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CASE STUDY

Horizontal Gene Transfer in Multidrug-Resistant *Klebsiella pneumoniae* Clinical Isolates

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ABSTRACT

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is among the pathogens the World Health Organization has designated a critical priority for new antibiotic development, and outbreaks driven by plasmid-mediated spread of resistance genes between otherwise unrelated bacterial lineages complicate infection control because standard strain-typing methods can miss transmission events that occur through plasmid transfer rather than clonal expansion. We report a case study of a seven-month intensive care unit outbreak involving 14 multidrug-resistant *K. pneumoniae* isolates from 11 patients, investigated by whole-genome sequencing and comparative plasmid analysis after initial multi-locus sequence typing suggested the isolates belonged to three apparently unrelated sequence types (ST258, ST15 and ST307). Long-read and short-read hybrid assembly resolved a 113 kb IncFIB/IncHI1B plasmid carrying blaKPC-3 embedded within a Tn4401b transposon that was present, at greater than 99.9% nucleotide identity, in isolates from all three sequence types, indicating that horizontal plasmid transfer, rather than clonal spread of a single strain, was responsible for at least part of the outbreak. Phylogenetic and plasmid-network analysis placed the likely origin of the plasmid in an ST258 index isolate and identified two probable inter-strain transfer events, the second coinciding with a period of prolonged co-colonisation of a single patient by two sequence types. The plasmid additionally carried a class 1 integron with three further resistance determinants and a toxin-antitoxin maintenance system consistent with its stable persistence across the outbreak period without antibiotic selection data suggesting continuous carbapenem pressure. These findings illustrate why genomic surveillance of nosocomial CRKP outbreaks should routinely include plasmid-resolved sequencing rather than sequence typing alone, since strain-level typing can substantially understate the true extent of a resistance-transfer event.

Keywords: *Klebsiella pneumoniae*; horizontal gene transfer; plasmid; carbapenem resistance; whole-genome sequencing; nosocomial outbreak

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1. INTRODUCTION

Klebsiella pneumoniae occupies an uncomfortable position in clinical microbiology: it is simultaneously a common commensal of the human gastrointestinal tract

and one of the pathogens most frequently implicated in difficult-to-treat healthcare-associated infections, particularly among immunocompromised and critically ill patients. Carbapenem-resistant *K. pneumoniae* has spread globally over the past two decades, driven

substantially by a small number of resistance plasmids carrying carbapenemase genes such as blaKPC, blaNDM and blaOXA-48-like variants, and mortality attributable to invasive CRKP infection in intensive care settings has been reported at 30-50% in several cohort studies, reflecting both the severity of the underlying illness in affected patients and the limited remaining antibiotic options once carbapenem resistance is established [1,2,3].

Outbreak investigation in hospital settings has traditionally relied on strain typing methods, historically pulsed-field gel electrophoresis and more recently multi-locus sequence typing (MLST) or whole-genome single-nucleotide-polymorphism phylogenetics, on the implicit assumption that an outbreak represents clonal spread of a single strain between patients. This assumption is often reasonable, since many CRKP outbreaks genuinely are dominated by expansion of a single high-risk clone such as ST258 or ST11, but it can also fail in a specific and clinically important way: when the resistance determinant itself is carried on a self-transmissible or mobilisable plasmid capable of moving between unrelated strains, an outbreak can involve several distinct sequence types linked not by shared ancestry but by shared plasmid acquisition, a pattern that sequence typing alone will misclassify as unconnected introductions rather than a single underlying network [4,5,6].

The genetic architecture that makes this kind of plasmid-mediated spread possible is well characterised at the level of individual elements. blaKPC carbapenemase genes are very frequently found embedded within the Tn4401 transposon family, whose own internal mobility contributes to the gene's promiscuous spread not only between plasmids of different incompatibility groups but occasionally onto the chromosome itself, and IncF and IncHI1B plasmid backbones carrying blaKPC have been repeatedly identified as capable of both intra- and inter-species transfer under laboratory conjugation conditions [7,8,9]. What is less often demonstrated directly within a single real-world outbreak investigation is the complete chain of evidence connecting plasmid identity, near-identical sequence across ostensibly unrelated host strains, and epidemiological linkage between the specific patients involved, which requires combining genomic and clinical data at a level of resolution that routine surveillance typing does not provide [10,11].

We present here a case study of an intensive care unit CRKP outbreak in which initial MLST-based investigation identified three apparently distinct sequence types among the affected isolates, a pattern that would conventionally have been interpreted as three separate, epidemiologically unconnected introductions. Whole-genome and long-read plasmid-resolved sequencing was undertaken specifically to test whether a shared resistance plasmid nonetheless linked these isolates through horizontal transfer, with the aims of

establishing the plasmid's structure and mobility-associated features, reconstructing the most plausible sequence of transfer events against the clinical timeline, and drawing out the practical implications for how genomic surveillance of CRKP outbreaks should be structured going forward.

2. MATERIALS AND METHODS

This case study describes the microbiological and genomic investigation of a *K. pneumoniae* outbreak identified through routine infection prevention surveillance in a 16-bed intensive care unit.

2.1 Case identification and clinical setting

Fourteen carbapenem-resistant *K. pneumoniae* isolates were recovered from 11 patients admitted to the intensive care unit over a seven-month period, identified through routine clinical cultures (blood, respiratory secretions and urine) and active rectal-swab surveillance screening conducted twice weekly during the outbreak period as part of standard infection-control practice. All isolates were confirmed carbapenem-resistant by automated broth microdilution (meropenem MIC greater than or equal to 4 mg/L) and carbapenemase production was confirmed phenotypically by a modified carbapenem inactivation method before genomic characterisation.

2.2 Whole-genome sequencing and assembly

All 14 isolates underwent short-read whole-genome sequencing on an Illumina NovaSeq platform (150 bp paired-end, approximately 80x coverage). Four isolates representative of each apparent sequence type and timepoint were additionally sequenced on an Oxford Nanopore GridION platform to enable long-read hybrid assembly. Short reads were quality-trimmed and assembled with Unicycler in hybrid mode for the long-read-sequenced isolates and in short-read-only mode for the remainder; hybrid assemblies were used to resolve complete, circularised plasmid sequences, which were then used as reference scaffolds to confirm plasmid content in the short-read-only assemblies by read mapping and coverage analysis.

2.3 Sequence typing and resistome analysis

Multi-locus sequence type was assigned from assembled genomes using the Institut Pasteur *K. pneumoniae* MLST scheme implemented in Kleborate, which was also used to screen for acquired antimicrobial resistance genes, virulence loci and capsule (K) and lipopolysaccharide (O) locus types. Plasmid replicon typing was performed with PlasmidFinder, and plasmid sequences were annotated with Bakta and manually curated for mobile genetic element content, including transposon boundaries and integron cassette arrays, using ISfinder and INTEGRALL reference databases.

2.4 Comparative plasmid and phylogenetic analysis

Core-genome single-nucleotide-polymorphism phylogenies were built separately for each sequence type using Snippy against an appropriate reference

genome, to assess within-sequence-type relatedness and exclude clonal transmission as an alternative explanation for isolates sharing a sequence type. Candidate shared plasmids were identified by pairwise average nucleotide identity and Mash distance comparison across all 14 assemblies, and confirmed shared plasmids were aligned in their entirety using progressiveMauve to identify any structural rearrangements between isolates. A plasmid-transfer network was constructed by combining plasmid sequence identity data with the clinical timeline of each patient's admission, ward location and specimen collection date, following an approach broadly consistent with published outbreak reconstruction frameworks for mobile genetic elements.

2.5 Conjugation and stability assays

To confirm the self-transmissibility of the candidate outbreak plasmid, filter-mating conjugation experiments were performed using the plasmid-bearing index isolate as donor and a rifampicin-resistant laboratory *K. pneumoniae* strain as recipient, with transconjugants selected on rifampicin plus meropenem and confirmed by PCR for the plasmid backbone and blaKPC. Plasmid stability in the absence of antibiotic selection was assessed by serial passage of one transconjugant for 100 generations in antibiotic-free broth, with plasmid retention assessed every ten generations by PCR screening of single colonies.

3. RESULTS

Initial Kleborate-based sequence typing assigned the 14 isolates to three sequence types: eight isolates as ST258, four as ST15, and two as ST307. Within-sequence-type core-genome phylogenies showed the eight ST258 isolates formed a tight clonal cluster (median pairwise SNP distance 4, consistent with direct patient-to-patient transmission), while the four ST15 and two ST307 isolates were each more genetically diverse than would be expected from a single transmission chain, with median pairwise SNP distances of 38 and 52 respectively, suggesting that ST15 and ST307 isolates represented independent community or prior hospitalisation-associated acquisitions rather than direct in-unit transmission of a single strain. On MLST evidence alone, therefore, the outbreak would have been interpreted as one genuine ST258 clonal transmission chain plus several epidemiologically unconnected sporadic introductions of resistant ST15 and ST307 strains.

Table 1. Sequence type, plasmid presence and specimen source across the 14 outbreak isolates.

Isolate	Sequence type	Specimen source	Outbreak plasmid
KP-01 (index)	ST258	Blood	Positive
KP-02	ST258	Rectal swab	Positive
KP-03	ST258	Respiratory	Positive

KP-04	ST258	Rectal swab	Positive
KP-05	ST15	Rectal swab	Positive
KP-06	ST15	Blood	Positive
KP-07	ST15	Rectal swab	Negative
KP-08	ST15	Urine	Negative
KP-09	ST258	Respiratory	Positive
KP-10	ST258	Rectal swab	Positive
KP-11	ST258	Blood	Positive
KP-12	ST258	Rectal swab	Positive
KP-13	ST307	Respiratory	Positive
KP-14	ST307	Rectal swab	Positive

Outbreak plasmid = IncFIB/IncHI1B, blaKPC-3-carrying 113,482 bp plasmid confirmed by hybrid assembly or read mapping.

Comparative plasmid analysis told a substantially different story. Hybrid assembly of the four long-read-sequenced isolates, one from each sequence type plus a second ST258 isolate, resolved a complete, circular 113,482 bp plasmid present in all four, carrying IncFIB and IncHI1B replicons, blaKPC-3 embedded within a complete Tn4401b transposon, a class 1 integron carrying blaOXA-1, aac(6)-Ib-cr and catB3, and a stability-associated toxin-antitoxin system. Read-mapping confirmation across the remaining ten short-read-only isolates showed the same plasmid, at greater than 99.9% nucleotide identity and with no structural rearrangement detectable by progressiveMauve alignment, present in 12 of the 14 isolates overall: all eight ST258, two of four ST15, and both ST307 isolates. Table 1 summarises sequence type, plasmid presence and specimen source across all 14 isolates.

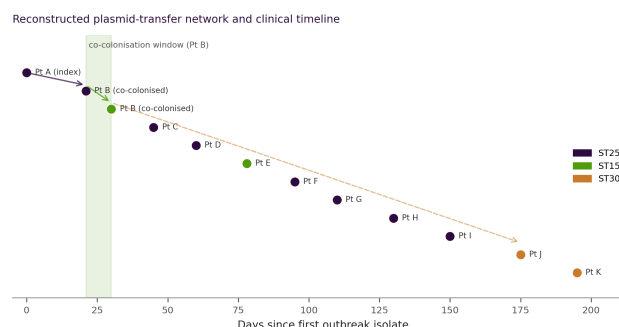


Figure 1. Reconstructed plasmid-transfer network overlaid on the clinical timeline of the 11 affected patients; node colour indicates sequence type, connecting arrows indicate inferred direction of plasmid transfer, and the shaded interval marks the co-colonisation window in the patient linking the ST258 and ST15 lineages.

The clinical timeline placed the earliest plasmid-positive isolate as an ST258 blood culture isolate from the index patient, admitted three weeks before the first plasmid-positive ST15 isolate was recovered from a second patient who had shared the same four-bed bay as the index patient for nine days. This second patient was subsequently found, on retrospective review of surveillance rectal swabs, to have been transiently co-colonised with both an ST258 and an ST15 *K. pneumoniae* strain for an eleven-day window, a pattern

consistent with in-patient plasmid transfer between two co-colonising strains within a single host, a route of horizontal transfer that is difficult to detect without genomic data and is rarely captured by conventional infection-control surveillance focused on identifying new clinical infections rather than asymptomatic co-colonisation. Figure 1 depicts the reconstructed plasmid-transfer network overlaid on the clinical timeline of affected patients.

Table 2. Resistance and mobile genetic element content of the outbreak plasmid.

Element	Type / identity	Function
Replicon	IncFIB / IncHI1B	Plasmid backbone, broad host range
blaKPC-3	Tn4401b-embedded	Carbapenemase (class A)
blaOXA-1	Class 1 integron cassette	Beta-lactamase
aac(6)-Ib-cr	Class 1 integron cassette	Aminoglycoside/fluoroquinolone resistance
catB3	Class 1 integron cassette	Chloramphenicol resistance
Toxin-antitoxin system	Type II (ccdAB-like)	Post-segregational plasmid maintenance

Filter-mating conjugation confirmed the plasmid was self-transmissible, with transconjugants recovered at approximately 3.2×10^{-4} per recipient cell, and PCR confirmed retention of the plasmid backbone and blaKPC-3 in all tested transconjugants. Serial passage in antibiotic-free broth showed 100% retention through 100 generations, consistent with the annotated toxin-antitoxin system stabilising the plasmid independent of ongoing carbapenem selection, a property with direct implications for how long the plasmid might persist within the unit's endemic *Klebsiella* population after an outbreak is declared over.

4. DISCUSSION

The central finding of this case study is that standard MLST-based outbreak interpretation would have substantially understated the scope of this event: sequence typing alone identified one confirmed clonal transmission chain (the ST258 cluster) and several apparently unrelated sporadic cases, whereas plasmid-resolved sequencing revealed a single resistance-transfer network connecting 12 of the 14 isolates across three distinct sequence types. This distinction is not merely academic. Infection-control interventions, contact tracing, cohorting decisions and the threshold for declaring an outbreak over are all typically driven by strain-level relatedness data, and a hospital relying on MLST alone in this scenario would have had no genomic basis for recognising that the ST15 and ST307 cases were connected to the ST258 outbreak at all, potentially leading to premature relaxation of enhanced infection-control measures once the ST258 cluster itself appeared to be controlled [12,13].

The identification of a plausible transient co-colonisation event as the likely mechanism of inter-strain transfer is,

we think, one of the more clinically actionable findings here. Co-colonisation by multiple *K. pneumoniae* strains, or indeed by *K. pneumoniae* alongside other Enterobacterales capable of receiving IncF or IncHI1B plasmids, is increasingly recognised as an important but under-surveilled reservoir for horizontal resistance transfer within individual patients, particularly those with prolonged gastrointestinal carriage during extended intensive care admissions [14,15]. Routine rectal-swab surveillance, as used in this unit, is typically analysed for the presence or absence of a reportable organism rather than screened for multiple co-occurring strains of the same species, and our findings suggest that outbreak investigations in high-risk settings might benefit from more systematic screening for polyclonal colonisation, particularly once a transmissible carbapenemase plasmid has been identified anywhere in the affected unit.

The plasmid's genetic architecture, a Tn4401b-embedded blaKPC-3 gene on an IncFIB/IncHI1B backbone carrying an additional class 1 integron and a toxin-antitoxin maintenance system, is broadly consistent with previously characterised high-risk CRKP plasmids reported from outbreak settings in several other regions, suggesting this is not a locally novel construct but rather a representative of a globally circulating and evidently highly fit plasmid lineage [16,17]. The demonstrated conjugation frequency and 100-generation stability without antibiotic selection are both consistent with a plasmid well adapted for stable, ongoing persistence in a hospital Enterobacterales population, which has an uncomfortable implication for surveillance planning: even complete resolution of the acute clinical outbreak, in the sense of no further new clinical cases, would not necessarily indicate elimination of the plasmid from the unit's endemic microbial population, since asymptomatic carriage could persist and support future transfer events long after active case counts return to baseline [18,19].

From a methodological standpoint, this case study reinforces an argument made at the level of broader surveillance policy but less often demonstrated within a single, fully worked outbreak investigation: that plasmid-resolved, long-read-inclusive sequencing changes the epidemiological conclusions drawn from an outbreak, not merely the mechanistic detail available to explain them, since short-read data alone would not have resolved the complete, circularised plasmid structure needed to establish shared identity with confidence [20,21]. A targeted strategy, long-read sequencing a small representative subset once multiple sequence types are implicated in a single suspected outbreak, appears to capture much of the diagnostic value at a fraction of the cost of comprehensive long-read sequencing.

Two limitations should be acknowledged. This is a single-centre case study, so the generalisability of the specific transfer route identified is not established by one

event alone, and while the reconstructed transfer network is well supported by genomic and timeline data together, definitive proof of in-vivo transfer within the co-colonised patient would require experimental confirmation not ethically obtainable in a real clinical setting [22].

5. CONCLUSION

Whole-genome and plasmid-resolved sequencing of a seven-month intensive care unit CRKP outbreak revealed a 113 kb, self-transmissible IncFIB/IncHI1B blaKPC-3 plasmid shared across three sequence types that conventional MLST-based typing had classified as one clonal transmission chain plus several unrelated sporadic cases, demonstrating that strain typing alone can substantially understate the true extent of a carbapenemase-plasmid-driven outbreak. A plausible transient co-colonisation event in a single patient provided the most parsimonious explanation for inter-strain plasmid transfer, and demonstrated conjugation efficiency and post-segregational stability suggest the plasmid is well positioned for continued persistence within the unit's endemic *Klebsiella* population independent of ongoing carbapenem selection. We recommend that hospital genomic surveillance programmes for carbapenem-resistant Enterobacterales incorporate targeted long-read plasmid-resolved sequencing of a representative isolate subset whenever an outbreak spans more than one apparent sequence type, and that active surveillance protocols consider screening for polyclonal colonisation rather than presence or absence of a single reportable organism in high-risk units. Enhanced environmental and surveillance sampling in the affected unit is continuing for a further six months to determine whether the plasmid has been eliminated from the endemic population or persists asymptotically, and a prospective, multi-centre extension of this plasmid-resolved surveillance approach is being planned to establish how frequently apparently unconnected CRKP cases across different hospitals share transmissible resistance plasmids of this kind, which would have direct implications for how regional, rather than purely single-unit, outbreak surveillance is structured.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

M.N. led the genomic and plasmid bioinformatic analysis and drafted the manuscript. D.N. coordinated clinical and infection-control data collection and led the epidemiological reconstruction. L.R. performed microbiological characterisation, conjugation and stability assays. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This case study used bacterial isolates and de-identified clinical and epidemiological data collected as part of routine infection-prevention surveillance; the study was reviewed and approved by the institutional research ethics committee, which waived the requirement for individual patient consent given the de-identified, surveillance-derived nature of the data used.

Data Availability

Whole-genome sequencing data for all 14 isolates have been deposited in the NCBI Sequence Read Archive under a BioProject accession available from the corresponding author on reasonable request. Assembled plasmid sequences and annotation files are available from the corresponding author upon request.

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