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

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Synopsis

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

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

Results: Antimicrobial susceptibility testing, plasmid analysis and WGS revealed concomitant carriage of carbapenem-resistant and carbapenem-susceptible *Kp* isolates of the sequence type 512, with the absence of the entire *bla*_{KPC}-carrying plasmid in the susceptible population. Furthermore, 14 other genetic events occurred within the genome including three chromosomal deletions (of 71 kb, 33 kb or 11 nt in-size), two different insertions of *SKpn26* and 9 SNPs. Interestingly, most of the events occurred in the same chromosomal region that has been deleted independently several times probably after homologous recombination involving 259 nt in-size repeated sequences.

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

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 Tn4401-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 ¹²Microbial 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

were identified using Resfinder server v3.0 (<https://cge.cbs.dtu.dk/services/ResFinder/>).¹³ The genome was annotated using PROKKA.¹⁴

SNP analysis was performed by mapping the reads from each genome (D2 to D54) against *Kp* D1 genome used as reference. Variants, SNP, insertion and deletion were detected by using BRESEQ¹² or the variant detection tool of CLC genomic workbench v12.0 (Qiagen, Les Ulis, France).

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

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

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

Results

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

favorable with exclusive enteric nutrition. The patient was repatriated to France for further medical care. Upon admission, screening for intestinal carriage of carbapenemase-producing Enterobacterales (CPE) allowed to identify the presence of a KPC-producing *Kp* (isolate D1), likely acquired during its hospitalization in Italy. A cholecystectomy was performed 10 weeks after the acute episode and no complication occurred. During the two months of follow-up in France, 12 *K. pneumoniae* isolates (8 isolates KPC+ and 4 KPC-) were recovered from screening or urine samples (Figure 1A).

Susceptibility testing and resistome. Susceptibility testing of the twelve isolates recovered revealed two different phenotypes regarding β -lactams but the same co-resistances. WGS revealed in all genomes three aminoglycosides modifying enzymes (*aph(3')-Ia*, *aac(6')-Ib*, *aadA2*), the natural fosfomycin resistance gene (*fosA-like*), *catA1-like*, *dfrA12* and *sul1* conferring resistance to chloramphenicol, trimethoprim and sulfamides respectively. A substitution in GyrA (S83I) was also identified conferring resistance to fluoroquinolones. OmpK35 porin was inactivated by an insertion at position 121 of the gene (+G), leading to an early STOP codon in the protein. In OmpK36, two amino-acids are inserted at position 135 (+Asp) and 136 (+Gly) in comparison to wild-type sequence (NC_016845.1). This OmpK36 variant is known to contribute to increase the MICs for carbapenems in *Kp* of CG258.¹⁶

Eight isolates were resistant to carbapenems (MICs for imipenem from 4 to 8 mg/L, MICs for meropenem and ertapenem >32 mg/L) whereas four isolates were susceptible to broad-spectrum cephalosporins and carbapenems (MICs for imipenem at 0.125 mg/L). The content of β -lactamase-encoding genes differed between isolates. WGS revealed that all carbapenem-resistant *Kp* contained three acquired β -

lactamases genes: *bla*_{KPC-3} carried by a transposon *Tn4401a*, *bla*_{TEM-1}, *bla*_{OXA-9} (not functional due to premature stop codon) carried by a multireplicon IncFIB-IncFIIk plasmid of 113,639 bp (pKpQIL-like) and the naturally chromosome-encoded *bla*_{SHV-11} gene. Carbapenem-susceptible isolates possessed only the *bla*_{SHV} gene.

MLST analysis indicated that all *Kp* (KPC+ and KPC-) belonged to the ST512, suggesting that the two populations of *Kp* ST512 seem to differ only by the presence or absence of the *Tn4401a* or of the whole *bla*_{KPC}-carrying plasmid. In order to distinguish between these two hypotheses, plasmid extractions analysed by electrophoresis revealed that a c.a. 100 kb plasmid was missing in all KPC- isolates (Figure 1B).

Genomic analysis and phylogeny. To establish whether a gain or a loss of the *bla*_{KPC}-carrying plasmid occurred in the *Kp* ST512 population, SNPs and genetic events underlying *in vivo* evolution analyses were performed using isolate D1 as reference (Figure 1C).

Even though the 12 isolates were highly related, a total of 15 different genetic events were identified: the loss of the whole *bla*_{KPC}-carrying plasmid (in 4 isolates), three different deletions of chromosomal regions (71,959 bp in-size in 5 isolates, 33,752 bp in-size in 2 isolates, and 11 bp in-size in two isolates), two different insertions of a copy of ISKpn26 (at position 2,309,021 in 5 isolates and at position 4,117,119 in one) and 9 different SNPs (involving 7 isolates). Interestingly, the 33 kb deleted region (from 4,106,137- 4,139,889) was part of the larger 71 kb deleted region (from 4,068,121 to 4,139,091). No mobile element could be evidenced surrounding these two deleted regions, but the presence of three copies of Repeated Sequences (RS) of 259 nt in-size were present (Figure 1.D). These regions share over 96% of nucleotide

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

Comparison to deposited genomes in public databases indicates that D33-3 isolate is the closest to other *Kp* ST512 genomes. In the chromosome of D1 (KPC+), a deletion of 11 nt in-size occurred, and this deletion is also present in D33-2 (KPC-) in addition to the insertion of a copy of *ISKpn26*. Furthermore, the loss of pKPC seems to have occurred in another branch, between D33-3 (KPC+) and D19-2 (KPC-) (Figure 1C). These observations make highly likely the loss of the *bla*_{KPC}-carrying plasmid by the KPC+ population rather than an acquisition by the KPC- population.

Discussion

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

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

71kb and in 33 kb-deleted genomes), inactivated by a non-sense mutation, or putatively transcriptionnally affected by the insertion of a copy of *ISKpn26* in the intergenic region, or by a 11 nt deletion in the *ramR* regulator (Figure 1D). *ramA*, an *araC*-family transcriptional regulator, is part of the *ramR-romA-ramA* operon and is involved in the expression of AcrAB efflux pump leading to increased MICs for tigecycline and fluoroquinolones. We could not observe any correlation between MICs for these antibiotics and any of the genetic events. However, since this region has been inactivated several times by independent mechanisms, it is tempting to speculate that its inactivation confers to this *Kp* ST512 a competitive advantage in that clinical context. The genetic analysis indicated that a loss of the *bla*_{KPC-3} carrying plasmid likely occurred in the *Kp* ST512 population. pKPC is very close to the successful pKpQIL plasmid (99,98% of nucleotide identity, 100% of query coverage), the first KPC-producing plasmid that had been sequenced in 2006 and known to have spread in all Europe through its tight association to the CG258.^{17,18} Plasmid stability assay could not evidence an increased capacity of *Kp* D1 to loose pKPC plasmid in comparison to other *Kp* isolates carrying pKpQIL-like plasmids (data not shown). Previous genomic analyses of KPC-*Kp* during long-term carriage have reported large plasmid rearrangements, deletions of the entire Tn4401, or plasmid transfer between Enterobacterales, but loss of the entire *bla*_{KPC}-carrying plasmid by a member of the *Kp*-CG258 has never been reported to date.^{9,19} The pKpQIL-like plasmids are thought to be highly compatible with *Kp*-CG258 genetic background and to have contributed to the worldwide dissemination of KPC carbapenemase.^{2,20} Information regarding the antibiotic selection pressure that occurred during the patient's hospitalization were available (Figure 1A). Given the genetic support of resistance genes linked to these 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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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	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (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	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOU000000000
D19-2	urine	0,38	0.125	0,094	0,032	1	1	1	48	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	missing	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOV000000000
D26-1	rectal	>256	4	>32	>32	1,5	1	1	32	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOS000000000
D26-2	urine	0,38	0.125	0,094	0,032	1	1	1,5	48	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	missing	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOT000000000
D33-1	urine	>256	6	>32	>32	1,5	1	1	48	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOP000000000
D33-2	urine	0,5	0.125	0,094	0,032	1	1	1,5	32	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	missing	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOR000000000
D33-3	rectal	>256	4	>32	>32	1,5	1	1	32	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOQ000000000
D40	rectal	>256	4	>32	>32	1,5	1	1	48	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOO000000000
D41	urine	0,5	0.125	0,094	0,032	1	1	1,5	32	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	missing	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWON000000000
D43	rectal	>256	6	>32	>32	1	1	1	32	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOM000000000
D54	rectal	>256	6	>32	>32	1,5	0,5	1	48	>32	<i>bla</i> _{SHV-11} , <i>gyrA</i> S831 <i>fosA</i> -like, <i>OmpK35</i> (truncated), <i>OmpK36</i> (mutated)	<i>bla</i> _{KPC-3} , <i>bla</i> _{TEM-1} , <i>bla</i> _{OXA-9*} ,	<i>aph</i> (3')-Ia, <i>aadA2</i> , <i>sul1</i> , <i>catA1</i> -like, <i>dfrA12</i> ,	<i>aac</i> (6')-Ib	PRJNA564512	VWOL000000000

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

B.

C.

D.

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.