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Interphylum dissemination of NDM-5-positive plasmids in hospital wastewater from Fuzhou, China: a single-centre, culture-independent, plasmid transmission study

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Summary

Background The global spread of plasmid-borne carbapenem resistance is an ongoing public health challenge; however, the nature of such horizontal gene transfer events among complex bacterial communities remains poorly understood. We examined the in-situ transfer of the globally dominant New Delhi metallo-β-lactamase (NDM)-5-positive IncX3 plasmid (denoted pX3_NDM-5) in hospital wastewater to simulate a real-world, One Health antimicrobial resistance context.

Methods For this transmission study, we tagged pX3_NDM-5 with the green fluorescent protein gene, gfp, using a CRISPR-based method and transferred the plasmid to a donor Escherichia coli strain. Bacteria were extracted from a hospital wastewater treatment plant (Fujian Provincial Maternity and Children’s Hospital, Fuzhou, China) as the bacterial recipient community. We mixed this recipient community with the E coli donor strain carrying the gfp-tagged plasmid, both with and without sodium hypochlorite (NaClO) as an environmental stressor, and conducted several culture-based and culture-independent conjugation assays. The conjugation events were observed microscopically and quantified by fluorescence-activated cell sorting. We analysed the taxonomic composition of the sorted transconjugal pool by 16S rRNA gene amplicon sequencing and assessed the stability of the plasmid in the isolated transconjugants and its ability to transfer back to E coli.

Findings We show that the plasmid pX3_NDM-5 has a broad host range and can transfer across various bacterial phyla, including between Gram-negative and Gram-positive bacteria. Although environmental stress with NaClO did not affect the overall plasmid transfer frequency, it reduced the breadth of the transconjugant pool. The taxonomic composition of the transconjugal pool was distinct from that of the recipient communities, and environmental stress modulated the permissiveness of some operational taxonomic units towards the acquisition of pX3_NDM-5. Notably, pX3_NDM-5 transconjugants included the Gram-positive pathogen Enterococcus faecalis, and the plasmid could subsequently be reconjugated back to E coli. These findings suggest that E faecalis could act as a natural shuttle vector for the wide dissemination of pX3_NDM-5 plasmids.

Interpretation Our culture-independent conjugation model simulates natural environmental conditions and challenges the established theory that Gram-negative and Gram-positive bacteria rarely exchange clinically important plasmids. The data show that plasmids disseminate more widely across genera and phyla than previously thought. These findings have substantial implications when considering the spread of antimicrobial resistance across One Health sectors.

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Introduction

Antimicrobial resistance has become a major global public health problem in the 21st century. Specifically, broad-range carbapenem resistance—driven by an increase in the prevalence of the New Delhi metallo-β-lactamase (NDM)-encoding genes (blaNDM)—has become globally ubiquitous, and carbapenem-resistant pathogens (such as Acinetobacter baumannii and Klebsiella pneumoniae) were reportedly responsible for between 50,000 and 100,000 deaths worldwide in 2019. The global spread of blaNDM genes is accelerated by plasmid-mediated horizontal gene transfer and, as a result, blaNDM genes are often observed in non-clinical environments—including surface water, hospital sewage, and wastewater treatment plants—highlighting the need for One Health antimicrobial resistance surveillance. Specifically, NDM-5 has raised concerns because of its high carbapenemase activity and its widespread dissemination via the...
Articles

Research in context

Evidence before this study
We searched PubMed, with no language restrictions, for reports published from database inception to Dec 31, 2022, using the search terms “culture independent conjugation” AND “carbapenem resistance”, OR “carbapenem resistance” and “interphylum dissemination”. We found no controlled studies examining the horizontal transfer of carbapenem-resistance genes using culture-independent methods. We extended the search using the terms “horizontal transfer”, “carbapenem resistance” AND “bacterial community”. Although 24 records were retrieved, none of these studies examined the transfer of carbapenem resistance in complex bacterial communities. To our knowledge, no studies to date have examined the diversity of transfer of New Delhi metallo-β-lactamase (NDM)-5-positive plasmids within bacterial communities or the transfer of these plasmids between bacterial phyla.

Added value of this study
To our knowledge, this is the first molecular study to examine the in-situ transfer of the NDM-5-positive IncX3 plasmid (denoted pX3_NDM-5)—the globally dominant antimicrobial resistance plasmid—in a complex bacterial community extracted from hospital wastewater. Rather than using culture-based methods, we developed a method based on fluorescence-activated cell sorting for the high-throughput screening and analysis of the transfer dynamics of pX3_NDM-5. We show that the clinically important plasmid pX3_NDM-5 has a broad host range and can transfer across many and varied bacterial phyla, including between Gram-negative and Gram-positive bacteria. We also examine, for the first time to our knowledge, the stability in Gram-positive bacteria of a plasmid from Gram-negative bacteria, and the ability of this plasmid to subsequently re-infect Gram-negative pathogens. These findings challenge established microbiological theories regarding the barriers to transfer between Gram-negative and Gram-positive bacteria.

Implications of all the available evidence
The global spread of plasmid-borne carbapenem resistance is an ongoing public health challenge, and resistant infections are often associated with increased mortality in clinical settings and longer hospital stays than susceptible infections. However, culture-based bacterial conjugation methods cannot accurately depict horizontal gene transfer events among complex bacterial communities. Our study combines both culture-independent cell-sorting methods and culture-based methods to efficiently track the transfer dynamics of the resistance-associated plasmid pX3_NDM-5 in bacterial communities from hospital sewage. Beyond increasing our knowledge of pX3_NDM-5 as a model plasmid, our results can be extrapolated to other clinically important resistance markers carried on globally dominant plasmids. These findings could help to steer the global conversation on how we understand the spread of antimicrobial resistance across One Health sectors.

Methods

Study design
In this plasmid transmission study, we examined the dissemination of pX3_NDM-5 in a simulated hospital wastewater treatment plant; such facilities have been recognised as potential hotspots for antimicrobial-resistance gene transfer. To mimic natural environmental conditions, bacterial communities were exposed to sodium hypochlorite (NaClO)—which is widely used as a disinfectant in hospital wastewater treatment systems—at concentrations of 40 mg/L and 100 mg/L. We established the identity of the transconjugants by PCR and 16S rRNA gene amplicon sequencing and isolated transconjugants of interest for stability studies and to investigate trans-Gram transfer. We developed a culture-independent, high-throughput cell-sorting approach to investigate the transfer dynamics of antimicrobial resistance plasmids under...
non-laboratory conditions and simulated environmental scenarios. This method was based on that described by Sørensen and colleagues\(^\text{10}\) with some modifications, including tagging with the green fluorescent protein gene \(gfp\), using a CRISPR RNA-guided method and culture-based characterisation of the isolated transconjugants (appendix p 2). The 46146 bp IncX3 plasmid (denoted \(pX3\_NDM-5\)) we selected for this study, originally isolated from chicken faeces, harbours only one resistance gene, \(\text{bla}\)\(^{\text{NDM-5}}\). The conjugation dynamics of the \(gfp\)-tagged plasmid in the complex bacterial community were monitored via a combination of culture-based and culture-independent methods, including transconjugant isolation, fluorescence-activated cell sorting (FACS), 16s rRNA gene amplicon sequencing, and bioinformatic analysis.

**Procedures**

**Plasmid \(gfp\)-tagging using a CRISPR RNA-guided method**

We developed a \(gfp\)-tagging strategy using a CRISPR RNA-guided method to enable us to track the transfer dynamics of \(pX3\_NDM-5\) in natural bacterial communities in a culture-independent manner. Detailed protocols for the construction of the \(gfp\)-tagged plasmid, as well as the strains and primers used, are listed in the appendix (p 6). The vector pSL1142 was used in the CRISPR RNA-guided method, following protocols described in a previous study.\(^\text{14}\) The \(gfp\)-mutated \(pX3\_NDM-5\) plasmid was then introduced into a donor strain \(Escherichia coli\) MG1655::\(lacI\)-\(pLpp\)-\(mCherry\)-\(kan\)\(^\text{4}\) by electroporation, in which \(gfp\) expression is repressed by the chromosomally encoded repressor \(lacI\).\(^\text{13}\) \(mCherry\) was constitutively expressed in this donor strain. This dual-labelling approach can effectively detect plasmid transfer without the requirement for selective agar plating and allows the rapid counting (>8000 per s) of the total number of transconjugants (culturable and non-culturable) in which \(gfp\) is expressed.

**Validation of conjugation assay for bacterial strains isolated from a hospital wastewater treatment plant**

To validate this culture-independent approach to measure the transfer of \(pX3\_NDM-5\) using flow cytometry, we isolated indigenous bacteria from a hospital wastewater treatment plant (Fujian Provincial Maternity and Children’s Hospital) using Urinary Tract Infections Chromogenic Agar (UTI; Hopebio, Qingdao, China) using Urinary Tract Infections Chromogenic Agar (UTI; Hopebio, Qingdao, China). Bacterial species were identified by 16s rRNA gene amplicon PCR followed by Sanger sequencing. We selected 86 bacterial strains, at random but ensuring that each belonged to a different bacterial species or phylum, isolated from the wastewater treatment plant as recipient strains, and individually mixed them with the donor strain \(E\ coli\) MG1655\ harbouiring \(pX3\_NDM-5\) for conjugation assays (appendix p 6). In brief, donor and recipient cells were grown at log phase before mixing in a 1:1 ratio in lysogeny broth (LB) and co-incubating overnight at 37°C. After 16 h mating, the mixtures were diluted and analysed on an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) with bacterial cell size and fluorescent signal threshold settings. Fluorescent controls of \(E\ coli\) MG1655::\(mCherry\) and \(E\ coli\) DH5α::\(gfp\) and a recipient community with no fluorescence were prepared to set appropriate photomultiplier tube voltages, as previously described,\(^\text{10}\) and to set appropriate gating for the green fluorescent transconjugant cells (excited at 488 nm) and the red fluorescent donor cells (excited at 561 nm). For each mating mixture, approximately 50000 events were recorded and the transfer frequency was approximated using the percentage of \(gfp\)-expressing transconjugants present in the mixed culture.

**Wastewater bacterial community extraction**

400 mL sewage water from the inlet of the hospital wastewater treatment plant at the Fujian Provincial Maternity and Children’s Hospital was collected on Dec 27, 2021, and kept at 4°C in a sterile 500 mL bottle until use. The 400 mL sample was then horizontally vortexed at 1500 rpm for 10 min, filtered through a 0.45 µm filter using a vacuum pump, and washed with 5–10 mL of sterilised sewage water by vortex for 3 min, followed by centrifugation at 5000 rpm for 10 min at 4°C. The bacterial pellets were resuspended in 10 mL residual sewage water and kept at 4°C until use. The sewage supernatant was sterilised for use as a bacterial growth liquid. To count bacteria in the extracted sewage sample, the cell suspension was diluted to ten times its initial volume in sterile saline and inoculated onto agar, the plates were incubated for 20 h at 30°C, and approximately 10⁶–10⁷ colony-forming units (CFU) per mL were used as recipients in the conjugation assay.

**Conjugation assay for bacterial community using FACS**

A schematic of the experimental setup is shown in the appendix (p 11). In brief, before the conjugation assay, the donor \(E\ coli\) MG1655 carrying the \(pX3\_NDM-5\) plasmid was incubated overnight at 37°C in LB. 100 µL of overnight culture was regrown in 5 mL LB for 6–8 h to reach the bacterial exponential phase and enumerated by plating and colony counting. Approximately 2.5 mL of the bacterial community extracted from wastewater was challenged with an equal volume of \(pX3\_NDM-5\)-positive donor \(E\ coli\) MG1655 with co-incubation for 16–20 h at 30°C. Conjugation assays were conducted without disinfectant stress (control group) or with NaClO supplementation at concentrations of 40 mg/L and 100 mg/L, as is commonly used for disinfection in hospital wastewater treatment plants.\(^\text{6}\) We did not use additional nutrient medium, instead using sterilised sewage water to mimic the natural environment during the mating incubation. Conjugation events were confirmed by confocal laser scanning microscopy (Leica TCS SP8X DLS; Wetzlar, Germany) and analysed by automated image analysis (Image Pro Plus; Media Cybernetics, Silver Spring, MD, USA).
We obtained the transconjugal pools (comprising all gfp-expressing transconjugants) using FACS. For each mating condition—control, 40 mg/L NaClO, or 100 mg/L NaClO—the contents of triplicate mating cultures were combined in one tube and transconjugant cells were analysed and sorted on the basis of their fluorescent signals using an Attune Nx flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA) and an S3e Cell Sorter (Bio-Rad, Hercules, CA, USA). Approximately 5 mL of mixed culture was collected for each condition, of which transconjugants made up between 2·34% and 4·87% of the 338,290 total recorded cells (appendix p 7). Sorted bacterial cells were collected, lysed, and subjected to 16S rRNA gene amplicon sequencing. We estimated the transfer frequency by calculating the percentage of gfp-expressing transconjugants present in the mixed culture.

Stability of newly formed transconjugants and transferability of pX3_NDM-5 from transconjugants
To examine the stability of pX3_NDM-5 in the new hosts, we conducted 15-day serial passages of 35 randomly selected transconjugants in meropenem-containing and meropenem-free LB in 96-well microtitre plates. Overnight cultures were diluted (1:200) into fresh LB with or without meropenem (1 mg/L) and incubated with vigorous shaking (220 rpm) for 24 h. To measure the percentage of NDM-5-positive strains, PCR was conducted on days 1, 3, 5, 7, 9, 11, 13, and 15. The bacterial density was measured using a microplate reader. All experiments were conducted in duplicate.

To investigate whether pX3_NDM-5 within the transconjugants could subsequently infect other bacteria, we selected streptomycin-resistant E coli C600 as the recipient strain and newly formed transconjugants from the sewage community as donors for conjugation assays. In brief, donor and recipient cells were grown at log phase before mixing in a 1:1 ratio in LB and co-incubating overnight at 37°C. After 16 h, the mixtures were diluted and plated on selective UTI agar plates containing meropenem (1 mg/L) and streptomycin (1000 mg/L). The resulting transconjugants were verified by blaNDM-5 and gfp gene PCR, and species were identified by 16S rRNA gene amplicon sequencing.

Data analysis
Details of 16S rRNA gene amplicon sequencing and bioinformatic analysis are described in the appendix (pp 3–4). The phylogenetic tree was obtained using ClustalW (version 2.0). The abundance of each operational taxonomic unit (OTU) was measured by dividing the number of reads per OTU by the total number of sequencing reads. For conjugation experiments, data were presented as mean (SD). We used GraphPad Prism 8.3 for data analysis and conducted unpaired t tests. p values less than 0·05 were considered to be significant.

Role of the funding source
The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results
To investigate the conjugation dynamics of the antimicrobial resistance plasmid pX3_NDM-5 in natural bacterial communities, we developed a dual-fluorescence labelling approach by tagging pX3_NDM-5 with gfp and the donor strain with mCherry (appendix pp 11–13). Transfer events can be identified by calculating the percentage of gfp-expressing cells (appendix p 7). To verify this approach, we used flow cytometry to investigate the ability of pX3_NDM-5 to transfer into 86 bacterial species obtained from hospital sewage water. 39 Gram-positive bacteria (Enterococcus spp) and 47 Gram-negative bacteria (28 Pseudomonas spp, six Burkholderia ubonensis, six Shigella flexneri, three Klebsiella spp, one Escherichia coli, one Elizabethkingia spp, and two Pantoea spp). The proportion of gfp-expressing transconjugants in mixed cultures varied, ranging from 0·00% to 63·78% (appendix pp 7, 14–16). This finding is consistent with previous observations that plasmid transfer is strongly influenced by donor–recipient interactions.9 In contrast to culture-based methods, no antibiotic-selective markers are required for this approach, which can efficiently detect the transfer of resistance plasmids in bacterial communities.

To establish the occurrence of pX3_NDM-5 plasmid transfer in natural complex bacterial communities, we challenged a microcosm extracted from a hospital wastewater treatment plant.
wastewater treatment plant with an *E. coli* donor strain carrying a pX3_NDM-5 plasmid. After overnight co-incubation of mating cultures in the absence or the presence of two different concentrations of the disinfectant NaClO as an environmental stressor, the gfp-expressing transconjugant cells were sorted from the mixed communities on the basis of size, presence of green fluorescence, and lack of red fluorescence. 338290 cells were counted per group, in which green fluorescence was detected in between 2·34% and 4·89%, corresponding to between 7909 and 16456 transconjugant cells per group (appendix p 8). These findings suggest that pX3_NDM-5 is capable of rapid and widespread dissemination through bacterial communities. In addition, conjugation events were observed by the presence of green fluorescent cells in confocal laser scanning micrographs (figure 1).

To further explore the taxonomic landscape of the pX3_NDM-5 transconjugant pools and recipient communities, we analysed transconjugants using deep 16S rRNA gene amplicon sequencing. Between 74264 and 77369 sequences per sample were acquired, and the phylogenetic structure of the transconjugant pools was analysed after the filtered sequences were clustered into OTUs at 97% similarity. We identified 231 transconjugant OTUs distributed over 12 phyla (figure 2). Pseudomonadota was the main host for pX3_NDM-5, and three classes of this phylum (γ, β, α) were identified. The additional 11 phyla comprised eight Gram-negative (Bacteroidota, Acidobacteriota, Nitrospirota, Planctomycetota, Verrucomicrobiota, Armamatimonadota, Ignavibacteriota, and Cyano bacteriota), and three Gram-positive (Bacillota, Actinomycetota, and Chloroflexota) phyla, some of which are known to be poorly cultivable. Compared with the culture-based method, a considerably broader host range of pX3_NDM-5 across Gram-negative and Gram-positive bacteria was detected. We also detected plasmid transfer to rare bacterial taxa within the recipient community using this method (appendix p 17). With the exception of the phyla Pseudomonadota and Bacillota, we observed a low abundance (OTU <0·001%) of transconjugal bacterial belonging to 15 other bacterial phyla in the NaClO-treated groups, including Planctomycetota, Verrucomicrobiota, Chloroflexota, Acidobacteriota, Candidatus Saccharibacteria, and Candidatus Parcubacteria. The involvement of these rare bacteria in the community in horizontal gene transfer suggests a broad reservoir pool for the pX3_NDM-5 plasmid.

At the phylum level, the phylogenic compositions of the transconjugant pools from both NaClO-treated groups were distinct from that of the non-treated group (appendix p 18). The relative abundance of Gram-positive Bacillota was higher in NaClO-treated groups than in the non-treated group (appendix p 18), suggesting that chemical stress might potentially influence the recipient community and subsequent uptake of pX3_NDM-5.

Compared with the control group, a decreased transconjugal phylogenetic breadth was observed in both NaClO-treated groups (appendix pp 19–21)—in particular the group exposed to 100 mg/L NaClO, in which the OTU diversity markedly decreased (from 231 to 92 OTUs), although the phylogenetic groups remained similar to those of the control group at the phylum level (appendix pp 19–21). To investigate whether NaClO stress has a modulating role in the permissiveness of OTUs, we analysed the relative changes in permissiveness (Δ) between the stressed and the non-stressed conditions for the top 50 transconjugal OTUs (figure 3A, appendix p 10). Two major bacterial phyla were found in the 50 most abundant OTUs, namely Pseudomonadota (n=20, 40% OTUs) and Bacillota (n=17, 34% OTUs). When challenged with 40 mg/L NaClO, 16 of the 50 OTUs showed a strong increase in bacterial permissiveness (log(Δ)>1; figure 3A)—particularly those belonging to the Lactobacillaceae family, in which permissiveness increased by approximately 500 times. These findings could explain the higher abundance of Bacillota in the stressed transconjugal group than in the control group. By contrast, the permissiveness decreased by between ten and 1000 times for 44 of the 50 OTUs when challenged with 100 mg/L NaClO. Only six OTUs showed a slightly increased permissiveness, with the exception of OTU1—belonging to the Lactobacillaceae family—in which permissiveness increased by approximately 584 times and the uptake of pX3_NDM-5 was highest. We note that 57 OTUs were detected across all three transconjugal groups (figure 3B), indicating that these species might act as a core, super-permissive community. This community mainly consisted of Pseudomonadaceae, Enterobacteriaceae, Lactobacillaceae, Rhizobiaceae, Aeromonadaceae, Streptococcaceae, and Moraxellaceae families (figure 3C).

To further characterise the pX3_NDM-5-positive transconjugants, we isolated the cultivable transconjugants using selective UTI agar plates supplemented with meropenem (1 mg/L). Approximately 10⁴ CFU per mL were isolated using this phenotypic selection method and many transconjugants expressed a green fluorescent signal under ultraviolet light (appendix p 22). The presence of *bla*<sub>NDM-5</sub> and *gfp* was further confirmed in those isolated colonies by PCR and bacterial species were identified using 16S rRNA gene amplicon sequencing. No *bla*<sub>NDM-5</sub> and *gfp* genes were detected in the recipient bacterial communities. 626 isolated transconjugants were identified and a phylogenetic tree was constructed from 16S rRNA gene amplicon sequences (figure 4, appendix p 8). The majority of transconjugants belonged to the genera *Klebsiella* (n=262, 41·5%), and *Pseudomonas* (n=174, 27·8%), followed by *Enterococcus* (n=61, 10·5%), *Enterobacter* (n=53, 8·5%), and *Escherichia* (n=41, 6·5%). The remaining transconjugants belonged to seven low-abundant genera: *Burkholderia* (n=7, 1·1%), *Citrobacter* (n=5, 0·8%), *Pantoea* (n=2, 0·3%), *Lelliottia* (n=2, 0·3%), *Aeromonas* (n=1, 0·2%), *Ligilactobacillus* (n=1, 0·2%),
and Elizabethkingia (n=1, 0.2%; appendix p 9). These data suggest that pX3_NDM-5 has a considerably broader host range than previously reported, spanning three phyla: Pseudomonada (γ and β, n=551, 88.0%), Bacillota (n=67, 10.7%), and the less abundant Bacteroidota (n=1, 0.2%; figure 4, appendix p 9).
We next assessed the persistence of pX3_NDM-5 in 35 of the newly formed transconjugants. The selected transconjugants were serially passaged in meropenem-containing (1 mg/L) or meropenem-free LB, and the presence of \textit{blaNDM-5} was evaluated by PCR. The persistence of pX3_NDM-5 varied in different hosts, although a marked decline was seen under antibiotic-free conditions (figure 5A). Notably, pX3_NDM-5 was stable for up to 15 days in three \textit{Enterococcus faecalis} transconjugants, whereas it was relatively unstable in the other tested \textit{E faecalis} transconjugants and was lost within 5 days (appendix p 10).

We also examined the ability of pX3_NDM-5 within the transconjugants to transfer back to Gram-negative bacteria. 40 isolated NDM-5-positive transconjugants, representative of the phylogenetic diversity and spanning six genera (figure 5B, C), were selected as donor strains with \textit{E coli} C600 as the recipient strain. 35 of the 40 transconjugants introduced the pX3_NDM-5 plasmid into \textit{E coli} C600, with transfer frequencies ranging from $10^{-4}$ to $10^{-9}$. The highest
Figure 4: High phylogenetic diversity of cultivable pX3_NDM-5-positive transconjugants

Phylogenetic tree of 16S rRNA gene amplicon sequences from 620 isolated transconjugants (left), with an expansion of the region containing the 67 sequences from the Gram-positive bacterial phylum Bacillota (right). Different bacterial phyla are indicated by distinct colours. Triangle nodes represent the collapsed clades with average branch length distance of less than 0.2. The number of collapsed strains is indicated in parentheses.
transfer frequency was observed for *Pseudomonas* (mean $6.34 \times 10^{-6}$ [SD $2.18 \times 10^{-5}$]), followed by *Burkholderia* spp, *Enterobacter* spp, and *Enterococcus* spp ($3.58 \times 10^{-8}$ [5.01 $\times 10^{-8}$]).

**Discussion**

The dynamics of plasmids and mobile genetic elements have an important role in the spread of antimicrobial-resistance genes throughout bacterial populations, which subsequently affects the incidence of antibiotic-resistant infections on a local, regional, and global level. In this study, we chose NDM-5 as a marker of emerging carbapenem resistance. Although typically associated with the plasmid IncX3, the association of *bla*$_{\text{NDM-5}}$ with other plasmid types has been reported. Most antimicrobial-resistance genes, including *bla*$_{\text{NDM-5}}$, are thought to exist on one side of a rarely breached Gram-negative–Gram-positive divide, contributing to the well established idea that Gram-negative and Gram-positive bacteria rarely exchange genetic material. One observational study has described the identification of *bla*$_{\text{KPC-2}}$ in *Enterococcus* spp from a hospital wastewater treatment plant, although no

![Graph](image-url)

**Figure 5**: Persistence of pX3_NDM-5 in transconjugants and transferability of pX3_NDM-5 back to *Escherichia coli*

(A) The persistence of pX3_NDM-5 in a set of 35 isolated transconjugants of different bacterial genera, maintained in the presence or absence of meropenem. Comparisons were measured by an unpaired t test. (B, C) 40 sorted transconjugants were selected as donor strains to assess their ability to transfer the plasmid pX3_NDM-5 into the recipient strain *E coli* C600: *Enterococcus faecalis* 7-19, *Enterococcus* spp 5, *Enterococcus* spp 4, *Enterobacter* huaxiensis 1-22, and *E faecalis* 7-5. T=transconjugant. D=donor.
additional information was given beyond the presence of the gene.

We show that the plasmid pX3_NDM-5 has built conjugative bridges between an E coli donor strain and organisms from hospital wastewater treatment plants in the presence and absence of chlorinated disinfectant as an environmental stressor. The transconjugal pools contained numerous OTUs belonging to 16 different phyla, challenging our understanding of the host range of IncX3 plasmids, which are classified as having an intermediate host range and were thought to be found typically within the Enterobacteriaceae family.23-26

Three key implications arise from these in-situ pX3_NDM-5 conjugation events. First, high conjugation rates (2.34–4.87%) of pX3_NDM-5 could compensate for the cost of plasmid carriage, in line with studies indicating that sufficient transfer rates can overcome the detrimental effect of plasmid fitness cost and enhance plasmid persistence.23,24 Second, our culture-independent transfer model reveals the high incidence of plasmid transfer to poorly cultivable or rare bacteria, including from the phyla Planctomycetota, Verrucomicrobiota, and Candidatus Parcubacteria. Such bacteria, which might typically be overlooked or disregarded, should be considered as potential reservoirs of pX3_NDM-5. These findings question the veracity of culture-based conjugation studies and our understanding of plasmid dynamics, which seem to have higher plasticity than previously considered. Although other studies have used similar technologies to track IncP plasmid transfer, we show—for the first time to our knowledge—the natural transfer of a plasmid conferring carbapenem resistance from an E coli donor to Enterococcus spp, in which the plasmid remains stable such that it can transfer back to E coli recipients. Our results indicate that the blaNDM-positive transconjugants could serve as natural shuttle vectors and build conjugative bridges between a wide range of recipients. These findings have considerable implications when considering the spread of antimicrobial resistance across One Health sectors as the majority of bacteria—particularly in the environment—are unculturable and we have very little understanding of how they act as reservoirs, potentially harbouring plasmids carrying multidrug-resistant genes. Here we used IncX3 with blaNDM, however, many plasmid Inc families could also be widely disseminated among these unculturable bacteria populations.

We further show that the chlorine disinfectant NaClO can modulate pX3_NDM-5 conjugation events. Our experimental conditions simulate the use of chlorine disinfectants in hospital wastewater treatment. The use of NaClO disinfectants was particularly widespread during the COVID-19 pandemic—for example, China dispensed at least 2000 tonnes of disinfectants in Wuhan city alone in March, 2020, resulting in high concentrations of free chlorine in hospital wastewater. Increasing evidence argues that chlorine disinfectants facilitate the spread of antimicrobial resistance; for example, bacteria treated with such disinfectants were found to be more likely than untreated bacteria to acquire resistant plasmids.23 Our results indicate that the variation in pX3_NDM-5 acquisition by different bacteria can be modulated by NaClO, with surprisingly high acquisition found in Bacillota, a phylum of Gram-positive bacteria, in the presence of NaClO.

We note some limitations of this study. First, we used a single donor strain with a specific plasmid, and therefore cannot extrapolate our data for all donor and plasmid combinations; however, using our culture-independent method, other combinations can be readily examined. Second, conjugation experiments were conducted under aerobic conditions, and repeating them under microaerophilic or anaerobic conditions could yield different conjugation results. Third, although we examined the effect of environmental stress on the permissiveness of pX3_NDM-5 in bacterial communities, the fitness cost of plasmid acquisition was not measured, and the genomic stability of the resistance region in serially passaged transconjugants merits further research. Finally, although the concentrations of and exposure times to NaClO were chosen to mimic common scenarios, these will vary between hospitals, regions, and countries.

In the past decade, antimicrobial resistance surveillance has undergone a substantial shift towards a One Health approach, incorporating human health, animal health, and environmental sampling. However, despite these impressive aspirations, conflating these complex sectors to show links and cause-and-effect relationships is challenging. Environmental samples, in particular, differ in terms of nutrient composition, temperature, precipitation, and propensity for flooding, among other factors. Our study, in which hospital waste was used to track the dynamics of carbapenem resistance, suggests that our current understanding of antimicrobial resistance population dynamics is limited by using only culture-based bacterial-conjugation experiments. Beyond increasing our knowledge of pX3_NDM-5 as a model plasmid, our methods can be applied to other clinically important resistance markers and their carrier plasmids. Furthermore, our data enhance the importance of a One Health approach to antimicrobial resistance surveillance, and suggest that what we are currently witnessing in terms of antimicrobial resistance plasmid dynamics could be only a small part of a much larger story.

Contributors

QFY and TRW conceived the project and designed the experiments. SZ contributed to the concept of the study. QFY, XM, LZ, MH, MZ, ML, and MW conducted the experiments. QW constructed the gfp-tagged plasmid and JSM contributed to the preparation of the plasmid construct. CL and LT contributed to the 16s rRNA gene sequencing analysis. DH and HL contributed to discussion and coordination to improve experimental assays. QFY and TRW wrote and revised the manuscript. QFY and XM accessed and verified all the data. All authors reviewed the manuscript, had access to the data reported in this study, and accept the responsibility to submit for publication.

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Declaration of interests
We declare no competing interests.

Data sharing
The data that support the findings of this study are provided within the Article and its appendix. 16s rRNA gene amplicon sequencing data (BioProject PRJNA68117; accession numbers SAMN30225285, SAMN30225273, and SAMN3022998) and the whole-genome sequences of nine isolated transconjugants (BioProject PRJNA905938; accession numbers SAMN3230346–SAMN3230349 and SAMN31888289–SAMN31888289) are available from the Sequence Read Archive of the National Center for Biotechnology Information.

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