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Community-acquired infection caused by the uncommon hypervirulent *Klebsiella pneumoniae* ST66-K2 lineage

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Abstract

Klebsiella pneumoniae (Kp) reference strain Kp52.145 is widely used in experimental *Klebsiella* pathophysiology. Since 1935, only one other strain of the same sublineage (sequence type ST66, capsular serotype K2) was isolated (AJ210, Australia). Here, we describe a community-acquired invasive infection caused by a ST66-K2 Kp strain in France. Four hypermucoviscous Kp isolates responsible for acute otitis media, meningitis, bacteraemia and bacteriuria, respectively, were obtained from a patient with a history of chronic alcoholism and diabetes mellitus, and infected with HIV. The isolates were characterized by phenotypic and genomic methods. The four genetically identical ST66-K2 isolates presented a full antimicrobial susceptibility profile, including to ampicillin, corresponding to a single strain (SB5881), which was more closely related to AJ210 (135 SNPs) than to Kp52.145 (388 SNPs). Colibactin and yersiniabactin gene clusters were present on the integrative and conjugative element ICE*Kp*10 in the chromosome. The two plasmids from Kp52.145 were detected in SB5881. In addition to carrying genes for virulence factors RmpA, aerobactin and salmochelin, plasmid II has acquired in SB5881, the conjugation machinery gene cluster from plasmid I. We report the first case of community-acquired infection caused by a hypervirulent ST66-K2 Kp strain in Europe. This demonstrates the long-term persistence of the high-virulence and laboratory model ST66-K2 sublineage. The combination of a conjugative apparatus and major virulence genes on a single plasmid may contribute to the co-occurrence of hypervirulence and multidrug resistance in single Kp strains.

DATA SUMMARY

Sequence read files for the four isolates and complete genome assembly of SB5881 have been deposited in the European Nucleotide Archive under the BioProject number PRJEB37472.

INTRODUCTION

Since the 1980s, an emergence of severe community-acquired infections (CAIs) driven by hypervirulent (Hv) *Klebsiella pneumoniae* (Kp) strains has been observed [1, 2]. Most common clinical manifestations of Kp CAIs include pyogenic liver abscess (in the absence of biliary disease), pneumonia, meningitis, brain abscess or endophthalmitis. These are often followed by bacteraemia, and the multiplicity of infection sites or the metastatic spread is a characteristic of HvKp infections [2]. HvKp infections are most prevalent in Asia (Taiwan, China, Hong Kong, Singapore and South Korea), with recent reports suggesting an increasing incidence worldwide (USA, South America, Australia and Europe) [1, 2]. In the first part of the twentieth century, CAIs involving HvKp were more common than today in the Western hemisphere

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Abbreviations: CAIs, community-acquired infections; cgMLST, core genome MLST; CGs, clonal groups; CNIL, Commission Nationale Informatique et Libertes; CSF, cerebro-spinal fluid; CT-scan, computed tomography; HIV, human immunodeficiency virus; Hv, hypervirulent; ICE, integrative conjugative element; IS, insertion sequence; kp, klebsiella pneumoniae; MLST, multilocus sequence typing; PCT, procalcitonin; WBC, white blood cells; WGS, whole genome sequencing.

Reads and genomic sequences of the isolates analysed in this study were deposited at the European Nucleotide Archive (accession number PRJEB37472).

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Three supplementary figures are available with the online version of this article.

and were associated with chronic alcoholism [2]. Population biology studies have shown that HvKp infections are caused by a limited number of lineages, such as clonal group (CG) 23, CG65 and CG380 [3]. These lineages often harbour a combination of chromosomal pathogenicity factors (K1 or K2 capsular types) and accessory virulence factors, in particular regulators of the mucoid phenotype (RmpA/RmpA2) and siderophores (aerobactin [*iro*] and salmochelin [*iut*]) carried by plasmids [4].

One of the main *Kp* laboratory reference strains is Kp52.145 (=a laboratory subculture of CIP 52.145=B5055) [5, 6]. It was isolated in 1935 in Indonesia, was selected as the reference strain of capsular serotype K2 [7] and belongs to sequence-type (ST) 66 [8]. Kp52.145 is a highly virulent strain (lethal dose 50 in mice, $<10^3$ c.f.u.) from which the virulence plasmid of *Klebsiella* and the role of aerobactin and RmpA in virulence were discovered [5, 6, 9]. Following these discoveries, the strain has been used as one of the main reference strains in *Klebsiella* physiopathology studies; e.g [10, 11]. Strain Kp52.145 harbours the most important Kp virulence factors: plasmid-carried *rmpA*/*rmpA2*, aerobactin cluster *iut/iuc,* salmochelin cluster *iro;* and chromosomal integrative conjugative elements (ICEs)-carried yersiniabactin [*ybt*] and colibactin [*clb*] clusters [6, 12, 13]. More recently an additional virulence factor, phospholipase D, was uncovered using this strain [6]. However, in sharp contrast with the prominent role of reference strain Kp52.145 in *Kp* laboratory work, sublineage ST66-K2 was almost never observed in clinical and epidemiological studies. For nearly one century, this sublineage has only been reported once, with the description of isolate AJ210 from Australia in 2002 [14]. Here, we describe the clinical and molecular characteristics of a recent ST66-K2 community-acquired infection.

Impact Statement

Klebsiella pneumoniae (Kp) represents nowadays one of the major antimicrobial-resistant bacterial pathogens that threaten public health. In addition to its notorious role as a nosocomial pathogen, Kp also causes severe community-acquired infections (CAIs). In recent years, population-structure studies have evidenced that Kp causing CAIs belong to a limited number of clonal groups (CGs), in contrast to the much larger diversity of classical Kp strains causing opportunistic nosocomial infections. In parallel, research into the molecular mechanisms of *Klebsiella* pathogenesis has relied on a few laboratory reference strains. Among these, strain Kp52.145, isolated in Indonesia in 1935 and of MLST type ST66/serotype K2, was central in the discovery of several important *Klebsiella* virulence factors, including its virulence plasmid, mucoid phenotype and iron acquisition capacities. However, somewhat mysteriously ST66 has almost never been reported in the literature among *Klebsiella* infections for nearly one century. Here, we describe a severe CAI caused by a Kp ST66/K2 strain, providing evidence for the persistence, even if largely silent, of this reference sublineage. Further, we show that the virulence plasmid of this 're-emerged' strain now possesses a conjugative apparatus gene cluster, prompting concern of possible dissemination of hypervirulence genes to other sublineages of Kp, including multidrug-resistant ones.

METHODS

Clinical case presentation

A patient who was infected with human immunodeficiency virus (HIV) and with a history of insulin-dependent diabetes mellitus and chronic alcoholism was admitted to the emergency room for alcohol withdrawal syndrome. No history of travel abroad or recent hospital admissions was reported. In the emergency room, the relevant clinical events were an episode of epileptic seizure spontaneous resolved, a body temperature at 311.15 K (38.5 °C) and a right otalgia/otorrhea. Laboratory tests revealed procalcitonin (PCT) at 3.54 µg l^{-1}, 5.7×10^9 l^{-1} white blood cells (WBC) and platelet count at 19×10^9 l^{-1}. Cranial computed tomography (CT-scan) concluded that there was no intracranial bleeding. Due to a neurological aggravation with vigilance disorders the patient was transferred to the intensive care unit and a lumbar puncture was performed. Cerebrospinal fluid (CSF) revealed bacterial meningitis with 5.3×10^9 l^{-1} WBC, 93% of polymorphonuclear neutrophils and with the presence of *K. pneumoniae*. In addition, bacteriological exams to urine, blood and an otorrhea samples revealed the presence of a hypermucoviscous *Kp*. Treatment with cefotaxime (neuromeningeal doses - 2 g IV every 4 h) was started, and complemented with ofloxacin IV and intra-auricular ofloxacin due to mastoid involvement suspicion, overturned by the CT-scan. Three days after, due to persistent fever, the dose of cefotaxime was increased to 3 g IV every 4 h. Neurological and clinical improvement was confirmed 5 days after. Treatment with cefotaxime was continued for 21 days. At the hospital discharge, the patient had no clinical sequelae.

Phenotypic and genotypic characterization of *K. pneumoniae* isolates

Four Kp isolates were recovered from blood (day 1: SB5881), urine (day 1: SB5882), CSF (day 2: SB5883) and otorrhea (day 2: SB5884) samples. They were involved in the different infectious site (bacteraemia, bacteriuria, meningitis and acute otitis media, respectively). The four isolates were analysed phenotypically and genotypically. Antimicrobial susceptibility testing to different antimicrobial classes (beta-lactams, aminoglycosides, fluoroquinolones and macrolides) was performed by disc diffusion following the CASFM/EUCAST 2017 guidelines (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf). Whole-genome sequencing (WGS) was performed after Nextera XT library preparation by Illumina (NextSeq-500, 2×150 nt), reads were assembled using SPAdes v3.12.0 [15]

and annotated with PROKKA (https://github.com/tseemann/prokka). The blood isolate (SB5881) was also subjected to long-read sequencing (Oxford Nanopore Technologies) and a hybrid assembly was obtained using Unicycler 0.4.4 [16]. Multilocus sequence typing (MLST, 7 genes) and core genome MLST (cgMLST, 629 genes) was performed using BIGSdb at the Institut Pasteur *Klebsiella* MLST website (http://bigsdb.pasteur.fr/klebsiella/) [3]. Antimicrobial resistance and virulence genes were detected using KLEBORATE (https://github.com/katholt/Kleborate) and BIGSdb. Plasmid replicons were searched using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and plasmids were compared using EasyFig 2.2.2. For phylogenetic analysis, short-reads from the four isolates and from AJ210 were mapped to the reference genome Kp52.145 using a Burrows–Wheeler aligner, and the final whole-genome alignment was used to infer a maximum-likelihood tree using IQ-TREE 1.6.3 (model K3Pu+F, 1000 bootstraps).

Ethical statement

In this study, the research was done on data that was already available. We used the leftover of the sample (the bacterial strains) to conduct our research. In no way was the patient's care changed by our study. Accordingly, in France this type of research is out of the scope of the decree no. 2016–1537 of 16 November 2016 implementing law no. 2012–300 of 5 March 2012 on research involving human subjects. The patient data has been included in a database called Outcomerea, for which a declaration for research was approved by the French competent body CNIL (Commission Nationale Informatique et Libertés), and received a CNIL certificate number 999262 v2. In this context, we have the authorization to use the patient's data.

RESULTS AND DISCUSSION

The four Kp isolates presented a hypermucoviscous phenotype (positive string test) and a fully susceptible antimicrobial profile, including for ampicillin. Genomic analyses revealed that they belonged to *K. pneumoniae sensu stricto* (Kp1 phylogroup) and to ST66-K2. To the best of our knowledge, this is the first time that ST66 is identified in Europe. Interestingly, none of the isolates carried the intrinsic bla_{SHV} typical of Kp1, as was the case for Kp52.145 or AJ210, explaining the exceptional susceptibility to ampicillin observed in our isolates. Genomic analysis of ST66 strains revealed that a 12 kb region between a diguanylate phosphodiesterase and the *lacZ* gene, containing the bla_{SHV} and its genetic surroundings, was missing from the ST66 strains (Fig. S1, available in the online version of this article). No acquired antimicrobial resistance gene was observed. cgMLST and SNP analyses revealed that the four isolates were genetically identical (0 cgMLST allelic mismatches and 0 SNPs; Fig. S2), thus corresponding to the same strain (which we name SB5881) disseminated in multiple body sites, a common characteristic of HvKp strains. Phylogenetic analysis (Fig. S2) showed that the SB5881 strain was more closely related to AJ210 strain (1.4% distinct alleles in cgMLST, 135 SNPs) than to Kp52.145 (5.4% distinct alleles, 388 SNPs). Chromosome sequence analysis evidenced a conserved structure with the presence of the K2 capsular gene cluster, colibactin (*clb* 1/CbST 9) and yersiniabactin (*ybt* 12/YbST 316) on an ICE*Kp*10 [12] mobile genetic element (Fig. S3). Other genomic islands previously identified in the reference strain Kp52.145 [6], as well as genes coding for type-III fimbriae (*mrk* cluster) and the phospholipase D (*pld1*), were also present. Contrary to other hypervirulent Kp sublineages such as CG23, no heavy-metal resistance (lead, copper, silver and tellurium) gene clusters were detected [4, 13].

Two plasmids were previously described in the Kp52.145 reference strain: plasmid I (95 kb, IncFIA[HI1], Fig. 1) and plasmid II (also called pKP-VP2; 121 kb, non-self-transmissible $IncFIB_K$, Fig. 1) [6, 13]. These plasmids were also detected in the four isolates, together with an additional small plasmid (3.7 kb). Plasmid I (homology of 99.98% with Kp52.145 plasmid I) harbored *rmpA2* and genes for the conjugation machinery (origin of transfer, relaxase, type-IV coupling protein and bacterial type-IV secretion system). Plasmid II (99.97% homology with the reference Kp52.145) harboured the genes for virulence factors RmpA (allele rmpA-9), aerobactin (*iuc*-2/AbST6) and salmochelin (*iro*-2/SmST22), typical for the pKP-VP2 [13]. However, different from plasmid II of Kp52.145, in strain SB5881 this plasmid also harboured a 40 kb region nearly identical to the conjugation machinery gene cluster from plasmid I (Fig. 1; 99.99% homology with SB5881 plasmid I, 4 SNPs at 3′ extremity of this gene cluster). The most likely mechanism of duplication of the conjugation gene cluster and its integration in plasmid II was the involvement of an insertion sequence (IS) belonging to the *IS*3 family (*IS*407 group) observed multiple times in the chromosome of SB5881 strain. These findings suggest an ability of virulence plasmid II to be self-conjugative and thereby disseminate its virulence genes horizontally. Our observation of pKP-VP2 in SB5881 strain demonstrates its long-term stability in the ST66 sublineage, as described for other HvKp sublineages [13]. However, the ability of pKP-VP2 to acquire a transfer apparatus machinery gene cluster might provide an explanation for the dissemination of pKP-VP2 in unrelated sublineages such as CG380 [13], and represents a risk for wider dissemination in other Kp lineages. More studies based on complete sequences of virulence plasmids are needed to define the prevalence and stability of potentially conjugative pKP-VP2 plasmids.

CONCLUSIONS

The clinical presentation was typical of community-acquired invasive infections caused by *K. pneumoniae* [2], with dissemination of the infectious strain to multiple organs. Our microbiological observations demonstrate the persistence and dissemination to Europe of the historical ST66-K2 sublineage. Besides, we show that the reported isolate differs from the archetypical reference strain Kp52.145 by the potential to conjugate and thereby disseminate plasmid-encoded virulence genes to other strains and sublineages. This work contributes a potential novel mechanistic explanation to the

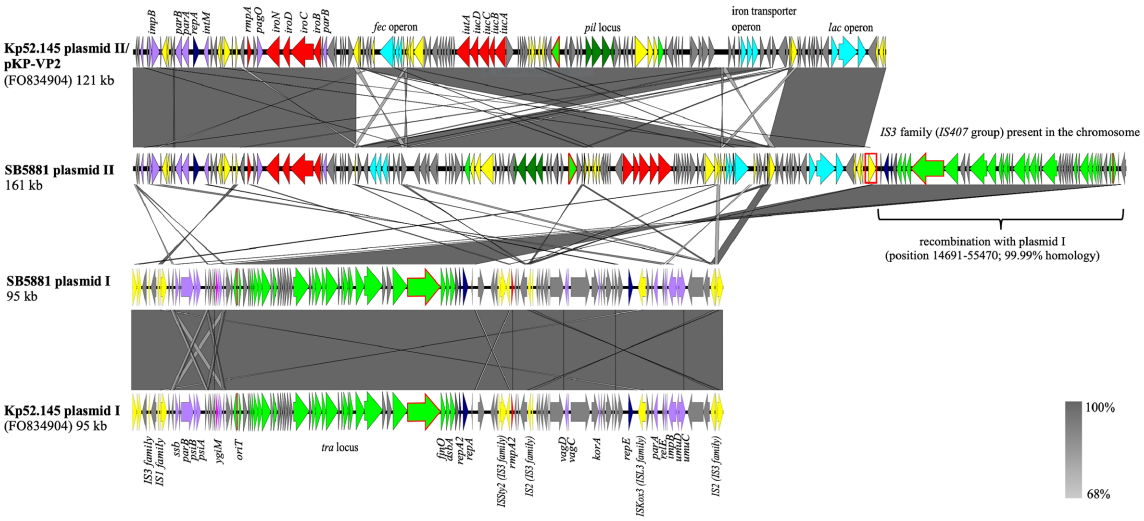

Fig. 1. Linear comparison of plasmid I and II from strain Kp52.145 and the ones from SB5881. The grey shading denotes shared regions of homology. ORFs are portrayed by arrows and coloured based on the predicted gene function: blue, replication; green; DNA transfer (associated with the *tra* locus – T4SS and T4CP systems); orange, origin of transfer site (*oriT*); light purple, partition, stability, and maintenance; yellow, mobile genetic elements; dark green, type IV pilus biosynthesis locus; red, virulence genes; cyan, iron transporter, lactose and *fec* operons; fuchsia, prophage-linked; light grey, other functions. Predicted relaxase is outlined in red. IS3 family (IS407 group) in the recombination region of plasmid I and II, originated from the chromosome of SB5881, is represented by a red box.

current worrisome scenario of virulence plasmids being transferred to multidrug-resistant Kp strains.

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Author contributions

Conceptualization of the study: S.B., E.R. performed the experiments: C.R., C.H., V.P. analysed the clinical data: C.H., E.R., G.P. analysed the genomic data: C.R., S.B. wrote the initial draft of the manuscript: C.R., C.H., S.B. commented on working versions of the manuscript and agreed on the final version of the manuscript: all.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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