



Characterization of hypervirulent *Klebsiella pneumoniae* isolates in Belgium

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Abstract

Hypervirulent *Klebsiella pneumoniae* (hvKp) raised concern worldwide. We studied 22 hvKp clinical invasive isolates referred to the Belgian national reference laboratory between 2014 and 2020. Sixty-four percent of the isolates expressed K2 capsular serotype and belonged to 7 different MLST lineages, while 32% expressed K1 (all belonging to ST23) and were associated with liver abscesses. Primary extra-hepatic infections were reported in 36% and sepsis for 95% of the patients with 30% of deaths. Improved clinical and microbiological diagnostics are required as hvKp may represent an underestimated cause of community-acquired invasive infections in Belgium.

Keywords *Klebsiella pneumoniae* · Hypervirulence · Hypermucoviscosity · Capsular serotype · Whole genome sequencing · Molecular epidemiology

Introduction

A hypervirulent variant of *Klebsiella pneumoniae* was reported in the mid-1980s and has raised concerns in recent years due to its extraordinary invasiveness, resulting in higher morbidity and mortality [1]. Typically, hypervirulent *K. pneumoniae* (hvKp) is responsible for severe monomicrobial community-acquired infections such as cryptogenic pyogenic liver abscesses [2] often associated with unusual septic

metastatic localizations, including endophthalmitis [3], or meningitis [4, 5], in immunocompetent hosts of any age.

According to the current knowledge, no single factor can fully define hvKp [6]. The diagnosis of hvKp infection is based on a combination of clinical suspicion and microbiological characteristics of hvKp distinguishable from classic *K. pneumoniae* (cKp). Hypermucoviscosity representing the major feature for hypervirulence results from the overexpression of capsular polysaccharides. It confers to hvKp higher resistance to phagocytosis and intracellular killing by neutrophils and bactericidal complements. The most common capsule locus associated with hvKp is K1, followed by K2, K5, and K57 [1, 7]. Most of hvKp strains of K1 serotype belong to clonal complex (CC) 23, while K2 serotype strains belong to multiple distinct multilocus sequence types (MLSTs) [8]. hvKp has acquired virulence genes present on large plasmids (e.g., pK2044 and pLVPK) and within integrative conjugative elements that participate to its hypervirulent phenotype such as the mucoid phenotype regulator involved in capsular synthesis and the iron acquisition systems and siderophores (e.g., aerobactin, salmochelin, yersiniabactin) involved in growth and survival of bacteria [1, 9].

While most cases have been initially described from South-East Asia, increasing reports have since evidenced

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hvKp infections worldwide. The estimated prevalence of hvKp among *K. pneumoniae* bacteraemia isolates was 8.2% in Canada [10], 6.3% in the USA [11], and 2.8% in Spain [12]. Yet, there have been no studies reported on the burden and characterization of hvKp in Belgium. We investigated retrospectively clinical and microbiological characteristics of hvKp strains collected by the Belgian National Reference Center for Antibiotic-Resistant Gram-Negative Bacilli (NRC) between 2014 and 2020.

Materials and methods

Between January 2014 and January 2020, 22 putative hyper-virulent *K. pneumoniae* isolates were referred to NRC based on clinical presentation. The bacterial isolates were collected from distinct patients dispersed in eight Belgian hospitals. Antimicrobial susceptibility testing was performed by disk diffusion using EUCAST interpretative breakpoints [13].

All isolates were tested for the hypermucoviscous phenotype by performing the “string test” (the formation of a viscous string > 5 mm in length when fresh bacterial colonies are stretched by an inoculation loop [14]) and analyzed by whole genome sequencing (WGS). Libraries were constructed with Nextera DNA Flex Library Prep Kit (Illumina Inc, CA, USA) and were sequenced on the Illumina iSeq according to the manufacturer’s protocol. *Klebsiella pneumoniae* taxonomy ID (573) was confirmed by Kraken 2 [15]. Resistance genes were identified with ResFinder (<https://cge.cbs.dtu.dk/services/ResFinder/>). Virulence genes, MLST, and strict core genome MLST (scg-MLST) were determined using Institut Pasteur MLST databases (<https://bigsdw.web.pasteur.fr/klebsiella/klebsiella.html>) [16] while capsule type using Kaptive (<http://kaptive.holtlab.net/>). Whole-genome MLST (wgMLST) was analyzed using BioNumerics (version 8.0, Applied-Maths, St Martens Latem, Belgium) with a scheme containing 19,729 loci for *K. pneumoniae*. To detect the virulence plasmids, the contigs of hvKp isolates were aligned with well-characterized virulence plasmids pLVPK (GenBank accession AY378100) [17], pK0244 (GenBank accession AP006726.1) [18], Kp52.145 plasmid I, and Kp52.145 plasmid II (GenBank accession FO834905.1) [19].

Results and discussion

Of the 22 hvKp clinical isolates, 7 and 14 isolates were identified as capsular genotypes K1 and K2, respectively. While studies in other countries have reported various prevalence rate of K57 serotype [20, 21], only one case was observed in this study.

Clinical records available are summarized in Table 1. All patients developed monomicrobial, community-acquired invasive infections. The median age was 61.3 years, with a majority of male patients (17/22). Underlying disorders such as diabetes mellitus, hepatobiliary diseases, active neoplasia, human immunodeficiency virus infection, alcoholism, and benign prostatic hyperplasia were reported in the majority (85%; 17/20; no information for two patients) of infected patient and are consistent with previous reports [2, 22–24]. Birthplace and travel information when available (19/22) indicated that one Asian patient traveled to Hong Kong before infection, one came from Romania, seven were from African countries, and ten were born in Belgium without notion of traveling abroad. Unlike previous reports [2, 23], Asian patients did not represent the main ethnic group (only one case (5%; 1/22) in our study vs. 50% (16/32) in cohorts in the USA [2]) suggesting the successful establishment of this pathogen in Belgium causing invasive infections among ethnicities other than Asian communities.

Anatomical origins of hvKp isolates included peripheral blood (68.2%; 15/22), hepato-biliary (18.2%; 4/22), ocular (4.5%; 1/22), genital (4.5%; 1/22), and soft tissues (4.5%; 1/22). The primary site of infection was a liver abscess for 63.6% (14/22) of the patients. Of note, the K1-isolates were exclusively associated with liver abscesses. Extra-hepatic manifestations in lung (pneumonia or lung abscess) were reported in five patients, while endophthalmitis, necrotising fasciitis, and orchiepididymitis were incriminated once each as the primary infection site. Sepsis occurred during the course of the infection for more than 95% of the patients. Septic metastatic foci were documented only for three patients, but could be underreported if extensive anatomical investigations were not conducted. Life-threatening conditions required admission to an intensive care unit during their hospital stay for nearly half (10/22) of the patients and 30% (6/20; no information for two patients) died. Of these deaths, five occurred in infections by non-K1 isolates and were associated with primary extrahepatic manifestations. Despite the lack of recommendations of the most suitable antibiotics for treating hvKp infections, patients were mostly treated with antibiotics such as penicillin/beta-lactamase inhibitor, cephalosporin, and fluoroquinolone alone or in combination, associated in ten cases with abscess drainage in accordance with previous studies [2].

MLST revealed that K1 isolates ($n = 7$) belonged exclusively to ST23 and K57 isolate belonged to ST592 whereas K2 isolates arose from seven distinct clonal lineages (ST380 ($n = 3$), ST2039 ($n = 3$), ST65 ($n = 3$), ST66 ($n = 2$), ST25 ($n = 1$), ST86 ($n = 1$), ST3240 ($n = 1$)), confirming the phylogenetic diversity within the K2 isolates (Fig. 1). In addition to those previously described [21, 25], we reported here a K2 isolate from ST3240. New hvKp STs continue to emerge, indicating the ongoing evolution of hvKp [8, 25, 26]. The

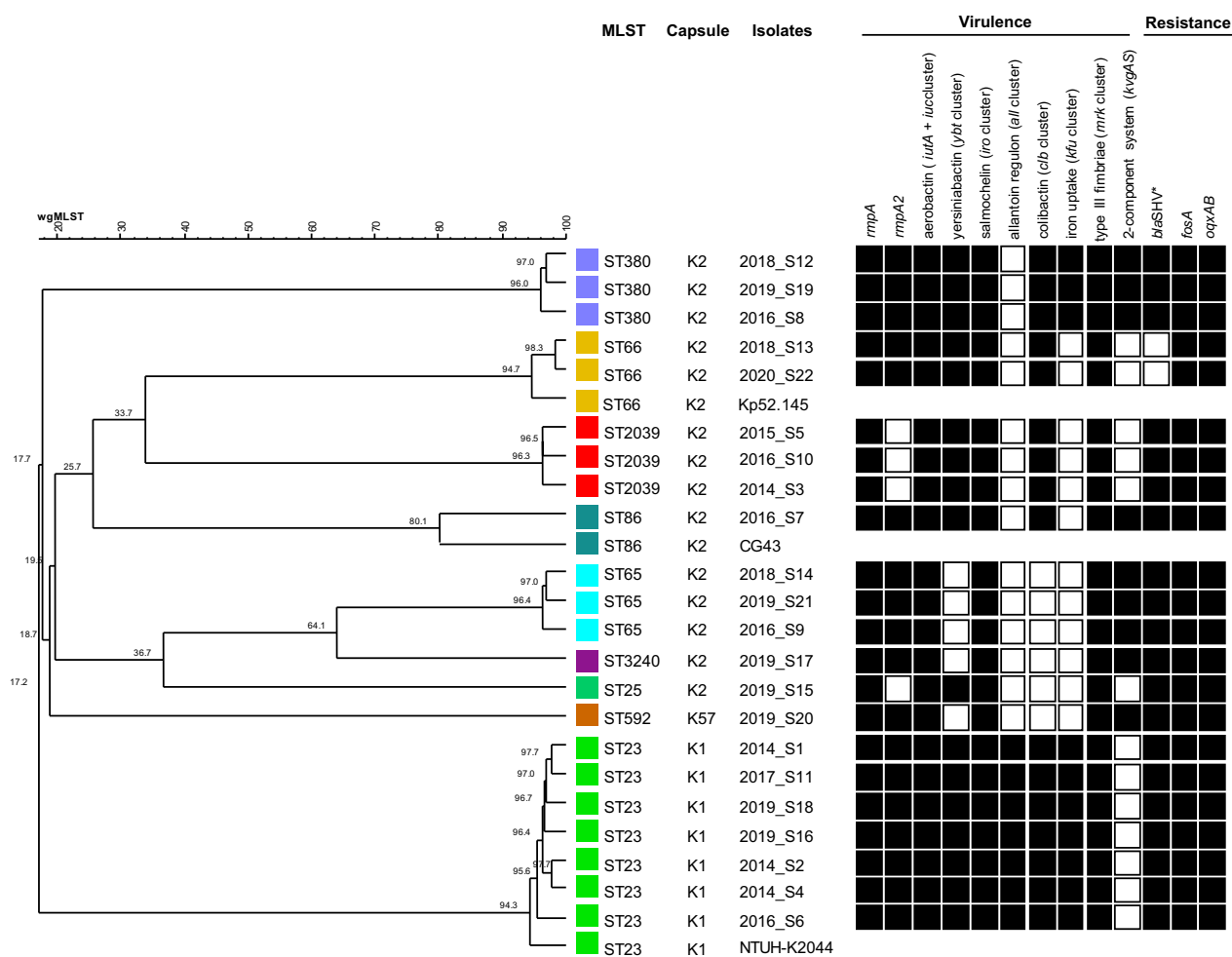
Table 1 Clinical characteristics of hypervirulent *K. pneumoniae* isolates

Isolates	MSLT	segMLST	Capsule	Sample year	Age	Gender	Birth place	Hospital/ province	Underlying condition	Anatomical site	Primary infection	Bacteremia	Metastasis	ICU	Treatment	Outcome
S1	ST23	scgST-26	K1	2014	71	M	Belgium	H1/Antwerp	Benign prostatic hyperplasia	Blood	Liver abscess	+	-	-	FQ	Cured
S2	ST23	scgST-26	K1	2014	NR	M	DR Congo	H2/Brussels	HIV	Blood	Liver abscess	+	-	+	TZP, C3G	Death
S3	ST2039	scgST-441	K2	2014	81	M	Belgium	H3/Brussels	Cholecystitis	Hepato/biliary	Liver abscess	-	-	+	AMC/SI	Cured
S4	ST23	scgST-26	K1	2014	62	M	DR Congo	H4/Brussels	HIV/diabetes	Blood	Liver abscess	+	Endophthalmitis	+	AMC, FQ/SI	Cured
S5	ST2039	scgST-441	K2	2015	57	F	Belgium	H5/Namur	Oropharyngeal carcinoma	Blood	Pneumoniae	+	-	+	AMC, TZP	Death
S6	ST23	scgST-26	K1	2016	68	M	Belgium	H2/Brussels	Colorectal adenocarcinoma	Ocular	Endophthalmitis	+	Liver/brain abscess	-	MEM, C3G	Cured
S7	ST86	scgST-47	K2	2016	27	M	Cameroon	H4/Brussels	Diabetes	Hepato/biliary	Liver abscess	+	-	-	C3G, FQ/SI	Cured
S8	ST380	scgST-53	K2	2016	52	M	Morocco	H4/Brussels	HIV/HCV/alcoholism	Blood	Pulmonary abscess	+	-	+	TZP, FQ	Death
S9	ST65	scgST-283	K2	2016	81	F	Belgium	H2/Brussels	-	Blood	Liver abscess	+	-	+	C3G, FQ/SI	Cured
S10	ST2039	scgST-441	K2	2016	80	M	Belgium	H5/Namur	Diabetes/COPD/prostatectomy	Testicle	Orchitis-epididymitis	+	Lung	-	C4G, TZP, SXT, C1G/SI	Death
S11	ST23	scgST-26	K1	2017	73	F	NR	H2/Brussels	NR	Blood	Liver abscess	+	NR	-	NR	NR
S12	ST380	scgST-53	K2	2017	72	M	Belgium	H3/Brussels	Diabetes/colorectal adenocarcinoma	Blood	Liver abscess	+	-	-	AMC, MEM, FQ/SI	Cured
S13	ST66	scgST-419	K2	2018	44	M	NR	H6/East Flanders	NR	Blood	Pulmonary abscess	+	NR	-	NR	NR
S14	ST65	scgST-283	K2	2018	47	M	Morocco	H7/Brussels	-	Blood	Liver abscess	+	-	-	TZP, FQ, C3G, SXT	Cured

Table 1 (continued)

Isolates	MSLT	scgMLST	Capsule	Sample year	Age	Gender	Birth place	Hospital/province	Underlying condition	Anatomical site	Primary infection	Bacteremia	Metastasis	ICU	Treatment	Outcome
S15	ST25	scgST-1533	K2	2019	39	M	Romania	H2/Brussels	Alcoholic steatohepatitis	Blood	Pulmonary abscess	+	-	+	MEM, C3G	Death
S16	ST23	scgST-26	K1	2019	75	F	Belgium	H1/Antwerp	Previous liver abscess/alcoholism	Blood	Liver abscess	+	-	+	FQ	Cured
S17	ST3240	scgST-55	K2	2019	60	M	Morocco	H5/Namur	Diabetes/partial Hepatectomy	Hepato/biliary	Liver abscess	+	-	-	AMC, C3G, FQ/SI	Cured
S18	ST23	scgST-26	K1	2019	67	M	China	H1/Antwerp	Diabetes/chronic myeloid leukemia	Hepato/biliary	Liver abscess	+	-	+	TZP, FQ	Cured
S19	ST380	scgST-53	K2	2019	67	M	Belgium	H5/Namur	Benign prostatic hyperplasia/cholelithiasis	Blood	Liver abscess	+	-	-	TZP, AMC, C3G, FQ, MEM/SI	Cured
S20	ST592	scgST-547	K57	2019	46	M	Guinea-Bissau	H8/Brussels	Diabetes/ HBV/ hepatic cirrhosis	Muscle	Necrotizing fasciitis	+	-	+	SI	Death
S21	ST65	scgST-283	K2	2019	55	M	NR	H8/Brussels	-	Blood	Liver abscess	+	-	-	AMC	Cured
S22	ST66	scgST-419	K2	2020	63	F	Belgium	H5/Namur	Alcoholism	Blood	Pulmonary abscess	+	-	-	TZP, FQ, C1G/SI	Cured

NR not reported, ICU intensive care unit admission, HIV human immunodeficiency virus, HCV hepatitis C virus, COPD chronic obstructive pulmonary disease, HBV hepatitis B virus, SI drainage or surgical intervention, FQ fluoroquinolone, TZP piperacillin/tazobactam, AMC amoxicillin/clavulanic acid, C1G first-generation cephalosporin, C3G third-generation cephalosporin, C4G fourth-generation cephalosporin, MEM meropenem, SXT trimethoprim/sulfamethoxazole



The wgMLST phylogenetic tree was generated with Bionumerics 8.0 using categorical values with a scaling factor of 100 (100*number of the loci in common/total number of loci in the complete comparison). The three hypervirulent *K. pneumoniae* reference strains CG43, NTUH-K2044, Kp52.145 were included in the analysis. MLST (ST) and capsular type of each isolate are indicated on the right of the dendrogram. A black square indicates presence of the corresponding gene. * *bla_{SHV-190}* for ST23; *bla_{SHV-110}* for ST25; *bla_{SHV-11}* for ST65; *bla_{SHV-28}* for ST86; *bla_{SHV-33}* for ST380; *bla_{SHV-26}* for ST592; *bla_{SHV-40}* for ST2039; *bla_{SHV-99}* for ST3240

Fig. 1 Clonal relationship of the hypervirulent *K. pneumoniae* isolates based on the whole-genome multilocus and distribution of virulence and resistance features

22 isolates were associated with hypermucoviscous phenotype and positive string test. Of note, hypermucoviscous cKp (non-hypervirulent) strains and hvKp strains without hypermucoviscosity have been described [27].

All K1/ST23 isolates were positive for the virulence genes: *rmpA* and *rmpA2* mucoid phenotype regulator, yersiniabactin (*ybt*), aerobactin (*iutA* and *iuc*) and salmochellin (*iro*) siderophores, *kfu* iron uptake, *clb* colibactin toxin, *mrk* type III fimbriae, and *allS* for allantoin metabolism. The

non-K1 isolates possessed also *rmpA* gene, *iuc*, *iut*, *iro*, and *mrk* loci but lacked *allS* gene. *rmpA2*, *ybt*, *clb*, and *kfu* loci were variably present in non-K1 isolates. Isolates belonging to a specific ST showed identical virulence gene profiles. Blast analysis identified the presence of highly similar ($\geq 99\%$ identity and $\geq 90\%$ coverage) virulence pK2044-like plasmid in ST23 and pLVPK-like plasmid in ST65, ST86, ST3240, and ST592 isolates. While two plasmids, similar to Kp52.145 plasmid I and Kp52.145 plasmid II [28], were

identified in ST66 and ST380 isolates, only the Kp52.145 plasmid II was detected in ST2039. None of these plasmids was identified in ST25 isolate. All the strains showed a wild-type in vitro susceptibility profile toward beta-lactams without any acquired resistance other than ampicillin resistance mediated by the presence of *bla*_{SHV}. Interestingly, the two ST66 isolates did not carry the intrinsic *bla*_{SHV} explaining the exceptional in vitro susceptibility to ampicillin observed in these isolates [28].

Increasing reports have described the emergence of multidrug-resistant hvKp strains (MDR-hvKp). Carbapenem-resistant hvKp, initially described in China, is reported all over the world with risk assessment required to evaluate and increase the awareness of the potential significant threat to public health [29, 30].

As hvKp isolates and clinical information for the corresponding patients were referred to the NRC on a voluntary basis by the hospital laboratories, our current study may not perfectly reflect the Belgian epidemiology. Nevertheless, our study evidenced the predominant circulation in Belgium of genetically diverse hvKp expressing capsular type K2 among local population unrelated to Asian ethnicity. These data suggest a potential underestimated spread of such invasive isolates. Further epidemiological studies of hvKp with a structured national survey are needed. Virulence factors should be investigated not only in *K. pneumoniae* isolates from invasive infections but also in carbapenemase-producing *K. pneumoniae* from all origins including screening samples to monitor the emergence of MDR-hvKp in Belgium [31].

Increased clinical awareness among physicians facing invasive or recurrent infections caused by *K. pneumoniae* should incite prompt microbiological diagnostic based on hypermucoviscous phenotype and differentiation of hvKp from cKp by molecular characterization of major virulence factors. Virulence genes commonly found in hvKp strains (e.g., *rmpA*) might serve as genotypic biomarkers for the early detection of hvKp strains in areas of low prevalence [32]. Management challenges included rapid initiation of therapy to prevent subsequent spread and sepsis, investigation for occult abscess to enable source control, and appropriate site-targeted therapy.

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Data availability Genome assemblies were submitted to NCBI Genome and are available under BioProject ID PRJNA817214.

Code availability Not applicable.

Declarations

Ethics approval All procedures were performed in accordance with the ethical standards of the national research committee and in accordance with the 1964 Helsinki Declaration. Information from microbiological and clinical files was anonymously analyzed and did not require patient's informed consent.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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