A novel Outer Membrane Vesicle-mediated mechanism of antimicrobial resistance of *Klebsiella pneumoniae*

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BACKGROUND: Antibiotic resistance can occur through mutations or the acquisition of resistance gene determinants via horizontal gene transfer (HGT). The latter is the most important factor in the current AMR pandemic. Recent evidence has reported OMVs as a novel mechanism of HGT, although, the mechanisms and factors that regulate this process are unclear. This study shows, for the first time, the transfer of plasmids containing beta-lactamase resistance genes via OMVs derived from *Klebsiella pneumoniae* (*K. pneumoniae*).

METHODS: Multisensitive standard *K. pneumoniae* (ATCC10031) was transformed through the calcium chloride method with pGR (high copy number) and PRM (low copy number) plasmids. OMVs were isolated from *K. pneumoniae*-pGR and -PRM colture and characterized via transmission electron microscopy (TEM), Dynamic Light Scattering (DLS) and mass spectrometry. The plasmid concentration in the vesicular lumen was obtained by Real-time PCR. For gene transfer assays, multi-sensitive standard *K. pneumoniae*, *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (P. *aeruginosa*), *Burkholderia cepacia* (*B. cepacia*) and *Salmonella enterica* (*S. enterica*) represent the recipient strains. These strains (10^7 CFU/mL) were incubated with 10 µg of OMV-*K. pneumoniae* for 8 hours at 37 °C and, then, plated on LB agar supplemented with ampicillin. The bacterial colonies grown were counted for transformation efficiency and subjected to colony-PCR to detect the resistance genes.

RESULTS: OMVs appeared on TEM as spherical electron-dense particles. DLS data showed that OMVs derived from *K. pneumoniae*-pGR and *K. pneumoniae*-PRM had diameters of 113.8±53.7 and 94.13±41.10, respectively (Figure 1). Mass spectra analysis detected 14 membrane-associated proteins, 3 periplasmic proteins, 32 cytosolic proteins and 21 enzymes (Figure 2). OMVs from *K. pneumoniae*-pGR contained 10.4±0.05 ng DNA/µg OMV, while *K. pneumoniae*-PRM had 08±0.62 ng DNA/µg OMV (Figure 3). Vesicles from *K. pneumoniae*-pGR and -PRM induced a transformation efficiency of $2.8±0.1x10^4$ CFU/µg and $7.8±0.9x10^3$ CFU/µg, respectively (Figures 4 and 5). OMVs derived from *K. pneumoniae*-pGR transferred DNA to *E. coli*, *S. enterica*, *P. aeruginosa* and *B. cepacia* with a transformation efficiency of $1.7±0.2x10^4$, $1.5±0.9x10^4$, $1.6±0.1x10^4$, $1.8±0.8x10^4$ CFU/µg, respectively (Figure 6).

CONCLUSIONS: These findings demonstrated the spread of beta-lactamase resistance via OMV-*K. pneumoniae*. This novel HGT mechanism was dependent on the identity of the genetic cargo and not related to the phylogenetic characteristics of the donor and the recipient species.

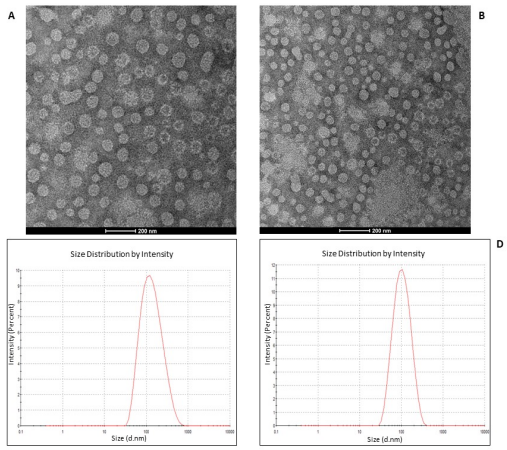


Figure 1. TEM of OMVs purified from *K. pneumoniae*-pGR (A) and *K. pneumoniae*-PRM (B). DLS intensity-weighed distribution of OMVs derived from *K. pneumoniae*-pGR (C) and *K. pneumoniae*-PRM (D).

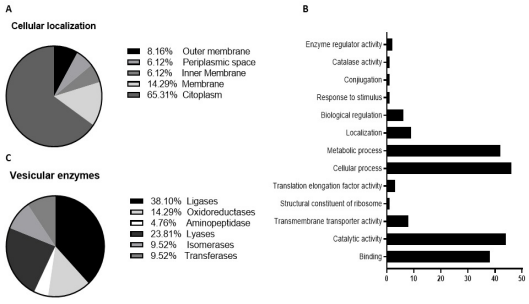
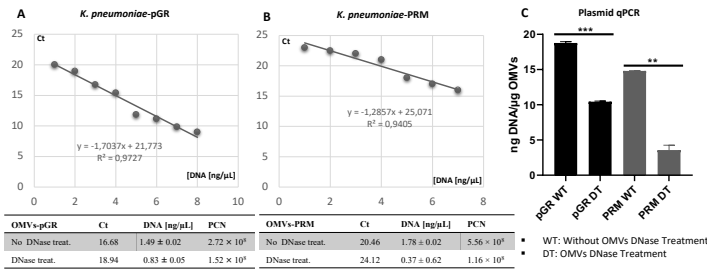


Figure 2. Classification of cellular localization (A), functional annotation (B) and enzymatic classes (C) of protein extracted from *K. pneumoniae*-OMVs.



OMVs-pGR	Ct	DNA [ng/µL]	PCN
No DNase treat.	16.68	1.49 ± 0.02	2.72×10^8
DNase treat.	18.94	0.83 ± 0.05	1.52×10^8

OMVs-PRM	Ct	DNA [ng/µL]	PCN
No DNase treat.	20.46	1.78 ± 0.02	5.56×10^8
DNase treat.	24.12	0.37 ± 0.62	1.16×10^9

Figure 3. Determination of PCN in OMVs, using quantitative PCR standard curves. The standard curves were generated by qPCR of the purified pGR (A) and PRM (B) plasmids. Histogram Graph of PGR and PRM cargo efficiency, before and after DNase treatment (C) (*P value < 0.05*).

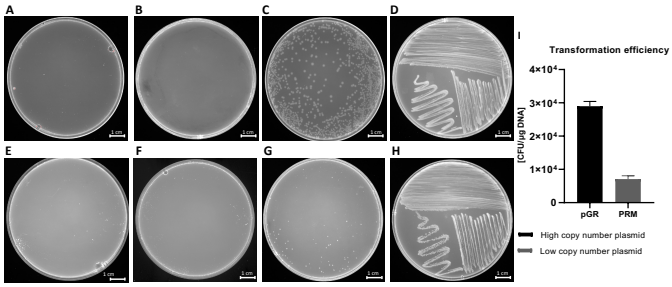


Figure 4: HGT via OMVs derived from *K. pneumoniae*-pGR and *K. pneumoniae*-PRM. Untreated cells (**A-E**) and cells treated with free plasmid (**B-F**) did not record transformants. *K. pneumoniae* ATCC treated with 10 µg of OMVs (**C-G**) and bacteria control on LB-plates (**D-H**). Intra-species HGT efficiency via OMVs purified from *K. pneumoniae*-pGR and *K. pneumoniae*-PRM (**I**)

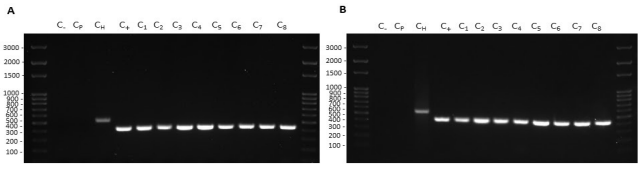


Figure 5. Colony-PCR from recipient cells treated with *K. pneumoniae* pGR (A) and *K. pneumoniae* PRM (B) OMVs. DNA gel showed PCR products with expected lengths: ß-lactamase product~ 424 bp ($C_{1–8}$), ribosomal 16S product ~ 550bp (C_H). Control water (C.) and untreated bacteria (C_p) did not show amplification.

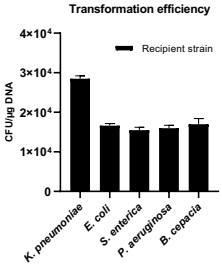


Figure 6. *K. pneumoniae*-pGR OMVs inter-species transformation efficiency (B).