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Extracellular Vesicles and Circulating Nucleic Acids

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Bacterial extracellular vesicles in priority pathogens: *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*

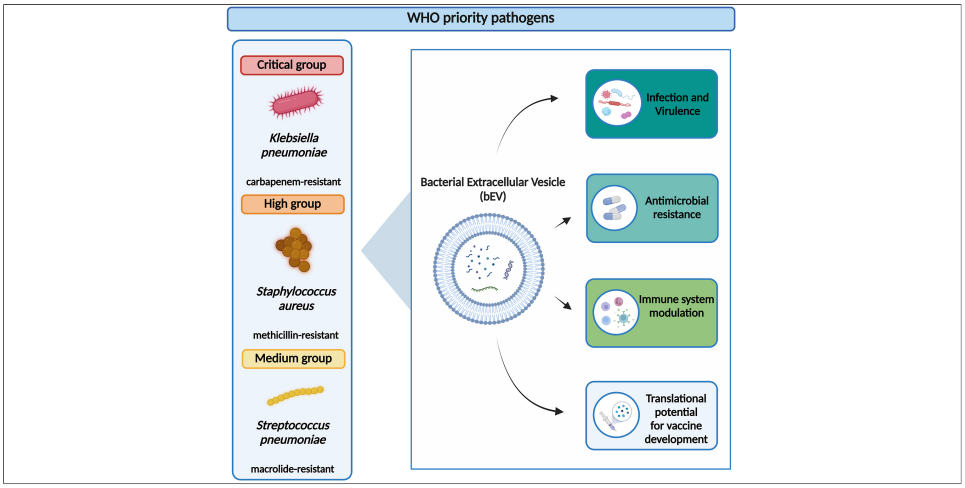
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Abstract

Antimicrobial resistance (AMR) is a major global health threat that requires innovative approaches to infection prevention and treatment. In 2024, the World Health Organization (WHO) updated its Bacterial Priority Pathogens List (BPPL) to guide research toward the pathogens of highest clinical relevance. Among these, carbapenem-resistant *Klebsiella pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and macrolide-resistant *Streptococcus pneumoniae* remain major contributors to infectious disease burden and AMR-associated mortality worldwide. Recent evidence identifies bacterial extracellular vesicles (bEVs) as important mediators of microbial adaptation, host interaction, and antimicrobial resistance. Released under physiological and stress conditions, bEVs carry proteins, lipids, and nucleic acids that can influence bacterial survival and infection outcomes. In this mini-review, we summarize current knowledge on bEVs produced by these WHO priority pathogens and discuss their emerging roles in infection biology. We highlight how bEVs contribute to antimicrobial resistance through the transport of resistance-associated proteins, the horizontal transfer of resistance genes and antibiotic-degrading activity. We further examine the involvement of bEVs in inflammation, host tissue damage and immune modulation, including their capacity to carry virulence factors or sequester antimicrobial agents. Finally, we discuss recent advances in the use of bEVs as

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vaccine platforms. We prioritize studies on extracellular vesicles (EVs) from antibiotic-resistant strains of the three WHO priority pathogens. When these were unavailable, we also included studies using antibiotic-susceptible strains, particularly for *S. pneumoniae*, for which data on macrolide-resistant strains are lacking. Overall, bEVs are increasingly recognized as active components of bacterial physiology and promising targets for developing new strategies against antimicrobial-resistant infections.

INTRODUCTION

Antimicrobial resistance (AMR) is a major threat to public health worldwide and was associated with approximately 5 million deaths in 2019[1]. By 2050, AMR-associated deaths are estimated to increase by 70%[2] and the global AMR cost to be around USD 100 trillion[3]. Despite this burden, only a small fraction of the antibiotics approved over the past 40 years belong to new compound classes[4].

AMR develops naturally when microorganisms adapt to overcome inhibitory agents, allowing them to survive and grow despite the presence of drugs[5]. Bacteria have developed diverse resistance mechanisms, such as reduced membrane permeability, efflux pumps, antibiotic degradation or target modification, which compromise the efficacy of most antibiotics[6,7]. Global Burden of Disease (GBD) estimates identify 33 bacterial pathogens (both resistant and susceptible to antimicrobials) responsible for 11 major infectious syndromes. Three of them, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*, are among the most significant contributors to deaths[8].

Accordingly, the 2024 World Health Organization (WHO) Bacterial Priority Pathogens List (BPPL) classifies carbapenem- and third-generation cephalosporin-resistant *K. pneumoniae*, methicillin-resistant *S. aureus* and macrolide-resistant *S. pneumoniae* as critical-, high- and medium-priority pathogens, respectively[1].

For these pathogens, treatment options are limited, or access to therapies is restricted in some populations or geographical areas[1]. Despite active research, no licensed vaccines are available for *K. pneumoniae* and *S. aureus*[5,9]. For *S. pneumoniae*, a serotype-independent vaccine is needed, since current pneumococcal polysaccharide and conjugate vaccines protect only against a fixed number of capsular serotypes and access remains limited in many settings[1,10,11].

Innovative strategies are needed to limit infections caused by these pathogens, particularly antibiotic-resistant strains. Recently, extracellular vesicles (EVs) have emerged as important mediators of cell-to-cell communication[12,13] and environmental adaptation[14-16], and many studies have highlighted their contribution to microbial resistance[7].

Bacterial extracellular vesicles (bEVs) are typically 40-400 nm in diameter and carry proteins, lipids and nucleic acids, including ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). In Gram-negative bacteria, EVs form by membrane blebbing, generating outer membrane vesicles (OMVs) and outer-inner membrane vesicles (OIMVs), or by explosive cell lysis, producing explosive outer membrane vesicles (EOMVs) and explosive outer-inner membrane vesicles (EOIMVs). In Gram-positive bacteria, EV biogenesis is less well understood and may involve membrane blebbing or bubbling cell death, producing cytoplasmic membrane vesicles (CMVs). Different vesiculation processes give rise to distinct EV types whose structure and content ultimately determine their function[17]. Bacterial EVs also contribute to antibiotic resistance by acting as decoys for membrane-targeting antibiotics, degrading antibiotics enzymatically or transferring resistance genes[7]. Moreover, bEVs have diagnostic potential as biomarkers of disease state and treatment efficacy, and they are being explored for vaccine development and targeted drug delivery[18].

In this review, we focus on EVs produced by *K. pneumoniae*, *S. aureus* and *S. pneumoniae,* summarizing recent findings that describe them as drivers of virulence, immune modulation, and antimicrobial resistance. Given the substantial global health burden posed by these pathogens, understanding the role of EVs in establishing infection, as well as their potential for vaccine development, is of particular interest. We searched PubMed for original research and review articles combining the terms "bacterial extracellular vesicles", "outer membrane vesicles", "*Klebsiella pneumoniae*", "*Staphylococcus aureus*", "*Streptococcus pneumoniae*", "antimicrobial resistance" and "vaccine". The reference lists of the retrieved articles were also manually checked for additional relevant studies. We prioritized recent, peer-reviewed studies on EVs from antibiotic-resistant strains of these WHO priority pathogens and, when these were unavailable, also considered studies on other strains. A recent book chapter covered bEVs from two Gram-negative, WHO critical-priority pathogens, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, focusing on antimicrobial resistance, pathogenicity and OMV-based vaccine strategies[19]. Our mini-review differs in scope: by covering pathogens from three WHO priority tiers (critical, high and medium), including both Gram-negative and Gram-positive organisms, it allows a comparison of EV-mediated resistance and immune modulation across the priority spectrum rather than within a single tier.

EVs FROM *KLEBSIELLA PNEUMONIAE*

K. pneumoniae is a commensal Gram-negative bacterium that colonizes the mucosal surfaces of the nose, throat, skin and gastrointestinal tract in healthy individuals[20]. It is an opportunistic pathogen that can cause pneumonia, urinary tract, bloodstream, soft tissue and surgical wound infections, particularly in immunocompromised individuals[20,21]. Its multidrug resistance, high virulence and high transmissibility pose a significant threat to public health[20].

K. pneumoniae infections have caused approximately 800,000 deaths worldwide, 80% of them associated with AMR[22]. Its clinical impact is largely attributable to the global spread of carbapenem-resistant *K. pneumoniae* (CRKP), which is classified in the critical priority group of the WHO 2024 BPPL[1,9,20,21].

K. pneumoniae produces EVs, classified as OMVs, under both physiological and stress conditions. Environmental conditions, including antibiotic treatment, influence the composition and function of *Klebsiella pneumoniae*-derived OMVs (KP OMVs)[21]. Using proteomics, Fan *et al.* identified thousands of proteins in OMVs released by meropenem-treated CRKP and reported an enrichment of proteins and enzymes associated with virulence, pathogenicity, drug resistance, stress, bacterial survival and cellular metabolism[23]. Resistance-related proteins included penicillin-binding protein 1B, EmrA, Na^+/antidrug transporter, ArnT, ArnA, ArnC, *blaKPC*, *blaSHV*, SHV-112, MacB, outer membrane channel protein, and outer membrane protein OmpK36[23]. The multidrug resistance-associated protein MdtQ was also detected in OMVs isolated from CRKP[24].

OMVs have also been recognized as mediators of horizontal gene transfer[7,17]. Li *et al.* demonstrated that OMVs of carbapenem-resistant hypervirulent *K. pneumoniae* (hvKP) contained genetic material, particularly carbapenemases and virulence genes, which could be transferred to recipient bacteria, enhancing their resistance-associated features[25]. CRKP OMVs were shown to mediate the transfer of the $bla_{\text{NDM-1}}$ gene (encoding New Delhi metallo-β-lactamase 1) to other *K. pneumoniae* strains, increasing the carbapenem minimum inhibitory concentration (MIC) of the transformants[26].

In addition to their role in antimicrobial resistance, OMVs are involved in host-pathogen interactions, inducing proinflammatory responses and modulating the host immune system[27,28]. KP OMVs, derived from hvKP and clinical *K. pneumoniae* strains, were shown to stimulate the release of proinflammatory interleukin-8 (IL-8) from human bronchial epithelial cells, through nuclear factor kappa B (NF-κB)

activation[29]. Ye *et al.* showed that treatment with imipenem, a widely used carbapenem antibiotic, enhanced the release of chaperonin GroEL-enriched OMVs from clinical multidrug-resistant *K. pneumoniae*. These OMVs were readily taken up by macrophages, inducing pyroptosis and promoting the release of proinflammatory cytokines [interleukin-1β (IL-1β), interleukin-18 (IL-18), and tumor necrosis factor-α (TNF-α)] in a mouse model of pneumonia[30]. Outer membrane proteins such as outer membrane protein A (OmpA) and outer membrane protein C (OmpC) are also components of KP OMVs (from *K. pneumoniae* ATCC 10031) that can induce an inflammatory response in host cells[31] and contribute to their immunogenicity[32].

OMVs are also being investigated as promising vaccine platforms[9,21,32]. Li *et al.* used high-pressure homogenization to induce CRKP to form stable, bacterial biomimetic vesicles (BBVs) with high yield and reduced nucleic acid content. These BBVs effectively stimulated dendritic cell maturation, elicited both humoral and cellular immune responses, and provided protection in mice against multidrug-resistant *K. pneumoniae* strains[33]. Wu *et al.* engineered a nanovaccine by reinforcing CRKP OMVs with size-controlled bovine serum albumin (BSA) nanoparticles, creating stable, homogeneous BSA-OMV nanoparticles. This formulation enhanced immune responses, including higher antibody titers, dendritic cell activation and protection against lethal CRKP infection in mice[34].

Overall, current evidence indicates that KP OMVs contribute to antimicrobial resistance, host interaction and immune modulation, and are potential candidates for innovative vaccines. However, these studies relied on a limited number of CRKP isolates, mostly using *in vitro* assays or short-term mouse models, and vesicle isolation protocols differed between them, which limits the comparison and generalization of the findings.

EVs FROM *STAPHYLOCOCCUS AUREUS*

S. aureus is a Gram-positive bacterium that asymptomatically colonizes the human host, primarily on mucosal and epithelial surfaces[35]. It can also cause a wide range of infections and life-threatening conditions, such as bacteremia, endocarditis, osteomyelitis and pneumonia[35,36]. *S. aureus* was associated with more than 1 million deaths in 2019[8], and its increasing antibiotic resistance complicates clinical management[35,36]. In particular, methicillin-resistant *S. aureus* (MRSA) strains are resistant to β-lactam antibiotics, including penicillin derivatives, which comprise many commonly used antibiotics[37]. Consequently, MRSA is listed in the high-priority group of the 2024 WHO BPPL[1].

The role of EVs in the pathogenesis of *S. aureus* has received increasing attention, particularly their biogenesis and involvement in the spread of antibiotic resistance[37]. EV production is strongly influenced by environmental conditions, and studies have shown that treating bacteria, including *S. aureus*, with β-lactam antibiotics increases EV production[37,38]. Moreover, EVs derived from ampicillin-treated MRSA were shown to possess β-lactamase activity and to degrade antibiotics such as amoxicillin and ampicillin[38]. Kim *et al.* reported that proteins related to β-lactam degradation, including penicillin-binding proteins (PBP1, -2, -3), β-lactamase (BlaZ) and the UPF0173 metal-dependent hydrolase (SA1529), were abundant in EVs released from stressed bacteria[38]. In addition, MRSA-derived EVs have been investigated for their ability to transfer resistance-related factors across bacterial species[39]. Lee *et al.* demonstrated that these EVs could deliver β-lactamase activity to susceptible *Escherichia coli*, increasing its resistance to β-lactam antibiotics. EVs from resistant *E. coli* strains contained higher levels of β-lactamase and other antimicrobial resistance-related proteins (OmpC, OmpA, OmpT, MipA, LpoA and LpoB)[39].

Staphylococcus aureus-derived EVs (SA EVs) are also involved in other resistance mechanisms, such as the sequestration of host-derived antimicrobial fatty acids (AFAs), which normally inhibit bacterial growth on skin and mucosal surfaces[40]. Kengmo Tchoupa *et al.* demonstrated that both methicillin-resistant and -susceptible *S. aureus* strains release EVs that act as decoys, reducing the binding of AFAs to bacterial cells[40].

To investigate the role of MRSA-derived EVs in modulating host immune responses, Asano *et al.* purified EVs from a clinical MRSA strain and found that they stimulate proinflammatory cytokine release [TNF-α and interleukin-6 (IL-6)] via Toll-like receptors (TLRs) 2, 4 and 9, and induce cell death in mouse macrophages[41]. SA EVs also contribute to skin infections[42]. Chen *et al.* showed that EVs from clinical MRSA strains inhibit proliferation and induce apoptosis in human adult low-calcium high-temperature keratinocyte (HaCaT) cells, potentially via upregulation of apoptosis-related genes such as B-cell lymphoma 2 (BCL2) antagonist/killer 1 (*BAK1*)[43].

SA EVs have also emerged as a promising vaccine platform, because they carry multiple immunogenic components, potentially overcoming the limitations of single-antigen vaccines[36]. Wang *et al.* genetically engineered *S. aureus* (JE2 strain) to produce detoxified EVs expressing non-toxic antigens, generating a vaccine candidate that elicited neutralizing antibodies and protected mice in a sepsis model[44]. Similarly, Chen *et al.* developed a multi-antigen vaccine by encapsulating SA EVs (from both drug-resistant and -susceptible strains) with magnetic mesoporous silica nanoparticles (MSNs) loaded with indocyanine green (ICG). Upon laser irradiation, this nanovaccine promoted dendritic cell maturation and enhanced cluster of differentiation 8-positive ($CD8^+$) T cell responses. *In vivo*, the nanovaccine was efficiently transported to lymph nodes, prevented superficial infections and reduced bacterial dissemination in mouse models[45].

Collectively, these findings show that *S. aureus*-derived EVs play multifaceted roles in antimicrobial resistance and host-pathogen interactions and are promising candidates for novel vaccines and therapeutic strategies. However, most mechanistic data come from a small number of clinical MRSA isolates studied *in vitro* or in short-term murine models, and it remains unclear how well they generalize across MRSA lineages or to natural infection.

EVs FROM *STREPTOCOCCUS PNEUMONIAE*

S. pneumoniae is a Gram-positive bacterium that asymptomatically colonizes the nasopharynx of healthy individuals. As an opportunistic pathogen, it can cause many diseases, including community-acquired pneumonia, meningitis, otitis media and bacteremia, particularly in the elderly, children and immunocompromised people[46,47]. Among children under 5 years old, *S. pneumoniae* was one of the pathogens responsible for the most AMR-attributable deaths in 2021[2]. The spread of antibiotic-resistant *S. pneumoniae* and non-vaccine serotypes is a major global concern, reflected in the classification of macrolide-resistant *S. pneumoniae* in the medium-priority group of the 2024 WHO BPPL[1,46,47].

Streptococcus pneumoniae-derived EVs (SP EVs) have been studied for their role in host-pathogen interactions, as carriers of virulence factors and as promising platforms for novel vaccines[47]. SP EV production is influenced by environmental cues, including temperature, pH, host-derived factors and nutrient availability[15,48]. The protein cargo of SP EVs (from *S. pneumoniae* D39) includes carbon metabolism-related proteins [e.g., enolase (Eno), glyceraldehyde-3-phosphate dehydrogenase A (GapA), non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GapN), phosphoglycerate mutase A (GpmA), phosphofructokinase A (PfkA), pyruvate kinase (PykF), and triosephosphate isomerase (Tpi)] and virulence factors, such as pneumolysin (Ply), lysozyme (LytC) and pneumococcal surface protein A (PspA)[15]. SP EVs are also implicated in gene transfer[49]. Werner Lass *et al.* demonstrated that SP EVs (from *S. pneumoniae* R6 and D39) are associated with bacterial DNA on their surface, which can be transferred to the transformation machinery of competent cells[49].

Recent research has further highlighted the contribution of SP EVs to pneumococcal pathogenicity[47], including host cell damage and lung epithelial barrier disruption[50]. Cui *et al.* showed that SP EVs (from *S. pneumoniae* D39) are internalized by alveolar epithelial cells, delivering virulence factors such as the eukaryotic-like serine/threonine-protein kinase (StkP), which activates autophagy through phosphorylation of beclin 1 (BECN1). This autophagy leads to degradation of the tight junction protein occludin (OCLN) and impairs alveolar barrier integrity[50].

SP EVs have also been studied in the context of hemolytic uremic syndrome (HUS), a severe kidney disease. Although HUS is most commonly associated with Shiga toxin-producing *E. coli* (STEC), it can also be a rare complication of invasive pneumococcal disease. EVs from *S. pneumoniae* strains isolated from patients with pneumococcus-associated HUS (HUS EVs) contain several virulence factors, including the 50S ribosomal subunit assembly factor (BipA), PspA, the lytic enzyme LytC, and several sugar utilization and fatty acid synthesis proteins. In addition, HUS EVs induce the release of proinflammatory cytokines (IL-1β, IL-6) and chemokines [chemokine C-C motif ligand 2 (CCL2), chemokine C-C motif ligand 3 (CCL3), and C-X-C motif chemokine ligand 1 (CXCL1)] by human monocytes[51].

Current pneumococcal vaccines provide limited serotype coverage: even the latest approved vaccine, pneumococcal conjugate vaccine 21 (PCV21), protects against only 21 serotypes[47,52]. Novel immunization strategies are therefore needed[47]. In this context, Dookie *et al.* demonstrated that SP EVs (isolated from pneumococci of serotype 4) have inherent adjuvant properties, mainly through the lipoproteins maltose/maltodextrin ATP-binding cassette (ABC) transporter (MalX) and chaperone cis-trans prolyl isomerase (PrsA), which activate Toll-like receptor 2 (TLR2) and induce a robust serotype-independent T helper 17 (Th17) response in both mice and humans. Moreover, intranasal immunization with SP EVs promoted lung macrophage expansion and conferred protection against pneumococcal colonization without additional adjuvants[53]. Similarly, Carneiro *et al.* showed that subcutaneous immunization with SP EVs (from *S. pneumoniae* R6) induces specific antibody responses, particularly against PspA, and confers protection in murine models of invasive pneumonia, with PspA playing a significant role even across heterologous PspA families[54].

To develop a stable and effective vaccine against pneumococcal infections, Mehanny *et al.* formulated spray-dried microparticles encapsulating SP EVs (from *S. pneumoniae* R6). These microparticles had suitable size, aerodynamic properties and stability for deep lung delivery. The encapsulated SP EVs did not compromise the viability of human macrophage-like cells derived from the human monocytic leukemia cell line (THP-1) and were readily internalized by them. They also induced the release of proinflammatory cytokines (TNF and IL-6) from primary human peripheral blood mononuclear cells (PBMCs)[55].

Taken together, these studies highlight the important role of SP EVs in pneumococcal pathogenesis and support their potential as vaccine candidates. However, most of the SP EV literature summarized here was generated using non-macrolide-resistant laboratory or clinical strains, and the biological properties and immunological functions of EVs released by macrolide-resistant *S. pneumoniae*, the WHO priority phenotype, remain largely unexplored. This aspect should be kept in mind when interpreting how far these findings translate to the WHO priority phenotype.

CONCLUSION AND FUTURE DIRECTIONS

AMR remains one of the most urgent challenges in infectious disease control, particularly for WHO priority pathogens such as *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. Current evidence indicates that bEVs from these pathogens are not passive by-products of bacterial growth, but active mediators of infection, immune modulation and antimicrobial resistance [Table 1].

Table 1. Characteristics and application of extracellular vesicles from the three selected pathogens: summary of the main studies included in this mini-review

Pathogen	EV cargo	EV biological effect	Application*
Klebsiella pneumoniae	• Proteins/enzymes associated with virulence, pathogenicity, drug resistance, stress, bacterial survival, and cellular metabolism in meropenem-treated CRKP OMVs[23] • MdtQ, outer membrane protein associated with multidrug resistance in CRKP OMVs[24] • Carbapenemases and virulence genes in carbapenem-resistant hvKP OMVs[25] • bla_{NDM-1} gene in CRKP OMVs[26]	• OMVs from hvKP and clinical KP strains induce proinflammatory IL-8 secretion from human bronchial epithelial cells, through NF-κB activation[29] • OMVs from imipenem-treated clinical multidrug-resistant KP, enriched in GroEL protein, induce pyroptosis in macrophages and release of proinflammatory cytokines (IL-1β, IL-18, TNF-α) in a mouse model of pneumonia[30] • KP OMVs (from KP ATCC 10031) containing OmpA and OmpC stimulate a proinflammatory response in host cells[31]	• BBVs (from CRKP) stimulate dendritic cell maturation, elicit both humoral and cellular immune responses, and provide protection in mice against multiple drug-resistant KP strains[33] • BSA-OMV nanoparticles (OMVs from CRKP with BSA) enhance immune responses, dendritic cell activation, and protection against CRKP infection in mice[34]
Staphylococcus aureus	• Proteins related to the degradation of β-lactam antibiotics, including PBP1, PBP2, PBP3 and BlaZ in EVs from ampicillin-treated MRSA[38] • Antibiotic resistance-related factors in MRSA EVs transferred to *E. coli* susceptible to β-lactam antibiotics[39]	• EVs from both methicillin-resistant and -susceptible SA act as decoys, reducing the binding of AFAs to bacterial cells[40] • Clinical MRSA-derived EVs stimulate proinflammatory cytokine release (TNF-α and IL-6) via TLR2, TLR4, and TLR9, and induce host cell death in mouse macrophages[41] • Clinical MRSA-derived EVs inhibit proliferation and induce apoptosis of HaCaT epithelial skin cells, via upregulation of apoptosis-related genes like *BAK1*[43]	• SA detoxified EVs (from JE2 strain) elicit neutralizing antibodies and provide protection in a mouse sepsis model[44] • SA EVs (from both drug-resistant and -susceptible strains) encapsulated with magnetic MSNs, loaded with ICG promote dendritic cell maturation and enhance $CD8^+$ T cell responses upon laser irradiation. *In vivo*, the nanovaccine prevents superficial infections, and reduces bacterial dissemination in mouse models[45]
Streptococcus pneumoniae+	• Carbon metabolism-related proteins (e.g., Eno, GapA, GapN, GpmA, PfkA, PykF, and Tpi) and virulence factors, such as Ply, LytC and PspA in SP EVs (from SP D39)[15] • Bacterial DNA on SP EVs (from SP R6 and D39) surface[49] • Virulence factor StkP in SP EVs (from SP D39)[50] • Several virulence factors (e.g., BipA, PspA, LytC) and sugar utilization and fatty acid synthesis proteins in HUS EVs (from clinical HUS strains)[51] • Lipoproteins MalX and PrsA in SP EVs (from SP serotype 4)[53]	• SP EVs (from SP D39) are internalized by alveolar epithelial cells and activate autophagy, leading to the degradation of OCLN and impairment of the alveolar barrier integrity[50] • HUS EVs (from clinical HUS strains) stimulate the release of proinflammatory cytokines (IL-1β, IL-6) and chemokines (CCL2, CCL3, CXCL1) by human monocytes[51] • SP EVs (from SP serotype 4) activate TLR2 and induce robust serotype-independent Th17 response in both mice and humans[53]	• Intranasal immunization with SP EVs (from SP serotype 4) promotes the expansion of lung macrophages, and confers protection against pneumococcal colonization[53] • Subcutaneous immunization with SP EVs (from SP R6) induces antibody response against PspA, and confers protection in murine models of invasive pneumonia[54] • Spray-dried microparticles encapsulating SP EVs (from SP R6) do not compromise the viability of THP-1 cells and induce the release of proinflammatory cytokines (TNF and IL-6) from PBMCs[55]

*All entries in the Application column reflect pre-clinical findings obtained in mouse models; none of the EV-based strategies discussed for *K. pneumoniae*, *S. aureus* or *S. pneumoniae* have yet entered human clinical trials; +Importantly, most of the *S. pneumoniae* EV literature summarized here was generated using non-macrolide-resistant laboratory or clinical strains. ABC: ATP-binding cassette; AFAs: antimicrobial fatty acids; ATCC: American Type Culture Collection; BAK1: BCL2 antagonist/killer 1; BCL2: B-cell lymphoma 2; BBVs: bacterial biomimetic vesicles; BipA: 50S ribosomal subunit assembly factor; BlaZ: β-lactamase; blaNDM-1: gene encoding New Delhi metallo-β-lactamase 1; BSA: bovine serum albumin; BSA-OMV: bovine serum albumin-reinforced outer membrane vesicle nanoparticles; CCL2: chemokine C-C motif ligand 2; CCL3: chemokine C-C motif ligand 3; $CD8^+$: cluster of differentiation 8-positive; CRKP: carbapenem-resistant *Klebsiella pneumoniae*; CXCL1: C-X-C motif chemokine ligand 1; E. coli: *Escherichia coli*; Eno: enolase; EVs: extracellular vesicles; GapA: glyceraldehyde-3-phosphate dehydrogenase A; GapN: non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase; GpmA: phosphoglycerate mutase A; GroEL: chaperonin GroEL; HaCaT: human adult low-calcium high-temperature keratinocytes; HUS: hemolytic uremic syndrome; hvKP: hypervirulent *Klebsiella pneumoniae*; ICG: indocyanine green; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-8: interleukin-8; IL-18: interleukin-18; KP: Klebsiella pneumoniae; LytC: lysozyme C; MalX: maltose/maltodextrin ABC transporter; MdtQ: multidrug resistance outer membrane protein; MDR: multidrug-resistant; MRSA: methicillin-resistant *Staphylococcus aureus*; MSNs: mesoporous silica nanoparticles; NF-κB: nuclear factor kappa B; OCLN: occludin; OmpA: outer membrane protein A; OmpC: outer membrane protein C; OMVs: outer membrane vesicles; PBMCs: peripheral blood mononuclear cells; PBP1: penicillin-binding protein 1; PBP2: penicillin-binding protein 2; PBP3: penicillin-binding protein 3; PfkA: phosphofructokinase A; Ply: pneumolysin; PrsA: cis-trans prolyl isomerase; PspA: pneumococcal surface protein A; PykF: pyruvate kinase; SA: *Staphylococcus aureus*; SP: *Streptococcus pneumoniae*; StkP: serine/threonine-protein kinase; Th17: T helper 17 cells; THP-1: human monocytic leukemia cell line; TLR2: Toll-like receptor 2; TLR4: Toll-like receptor 4; TLR9: Toll-like receptor 9; TNF: tumor necrosis factor; TNF-α: tumor necrosis factor-α; Tpi: triosephosphate isomerase.

Although *K. pneumoniae*, *S. aureus* and *S. pneumoniae* differ in vesicle biogenesis and cell wall structure, their EVs share common downstream roles: all three carry virulence- and resistance-associated cargo, modulate host immune responses, and are being explored as vaccine platforms. The maturity of the evidence

differs considerably, being most developed for *K. pneumoniae* and *S. aureus* and comparatively limited for *S. pneumoniae*, particularly for the macrolide-resistant phenotype prioritized by WHO. These similarities and differences suggest that lessons from the better-studied Gram-negative and Gram-positive models could guide future work on less characterized pathogens and phenotypes. Important knowledge gaps remain across all three pathogens. For *K. pneumoniae* and *S. aureus*, current evidence is based on a limited number of resistant isolates, whereas EVs from macrolide-resistant *S. pneumoniae* remain largely unexplored, and closing this gap should be a priority. Further *in vivo* studies using clinically relevant strains are needed to determine the robustness and translational relevance of these findings. In addition, differences in bacterial culture conditions, antibiotic exposure, vesicle isolation protocols and downstream analyses can strongly affect the observed EV composition and function[18,56]. Standardized methodologies for bEV isolation, purification and characterization are therefore needed to improve reproducibility and comparability between studies. In this regard, the 2023 Minimal Information for Studies of Extracellular Vesicles (MISEV2023) guidelines of the International Society for Extracellular Vesicles (ISEV) provide minimal information standards for EV studies[57]. In parallel, EV-based vaccine strategies appear promising, but their safety, scalability, stability and protective efficacy need careful assessment before clinical application[18]. Because bEVs can carry virulence factors, toxins and proinflammatory components [e.g., lipopolysaccharide (LPS), pore-forming toxins, superantigens], EV-based vaccine candidates carry an intrinsic risk of reactogenicity or off-target inflammation, and removing or inactivating these components without compromising immunogenicity remains a key safety challenge. Currently, the only licensed OMV-based vaccines target *Neisseria meningitidis* serogroup B (MenB) and *Haemophilus influenzae* type b (Hib). OMV vaccines against other Gram-negative pathogens, including *Shigella* and non-typhoidal *Salmonella*, are progressing clinically, and meningococcal OMV vaccines have shown 31%-46% cross-protection against *Neisseria gonorrhoeae* in observational studies, supporting further gonococcal vaccine development[58,59]. This contrasts with the largely pre-clinical evidence for vesicle-based vaccines against *K. pneumoniae*, *S. aureus* and *S. pneumoniae*.

In conclusion, recent findings support the idea that bEVs are part of the broader pathogen response to antimicrobial pressure. A deeper understanding of their biology may help identify new therapeutic strategies against antibiotic-resistant infections, and studying EVs from WHO priority pathogens may therefore offer important opportunities to counteract AMR and improve infectious disease management.

DECLARATIONS

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Authors' contributions

Conceptualized the work, performed the literature search, analysis and interpretation, drafted the manuscript, and revised the manuscript: Battista M

Conceptualized the work, performed the literature search, revised the manuscript, and approved the final version: Vilhena C

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