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Concomitant carriage of KPC-producing and non-KPC-producing *Klebsiella pneumoniae* ST512 within a single patient

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33 **Synopsis**

34

35 **Background**: KPC-producing *Klebsiella pneumoniae* (*Kp*) of the clonal group 258 are
36 prominent in the health care settings in many regions of the world. The bla_{KPC} gene is
37 mostly carried by a multi-replicon IncFIIk-IncFI plasmid suspected to be highly
38 compatible and stable in this genetic background. Here, we analysed the genetic
39 diversity of a ST512 *Kp* population in a single patient.

40 **Material and methods**: Twelve *Kp* isolates (n=5 from urine samples and n=7 from
41 rectal swabs) were recovered from one patient over a two-months period. Antimicrobial
42 susceptibility testing, plasmid extraction and WGS were performed on all isolates. The
43 first *Kp* isolate D1 was used as reference for phylogenetic analysis.

44 **Results**: Antimicrobial susceptibility testing, plasmid analysis and WGS revealed
45 concomitant carriage of carbapenem-resistant and carbapenem-susceptible *Kp*
46 isolates of the sequence type 512, with the absence of the entire bla_{KPC}-carrying
47 plasmid in the susceptible population. Furthermore, 14 other genetic events occurred
48 within the genome including three chromosomal deletions (of 71 kb, 33 kb or 11 nt in-
49 size), two different insertions of S*Kpn*26 and 9 SNPs. Interestingly, most of the events
50 occurred in the same chromosomal region that has been deleted independently
51 several times probably after homologous recombination involving 259 nt in-size
52 repeated sequences.

53 **Conclusion**s: Our study revealed the first case of in-vivo bla_{KPC}-carrying plasmid
54 curing and a wide in-patient genetic diversity of a single *Kp* ST512 clone over a short
55 period of carriage. This within-patient diversity must be taken into account when
56 characterizing transmission chains using WGS during nosocomial outbreaks.

57

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* has emerged as a formidable threat in health care facilities.[1] *Klebsiella pneumoniae* carbapenemase (KPC) belongs to Ambler's Class A and has emerged globally in Enterobacterales in the early 2000's.[2] The worldwide spread of KPC is multifactorial and has been related to the diffusion of a particular clonal group (CG), the CG258 defined by few single locus variants (SLV), ST258, ST11, and ST512, being the most predominant ones.[3] Notably, the emergence of *bla*$_{KPC-3}$ gene in Italy has been related to the spread of the ST512 isolates.[4,5] Other genetic features associated to its spread are related to the genetic structure, a class II transposon containing the *bla*$_{KPC}$ gene named Tn*4401*-like, and a conjugative plasmid of IncFII$_{K}$ type.[2,6,7]

After the worldwide dissemination of these successful clones and clonal-complex since 2000, all reports of *K. pneumoniae* (*Kp*) of the CC258 refer to KPC-producing strains with the exception of a study that retrospectively analysed carbapenem-susceptible *Kp* collection strains isolated between 1999 and 2013 in New York City and some of them belonged to the ST258.[8]

Whole-Genome Sequencing has emerged as a powerful tool to study bacterial evolution. Evolution of the genome of KPC-*Kp*-ST258 during long-term human colonization revealed complex plasmid rearrangements and genome plasticity.[9,10] However, little is known about the genetic diversity that resides in the gastro-intestinal tract within a single bacterial population at a specific time point.

Here, we described the concomitant carriage of *bla*$_{KPC}$-positive carbapenem-resistant and *bla*$_{KPC}$-negative carbapenem-susceptible *Kp*-ST512 clinical isolates in a single patient. Our objectives were to analyse the genetic diversity of a KPC-*Kp* isolate and to appreciate the cohabitation of several sub-populations of *Kp*-ST512.

83

84 **Material and methods**

85 **Bacterial isolates, MICs and growth conditions.** Twelve clinical isolates of *K.*
86 pneumoniae were isolated from a single patient over a two-months period. The isolates
87 were obtained from rectal swabs (n=7) or urine samples (n=5) and were named after
88 the sampling date, D1 being the first isolate and D54 the one being isolated 54 days
89 later. From rectal swabs, *K. pneumoniae* were obtained after growth on selective
90 media supplemented with carbapenems (ChromID ® CARBA SMART, bioMérieux,
91 Marcy L'Etoile, France) whereas urine samples were spread on non- selective
92 chromogenic media (UriSelect™ Bio-Rad, Marnes-La-Coquette, France).
93 Antimicrobial susceptibilities were determined by the disc diffusion method on Mueller-
94 Hinton (MH) agar (Bio-Rad), and were interpreted according to EUCAST guidelines.[11]
95 MICs for carbapenems, ceftazidime, tigecycline, ciprofloxacin and aminoglycosides
96 were determined by Etest (bioMérieux), and for colistin by broth microdilution
97 (Sensititre™ Thermofisher, France).

98

99 **Genomic analysis.** Total DNA was extracted from colonies using the Ultraclean
100 [12]Microbial DNA Isolation Kit (MO BIO Laboratories, Ozyme, Saint-Quentin, France)
101 following the manufacturer's instructions. The DNA library was prepared using the
102 Nextera XT-v2 kit (Illumina, Paris, France) and then run on HiSeq Illumina system
103 (2x150-bp paired-end). Full sequence of *Kp* D1 genome was obtained using PacBio
104 and Illumina's sequencing technologies. Assembly was performed using the
105 RS_HGAP_Assembly.3 protocol from the SMRT analysis toolkit v2.3 and with Canu.[12]
106 The consensus sequence was polished with Quiver, and manually corrected by
107 mapping Illumina reads using Breseq.[12] The acquired antimicrobial resistance genes

108 were identified using Resfinder server v3.0
109 (https://cge.cbs.dtu.dk/services/ResFinder/).[13] The genome was annotated using
110 PROKKA.[14]

111 SNP analysis was performed by mapping the reads from each genome (D2 to
112 D54) against *Kp* D1 genome used as reference. Variants, SNP, insertion and deletion
113 were detected by using BRESEQ[12] or the variant detection tool of CLC genomic
114 workbench v12.0 (Qiagen, Les Ulis, France).

115

116 **Plasmid content analysis.** Plasmids were extracted using the Kieser's extraction
117 method and subsequently analysed by electrophoresis on a 0.7% agarose gel.[15]

118

119 **Nucleotide sequence accession number.** The whole genome sequences
120 generated in the study have been submitted to the Genbank nucleotide sequence
121 database under accession numbers detailed in Table 1.

122

123 **Ethics.** This study was conducted in accordance with the Declaration of Helsinki
124 and national standards. Signed statement of informed consent was obtained from the
125 patient.

126

127

128 **Results**

129 ***Case report.*** In 2015, a patient suffered from acute pancreatitis (Balthazar
130 score E) due to gallstones during a stay in Italy. The patient was hospitalized in Italy
131 for ten days, during which no nutrition was given to rest the pancreas and bowels.
132 Food was then reintroduced through a nasogastric tube, and the evolution was

133 favorable with exclusive enteric nutrition. The patient was repatriated to France for
134 further medical care. Upon admission, screening for intestinal carriage of
135 carbapenemase-producing Enterobacterales (CPE) allowed to identify the presence
136 of a KPC-producing *Kp* (isolate D1), likely acquired during its hospitalization in Italy. A
137 cholecystectomy was performed 10 weeks after the acute episode and no complication
138 occurred. During the two months of follow-up in France,12 *K. pneumoniae* isolates (8
139 isolates KPC+ and 4 KPC-) were recovered from screening or urine samples (Figure
140 1A).
141
142 ***Susceptibility testing and resistome***. Susceptibility testing of the twelve
143 isolates recovered revealed two different phenotypes regarding β-lactams but the
144 same co-resistances. WGS revealed in all genomes three aminoglycosides modifying
145 enzymes (*aph(3')-Ia, aac(6')-Ib, aadA2),* the natural fosfomycin resistance gene *(fosA-*
146 *like), catA1-like, dfrA12 and sul1* conferring resistance to chloramphenicol,
147 trimethoprime and sulfamides respectively. A substitution in GyrA (S83I) was also
148 identified conferring resistance to fluoroquinolones. OmpK35 porin was inactivated by
149 an insertion at position 121 of the gene (+G), leading to an early STOP codon in the
150 protein. In OmpK36, two amino-acids are inserted at position 135 (+Asp) and 136
151 (+Gly) in comparison to wild-type sequence (NC_016845.1). This OmpK36 variant is
152 known to contribute to increase the MICs for carbapenems in *Kp* of CG258.[16]
153 Eight isolates were resistant to carbapenems (MICs for imipenem from 4 to 8
154 mg/L, MICs for meropenem and ertapenem >32 mg/L) whereas four isolates were
155 susceptible to broad-spectrum cephalosporins and carbapenems (MICs for imipenem
156 at 0.125 mg/L). The content of β-lactamase-encoding genes differed between isolates.
157 WGS revealed that all carbapenem-resistant *Kp* contained three acquired β-

158 lactamases genes: *bla*$_{KPC-3}$ carried by a transposon Tn*4401a*, *bla*$_{TEM-1}$, *bla*$_{OXA-9}$ (not
159 functional due to premature stop codon) carried by a multireplicon IncFIB-IncFIIk
160 plasmid of 113,639 bp (pKpQIL-like) and the naturally chromosome-encoded *bla*$_{SHV-11}$
161 gene. Carbapenem-susceptible isolates possessed only the *bla*$_{SHV}$ gene.
162 MLST analysis indicated that all *Kp* (KPC+ and KPC-) belonged to the ST512,
163 suggesting that the two populations of *Kp* ST512 seem to differ only by the presence
164 or absence of the Tn*4401a* or of the whole *bla*$_{KPC}$-carrying plasmid. In order to
165 distinguish between these two hypotheses, plasmid extractions analysed by
166 electrophoresis revealed that a c.a. 100 kb plasmid was missing in all KPC- isolates
167 (Figure 1B).
168
169 **Genomic analysis and phylogeny**. To establish whether a gain or a loss of
170 the *bla*$_{KPC}$-carrying plasmid occurred in the *Kp* ST512 population, SNPs and genetic
171 events underlying *in vivo* evolution analyses were performed using isolate D1 as
172 reference (Figure 1C).
173 Even though the 12 isolates were highly related, a total of 15 different genetic
174 events were identified: the loss of the whole *bla*$_{KPC}$-carrying plasmid (in 4 isolates),
175 three different deletions of chromosomal regions (71,959 bp in-size in 5 isolates,
176 33,752 bp in-size in 2 isolates, and 11 bp in-size in two isolates), two different insertions
177 of a copy of ISKpn26 (at position 2,309,021 in 5 isolates and at position 4,117,119 in
178 one) and 9 different SNPs (involving 7 isolates). Interestingly, the 33 kb deleted region
179 (from 4,106,137- 4,139,889) was part of the larger 71 kb deleted region (from
180 4,068,121 to 4,139,091). No mobile element could be evidenced surrounding these
181 two deleted regions, but the presence of three copies of Repeated Sequences (RS) of
182 259 nt in-size were present (Figure 1.D). These regions share over 96% of nucleotide

183 identity (Supplementary Figure). Recombination events involving RS1 and RS3, and
184 RS2 and RS3 are likely responsible for the deletions of the 71 kb and the 33 kb in-size
185 fragments respectively.

186 Comparison to deposited genomes in public databases indicates that D33-3
187 isolate is the closest to other *Kp* ST512 genomes. In the chromosome of D1 (KPC+),
188 a deletion of 11 nt in-size occurred, and this deletion is also present in D33-2 (KPC-)
189 in addition to the insertion of a copy of IS*Kpn*26. Furthermore, the loss of pKPC seems
190 to have occurred in another branch, between D33-3 (KPC+) and D19-2 (KPC-) (Figure
191 1C). These observations make highly likely the loss of the bla_{KPC}-carrying plasmid by
192 the KPC+ population rather than an acquisition by the KPC- population.

193

194 **Discussion**

195 An unexpected genetic and phenotypic variability of *Kp* ST512 in a single patient
196 was observed as a result of several unrelated genetic events. Concomitant isolation
197 of carbapenem-resistant and -susceptible isolates recovered over a short period of
198 time (two months) was due to the presence or not of the bla_{KPC-3} carrying plasmid
199 (pKPC). In our study, this diversity was clearly underestimated by the use of selective
200 media for rectal samples that allow only the growth of carbapenem-resistant bacteria.
201 Hence the carbapenem -susceptible population could be identified only from urine
202 samples. Despite this major bias, three isolates from different branches of the
203 distribution were recovered the same day (D33) as a proof that this genetic diversity is
204 present in the patient's microbiota.

205 Interestingly, over the 15 genetic events described, 5 involved the same chromosomal
206 region (from 4068121 – 4139091) in 10 isolates. This region included over 60 CDS
207 and among them, the *ramA* gene (Accession KC843634) is either entirely deleted (in

208 71kb and in 33 kb-deleted genomes), inactivated by a non-sense mutation, or
209 putatively transcriptionnally affected by the insertion of a copy of IS*Kpn*26 in the
210 intergenic region, or by a 11 nt deletion in the *ramR* regulator (Figure 1D). *ramA*, an
211 *araC*-family transcriptional regulator, is part of the *ramR-romA-ramA* operon and is
212 involved in the expression of AcrAB efflux pump leading to increased MICs for
213 tigecycline and fluoroquinolones. We could not observe any correlation between MICs
214 for these antibiotics and any of the genetic events. However, since this region has been
215 inactivated several times by independent mechanisms, it is tempting to speculate that
216 its inactivation confers to this *Kp* ST512 a competitive advantage in that clinical context.
217 The genetic analysis indicated that a loss of the bla_{KPC-3} carrying plasmid likely
218 occurred in the *Kp* ST512 population. pKPC is very close to the successful pKpQIL
219 plasmid (99,98% of nucleotide identity, 100% of query coverage), the first KPC-
220 producing plasmid that had been sequenced in 2006 and known to have spread in all
221 Europe through its tight association to the CG258.[17,18] Plasmid stability assay could
222 not evidence an increased capacity of *Kp* D1 to loose pKPC plasmid in comparison to
223 other *Kp* isolates carrying pKpQIL-like plasmids (data not shown). Previous genomic
224 analyses of KPC-*Kp* during long-term carriage have reported large plasmid
225 rearrangements, deletions of the entire Tn*4401*, or plasmid transfer between
226 Enterobacterales, but loss of the entire bla_{KPC}-carrying plasmid by a member of the
227 *Kp*-CG258 has never been reported to date.[9,19] The pKpQIL-like plasmids are thought
228 to be highly compatible with *Kp*-CG258 genetic background and to have contributed to
229 the worldwide dissemination of KPC carbapenemase.[2,20] Information regarding the
230 antibiotic selection pressure that occurred during the patient's hospitalization were
231 available (Figure 1A). Given the genetic support of resistance genes linked to these
232 antimicrobial agents, cefixime seems to be the solely molecule capable to maintain a

selective pressure to prevent the loss of the KPC plasmid. But it has been prescribed at Day 44, far after the isolation of the first non KPC-producer at Day 19. So most of the patient's bacterial follow-up was done when no antimicrobial selective pressure occurred, and we witnessed the natural history of a colonizing *Kp* ST512 isolate.

Overall, we report here a wide genetic diversity of *Kp* ST512 in a single patient that underwent low antimicrobial selective pressure. This diversity must be taken in account when trying to infer transmission routes using WGS during nosocomial outbreaks.

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Transparency declarations

None to declare.

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258

259

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261
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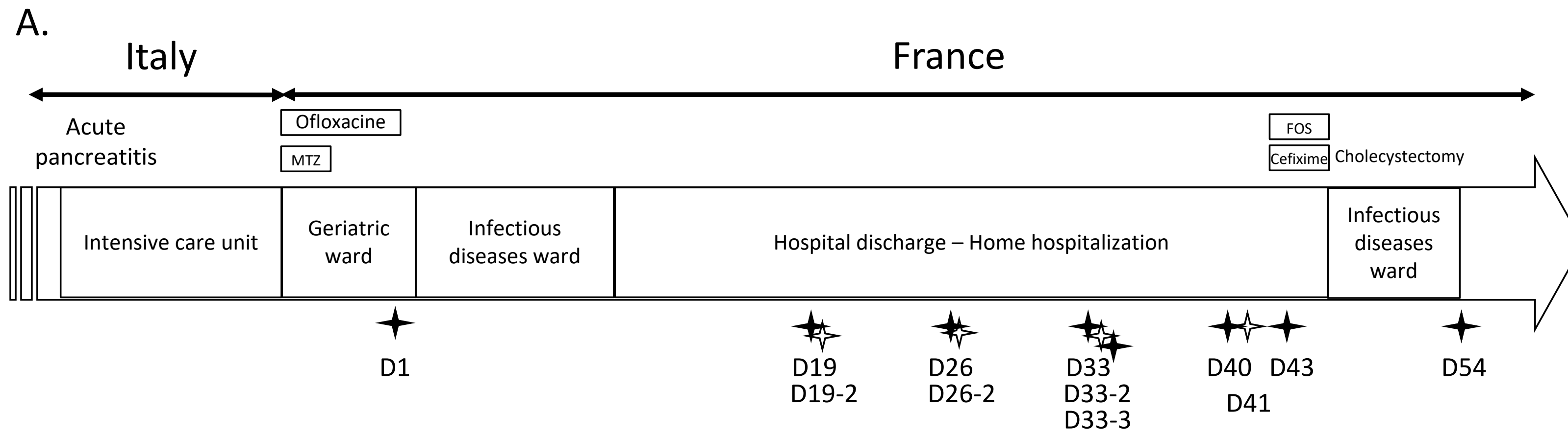
317

Table 1: Clinical features, antimicrobial susceptibility, resistomes and accession numbers of *K. pneumoniae* isolates

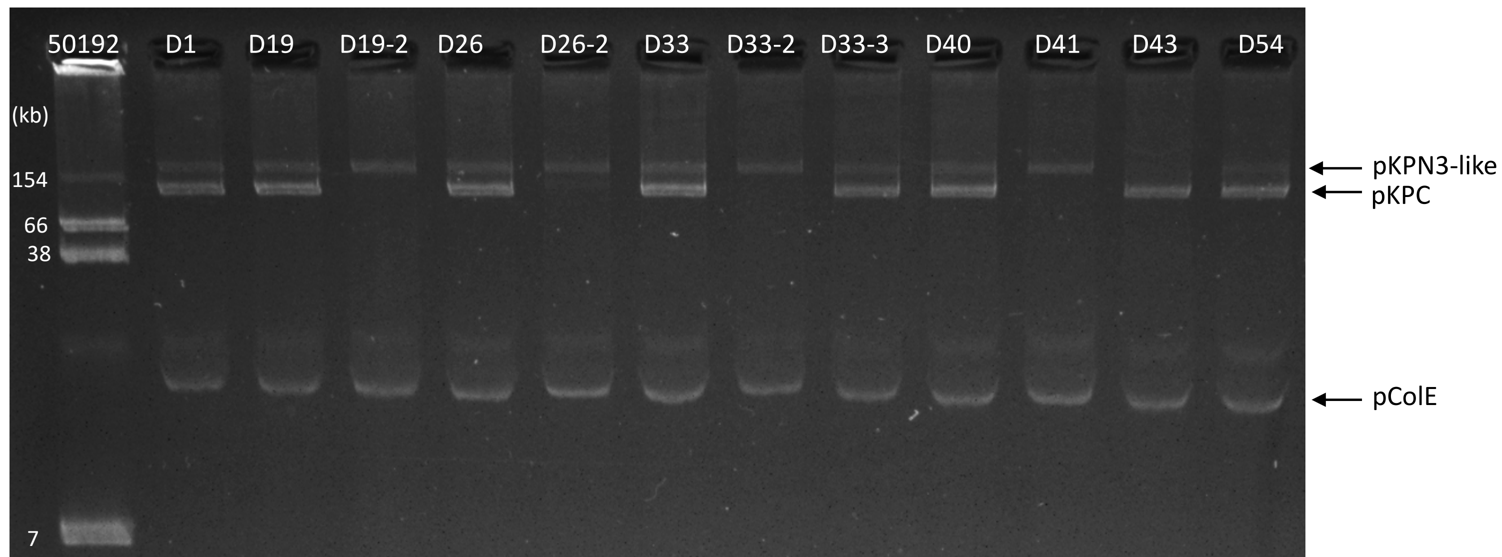
Isolates	Source	Susceptibility testing (mg/L)									Resistome				BioProject	Genbank Accession numbers
		CAZ	IPM	ERT	MER	TGC	COL	GN	AK	CIP	Chromosome	pKPC	pKPN3-like	pColE		
D1	rectal	128	4	>32	>32	4	1	1	32	***>32***	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	Chromosome CP043969 pKPN3-like CP043970 pD1-KPC CP043971 pColE CP043972
D19-1	rectal	>256	8	>32	>32	4	0,5	1	32	***>32***	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOU00000000
D19-2	urine	0,38	0.125	0,094	0,032	1	1	1	48	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	missing	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOV00000000
D26-1	rectal	>256	4	>32	>32	1,5	1	1	32	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOS00000000
D26-2	urine	0,38	0.125	0,094	0,032	1	1	1,5	48	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	missing	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOT00000000
D33-1	urine	>256	6	>32	>32	1,5	1	1	***48***	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOP00000000
D33-2	urine	0,5	0.125	0,094	0,032	1	1	1,5	32	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	missing	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOR00000000
D33-3	rectal	>256	4	>32	>32	1,5	1	1	32	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOQ00000000
D40	rectal	>256	4	>32	>32	1,5	1	1	***48***	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOO00000000
D41	urine	0,5	0.125	0,094	0,032	1	1	1,5	32	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	missing	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWON00000000
D43	rectal	>256	6	>32	>32	1	1	1	***32***	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOM00000000
D54	rectal	>256	6	>32	>32	1,5	0,5	1	***48***	*>32*	bla_{SHV-11}, *gyrA* S83I *fosA -like*, OmpK35 (truncated), OmpK36 (mutated)	$\mathbf{bla_{KPC-3}}$, bla_{TEM-1}, bla_{OXA-9^*},	*aph(3')-Ia, aadA2, sul1, catA1-like, dfrA12,*	*aac(6')-Ib*	PRJNA564512	VWOL00000000

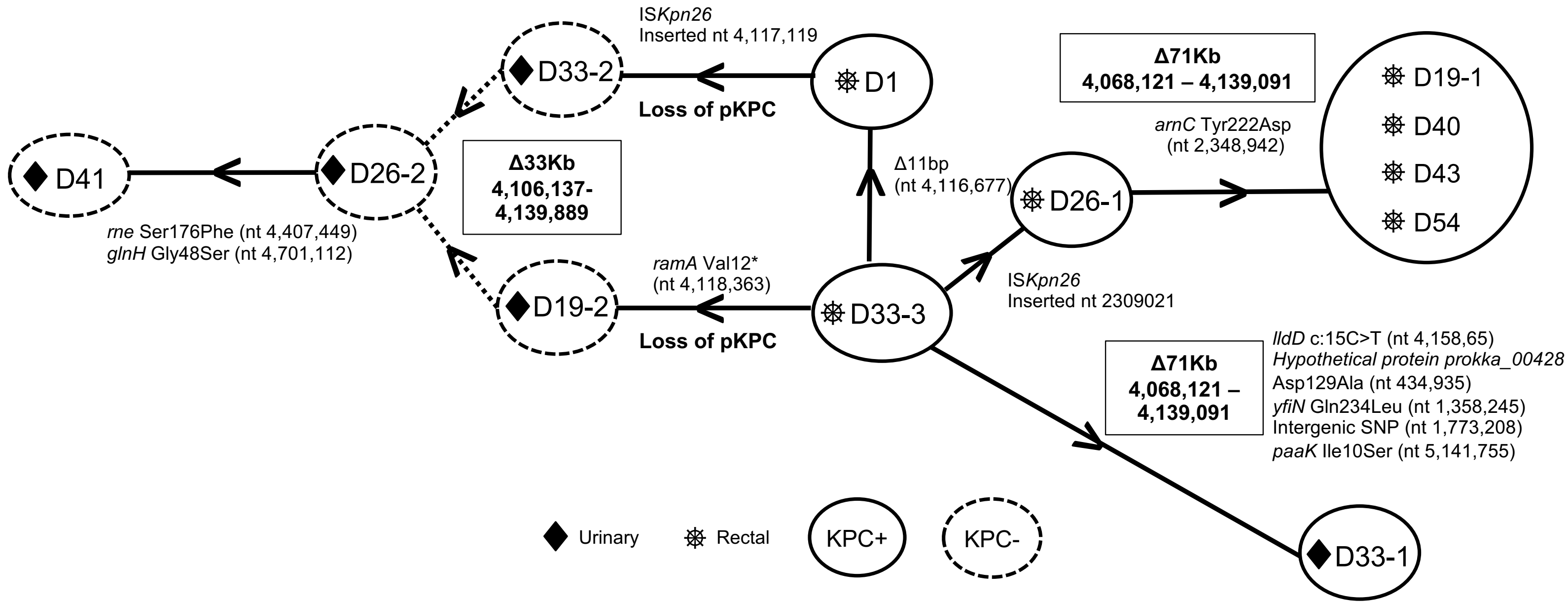
CAZ: ceftazidime; IPM: imipenem; ERT: ertapenem; MER: meropenem; TGC: tigecyline; GN: gentamicin ; AK: amikacin ; CIP: ciprofloxacin

A.

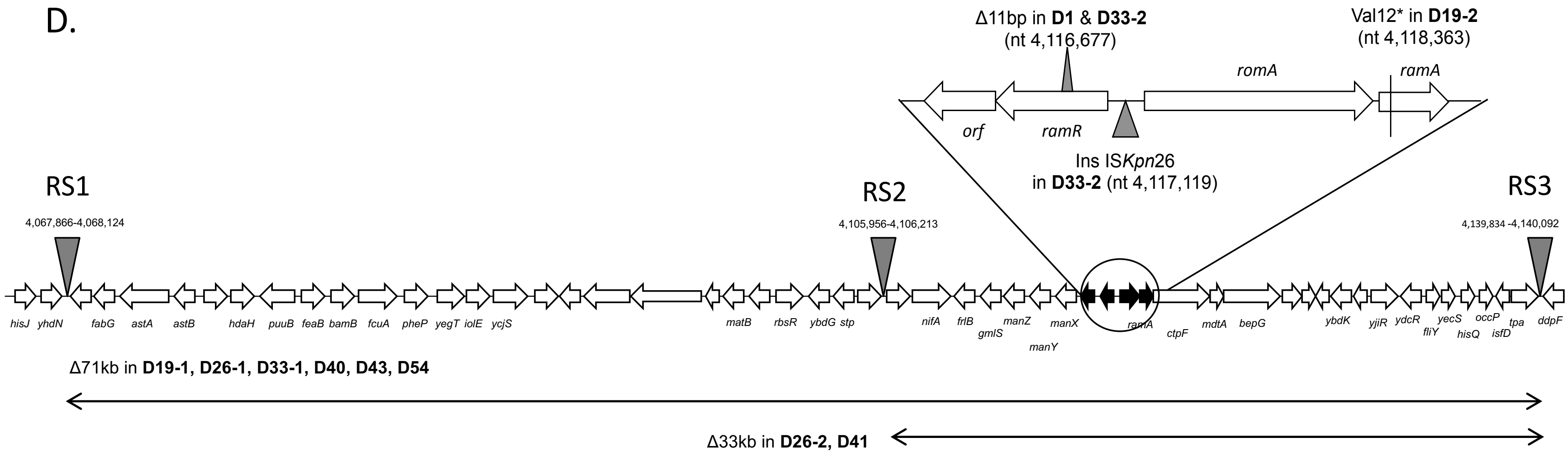


B.





C.
ISKpn26
Inserted nt 4,117,119
Loss of pKPC
D33-2
D1
D41
D26-2
Δ33Kb
4,106,137-
4,139,889
Δ11bp
(nt 4,116,677)
Δ71Kb
4,068,121 – 4,139,091
arnC Tyr222Asp
(nt 2,348,942)
D26-1
D19-1
D40
D43
D54
rne Ser176Phe (nt 4,407,449)
glnH Gly48Ser (nt 4,701,112)
ramA Val12*
(nt 4,118,363)
D19-2
Loss of pKPC
D33-3
ISKpn26
Inserted nt 2309021
lldD c:15C>T (nt 4,158,65)
Hypothetical protein prokka_00428
Asp129Ala (nt 434,935)
yfiN Gln234Leu (nt 1,358,245)
Intergenic SNP (nt 1,773,208)
paaK Ile10Ser (nt 5,141,755)
Δ71Kb
4,068,121 –
4,139,091
Urinary
Rectal
KPC+
KPC-
D33-1



D.
Δ11bp in D1 & D33-2
(nt 4,116,677)
Val12* in D19-2
(nt 4,118,363)
romA
ramA
orf
ramR
Ins ISKpn26
in D33-2 (nt 4,117,119)
RS1
4,067,866-4,068,124
RS2
4,105,956-4,106,213
RS3
4,139,834 -4,140,092
hisJ yhdN fabG astA astB hdaH puuB feaB bamB fcuA pheP yegT iolE ycjS
matB rbsR ybdG stp nifA frlB gmlS manZ manX manY ramA ctpF mdtA bepG
ybdK yjiR ydcR fliY yecS hisQ occP isfD tpa ddpF
Δ71kb in D19-1, D26-1, D33-1, D40, D43, D54
Δ33kb in D26-2, D41

Figure's legend:

Figure 1. A. Patient's clinical case. This timeline indicates the patient's medical history during the two-months of follow-up. Antibiotics prescribed have been written within boxes. KPC+ and KPC - *Kp* are indicated by black and white stars respectively. At hospital admission in France, six days of ofloxacin per os combined with intravenous metronidazole for two days were given. After the identification of the first KPC-*Kp* D1 from rectal swab and since no pancreatitis necrosis could be evidenced, this antimicrobial chemotherapy was stopped. The patient received no antimicrobial treatment until its hospital discharge, when an episode of asymptomatic bacteriuria was treated using Fosfomycin and Cefixime per os between day 44 and day 47 of the follow-up. The timeline was drawn at scale.

B. Plasmid extraction analysed by electrophoresis on a 0.7% agarose gel. *E. coli* 50192 was used as reference for plasmid size. The arrows indicate the position of the three plasmids: pKPN3-like, pKPC (missing in KPC- isolates) and pColE.

C. Phylogenetic analysis of isolates recovered over the two-months period. The circles are at scale with the number of isolates. KPC+ and KPC- *Kp* are indicated in full or in dashed circles respectively. The length of the branches is proportional to the number of genetic events (insertions, deletions, loss of pKPC plasmid, SNPs). Dashed lines indicate two different putative evolutionary pathways.

D. Genetic environment of the deleted regions. Repeated sequences (RS) are indicated by grey triangles. Losses of the 71 kb and 33 kb fragments likely occurred after homologous recombination implicating RS1/RS3 and RS2/RS3 respectively. A total of five independent genetic events occurred in this region that contains the *ramR-romA-ramA* genes.