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

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Background: KPC-producing *Klebsiella pneumoniae* of clonal group 258 are prominent in healthcare settings in many regions of the world. The *bla*KPC gene is mostly carried by a multireplicon IncFIIk-IncFI plasmid suspected to be highly compatible and stable in this genetic background. Here, we analysed the genetic diversity of an ST512 *K. pneumoniae* population in a single patient.

Methods: Twelve *K. pneumoniae* isolates (*n* = 5 from urine samples and *n* = 7 from rectal swabs) were recovered from one patient over a 2 month period. Antimicrobial susceptibility testing, plasmid extraction and WGS were performed on all isolates. The first *K. pneumoniae* isolate, D1, was used as a reference for phylogenetic analysis.

Results: Antimicrobial susceptibility testing, plasmid analysis and WGS revealed concomitant carriage of carbapenem-resistant and carbapenem-susceptible *K. pneumoniae* isolates of ST512, with the absence of the entire *bla*KPC-carrying plasmid in the susceptible population. Furthermore, 14 other genetic events occurred within the genome, including 3 chromosomal deletions (of 71 kb, 33 kb and 11 bp), 2 different insertions of IS*Kpn*26 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 bp repeated sequences.

Conclusions: Our study revealed (to the best of our knowledge) the first case of *in vivo* *bla*KPC-carrying plasmid curing and a wide within-patient genetic diversity of a single *K. pneumoniae* 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 healthcare facilities. KPC belongs to Ambler class A and emerged globally in Enterobacterales in the early 2000s. The worldwide spread of KPC is multifactorial and has been related to the diffusion of a particular clonal group (CG), i.e. CG258, defined by a few single-locus variants, with ST258, ST11 and ST512 being the most predominant. Notably, the emergence of the *bla*KPC gene in Italy has been related to the spread of ST512 isolates. Other genetic features associated with their spread are related to the genetic structure, a class II transposon containing the *bla*KPC gene (named Tn4401-like) and an IncFII-type conjugative plasmid. After the worldwide dissemination of these successful clones and CG since 2000, all reports of *K. pneumoniae* of CG258 refer to KPC-producing strains, with the exception of a study that retrospectively analysed carbapenem-susceptible *K. pneumoniae* collection strains isolated between 1999 and 2013 in New York City and some of these belonged to ST258.

WGS has emerged as a powerful tool to study bacterial evolution. Evolution of the genome of KPC-*K. pneumoniae* ST258 during long-term human colonization revealed complex plasmid rearrangements and genome plasticity. However, little is known about the genetic diversity that resides in the gastrointestinal tract within a single bacterial population at a specific timepoint. Here, we describe the concomitant carriage of *bla*KPC-positive carbapenem-resistant and *bla*KPC-negative carbapenem-susceptible...
K. pneumoniae ST512 clinical isolates in a single patient. Our objectives were to analyse the genetic diversity of KPC-K. pneumoniae isolates and to evaluate the cohabitation of several subpopulations of K. pneumoniae ST512.

Methods

Bacterial isolates, MICs and growth conditions

Twelve clinical isolates of K. pneumoniae were isolated from a single patient over a 2 month period. The isolates were obtained from rectal swabs (n = 7) and urine samples (n = 5) and were named after the sampling date, D1 being the first isolate and D54 being the one isolated 53 days later. From rectal swabs, K. pneumoniae isolates were obtained after growth on selective medium supplemented with carbapenems (ChromID 

The whole-genome sequences generated in the study have been submitted to the GenBank nucleotide sequences database under the accession numbers detailed in Table 1.

Ethics

This study was conducted in accordance with the Declaration of Helsinki and national standards. 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 10 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 favourable with exclusive enteric nutrition. The patient was repatriated to France for further medical care. Upon admission, screening for intestinal carriage of carbapenemase-producing Enterobacterales allowed identification of the presence of a KPC-producing K. pneumoniae (isolate D1), likely acquired during hospitalization in Italy. A cholecystectomy was performed 10 weeks after the acute episode and no complications occurred. During the 2 months of follow-up in France, 12 K. pneumoniae isolates (8 KPC-positive isolates and 4 KPC-negative isolates) were recovered from rectal swabs or urine samples (Figure 1a).

Susceptibility testing and resistome

Susceptibility testing of the 12 isolates recovered revealed two different phenotypes regarding β-lactams, but the same co-resistances. WGS revealed in all genomes three genes encoding aminoglycoside-modifying enzymes [aph(3’)-Ia, aac(6’)-Ib and aadA2], the natural fosfomycin resistance gene (fosA-like) and catA1-like, dfpA12 and sulI conferring resistance to chloramphenicol, trimethoprim and sulphonamides, respectively. A substitution in gyrA (S83I) was also identified, conferring resistance to fluoroquinolones. The 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 were inserted at positions 135 (+Asp) and 136 (+Gly) in comparison with the WT sequence (NC_016845.1). This OmpK36 variant is known to contribute to increased MICs of carbapenems for K. pneumoniae of CG258.

Eight isolates were resistant to carbapenems (imipenem MICs of 4–8 mg/L and meropenem and ertapenem MICs of >32 mg/L), whereas four isolates were susceptible to broad-spectrum cephalosporins and carbapenems (imipenem MICs of 0.125 mg/L). The content of β-lactamase-encoding genes differed between isolates. WGS revealed that all carbapenem-resistant K. pneumoniae isolates contained four β-lactamase genes: blaKPC-3 carried by transposon Tn4401a, blaTEM-1, blaOXA-3 (not functional due to a premature stop codon) carried by a multireplicon IncFIB-IncFII plasmid of 113 639 bp (pKpQ1-like) and the naturally chromosome-encoded blaSHV-11 gene. Carbapenem-susceptible isolates possessed only the blaSHV gene of the β-lactamase-encoding genes.

MLST analysis indicated that all K. pneumoniae (KPC positive and KPC negative) belonged to ST512, suggesting that the two populations of K. pneumoniae ST512 seem to differ only by the presence or absence of Tn4401a or of the whole blakpc-carrying plasmid. In order to distinguish between these two hypotheses, plasmid extractions analysed by electrophoresis revealed that an ~100 kb plasmid was missing in all KPC-negative isolates (Figure 1b).

Genome analysis

Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Ozyme, Saint-Quentin, France) following the manufacturer’s instructions. The DNA library was prepared using the Nextera XT-v2 Kit (Illumina, Paris, France) and then run on a HiSeq Illumina according to the manufacturer’s instructions. The DNA library was prepared using the RS_HGAP_Assembly.3 protocol and the genome was obtained using PacBio and Illumina’s sequencing technologies. Assembly was performed using the CLC genomic workbench v12.0 (QIAGEN, Marcy-l’Etoile, France), whereas urine samples were spread on non-selective chromogenic medium (UriSelect 10; Bio-Rad, Marnes-La-Coquette, France).

Antimicrobial susceptibility testing was performed using the disc diffusion method on Mueller–Hinton agar (Bio-Rad) and results were interpreted according to EUCAST guidelines. MICs of carbapenems, ceftazidime, tigecycline, rifampicin and aminoglycosides were determined by Etest (bioMérieux, Marcy-l’Etoile, France) and MICs of colistin were determined by broth microdilution (SensiTitre; Thermo Fisher, France).

Plasmid content analysis

Plasmids were extracted using Kieser’s extraction method and subsequently analysed by electrophoresis on a 0.7% agarose gel. Plasmid extractions analysed by electrophoresis revealed that the variant detection tool of CLC genomic workbench v12.0 (QIAGEN, Les Ulis, France).

Nucleotide sequence accession numbers

The whole-genome sequences generated in the study have been submitted to the GenBank nucleotide sequences database under the accession numbers detailed in Table 1.
| Isolate | Source | CAZ | IPM | ETP | MEM | TGC | CST | GEN | AMK | CIP | Resistome | GenBank accession number(s) |
|---------|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----------|-----------------------------|
| D1      | rectal | >128 | 4   | >32 | 4   | 1   | 1   | 32  | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | chromosome CP043969; pKPC-3-like CP043970; pD1-KPC CP043971; pColE CP043972 |
| D19-1   | rectal | >256 | 8   | >32 | 4   | 0.5 | 1   | 32  | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D19-2   | urine  | 0.38 | 0.125 | 0.094 | 0.032 | 1 | 1 | 1 | 48 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D26-1   | rectal | >256 | 4   | >32 | 1.5 | 1 | 1 | 32 | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D26-2   | urine  | 0.38 | 0.125 | 0.094 | 0.032 | 1 | 1 | 1 | 48 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D33-1   | urine  | >256 | 6   | >32 | 1.5 | 1 | 1 | 32 | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D33-2   | urine  | 0.5 | 0.125 | 0.094 | 0.032 | 1 | 1 | 1 | 32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D33-3   | rectal | >256 | 4   | >32 | 1.5 | 1 | 1 | 32 | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D40     | rectal | >256 | 4   | >32 | 1.5 | 1 | 1 | 32 | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D41     | urine  | 0.5 | 0.125 | 0.094 | 0.032 | 1 | 1 | 1 | 32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D43     | rectal | >256 | 6   | >32 | 1 | 1 | 1 | 32 | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |
| D54     | rectal | >256 | 6   | >32 | 1.5 | 0.5 | 1 | 32 | >32 | >32 | blao_{IV-11}, gyrA S83I, fosA-like, OmpK35 (truncated), OmpK36 (mutated) | PRJNA564512 |

CAZ, ceftazidime; IPM, imipenem; ETP, ertapenem; MEM, meropenem; TGC, tigecycline; CST, colistin; GEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin.

An asterisk indicates the presence of a nonsense mutation in the β-lactamase-encoding gene.
Figure 1. (a) Patient’s clinical case. This timeline indicates the patient’s medical history during the 2 months of follow-up. Antibiotics prescribed have been written within boxes. KPC-positive and KPC-negative K. pneumoniae are indicated by black and white stars, respectively. At hospital admission in France, 6 days of oral ofloxacin was given, combined with IV metronidazole (MTZ) for 2 days. After the identification of the first KPC-K. pneumoniae isolate (D1) from a rectal swab, and since there was no evidence of necrotizing pancreatitis, this antimicrobial chemotherapy was stopped. The patient received no further antimicrobial treatment until hospital discharge, when an episode of asymptomatic bacteriuria was treated using fosfomycin (FOS) and cefixime orally between Day 44 and Day 47 of the follow-up. The timeline is drawn to scale. (b) Plasmid extraction analysed by electrophoresis on a 0.7% agarose gel. Escherichia coli 50192 was used as a reference for plasmid size. The arrows indicate the positions of the three plasmids: pKPN3-like, pKPC (missing in KPC-negative isolates) and pColE. (c) Phylogenetic analysis of isolates recovered over the 2 month period. The circles are to scale with the number of isolates. KPC-positive and KPC-negative K. pneumoniae are indicated in full and dashed circles, respectively. The length of the branches is proportional to the number of genetic events (insertions, deletions, loss of pKPC plasmid and SNPs). Dashed lines indicate two different putative evolutionary pathways. (d) Genetic environment of the deleted regions. RSs are indicated by grey triangles. Losses of the 71 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-ramA-ramA genes.
Genomic analysis and phylogeny
To establish whether a gain or loss of the bla_{KPC}-carrying plasmid occurred in the K. pneumoniae ST512 population, SNPs and genetic events underlying in vivo evolution analyses were explored using isolate D1 as a 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), 3 different deletions of chromosomal regions (71 959 bp in 5 isolates, 33 752 bp in 2 isolates and 11 bp in 2 isolates), 2 different insertions of a copy of ISKpn26 (at position 2309021 in 5 isolates and at position 4117119 in 1 isolate) and 9 different SNPs (involving 7 isolates). Interestingly, the 33 kb deleted region (4106137–4139889) was part of the larger 71 kb deleted region (4068121–4139091). No mobile element could be found surrounding these two deleted regions, but the presence of three copies of repeated sequences (RSs) of 259 bp were present (Figure 1d). These regions share over 96% nucleotide identity (Figure S1, available as Supplementary data at JAC Online). Recombination events involving RS1/RS3 and RS2/RS3 are likely responsible for the deletions of the 71 and 33 kb fragments, respectively.

Comparison with deposited genomes in public databases indicates that isolate D33-3 was the closest to other K. pneumoniae ST512 genomes. In the chromosome of D1 (KPC positive), a deletion of 11 bp occurred and this deletion was also present in D33-2 (KPC negative), 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 positive) and D19-2 (KPC negative) (Figure 1c). These observations make it highly likely that there was loss of the bla_{KPC}-carrying plasmid by the KPC-positive population rather than its acquisition by the KPC-negative population.

Discussion
An unexpected genetic and phenotypic variability of K. pneumoniae 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 (2 months) was due to the presence or not of the bla_{KPC}-carrying plasmid (pKPC). In our study, this diversity was clearly underestimted by the use of a selective medium for rectal samples that only allowed the growth of carbapenem-resistant bacteria. Hence the carbapenem-susceptible population could only be identified from urine samples. Despite this major bias, three isolates from different branches of the distribution were recovered on the same day (Day 33) as proof that this genetic diversity was 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 coding sequences and, among them, the ramA gene (accession number KC843634) was entirely deleted (in 71 and 33 kb deleted genomes), inactivated by a nonsense mutation or putatively transcriptionally affected by the insertion of a copy of ISKpn26 in the intergenic region or by an 11 bp deletion in the ramR regulator (Figure 1d). ramA, an aacC-family transcriptional regulator, is part of the ramR-ramA-ramA operon and is involved in the expression of the AcrAB efflux pump, leading to increased MICs of tigecycline and fluoroquinolones. We could not observe any correlation between MICs of these antibiotics and any of the genetic events. However, since this region was inactivated several times by independent mechanisms, it is tempting to speculate that its inactivation confers to this K. pneumoniae 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 K. pneumoniae ST512 population. pKPC is very closely related to the successful pKpQIL plasmid (99.98% nucleotide identity and 100% query coverage), the first KPC-producing plasmid that was sequenced in 2006 and known to have spread throughout all of Europe by its tight association with CG258.7,18 A plasmid stability assay did not find an increased capacity of K. pneumoniae D1 to lose the pKPC plasmid in comparison with other K. pneumoniae isolates carrying pKpQIL-like plasmids (data not shown). Previous genomic analyses of KPC-K. pneumoniae 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 K. pneumoniae CG258 has never been reported to date.9,19 The pKpQIL-like plasmids are thought to be highly compatible with the K. pneumoniae 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 was available (Figure 1a).Given the genetic support of resistance genes linked to these antimicrobial agents, cefixime seems to be the sole molecule capable of maintaining selective pressure to prevent the loss of the KPC plasmid; however, it was prescribed on Day 44, far after the isolation of the first non-KPC producer on Day 19. Thus, 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 K. pneumoniae ST512 isolate.

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

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Transparency declarations
None to declare.
Supplementary data

Figure S1 is available as Supplementary data at JAC Online.

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