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ORIGINAL RESEARCH

Circulating Outer Membrane Vesicles from Gut-Colonized Carbapenem-Resistant *Enterobacterales* Degrade Antibiotics and Promote Bacterial Survival

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Purpose: Gut colonization of carbapenem-resistant Enterobacterales (CRE) poses a significant risk for systemic infections, but the mechanisms driving resistance dissemination are poorly understood. This study aimed to investigate whether outer membrane vesicles (OMVs) secreted by gut-colonized CRE can enter the human circulatory system and mediate extracellular antibiotic resistance through functional carbapenemases and resistance genes.

Patients and Methods: We conducted comparative proteomic analyses of OMVs isolated from parental CRE strains and patient plasma samples. Antibiotic degradation assays were performed to evaluate OMV-mediated hydrolysis of imipenem and meropenem. In vitro experiments assessed the protective effects of OMVs on carbapenem-susceptible Escherichia coli and Pseudomonas aeruginosa. Additionally, a Galleria mellonella infection model was used to examine OMV-mediated bacterial survival under carbapenem pressure.

Results: Plasma-derived OMVs exhibited proteomic profiles similar to bacterial OMVs, including carbapenemase components, and demonstrated comparable antibiotic-degrading activity. These OMVs hydrolyzed 60–75% of imipenem and meropenem within 24 hours, protecting susceptible bacteria from growth inhibition in vitro. Although no horizontal gene transfer was observed, OMVs enhanced *Klebsiella pneumoniae* survival under carbapenem pressure in the G. mellonella model, increasing larval survival rates by 25%.

Conclusion: Our findings reveal a novel OMV-mediated extracellular resistance mechanism that operates independently of genetic transfer, promoting bacterial persistence in the bloodstream. This study provides key insights into the role of OMVs in clinical treatment failure and identifies potential therapeutic targets to combat antibiotic resistance dissemination.

Keywords: outer membrane vesicles, carbapenem-resistant enterobacterales, CRE, carbapenemase, antibiotic degradation, intestinal colonization

Introduction

CRE have emerged as a critical threat to global public health,with mortality rates exceeding 30% in infected patients due to limited therapeutic options[1,2] While carbapenems remain last-line antibiotics for multidrug-resistant infections, the rapid spread of CRE—particularly in high-risk settings like intensive care unit (ICU)—has rendered these drugs increasingly ineffective.[3,4] A key reservoir for CRE is the human gastrointestinal tract, where colonization rates reach up to 6.8%45.4% in immunocompromised populations.[5–9] Crucially, intestinal CRE carriage elevates the risk of subsequent bloodstream infections by 10-fold, yet the mechanisms facilitating systemic resistance dissemination remain poorly understood.[10]

Recent studies highlight outer membrane vesicles (OMVs) as potent mediators of bacterial communication and antibiotic resistance.[11–13] These nanosized proteoliposomes, secreted by Gram-negative bacteria, transport functional

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proteins, nucleic acids, and enzymes across biological barriers, enabling intercellular interactions without direct contact.[14] Notably, OMVs from β-lactamase-producing strains degrade antibiotics extracellularly and protect susceptible bacteria, suggesting a non-genetic route for resistance spread[15.] We hypothesized that OMVs derived from gut-colonized CRE could translocate into the bloodstream, where their cargo of carbapenemases could degrade antibiotics systemically, thereby promoting bacterial survival—a novel extracellular resistance mechanism independent of genetic transfer. This hypothesis is critical for two reasons. First, the gut microbiota generates approximately 4×10^{14} OMVs daily, which may breach intestinal barriers under physiological conditions.[16] Second, carbapenemase-loaded OMVs could theoretically degrade antibiotics in systemic circulation, fostering bacterial persistence even in the absence of horizontal gene transfer. Supporting this, proteomic and functional assays have been successfully used to identify and characterize OMVs from complex clinical samples like plasma,[16] providing a means to test our hypothesis.

Here, we provide the first evidence that OMVs secreted by gut-colonized CRE translocate into human blood, carrying active carbapenemases and resistance genes. Using proteomic and functional assays, we demonstrate that plasma-derived OMVs degrade imipenem and meropenem, rescuing susceptible bacteria in vitro and in a Galleria mellonella infection model. Importantly, while these OMVs failed to transfer resistance genes horizontally, their enzymatic activity significantly reduced antibiotic efficacy, revealing a novel extracellular resistance mechanism. Our findings redefine the paradigm of carbapenem resistance dissemination and underscore OMVs as potential therapeutic targets to combat treatment failure.

Materials and Methods

Patients

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (Approval number: 2024–709). All participants provided informed written consent prior to participation and sample collection (stool and serum). The consent process included detailed information about the study aims, procedures, potential risks and benefits, confidentiality measures, and the voluntary nature of participation, including the right to withdraw at any time without penalty. Fecal or rectal swab samples were collected from patients admitted to the ICU for more than 48 hours at the Second Affiliated Hospital of Fujian Medical University between January and June 2024 (Table 1). One specimen was collected per patient, and duplicate samples from the same patient were excluded.

Inclusion criteria: Patients who tested positive for CRE in their fecal or rectal swab samples but did not show any clinical signs of infection were considered to be colonized with CRE.

Exclusion criteria: Patients who did not sign an informed consent form, patients who could not cooperate, and patients with diarrhea were excluded from the study.

Table 1 Demographic Features of Patients and the Clinical Characteristics of Intestinal Colonized CRE in This Study

	Patients				
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**
Isolates	KP-R1	KP-R2	KP-R3	KP-R4	KP-R5
Strain type	Kpn	Kpn	Kpn	Kpn	Kpn
Enzyme type	NDM	KPC	KPC	KPC	KPC
Age	58	63	87	82	75
Gender	Male	Female	Male	Female	Male
Date	31-Dec-23	13-Jan-24	5-Jan-24	8-Jan-24	18-Jan-24
Ward	ICU	ICU	ICU	ICU	ICU
Diagnosis	Severe pneumonia	Hemorrhagic shock	Severe pneumonia	Coronary Heart Disease	Acute Heart Failure
Specimens	Feces	Feces	Feces	Feces	Feces
Outcome	Recovered	Recovered	Died	Died	Died

Ethical Statement

This study was conducted in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Fujian Medical University (Approval number: 2024–709; Date: 16 December 2023). Written informed consent was obtained from all participants involved in the study.

Bacterial Strains

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains KP-R1, carrying the blaNDM gene, and KP-R2, carrying the blaKPC gene, were used for the isolation and purification of outer membrane vesicles (OMVs). The bacterial isolates were identified using MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany), and the minimal inhibitory concentrations (MICs) were determined by the microdilution broth method. MICs were tested against a panel of antibiotics including ampicillin, amoxicillin-clavulanic acid, piperacillin-tazobactam, ceftriaxone, cefepime, aztreonam, meropenem, ertapenem, imipenem, amikacin, tobramycin, ciprofloxacin, levofloxacin, tigecycline, sulfamethoxazole-trimethoprim, cefoperazone-sulbactam, ceftazidime-avibactam, and colistin (see Table S1 for complete profiles). The presence of the bla_{KPC} and bla_{NDM} genes was reconfirmed by PCR. Table 2 lists the specific primers and product sizes. The recipient strains used in the transformation experiments were *Klebsiella pneumoniae* ATCC 700603 and Hypervirulent *K. pneumoniae* (hvKP)NTUH-K2044.

OMV Purification

Outer membrane vesicles (OMVs) were purified from bacterial cultures and patient plasma as previously described, with modifications.[15] Briefly, the CRKP strains were incubated in Luria-Bertani (LB) medium at 37 °C and 200 rpm for 12 hours. They were then centrifuged at 5,400×g for 10 minutes at 4 °C, and the pellet was discarded. The supernatant was concentrated using a 100-kDa, 50-mL ultrafiltration tube (Millipore), followed by ultracentrifugation at 100,000×g for 3 hours at 4 °C. The supernatant was discarded, and the pellet was washed with sterile phosphate-buffered saline (PBS) (pH 7.4). The OMV-containing pellet was resuspended in 200 mL of PBS.

To isolate OMVs from peripheral blood, 20 mL of peripheral blood was collected in EDTA tubes and centrifuged at 1,500×g for 20 minutes to obtain platelet-free plasma. The plasma was then ultracentrifuged at 100,000×g for 70 minutes to pellet the OMVs. The OMVs were washed with PBS and concentrated again at 100,000×g for an additional 70 minutes. The protein concentration of purified OMVs was determined using a bicinchoninic acid (BCA) assay kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. Finally, the OMV pellet was resuspended in 200 μL of PBS, aliquoted and stored at −80°C until use.

Transmission Electron Microscopy (TEM)

The morphology of OMVs was examined by transmission electron microscopy (TEM) using a standard negative staining protocol. Briefly, OMVs were resuspended in PBS and applied to 400-mesh formvar-coated copper grids. After adsorption for 1 minute, the grids were negatively stained with a 2*% uranyl acetate solution for 1 minute. The samples were then observed under a transmission electron microscope (HT7700, Hitachi) operating at an accelerating voltage of 60 kV.*

Table 2 Oligonucleotides Used in This Study

Primer	Sequence (5'–3')	Length (bp)	Use
NDM-1 Forw	GGGCAGTCGCTTCCAACGGT	476	PCR
NDM-1 Rev	GTAGTGCTCAGTGTCGGCAT		
KPC-2 Forw	TGTCACTGTATCGCCGTC	1024	PCR
KPC-2 Rev	CTCAGTGCTCTACAGAAAACC		
Phoe Forw	TTCAACAGCGACGCAGGCAGC	277	PCR
PhoE Rev	GCCGTAGTTCTTCAGCTTC		
REP Forw	IIIGCGCCGICATCAGGC	N/A	REP-PCR
REP Rev	ACGTCTTATCAGGCCTAC	N/A	

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Nanoparticle Tracking Analysis (NTA)

The OMVs were diluted in PBS to a concentration of 10^8 particles/mL and then analyzed using the NanoSight NTA instrument and software. The analysis yielded the particle size distribution and concentration.

REP-PCR

Repetitive extragenic palindromic PCR (REP-PCR) was performed as previously described.[17] The primers used for REP-PCR are listed in Table 2. If two strains exhibit the same banding pattern, they are considered to belong to the same genotype, with a maximum difference of two bands being acceptable.

Proteomics Analysis of OMVs

Two sets of purified OMVs were sent to Beijing Biotech Pack Biotechnology Co., Ltd. First, proteins were extracted from the samples and subjected to SDS-PAGE. The corresponding gel bands were then excised and destained. The destained gel pieces were subsequently subjected to trypsin digestion. The digested samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify the OMV proteins. The properties and functions of these proteins were further characterized using the website https://www.ncbi.nlm.nih.gov/guide/proteins/.To confirm the absence of bacterial contamination, purified OMV suspensions were plated on LB agar plates and incubated at 37°C for 24 hours. No bacterial growth was observed in any OMV preparation used for subsequent experiments.

Detection of Carbapenem Antibiotic Concentration

The concentrations of meropenem (MEM) and imipenem (IPM) were determined using a potassium permanganate spectrophotometer. First, a standard curve was fitted. Then, 10^5 particles of CRKP-OMVs and plasma OMVs were separately added to 200 μL of different concentrations of MEM and IPM and incubated for 12 and 24 hours. Finally, the concentrations of the residual MEM and IPM in the solution were measured.[18]

Horizontal Gene Transfer (HGT) Assay

The potential for horizontal gene transfer via OMVs was assessed by co-culturing carbapenem-susceptible recipient strains (*K. pneumoniae* ATCC 700603 and NTUH-K2044) with purified OMVs (50 μg/mL) in LB broth at 37°C for 24 hours. After incubation, the cultures were plated onto LB agar plates containing selective concentrations of meropenem (2 μg/mL) or imipenem (2 μg/mL). The plates were examined for bacterial growth after 48 hours of incubation. Any potential transformants were further analyzed by PCR for the presence of bla_{KPC} and bla_{NDM} genes.

Galleria Mellonella Larvae Infection Experiments

The in vivo protective efficacy of OMVs was evaluated using a *Galleria mellonella* survival assay, as previously described with minor modifications.[18] Briefly, 10 μL of NTUH-K2044 (approximately 1×10^4 CFU) and 0.08 μg of meropenem were injected into the last left proleg of the larva. After 1.5 hours, 10 μL of KPC-loaded OMVs (from strain KP-R2) at a concentration of 6.25 μg/mL was administered into the last right proleg of the larva; plasma OMVs were also subjected to the same treatment. The control groups included larvae infected with NTUH-K2044 treated solely with meropenem in the Galleria mellonella infection model. The larvae were incubated at 37 °C for 48 hours, and survival was monitored at the indicated time points.

Statistical Analysis

Data were analyzed using GraphPad Prism 10. Specific statistical tests are detailed in the figure legends and included unpaired t-tests for two-group comparisons and two-way ANOVA with Tukey's or Dunnett's post hoc test for multiple comparisons. A p-value of < 0.05 was considered statistically significant.

Results

Size Heterogeneity and Shared Carbapenemases Define OMVs from Gut-Colonized CRKP and Circulating Plasma

To characterize OMVs from clinical CRKP strains and patient plasma, we performed morphological and quantitative analyses. Transmission electron microscopy (TEM) revealed that both CRKP-derived OMVs (CRKP-OMVs) and plasma-derived OMVs (Plasma-OMVs) exhibited spherical nanostructures with intact bilayered membranes (Figure 1A and B). However, nanoparticle tracking analysis (NTA) demonstrated a significant size difference: CRKP-OMVs had a median diameter of 313.1 nm (range: 120–450 nm), whereas Plasma-OMVs were smaller (median: 141.2 nm; range: 80–220 nm; Figure 1C and D). This discrepancy may reflect host environmental influences, such as blood shear forces or interactions with immune components, which could modify vesicle size during systemic circulation.

Shared Genetic Markers and Functional Enzymes in Plasma-OMVs and CRKP-OMVs Link Gut Colonization to Systemic Resistance

To determine whether OMVs isolated from patient plasma originated from intestinal CRKP strains, we analyzed their genetic and proteomic profiles. PCR amplification confirmed that both Plasma-OMVs and CRKP-OMVs harbored the *phoE* gene, a species-specific marker for *Klebsiella pneumoniae* (Figure 2A). REP-PCR genotyping further revealed identical banding patterns between Plasma-OMVs and their parental CRKP strains (Figure 2B), establishing a direct genetic lineage. Proteomic profiling identified 71 proteins in Plasma-OMVs, 12 of which overlapped with CRKP-OMVs (Figure 2C). Functional annotation classified these shared proteins into three categories: Antibiotic resistance, Metabolic activity and Catalytic function (Figure 2D and E), corroborating the functional similarity between the two OMVs types. In addition, critical quality control measures confirmed the absence of bacterial contamination in OMV preparations. Gram staining and culture-based assays showed no residual bacteria in purified OMV suspensions (data not show).

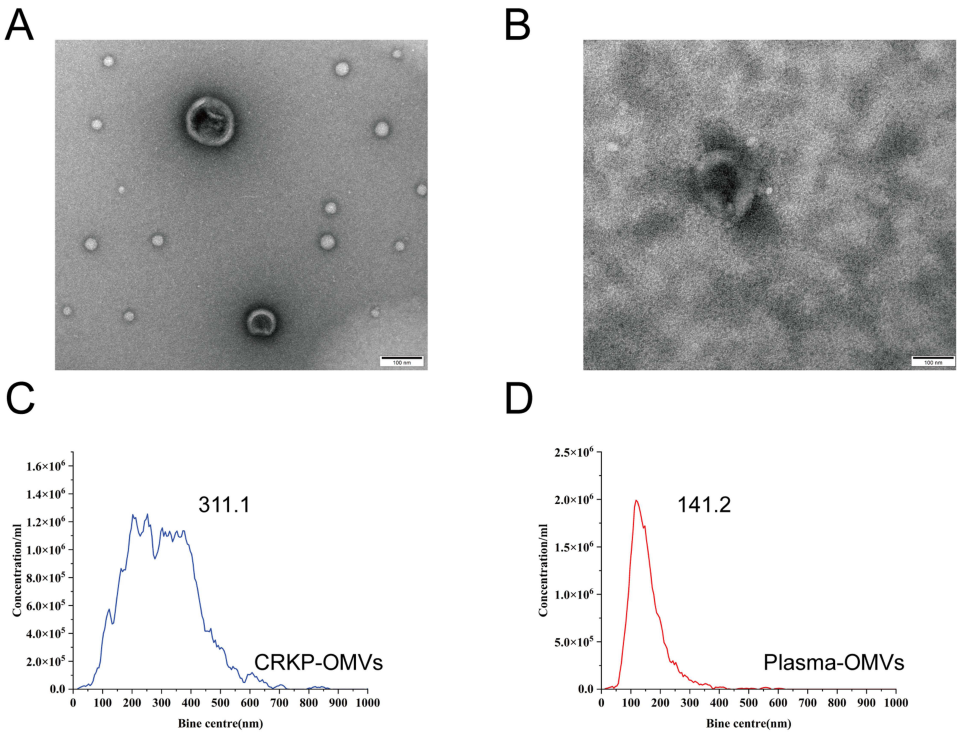

Figure 1 Isolation and characterization of OMVs from CRKP and patient plasma. (**A** and **B**) Transmission electron microscopy (TEM) images of OMVs isolated from *Klebsiella pneumoniae* culture supernatants (**A**) and plasma of a CRE-colonized patient (**B**). Scale bars: 100 nm. (**C** and **D**) Nanoparticle tracking analysis (NTA) of CRKP-OMVs (**C**) and plasma-OMVs (**D**). Particle size distributions (mean ± SD) revealed significantly larger diameters for CRKP-OMVs (313.1 ± 42.3 nm) compared to plasma-OMVs (141.2 ± 28.7 nm). Data represent three independent experiments (n = 3).

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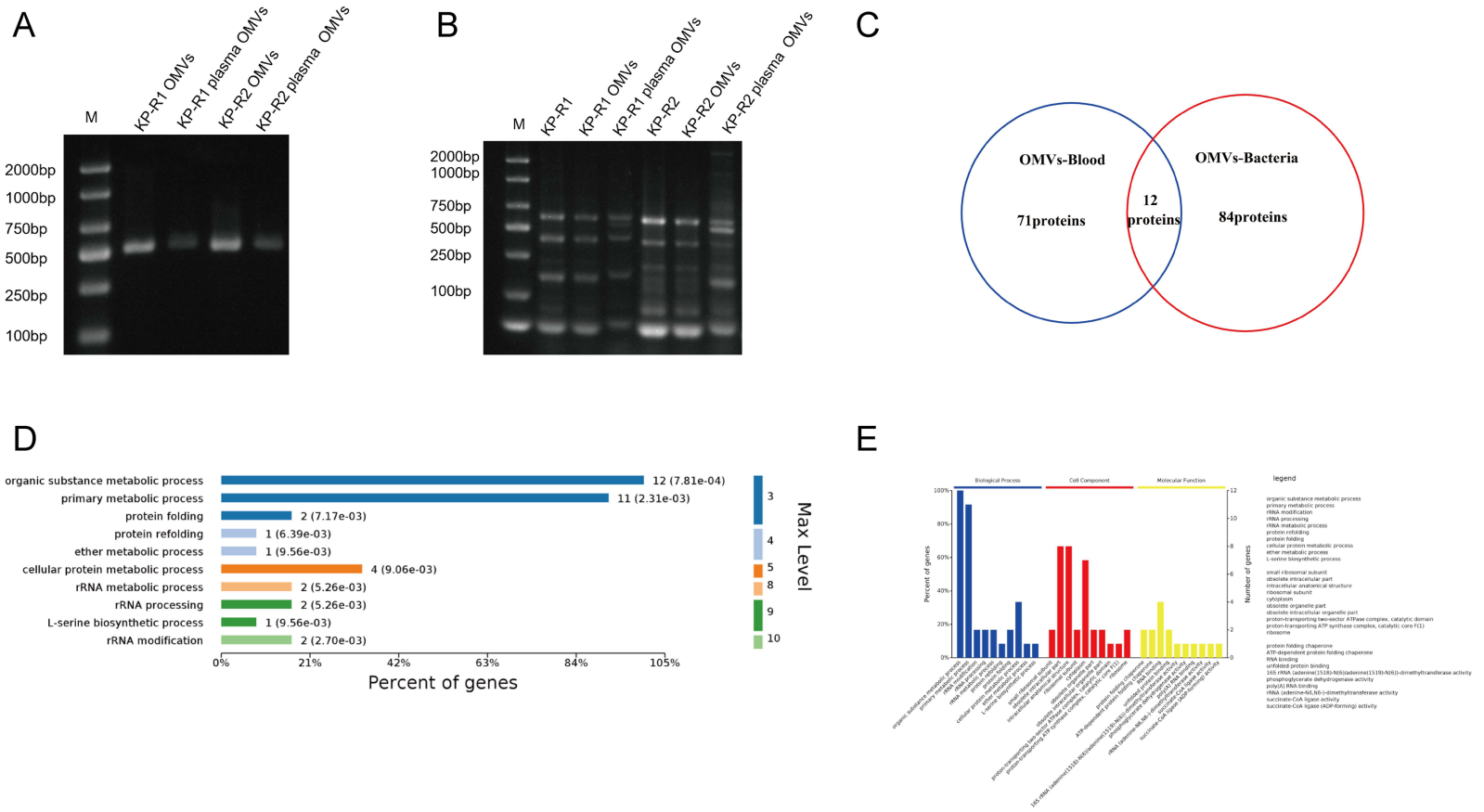

Figure 2 Comparative analysis of OMVs from CRKP and patient plasma **(A)** PCR amplification of *phoE* (547 bp) in DNA extracted from CRKP-OMVs and plasma-OMVs. Lane M: DNA ladder; Lane 1, 3: CRKP-OMVs; Lane 2, 4: Plasma-OMVs. **(B)** REP-PCR genotyping profiles of parental CRKP strains (Lane 1,4), CRKP-OMVs (Lane 2, 5), and plasma-OMVs (Lane 3, 6). Identical banding patterns confirm the intestinal origin of plasma-OMVs.**(C)** Venn diagram of proteins identified in CRKP-OMVs and plasma-OMVs. Of 71 proteins in plasma-OMVs, 12 overlapped with CRKP-OMVs.**(D)** KEGG pathway enrichment analysis of shared proteins. **(E)** Biological Process (left), Cellular Component (middle), and Molecular Function (right) of Common Proteins in CRKP-OMVs and Plasma-OMVs.

Plasma-OMVs Carry Functional Carbapenemases and Resistance Genes but Fail to Transfer Genetic Material

To assess whether Plasma-OMVs carry functional carbapenemases, we performed PCR and enzymatic activity assays. PCR amplification confirmed the presence of bla_{KPC} and bla_{NDM} genes in both Plasma-OMVs and CRKP-OMVs (Figure 3A). Immunoblotting further validated the expression of KPC and NDM enzymes in these vesicles (Figure 3B). Despite harboring bla_{KPC} and bla_{NDM} genes, these Plasma-OMVs failed to transfer resistance genes to susceptible *K. pneumoniae* strains (ATCC 700603 and NTUH-K2044) in horizontal gene transfer assays, suggesting that enzymatic payloads—rather than genetic material—mediate their role in antibiotic resistance.

Carbapenemase-Loaded OMVs Hydrolyze Imipenem and Meropenem

To determine whether OMV-associated carbapenemases retain enzymatic activity in blood—resisting degradation by host proteases or neutralization by IgG—we quantified their antibiotic hydrolysis capacity. Standard curves for imipenem and meropenem were established using HPLC. Plasma-OMVs and CRKP-OMVs (10^5 particles/mL) were incubated with each antibiotic (20 μg/mL imipenem; 5 μg/mL meropenem) for 24 h. Residual drug concentrations revealed that Plasma-OMVs degraded 58.7 ± 3.8% of imipenem and 52.4 ± 4.2% of meropenem, while CRKP-OMVs exhibited higher activity (72.1 ± 5.1% and 68.3 ± 4.7%, respectively; $P < 0.01$, Figure 4A and B).

Plasma-OMVs Protect Susceptible Bacteria Against Carbapenems via Enzymatic Antibiotic Degradation

To investigate whether Plasma-OMVs confer protection to carbapenem-susceptible bacteria under carbapenem pressure, we conducted in vitro assays. Co-culture of *Escherichia coli* ATCC 25922 with Plasma-OMVs (25 μg/mL) in the presence of imipenem or meropenem (1 μg/mL) increased bacterial survival by 42.5 ± 5.3% compared to OMV-free controls ($P < 0.001$; Figure 5A and B). Similar results were observed for *Pseudomonas aeruginosa* ATCC 27853, with Plasma-OMVs rescuing 38.1 ± 4.7% of cells from meropenem-induced death ($P < 0.01$; Figure 5C and D). This protective effect exhibited dose dependency: higher OMV concentrations (50 μg/mL) enhanced survival rates to 67.8 ± 6.1% (*E. coli*) and 59.4 ± 5.5% (*P. aeruginosa*).

Plasma-OMVs Protect Carbapenem-Susceptible Klebsiella pneumoniae via Enzymatic Antibiotic Degradation in a Galleria mellonella Infection Model

In order to validate the in vivo protective role of Plasma-OMVs, we employed a *Galleria mellonella* infection model. Larvae injected with carbapenem-susceptible *Klebsiella pneumoniae* NTUH-K2044 (10^4 CFU/larva) alone exhibited 100% mortality

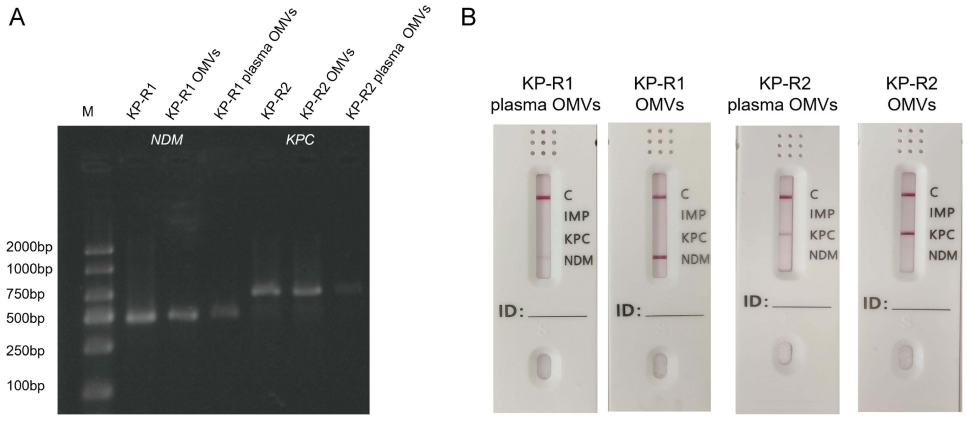

Figure 3 Carbapenemase and resistance gene profiling in OMVs.**(A)** PCR amplification of carbapenemase genes bla_{NDM} (476 bp) or bla_{KPC} (1024 bp) in DNA extracted from parental CRKP strains (Lane 1,4), CRKP-OMVs (Lane 2, 5), and plasma-OMVs (Lane 3, 6). Lane M: DNA ladder. **(B)** Immunoblot detection of carbapenemases (KPC and NDM) in OMVs. Data represent three biological replicates (n = 3).

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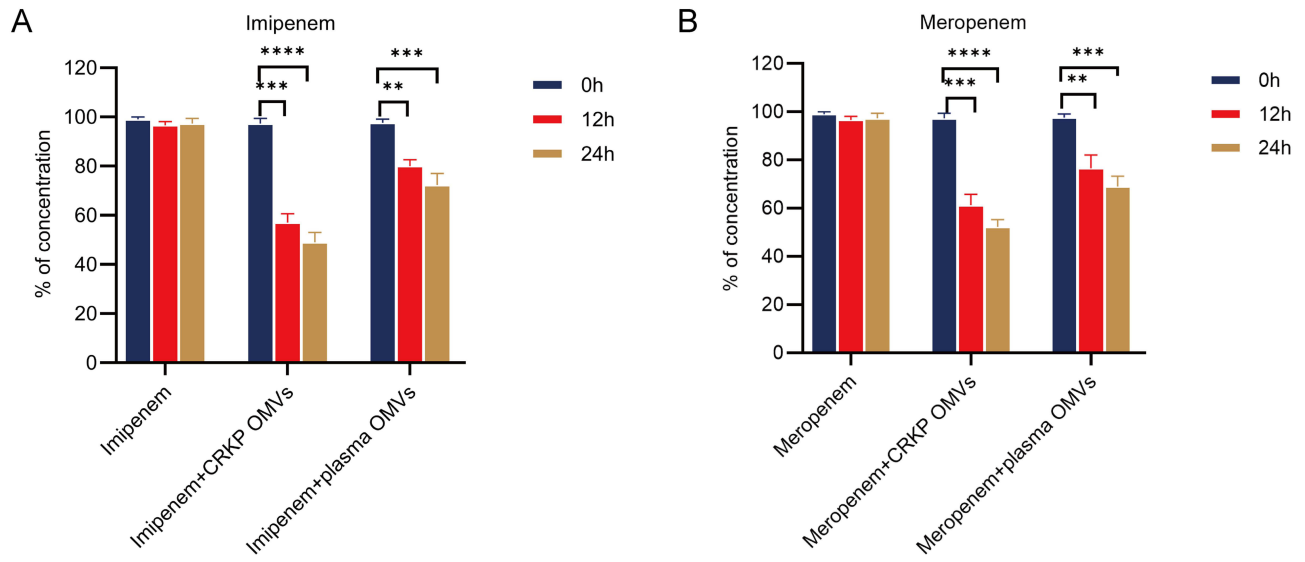

Figure 4 Carbapenems degradation by CRKP-OMVs and plasma-OMVs. (**A** and **B**) Hydrolysis of imipenem (20 μg/mL, (**A**) and meropenem (5 μg/mL, (**B**) by CRKP-OMVs (10 μg/mL) or plasma-OMVs (10 μg/mL) in PBS at 37°C with shaking (150 rpm). Filtered PBS containing antibiotics without OMVs served as the 100% activity control. Residual antibiotic concentrations were quantified by HPLC at 12 h and 24 h. Data are presented as mean ± SD (n = 3). **P < 0.01, ***P < 0.001, ****P < 0.0001.

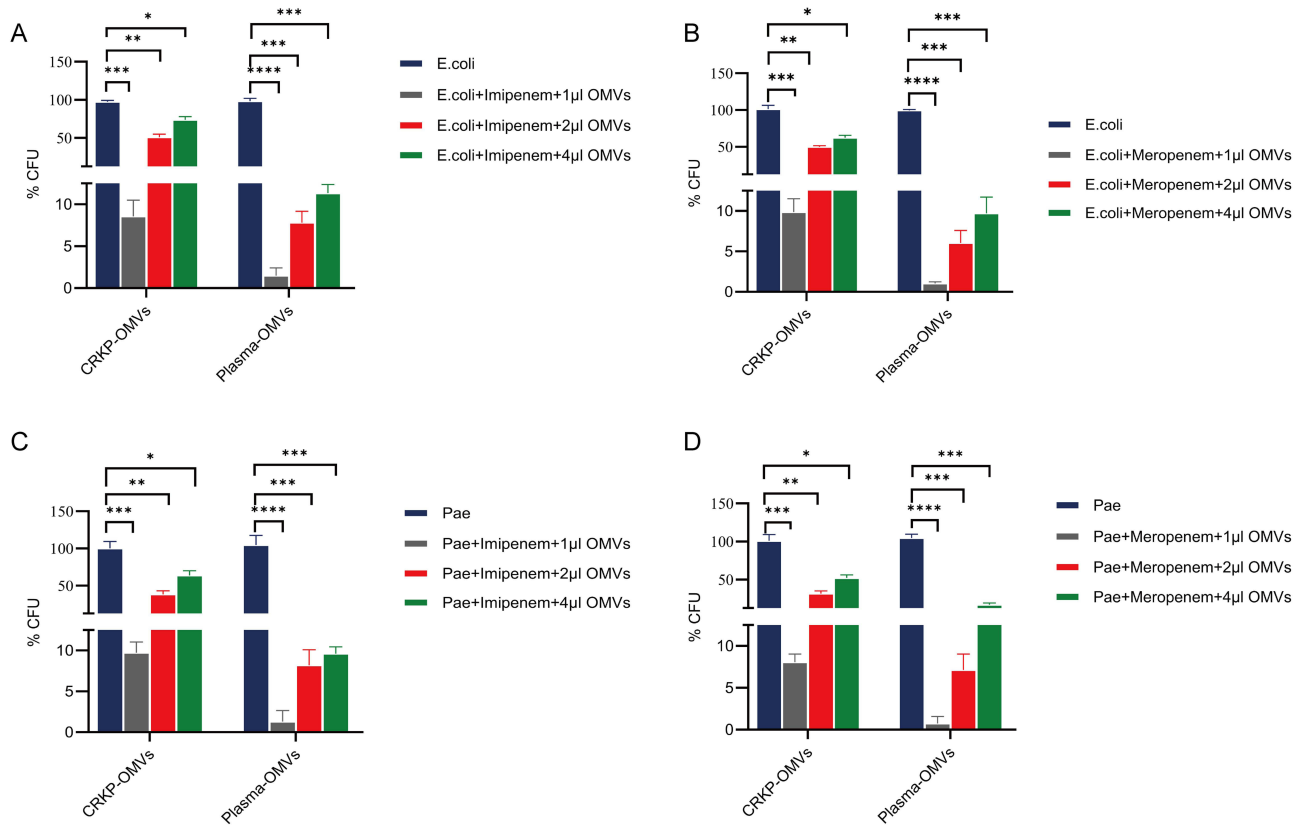

Figure 5 Plasma-OMVs protect carbapenem-susceptible bacteria from growth inhibition. (**A** and **B**) Survival rates of *Escherichia coli* ATCC 25922 co-cultured with imipenem (0.8 μg/mL, (**A**) or meropenem (0.8 μg/mL, (**B**) and increasing concentrations of CRKP-OMVs or plasma-OMVs for 24 h. (**C** and **D**) Survival rates of *Pseudomonas aeruginosa* ATCC 27853 under identical treatment conditions. Data are presented as mean ± SEM (n = 3). Statistical significance was determined by two-way ANOVA with Dunnett's post hoc test versus the OMV-free control group. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

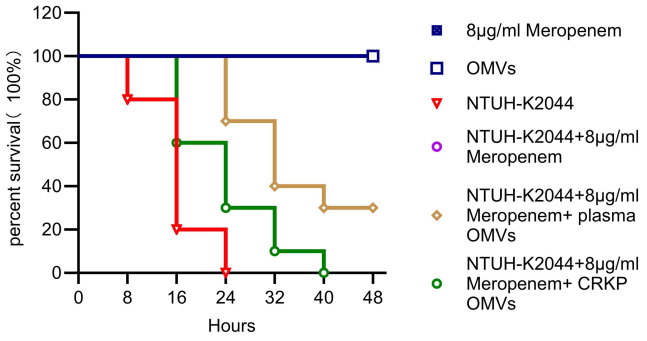

Figure 6 Plasma-OMVs protect carbapenem-susceptible *Klebsiella pneumoniae* via meropenem degradation in a *Galleria mellonella* infection model.Larvae were injected with *K. pneumoniae* NTUH-K2044 (10^4 CFU/larva) and meropenem (8 μg/mL), followed by administration of plasma-OMVs (6.25 μg/mL) or CRKP-OMVs (6.25 μg/mL) at 1.5 h post-infection. Survival was monitored for 48 h. Co-administration of plasma-OMVs significantly reduced survival rates to 25% compared to the meropenem-only control (100% survival). CRKP-OMVs exhibited stronger protection (0% survival at 40 h). No mortality occurred in larvae treated with OMVs alone, confirming their low toxicity. Data represent three independent experiments (n = 15 larvae/group).

within 24 h. Treatment with imipenem (8 μg/mL) alone completely rescued larval survival (100% at 48 h). However, co-administration of Plasma-OMVs (6.25 μg/mL) with NTUH-K2044 and imipenem significantly reduced survival rates to 25% at 48 h ($P < 0.001$ vs imipenem-only group; Figure 6). This result paralleled the in vitro antibiotic degradation activity of Plasma-OMVs, confirming their ability to neutralize carbapenems in a living host.

Control experiments demonstrated that Plasma-OMVs alone caused no larval mortality, ruling out nonspecific toxicity. Importantly, Plasma-OMVs isolated from non-CRE colonized patients failed to protect NTUH-K2044 under identical conditions (date not shown), underscoring the specificity of carbapenemase-dependent protection.

Discussion

CRE intestinal colonization has been recognized as a pivotal risk factor for systemic infections, with studies reporting up to 56.8% of colonized ICU patients progressing to CRE-associated bacteremia within 30 days.[19,20] Despite this clinical urgency, the mechanisms linking gut colonization to systemic antibiotic resistance remain elusive. Conventional research has focused on horizontal gene transfer (HGT) via plasmids or transposons as the primary driver of carbapenem resistance dissemination.[21,22] However, this paradigm fails to explain why carbapenem-susceptible pathogens frequently persist in colonized patients receiving appropriate antibiotic therapy—a phenomenon observed in 18.4% of CRE-colonized cohorts.[23] Recent advances in vesicle biology suggest that OMVs, secreted by Gram-negative bacteria, may serve as alternative vectors for resistance propagation. For instance, OMVs from *Haemophilus influenzae* and *Acinetobacter baumannii* have been shown to deliver β-lactamases and efflux pumps, protecting neighboring bacteria in biofilms.[24,25] Particularly, previous studies demonstrate that antibiotic resistance enzymes, such as β-lactamases, are shielded from enzymatic degradation and IgG-mediated neutralization during interbacterial transfer via OMVs.[26] Furthermore, plasmid DNA encapsulated within OMVs resists DNase digestion, maintaining structural integrity even after enzymatic treatment.[27] These findings underscore the remarkable stability of OMVs, enabling efficient long-distance dissemination of virulence factors and resistance genes within the host. Yet, critical questions remain unanswered: Can OMVs from intestinal CRE translocate into the bloodstream? If so, do they retain enzymatic activity in systemic circulation, and how do they influence clinical outcomes?

To address these gaps, we focused on the search for OMVs in the blood of CRE-colonized patients. Our study provides the first evidence that OMVs secreted by gut-colonized CRE translocate into the bloodstream. Using OMVs isolated from three CRE-colonized patients' serum, we observed that Plasma-OMVs and their parental CRKP-OMVs shared identical genetic fingerprints (Figure 2B) and conserved carbapenemase components (Figure 3), despite morphological divergences. While CRKP-OMVs exhibited larger median diameters (Figure 1C and D), both vesicle types maintained structural integrity (Figure 1A and B) and resisted host-mediated degradation—critical attributes enabling systemic antibiotic resistance dissemination.

Recent studies have indicated that bacterial OMVs can absorb the surrounding antibiotics through porins, and then degrade the antibiotics via the action of internal β-lactamases.[28,29] Our findings extend this mechanism to circulating

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Plasma-OMVs, demonstrating that both Plasma-OMVs and CRKP-OMVs carry functional carbapenemases (KPC/NDM) and corresponding resistance genes (bla_{KPC}, bla_{NDM}), enabling sustained imipenem degradation Figure 4). This enzymatic shield explains the survival of carbapenem-susceptible bacteria under OMVs treatment, as observed in *E. coli* and *P. aeruginosa* co-culture assays (Figure 5). Critically, our data suggest that antibiotic overuse may exacerbate resistance by triggering hyperproduction of OMVs loaded with inactivating enzymes—a vicious cycle that undermines therapeutic efficacy and worsens clinical outcomes.

Notably, while OMVs are known to facilitate HGT of resistance determinants,[30,31] our horizontal transfer assays revealed no acquisition of bla_{KPC} or bla_{NDM} by recipient strains (*K. pneumoniae* ATCC 700603/NTUH-K2044), despite PCR-confirmed gene packaging in OMVs (Figure 3A). This dichotomy highlights a key distinction: CRE-derived OMVs prioritize enzymatic antibiotic resistance over genetic dissemination, suggesting their evolutionary adaptation to evade host immune surveillance targeting foreign DNA. This study has some limitations. Firstly, the patient cohort was relatively small as this represents an initial proof-of-concept investigation. Future studies with larger sample sizes are needed to validate the clinical prevalence and impact of this mechanism.

Conclusion

In conclusion, we have identified a potent extracellular resistance mechanism facilitated by OMVs derived from gut-colonized CRE. These vesicles translocate into the bloodstream, harboring active carbapenemases that degrade antibiotics extracellularly, thus protecting susceptible bacteria and enhancing their survival independent of genetic exchange. This finding critically explains the high mortality associated with CRE co-infections and challenges the prevailing gene-centric view of resistance spread.

From a clinical perspective, our results underscore the necessity of routine CRE surveillance in high-risk populations. For colonized patients, therapeutic strategies should eschew carbapenem monotherapy and prioritize β-lactamase inhibitor combinations to neutralize this OMV-mediated threat. Ultimately, targeting OMVs may offer a novel avenue for restoring antibiotic efficacy and improving patient outcomes.

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Disclosure

The authors report no conflicts of interest in this work.

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