithelial cells and initiating pro-inflammatory responses [46]. Therefore, further research in this area is necessary to reveal the precise effects of OMV particle size on the induction of immune responses, which might facilitate the development of OMV-based vaccine strategies and therapeutics.

A proinflammatory response in epithelial cells was induced by *K. pneumoniae* OMVs [37] (Table 1, Figure 3). The expression of proinflammatory cytokines IL-1β and IL-8 in epithelial HEp-2 cells increased in a dose-dependent manner when treated with different amounts of *K. pneumoniae* OMVs. Meanwhile, *K. pneumoniae* OMVs caused lung pathology in neutropenic mice, with more severe pathological changes than live bacterial infection. You et al. also reported OMV-induced alterations in the expression of immune-related genes in epithelial cells and mast cells [47]. OMVs produced by *K. pneumoniae* ATCC 13883 could induce increased expression of immune response genes including IL-8, CTGF, DNAJB1, HMOX1, and HSPA1A in HEp-2 cells and CYP1A1, SC4MOL, IL-24, IL-1β, and ALDH5A1 in HMC-1 cells. The immune responses may be induced by the OMV components including LPS, the outer membrane proteins, and the porins, including OmpA, OmpC, and OmpX. Martora et al. demonstrated *K. pneumoniae* OMV (5 μg/ml)-induced expression of cytokine genes in human bronchial epithelial cells BEAS-2B [48]. Increased expression of IL-8, IL-6, IL-1β, and TNF-α was indicated by qPCR analysis, whereas only a significant increase of IL-8 and IL-6 secretion was indicated by the enzyme-linked immunosorbent assay (ELISA). This may indicate other regulation mechanisms in the expression of cytokine genes. OMVs isolated from *K. pneumoniae* ATCC 10031 induced 41.15 % early apoptosis and 41.14 % late apoptosis in treated BEAS-2B cells, higher than that treated with LPS and the untreated controls [49]. The expression analysis of apoptosis-related proteins affected by OMVs indicated the activation of initiator caspase-9 and caspase-3 effectors, the dysregulation of mitochondrial proteins BAX, B-cell lymphoma-2 extra-large (Bcl-xL), and Bcl-2 Interacting Mediator of cell death (BIM) proteins, and increased expression of endoplasmic reticulum stress markers such as CHOP and phospho-AKT1.

The impact of OMVs varied among different types of host cells. HvKP OMVs induced different cytotoxicity against human lung cell lines (A549 and BEAS-2B) and liver cell lines (L02), which promoted A549 cell proliferation but inhibited L02 cell growth [50]. Surprisingly, hvKP OMVs promoted BEAS-2B cell proliferation at lower concentration (0.25 mg/mL), whereas prevented cell growth at higher concentration (0.5 mg/mL). Expression of IL-6 and IL-8 increased in L02, L×2, and BEAS-2B cells in response to OMVs (Table 1). Proinflammatory chemokines, including IL-6, IL-8, and IL-1β, did not increase expression in response to OMVs either in A549 or in HepG2 cancer cells, while IL-1β was not induced in all cell types analysed. HvKP OMVs are able to induce lung inflammatory responses by increasing the expression of IL-6, IL-8, and TNF-α in C57 wild-type mice model *in vivo*.

OMVs could also mediate host immune responses through affecting the transcription levels of host miRNAs. Changed miRNA expression in BEAS-2B cells treated with OMVs produced by standard and clinical *K. pneumoniae* strains was reported [51]. *K. pneumoniae* ATCC 10031-derived OMVs altered the expression of 94 miRNAs (81 upregulated and 13 downregulated), the OMVs of a multi-sensitive clinical strain *K. pneumoniae* altered the expression of 73 miRNAs (57 upregulated and 16 downregulated), and KPC-producing *K. pneumoniae*-derived OMVs altered the expression of 71 miRNAs (58 upregulated and 13 downregulated). These miRNAs may regulate host immune responses through NF-κB (miR-223), TLR4 (hsa-miR-21), cytokine (hsa-miR-25), and IL-6 (hsa-let-7 g miRNA) signal pathways.

K. pneumoniae OMVs can not only activate proinfammatory signalling but also induce antiviral immunity in macrophages, alveolar epithelial cells (AECs), and lung tissue [52]. *K. pneumoniae* OMVs activated the innate immune response of human primary blood-

Table 1. Summary of *K. pneumoniae* OMV interactions with host cells and induced immune responses.

Strain types	Cell types or animal model	Immune responses	References
K. pneumoniae	Epithelial HEp-2 cells; neutropenic mice	• Increased expression of IL-1β and IL-8 • More severe pathological changes than live bacterial infection	[37]
K. pneumoniae ATCC 13883	Epithelial cell HEp-2 and mast cell HMC-1	• Increased expression of IL-8, CTGF, DNAJB1, HMOX1, and HSPA1A in HEp-2 cells and CYP1A1, SC4MOL, IL-24, IL-1β, and ALDH5A1 in HMC-1 cells.	[47]
K. pneumoniae	Human bronchial epithelial cells, BEAS-2B	• Increased expression of IL-8, IL-6, IL-1β, and TNF-α by qPCR analysis; increase of IL-8 and IL-6 at protein level	[48]
K. pneumoniae ATCC 10031	BEAS-2B cells	• Induced 41.15 % early apoptosis and 41.14 % late apoptosis • The activation of initiator caspase-9 and caspase-3 effectors • The dysregulation of mitochondrial proteins BAX, Bcl-xL, Bcl-2, and BIM proteins • Increased expression of endoplasmic reticulum stress markers such as CHOP and phospho-AKT1 • Decreased expression of eIF2α, SOD-1, CAT-1, and GPX-1.	[49]
HvKP	Human lung cell lines (A549, BEAS-2B) and liver cell lines (L02); C57 wild-type mice model	• OMVs promoted BEAS-2B cell proliferation at 0.25 mg/mL, whereas they prevented cell growth at 0.5 mg/mL • Increased expression of IL-6 and IL-8 in L02, L×2, and BEAS-2B cells in response to OMVs. • Proinflammatory chemokines were not induced by OMVs either in A549 or in HepG2 cancer cells • Induce lung inflammatory responses by increasing the expression of IL-6, IL-8, and TNF-α in the C57 wild-type mice model *in vivo*.	[50]
K. pneumoniae ATCC 10031 and clinical strains	BEAS-2B	• *K. pneumoniae* ATCC 10031 derived OMVs altered the expression of 94 miRNAs (81 upregulated and 13 downregulated) • OMVs of a multi-sensitive clinical strain *K. pneumoniae* altered the expression of 73 miRNAs (57 upregulated and 16 downregulated) • KPC-producing *K. pneumoniae*-derived OMVs altered the expression of 71 miRNAs (58 upregulated and 13 downregulated) • These miRNAs may regulate host immune responses through NF-kB (miR-223), TLR4 (hsa-miR-21), cytokine (hsa-miR-25), and IL-6 (hsa-let-7 g miRNA) signal pathways.	[51]
K. pneumoniae	Macrophages, alveolar epithelial cells (AEC) and lung tissue	• OMVs activated the innate immune response of human primary blood-derived macrophages (BDM) including CXCL8, IL1B, IL12B, IFNI phosphorylation of STAT1, and the expression of interferon-stimulated genes (ISGs; IFIT1, IFI44, and Mx1). Pre-treatment with Mx1-inducing *K. pneumoniae* OMVs blocked influenza A virus and vesicular stomatitis virus replication in THP-1 cells in a TLR4-TRIF-dependent manner.	[52]
HvKp and cKP-	Human bronchial epithelial (HBE) cells	• Both hvKp-derived OMVs and cKP-derived OMVs induced similar expression levels of IL-8 by activating the nuclear factor NF-κB	[53]
Multidrug-resistant *K. pneumoniae*	Mouse sepsis and pneumonia model	• Inappropriate use of antibiotics resulted in upregulation of the membrane presentation of heat shock protein GroEL of OMVs, which promotes the absorption of OMVs in macrophages, induces pyroptosis, and the release of proinflammatory cytokines and mediators (IL-1β, IL-18, and TNF-α).	[11]
K. pneumoniae	MCF7 cells	• Enhanced anti-hormonal effects of tamoxifen in MCF7 cells via Cyclin E2 and p-ERK	[54]

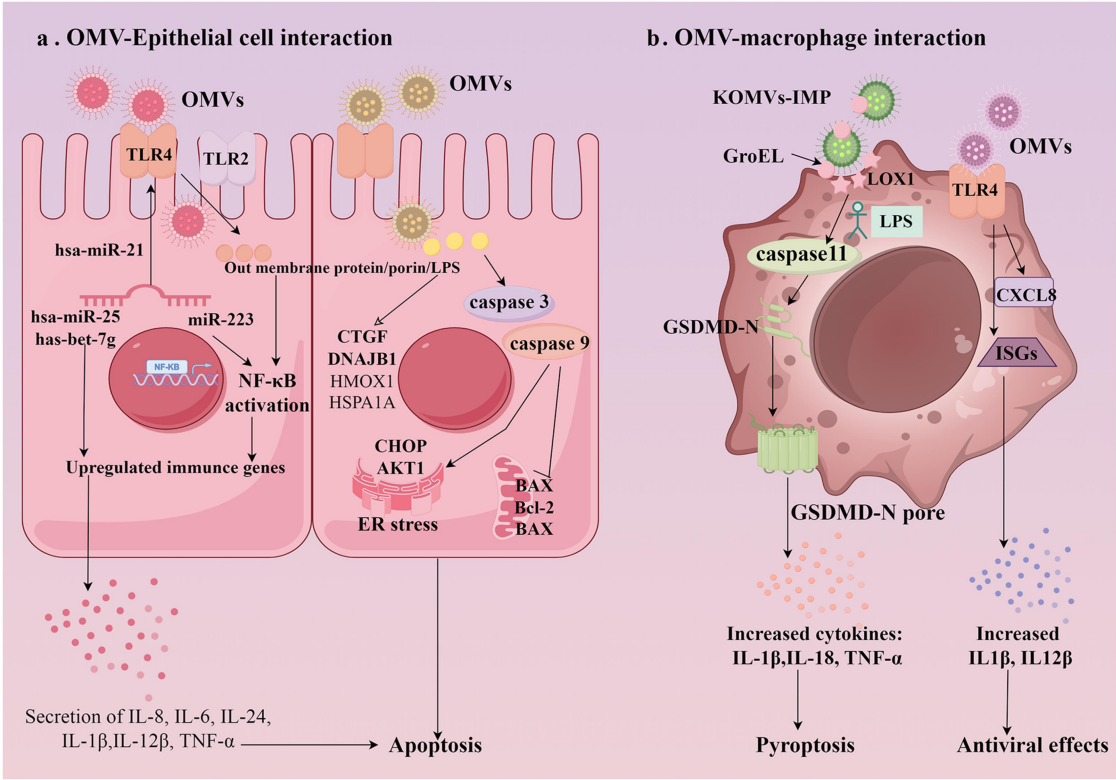

Figure 3. Immune responses of host cells against *Klebsiella pneumoniae* outer membrane vesicles. (a) OMV–epithelial cell interaction. (b) OMV–macrophage interaction. OMVs consist of lipopolysaccharide (LPS), outer membrane proteins, porin, bacterial DNA, etc., which are recognized by host pathogen recognition receptors, such as the membrane-bound toll-like receptor 4 (TLR4), and induce a downstream immune response and the production of proinflammatory cytokines. The immune responses mainly include the activation of pattern recognition receptors, regulation of cytokine release pathways, inflammasome activation, and miRNA-mediated pathways. Abbreviations: OMV, out membrane vesicle; toll-like receptor 4, TLR4; OMVs isolated from multidrug-resistant *K. pneumoniae* treated with imipenem, KOMVs-imp. Drawn by Figdraw.

derived macrophages (BDM), including CXCL8, IL1β, IL12β, IFNI phosphorylation of STAT1, and expression of interferon-stimulated genes (ISGs; IFIT1, IFI44, and Mx1). Pretreatment with Mx1-inducing *K. pneumoniae* OMVs blocked influenza A virus and vesicular stomatitis virus replication in THP-1 cells in a TLR4-TRIF-dependent manner. In addition, the antiviral and immunity regulation effects of *K. pneumoniae* OMVs were validated using an *ex vivo* infection model of human precision cut lung slices (PCLSs). Both hvKp-derived OMVs and cKP-derived OMVs induced similar expression levels of IL-8 by activating the nuclear factor NF-κB in human bronchial epithelial (HBE) cells, which could be reduced by the NK-κB inhibitor [53].

In addition, OMVs participated in the inflammatory responses caused by the inappropriate use of antibiotic. OMVs isolated from multidrug-resistant *K. pneumoniae* treated with imipenem (KOMVs-IMP) caused higher mouse mortality in a mouse sepsis model and exacerbated inflammatory responses in the BALFs of pneumonia mice [11]. Inappropriate use of antibiotics resulted in upregulated the membrane presentation of heat shock protein GroEL of OMVs, which promotes the absorption of OMVs in macrophages and induces pyroptosis and the release of proinflammatory cytokines and mediators (IL-1β, IL-18, and TNF-α). Binding between GroEL and its receptor LOX-1 can promote the uptake of OMVs by macrophages. The intracellular LPS signals released by OMV can be sensed by caspase-11, which is the crucial sensor of intracellular LPS. KOMV-IMP resulted in an increased protein expression of the caspase-11-cleaved GSDMD-NT fragment. Hence, a proposed regulatory mechanism of inflammatory damage was indicated (Figure 3). An et al. reported that the addition of *K. pneumoniae* extracellular vesicles enhanced the anti-hormonal effects of tamoxifen in MCF7 cells via cyclin E2 and p-ERK, indicating new insights into the hormone therapy of breast cancer [54].

In summary, participation of *K. pneumoniae* OMVs in the host immune responses were indicated both in

Table 2. OMVs-mediated horizontal gene transfer of antibiotic resistance and virulence genes.

Resources of OMV	Genes transferred	Recipient bacteria	Phenotypes of the recipient bacteria	References
K. pneumoniae	drug-resistant plasmids	*Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*	resistance to β-lactam antibiotics	[16]
HvKP	virulence genes	extended-spectrum β-lactamase (ESBL)-producing classical *K. pneumoniae*	antibiotic-resistant and hypervirulent	[57]
Carbapenem-resistant *K. pneumoniae* CRK3022	two resistance plasmids	*K. pneumoniae* and *E. coli*	carbapenem resistance	[39]
*bla*NDM-1 CRKP	*bla*NDM-1	cKP (ATCC 10031) and hvKP (NTUH-K2044)	carbapenem resistance	[41]
*bla*KPC-2 CRKP	*bla*KPC-2	*K. pneumoniae*	imipenem and meropenem resistance	[15]

the cell and the animal model, which can act through different intracellular pathways including the activation of pattern recognition receptors, regulation of cytokine release pathways and inflammasome activation. The cytokines released varied in different cell types and in response to OMVs derived from different sources.

OMV-mediated antibiotic resistance

The horizontal gene transfer (HGT) important mechanism mediates the transfer of drug resistance and virulence genes [55]. Bacterial OMVs are a new horizontal gene transfer path, which played roles in the spread of virulence or drug-resistant genes [40,56], and related researches on *K. pneumoniae* OMVs were summarized in Table 2. Dell'Annunziata et al. reported that KP-OMVs could transfer drug-resistant plasmids horizontally in a plasmid copy number-dependent manner, which could transfer plasmids to different bacterial species including *Escherichia coli*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* [16]. After the OMV-HGT, the resistance to β-lactam antibiotics of the transformed strains was verified through the antibiotic susceptibility assay. Hua et al. demonstrated that virulence genes encapsulated in hvKP-OMVs could be transferred to the extended-spectrum β-lactamase (ESBL)-producing classical *K. pneumoniae*, generating antibiotic-resistant and hypervirulent transformants, and their phenotypes were verified *in vitro* and *in vivo* [57]. Wang et al. demonstrated that *K. pneumoniae* OMVs could mediate both the intraspecific and interspecific horizontal transfer of virulence or drug-resistant plasmids [39]. OMVs isolated from carbapenem-resistant *K. pneumoniae* CRK3022 could simultaneously transfer two resistance plasmids into recipient strains including *K. pneumoniae* and *E. coli*. OMVs could mediate the transfer of the hypervirulent plasmid to the carbapenem-resistant *K. pneumoniae*, leading to the formation of CR-hvKp.

OMVs could not only mediate the transfer of antimicrobial resistance genes but also carry the carbapenem enzymes, which have antibiotic hydrolytic activity. *Klebsiella pneumoniae* carbapenemase (KPC) was detected in CRKP OMVs, and OMVs from *bla*KPC-positive CRKP strains can be released by Triton X-100 treatment and hydrolyse meropenem, indicating the roles of OMVs in the antimicrobial resistance [58]. Tang et al. demonstrated that OMVs from CRKP contained *bla*NDM-1 and medicated resistance gene transfer [41]. OMVs from CRKP could transfer the $bla_{\text{NDM-1}}$ gene to cKP (ATCC 10,031) and hvKP (NTUH-K2044), thus increasing the carbapenem resistance of the transformants. The transfer of carbapenem genes into hypervirulence strains may promote the emergence and spread of CR-hvKP. Enzyme NDM-1 detected in CRKP OMVs can hydrolyse meropenem *in vitro*. Chen et al. reported the transfer of the resistance gene *bla*KPC-2 via CRKP OMVs to *K. pneumoniae*, and the resistant phenotype of the recipient strain to imipenem and meropenem was verified, whereas negative results were found for the *E. coli* treated with CRKP OMVs under similar conditions, indicating a comprehensive resistance transmission mechanism [15]. Hence, OMV-mediated gene transfer could lead to the transmission of antibiotic resistance and virulence phenotype, resulting in more complex and severe problems for the prevention and control of *K. pneumoniae*-related infections.

Potential application of *K. pneumoniae* OMVs in the prevention, diagnosis, and treatment of related infections

Application of OMVs in vaccine development

In addition to antibiotics, vaccines represent a potent strategy for combating bacterial infections. The fundamental characteristics of vaccines encompass antigenicity, the capacity to elicit an immune response, safety profiles, and the presence of adjuvants. OMV is antigenic, not infectious, and non-toxic to cells and can easily be genetically manipulated, rendering it

a promising vaccine candidate [59]. There are bacterial OMV vaccines such as *Neisseria meningitides*-derived OMV vaccines and meningococcal group B bacteria OMV vaccines that have been successfully applied to clinical practice, indicating the safety and clinical potential of OMV vaccines [60,61]. Currently, vaccines in development against *K. pneumoniae* include inactivated vaccine, polysaccharide vaccine, conjugate vaccine, protein vaccine, nucleic acid vaccine, nanovaccines, and outer membrane vesicle (OMV) vaccine [62–65]. Lee et al. showed that vaccination with *K. pneumoniae*-derived OMVs effectively protected bacteria-induced lethality in a murine sepsis model via both humoral and cellular immunity [66]. The vaccination of mice with 10, 100, or 1000 ng *K. pneumoniae* EVs induced the production of specific IgG antibodies; the specific T-cell responses including the production of IFN-γ, IL-17, and IL-4 in $CD4^+$ cells; and the production of key cytokine by Th1 cells.

The stability and integrity of OMVs profoundly influence the protective effect of vaccines; hence, nanotechnology was employed to enhance the effects of OMV-based nanovaccines by reinforcing the OMV structure and homogenizing their size. Wu et al. reported the modification of the hollow OMVs onto bovine serum albumin (BSA) NPs (BN), which showed improved stability, integrity, and immune efficacy after structure optimization [67]. The BSA-OMV nanoparticles (BN-OMVs) were homogenous and had a size of around 100 nm, reinforced by size-controlled BSA nanoparticles [67]. Subcutaneous BN-OMV vaccination showed significantly higher CRKP specific antibody titres, increased recruitment and maturation of dendritic cells, and significantly increased the survival rate of the mice infected with a lethal dose of CRKP. The protective effect of BN-OMVs was dependent on humoral and cellular immunity indicated by the adoptive transfer experiment. Inspiringly, Li et al. established a technology platform to produce high yield, safe and stable artificial bacterial biomimetic vesicles (BBVs) from drug-resistant *K. pneumoniae*, which had little to no bacterial intracellular protein or nucleic acid [63]. BBVs can stimulate the maturation of dendritic cells and induce bacteria-specific humoral and cellular immune responses, thus increasing the survival rate of infected mice through reducing pulmonary inflammation and bacterial loads. Compared to lysate vaccines, BBV vaccines induced stronger bacteria-specific IgG antibody responses and significant bacteria-specific $CD3^+CD4^-CD8^+$ T cellular immune responses with increased expression of IFN-γ, IL-4, IL-17A, IL-10, and IL-2. Immune protection roles of BBVs against different antibiotic-resistant *K. pneumoniae* strains were verified *in vivo* using the bacteria-induced mice sepsis model and the pneumonia model. In summary, OMV-based nanovaccine can broaden our horizons in the development of drug-resistant vaccines to combat the infections caused by antibiotic-resistant *K. pneumoniae* strains.

Possible application of OMVs in the diagnosis of infectious diseases

Techniques used to identify bacterial infections, such as bacterial culture and immunofluorescence detection, are time-consuming and inaccurate [68]. OMVs can be found in body fluids such as blood, bronchoalveolar lavage fluid, sputum, and urine and can express several inherent pathogen components simultaneously; hence, OMV is an ideal candidate biomarker to identify bacterial infection [19,69]. Bacterial vesicles were detected in the cerebrospinal fluid of an infant with meningitis caused by *Neisseria meningitides* and in the gastric juices of patients infected with *Helicobacter pylori* [70,71]. The presence of OMVs was detected by enzyme-linked immunosorbent assay using anti-LPS antibodies, and significant quantities were present in faeces from both mice and humans, which could induce local and systemic inflammatory responses and could be attenuated in mice lacking TLR2 [72].

OMVs contain diverse biomolecules, including lipids, proteins, nucleic acids, and various metabolites that can reflect the physiological state and pathogenic potential of their parent bacteria. This unique composition makes OMVs attractive candidates for biomarker discovery and diagnostics. Numerous studies have indicated that the lipid composition of BEVs might fluctuate in accordance with the bacterial type. OMVs secreted by gram-negative bacteria are abundant in lipopolysaccharide (LPS) [73]. LPS-positive bacterial EVs are present in the plasma of the patients with impaired barrier integrity. The lipid composition of OMVs varies among different bacteria. In *Porphyromonas gingivalis*, OMVs carry LPS molecules with long sugar chains and deacylated lipid A [74], whereas deacylated lipid A accumulates in OMVs in *Salmonella* [75]. In *Escherichia coli*, OMVs contain lipids involved in membrane folding, such as glycerophospholipids, phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin [74]. Lipidomic analysis revealed that the lipid composition of *K. pneumoniae*-derived OMVs was predominantly constituted by glycerophospholipids (35%), fatty acids (33%), and sphingolipids (20%) [38]. Additionally, outer membrane proteins, periplasmic proteins, and other specific proteins can identify OMVs. Gram-negative bacteria release OMVs rich in outer membrane proteins such

as OmpA, OmpC, and OmpF, as well as periplasmic proteins and alkaline phosphatase [74]. Prosseda et al. used outer membrane proteins OmpF and OmpC to relatively quantify *E. coli* OMVs [76]. The outer membrane protein X also exhibited a high level of expression in *K. pneumoniae*-derived OMVs [37]. Virulence and resistance-related proteins were also detected in *K. pneumoniae*-derived OMVs. Several virulence genes, such as *rmpA*, *rmpA2*, *iucA*, *iroB*, and *peg344*, were detected in the OMVs of the hypervirulent *K. pneumoniae* (HvKP) strain [39]. These genes are commonly employed as hypervirulence biomarkers of HvKP. Meanwhile, resistance genes such as *bla*KPC-2, *bla*CTX-M-1, and *mph(A)* were detected in CRKP-derived OMVs. The composition of OMVs cargo varies depending on the growth conditions, the strain phenotype, and antibiotic induction; hence, stable and specific biomarkers await further research. Changes in the quantity or cargo content of circulating OMVs following antibiotic therapy could offer valuable information about the treatment efficacy or emergence of drug resistance.

The application of OMVs in *K. pneumoniae* infection diagnosis remains unreported. Further investigations are requisite to comprehend the systemic functions of circulating bacterial extracellular vesicles to explore their biomarker potential and identify specific molecules as biomarkers. Nevertheless, there remain certain challenges before OMVs can be extensively utilized as biomarkers. For instance, the technology for the isolation and purification of OMVs requires further optimization to enhance purity and yield; the precise detection and quantification of cargo within OMVs also need to be perpetually enhanced and standardized. Overall, exploring the application of bacterial OMVs in biomarker discovery presents an exciting opportunity to harness their unique properties for improving diagnostics across various fields.

Application of OMVs in the delivery of drugs

The cell-targeting capabilities of bacterial vehicles have also been explored to deliver therapeutic drugs to specific cells or tissues [77,78]. Compared to the most common and well-studied synthetic nanocarriers (i.e. liposomes), bacterial vehicles are similar in size, shape, and structure, containing a variety of lipids, proteins, and other components [79]. Kuerban et al. reported that *K. pneumoniae* outer-membrane vesicles were used as the delivery vector of the doxorubicin, which exerted enhanced anti-tumour efficacy against non-small-cell lung cancer [80]. OMVs released by attenuated *K. pneumoniae* were used to prepare doxorubicin-loaded O0MVs (DOX-OMV). DOX-OMV can be efficiently transported into NSCLC A549 cells indicated by confocal microscopy and *in vivo* distribution analysis, which can lead to intensive cytotoxic effects and cell apoptosis. DOX-OMV exhibited a considerable tumour growth inhibition in A549 tumour-bearing BALB/c nude mice. Moreover, OMVs possess appropriate immunogenicity to recruit macrophages in the tumour microenvironment, thereby synergizing with doxorubicin *in vivo*. Therefore, *K. pneumoniae* OMVs can not only serve as drug nanocarriers but also induce appropriate immune responses, thus having great potential in the targeted therapy of tumour. More research is required to evaluate the safety and efficacy of OMVs as nanocarriers of different drugs against different diseases.

Conclusion and future prospects

Infections caused by multidrug-resistant gram-negative bacteria are the global and major public health problems. In recent decades, there have been increasing concerns regarding the gram-negative opportunistic pathogen *K. pneumoniae* due to the emergence of hypervirulent and antibiotic resistant isolates. Outer membrane vesicles contain a variety of bioactive substances, including lipopolysaccharide, peptidoglycan, periplasmic, and cytoplasmic proteins, toxins, and nucleic acids, which have been reported to participate in bacterial virulence, pathogenesis, antibiotic resistance, stress adaptation, and host interactions. OMVs as a promising target may provide new ways to explore the pathogenesis, antibiotic resistance and treatment of *K. pneumoniae* related infections. *K. pneumoniae* OMVs can activate proinflammatory cytokines through different intracellular pathways including the activation of pattern recognition receptors, regulation of cytokine release pathways, and activation of inflammasome pathway, resulting in various inflammatory responses in host cells. As a new horizontal gene transfer path, *K. pneumoniae* OMVs could mediate both the intraspecific and interspecific transfer of drug-resistant plasmids, thus leading to the transfer of bacterial drug resistance. Since its discovery, OMVs have been extensively explored for numerous applications, while the majority of research has focused on vaccination, drug delivery, and diagnosis markers. Vaccination with *K. pneumoniae*-derived OMVs could effectively protect bacteria-induced lethality in a murine sepsis model via both humoral and cellular immunity. Nanotechnology was employed to improve the efficiency of OMV-based nanovaccines by reinforcing the structure of OMVs and uniforming their size. Although preliminary data of

K. pneumoniae OMVs were inspiring, further studies are required to exploit their properties in a more flexible way. Given the wide range of positive and negative effects of OMVs on host immunity, bacterial virulence, and antibiotic resistance and its potential therapeutic approaches, OMVs will undoubtedly become major research focus in future.

Nevertheless, numerous aspects of OMVs still require further investigation. Firstly, the biogenesis of OMVs could be examined in detail to understand the molecular pathways involved in their formation and release from bacterial cells. This includes investigating regulatory mechanisms at both genetic and environmental levels that influence OMVs' production under different growth conditions or stresses. Next, the cargo content were carried by OMVs and their delivery mechanisms. Understanding what types of molecules are encapsulated within OMVs and how they are selectively sorted during vesicle formation is critical for deciphering their functions. Furthermore, exploring how these cargoes interact with recipient cells could provide insights into novel therapeutic strategies. Additionally, the interactions between OMV and host cells represent another vital research area for exploration. Although OMVs can carry virulence factors that contribute to pathogenicity, the specific mechanisms through which the factors influence host cell responses remain poorly understood. Moreover, the biophysical properties of OMVs such as size distribution can affect their interaction with target cells; thus far, research has yet to fully clarify how these characteristics affect their biological functions. Investigating how different types of host cells respond to exposure to various bacterial OMVs may reveal important information about pathogen–host interactions. Lastly, since OMVs show potential in the clinical application in the vaccine development, as biomarkers, and in the delivery of drugs, the establishment of high-efficiency and high-yields methods for the separation and purification of OMVs constitute an urgent issue that demands researches, and the construction of standardized detection platforms and bioinformatics analysis platforms is equally significant.

Disclosure statement

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Author contribution

Lifeng Li: conceptualization, Writing – original draft, funding acquisition; Xinxiu Xu: data curation, writing – review and editing; Ping Cheng: writing – review and editing; Zengyuan Yu: Writing – review and editing; Mingchao Li: Writing – review and editing; Zhidan Yu: Writing – review and editing; Weyland Cheng: Writing – review and editing; Wancun Zhang: supervision, writing – review and editing; Huiqing Sun: supervision, writing – review and editing; Xiaorui Song: conceptualization, writing - review and editing. All authors revised the manuscript critically and approved the submission of the manuscript.

Data availability statement

Data sharing is not applicable to this review article as no new data were created or analysed in this study.

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