

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

Carla Rodrigues<sup>1</sup>†, Camille d'Humières<sup>2,3,4,5,6</sup>†, Grégory Papin<sup>7</sup>, Virginie Passet<sup>1</sup>, Etienne Ruppé<sup>2,3,4</sup> and Sylvain Brisse<sup>1,\*</sup>

## 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 ICEKp10 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

Received 27 May 2020; Accepted 17 July 2020; Published 04 August 2020

**Author affiliations:** <sup>1</sup>Institut Pasteur, Biodiversity and Epidemiology of Bacterial Pathogens, 75015 Paris, France; <sup>2</sup>INSERM, IAME, UMR 1137, 75018 Paris, France; <sup>3</sup>Université de Paris, Paris, France; <sup>4</sup>AP-HP, Hôpitaux Universitaires Paris Nord Val-de-Seine, Laboratoire de Bactériologie, Site Bichat, 75018 Paris, France; <sup>5</sup>Microbial Evolutionary Genomics, Institut Pasteur, 75015 Paris, France; <sup>6</sup>CNRS, UMR3525, 75015 Paris, France; <sup>7</sup>AP-HP, Hôpitaux Universitaires Paris Nord Val-de-Seine, Service de Réanimation Médicale, Site Bichat, F-75018 Paris, France.

\*Correspondence: Sylvain Brisse, sbrisse@pasteur.fr

**Keywords:** Europe; community-acquired infection; hypervirulence; genomics; recombination; virulence plasmid.

**Abbreviations:** CAIs, community-acquired infections; cgMLST, core genome MLST; CGs, clonal groups; CNIL, Commission Nationale Informatique et Libertés; 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, prolactin; 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).

†These authors contributed equally to this work

**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.

000419 © 2020 The Authors



This is an open-access article distributed under the terms of the Creative Commons Attribution NonCommercial License.

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.

## 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 spontaneously resolved, a body temperature at 311.15 K (38.5°C) and a right otalgia/otorrhea. Laboratory tests revealed procalcitonin (PCT) at  $3.54 \mu\text{g l}^{-1}$ ,  $5.7 \times 10^9 \text{ l}^{-1}$  white blood cells (WBC) and platelet count at  $19 \times 10^9 \text{ l}^{-1}$ . 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  $5.3 \times 10^9 \text{ l}^{-1}$  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

### 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.

IV every 4 h) was started, and complemented with ofloxacin IV and intra-auditory 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](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

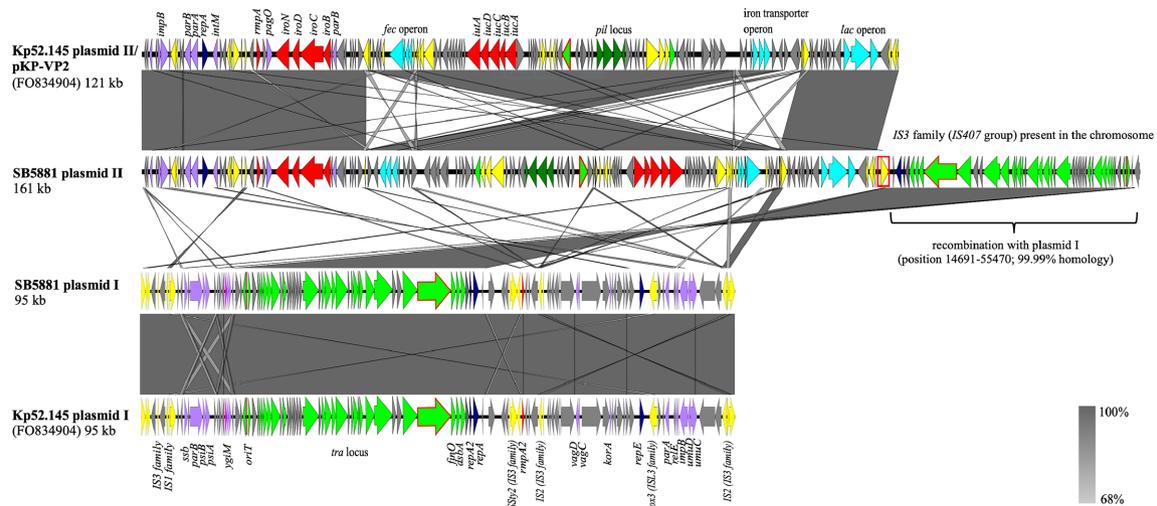
The four Kp isolates presented a hypermucoviscous phenotype (positive string test) and a fully susceptible antimicrobial profile, including for ampicillin. Genomic analyses revealed that they belonged to *K. pneumoniae sensu stricto* (Kp1 phylogroup) and to ST66–K2. To the best of our knowledge, this is the first time that ST66 is identified in Europe. Interestingly, none of the isolates carried the intrinsic *bla*<sub>SHV</sub> 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*<sub>SHV</sub> 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 ICEKp10 [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<sub>K</sub>, 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 IS3 family (IS407 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.

#### Funding information

This work was supported financially by the French Government's Investissement d'Avenir program Laboratoire d'Excellence 'Integrative Biology of Emerging Infectious Diseases' (ANR-10-LABX-62-IBEID). C.R. was financially supported by the MedVetKlebs project, a component of European Joint Programme One Health EP, which has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No. 773830.

#### Acknowledgements

We thank the 'Plateforme de Microbiologie Mutualisée' of Institut Pasteur for genomic sequencing.

#### 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.

#### References

- Siu LK, Yeh K-M, Lin J-C, Fung C-P, Chang F-Y. *Klebsiella pneumoniae* liver abscess: a new invasive syndrome. *Lancet Infect Dis* 2012;12:881–887.
- Russo TA, Marr CM. Hypervirulent *Klebsiella pneumoniae*. *Clin Microbiol Rev* 2019;32.
- Bialek-Davenet S, Criscuolo A, Ailloud F, Passet V, Jones L et al. Genomic definition of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* clonal groups. *Emerg Infect Dis* 2014;20:1812–1820.
- Wyres KL, Lam MMC, Holt KE. Population genomics of *Klebsiella pneumoniae*. *Nat Rev Microbiol* 2020;18:344–359.
- Nassif X, Sansonetti PJ. Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with the presence of a plasmid encoding aerobactin. *Infect Immun* 1986;54:603–608.
- Lery LMS, Frangeul L, Tomas A, Passet V, Almeida AS et al. Comparative analysis of *Klebsiella pneumoniae* genomes identifies a phospholipase D family protein as a novel virulence factor. *BMC Biol* 2014;12:41.
- Ørskov I, Ørskov F. 4 serotyping of *Klebsiella*. *Methods Microbiol* 1984;14:143–164.
- Brisse S, Fevre C, Passet V, Issenhuth-Jeanjean S, Tournèze R et al. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. *PLoS One* 2009;4:e4982.
- Nassif X, Honoré N, Vasselon T, Cole ST, Sansonetti PJ. Positive control of colanic acid synthesis in *Escherichia coli* by *rmpA* and *rmpB*, two virulence-plasmid genes of *Klebsiella pneumoniae*. *Mol Microbiol* 1989;3:1349–1359.
- Frank CG, Regueiro V, Rother M, Moranta D, Maeurer AP et al. *Klebsiella pneumoniae* targets an EGF receptor-dependent pathway to subvert inflammation. *Cell Microbiol* 2013;15:1212–1233.
- March C, Moranta D, Regueiro V, Llobet E, Tomás A et al. *Klebsiella pneumoniae* outer membrane protein A is required to prevent the activation of airway epithelial cells. *J Biol Chem* 2011;286:9956–9967.
- Lam MMC, Wick RR, Wyres KL, Gorrie CL, Judd LM et al. Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICEKp in *Klebsiella pneumoniae* populations. *Microb Genom* 2018;4.
- MMC L, Wyres KL, Judd LM, Wick RR, Jenney A et al. Tracking key virulence loci encoding aerobactin and salmochelin siderophore synthesis in *Klebsiella pneumoniae*. *Genome Med* 2018;10:1–15.
- Holt KE, Wertheim H, Zadoks RN, Baker S, Whitehouse CA et al. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proc Natl Acad Sci U S A* 2015;112:E3574–E3581.

15. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M *et al.* SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012;19:455–477.
16. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput Biol* 2017;13:e1005595.

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

**Find out more and submit your article at [microbiologyresearch.org](http://microbiologyresearch.org).**