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Genomic epidemiology of a complex, multi-species plasmid-borne *bla*$_{KPC}$ carbapenemase outbreak in Enterobacterales in the UK, 2009-2014

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ABSTRACT

Carbapenem resistance in Enterobacterales is a public health threat. *Klebsiella pneumoniae* carbapenemase (encoded by alleles of the bla_{KPC} family) is one of the commonest transmissible carbapenem resistance mechanisms worldwide. The dissemination of bla_{KPC} has historically been associated with distinct *K. pneumoniae* lineages (clonal group 258 [CG258]), a particular plasmid family (pKpQIL), and a composite transposon (Tn*4401*). In the UK, bla_{KPC} has caused a large-scale, persistent outbreak focused on hospitals in North-West England. This outbreak has evolved to be polyclonal and poly-species, but the genetic mechanisms underpinning this evolution have not been elucidated in detail; this study used short-read whole genome sequencing of 604 bla_{KPC}-positive isolates (Illumina) and long-read assembly (PacBio)/polishing (Illumina) of 21 isolates for characterisation. We observed the dissemination of bla_{KPC} (predominantly bla_{KPC-2}; 573/604 [95%] isolates) across eight species and more than 100 known sequence types. Although there was some variation at the transposon level (mostly Tn*4401*a, 584/604 (97%) isolates; predominantly with ATTGA-ATTGA target site duplications, 465/604 [77%] isolates), bla_{KPC} spread appears to have been supported by highly fluid, modular exchange of larger genetic segments amongst plasmid populations dominated by IncFIB (580/604 isolates), IncFII (545/604 isolates) and IncR replicons (252/604 isolates). The subset of reconstructed plasmid sequences also highlighted modular exchange amongst non-bla_{KPC} and bla_{KPC} plasmids, and the common presence of multiple replicons within bla_{KPC} plasmid structures (>60%). The substantial genomic plasticity observed has important implications for our understanding of the epidemiology of transmissible carbapenem resistance in Enterobacterales, for the implementation of adequate surveillance approaches, and for control.

IMPORTANCE

Antimicrobial resistance is a major threat to the management of infections, and resistance to carbapenems, one of the "last line" antibiotics available for managing drug-resistant infections, is a significant problem. This study used large-scale whole genome sequencing over a five-year period in the UK to highlight the complexity of genetic structures facilitating the spread of an important carbapenem resistance gene (bla_{KPC}) amongst a number of bacterial species that cause disease in humans. In contrast to a recent pan-European study from 2012-2013(1), which demonstrated the major role of spread of clonal bla_{KPC}-*Klebsiella pneumoniae* lineages in continental Europe, our study highlights the substantial plasticity in genetic mechanisms underpinning the dissemination of bla_{KPC}. This genetic flux has important implications for: the surveillance of drug resistance (i.e. making surveillance more difficult); detection of outbreaks and tracking hospital transmission; generalizability of surveillance findings over time and for different regions; and for the implementation and evaluation of control interventions.

INTRODUCTION

Antimicrobial resistance (AMR) in Enterobacterales is a critical public health threat. Carbapenem resistance is of particular concern, and outbreaks involving multiple species of carbapenemase-producing Enterobacterales (CPE) are increasingly reported(2-5). Exchange of AMR genes, including carbapenem resistance genes, happens at multiple genetic levels(6), and is often facilitated by their presence on plasmids [circular DNA structures of variable size (2kb~>1Mb)], and/or other smaller mobile genetic elements (MGEs) such as transposons and insertion sequences (IS), that form part of the accessory genome.

Whole genome sequencing (WGS) has significantly improved our understanding of infectious diseases epidemiology and is used in both community-associated and nosocomial transmission analyses(7, 8). Although useful for delineating transmission routes in clonal, strain-based outbreaks, standard phylogenetic approaches and comparative analyses have been more difficult for outbreaks involving multiple bacterial strains/species and transmissible resistance genes(6). Reconstruction of the genetic structures of plasmids carrying relevant antimicrobial resistance genes using long-read sequencing has improved our understanding of the genetic complexity of these resistance gene outbreaks, but has been difficult to undertake on a large scale.

Although approximately 40 *Klebsiella pneumoniae* carbapenemase (KPC; encoded by bla_{KPC}) variants have now been described (as per NCBI's AMR reference gene catalogue, available at https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/), only two have been most widely reported globally, namely KPC-2 and KPC-3 (H272Y with respect to KPC-2; single nucleotide difference in bla_{KPC} [C814T])(9,

10). In the UK, the first KPC isolate identified was a KPC-4-containing *Enterobacter* sp. isolated in Scotland in 2003(11), with subsequent identification of KPC-3 in isolates in the UK in 2007. From 2007, increasing numbers of suspected KPC isolates were referred to Public Health England (PHE's) Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, with the majority of confirmed KPC-producers (>95%) coming from an evolving KPC-2-associated outbreak in hospitals in North-West England, first recognised in 2008(12). These isolates were predominantly *bla*$_{KPC}$-positive Enterobacterales cultured from patients in the Central Manchester University Hospitals NHS Foundation Trust (CMFT; now part of Manchester University NHS Foundation Trust)(13). *bla*$_{KPC}$ is thought to have been introduced into the region via a pKpQIL-like plasmid(14, 15), a plasmid backbone previously associated with the global dissemination of *bla*$_{KPC}$ in *K. pneumoniae* clonal group 258, and already observed in other *K. pneumoniae* sequence types (STs) and species in an analysis of 44 UK KPC-Enterobacterales from 2008-2010(15).

We used WGS to undertake a large-scale retrospective study of this multi-species, polyclonal, *bla*$_{KPC}$ outbreak in North-West England from 2009, generating complete genome structures, including *bla*$_{KPC}$ plasmids, for a subset of isolates. We contextualised our analysis of regional outbreak strains using isolates from a national *bla*$_{KPC}$ surveillance programme, with the goal of understanding the genetic structures associated with the regional emergence of *bla*$_{KPC}$ in this setting.

RESULTS

Of 742 isolates identified for sequencing, 60 (8%) were not retrievable or cultivable from the laboratory archives. After de-duplicating by taking the first *bla*$_{KPC}$-positive Enterobacterales (KPC-E) per patient, and excluding sequencing failures, any sequences without *bla*$_{KPC}$ (assumed lost in culture), and mixtures (identified from genomic data analysis, see Methods), 604 evaluable isolate sequences were included. These included: 327 archived isolates (54%) from inpatients in the early stages of the observed outbreak (2009-2011), of which 309 and 18 isolates were from CMFT and the University Hospital of South Manchester NHS Foundation Trust (UHSM; now part of Manchester University NHS Foundation Trust) respectively; 78 (13%) later isolates from CMFT/UHSM (2012-2014); 119 (20%) isolates from other hospitals (n=15 hospitals) in North-West England (2009-2014, excluding CMFT and UHSM, up to the first 25 consecutive KPC-E isolates per hospital); 72 (12%) isolates from UK and Republic of Ireland hospitals (n=72 locations [n=4 from the Republic of Ireland]) outside the North-West (2009-2014) (first KPC-E isolate per hospital); and 8 (1%) isolates from English outpatient/primary care settings.

Although three *bla*$_{KPC}$ variants were observed in the 604 included isolates, *bla*$_{KPC-2}$ dominated (n=573, 95%); *bla*$_{KPC-3}$ [n=27, 4%] and *bla*$_{KPC-4}$ [n=4, 1%]) were also observed. Two isolates (0.3%; trace524, trace534) showed evidence of mixed populations of *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$. The median *bla*$_{KPC}$ copy number estimate was 1.8 (IQR: 1.6-2.1), with a maximum of 8.2. Across the three main species, *bla*$_{KPC}$ copy numbers were higher in *K. pneumoniae* (n=525 [87%], median 1.8 [IQR: 1.6-2.1]), than *E. coli* (40 [7%]: 1.7 [1.5-1.9]) or *E. cloacae* (26 [4%], 1.6 [1.4-2.0]) (Kruskal-Wallis; p=0.0003; Fig.1A). Amongst common STs, copy number was highest in *K.*

pneumoniae ST258 (n=65 [11%], median 2.4 [IQR: 1.8-2.9]) versus other species/STs (n=531 [89%], median 1.8 [1.6, 2.0]) (Kruskal-Wallis; p=0.0001; Fig.1B, Fig.S1).

Other broad or extended-spectrum beta-lactamase genes were also commonly present across isolates, including: bla_{TEM} (n=452, all $bla_{\text{TEM-1}}$), bla_{OXA} (n=492; $\Delta bla_{\text{OXA-9}}$ [n=425], $bla_{\text{OXA-1}}$ [n=138]), bla_{SHV} (n=497) and $bla_{\text{CTX-M}}$ (n=89; $bla_{\text{CTX-M-15}}$ [n=57], $bla_{\text{CTX-M-9}}$ [n=28]). Aminoglycoside resistance genes were also widely prevalent: *aac* (n=243), *aph* (n=196), *ant* (n=93) and *aadA* (n=280). In terms of acquired quinolone resistance, 160 isolates contained *qnr* variants, and 137 isolates contained *aac(6')-Ib-cr*; no *qep* variants were seen.

Insertion sequences (ISs) have been shown to be key in the reshaping and streamlining of bacterial genomes, as well as exerting more subtle effects in the regulation of gene expression(16). The median number of different IS types in isolates was 15 (IQR: 13-16), with a maximum of 32. Four hundred distinct IS profiles were identified amongst the 604 isolates, with only five identical profiles shared amongst ≥10 isolates - these included a distinct profile seen only in national *K. pneumoniae* ST258 isolates (IS*1F*, IS*1R*, IS*3000*, IS*6100*, IS*903B*, IS*Ecl1*, IS*Kpn1*, IS*Kpn14*, IS*Kpn18*, IS*Kpn25*, IS*Kpn26*, IS*Kpn28*, IS*Kpn31*, IS*Kpn6*, IS*Kpn7*), and other unique profiles seen in small groups of *K. pneumoniae* ST588, ST11, ST321 and ST54. This highlights the significant flux of small mobile genetic elements within and between lineages in our dataset.

Tn*4401* is a ~10kb transposon that has been the major transposable context for bla_{KPC} to date(17, 18). A predominant Tn*4401* isoform was associated with both $bla_{\text{KPC-2}}$ and

176 *bla*$_{KPC-3}$ in this study, namely Tn*4401*a(17), which occurred in 584/604 (97%)
177 isolates. Other known variants included Tn*4401*b (n=7) and Tn*4401*d (n=3). Only
178 20/584 (3%) isolates demonstrated evidence of SNV-level variation in Tn*4401*a
179 (homozygous calls at 6 positions; heterozygous calls [i.e. mixed populations] at 3
180 positions). *bla*$_{KPC-2}$-Tn*4401*a (n=539 isolates) was predominantly flanked by a 5-bp
181 target site duplication (TSD) ATTGA, with 465/604 (77%; 465/539 [86%] of this
182 sub-type) isolates with this Tn*4401*/TSD combination (Fig.2A). In 74 other *bla*$_{KPC-2}$-
183 Tn*4401*a isolates, the Tn*4401*a was flanked by other target site sequence (TSS)
184 combinations, consistent with additional transposition events. Thirty-two of these
185 were TSDs (16 AATAT-AATAT, 16 AGTTG-AGTTG), which have been described
186 as more consistent with inter-plasmid transposition of Tn*4401*(19), and 35 were non-
187 duplicate TSS combinations (ATTGA with either ATATA, TGGTA, CTGCC,
188 AATAA, AGGAT), described as more consistent with intra-plasmid transposition.
189 Evidence of multiple TSSs around *bla*$_{KPC-2}$-Tn*4401*a within single isolates was seen
190 in 6 cases (i.e. multiple right and/or left Tn*4401* TSSs); 1 case had a right TSS
191 present, but no left TSS identified.
192
193 The 604 isolates contained 91 unique combinations of plasmid Inc types (a crude
194 proxy of plasmid populations present); no isolate was replicon negative. However,
195 there were seven predominant combinations (Fig.2B) represented in 443/604 (73%)
196 isolates, and these included six major Inc types, namely IncF (FIB [found in n=580
197 isolates], FII [n=545]), FIA (n=103), IncR (n=252), ColRNAI (n=86), and IncX3
198 (n=60). For many of the plasmid families, several different reference replicon
199 sequences exist in the PlasmidFinder database, with a degree of homology amongst
200 sequences in the same family, making it difficult to establish exactly which exact sub-

type of replicon is present. However, restricting to 100% matches to reference replicon types for these common families, top matches included: IncFIB(pQil)_JN233705 (n=300) and IncFIB(K)_1_Kpn3_JN233704 (n=107); ColRNAI_1_DQ298019 (n=84); IncR_1_DQ449578 (n=70); and IncFII_1_pKP91_CP000966 (n=62; plasmid MLST $IncFII_{K4}$), IncFII(K)_1_CP000648 (n=51; plasmid MLST $IncFII_{K1}$) and IncFII_1_AY458016 (n=19; plasmid MLST $IncFII_{K2}$).

Species and lineage diversity in the outbreak was substantial, with eight different species amongst sequenced isolates, and many different known STs, including: *K. pneumoniae* (n=525 isolates, 70 known STs), *E. coli* (n=40, 20 known STs), *Enterobacter cloacae* (n=26, 9 known STs), *Klebsiella oxytoca* (n=6, 3 known STs), *Raoultella ornithinolytica* (n=4), *Enterobacter aerogenes* (n=2), *Serratia marcescens* (n=1) and *Kluyvera ascorbata* (n=1). The most common STs were all *K. pneumoniae*, including ST258 (n=66), ST11 (n=35), ST491 (n=31), ST1162 (n=29) and ST54 (n=27) (Fig.2C). Therefore, although some of the earliest sequenced isolates were KPC-*K. pneumoniae* ST258 and ST11 (both in 2009) [two major KPC strains from CG258 circulating globally and in China at the time(9, 20)] and although KPC-producing *K. pneumoniae* ST258 appears to have been one of the earliest strains observed in CMFT and UHSM, multiple diverse STs and species were subsequently rapidly recruited to the outbreak in 2010 and 2011. This was most likely by the widespread sharing of a bla_{KPC-2}-Tn*4401*a-ATTGA-ATTGA transposon within and between IncFIB, IncFII and IncR plasmid populations (Fig.2B, 2C).

Long-read sequencing analyses

In addition to short-read data, to resolve genetic structures fully we obtained long-read PacBio data for 23 isolates, chosen to maximise the *bla*$_{KPC}$ plasmid diversity assayed and focussing on isolates collected from the two main Manchester hospitals (12 CMFT isolates, 5 UHSM; plus 2 from other hospitals in North-West England, 4 from other UK locations). These included the two presumed earliest *bla*$_{KPC}$ isolates from both CMFT and UHSM, as well as isolates sharing the same species/ST but with different plasmid replicon combinations or from North West regional versus national locations, same-species isolates with different STs, and isolates of different species. One PacBio sequencing dataset represented a clear isolate mixture (trace597 [UHSM] of *E. cloacae* ST133 and *K. pnemoniae* ST258), and for one isolate (trace457 [CMFT]), there were discrepancies between the short-read and long-read sequencing datasets, suggesting a laboratory error (*E. cloacae* ST45 long-read, *E. coli* ST88 short-read). These two assemblies were excluded, leaving 21 assemblies for further analysis (Table S1).

Of the 153 contigs from these 21 assemblies, 30 were clearly chromosomal, 77 plasmid, one chromosomal with an integrated plasmid, and 45 with unclear provenance (i.e. possibly phage, plasmid, or chromosomal). Overall 78/153 [51%] contigs were circularised, including 56/77 [73%] clear plasmid sequences. Thirty-one contigs (21 (68%) circularised) harboured *bla*$_{KPC}$, of which one (trace552, *K. pneumoniae* ST11) had *bla*$_{KPC}$ integrated into the chromosome. Four isolates had multiple copies of *bla*$_{KPC}$ (trace205 [*K. pneumoniae*, ST468; 2 copies, 1 contig], trace457 [*E. cloacae*, ST45; 5 copies [1 truncated], 2 contigs], trace75 [*K. pneumoniae*, ST252; 2 copies, 2 contigs], trace149 [9 copies, 9 contigs]). Trace149 (*E. coli*, ST1642), which had nine copies of *bla*$_{KPC}$, had one copy each on: a long,

incomplete *bla*$_{KPC}$ contig (203kb), seven nearly identical complete *bla*$_{KPC}$-containing circularised sequences of size ~10kb (possibly representing circularised translocatable intermediates(21)), and a short linear *bla*$_{KPC}$ contig (~18kb).

We observed *bla*$_{KPC}$ in multiple plasmid backgrounds (Fig.3), including a majority of *bla*$_{KPC}$ plasmids with multiple replicons (13/21 [60%] clear plasmid contigs, as represented in Fig.3), particularly with IncFIB/IncFII and/or IncR, consistent with replicon patterns in the isolates overall (Fig.2). For the IncFII group, for which we had 17 plasmid sequences with an IncFII(K)_CP000648-like replicon (plasmidFinder match; 5 *bla*$_{KPC}$-negative [i.e. not represented in Fig.3] and 12 *bla*$_{KPC}$-positive), there was evidence of significant exchange and rearrangement of plasmid components between both *bla*$_{KPC}$-positive and *bla*$_{KPC}$-negative plasmids, integration of IncFII$_K$ and IncR plasmids, and gene duplication events of Tn*4401*/*bla*$_{KPC}$, as well as sharing between STs and species (Fig.4).

In addition to their plasticity, part of the success of these *bla*$_{KPC}$ plasmids may also be attributable to the presence of toxin-antitoxin plasmid addiction systems (*ccdA*/*ccdB* n=4 *bla*$_{KPC}$ plasmids; *higA* n=6; *vapB*/*vapC* n=11); anti-restriction mechanisms (*klcA* n=16, previously shown to promote *bla*$_{KPC}$ dissemination(22)); and heavy metal resistance (*terB* [tellurite] n=3; *ars* operon [arsenicals] n=3; chromate resistance n=1; *cop* operon/*pcoC*/*pcoE* [copper] n=7; *mer* operon [mercury] n=10).

bla$_{KPC}$ *plasmid typing*

Attempts to identify complete plasmids (as opposed to plasmid replicon typing) from short-read data by comparison to a reference plasmid database has been estimated as

being correct in only ~45%-85% of cases in previous studies(6, 23). However, 13/14 (93%) of isolates for which we had hybrid assemblies with only one completely reconstructed *bla*$_{KPC}$ plasmid had the correct top match using our *bla*$_{KPC}$ plasmid typing method (Table S2). Noting that any complete plasmid typing approach from short-read data is sub-optimal, we compared all short-read sequences with our reference *bla*$_{KPC}$ plasmid database (see Methods); matches to one or more reference *bla*$_{KPC}$ plasmid sequences were identified in 554/604 (92%) isolates. Filtering the single match with the highest score at the predefined ≥0.80 threshold left a subset of 428/554 (77%) for evaluation. These 428 isolates had matches to 12 *bla*$_{KPC}$ plasmid clusters.

Based on classification by these top plasmid-cluster matches, *bla*$_{KPC}$ plasmid clusters were shared across a median (IQR) of 3 (1-6) STs, with pKpQIL-like plasmids being most widespread across species/STs (7 species, 75 STs), and clearly playing a major role in the North-West England outbreak, as well as being spread regionally and nationally (Fig.5). Other plasmid types identified as top-matches across the entire dataset included those fully resolved by long-read sequencing performed within this study, some of which were seen in ≥5% of study isolates (e.g. pKPC-trace75 [a non-typeable replicon]), and in non-North-West settings, likely reflecting recombination and generation of new *bla*$_{KPC}$ plasmid variants in North-West England and their subsequent dissemination.

DISCUSSION

We present the largest WGS-based analysis of *bla*$_{KPC}$-positive isolates (n=604) to our knowledge, focused on assessing genetic diversity around the carbapenemase gene

itself rather than limiting the analysis based on species type, and incorporating a sampling frame from UK regional and national collections, over five years. *bla*$_{KPC}$ remains one of the three most common carbapenemases observed in the UK, accounting for ~15% of cases referred to the AMRHAI Reference Unit in 2017(24), and presenting a significant challenge to hospitals in North-West England, including Manchester, where it accounted for >97% of carbapenem resistance through 2015(25).

Our study provides an interesting context in which to consider the findings of a recently published pan-European survey of carbapenem non-susceptible *K. pneumoniae* (the EuSCAPE study; 6 months, 2013-2014; 244 hospitals, 32 countries)(1). In EuSCAPE, 684 carbapenemase-producing *Klebsiella* spp. isolates were Illumina sequenced, and similar to our study, most cases were healthcare-exposed (<2% from outpatients). EuSCAPE carbapenemase-producing isolates were also predominantly *bla*$_{KPC}$ (~45%, n=311 isolates), but mostly *bla*$_{KPC-3}$ (232/311 [75%] versus 27/604 [5%] in our study), and ST258/ST512 (226/311 [73%] versus 107/525 (20%) of *K. pneumoniae* overall in our study). Based on identifying genetic "nearest-neighbours" in their data, the EuSCAPE team found 51% of *bla*$_{KPC}$-*K. pneumoniae* were most closely related to another isolate from the same hospital. The authors concluded that there was strong geographic structuring of strains, and that the expansion of a handful of clonal lineages was predominantly responsible for the spread of carbapenemases in *K. pneumoniae* in Europe, with onward nosocomial transmission. Like *bla*$_{KPC-3}$ in EuSCAPE, *bla*$_{KPC-2}$ has also been linked with the clonal expansion of ST258 in Australia(26), where 48% of 176 *K. pneumoniae* isolates sequenced were *bla*$_{KPC-2}$-containing ST258.

326

327 However, instead of clonal expansion, in our study we found rapid dissemination of
328 mobile backgrounds supporting *bla*$_{KPC-2}$, similar to observations from sequencing of
329 other polyclonal *bla*$_{KPC}$ outbreaks reported elsewhere, including the US(6, 27).
330 Tn*4401*a, associated with high levels of *bla*$_{KPC}$ expression(28), has been previously
331 predominantly seen in *K. pneumoniae*, and in isolates from the US, Israel and Italy,
332 and similarly most commonly with an ATTGA-ATTGA TSD(10). Thus our findings
333 are consistent with the importation of the predominant *bla*$_{KPC-2}$-Tn*4401*a-ATTGA-
334 ATTGA motif into CMFT/North-West England and subsequent horizontal spread.
335 Notably, as in EuSCAPE, 46/72 (64%) singleton isolates we sampled from UK
336 hospitals were also CG258, but our detailed sampling within a region reflected a very
337 different molecular epidemiology. Although the EuSCAPE study is large and
338 impressive, its breadth may have been limiting in understanding regional diversity -
339 for example, the subset of *bla*$_{KPC}$-*K. pneumoniae* from the UK that were analysed in
340 EuSCAPE consisted of 11 isolates submitted from six centres
341 (https://microreact.org/project/EuSCAPE_UK). The focus was also more on analysing
342 species-specific clonal relationships, with no analysis of other species or MGEs.
343
344 Although in our study diversification occurred at all genetic levels (Tn*4401*+TSSs,
345 plasmids, plasmid populations, strains, species), there was more limited variation
346 observed within the transposon and its flanking regions, and the spread of *bla*$_{KPC}$
347 appears to have been supported by highly plastic modular exchange of larger genetic
348 segments within a distinct plasmid population, particularly IncFIB/IncFII (found in
349 580 and 545 of the 604 isolates respectively) and IncR replicons (252/604 isolates). A
350 previous study, in which 11 transformed *bla*$_{KPC}$ plasmids from the UK (2008-2010)

were sequenced (Roche 454/assembly, PCR+sequencing based gap closure), identified a UK variant of the pKpQIL plasmid, designated pKpQIL-UK (IncFII$_{K2}$ by plasmid MLST), that was highly similar to pKpQIL (maximum 32 SNVs diversity), and several other IncFII$_{K2}$ pKpQIL-like plasmids, but with novel segmental genetic rearrangements (gains/losses; pKpQIL-D1, pKpQIL-D2)(15). Our data support the importance of IncFII$_{K2}$-like plasmids in this *bla*$_{KPC}$ outbreak too, but also that other IncFII$_{K}$-like plasmids (e.g. IncFII-$_{K1, -K4, -K7, -K15}$) and replicons (IncFIB, IncR) have also been a significant feature. In addition to their plasticity, the plasmids identified frequently harboured AMR genes other than *bla*$_{KPC}$ which might offer a selective advantage, alongside heavy metal resistance genes, and plasmid toxin-antitoxin addiction systems. The plasticity and association of IncFII$_{K}$ plasmids with resistance genes and IncFIB replicons has been supported by findings of a recent analysis of IncFII$_{K}$ plasmids(29).

The problem of accurately classifying plasmid populations from short-read data was exemplified in this analysis, and highlighted by our smaller long-read/short-read hybrid assembly-based analysis, which demonstrated significant diversity within structures assigned as similar by short-read based typing approaches. With this caveat, it was interesting that even with relatively relaxed thresholds, 29% of isolates did not have a match to our reference *bla*$_{KPC}$ plasmid database (based on clustering of all publicly available reference sequences, as in Methods), consistent with rapid diversification in the genetic background of Tn*4401*/*bla*$_{KPC}$ elements in this setting.

Our findings demonstrated that it is also important to consider plasmids without the resistance gene of interest in a population, as these may be relevant to a wider

376 understanding of the transmission and evolution of smaller mobile genetic elements
377 harbouring resistance genes (Fig.4). This was also shown to be relevant in a previous
378 analysis of a large KPC-*E. coli* outbreak in the same setting in 2015-2016, in which a
379 circulating *bla*$_{KPC}$-negative plasmid, pCAD3 (IncFIB/FII), acquired Tn*4401* from a
380 IncHI2/HI2A *bla*$_{KPC}$-positive plasmid, and went on to dominate within a clonal *E. coli*
381 lineage(25). Most studies in general however tend to focus on analysing AMR
382 plasmids of interest. Fortunately, long-read sequencing is becoming increasingly low
383 cost and high-throughput, and hybrid assembly is able to reconstruct plasmid
384 sequences in Enterobacterales(30, 31). New developments in large-scale comparative
385 genomics of complete genomes, including plasmid structures, are essential for future
386 large-scale analysis of AMR gene outbreaks.
387
388 There are several limitations to our study. The reconstructed genomes generated using
389 long-read PacBio data remained incomplete (49% of all contigs uncircularised).
390 Improvements in long-read technology and assembly approaches will likely overcome
391 this(30). Our short-read and long-read datasets were generated from the same frozen
392 stocks of isolates, but from separate sub-cultures (because we used the short-read data
393 to inform selection for long-read sequencing); ideally they would have been generated
394 from the same DNA extract. PacBio sequencing library preparation incorporates size
395 selection, and this may have led to short plasmid sequences (<15kb) being lost. Our
396 interpretation of the evolution of backgrounds supporting *bla*$_{KPC}$ was limited by the
397 diversity present, and the inability to capture sequential evolutionary events, even
398 with this large study. Lastly, very limited epidemiological data linked to the isolates
399 were available, meaning that we were unable to ascertain any epidemiological drivers
400 which might be contributing to the enormous heterogeneity of *bla*$_{KPC}$ transmission

401 over apparently short timeframes; the latter finding also precluded the useful
402 application of standard phylogenetic approaches based on identifying variants core to
403 and within species. In addition, the collection of isolates by PHE as part of regional
404 and national surveillance was dictated by referral patterns of isolates from the
405 hospitals surveyed, and we do not have any denominator information on cultures
406 (either *bla*$_{KPC}$-positive or *bla*$_{KPC}$-negative) to corroborate details on the robustness of
407 this referral process.
408
409 In conclusion, our large analysis highlights the difficulty and complexity of these
410 outbreaks once important AMR genes have "escaped" the genetic confines of
411 particular mobile genetic elements and bacterial species/lineages, with important
412 implications for surveillance. These include the need to consider multiple bacterial
413 species and plasmids as potential hosts of *bla*$_{KPC}$, and invest resource in sequencing
414 approaches to adequately reconstruct genetic structures and avoid misinterpreting the
415 molecular epidemiology. It also demonstrates that regional differences in AMR gene
416 epidemiology may be quite marked, which may affect the generalizability of control
417 methods. Finally, it is important to consider the wider genetic background of host
418 strains and plasmids in understanding the evolution and dissemination of important
419 AMR genes, as AMR gene transfer between plasmid backgrounds within bacteria
420 may occur over short timescales, and the interaction of several plasmids (i.e. not just
421 those harbouring the AMR gene of interest at any given time) in a population may be
422 highly relevant to the persistence and dissemination of the AMR gene itself.
423

424 **MATERIAL AND METHODS**

425 *Study isolates and setting*

We sequenced archived carbapenem-resistant Enterobacterales isolates from two hospital groups in Manchester (formerly known as CMFT and UHSM), aiming to include all inpatient isolates archived in the early stages of the observed outbreak, 2009-2011, and a subset of *bla*$_{KPC}$-positive Enterobacterales (KPC-E) isolates sequenced as part of regional and national surveillance undertaken by Public Health England (PHE, 2009-2014). The PHE set included: (i) up to the first 25 consecutive KPC-E isolates from any hospital in North-West England (2009-2014) and referred to the PHE reference laboratory (2009-2014); (ii) the first KPC-E isolate from any other hospital in the UK and the Republic of Ireland referred to PHE (2009-2014); and, (iii) any KPC-E isolates from outpatient/primary care settings in the UK referred to PHE (2009-2014).

Ethical approval was not required as only bacterial isolates were sequenced, and their collection was part of outbreak investigation and management.

DNA extraction and sequencing

For short-read Illumina sequencing (HiSeq 2500, 150bp PE reads), DNA was extracted using Quickgene (Fujifilm, Japan), with an additional mechanical lysis step following chemical lysis (FastPrep, MP Biomedicals, USA). Sequencing libraries were constructed using the NEBNext Ultra DNA Sample Prep Master Mix Kit (NEB) with minor modifications and a custom automated protocol on a Biomek FX (Beckman). Ligation of adapters was performed using Illumina Multiplex Adapters, and ligated libraries were size-selected using Ampure magnetic beads (Agencourt). Each library was PCR-enriched with custom primers (Index primer plus dual index PCR primer) (32). Enrichment and adapter extension of each preparation was

obtained using 9ul of size-selected library in a 50ul PCR reaction. Reactions were then purified with Ampure beads (Agencourt/Beckman) on a Biomek NXp after 10 cycles of amplification (as per Illumina recommendations). Final size distributions of libraries were determined using a Tapestation 1DK system (Agilent/Lab901), and quantified by Qubit fluorometry (Thermofisher).

For long-read sequencing (PacBio [n=28]), DNA was extracted using the Qiagen Genomic tip 100/G kit (Qiagen, Netherlands). DNA extracts were initially sheared to an average length of 15kb using g-tubes, as specified by the manufacturer (Covaris). Sheared DNA was used in SMRTbell library preparation, as recommended by the manufacturer. Quantity and quality of the SMRTbell libraries were evaluated using the High Sensitivity dsDNA kit and Qubit Fluorimeter (Thermo Fisher Scientific) and DNA 12000 kit on the 2100 Bioanalyzer (Agilent). To obtain the longest possible SMRTbell libraries for sequencing (as recommended by the manufacturer), a further size selection step was performed using the PippinHT pulsed-field gel electrophoresis system (Sage Science), enriching for the SMRTbell libraries >15kb for loading onto the instrument. Sequencing primer and P6 polymerase were annealed and bound to the SMRTbell libraries, and each library was sequenced using a single SMRT cell on the PacBio RSII sequencing system.

Sequencing data have been deposited in the NCBI (BioProject Accession: PRJNA564424).

Sequence data processing and assembly

We used Kraken(33) to assign species to sequenced isolates. SPAdes(34) v3.6 was used to *de novo* assemble reads (default options; subsequent removal of contigs shorter than 500bp and assembly coverage <2X). Isolates with sequence assemblies >6.5Mb were excluded to ensure that potentially mixed sequences were not included in the analyses. MLST was derived in silico by blasting de novo assemblies against publicly available MLST databases for *E. coli* (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), *K. pneumoniae, E. cloacae and K. oxytoca* (https://pubmlst.org/). Isolates with mixed MLST outputs were excluded. Antimicrobial resistance (AMR) genes, plasmid replicon (Inc) types and insertion sequences (IS) were identified using resistType (https://github.com/hangphan/resistType_docker; curated AMR gene database as in(35), plasmid replicon reference sequences from PlasmidFinder(36), ISs from the ISFinder platform(37); ≥80% identity used as a threshold). bla_{KPC} copy number for each isolate was estimated by dividing coverage of the contig containing bla_{KPC} by the average coverage for the assembly. Plasmid MLST for common family types identified in short read data and for resolved genomes (i.e. based on PacBio and Pilon assemblies - see below) was confirmed by 100% sequence matches to reference alleles for families catalogued in the plasmidMLST website (https://pubmlst.org/plasmid/; IncA/C, IncHI1/2, IncN).

PacBio sequencing data were assembled using the HGAP pipeline(38), and polished with the corresponding Illumina datasets using Pilon (version 1.18, default parameters)(39). Chromosomal sequences and plasmid sequences were then manually curated where possible to create complete, closed, circular structures by using BLASTn to identify overlaps at the end of assembled contigs. Those with overlapping

ends larger than 1000bp with sequence identity >99% were considered circularised/complete, and trimmed appropriately for resolution. Complete sequences were annotated using PROKKA (version 1.11)(40); annotations were used to determine genes known to encode toxin-antitoxin systems, heavy metal resistance, and anti-restriction mechanisms.

Tn4401 typing

Tn*4401* typing was performed using TETyper(10), using the Tn*4401*, SNP and structural profile reference files included with the package (https://github.com/aesheppard/TETyper; Aug 2019), and a flanking length of 5bp, representative of the known target site signature sequence indicative of Tn*4401* transposition(41).

Plasmid database for bla_{KPC} plasmid typing

A reference bla_{KPC} plasmid sequence database composed of bla_{KPC}-harbouring contigs/plasmids from long-read sequencing of isolates in this study and all complete bla_{KPC} plasmids from (42-44) (August 2018) was used for bla_{KPC} plasmid typing within this study. To construct this database, all 279/6018 evaluable plasmid sequences carrying bla_{KPC} were first compared using *dnadiff*(45) to obtain the pairwise similarity between any two plasmid sequences p_i and p_j. The similarity was defined as a function of their lengths l_i, l_j, and the aligned bases l_{ij}, l_{ji} as reported by:

$$(p_i, p_j) = \frac{1}{2}\left(\frac{l_{ij}}{l_i} + \frac{l_{ji}}{l_j}\right) \times \min\left(\frac{l_i}{l_j}, \frac{l_j}{l_i}\right)$$

The score was designed to penalise differences in length of the compared sequences, i.e. to make sequences of different lengths proportionately more different. The

resulting similarity matrix was used to perform clustering of plasmid sequences using the affinity propagation clustering technique, suitable for graph clustering problems with sparse similarity matrix and uneven cluster size and cluster number(46), and resulted in 34 clusters of 1-43 plasmids per cluster (Table S3). The largest cluster was the set of pKpQIL-like plasmids comprising 43 related sequences. Representative sequences of each *bla*$_{KPC}$ plasmid cluster in this network were chosen randomly, to generate a set (*KPC-pDB*) of plasmids ranging from 7,995bp (NC_022345.1; plasmid pAP-2) to 447,095bp (NZ_CP029436.1; plasmid pKPC_CAV2013) in the final database used for *bla*$_{KPC}$ plasmid typing in this study.

Subsequently, *bla*$_{KPC}$ plasmid typing for each study isolate sequence was performed as follows: (1) assembled sequences for each isolate were BLASTed (BLASTn) against *KPC-pDB*; (2) any >1kb contig with >90% nucleotide identity and >80% total coverage match to sequences in *KPC-pDB* was retained; (3) for any sequence p_i in *KPC-pDB*, a score s_i was calculated by dividing the total matched bases of all contigs matched to p_i by p_i's length; and (4) an isolate was assumed to plausibly carry p_i if $s_i \geq 0.80$. An isolate could have several *bla*$_{KPC}$ plasmid matches; we restricted to the top match for each isolate in our analyses.

Statistical analysis and data visualisation

Statistical analysis (Kruskal-Wallis testing) was carried out in Stata 14.2. Plots for figures 1, 2, 5 and S1 were generated using ggplot2 in R (version 1.1.463). Figure 4 was generated using the GenomeDiagram package(47) in Biopython(48).

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569 investigators are listed alphabetically, and include several of the authors also listed by
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776

Figure legends

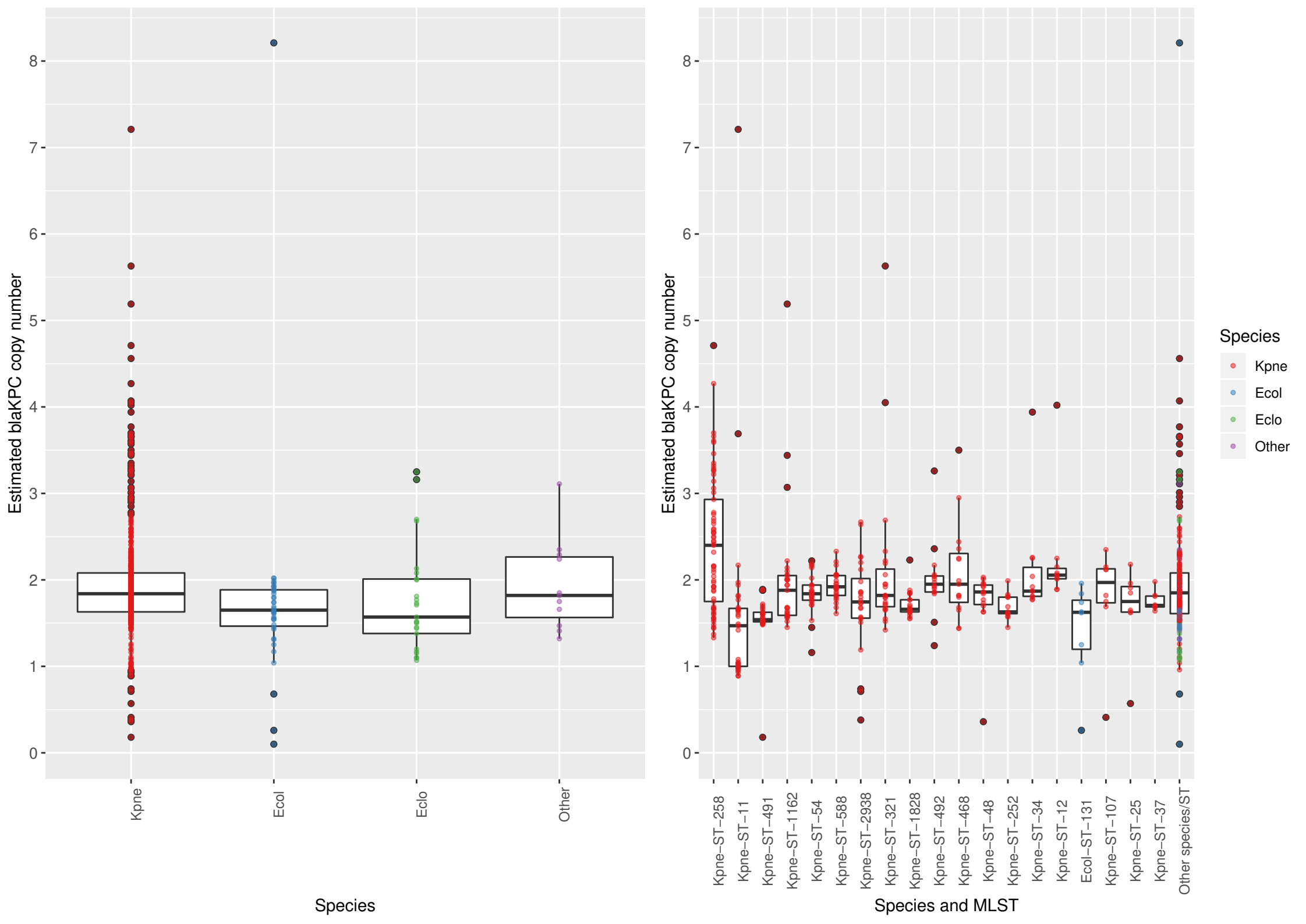
Figure 1. Estimated *bla*$_{KPC}$ copy number distributions within major species (Fig.1A), and the top nineteen commonest species/ST combinations (Fig.1B) observed within the study (other ST/species combinations assigned as "Other" or "Other species/ST" respectively). Dots represent estimated copy number for single isolates; boxplots represent median estimated *bla*$_{KPC}$ copy number +/- 1.58*IQR/sqrt(n). Boxplots are ordered by most common species and species/ST categories, left-to-right, except for the "Other", "Other species/ST", assigned to the right of the plots. For species assignations, "Kpne" = *Klebsiella pneumoniae*, "Ecol" = *Escherichia coli*, and "Eclo" = *Enterobacter cloacae*.

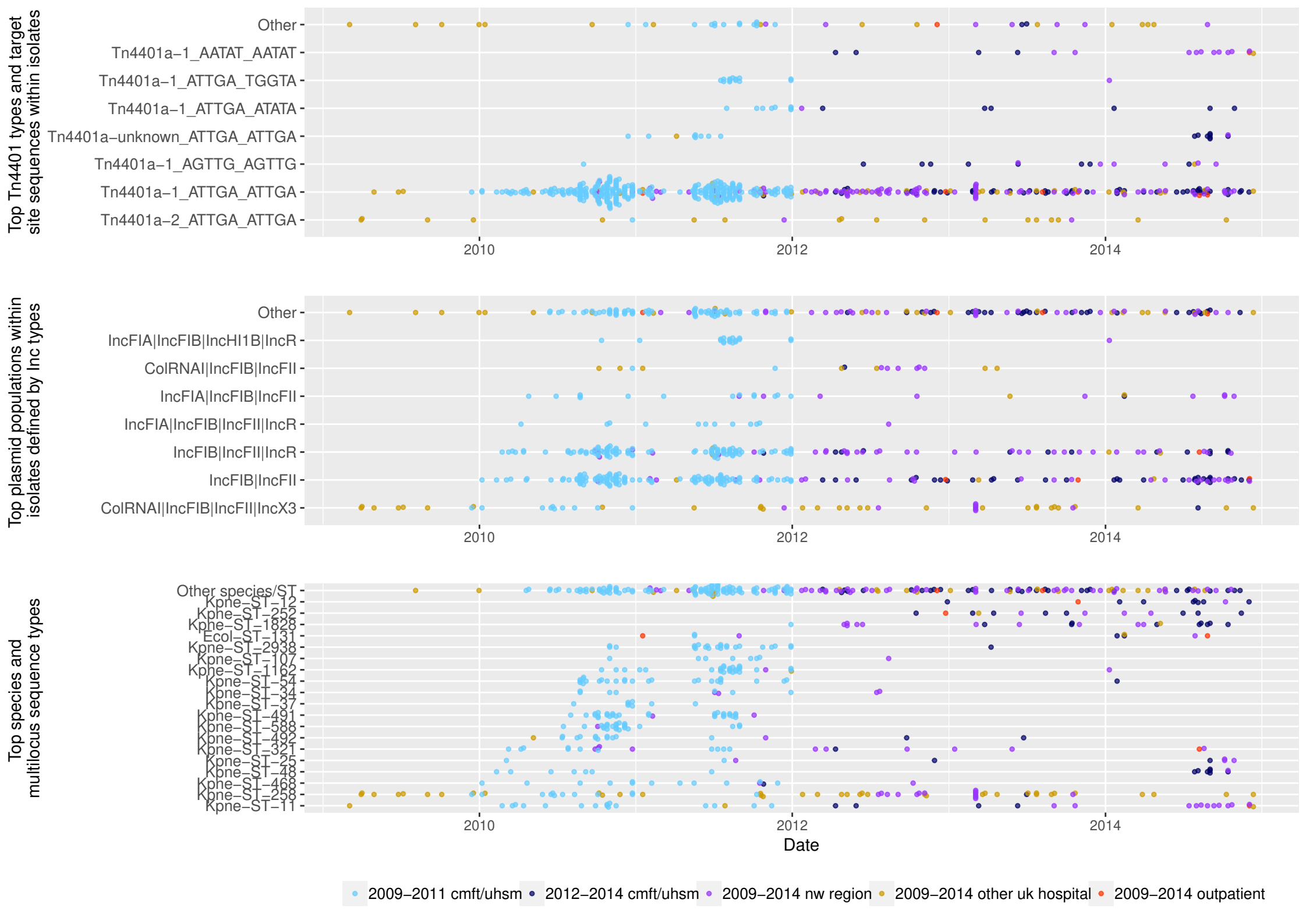
Figure 2. Incidence plots for 604 isolates included in the analysis. Dots are coloured by location of isolate collection, as defined in Methods. (A) Incidence plot of Tn*4401*/target site sequence (TSS; categories including ≥10 isolates); Tn*4401*a-1 is *bla*$_{KPC-2}$/Tn*4401*a, Tn*4401*a-2 is *bla*$_{KPC-3}$/Tn*4401*a; Tn*4401*a-unknown comprises a set of Tn*4401*a (n=18) with mutational variation including C684T, G962A C3042Y|G4739R, C4121T, G5583A and C7187T. (B) Incidence plot of plasmid replicon combinations (categories including ≥10 isolates). (C) Incidence plot of species/ST (top 20 common categories as in Fig.1).

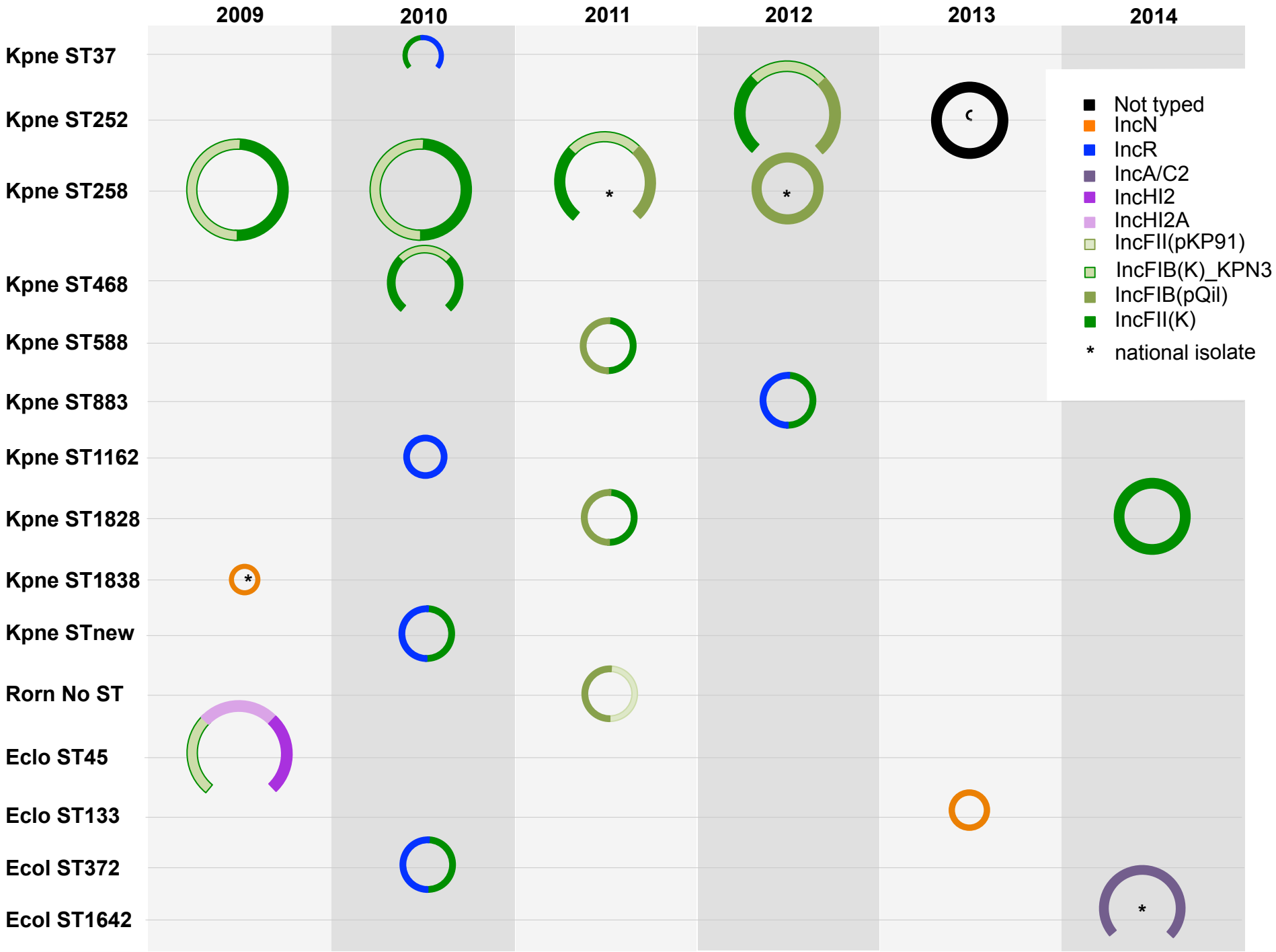
Figure 3. Schematic of *bla*$_{KPC}$ plasmid types and sizes identified from long-read/short-read hybrid sequencing approach by species/ST and year of collection (NB only 21 contigs clearly designated as plasmid are represented). Closed circles denote circularised contigs (i.e. complete plasmids); circle colours denote replicon types assigned to each plasmid sequence (i.e. multiple colours represent multi-replicon

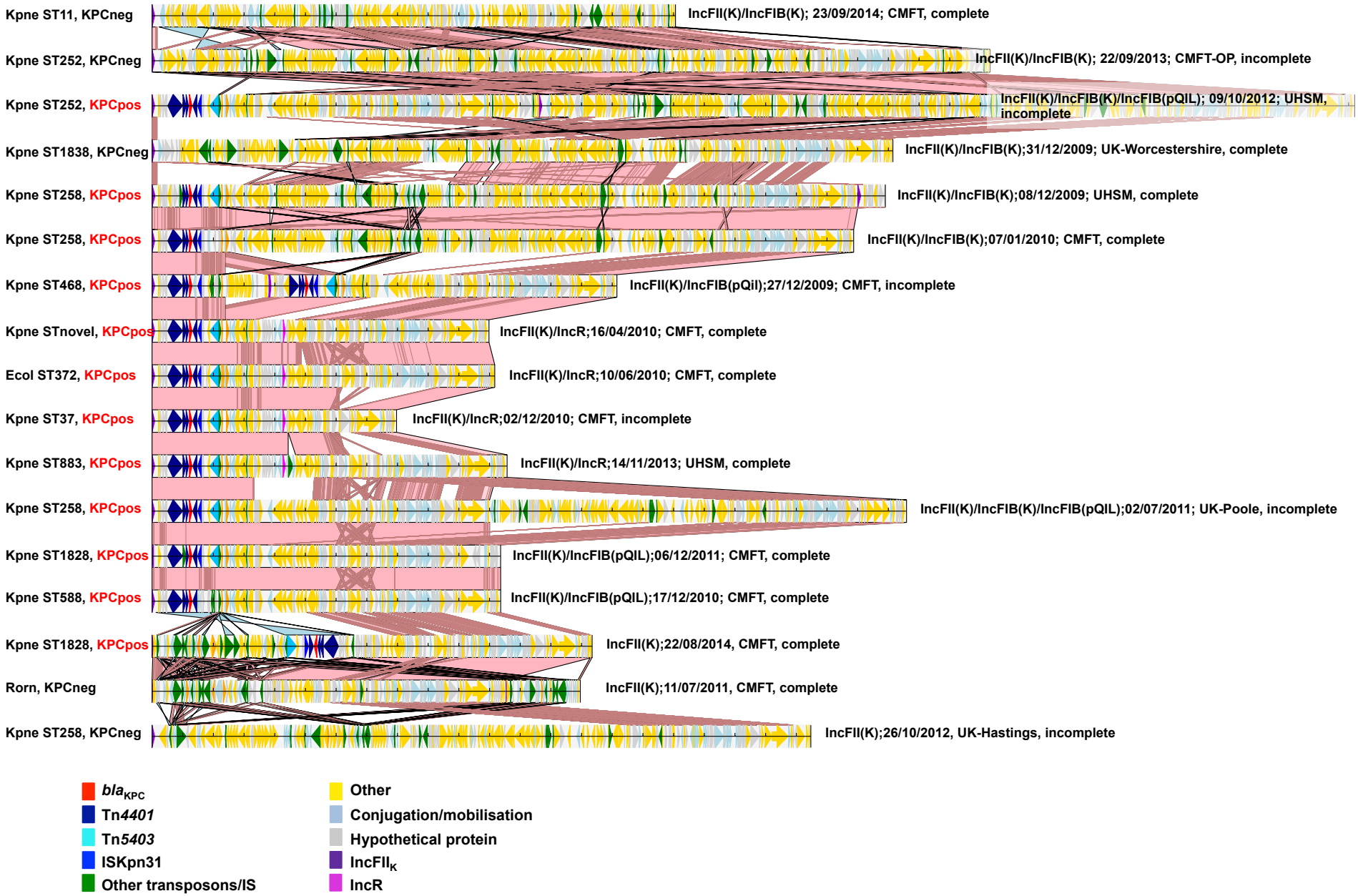
802 plasmids). Plasmids from isolates from the wider UK collection are denoted with a
803 "*".
804
805 **Figure 4**. Alignments of plasmid sequences harbouring an IncFII(K)_1_CP000648-
806 like replicon, includes *bla*$_{KPC}$-negative and *bla*$_{KPC}$-positive sequences. All sequences
807 were re-orientated to start at IncFII for the purposes of alignment visualization (this
808 also includes incomplete sequences, for which the exact structure and order may
809 therefore be a proxy only). Loci of interest have been coloured and annotated as
810 shown. Shading between sequences denotes regions of homology, with light pink
811 shading denoting areas ≥90% nucleotide identity, dark pink areas ≥50% nucleotide
812 identity, and light blue areas ≥90% nucleotide identity in reverse orientation. The
813 order of sequences is adjusted to highlight genetic overlap between sequences, but not
814 to imply any specific direct exchange events.
815
816 **Figure 5**. Short-read *bla*$_{KPC}$ plasmid typing results (top match) for isolates by species
817 and date. Dots are coloured by location of isolate collection, as defined in Methods.
818
819 **Supplementary Tables and Figures**
820 **Figure S1.** Estimated *bla*$_{KPC}$ copy number distributions for species/ST combinations.
821 Dots represent estimated copy number for single isolates; boxplots represent median
822 estimated *bla*$_{KPC}$ copy number +/- 1.58*IQR/sqrt(n). For species assignations, "Eclo"
823 = *Enterobacter cloacae*, "Ecol" = *Escherichia coli*, "Ente" = *Enterobacter* spp.,
824 "Kluy" = *Kluvera* spp., "Koxy" = *Klebsiella oxytoca*, "Kpne" = *Klebsiella*
825 *pneumoniae*, "Raou" = *Raoultella ornithinolytica*, "Serr" = *Serratia marcescens*.

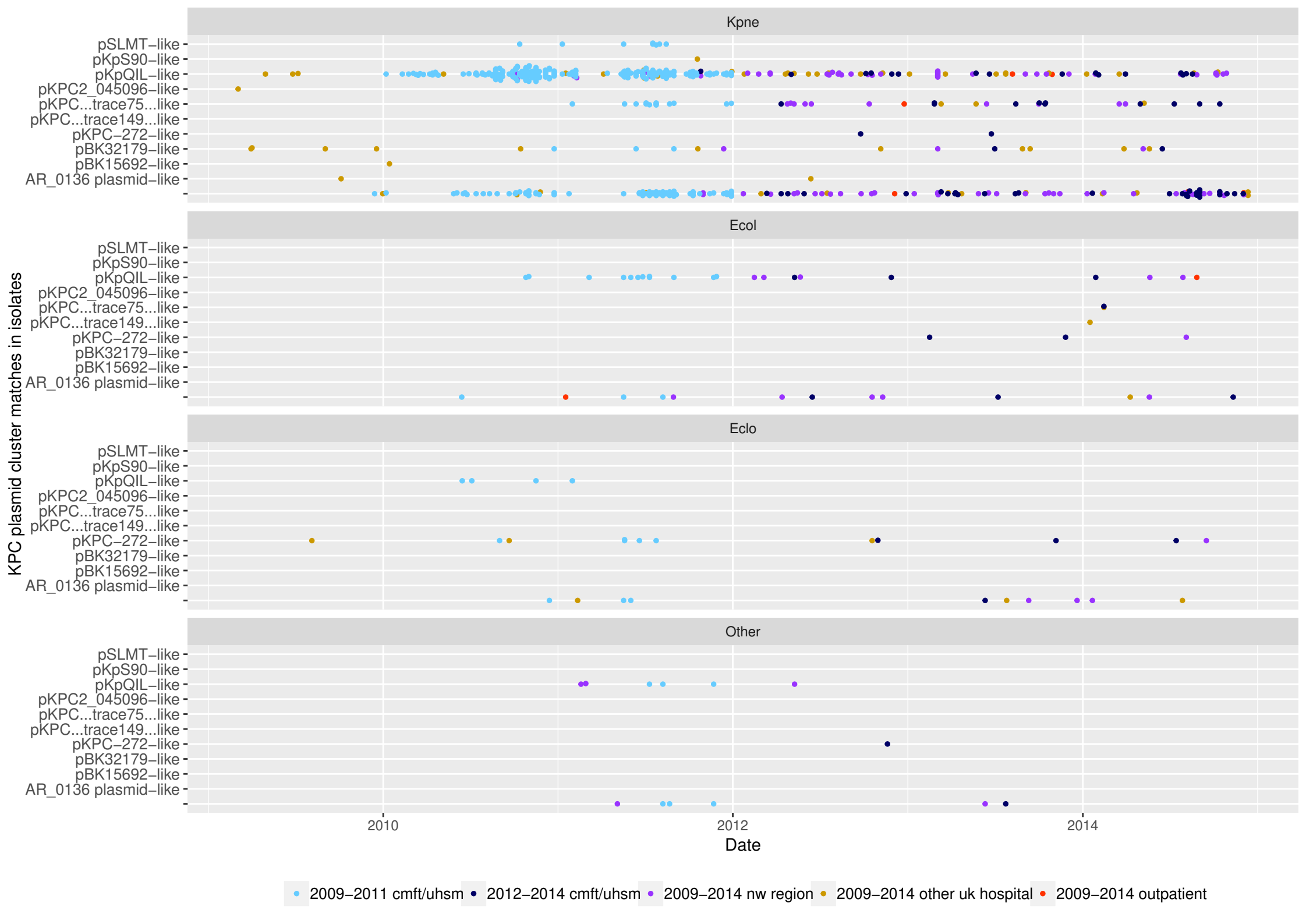
826 **Table S1.** Details of isolates assembled using short-read (Illumina) and long-read
827 (PacBio) datasets.
828
829 **Table S2.** Plasmid typing matches for isolates with short-read (Illumina) and long-
830 read (PacBio) assemblies and reconstructed plasmid structures.
831
832 **Table S3.** Assignation of *bla*$_{KPC}$ plasmids in study reference database to clusters for
833 plasmid typing.


Estimated blaKPC copy number
0
1
2
3
4
5
6
7
8
Kpne
Ecol
Eclo
Other
Species
Species and MLST
Kpne–ST–258
Kpne–ST–11
Kpne–ST–491
Kpne–ST–1162
Kpne–ST–54
Kpne–ST–588
Kpne–ST–2938
Kpne–ST–321
Kpne–ST–1828
Kpne–ST–492
Kpne–ST–468
Kpne–ST–48
Kpne–ST–252
Kpne–ST–34
Kpne–ST–12
Ecol–ST–131
Kpne–ST–107
Kpne–ST–25
Kpne–ST–37
Other species/ST
Species
Kpne
Ecol
Eclo
Other


Top Tn4401 types and target site sequences within isolates
Other
Tn4401a−1_AATAT_AATAT
Tn4401a−1_ATTGA_TGGTA
Tn4401a−1_ATTGA_ATATA
Tn4401a−unknown_ATTGA_ATTGA
Tn4401a−1_AGTTG_AGTTG
Tn4401a−1_ATTGA_ATTGA
Tn4401a−2_ATTGA_ATTGA
2010
2012
2014
Top plasmid populations within isolates defined by Inc types
Other
IncFIA|IncFIB|IncHI1B|IncR
ColRNAI|IncFIB|IncFII
IncFIA|IncFIB|IncFII
IncFIA|IncFIB|IncFII|IncR
IncFIB|IncFII|IncR
IncFIB|IncFII
ColRNAI|IncFIB|IncFII|IncX3
2010
2012
2014
Top species and multilocus sequence types
Other species/ST
Kpne−ST−12
Kpne−ST−252
Kpne−ST−1828
Ecol−ST−131
Kpne−ST−2938
Kpne−ST−107
Kpne−ST−1162
Kpne−ST−54
Kpne−ST−34
Kpne−ST−37
Kpne−ST−491
Kpne−ST−588
Kpne−ST−492
Kpne−ST−321
Kpne−ST−25
Kpne−ST−48
Kpne−ST−468
Kpne−ST−258
Kpne−ST−11
2010
2012
2014
Date
2009−2011 cmft/uhsm
2012−2014 cmft/uhsm
2009−2014 nw region
2009−2014 other uk hospital
2009−2014 outpatient


2009
2010
2011
2012
2013
2014
Kpne ST37
Kpne ST252
Kpne ST258
Kpne ST468
Kpne ST588
Kpne ST883
Kpne ST1162
Kpne ST1828
Kpne ST1838
Kpne STnew
Rorn No ST
Eclo ST45
Eclo ST133
Ecol ST372
Ecol ST1642
Not typed
IncN
IncR
IncA/C2
IncHI2
IncHI2A
IncFII(pKP91)
IncFIB(K)_KPN3
IncFIB(pQil)
IncFII(K)
* national isolate


Kpne ST11, KPCneg
IncFII(K)/IncFIB(K); 23/09/2014; CMFT, complete
Kpne ST252, KPCneg
IncFII(K)/IncFIB(K); 22/09/2013; CMFT-OP, incomplete
Kpne ST252, KPCpos
IncFII(K)/IncFIB(K)/IncFIB(pQIL); 09/10/2012; UHSM, incomplete
Kpne ST1838, KPCneg
IncFII(K)/IncFIB(K);31/12/2009; UK-Worcestershire, complete
Kpne ST258, KPCpos
IncFII(K)/IncFIB(K);08/12/2009; UHSM, complete
Kpne ST258, KPCpos
IncFII(K)/IncFIB(K);07/01/2010; CMFT, complete
Kpne ST468, KPCpos
IncFII(K)/IncFIB(pQil);27/12/2009; CMFT, incomplete
Kpne STnovel, KPCpos
IncFII(K)/IncR;16/04/2010; CMFT, complete
Ecol ST372, KPCpos
IncFII(K)/IncR;10/06/2010; CMFT, complete
Kpne ST37, KPCpos
IncFII(K)/IncR;02/12/2010; CMFT, incomplete
Kpne ST883, KPCpos
IncFII(K)/IncR;14/11/2013; UHSM, complete
Kpne ST258, KPCpos
IncFII(K)/IncFIB(K)/IncFIB(pQIL);02/07/2011; UK-Poole, incomplete
Kpne ST1828, KPCpos
IncFII(K)/IncFIB(pQIL);06/12/2011; CMFT, complete
Kpne ST588, KPCpos
IncFII(K)/IncFIB(pQIL);17/12/2010; CMFT, complete
Kpne ST1828, KPCpos
IncFII(K);22/08/2014, CMFT, complete
Rorn, KPCneg
IncFII(K);11/07/2011, CMFT, complete
Kpne ST258, KPCneg
IncFII(K);26/10/2012, UK-Hastings, incomplete
blaKPC
Tn4401
Tn5403
ISKpn31
Other transposons/IS
Other
Conjugation/mobilisation
Hypothetical protein
IncFIIK
IncR


Kpne
Ecol
Eclo
Other
pSLMT−like
pKpS90−like
pKpQIL−like
pKPC2_045096−like
pKPC...trace75...like
pKPC...trace149...like
pKPC−272−like
pBK32179−like
pBK15692−like
AR_0136 plasmid−like
KPC plasmid cluster matches in isolates
2010
2012
2014
Date
2009−2011 cmft/uhsm
2012−2014 cmft/uhsm
2009−2014 nw region
2009−2014 other uk hospital
2009−2014 outpatient