Long read sequencing provides an insight into plasmids found among carbapenemase producing *Enterobacterales* from hospitals in the United Kingdom during 2021 to 2023

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Carbapenemases, plasmids, nanopore sequencing

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Abstract

107 isolates of *Enterobacterales* consisting of *Klebsiella pneumoniae* (n=90), *Escherichia coli* (n=7), *Enterobacter cloacae* complex (n=6), *Klebsiella oxytoca* complex (n=3) and *Citrobacter freundi* (n=1) and additionally an isolate of *Acinetobacter baumannii* carrying genes encoding NDM (NDM-1, NDM-5 and NDM-14), KPC (KPC-2 and KPC-3), OXA-48-like (OXA-48, OXA-181 and OXA-232) and IMP (IMP-1 and IMP-4) carbapenemases were sequenced using q20 nanopore chemistry to provide complete/near-complete assemblies and relevant plasmids compared. Investigation of potential 'plasmid outbreaks' in individual hospitals among isolates of different types and species revealed a mixed situation with some isolates carrying similar plasmids, but with segments missing/added and some plasmids that were clearly distinct.

While most plasmids carrying $bla_{\text{OXA-48}}$ were typical IncL plasmids of approximately 60 kb that are widely described, there was some variation among these. One isolate carried an IncR plasmid that had only limited homology with the others. Identical 51,479 bp ColKP3/IncX3 plasmids carrying $bla_{\text{OXA-181}}$ were found from isolates from different hospitals that exactly matched those on GenBank from other countries. In other isolates $bla_{\text{OXA-181}}$ was carried on IncFII plasmids. $bla_{\text{OXA-232}}$ was found in highly conserved small ColKP3 plasmids that matched those found in other countries and continents. These observations highlight the importance of understanding the wider distribution of plasmids of concern.

IncHI2/IncHI2A plasmids were important vehicles for carbapenemase genes and were found with $bla_{\text{KPC-2}}$, $bla_{\text{IMP-1}}$, $bla_{\text{IMP-4}}$ or $bla_{\text{NDM-1}}$, sometimes with the colistin resistance gene *mcr-9* in addition. Representatives of *K. pneumoniae* sequence type (ST) 147 from seven hospitals carried IncFIB(pNDM-Mar)/IncHI1B(pNDM-MAR) hybrid virulence resistance plasmids of 325 to 352 kb that combined $bla_{\text{NDM-5}}$ and other resistance genes with genes found in virulence plasmids. A similar plasmid was also found in an isolate of *K. pneumoniae* ST1558 and has been described in representatives of ST383.

Nanopore sequencing has been instrumental in improving our knowledge of plasmids carrying carbapenemase genes leading to a better understanding of their epidemiology.

Introduction

Carbapenems have been considered 'last-resort' antibiotics for the treatment of infections caused by members of the *Enterobacterales* belonging to genera such as *Klebsiella*, *Enterobacter*, *Escherichia* and *Citrobacter*. However, the dramatic rise in incidence of carbapenemase enzymes, especially belonging to the NDM, OXA-48-like, KPC, IMP and VIM families, that can degrade these antibiotics among these organisms seriously threatens their effectiveness [1]. The genes encoding these carbapenemases are mostly carried in plasmids, but in many instances, these remain uncharacterised.

The advent of q20 high accuracy nanopore sequencing [2] has facilitated complete assembly of bacterial chromosome and their plasmids, providing an opportunity to investigate and describe plasmids, including those carrying carbapenemase genes. This not only provides a better understanding of their genetic environment but enables an assessment of the extent to which plasmids or other elements carrying carbapenemase genes may be shared between organisms of different species from the same and different hospitals. Here we describe sequences from isolates of *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae* complex, *Klebsiella oxytoca* complex, *Citrobacter freundi* and additionally an isolate of *Acinetobacter baumannii* carrying a variety of carbapenemase genes encoding NDM (NDM-1, NDM-5 and NDM-14), KPC (KPC-2 and KPC-3), OXA-48-like (OXA-48, OXA-181 and OXA-232) and IMP (IMP-1 and IMP-4) carbapenemases. In order to answer various questions around potentially shared plasmids and around suitability of the method for typing of *Klebsiella pneumoniae*, there is a focus on isolates from particular hospitals and on particular types, especially *K. pneumoniae* Sequence Types (ST) 147, 307 and 20, but the 108 isolates included consisted of many types and were from a total of 30 hospital laboratories. This work provides an insight into plasmids circulating among *Enterobacterales* in UK hospitals between 2021 and 2023.

Methods

Isolates were referred to the UK reference laboratory for typing from hospitals in the UK and were typed by Variable Number Tandem Repeat (VNTR) analysis at 11 loci (*Klebsiella pneumoniae sensu stricto*) or pulsed-field gel electrophoresis of XbaI-digested genomic DNA (all other species). They were labelled by an organism code (KP for *K. pneumoniae*, EB for *E. cloacae* complex, ES for *E. coli*, CF for *C. freundii*, KO for *K. oxytoca* complex and AB for *Acinetobacter baumannii*) followed by a unique number for that organism, hospital code, the week and year of receipt, their sequence type and the carbapenemase genes that they carried (e.g., KP2_L3_37.21_ST147_NDM5). Hospitals were labelled according to region and number within that region (L, London; SE, South East England; SW, South West

79 England; WM, West Midlands; EM, East Midlands; NE, North East London; NW, North West
80 England; YH, Yorkshire and Humber). Patient duplicates (i.e. more than one isolate of the
81 same type from the same patient) were excluded. All isolates are listed in Table S1.

82 **Nanopore sequencing**

83 DNA from growth from single colony picks was extracted using a GeneJet genomic DNA kit
84 (ThermoFisher, UK) and sequenced on R10.4.1 flow cells on a minION Mk1C (or gridION for
85 run q20run8) following library preparation using the rapid barcoding kit SQK-RBK114.24
86 (Oxford Nanopore Technologies, UK). Library preparation was carried out following the
87 protocol provided by Oxford Nanopore Technologies, with concentration of the pooled
88 barcoded libraries using 'clumping buffer' as described previously [3] (rrp8, rrp9, q20runs 3-
89 7) or Ampure beads (q20runs 8-16). Basecalling was by the high accuracy basecalling
90 option with barcode trimming. MinKNOW versions were 22.10.7 for q20 run 2 (rrp9), 22.12.5
91 (bionic) for q20 runs 5, 7, 8, 10, 13 and 14 and 23.04.5 (bionic) for q20 runs 15 and 16. A
92 speed of 260 bp/s was used unless only a 400 bp/s option was available. Assemblies were
93 done using flye 2.9.1-b1780 [4] and medaka 1.7.2
94 (https://github.com/nanoporetech/medaka). Resistance genes, plasmid replicon types,
95 plasmid sequence types (pST) and MLST types were sought using ResFinder 4.3.3,
96 PlasmidFinder 2.1, pMLST 2.0 and MLST 2.0 on the Center for Genomic Epidemiology
97 website (https://cge.food.dtu.dk/) [5, 6, 7].

98 **Resistance genes**

99 Resistance genes were as detected on ResFinder. Where there was no exact match, the
100 closest allele(s) are provided. As a guide, the antibiotic classes to which the most common
101 acquired genes found in this study confer resistance are summarised in Table 1, but fuller
102 information is available on ResFinder (http://genepi.food.dtu.dk/resfinder).

103

Resistance class	Resistance genes beginning
aminoglycoside	*aac*, *aad*, *ant*, *aph*, *armA*, *rmt*
quinolone	*qnr*
Beta-lactam	bla_{KPC}, $bla_{CTX\text{-}M}$, bla_{TEM}, bla_{SHV}, bla_{IMP}, bla_{NDM}, bla_{ACT}, bla_{CMY}, bla_{OXA}, bla_{ADC}
folate pathway antagonist	*sul*, *dfrA*
tetracycline	*tet*
Streptogramins	*msr*
macrolides	*ere*, *erm*, *mph*
polymyxin	*mcr*
amphenicol	*cat*

104 Table 1. Guide to antibiotic resistance classes to which acquired resistance genes found in
105 this study confer resistance. Fuller information is available from ResFinder
106 (http://genepi.food.dtu.dk/resfinder).

107 **Sequences**

108 Sequences were deposited on GenBank under project PRJNA1010831.

109 **Results and Discussion**

110 **Isolates from hospitals querying 'plasmid outbreaks'**

111 Many of these isolates were referred by hospitals concerned that they may be experiencing
112 plasmid outbreaks and often consisted of sets containing multiple species of
113 *Enterobacterales*. Here we describe the results of investigation of three such sets.

114 a) Isolates from hospital WM3 carrying bla_{KPC-2}

115 Thirteen isolates, all carrying bla_{KPC-2}, were sequenced, and carried a mixture of plasmid
116 types. Four isolates (EB4_WM3_44.22_NV1_KPC2, KO1_WM3_47.22_ST183_KPC2,
117 KO3_WM3_07.23_ST77_KPC2 and KP111_WM3_19.23_ST846_KPC2) belonging to three
118 different species (*Enterobacter hormaechei*, *Klebsiella michiganensis* and *K. pneumoniae*)
119 and all of different types carried an IncN plasmid of pST15 of 63-69 kb carrying bla_{KPC-2},
120 *aph(6)-Id*, *aph(3")-Ib*, bla_{TEM-1B}, *qnrB2*, *sul1* and *dfrA19* (e.g. CP141849) (Fig. 1). On
121 GenBank the most similar plasmid was also in an isolate (S11_16) of *E. hormaechei* from
122 the UK (CP035387) collected in 2016. However, other isolates
123 (EB6_WM3_49.22_ST66_KPC2 and ES6_WM3_50.22_ST95_KPC2) carried an
124 IncHI2/IncHI2A plasmid of approximately 300 kb with bla_{KPC-2}, *aadA2b*, *ant(2")-Ia*, $bla_{CTX-M-9}$,
125 *qnrA1*, *sul1*, *tet(A)*, *dfrA16* (e.g. CP133857); a further isolate
126 (KP71_WM3_51.22_ST462_KPC2) carried a larger IncHI2/IncHI2A plasmid (461 kb)
127 containing those same elements. Two isolates appeared to carry these elements
128 chromosomally (KP66_WM3_49.22_ST307_KPC2 and ES7_WM3_51.22_ST5295_KPC2);
129 this was the case for two separate runs carried out on these isolates. At least two other
130 plasmid types were also detected among the set, a 220 kb IncHI1B(pNDM-MAR)/
131 IncFIB(pNDM-Mar) plasmid carrying bla_{KPC-2}, *aph(3")-Ib*, *aph(6)-Id*, bla_{TEM-1B}, *qnrB2*, *sul1* and
132 *dfrA19*, (in KP64_WM3_49.22_ST307_KPC2) (CP141558) and a 270 kb IncFIB(pQil)
133 plasmid (in KP49_WM3_45.22_ST258_KPC2).

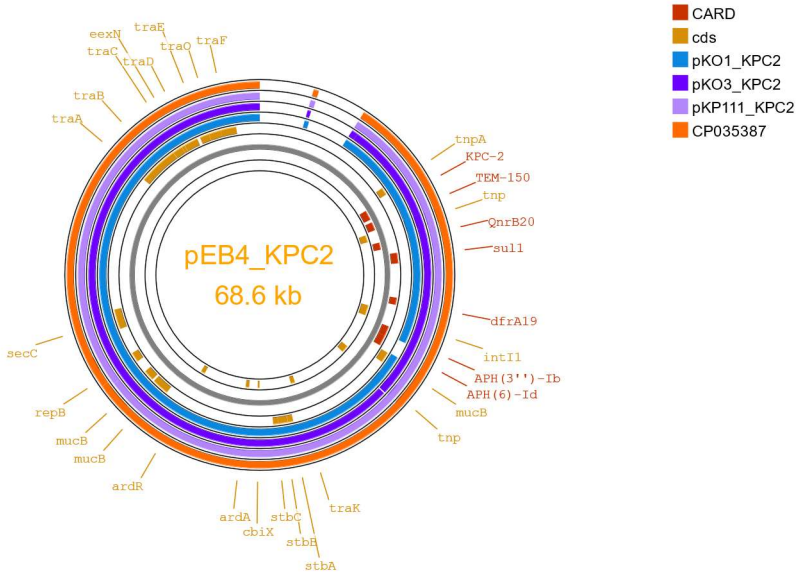


Fig. 1. BLAST comparison of IncN plasmids of pST15 carrying *bla*$_{KPC-2}$ from isolates from hospital WM3 and with that of *E. hormaechei* S11_16 (CP035387). Plasmids were compared with that of isolate EB4_WM3_44.22_NV1_KPC2 (pEB4_KPC2). Regions of homology are shown in colour. Plasmids were from isolates of three different species (EB, *Enterobacter cloacae* complex; KO, *Klebsiella oxytoca* group and KP, *Klebsiella pneumoniae*) and were compared by Proksee [8]. Resistance genes were identified by the Comprehensive Antibiotic Resistance Database (CARD) on Proksee. NV1 refers to a novel sequence type of the *Enterobacter cloacae* complex of allelic profile 59,9,12,9,242,37,6. Only named coding sequences (cds) are shown.

b) Isolates from hospital L5 carrying *bla*$_{NDM}$

Many of the isolates from this investigation were clonally related isolates of *K. pneumoniae* ST20 carrying *bla*$_{NDM-1}$. These carried an IncHI2/ IncHI2A plasmid of approximately 340 kb carrying *bla*$_{NDM-1}$, *aadA5*, *aac(6')-Ib-cr*, *aac(6')-IIc*, *bla*$_{DHA-1}$, *bla*$_{OXA-1}$, *ere(A)*, *mph(A)*, *catB3*, *qnrB4*, *sul1*, *dfrA17* (e.g. CP141854 from KP34_L5_41.22_ST20_NDM1). However, there were also isolates of *K. pneumoniae* of ST16 carrying IncX3 plasmids of 46 kb carrying *bla* $_{NDM-5}$ with no other resistance elements detected; these isolates also carried *bla*$_{OXA-232}$ in a relatively small (12.3 kb) ColKP3 plasmid (KP21_L5_33.22_ST16_NDM5_OXA232 and KP24_L5_34.22_ST16_NDM5_OXA232). There were also two further types of *K. pneumoniae* (ST147 and ST3096) carrying *bla*$_{NDM-5}$, each with further distinct plasmids, the two representatives of ST147 carrying a 156.5 kb IncFII/IncR plasmid with *bla*$_{NDM-5}$, *aadA2*, *aadA1*, *armA*, *rmtB*, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *msr(E)*, *ere(A)*, *mph(A)*, *mph(E)*, *erm(B)*, *cmlA1*, *qnrB1*, *ARR-2*, *sul1* and *dfrA12* (KP104_L5_18.23_ST147_NDM5_OXA232) and a 343.0 kb IncFIB(pNDM-Mar)/IncHI1B(pNDM-MAR) hybrid virulence/resistance plasmid (carrying

159 *bla*$_{NDM-5}$, *aadA1*, *aph(3')-VI*, *aac(6')-Ib*, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1C/1B}$, *bla*$_{OXA-9}$, *mph(A)*, truncated
160 *catA1*, *qnrS1*, *sul1*, *sul2*, *dfrA5* together with various genes associated with virulence
161 plasmids (*rmpA*/*A2*, *terABCDEWXYZ*, *iucABCD*,*iutA*, hemin, *shiF*, lysozyme inhibitor,
162 *cobW*)) (KP124_L5_21.23_ST147_NDM5) (CP137392), respectively, while the isolate of
163 ST3096 (KP52_L5_46.22_ST3096_NDM5_OXA48) carried a 143 kb IncC plasmid with
164 *bla*$_{NDM-5}$, *aac(6')-Ib3*, *bla*$_{CMY-23}$ and *sul1* (CP141561); this isolate also carried a 78.9 kb IncL
165 plasmid with *bla*$_{OXA-48}$, *aac(6')-Ib-cr*, *bla*$_{TEM-1D}$, *bla*$_{OXA-1}$, truncated *catB3* and *qnrS1*
166 (CP141563).

167 c) Isolates from hospital WM2 carrying *bla*$_{OXA-48}$ and/or *bla*$_{NDM-5}$

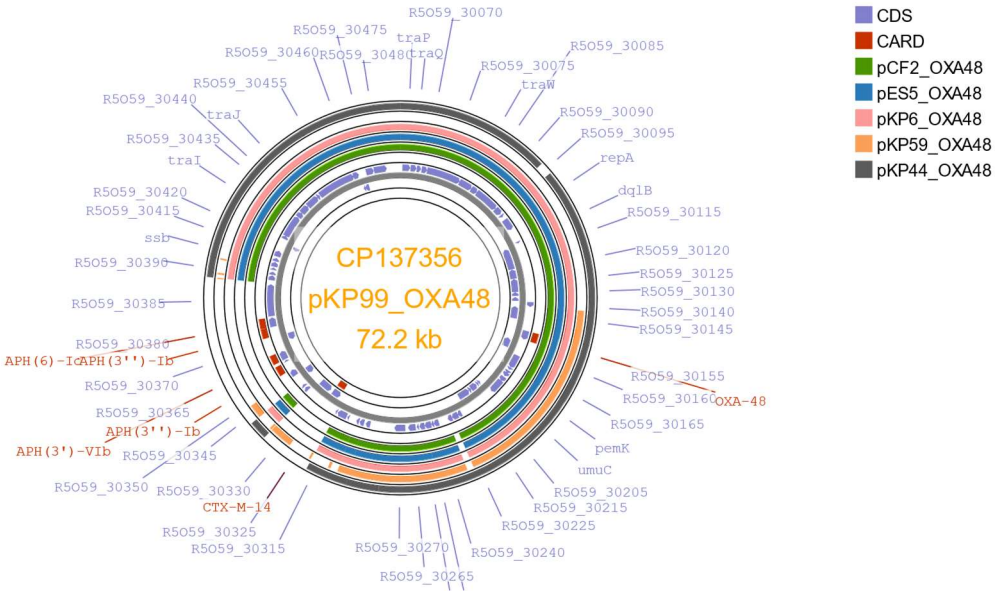
168 A set of seven isolates belonging to four different genera were sequenced, five of which
169 carried *bla*$_{OXA-48}$. Four of the five carried IncL plasmids (CF2_WM2_48.22_ST162_OXA48,
170 EB5_WM2_48.22_ST108_OXA48, ES5_WM2_48.22_ST1607_OXA48 and
171 KP58_WM2_48.22_ST147_NDM5_OXA48) (CP133849, CP133863, CP133873 and
172 CP137377, respectively) only one of which carried other resistance determinants in addition
173 to *bla*$_{OXA-48}$ (*aph(3'')-Ib*, *aph(6)-Id*, *bla*$_{CTX-M-14b}$ in CP137377). The remaining isolate
174 (KP59_WM2_48.22_ST395_OXA48) carried a 54.8 kb IncR plasmid that carried *catA1* in
175 addition to *bla*$_{OXA-48}$. Three isolates carried *bla*$_{NDM-5}$, one in a 459.9 kb IncFIB(pNDM-
176 Mar)/IncHI1B(pNDM-MAR) hybrid virulence/resistance plasmid (carrying *bla*$_{NDM-5}$, *aac(6')-Ib-*
177 *cr*, *aadA1*, *aph(3')-VI*, *aac(6')-Ib*, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1C/1B}$, *bla*$_{OXA-9}$, *mph(A)*, truncated *catA1*,
178 *qnrS1*, *sul1* and *dfrA5* together with virulence plasmid elements *rmpA*/*A2*, *terABCDEWXYZ*,
179 *iucABCD*, *iutA*, hemin, *shiF*, lysozyme inhibitor and *cobW*) (CP137375). The remaining
180 isolates carrying *bla*$_{NDM-5}$ were both representatives of *E. coli* ST167 with multi-replicon IncF
181 (IncFIA /IncFIB(AP001918)/IncFII) *bla*$_{NDM-5}$ plasmids (e.g. CP133853) widely found in this
182 organism [9, 10, 11]; they were highly similar to one another, one of 143.5 kb and the other
183 of 141.9 kb, both with *bla*$_{NDM-5}$, *aac(6')-Ib-cr*, *aadA2*, *sul1*, *dfrA12*, *sitABCD*, *tet(A)*, *bla*$_{CTX-M-15}$,
184 *bla*$_{OXA-1}$, *mph(A)*, truncated *qacE* and *catB3* (ES3_WM2_48.22_ST167_NDM5 and
185 ES4_WM2_48.22_ST167_NDM5).

186 In all three cases, a mixed scenario was observed with some isolates carrying highly similar
187 plasmids, while there were also clear examples where the plasmids were distinct.

188 **OXA-48 plasmids**

189 21 isolates in the set from eight hospital groups carried *bla*$_{OXA-48}$, the vast majority in IncL
190 plasmids of approximately 64 kb that contained no other resistance genes (e.g. CP133849,
191 CP133873, CP133846). These have a large number of matches on GenBank with these
192 plasmids being widely distributed. In some cases the IncL plasmid was larger (e.g.
193 CP133863 (130 kb), CP137356 (72.2 kb)) (Fig. 2). The latter included an extra segment that

194 carried *aph(6)-Id, aph(3")-Ib, aph(3')-Vib* and *bla*$_{CTX-M-14b}$. There were 5 examples of isolates
195 with a 61.5 kb IncM1 *bla*$_{OXA-48}$ plasmid with no other resistance genes; these were all
196 representatives of *K. pneumoniae* ST307 from the same hospital related in space and time
197 (KP44 to KP48 inclusive all followed by _NW4_44.22_ST307_OXA48) (e.g. CP141564 in
198 KP44_NW4_44.22_ST307_OXA48). KP59_WM2_48.22_ST395_OXA48 carried a 54.8 kb
199 IncR *bla*$_{OXA-48}$ plasmid also containing *catA1*, while KP17_NW3_25.22_ST395_OXA48
200 carried a 78.1 kb IncM2 *bla*$_{OXA-48}$ plasmid also carrying *msr(E)*, *armA*, *mph(E)*, *bla*$_{TEM-1B}$
201 resistance genes (CP133747). The IncR plasmid (pKP59_OXA48) shown in orange in Fig.
202 2) only had limited homology with the IncL, IncM1 and IncM2 plasmids (Fig. 2).



203

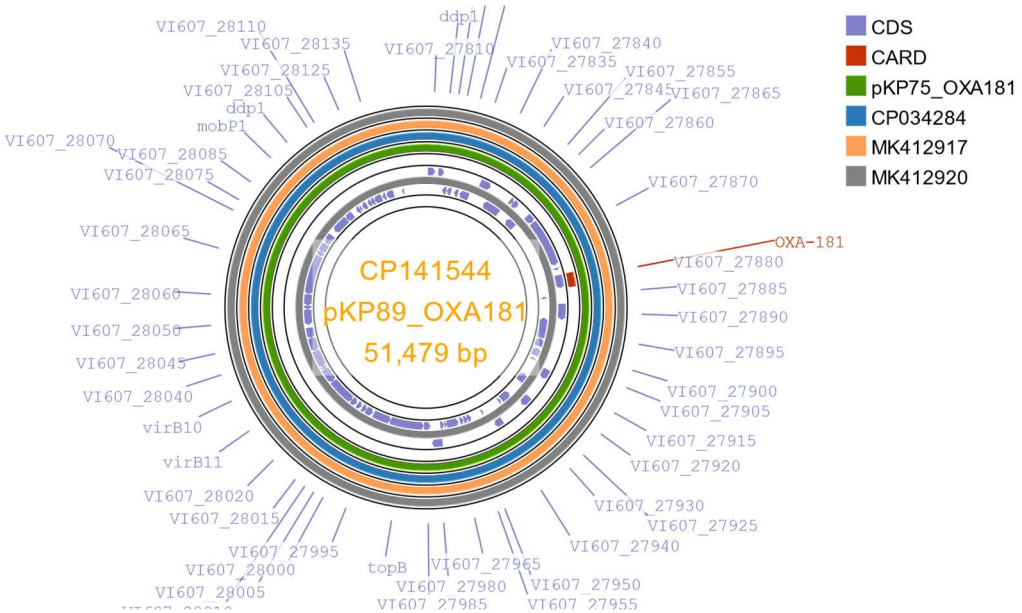
204 Fig. 2. BLAST comparison of plasmids carrying *bla*$_{OXA-48}$. Coloured regions show areas of
205 homology. Most isolates carried IncL plasmids of approximately 64 kb (e.g. pCF2_OXA48,
206 pES5_OXA48 and pKP59_OXA48), while pKP99_OXA48 had an extra segment carrying
207 additional resistance genes. KP44_NW4_44.22_ST307_OXA48 had an IncM1 plasmid of
208 61.5 kb (pKP44_OXA48). The IncR plasmid pKP59_OXA48 (54.8 kb) had only partial
209 homology with the IncL/M plasmids. Isolates compared were from four different hospitals,
210 with pCF2_OXA48, pES5_OXA48 and pKP59_OXA48 being from isolates from the same
211 hospital group. Plasmids were compared using Proksee [8]. Resistance genes were
212 identified by the Comprehensive Antibiotic Resistance Database (CARD) on Proksee. CDS,
213 coding sequences.

214 In agreement with our results, Boyd et al [12] also commented in a recent review that *bla*$_{OXA-}$
215 $_{48}$ is usually found in self-conjugative 60- to 70-kb plasmids with an IncL scaffold, but that
216 there is some variation among them, with some plasmids also carrying other resistance
217 genes. Notably, the *bla*$_{OXA-48}$ gene is not associated with class 1 integrons in these plasmids,

218 and is typically carried in transposon Tn*1999* with an upstream, and often a downstream,
219 IS*1999* insertion sequence [13].
220

221 **OXA-181 plasmids**

222 The set included nine isolates carrying *bla*$_{OXA-181}$ from six different hospitals. Most carried
223 ColKP3 replicons (e.g. CP137409 in KP102_WM3_17.23_ST147_OXA181, CP141549 in
224 KP75_L14_02.23_ST20_OXA181 and CP141544 in KP89_L16_06.23_ST307_OXA181)
225 and either no, or only *qnrS1* resistance gene in addition. The 51,479 bp ColKP3/IncX3
226 plasmids pKP75_OXA181 and pKP89_OXA181 matched (100 % identity, 100 % coverage)
227 one another and many others on GenBank (e.g. CP034284, MK412917, MK412920,
228 CP114995 and CP110780) from isolates of *K. pneumoniae*, *E. coli*, the *Enterobacter cloacae*
229 complex and *Citrobacter freundii* from various countries (e.g. USA, United Arab Emirates,
230 Italy) (Fig. 3). However, *bla*$_{OXA-181}$ was associated with IncFII replicon types in some isolates
231 (e.g. KP4_L4_52.21_ST147_OXA181). Li *et al*. [14] who studied 191 *bla*$_{OXA-48-like}$ plasmids in
232 *K. pneumoniae* also noted that *bla* $_{OXA-181}$ was often carried by 50-kb ColKP3-IncX3
233 conjugative plasmids, which were found in multiple countries, and in small ColKP3-type
234 mobilizable plasmids, mirroring our results.



235

236 Fig. 3. BLAST comparison of 51.5 kb ColKP3/IncX3 plasmids from isolates of *K.*
237 *pneumoniae* from two different hospitals and belonging to different sequence types from this
238 study with examples from GenBank from isolates of *K. pneumoniae* (CP034284 and
239 MK412920) and *E. coli* (MK412917) from USA and United Arab Emirates. All the plasmids
240 matched one another (100 % identity, 100 % coverage). CDS, coding sequences; CARD,
241 Comprehensive Antibiotic Resistance Database
242

In common with our results finding identical ColKP3/IncX3 plasmids carrying *bla*$_{OXA-181}$ from isolates of different types and genera and from various countries, others have also observed that *bla*$_{OXA-181}$ are predominantly associated with IncX3 and ColKP3 plasmids and that these are near identical among isolates from Jordan, Egypt, Turkey, South Korea, Thailand, South Africa, Kuwait, China and Angola [15,16].

OXA-232 plasmids

The set included nine isolates carrying *bla*$_{OXA-232}$ from seven different hospitals. As with the *bla*$_{OXA-181}$ plasmids, those carrying *bla*$_{OXA-232}$ were often associated with ColKP3 replicons (e.g. CP140296, CP141573, CP137419, CP141552). They carried no other resistance genes. The original assemblies indicated that three isolates carried a ColKP3 plasmid of 6.14 kb (KP28_L6_38.22_ST147_NDM5_OXA232, KP67_L13_49.22_ST437_NDM5_OXA232 (CP141552) and KP104_L5_18.23_ST147_NDM5_OXA232), 5 carried 12.3 kb plasmids (KP21_L5_33.22_ST16_NDM5_OXA232, , KP33_NE2_41.22_ST2096_OXA232 (CP140296), KP35_L8_42.22_ST2096_OXA232 (CP141573), KP54_WM4_48.22_ST147_OXA232 and KP101_SE2_14.23_ST147_NDM5_OXA232 (CP137419)) and one a 18.4 kb plasmid (KP24_L5_34.22_ST16_NDM5_OXA232), but closer examination showed that the 12.3 kb plasmids consisted of 2 copies of the 6.14 kb plasmid and the 18.4 kb plasmid 3 copies, perhaps because of an assembly issue with flye with high copy number reads. Analysis of the reads themselves showed that they mostly exist as 6.14 kb single copy plasmids although they may also be present as double and triple copy plasmids. There were many matches/close matches to these plasmids on GenBank (e.g. CP01562, JX423831, OY856406). The original description of OXA-232 described the gene in 6.1 kb plasmids [17] (e.g. JX423831), but there are also examples on GenBank of the 12.3 kb double version (e.g. in FDAARGOS_440 CP023924). Li *et al*. [14] also commented that the *bla*$_{OXA-232}$ gene was mainly found in small (6.1-kb) ColKP3-type plasmids, largely found in India in their study.

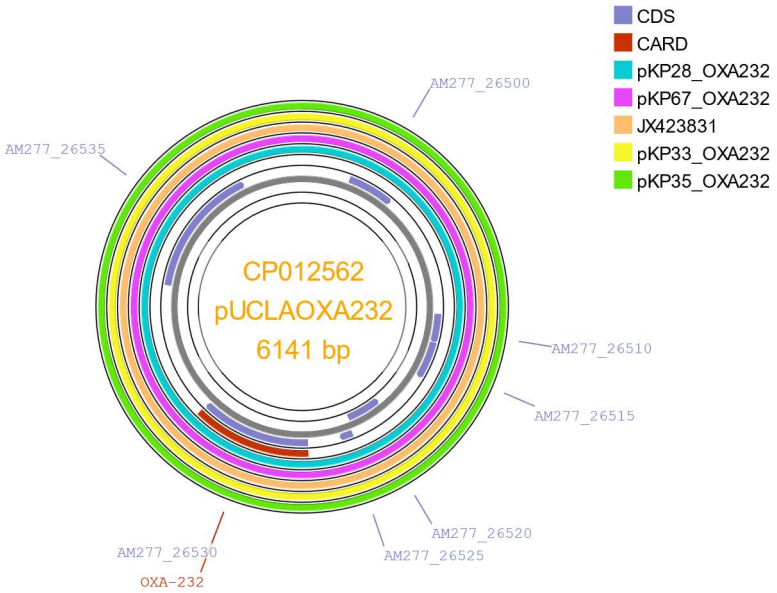


Fig. 4 BLAST comparison of ColKP3 plasmids carrying *bla*$_{OXA-232}$ in isolates of *K. pneumoniae* from this study with CP012562 (pUCLAOXA232-1) from an isolate of *K. pneumoniae* from California, USA and JX423831 from an isolate of *E. coli* from France (transferred from India). Plasmids compared are from KP28_L6_38.22_ST147_NDM5_OXA232, KP33_NE2_41.22_ST2096_OXA232, KP35_L8_42.22_ST2096_OXA232 and KP67_L13_49.22_ST437_NDM5_OXA232, which are from four different hospitals. Plasmids were compared using Proksee [8]. Resistance genes were identified by the Comprehensive Antibiotic Resistance Database (CARD) on Proksee. CDS, coding sequences.

KPC-2 plasmids

The majority of the isolates carrying *bla*$_{KPC-2}$ in this study were from hospital WM3 and have been described earlier; they carried mainly IncN or IncHI2/ IncHI2A *bla*$_{KPC-2}$ plasmids (Fig. 1). The set also included five isolates from a further two hospital groups. Those from hospital WM4 all belonged to *K. pneumoniae* ST307 and all carried a 52.3 kb IncN plasmid containing *bla*$_{KPC-2}$ and *bla*$_{TEM-1B}$ (e.g. CP133741). They lacked the segment carrying *aph(6)-Id*, *aph(3")-Ib*, *qnrB2*, *sul1* and *dfrA19* found in the IncN plasmids from hospital WM3. That from hospital YH1 carried a 99.3 kb IncR/IncN plasmid with *bla*$_{KPC-2}$, *bla*$_{TEM-1A}$, *bla*$_{OXA-9}$ and *dfrA14* (CP133867).

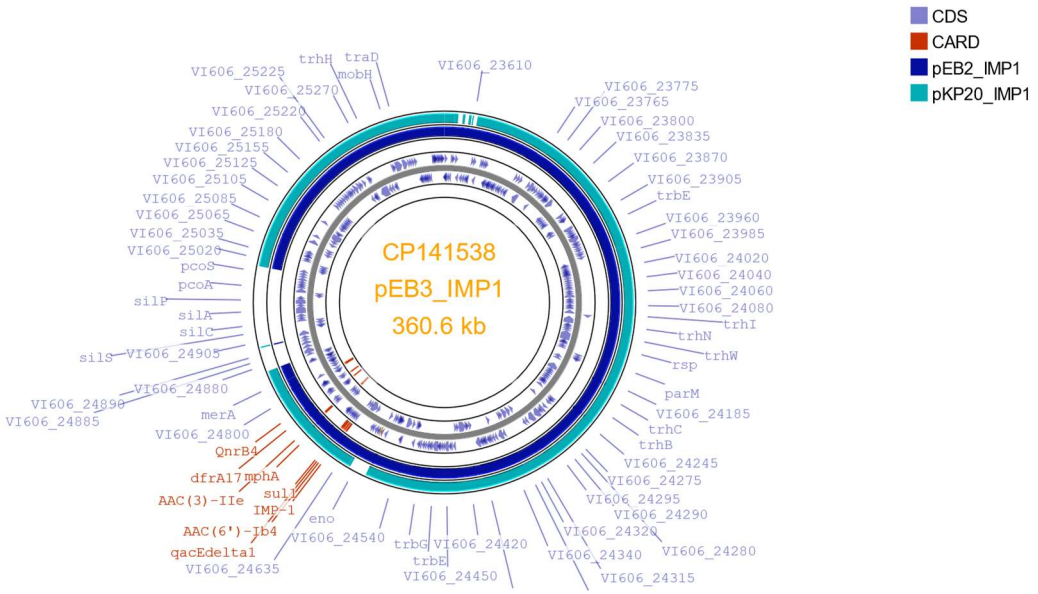
KPC-3 plasmids

Four isolates in the set from two hospital groups carried plasmids with *bla*$_{KPC-3}$. All belonged to *K. pneumoniae* ST307 and their *bla*$_{KPC-3}$ plasmids were 90 to 97 kb in size with those from hospital L9 carrying *bla*$_{TEM-1A}$ and *bla*$_{OXA-9}$ in addition, while those from hospital WM1 carried

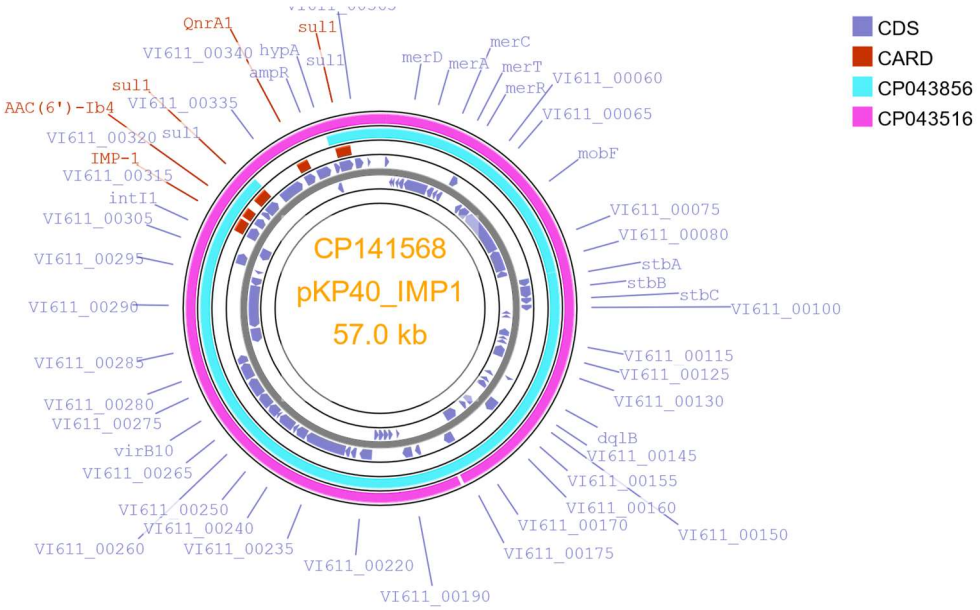
no additional resistance genes (e.g. KP19_WM1_28.22_ST307_KPC3). All were of IncFIB(pQil)/IncFII(K) replicon types. The closest matches on GenBank were slightly bigger plasmids (109 to 118 kb) that shared 99 % identity and 99 % coverage with pKP19_KPC3 (e.g. OQ821101).

IMP-1 plasmids

The set included 13 isolates carrying *bla*$_{IMP-1}$ from six different hospitals, with six isolates belonging to *K. pneumoniae* novel sequence type ST6601 from 2 hospitals (L1 and L6) between which patient transfers are known to have occurred. These isolates carried IncHI2/IncHI2A plasmids of approximately 270 kb carrying *bla*$_{IMP-1}$, *aac(6')-Ib3*, *bla*$_{SHV-12}$, *sul1* and *qnrA1*. Isolates KP20_YH2_29.22_ST54_IMP1, EB1_SW1_27.22_ST104_IMP1, EB2_SW1_42.22_ST145_IMP1 and EB3_SW1_43.22_ST134_IMP1 all carried IncHI1A(NDM-CIT)/IncHI1B(pNDM-CIT) plasmids of 334 to 361 kb in size carrying *bla*$_{IMP-1}$, *aac(3)-IIa*, *aac(6')-Ib3*, *bla*$_{DHA-1}$, *mph(A)*, *qnrB4*, *sul1*, *dfrA17* (e.g. CP141538 in EB3_SW1_43.22_ST134_IMP1), despite being isolated from two different regions of the UK and all belonging to different types. Interestingly, the plasmids from the EB2 and KP20 isolates lacked the copper and silver heavy metal resistance genes found in the plasmid from the EB3 isolate (Fig. 5). KO2_WM3_48.22_STNV2_IMP1 carried a 170.5 kb IncC plasmid containing *bla*$_{IMP-1}$, *aac(6')-Ib-cr*, *aac(6')-Ib3*, *qnrA1*, *sul1* and dfrA1, while KP40_L1_43.22_ST20_IMP1 had a 57.0 kb IncN3 plasmid containing *bla*$_{IMP-1}$, *aac(6')-Ib3*, *qnrA1* and *sul1* (CP141568 in KP40_L1_43.22_ST20_IMP1), which was highly similar to those we have reported previously from *Enterobacter* isolates from London hospitals from 2019 (CP043856 and CP043516) [18] (Fig. 6). *Acinetobacter baumannii* isolate AB1_L10_11.23_ST203_IMP1 carried *bla*$_{IMP-1}$, *aadA5*, *aac(6')-IIa*, *msr(E)*, *mph(E)*, *floR*, *sul1* and *sul2* in a 326.9 kb plasmid with no plasmid hits on PlasmidFinder.



322 Fig. 5. BLAST comparison of the IncHI1A(NDM-CIT)/IncHI1B(pNDM-CIT) plasmids carrying
323 *bla*$_{IMP-1}$ from *E. cloacae* complex and *K. pneumoniae* isolates from two different hospitals
324 (pEB2_IMP1 and pEB3_IMP1 from hospital SW1, pKP20_IMP1 from hospital YH2). The
325 *Enterobacter* isolates belonged to distinct sequence types (STs 145 and 134). CDS, coding
326 sequences; CARD, Comprehensive Antibiotic Resistance Database.
327



328
329 Fig. 6. BLAST comparison of the IncN3 plasmid from *K. pneumoniae*
330 KP40_L1_43.22_ST20_IMP1 with IncN3 plasmids from *Enterobacter* isolates from London
331 hospitals from 2019 (CP043856 and CP043516). CDS, coding sequences; CARD,
332 Comprehensive Antibiotic Resistance Database,
333

334 **IMP-4 plasmids**

335 The set included just two isolates carrying *bla*$_{IMP-4}$ which were both from the same hospital
336 and belonged to the same type (KP12_NE1_13.22_ST54_IMP4 and
337 KP14_NE1_17.22_ST54_IMP4). Both had IncHI2/IncHI2A plasmids of approximately 326 kb
338 containing *bla*$_{IMP-4}$, *aph(3")-Ib*, *aac(6')-Ib3*, *aac(6')-Ib-cr*, *aph(6)-Id*, *mcr-9*, *bla*$_{SHV-12}$, *bla*TEM-1B,
339 *bla*$_{OXA-1}$, *mph(A)*, *qnrB2*, *sul1*, *dfrA19*, *ARR-3*, *formA*, truncated *qacE* and *catB3*. Although
340 not well-represented in this set, we are aware that large IncHI2/IncHI2A plasmids carrying
341 both *bla*$_{IMP-4}$ and *mcr-9* are found in an increasing proportion of IMP positive isolates in the
342 UK [19], with most isolates referred to the reference laboratory during the latter part of 2023
343 carrying them. Such plasmids are widely distributed and have been described in other
344 organisms and countries/continents (e.g. KX810825 in *Salmonella enterica* MU1, CP022533
345 in *Enterobacter hormaechei* MS7884A both from Australia) [20] (Fig. 7).
346

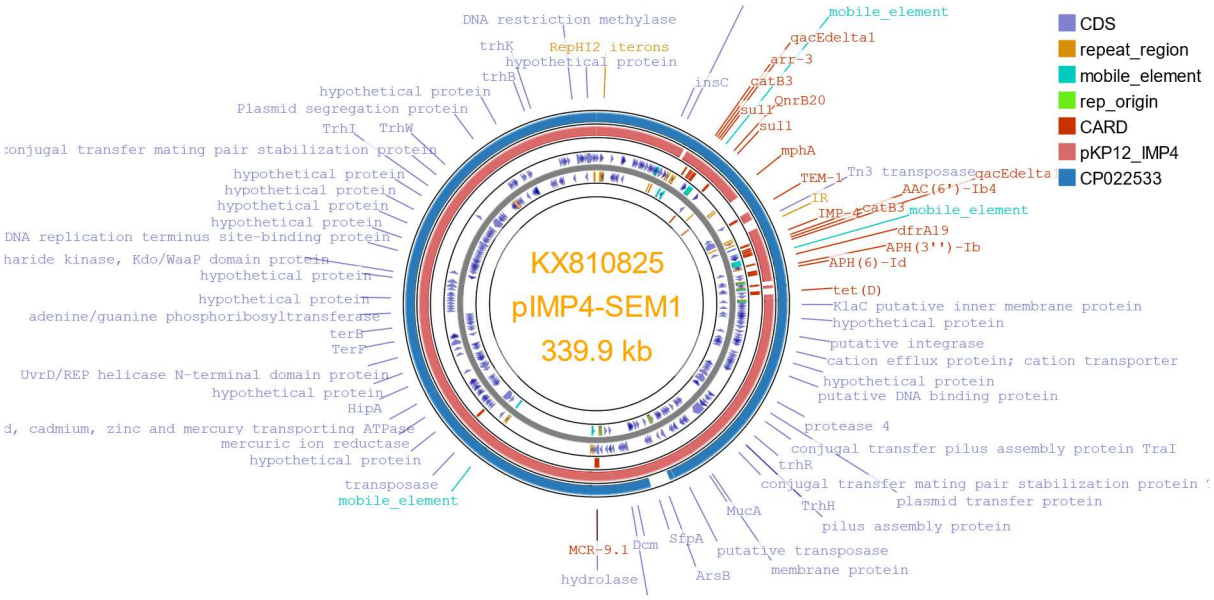


Fig. 7. BLAST comparison of the IncHI2/IncHI2A *bla*$_{IMP-4}$ plasmid in *K. pneumoniae* KP12_NE1_13.22_ST54_IMP4 with those from *Salmonella enterica* MU1 (KX810825) and *Enterobacter hormaechei* MS7884A (CP022533) from Australia. CDS, coding sequences; CARD, Comprehensive Antibiotic Resistance Database.

NDM-1 plasmids

Six of the 12 isolates with *bla*$_{NDM-1}$ sequenced were representatives of *K. pneumoniae* ST20 from hospital L5 described earlier which had IncHI2/IncHI2A plasmids of approximately 340 kb containing *bla*$_{NDM-1}$, *aadA5*, *aac(6')-Ib-cr*, *aac(6')-IIc*, *bla*$_{DHA-1}$, *bla*$_{OXA-1}$, *ere(A)*, *mph(A)*, *catB3*, *qnrB4*, *sul1* and *dfrA17* (e.g. CP141854). KP118_NW3_20.23_ST101_NDM1 similarly carried an IncHI2/IncHI2A *bla*$_{NDM-1}$ plasmid, which was slightly larger (365.7 kb) and contained *aph(6)-Id*, *aph(3'')-Ib*, *bla*$_{SHV-12}$, *bla*$_{TEM-1B}$ and *catA2* resistance genes in addition to *bla*$_{NDM-1}$. A further isolate (KP36_NW3_42.22_ST834_NDM1) had a 131.2 kb IncFIB(pQil) plasmid with *bla*$_{NDM-1}$, *aac(3)-IIa*, *aac(6')-Ib-cr*, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, truncated *catB3* and *qnrB1*. The remaining isolates were representatives of *K. pneumoniae* ST147, three of which (from two different hospitals) had 54 kb IncFIB(pQil) plasmids with *bla*$_{NDM-1}$, *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *ARR-3*, *sul1*, *qnrS1*, *aac(6')-Ib-cr*, *aph(3')-VI*, *catB3* and truncated *qacE* (e.g. CP137381). The remaining representative of ST147 from a further hospital had a 352.3 kb IncFIB(pNDM-Mar)/IncHI1B(pNDM-MAR) hybrid virulence resistance plasmid carrying *bla*$_{NDM-1}$, *aph(3')-Ia*, *aph(3')-VI*, *armA*, *msr(E)*, *mph(A)*, *mph(E)*, *qnrS1*, *sul1*, *sul2* and *dfrA5* and virulence plasmid elements aerobactin (*iutA*, *iucABCD*), *rmpA*/*A2*, *terABCDEWXYZ*, hemin, *luxR*, *pagO*, *shiF*, lysozyme inhibitor (CP137400).

NDM-5 plasmids

370 The rise in frequency of this NDM variant, particularly in *E. coli*, but also in *K. pneumoniae*,
371 has been marked and has been noted by many authors [9, 10, 11]. Our set included two
372 representatives of *E. coli* ST167 with multi-replicon IncF plasmids carrying bla_{NDM-5} (e.g.
373 CP133853) along with *aac(6')-Ib-cr*, *aadA2*, *sul1*, *dfrA12*, *sitABCD*, *tet(A)*, $bla_{CTX-M-15}$, bla_{OXA-}
374 $_{1}$, *mph(A)*, truncated *qacE* and *catB3* (ES3_WM2_48.22_ST167_NDM5 and
375 ES4_WM2_48.22_ST167_NDM5). Other isolates carrying bla_{NDM-5} included in this study
376 belonged largely to *K. pneumoniae* ST147 (or ST6796, a single locus variant of ST147)
377 (n=15), which was a particular focus and are described more fully elsewhere [21]. All but five
378 of these carried an IncFIB(pNDM-Mar)/IncHI1B(pNDM-MAR) hybrid virulence resistance
379 plasmid that combined bla_{NDM-5} and other resistance genes with genes associated with
380 virulence plasmids (aerobactin (*iutA*, *iucABCD*), *rmpA*/*A2*, *terABCDEWXYZ*, hemin, *shiF*,
381 lysozyme inhibitor) (e.g. CP137386 in KP39_L11_43.22_ST147_NDM5, CP137375 in
382 KP58_WM2_48.22_ST147_NDM5_OXA48, CP137372 in KP81_L15_03.23_ST147_NDM5,
383 CP137435 in KP82_L15_04.23_ST147_NDM5, CP137360 in
384 KP97_L17_12.23_ST147_NDM5, CP137352 in KP99_L14_14.23_ST147_NDM5_OXA48
385 and CP137392 in KP124_L5_21.23_ST147_NDM5), despite being from seven different
386 hospital groups. The remaining five representatives of ST147 did not have a virulence
387 plasmid and carried bla_{NDM-5} in 96 kb IncFII plasmids also with *aadA2*, *rmtB*, bla_{TEM-1B},
388 truncated *qacE*, *erm(B)*, *dfrA12*, *sul1* and *mph(A)* (KP2_L3_37.21_ST147_NDM5 and
389 KP30_YH2_39.22_ST147_NDM5_OXA181), or in a 156.5 kb IncFII/IncR plasmid also
390 carrying *aadA2*, *aadA1*, *armA*, *rmtB*, $bla_{CTX-M-15}$, bla_{TEM-1B}, *msr(E)*, *ere(A)*, *mph(A)*, *mph(E)*,
391 *erm(B)*, *cmlA1*, *qnrB1*, *ARR-2*, *sul1* and *dfrA12* (KP104_L5_18.23_ST147_NDM5_OXA232)
392 or chromosomally (KP29_L6_38.22_ST147_NDM5 and
393 KP28_L6_38.22_ST147_NDM5_OXA232), the latter result being obtained on two separate
394 occasions for both isolates.

395 Five further isolates of bla_{NDM-5} positive *K. pneumoniae* belonging to four further STs were
396 included; the two isolates belonging to ST16 (both from hospital L5) had a 46.1 kb IncX3
397 plasmid carrying no other resistance gene, while that of ST3096, also from hospital L5,
398 carried bla_{NDM-5} in a 143 kb IncC plasmid that also contained *aac(6')-Ib3*, bla_{CMY-23} and *sul1*.
399 That belonging to ST1558 (KP77_WM1_02.23_ST1558_NDM5) had bla_{NDM-5} in a 348 kb
400 IncFIB(pNDM-Mar)/IncHI1B(pNDM-MAR) hybrid virulence resistance plasmid also carrying
401 *aph(3')-Ia*, *aadA1*, *aph(3')-VI*, *aac(6')-Ib*, $bla_{CTX-M-15}$, $bla_{TEM-1C/1B}$, bla_{OXA-9}, *mph(A)*, truncated
402 *catA1*, *sul1* and *dfrA5*, aerobactin genes, *rmpA*/*A2*, tellurite resistance genes, *shiF*,
403 lysozyme inhibitor and *cobW* (CP141546) that was highly similar to those seen in
404 representatives of ST147, with most similarity to CP137375 from
405 KP58_WM2_48.22_ST147_NDM5_OXA48. Similar plasmids have also been seen in *K.*

406 *pneumoniae* ST383 (e.g. CP034201 and CP091814) and it is worrying that they have been
407 seen in multiple types and among geographically unrelated isolates. Finally, that belonging
408 to ST437, from a further hospital (L13) had a 106.8 kb IncFIB(pQil)/IncFII plasmid carrying
409 bla_{NDM-5} together with $bla_{CTX-M-15}$, *erm(B)*, *sul1* and *dfrA30*.

410 **NDM-14 plasmids**

411 Just three isolates in the set, which were clonally and epidemiologically related, carried this
412 unusual NDM allele (KP86-KP88 _L3_06.23_ST147_NDM14). All belonged to *K.*
413 *pneumoniae* ST147 and carried a 54.1 kb IncFIB(pQil) plasmid containing bla_{NDM-14}, *aph(3')-*
414 *VI*, *aac(6')-Ib-cr*, $bla_{CTX-M-15}$, bla_{OXA-1}, *catB3*, *qnrS1*, *ARR-3* and *sul1* (e.g. CP137365,
415 CP137425). All also carried a separate hybrid virulence/resistance plasmid (e.g. CP137363,
416 CP137424). There are a number of highly similar plasmids in isolates of *K. pneumoniae* on
417 GenBank of the same length (99-100 % coverage, 99.99-100 % identity) e.g. CP021947,
418 CP014757, OQ785270, CP014757 and AP018834 from isolates from the USA, South Korea
419 and Myanmar, although most carried bla_{NDM-1} rather than bla_{NDM-14}.

420 **Virulence/resistance plasmids in *K. pneumoniae***

421 In addition to the hybrid virulence resistance plasmids described in representatives of ST147
422 (and single locus variant (SLV) ST6796) [21], we have described hybrid virulence resistance
423 plasmids in other, less common types in this study; ST1558 (CP141546)
424 (KP77_WM1_02.23_ST1558_NDM5), ST2096 (CP140295)
425 (KP33_NE2_41.22_ST2096_OXA232) (an SLV of ST14) and ST395 (CP133748)
426 (KP17_NW3_25.22_ST395_OXA48 and KP59_WM2_48.22_ST395_OXA48). In the case of
427 the latter three isolates, these plasmids did not include a carbapenemase gene, which was
428 carried in another plasmid in both cases. In common with other non-NDM hybrid resistance
429 virulence plasmids found in representatives of ST147 they were large (approximately 310
430 kb) IncFIB(pNDM-Mar)/ IncHI1B(pNDM-MAR) plasmids carrying *armA*, *msrE*, *mphE*, *sul2*
431 and *catA1* (KP33) or *armA*, *aadA2*, $bla_{CTX-M-15}$, bla_{TEM1A}, *msrE*, *dfrA12*, *sul1*, *tetD*, *mphE*
432 (KP17) in addition to aerobactin genes, *rmpA*/*rmpA2*, tellurite resistance genes, *shiF*,
433 lysozyme inhibitor and *cobW*. That of KP77_WM1_02.23_ST1558_NDM5 carried bla_{NDM-5}
434 and was highly similar to the NDM-5 virulence resistance plasmids found in representatives
435 of ST147 (e.g. CP137375).
436

437 **Carbapenemase genes among representatives of *K. pneumoniae* ST307**

438 *K. pneumoniae* ST307 is a 'high-risk' clone for resistance that is commonly received by the
439 reference laboratory from hospitals and has been used as an exemplar for comparison of
440 plasmids found within the same type within and between hospitals. We typed 144 non-

441 duplicate isolates of this type from 2021-2023 of which at least 32 were received as
442 carbapenemase producers.

443 The set included 22 representatives of ST307 from nine hospital laboratories and four
444 regions carrying a variety of carbapenemase genes; *bla*$_{KPC-2}$ (n=10), *bla*$_{KPC-3}$ (n=4), *bla*$_{OXA-181}$
445 (n=2) and *bla*$_{OXA-48}$ (n=6). Largely, the plasmids found were consistent within a hospital set
446 and distinct from those from other hospitals carrying the same carbapenemase gene; for
447 example, the 3 KPC-positive isolates from hospital WM4 (KP68_WM4_50.22_ST307_KPC2,
448 KP72_WM4_01.23_ST307_KPC2 and KP73_WM4_01.23_ST307_KPC2) all had a 52.3 kb
449 IncN plasmid carrying *bla*$_{KPC-2}$ and *bla*$_{TEM-1B}$, while that from hospital YH1 carried *bla*$_{KPC-2}$ in a
450 99.3 kb IncR/IncN plasmid with *bla*$_{TEM-1A}$, *bla*$_{OXA-9}$ and *dfrA14*. Another set of isolates from
451 hospital NW4 from five patients all carried *bla*$_{OXA-48}$ in 61.5 kb IncM1 plasmids with no other
452 resistance genes, and all carried an additional IncFII(K)/IncFIB(K) plasmid with *aac(3)-IIa*,
453 *aph(6)-Id*, *aph(3")-Ib*, *aac(6')-Ib-cr*, *bla*$_{CTX-M-15}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-1}$, truncated *catB3*, *qnrB1*,
454 *sul2*, *tetA* and *dfrA14*, supporting that they are linked. Both isolates from hospital WM1 with
455 *bla*$_{KPC-3}$ carried 92.9 kb IncFIB(pQil)/IncFII(K) plasmids.

456 These results are in contrast with those of representatives of ST147, many of which shared
457 highly similar hybrid virulence plasmids despite coming from different hospital groups.

458 **Further Comments and Conclusions**

459 It is clear that comparison of plasmids to investigate if 'plasmid outbreaks' are occurring is
460 far from straightforward. Even where similar plasmids were found among isolates that were
461 related in time and space there were often minor differences between them with segments
462 missing/additional segments (e.g. Fig. 1) leading to potential difficulties in interpretation. In
463 addition, many plasmids are widely distributed, and finding similar plasmids among isolates
464 carrying them does not necessarily indicate plasmid spread between them. Examples are
465 the widespread IncL pOXA48a and IncHI2/IncHI2A *bla*IMP-4 plasmids that have been found
466 in many countries [19, 20, 22]. The widely found ColKP3/IncX3 OXA-181 plasmids and
467 ColKP3 *bla*$_{OXA-232}$ plasmids were remarkably conserved (to the base) and seen in different
468 organisms and many different geographical locations. It is clearly important to know the
469 context and wider distribution of a relevant plasmid before considering whether a plasmid
470 outbreak may be occurring. All three hospital sets we examined revealed a complex picture.
471 In all of them, the situation was mixed with some evidence for plasmid transfer, but also
472 clear examples where the plasmids were different.
473
474 IncHI2/HI2A plasmids are important vehicles for carbapenemase genes; we observed
475 plasmids of this type carrying *bla*$_{KPC-2}$, *bla*$_{IMP-1}$, *bla*$_{IMP-4}$ or *bla*$_{NDM-1}$. Where we observed the

476 colistin resistance gene *mcr-9* it was always in IncHI2/IncHI2A plasmids e.g. CP141854,
477 although not all carried this gene. It is likely that the gene is carried silently and is not
478 expressed in these isolates [23].
479
480 This study concentrated on the plasmids carrying carbapenemase genes, but most of these
481 isolates carried multiple plasmids with the others often also carrying resistance genes. For
482 example, not only did KP72_WM4_01.23_ST307_KPC2 carry a KPC plasmid (CP141579),
483 but it also had a further plasmid containing *aac(3)-IIa*, *aph(6)-Id*, *aph(3'')-Ib*, *aac(6')-Ib-cr*,
484 $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, $bla_{OXA\text{-}1}$, truncated *catB3*, *qnrB1*, *sul2*, *tet(A)* and *dfrA14* (CP141580).
485 This study also only looked at these plasmids at a relatively superficial level and a deeper
486 analysis could achieve greater insight.
487
488 While most plasmids carrying $bla_{OXA\text{-}48}$ did so in a typical IncL plasmid of approximately 60-
489 70 kb, there was some variation among these plasmids and some very different plasmids
490 were found (e.g. the IncR plasmid pKP59_OXA48), showing that there can be value in
491 analysing plasmids carrying $bla_{OXA\text{-}48}$ where a plasmid outbreak is suspected.
492
493 Nanopore sequencing was essential in providing complete or near complete assemblies of
494 these isolates, facilitating investigation of their plasmids. Wide use of nanopore sequencing
495 is providing information not previously available leading to a greatly increased understanding
496 of the global epidemiology of plasmids carrying carbapenemase genes.
497

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587 Table S1. Carbapenemase producers included in the study and their resistance genes.
588 Isolates were labelled with a unique organism number (beginning KP for *K. pneumoniae*, EB
589 for *E. cloacae* complex, ES for *E. coli*, CF for *C. freundii*, KO for *K. oxytoca* complex and AB
590 for *Acinetobacter baumannii*), followed by a hospital code, the week and year of receipt by
591 the laboratory, their sequence type (ST) and the carbapenemase genes that they carried.
592 Hospitals were labelled according to region in the UK and number within that region (L,
593 London; SE, South East England; SW, South West England; WM, West Midlands; EM, East
594 Midlands; NE, North East London; NW, North West England; YH, Yorkshire and Humber).
595 Contigs are labelled as circular (c) or linear (l). An asterisk indicates a truncated gene or (in
596 the allelic profile column) a novel allele. Where there was no exact match to a resistance
597 gene, the closest alternative(s) are given. NV refers to novel sequence types. Where more
598 than one run was carried out on an isolate, run identifications highlighted in turquoise
599 indicate that the same extract was used; yellow highlighting indicates that a different extract
600 was used.