

1 Genomic epidemiology and longitudinal sampling of ward 2 wastewater environments and patients reveals complexity 3 of the transmission dynamics of *bla*_{KPC}-carbapenemase- 4 producing Enterobacterales in a hospital setting

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31 1.4 Keywords

32 Carbapenemase-producing Enterobacterales, genomic epidemiology, bla_{KPC}, wastewater sites,
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35 Sequence data have been deposited in NCBI's GenBank (BioProject accessions: PRJNA768622 and
36 PRJNA514245).

37 2. Abstract

38 Healthcare-associated wastewater reservoirs and asymptomatic gastrointestinal patient colonisation
39 by carbapenemase-producing Enterobacterales (CPE) contribute to nosocomial CPE dissemination.
40 We systematically sampled wastewater sites (n=4488 sampling events; 349 sites) and patients
41 (n=1247) across six wards over 6-12 months in 2016 to better understand bla_{KPC}-associated CPE
42 (KPC-E) diversity within these niches and transmission potential in an endemic healthcare setting. Up
43 to five isolates in KPC-E-positive samples were sequenced (Illumina). Recombination-adjusted
44 phylogenies were used to define genetically related strains; assembly and mapping-based typing
45 approaches were used to characterise antimicrobial resistance genes, insertion sequences, and
46 Tn4401 types/target site sequences. The wider accessory genome was evaluated in a subset of the
47 largest clusters, and those crossing niches.

48

49 Wastewater site KPC-E-positivity was substantial (101/349 sites [28.9%] positive); 228/5,601 (4.1%)
50 patients cultured were CPE culture-positive over the same timeframe. At a genomic-level, 13 KPC-E
51 species and 109 strains were identified, and 24% of wastewater and 26% of patient KPC-E-positive
52 samples harboured ≥1 strain. Most diversity was explained by the individual niche, suggesting
53 localised factors are important in selection and spread. Tn4401+target site sequence diversity was
54 greater in wastewater sites (p<0.001), which might favour Tn4401-associated
55 transposition/evolution. Shower/bath and sluice/mop-associated sites were more likely to be KPC-E-
56 positive (Adjusted Odds Ratio [95% CI]: 2.69 [1.44-5.01], p=0.0019 and 2.60 [1.04-6.52], p=0.0410,
57 respectively). Different strains had different transmission and bla_{KPC} dissemination dynamics.

58

59 There may be substantial KPC-E colonisation of wastewater sites and patients in KPC-E-endemic
60 healthcare settings. Niche-specific factors (e.g. microbial interactions, selection pressure) likely
61 affect carbapenemase gene persistence and evolution, and different strains and mobile genetic
62 elements with different transmission dynamics influence carbapenemase gene dissemination; these
63 factors should be considered in surveillance and control strategies.

64 3. Impact statement

65 Antibiotic resistance is a major healthcare problem, and common infection-causing bacteria known
66 as Enterobacterales have developed resistance to one of the “last-line” antibiotic classes used for
67 treatment, namely carbapenems. Carbapenem resistance in Enterobacterales is largely caused by
68 the presence of carbapenemase enzymes, such as “KPC” (*Klebsiella pneumoniae* carbapenemase),
69 which has caused outbreaks of antibiotic-resistant healthcare-associated infection in the USA and
70 UK. Many infection-associated Enterobacterales species can also live asymptotically in the human
71 gut and environmental niches, such as hospital wastewater sites, which act as potential reservoirs
72 for the exchange of the gene encoding for KPC (*bla_{KPC}*). Diversity within and transmission between
73 these reservoirs remains poorly characterised. We therefore systematically and densely sampled
74 humans and wastewater sites (e.g. sink/shower drains, toilets etc.) on six wards in 2016-2017 and
75 sequenced 1646 isolates in a prospective study in a hospital affected by outbreaks of KPC-
76 Enterobacterales-associated infections. We found that 1 in 20 patients and nearly a third of all
77 wastewater sites were colonised with KPC-Enterobacterales. Samples frequently contained multiple
78 different KPC-positive species or strains at any given time, and different transmission patterns were
79 evident. This study has major implications for surveillance of carbapenem-resistant Enterobacterales
80 in hospitals, and for designing strategies to mitigate their spread.

81 4. Data summary

82 The authors confirm all sequencing data and available metadata has been provided in NCBI
83 BioProjects PRJNA768622 and PRJNA514245 and in supplementary dataset 2.

85 5. Introduction

86 Carbapenemase-producing Enterobacterales (CPE) are a global health threat¹, and commonly
87 associated with multi-drug resistance, rendering treatment of CPE infections difficult. Major
88 transmissible global carbapenemases include the metallo-beta-lactamases (*bla_{NDM}*, *bla_{VIM}*, *bla_{IMP}*),
89 some oxacillinases (*bla_{OXA-48/48-like}* variants), and the *Klebsiella pneumoniae* carbapenemase (KPC,
90 encoded by *bla_{KPC}*)². Intra- and inter-species horizontal transfer of these genes is facilitated by
91 mobile genetic elements such as transposons and plasmids, resulting in rapid dissemination of
92 carbapenem resistance^{3, 4}.

94 CPE have been isolated from human and animal gastrointestinal tracts, and from sewage, rivers and
95 sink drains⁵⁻⁷. Recent studies have highlighted that hospital wastewater sites may act as a CPE
96 reservoir⁵, but the diversity within these niches, genetic overlap with patient isolates and likely
97 directionality and rates of transmission remain to be elucidated. Studies evaluating the contribution
98 of patient-to-patient transmission as the sole explanatory factor explaining transmission have
99 generally been unable to robustly explain the majority of transmission events, suggesting that
100 patient, staff and/or environmental reservoirs remain insufficiently sampled to account for the

diversity observed⁸. Few studies have considered within-niche diversity of strains by sampling multiple isolates per individual and/or site⁹⁻¹¹.

The Manchester University NHS Foundation Trust (MFT; Manchester, UK), has experienced on-going transmission and patient acquisition of multi-species CPE, predominantly bla_{KPC}-positive Enterobacterales (KPC-E), since 2009. Following a bla_{KPC-2}-ST216-*Escherichia coli* outbreak on two cardiac wards in the Manchester Royal Infirmary (MRI; part of MFT) and subsequent ward closure and plumbing infrastructure replacement, early environmental sampling after the ward reopened suggested rapid re-colonisation of wastewater sites with KPC-E, likely from transfers of colonised patients to the ward and ongoing evolution of new KPC-E strains in the environment¹². We therefore undertook a prospective study systematically sampling all wastewater sites on six wards for 6-12 months alongside patient rectal screening/clinical sampling, and used an anonymised electronic database to characterise patient admission, ward movement, sampling profiles and culture results. Multiple colonies were isolated from KPC-E-positive samples to define within-niche diversity, and whole genome sequencing (Illumina) of individual isolates was used to characterise this diversity and consider modes of evolution and transmission.

6. Methods

Study setting and epidemiological data

MFT is a large, regional referral centre in northwest England, UK, managing >10,000 patients/year. In response to the regional emergence of KPC-E¹³, the Trust implemented an extensive Infection Prevention and Control (IPC) programme, consistent with UK CPE guidelines (published in 2014)¹⁴. Despite this, in April 2015 there was a large bla_{KPC}-*E. coli* outbreak in the cardiac unit¹², leading to its closure (September 2015-January 2016) and complete refurbishment, including plumbing replacement. Subsequently systematic, prospective wastewater site sampling was undertaken (described further below).

To evaluate patient-level microbiological and admissions data, MFT electronic bacteriology records were linked, based on NHS numbers, to patient administration data, and anonymised. For this study, we analysed anonymised patient data from 1st January 2016-31st December 2016 inclusive, and included only those patients with exposure to any one of six wards within the three study units (acute medicine, cardiology, geratology) during that time.

Environmental sampling and laboratory processing

All sink/drain/wastewater sites on six wards in three units were sampled: the cardiac unit (wards 3 [W3] and 4 [W4]); the geratology unit (wards 45 [W45] and 46 [W46]); and the acute medicine unit

(wards AM1 and AM2) (Table S1 shows sampling site designations). All wastewater sites on both W3 and W4 were sampled fortnightly on rotation from 8th January 2016-28th December 2016. W45, W46, AM1, AM2 wastewater sites were similarly sampled fortnightly, but from 18th July 2016-31st December 2016. Wastewater site types are described in the Supplementary methods.

Environmental sampling was carried out by aspirating ~20mls of wastewater from sink P-traps, shower drains or toilets and performing enrichment-based culture (see Supplementary methods). Multiplex, real-time, quantitative PCR (for *bla*_{NDM}, *bla*_{KPC}, *bla*_{OXA-48}¹²) was performed on broths following incubation. 10µl of any *bla*_{KPC}-positive-broth were streaked onto CPE-selective agar plates (Chromid CARBA) and re-incubated (aerobically, 37°C).

Patient sampling and laboratory processing

Routinely collected patient clinical samples for the diagnosis of infection were processed using standard operating procedures in line with UK Standards¹⁵. In addition, a rectal CRE screening programme was in place from 2014 in line with national guidance: This included routine screening of high-risk patients (i.e. previously CRE-positive and/or a history of hospitalization abroad or in UK hospital with a known CRE problem in the last 12 months) on admission and weekly; screening of all patients on wards where CRE-positive patients were identified (either colonised or infected cases); ward closure and twice weekly screening if more than two cases were identified on a ward until no new cases were identified; isolation and/or cohorting of CRE-positive patients. Rectal swabs were screened for CPE using the Cepheid Xpert Carba-R assay or an in-house multiplex PCR (identifying *bla*_{KPC}, *bla*_{NDM}, and *bla*_{OXA-48}). Species identification of isolates was performed by MALDI-ToF (Bruker Biotyper, Bruker MD); antimicrobial susceptibility testing was performed as per EUCAST guidelines¹⁶. Enterobacterales isolates that had resistant or intermediate susceptibility to any carbapenem were classified as a carbapenem-resistant Enterobacterales (CRE).

Analyses of within-niche diversity

To evaluate within-niche diversity in the environment and patients, up to five different CRE colonies were individually sub-cultured; *bla*_{KPC}-positivity in sub-cultures was re-confirmed by PCR and species identification performed by MALDI-ToF prior to storage in nutrient broth at -80°C.

DNA extraction and isolate sequencing

DNA was extracted from frozen sub-culture stocks with the QuickGene kit, as per the manufacturer's instructions, with an additional mechanical lysis step post-chemical lysis (6m/s at 40secs x2 FastPrep, MPBio). Isolates were sequenced on the Illumina HiSeq 4000 generating 150bp paired-end reads.

172

173 *Sequence data processing and analysis*

174 Sequencing reads were initially processed using an in-house pipeline¹⁷ (details in Supplementary
175 methods); species identification was performed with Kraken2 (default settings)¹⁸. If at least 50
176 sequenced isolates were of a single species, reads were mapped to species-specific references.
177 Variant calling and filtering was performed, and consensus fasta sequences of variant calls with
178 respect to the reference generated. Recombination-adjusted phylogenies by species were created
179 from core chromosomal single nucleotide variants (SNVs; padded to the length of the reference
180 genome prior to analysis) using IQtree¹⁹ (flags: -m GTR+G -blmin 0.00000001 -t PARS) and then
181 ClonalFrameML²⁰ (default parameters). A distance of ≤ 400 SNVs was used as a threshold to define
182 clusters based on previous work (threshold used similar to²¹; distribution of SNV distances within
183 clusters displayed in Fig.S2A-E for the most common species identified); indels were not considered
184 in our evaluation of relatedness. For detailed sub-strain analysis for *K. pneumoniae* strain 9 (see
185 Results), sub-strains were defined on the basis of accessory genome clusters characterised using
186 Panaroo (default parameters including --clean-mode strict²²) and clustered using the R heatmap
187 package.

188

189 Isolates were assembled using SPAdes (v3.6; default parameters)²³. Multi-locus sequence types were
190 derived using BLASTn and species-specific databases (<https://pubmlst.org>; Achtman 7-gene MLST for
191 *E. coli*). AMR genes were identified using ARIBA²⁴ with the CARD database²⁵ and insertion sequences
192 using ISFinder (<https://isfinder.biotoul.fr>). *bla*_{KPC} genes, Tn4401 transposon contexts and the 5-bp
193 target site sequences (TSSs) on either side of the Tn4401 transposon reflecting Tn4401 transposition
194 signatures were characterised using TETyper²⁶, a Tn4401b reference sequence, a 5bp flank length
195 and the default structural/SNV profiles. Arbitrary profile numbers were assigned based on the
196 presence of unique profiles of AMR genes, plasmid replicons, IS sequences (see Supplementary
197 dataset 1 for details of which AMR gene variants constitute a given AMR gene profile, and
198 Supplementary dataset 2 for details on plasmid replicon and IS combinations identified).

199

200 For the analysis of *K. pneumoniae* strain 9 (see Results), putative transmission networks were
201 inferred first using SCOTTI²⁷ (Supplementary methods), and then heuristically, linking isolates
202 identified as clusters on the basis of accessory component profiles and geographic/temporal overlap
203 (Supplementary methods).

204

205 *Statistical analyses and data visualisation*

206 Descriptive statistics were calculated in R v3.6.2 or STATA v16.1. To conduct permutational analyses
207 of variance, we used the *adonis* function (vegan R package, v2.5-7)²⁸ on a matrix of pairwise Gower
208 distances based on genetic features (i.e. composite of species-strain type, plasmid replicon profile,

AMR gene profile, IS profile, and Tn4401+flanking TSS profiles), which was calculated using the *daisy* function (R cluster package). To evaluate the impact of unit location or environmental site type on the odds of CPE positivity, we used logistic regression with robust standard errors clustered by environmental site sampled (R rms package, v6.2-0)²⁹. Data visualisation was using the ggplot2³⁰ and scatterpie³¹ (v.0.1.5) R packages, and BioRender (www.biorender.com).

Data availability

Sequence data have been deposited in NCBI's GenBank (BioProject accession PRJNA768622 and PRJNA514245; accession numbers and metadata for successfully sequenced isolates are in Supplementary dataset 2). We are not able to share the complete database of patient tests, results and admissions episodes; dates for patient admission/sampling are given as month-year only. Of note, labels represented in the figures/supplementary data are anonymised study-specific labels that are not patient-identifiable.

7. Results

Environmental sampling revealed high prevalence and clustering of wastewater site KPC-E colonisation

349 sites across the six study wards (i.e. three units) were sampled a total of 4,488 times (Fig.1): 101/349 (28.9%) sites and 319/4,488 (7.1%) sampling events were KPC-E-positive, with no difference by unit (Fig.2, $p=0.908$), or over time. Adjusting for environmental site type and unit location, shower/bath drain sites and sluices/sluice sinks/mop sinks were significantly more likely to be positive for KPC-E than others (Adjusted Odds Ratio [AOR] (95% CI): 2.69 (1.44-5.01), $p=0.0019$ and 2.60 (1.04-6.52), $p=0.041$, respectively), and toilet water sampling less likely to be positive (AOR (95% CI): 0.28 (0.10-0.78), $p=0.015$); there was no evidence of a unit-dependent difference (Table 1). 7/349 (2.0%) sites were persistently KPC-E positive (≥ 10 consecutive KPC-E-positive sampling events); interestingly five of these were medicines/treatment room handwash basin drains (of eight medicines/treatment room sinks in total) likely only accessible to healthcare staff (Table S1, Fig.S3).

Approximately one in twenty-five patients was affected by carbapenemase-producing Enterobacterales (CPE), with most cases reflecting rectal colonisation with KPC-producing K. pneumoniae, E. coli or E. cloacae

Of 2,529 Enterobacterales positive-samples (2,622 Enterobacterales isolates, 1,247 patients), 439/2,529 (17.4%) samples cultured at least one carbapenem-resistant Enterobacterales (CRE, $n=478$ isolates in total) (Figs.1, S4). For 344/439 (78.4%) CRE-positive samples on which carbapenemase PCR data were also available, all contained at least one known carbapenemase gene, of which 303/344 (88.0%) were bla_{KPC} (Table S2, CRE therefore henceforth denoted as CPE for

simplification). CPEs were cultured from 228 patients (4.1% of all patients cultured, 18.2% of all patients with an Enterobacterales-positive culture) during the study period (median=1, IQR: 1-2, range: 1-17 CPE isolates/patient).

The majority of CPE-positive isolates came from rectal screens (n=387/478 [81.0%]); others came from non-screening clinical specimens (91/478 [19.0%]) (Table S3). Most CPE-positive patients (178/228 [78%]) were CPE-positive only on rectal screen, but 23/228 (10%) individuals were positive only on clinical culture, and 27 (12%) on both. By routine laboratory species identification methods, >20 species and eight genera were represented amongst CPEs, with *K. pneumoniae*, *E. coli* and *E. cloacae* complex predominating (434/478 [90.8%]). Notably, the proportions of carbapenem-resistant isolates within these top three carbapenem resistance-associated species varied (n=284/593 [48%] vs 106/1,404 [8%] vs 44/110 [40%] respectively; Fisher's exact test, p<0.001, Fig.S4).

The proportion of patient CPE-positive cultures was lower than that of environmental site samples, typically ~3% (Fig. 3, bottom panel). Occasional peaks in prevalence were observed, consistent with outbreaks (e.g. week 31, acute medicine unit). CPE prevalence amongst patient cultures on the refurbished cardiology unit was 0% (97.5% CI: 0-0.02) for the first 10 weeks post-reopening, although two utility room sink drains on were positive for KPC-E a day after W3 reopened to patient admissions (11th January 2016) following closure and complete plumbing replacement over three months; thereafter the unit was rapidly re-colonised (Fig.2).

Structuring of diversity by patient and environmental niche, with most diversity explained by the individual niche

1,646 CPE isolates were successfully sequenced (1,275 environmental, 371 patient isolates; Fig.1, Supplementary dataset 2), of which all contained bla_{KPC-2} as the carbapenem resistance variant, and 13 bla_{KPC-2}-Enterobacterales species were identified across patients and environmental reservoirs. Amongst five species with ≥50 sequenced isolates which were evaluated at the strain-level, 109 unique strains were represented. Of the 109 strains, 61 (56%) were found only in the environment, 27 (24%) only in patients and 21 (19%) in both patients and the environment (Fig.4 for the major species); the median strain cluster size was 5 isolates (range: 1-122), and the median number of unique niches (i.e. unique patients or wastewater sites) affected by a strain was 2 (range: 1-23).

Amongst sequenced isolates, 100 plasmid replicon profiles, 754 IS profiles, 939 AMR gene profiles, and 70 Tn4401/TSS types were identified (Supplementary dataset 2). A much greater proportion of all identified Tn4401/TSS types was observed in the environment versus patients (66/70 [94%] of types versus 12/70 [17%] respectively; Fisher's exact test, p<0.001), and all the isolates with >1 set of

TSSs were seen in environmental sites, consistent with these representing a more favourable niche for Tn4401-associated transposition/evolution and dissemination.

For colonised niches (i.e. patient or environmental), using a metric of genomic diversity based on strain, plasmid replicon profile, AMR gene profile, IS profile, and Tn4401+5bp TSS profiles, most variance was explained by the specific individual site sampled ($R^2=50.5\%$; $p<0.001$), versus small but significant contributions made by niche type (i.e. patient vs environmental niche; $R^2=5.6\%$; $p<0.001$) and unit location ($R^2=2.8\%$; $p<0.001$).

For the fifteen scenarios where exactly the same strain, AMR gene profile, IS profile, plasmid profile and Tn4401/TSS type were seen in patient and environmental niches (Fig.S5), and considering only the event-pairs where the chromosomal SNP distances were within 5 SNPs of each other and there was a change in sampling site, the temporal relationship of these supported: patient-to-environment transmission in 13/29 (45%) events, environment-to-patient transmission in 2/29 (7%) events, environment-to-environment transmission in 1/29 (3%) events, and patient-to-patient transmission in 13/29 (45%) events (test of equality of proportions, $p<0.001$).

Environmental and patient niches harbour diverse KPC-E within single samples and over time

Environmental sites showed evidence of major strain-level diversity: from the 101/349 sites from which bla_{KPC-2}-Enterobacterales isolates were successfully sequenced at any timepoint, 78/319 (24.4%) KPC-E-positive samples had >1 bla_{KPC-2}-Enterobacterales strain, namely 52/319 (16.3%) with two strains, 16/319 (5.0%) with three, 9/319 (2.8%) with four and 1/319 (0.3%) with five strains (Figs.5, S6). Over the study period, colonised environmental sites had a median of 3 (range: 1-17, IQR: 2-5) bla_{KPC-2}-Enterobacterales strains. 49/101 sites (49%) were apparently colonised by a single bla_{KPC-2}-Enterobacterales strain only, of which 38 were at single sampling-points, and 11 sites positive with the same strain at ≥ 2 timepoints.

For patient samples, based on routine laboratory microbiology, evidence of mixed-species KPC-E colonisation/infection was found in 35/439 (8%) samples from 26/228 (11%) patients; these were mostly *E. coli*/*K. pneumoniae* mixtures (n=17 samples; maximum two species identified). To evaluate within-patient diversity with genomic rather than species-level resolution, in a subset of 76 patients, strains were sequenced successfully from at least one CPE-positive sample during the study period; for 97 samples, multiple colonies were picked from single sample sub-cultures. In total 457 sequenced colonies from 97 samples were available for evaluation of within-patient diversity; 25/97 samples (25.7%) had more than one strain per sample, namely 17/97 samples (17.5%) with two bla_{KPC-2}-Enterobacterales strains, 6/97 (6.2%) with three strains and 2/97 (2.0%) with four strains

(Figs.5, S6) - i.e. considerably more diversity identified than based on routine laboratory species identification data (26% vs 8% samples; Fisher's exact test, $p < 0.001$).

For a subset of 14 patients and 53 wastewater sites where within-sample diversity was evaluated by sequencing multiple isolates from individual samples and ≥ 2 longitudinal samples were positive, longitudinal switches in *bla*_{KPC-2}-Enterobacterales species-strain composition was common, occurring in 7/14 (50%) of patients and 41/53 (77%) wastewater sites (Figs.5, S6).

Genomic analysis reveals distinct population biology for different bla_{KPC-2}-Enterobacterales strains

To investigate different modes of *bla*_{KPC-2} dissemination, we characterised in detail several sequenced KPC-E strain clusters, including the largest environmental-only cluster (*E. cloacae* strain 10 [n=122 isolates]), a large cluster involving patients and the environment (*K. pneumoniae* strain 9 [n=106 isolates]), and a cluster in which signatures of Tn4401 transposition appeared highly frequent (*K. pneumoniae* strain 11 [n=96 isolates]).

Scenario 1: Dynamic changes in AMR gene, IS, plasmid replicon and Tn4401 profiles in E. cloacae strain 10 in sink drain sites

E. cloacae strain 10 (eclo10, ST32) appeared a site-restricted but persistent coloniser of three co-located sink drains on a geratology ward (in a drug room clinical handwash basin drain [F45], utility sink drain [F46], and a separate treatment room clinical handwash basin drain [F47]) over ~5 months (18/Jul/2016-20/Dec/2016). At the core chromosomal level, strains appeared largely structured by site, with 7 SNVs separating the majority of isolates in F45 from those in F47, and 14 SNVs those in F46 from F45. Three isolates in F47 and one isolate in F46 clustered with the F45-associated isolates; sampling dates suggest that these represented transmission events from F45 (Fig.6A). There was evidence of rapid churn in AMR gene and IS content (111 and 36 different profiles respectively; Fig.6Bi and 6Bii) in the relatively stable strain background, and almost all isolates in F47 had acquired an additional set of IncHI2/HI2A replicons on a background of FIB/FII, consistent with plasmid gain/loss (Fig.6C). Although the Tn4401 type and flanking sequences were largely identical (Tn4401a-ATTGA-ATTGA in 105/122 (86%) isolates across sinks), there was evidence of unique additional Tn4401 deletions occurring in multiple isolates at several distinct timepoints in the three sink sites (Fig.6).

Scenario 2: Rapid clonal patient-patient transmission of K. pneumoniae strain 9

K. pneumoniae strain 9 (kpne9, ST252) was highly related (Fig.S7; maximum 6 SNVs across strain, 68 isolates with 0 SNVs between them), and spread amongst 21 patients across three wards on two units (acute medicine AM1 and AM2 and geratology W46), with a dense outbreak in Aug 2016, most

consistent, at least initially, with direct/indirect patient-patient transmission (Fig.7). *bla_{KPC}* was consistently nested in Tn4401-ATTGA-ATTGA, except for one isolate which acquired a Tn4401-associated mutation (isolate: 2216698_14, C4620T), and another in which the right target site sequence (TSS) could not be identified (isolate: 2035791_11). Amongst the 106 isolates there was evidence of four transient plasmid replicon acquisitions on a background of the stable presence of a set of IncFIB/IncFII replicons (Fig.S7). Transmission probabilities inferred by SCOTTI were weak (see Supplementary material; Fig.S8), as seen in other studies⁸, but using accessory cluster profiling to further discriminate amongst strains suggested that there were several discrete transmission clusters (Fig.S9), with some individuals involved in several of these (i.e. harbouring multiple distinct sub-lineages simultaneously, Fig.7). A single shower drain site was involved (A13), which was likely a seeded bystander from affected patients, but may have contributed to transmission of sub-strain 3g (Fig.7).

Scenario 3: Cross-unit dissemination with evidence of significant Tn4401-associated *bla_{KPC}* transposition in *K. pneumoniae* strain 11

Given that Tn4401 transposition is associated with *bla_{KPC}* dissemination²¹, we also evaluated whether the number of different Tn4401-TSS types was different across strains, noting that the observed frequency of different Tn4401-TSS combinations was higher in *K. pneumoniae* strain 11 (kpne11, ST11) than in other strains (Fig.S10). On further analysis, Kpne11 represented two sub-clusters, separated by ~40SNVs (Fig.8, designated “cluster 1” and “cluster 2”; Fig.S11), both of which were isolated from patients and wastewater sites across ward settings, including the cardiac unit after plumbing replacement. Within both clusters of isolates there were ten different Tn4401a flanking signatures observed, only one of which was seen in the other >1600 isolates sequenced in the study (Tn4401a-1_ATTGA_ATTGA, the most common flanking signature observed overall), and therefore most strongly consistent with the occurrence of an atypical and increased number of transposition events within this *K. pneumoniae* strain. Although all isolates contained IncFIB and IncFII plasmid replicons, evidence of plasmid gain/loss events as demonstrated by changes in plasmid replicon profiles also appeared common (Fig.8 for cluster 1).

8. Figures and tables

Figure 1. Sampling and sequencing flowchart for the study.

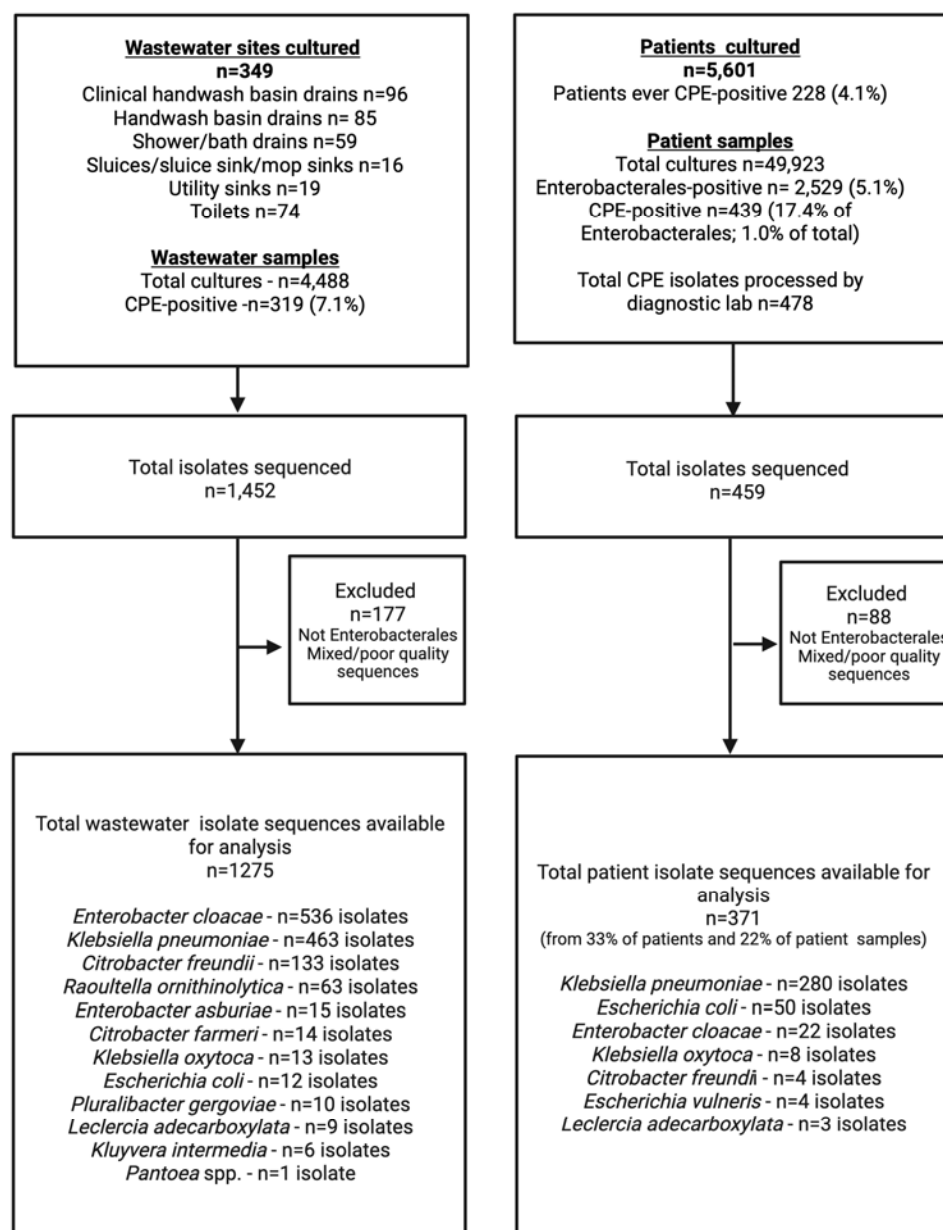


Figure 2. bla_{KPC}-Enterobacterales (KPC-E)-positive environmental sites by week in 2016 and hospital unit. The number of environmental site samples performed and KPC-E-positive samples by week of sampling, stratified by unit (top three panels), and the proportion of KPC-E-positive sites by week of sampling, again stratified by unit (bottom panel).

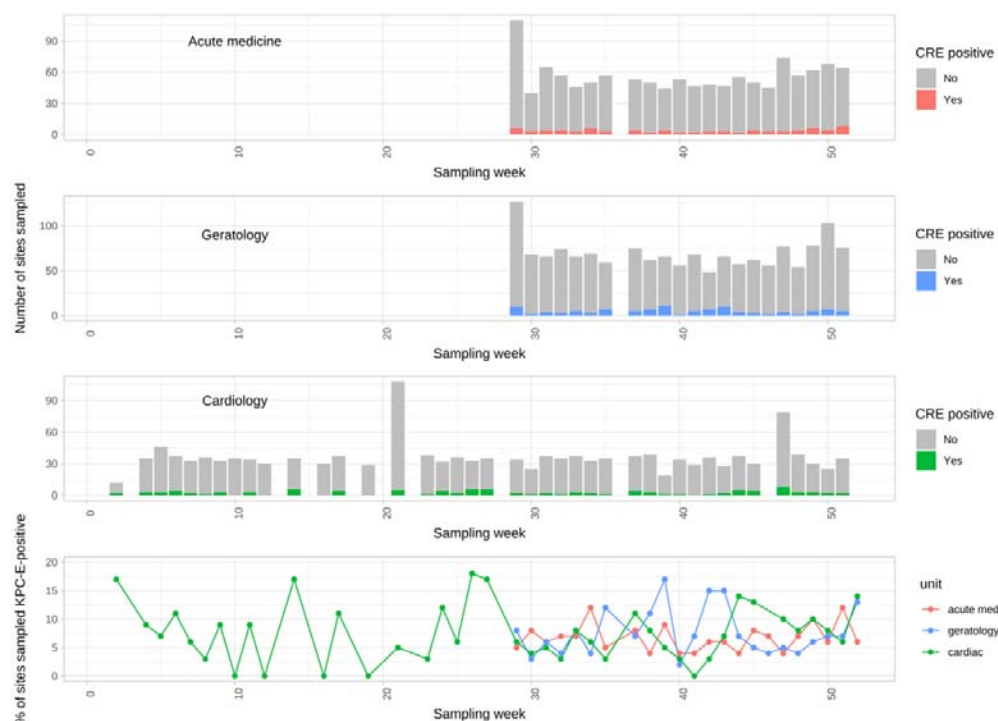


Figure 3. Carbapenem-susceptible Enterobacterales (CSE) and carbapenem-resistant-Enterobacterales (CRE) patient cultures by week in 2016 and hospital unit. Top three panels show counts of Enterobacterales culture-positive samples stratified by unit and carbapenem susceptibility; bottom panel shows the proportion of all cultures taken that were CRE, again stratified by unit.

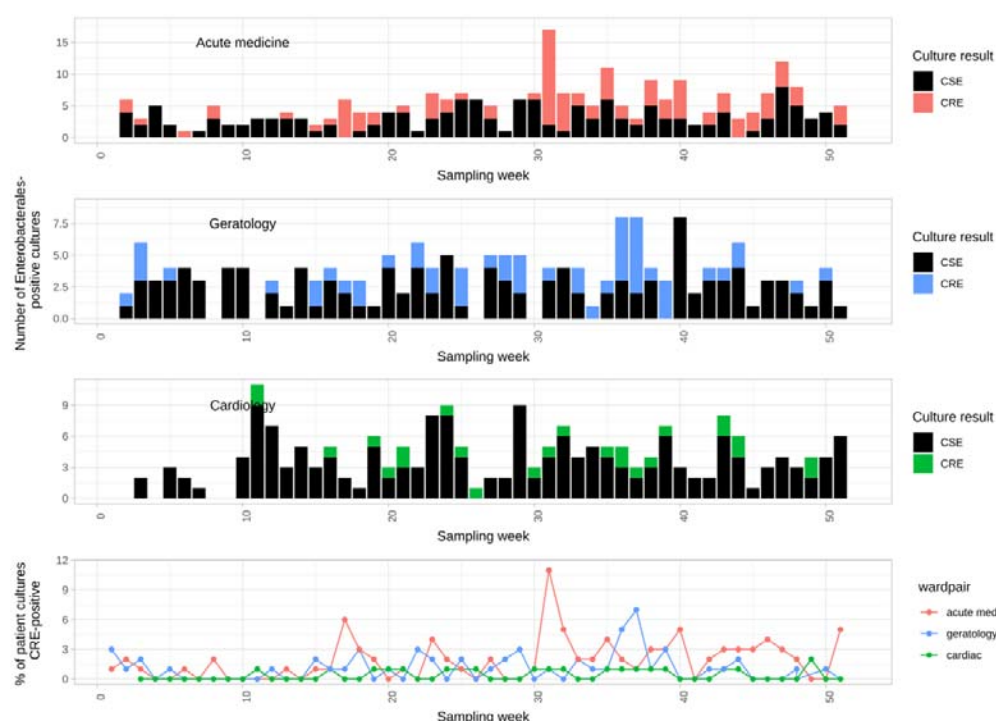


Figure 4. Number of unique patient/environmental niches colonised by common *bla*_{KPC-2} Enterobacterales species/strains and the total number of isolates for each cluster. Includes species/strains if >50 isolates of a species were identified [see Methods]). Orange stars denote patient niches, black circles wastewater niches; larger shape size denotes a larger number of isolates sequenced. Strains observed in both niches are either overlapping shapes (same number of niches), or joined by a line (different numbers of niches).

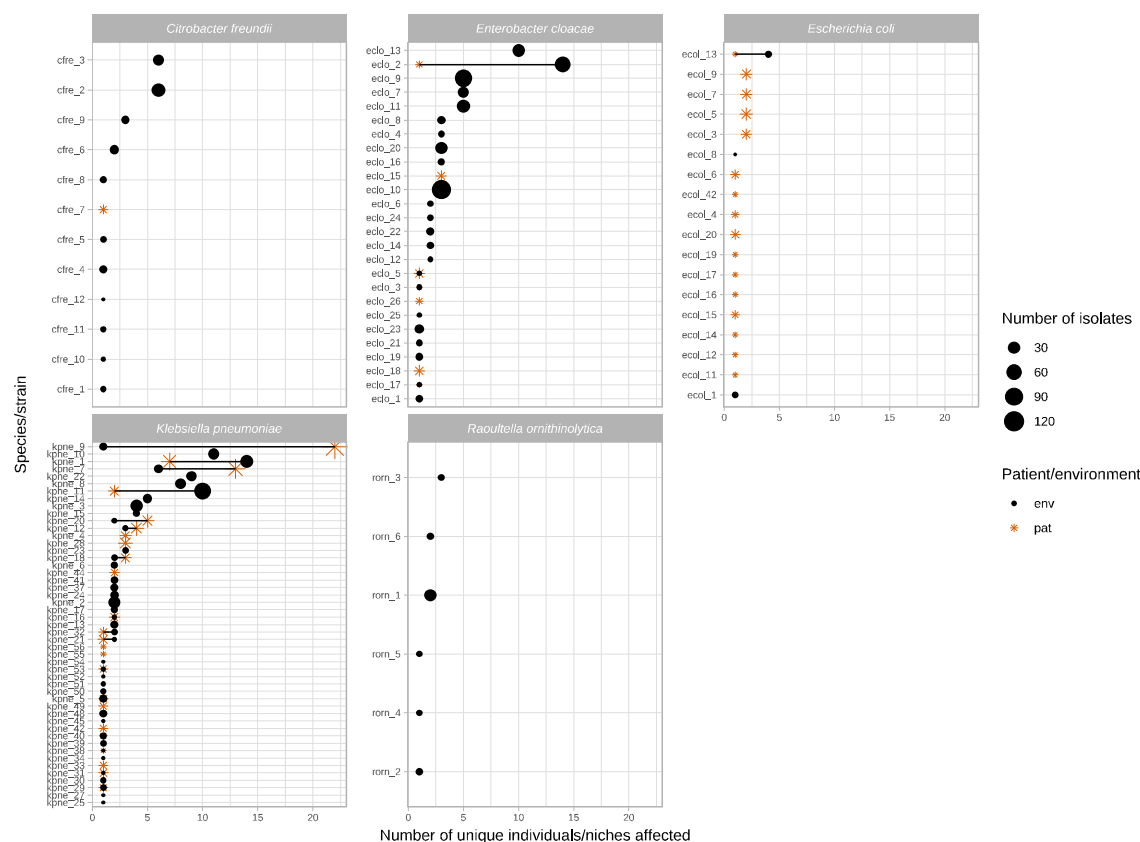


Figure 5. Summary of within-niche and KPC-E sample diversity using genomics.

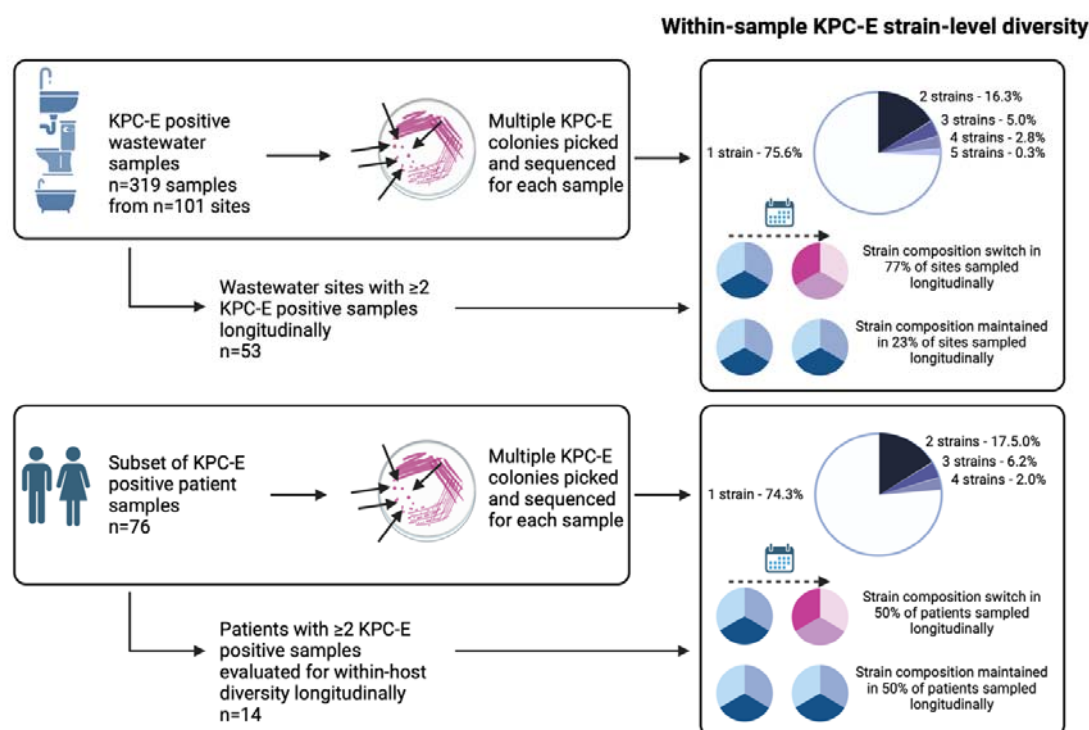


Figure 6. *Enterobacter cloacae* strain 10 - features of population structure and transmission dynamics - niche restriction, persistence and genetic turnover. Summaries of A) Chromosomal SNP distances for *E. cloacae* strain 10 across the three sites it colonised; Bi) AMR and Bii) IS profiles, C) plasmid replicon profiles, and D) deletions in Tn4401a in isolates with each sequenced isolate plotted as a dot reflecting within-sample diversity at given timepoints and longitudinally.

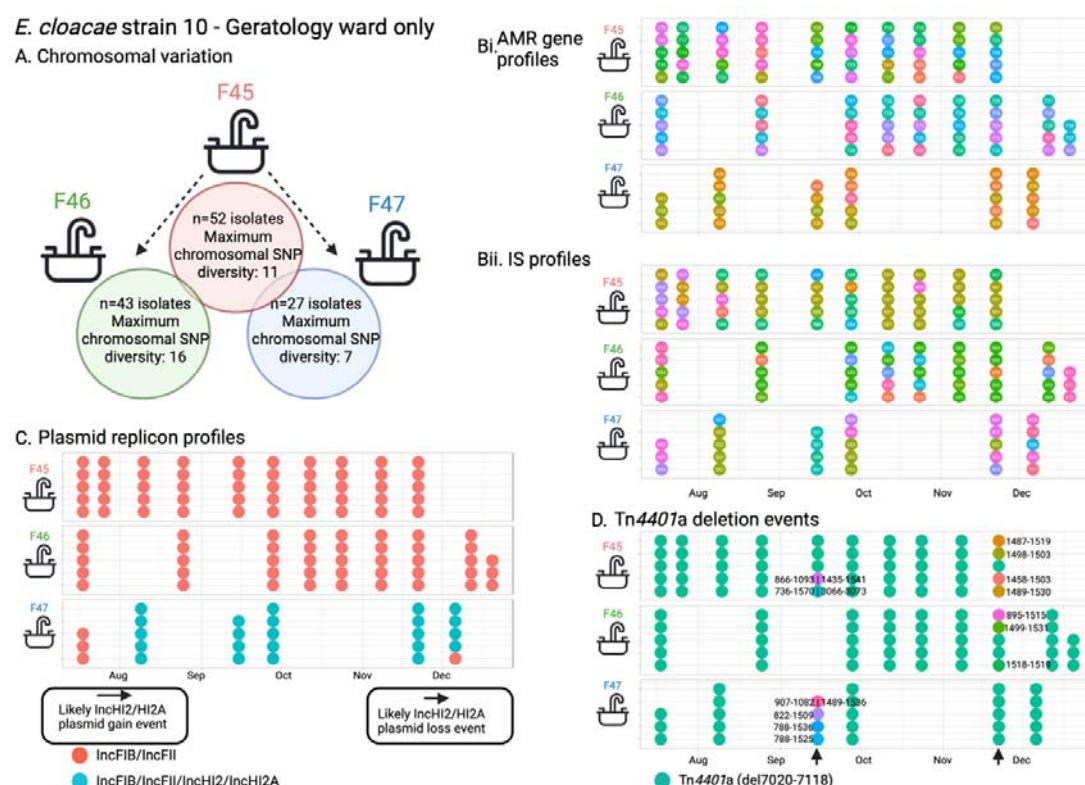


Figure 7. *Klebsiella pneumoniae* strain 9 - features of population structure and transmission dynamics - rapid patient-patient transmission. Putative transmission network for sub-lineages of *K. pneumoniae* strain 9, defined by accessory genome clustering (clusters 1, 2, 3a-h). Horizontal bars represent admission episodes to study wards for each patient (pale blue=W45, geratology; pale pink=AM1, acute medicine; pale purple=AM2, acute medicine; A13 represents the only environmental (shower) site involved). Empty black triangles denote CPE-negative rectal screens on patients. Arrows denote possible transmission links (dashed arrows denote events that have no geographic overlap or could be associated with two links based on negative screen, geographic location and timing). For clarity, arrows are not drawn for clusters 3g and 3h, which affected the most patients - these are shown in detail as an inset panel at the bottom right. In some cases patients were colonised by other species-strains contemporaneously (not shown here).

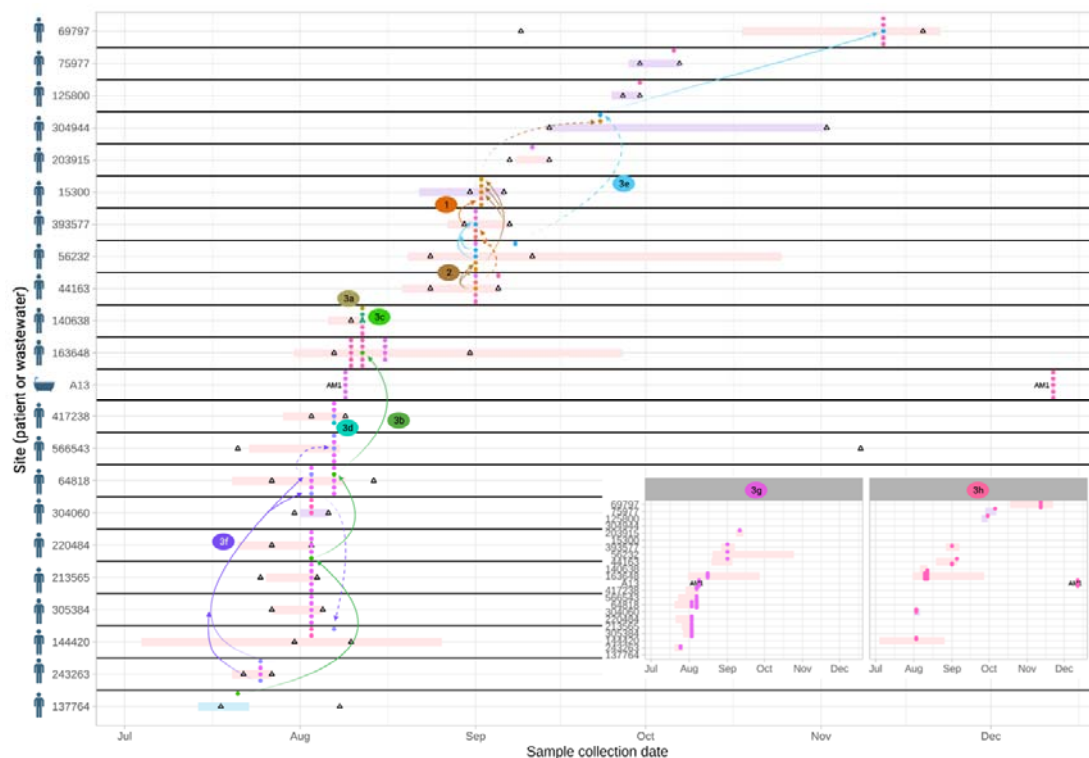


Figure 8. *Klebsiella pneumoniae* strain 11 cluster - features of population structure and transmission dynamics – multiple Tn4401 transposition events. Examples of possible transmission/genetic events are annotated on the figure. Each isolate sequenced is represented as a dot, with colour denoting the TSS profiles observed. Plasmid replicon profiles are annotated as text.

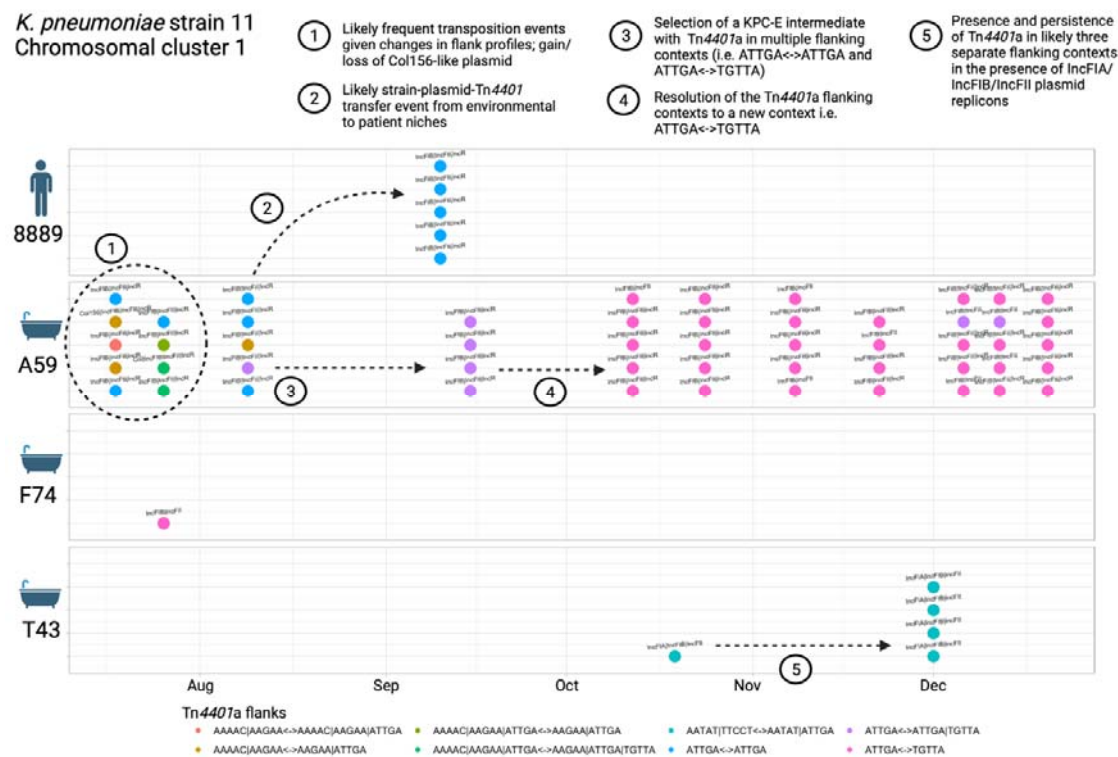


Table 1. Association of environmental site type and unit location with *bla*_{KPC}-Enterobacterales (KPC-E) positivity in environmental samples. Outputs of a multivariable logistic regression model with robust standard errors clustered by environmental site type and unit location; no other variables were considered in the model. P-values <0.05 were considered significant and are highlighted in bold. No interactions with an interaction Wald p <0.05 were observed.

Environmental site	Number of sites	Number of sampling events KPC-E-positive (% of total sampling events)	Number of sampling events KPC-E-negative	OR (95% CI)	p
Environmental site type					
Clinical handwash basin drain	96	79 (5.9%)	1270	Reference	
Handwash basin drain	85	52 (4.6%)	1077	0.99 (0.51-1.93)	0.9735
Shower/bath drain	59	100 (19.2%)	421	2.69 (1.44-5.01)	0.0019
Sluice/sluice sink/mop sink	16	47 (17.9%)	215	2.60 (1.04-6.52)	0.0410
Toilet	74	5 (0.5%)	931	0.28 (0.10-0.78)	0.0152
Utility sink	19	36 (12.4%)	255	1.32 (0.47-3.68)	0.5965
Environmental site location					
Acute medicine unit	129	86 (6.6%)	1223	Reference	
Cardiac unit	76	111 (7.0%)	1453	1.07 (0.57-2.00)	0.8412
Geratology unit	144	122 (7.6%)	1493	0.88 (0.52-1.48)	0.6243

9. Discussion

This work highlights the substantial KPC-E diversity and flux that can occur in both human and wastewater niches in healthcare settings over variable timeframes, in this case in relation to a large bla_{KPC-2}-associated carbapenemase gene outbreak. KPC-E strains have features likely to make generalising results regarding modes of transmission, evolution and gene sharing of bla_{KPC} amongst these species very challenging. Although structuring at the strain-level was observed by niche and species (Fig.4), likely transmissions occurred in a non-negligible manner in all directions from patients to other patients and the environment, and from the environment to patients and other environmental sites. Environmental niches and certain lineage contexts appeared to amplify bla_{KPC-2} transposition events.

Although some environmental sites were persistently colonised with individual strains over months, many appeared to be only transiently colonised. Sites particularly associated with KPC-E colonisation included sluices, sluice sinks, mop sinks and shower/bath drains, supported by other studies⁵, and consistent with these representing a major interface between human faeces and premise plumbing. Toilets were less likely to be KPC-E-positive, perhaps because of regular flushing or particular cleaning protocols; consistent with this, several studies have demonstrated that daily bleach application decontaminated wastewater sites^{32, 33}.

Our study underscores the detailed and dense sampling effort required to understand within-sample diversity and longitudinal flux, and highlights the difficulty in accurately tracking the dissemination of AMR genes and strains across niches. This problem has also been demonstrated in other recent work investigating bla_{OXA-48}, where only patient-based sampling was undertaken and most bla_{OXA-48}-associated transmission events were non-delineated or only weakly supported by joint phylogenetic/epidemiological inference⁸. Our study clearly shows that different species and lineages represent different epidemiological risks and have their own behaviours, and a focus on single, clonal strains as mediators of AMR gene transmission is irrelevant in this and potentially many other settings where important AMR genes have become endemic.

The importance of evaluating asymptomatic carriage, characterising within-host diversity of strains, considering rapid acquisition/loss of strains over short timeframes, and including environmental sites in transmission networks has been partly demonstrated in previous studies investigating ESBL-*E. coli*^{10, 34} and *Enterococcus* spp.³⁵, but generally these features are not jointly investigated because doing so is highly resource-intensive, and there are no robust analysis methods for doing this to our knowledge. However, without this effort, such as in studies which consider only single or infrequent sampling timepoints, and do not investigate asymptomatic colonisation or consider within-site diversity³⁶, our study highlights that relevant events may easily be missed.

There are several limitations to this work. CPE-sample capture for patients for this study was not continuous, and we therefore only evaluated a subset of CPE-positive patients and samples using genomics (33% and 22% respectively); we anticipate many transmission events were missed, but our sampling already reveals the high complexity of *bla*_{KPC} transmission. We were not resourced to perform long-read sequencing to enable plasmid sequence reconstruction, and plasmid replicon or *bla*_{KPC}-contig profiling is relatively low-resolution and can be misleading²¹; we therefore avoided a detailed plasmid analysis which would be important in quantifying horizontal *bla*_{KPC} transmission. However, the diversity we observed in genetic contexts supporting *bla*_{KPC} was substantial, consistent with high rates of HGT. A further limitation is the absence of metadata capturing relevant selection pressures in each niche, including cleaning protocols for environmental reservoirs and drug prescription/antimicrobial stewardship data for patients. We only investigated one healthcare setting, but the same polyspecies, polyclonal phenomenon has been seen elsewhere and with other carbapenemase genes^{4,8}. We focused only on Enterobacterales and may have missed wider HGT of carbapenemase genes to other bacteria. In line with this, at least 23 *Pseudomonas* spp., five *Stenotrophomonas maltophilia* and two *Acinetobacter* spp. that were PCR-positive for *bla*_{KPC} or *bla*_{NDM} were inadvertently picked up on rectal screening. Our approaches to evaluating some of the putative genetic changes and transmission events observed were heuristic and manual, partly because the data were highly heterogeneous and complex. The study is now relatively historic, but these types of polyspecies, polyclonal carbapenemase gene outbreaks associated with hospital wastewater sites continue to be described and as such we believe the findings remain of interest.

Further optimisation of methods to evaluate multi-level genetic transmission and within-sample diversity without individual colony-level characterisation is needed. Holistic approaches using fully reconstructed chromosome/plasmid assemblies to quantify the diverse types of genomic transmission (including clonal transmission, horizontal transfer at the gene, transposon and plasmid-level) are also required to better understand what facilitates emergence, selection and persistence of different lineages and genetic vectors of *bla*_{KPC}. This information will improve our understanding of how to intervene to limit the transmission of drug-resistant Enterobacterales in healthcare settings, and avoid incorrect inference.

10. Author statements

10.1 Conflicts of interest

DWE has received lecture fees from Gilead, outside the submitted work. The other authors declare that there are no conflicts of interest.

10.2 Funding information

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10.3 Ethical approval

This study was performed as part of a Trust board-approved and UK Health Security Agency (formerly Public Health England) long-term outbreak response, and ethics approval was not required under NHS governance arrangements; all analyses were undertaken using anonymised data. This was reviewed and agreed by the Joint Research Office of the Oxford University Hospitals NHS Foundation Trust and the University of Oxford (who oversee research governance, ethics and assurance for our institution), and who deemed that neither sponsorship nor research ethics review was required.

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