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Comparative analysis of *Klebsiella pneumoniae* strains isolated in 2012–2016 that differ by antibiotic resistance genes and virulence genes profiles

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ABSTRACT

The antibacterial resistance and virulence genotypes and phenotypes of 148 non-duplicate *Klebsiella pneumoniae* strains collected from 112 patients in Moscow hospitals in 2012–2016 including isolates from the respiratory system (57%), urine (30%), wounds (5%), cerebrospinal fluid (4%), blood (3%), and rectal swab (1%) were determined. The majority (98%) were multidrug resistant (MDR) strains carrying bla_{SHV} (91%), bla_{CTX-M} (74%), bla_{TEM} (51%), bla_{OXA} (38%), and bla_{NDM} (1%) beta-lactamase genes, class 1 integrons (38%), and the porin protein gene *ompK36* (96%). The beta-lactamase genes bla_{TEM-1}, bla_{SHV-1}, bla_{SHV-11}, $bla_{SHV-110}$, $bla_{SHV-190}$, $bla_{CTX-M-15}$, $bla_{CTX-M-3}$, $bla_{CTX-M-55}$, bla_{OXA-48}, $bla_{OXA-244}$, and bla_{NDM-1} were detected; class 1 integron gene cassette arrays (*aadA1*), (*dfrA7*), (*dfrA1-orfC*), (*aadB-aadA1*), (*dfrA17-aadA5*), and (*dfrA12-orfF-aadA2*) were identified. Twenty-two (15%) of clinical *K. pneumoniae* strains had hypermucoviscous (HV) phenotype defined as string test positive. The *rmpA* gene associated with HV phenotype was detected in 24% of strains. The intrapersonal mutation of *rmpA* gene (deletion of one nucleotide at the polyG tract) was a reason for negative hypermucoviscosity phenotype and low virulence of *rmpA*-positive *K. pneumoniae* strain KPB584. Eighteen virulent for mice strains with $LD_{50} \leq 10^4$ CFU were attributed to sequence types ST23, ST86, ST218, ST65, ST2174, and ST2280 and to capsular types K1, K2, and K57. This study is the first report about hypervirulent *K. pneumoniae* strain KPB2580-14 of $ST23^{K1}$ harboring extended-spectrum beta-lactamase CTX-M-15 and carbapenemase OXA-48 genes located on pCTX-M-15-like and pOXA-48-like plasmids correspondingly.

KEYWORDS

Klebsiella pneumoniae; antibacterial resistance; virulence; MLST; K-serotype

Introduction

Klebsiella pneumoniae is one of the main pathogens of hospital infections and causes bacteremia and infections of the respiratory tract, skin, urinary tract and central nervous system. Two independent evolutionary branches of *K. pneumoniae* have been described: classical (cKP) and hypervirulent (hvKP). Modern cKP strains are causative agents of pneumonia, urinary tract infections, and other infections in people with low immune status; these strains are characterized by multi drug resistance (MDR) associated with the production of beta-lactamases (including extended spectrum beta-lactamases (ESBLs) and carbapenemases), activation of efflux pumps, and changes in the structure of porin proteins [1]. *K. pneumoniae* hvKP causes primary infections in people with normal immunity (acute liver abscesses, septicemia, and meningitis) and can metastasize from the primary focus of infection to other organs and tissues of the body [2–4]. Infections caused by hvKP have a high mortality rate of up to 31% [5]. Virulence factors of hvKP include a hypermucoviscous phenotype, iron assimilation, allantoin recycling, lipopolysaccharide and polysaccharide capsule synthesis and type I fimbriae [1,6]. Most virulent *K. pneumoniae* strains are hypermucoviscous and belong to the K1, K2, K5, K20, K54, and K57 capsular serotypes and to ST23, ST29, ST65, ST86, ST268, ST375, ST412, and ST420 sequence types [3,5]. It was currently considered, that clonal complexes of hvKP and MDR strains are non-overlapping [7]. However, the acquisition of multiple antibacterial resistance by epidemic hvKP strains has been reported in the last decade, including a hvKP $ST23^{K1}$ clone harboring the bla_{KPC-2} gene in Argentine, $bla_{CTX-M-3}$ gene in France, and $bla_{CTX-M-15}$ gene in South Korea; a hvKP $ST1797^{K1}$ clone acquired carbapenemase gene bla_{KPC-2} in China; and a hvKP ST86 clone acquired cephalosporinase $bla_{CTX-M-14/15}$ gene in Australia [8–12].

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The aims of this study were (i) to determine the virulence and antibacterial resistance phenotypes and genotypes of clinical *K. pneumoniae* strains isolated in Russia in 2012–2016 with a particular emphasis on the investigation of virulent strains and their distinctness from other strains; (ii) to characterize the virulent strains with respect to capsular type, sequence type, and experimental virulence in mice. (iii) to determine the distribution of antibiotic resistance genes among *K. pneumoniae* with different virulence gene profiles.

Materials and methods

Bioethical requirements

In accordance with the requirements of the Russian Federation Bioethical Committee, each patient signed an agreement with the hospital consenting to treatment and laboratory examination. This agreement was filed in the medical history of each patient. The materials used in the study did not contain personal data of patients because the labeling of the clinical isolates did not include name, date of birth, address, disease history, or other personal information. Personal data were erased when the monitoring tables were generated. Overall, the study was a prospective observational study; all patients received the same medical treatment according to clinical indications.

Bacterial strains, identification, and growth conditions

K. pneumoniae strains used in this study were isolated from clinical samples, including patient specimens (respiratory system, urine, wounds, cerebrospinal fluid, blood, and rectal swab) collected in the Burdenko Neurosurgery Institution (n = 106), Moscow Infectious Hospital No. 1 (n = 25), and other sources (n = 17). Additionally, one *K. pneumoniae* strain, KPM9, was isolated from the environment (fresh-water) in the Krasnodar Region of Russia in 2011 during mice epizooty; this strain was used as a reference strain virulent for mice [13].

Bacterial identification was performed using a Vitek-2 Compact instrument with a VITEK® 2 Gram-negative (GN) ID card (SKU number 21341; BioMérieux, Paris, France) and a MALDI-TOF Biotyper (Bruker Daltonics, Bremen, Germany) instrument capable of distinguishing among *K. oxytoca*, *K. pneumoniae* ssp. *ozaenae*, *K. pneumoniae* ssp. *pneumoniae*, *K. pneumoniae* ssp. *rhinoscleromatis*, and *K. variicola*. Bacterial isolates were stored in 15% glycerol at minus 80 °C.

Bacterial cultures were grown at 37 °C on Nutrient Medium No. 1 (SRCAMB, Obolensk, Russia), Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) and Muller-Hinton broth (Himedia, Mumbai, Maharashtra, India).

Hypermucoviscous *K. pneumoniae* strains were identified using the string test [6]. The test was considered positive if a colony of *K. pneumoniae* could be 'stretched' more than 5 mm using a standard bacteriological loop.

Susceptibility to antibacterial agents

Minimal inhibitory concentrations (MICs) of antibacterials belonging to eight functional classes (beta-lactams, tetracyclines, fluoroquinolones, phenicols, aminoglycosides, sulfonamides, nitrofurans, and phosphomycins) were determined using a Vitek-2 device with VITEK-2 AST N-101 and AST N-102 cards (BioMérieux, Paris, France). The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/clinical_breakpoints/). *E. coli* strains ATCC 25922 and ATCC 35218 were used for quality control.

Virulence assessment of K. pneumoniae strains

All protocols for animal experiments were approved by the State Research Center for Applied Microbiology and Biotechnology Bioethics Committee (Permit No: VP-2014/1). *K. pneumoniae* virulence (LD_{50}) was evaluated in female outbred white mice weighing 18–22 g obtained from the 'Andreevka' Laboratory Animals Nursery (SCBT, Moscow region, Russia). The mice were housed under standard conditions in accordance with international standards and requirements [14]. The mice were infected intraperitoneally with *K. pneumoniae* cells using six mice per infective dose. The actual number of bacteria present was determined by plating on agar medium. The specificity of *K. pneumoniae* infection was confirmed by isolation of *K. pneumoniae* from the liver, spleen, and lungs. The LD_{50} was calculated using the Karber method [15].

PCR detection of antibacterial resistance, virulence, and K serotype-specific genes

Genes associated with *K. pneumoniae* virulence (*rmpA*, *aer*, *uge2*, *wabG*, *kfu*, *fimH,* and *allR*) or antibacterial resistance (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, $bla_{OXA-48-like}$, bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, *ompK36,* and class 1 and 2 integrons) were detected by PCR using previously described specific primers [16–22]. The capsular serotypes of the *K. pneumoniae* strains were determined using specific primers for *wzy* genes associated with K serotypes K1, K2, K5, K20, K54, and K57 and by *wzi* gene sequencing (Supplementary Data Table 1). Bacterial thermolysates were used as DNA templates for amplification using a GradientPalmCycler (Corbert Research, Sydney, NSW, Australia) and Thercyc cycler (DNA-Technology, Protvino, Moscow region, Russia) [23]. The PCR products were analyzed by electrophoresis on 1.5% agarose gels.

DNA sequencing and bioinformatics analysis

Whole-genome sequencing was performed using the Illumina MiSeq instrument according to the manufacturer's instruction (Illumina, San Diego, CA, USA). DNA libraries were prepared using Nextera DNA Library Preparation Kit. Miseq Reagent Kit v3 (300 cycles) was used for sequencing. Reads were *de novo* assembled using SPAdes v. 3.9 (http://bioinf.spbau.ru/spades). Basic Local Alignment Search – BLAST (https://blast.ncbi.nlm.nih.gov/) was used for searching homologous sequences. Program MAUVE (http://darlinglab.org/mauve/mauve.html) was used for multiple genome alignments.

Sequencing of separate genes and DNA fragments was carried out using the ABI PRISM BigDye Terminator v.3.1 kit (Applied Biosystems, Foster City, CA, USA). Purified products were analyzed on an ABI PRISM 3100-Avant automated DNA Sequencer at the SINTOL Center for collective use (Moscow, Russia). DNA sequences were analyzed using Vector NTI9 (Invitrogen, USA) and BLAST. Class 1 and 2 integrons were analyzed using the INTEGRAL database (http://integrall.bio.ua.pt/?). The DNA sequences of 411 genes have been deposited in the GenBank database (Supplementary Data Table 2).

Multilocus sequence typing (MLST) of *K. pneumoniae* strains carrying different sets of virulence genes and exhibiting different virulence in mice was performed by determining the nucleotide sequences of seven housekeeping genes as described previously [24]. The features of the 38 *K. pneumoniae* strains attributed to 12 sequence types have been deposited in the MLST PASTEUR database (Supplementary Data Table 3).

Phylogenetic analysis

Complete genome sequence data of 65 *K. pneumoniae* strains from the DDBJ/EMBL/GenBank database isolated in different geographical areas and KPB2580 strain were used to generate bacterial core genome SNPs by the Wombac 2.0 software (http://www.mybiosoftware.com/wombac-1-1-bacterial-core-genome-snps-phylogenomic-trees-ngs-reads-andor-draft-genomes.html). The trees were constructed by using the neighbor-joining method with 1000 bootstraps implemented in SplitsTree [25].

Results and discussion

Bacterial strains

One hundred and forty-eight *K. pneumoniae* strains were isolated in 2012-16 from 112 patients from the respiratory system (57%), urine (30%), wounds (5%), cerebrospinal fluid (4%), blood (3%), and rectal swab (1%). It should be noted, *K. pneumoniae* was one of the dominant pathogens among bacteria causing hospital infections in Moscow intensive care units (ICUs) under study during this period.

Antibacterial susceptibility and antibacterial resistance gene profiles

Most of *K. pneumoniae* strains (94%) in this study expressed the MDR phenotype, i.e. they were resistant to three or more classes of antibacterials, accordingly to Magiorakos et al. definitions [26]. Specifically, 31% strains were resistant to seven functional classes of antibacterials, 20% to six classes, 22% to five classes, 16% to four classes, and 5% to three classes. Most of the strains were resistant to beta-lactams, nitrofurans, fluoroquinolones, phenicols, and aminoglycosides. Of particular concern is the high level of resistance to reserve drugs: more than 1/3 of the strains were resistant to tigecycline with MIC > 2 mg/L and to imipenem with MIC ≥ 4 mg/L; almost half the strains were resistant to amikacin with MIC ≥ 16 mg/L (Figure 1(A)).

Beta-lactamase genes of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, and bla_{NDM} types were detected in the strains; bla_{KPC}, bla_{VIM}, and bla_{IMP} types were not detected (Figure 1(B)). The following alleles of beta-lactamase genes were identified: bla_{TEM-1}, bla_{SHV-1}, bla_{SHV-11}, $bla_{SHV-110}$, $bla_{SHV-190}$, $bla_{CTX-M-15}$, $bla_{CTX-M-3}$, $bla_{CTX-M-55}$, bla_{OXA-48}, $bla_{OXA-244}$, and bla_{NDM-1}. Ten combinations of five beta-lactamase gene types were identified in the strain collection (Table 1). Major (37% strains) carried three types, rarer (21, 20 and 16% strains) carried four, two, and one beta-lactamase gene types correspondingly. One strain (isolated in March of 2016 from the endotracheal aspirate of a patient on mechanical ventilation) *K. pneumoniae* KPB417-16 belonged to sequence type ST147, carried five beta-lactamase genes (bla_{TEM-1}, bla_{SHV-11}, $bla_{CTX-M-15}$, bla_{OXA-48}, and bla_{NDM-1}). Herewith the bla_{NDM-1} gene was first detected in Moscow hospital [27], although previously such gene was detected in *K. pneumoniae* of ST147 in the Northwest region of Russia in 2015 [28].

Minimal inhibitory concentrations (MICs) of beta-lactams for *K. pneumoniae* strains were different for various sets of beta-lactamase genes. Resistance to beta-lactams detected for the strains that have no beta-lactamase genes indicate the presence of additional molecular mechanisms, possibly other types of beta-lactamases, porin mutations and/or efflux pumps (Table 1). For example, recently we described *K. pneumoniae* strains carrying the insertions of IS1R and IS10R elements that inactivated the *ompK36* gene, resulting in loss of the OmpK36 porin and raising resistance to imipenem [29]. Class 1 integrons detected in 38% of *K. pneumoniae* strains undoubtedly contributed to the resistance phenotype of these strains. Gene cassette arrays (*aadA1*), (*dfrA7*), (*dfrA1-orfC*), (*aadB-aadA1*), (*dfrA17-aadA5*), and (*dfrA12-orfF-aadA2*) were identified in 45% of class 1 integrons (Supplementary Data Table 3).

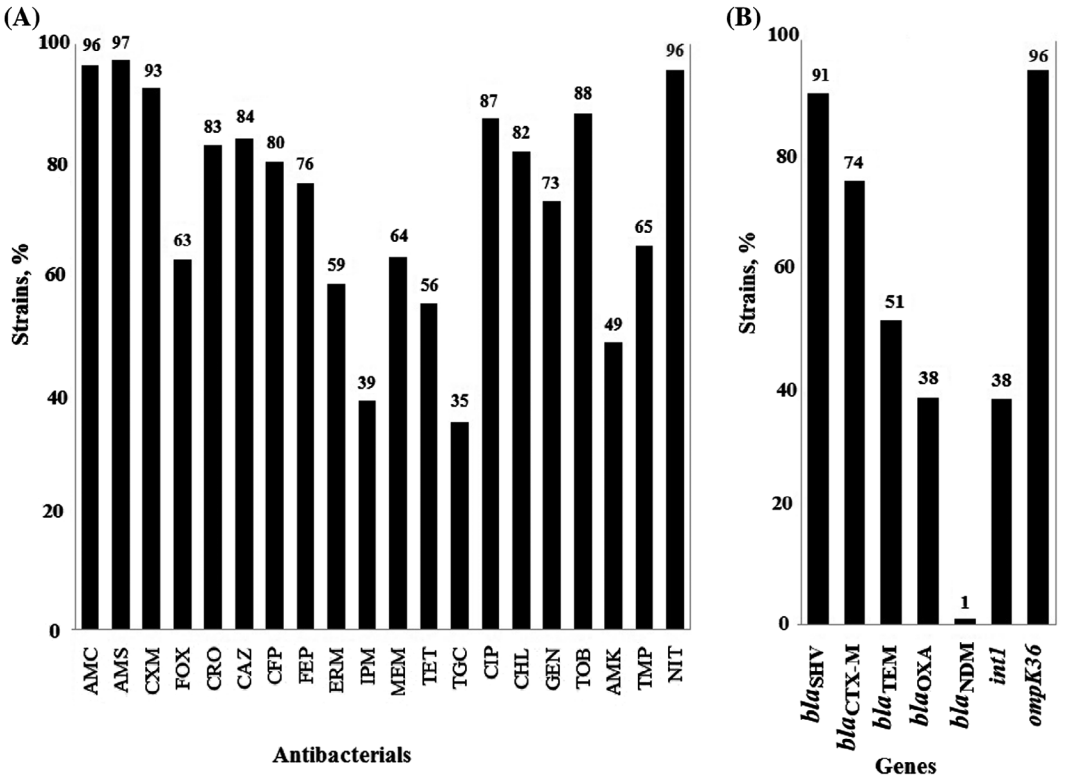

Figure 1. Antibacterial resistance and resistance genes of 148 *K. pneumoniae* strains. (A) Rate of the strains resistant to antibacterials amoxicillin/clavulanic acid, AMC; amoxicillin/sulbactam, AMS; cefuroxime, CXM; cefoxitin, FOX; ceftriaxone, CRO; ceftazidime, CAZ; cefoperazone/sulbactam, CFP; cefepime, FEP; ertapenem, ERM; imipenem, IPM; meropenem, MEM; tetracycline, TET; tigecycline, TGC; ciprofloxacin, CIP; chloramphenicol, CHL; gentamicin, GEN; tobramycin, TOB; amikacin, AMK; trimethoprim, TMP; and nitrofurantoin, NIT; (B) Rate of the strains carrying beta-lactamase genes of bla_{SHV}-type (bla_{SHV-1}, bla_{SHV-11}, $bla_{SHV-110}$, $bla_{SHV-190}$); bla_{CTX-M}-type ($bla_{CTX-M-15}$, $bla_{CTX-M-3}$, $bla_{CTX-M-55}$); bla_{TEM}-type (bla_{TEM-1}); bla_{OXA}-type (bla_{OXA-48}, $bla_{OXA-244}$); bla_{NDM}-type (bla_{NDM-1}); class 1 integrons; and *ompK36* porin protein gene.

Virulence genes, hypermucoviscosity, capsular types, and virulence in mice

Seven *K. pneumoniae* genes associated with *Klebsiella* virulence factors [6,30] were detected by PCR. The most prevalent were intrinsic genes *wabG* (94% of strains) and *uge2* (81%), which are involved in the synthesis of the polysaccharide capsule and lipopolysaccharide (LPS), and *fimH* (91%), which encodes type I fimbria. Less common were *kfu* (29%) and *aer* (21%), which are associated with utilization of trivalent iron, and *rmpA* (24%), which is associated with a hypermucoviscous phenotype. The allantoin regulon gene *allR* was rarely detected in 7% of strains (Table 2). Twenty-two of clinical *K. pneumoniae* strains had hypermucoviscous (HV) phenotype defined as string test positive. They belonged to capsular types K1 ($n = 10$), K2 ($n = 7$), and K57 ($n = 5$). Moreover, capsular types K14, K47, K62, K60, K27, K28, and K420 were identified among HV-negative strains (Table 3).

To compare *in vivo* virulence of the strains an experimental *K. pneumoniae* infection model on outbred white mice was used. Positive control for hypervirulence in mice *K. pneumoniae* strain KPM9 was attributed to capsular type K20 [13]. Thirty-seven clinical strains carrying different arrays of virulence genes and belonged to different K-types were selected. The strains were isolated from the respiratory system ($n = 18$), urine ($n = 13$), cerebrospinal fluid ($n = 3$) and wounds ($n = 3$) of the patients. According to the data obtained, the clinical strains were divided into three groups: hypervirulent (LD_{50} < 100 CFU), virulent (LD_{50} from 3×10^2 to 10^4 CFU) and non-virulent ($LD_{50} > 10^6$ CFU) in mice (Figure 2). It should be noted, virulent and hypervirulent in mice strains were isolated from the patients with severe infections of the central nervous system (meningitides), respiratory system (ventilator-associated pneumonia) or urinary tract catheter-associated infections. Five *K. pneumoniae* strains (KPB1103-14, KPB1802K, KPB2580-14, KPB4010, and KPS73) were associated with patient death.

Strains of different capsular types exhibited different degrees of virulence in mice (Figure 3). All 10 virulent or hypervirulent in mice clinical strains of K1-type carried a complete array of selected 7 virulence genes: *rmpA, aer, uge2, wabG, kfu, fimH,* and *allR*. The strains of K2-type had 6 or 5 virulence genes – *allR* and *kfu* or *rmpA* genes were missed in their arrays. One virulent strain of K57-type carried 5 virulence genes: *rmpA, aer, wabG, kfu,* and *fimH*. Non-virulent strains carried fewer (0–4) virulence genes. In our opinion, the importance of genes for the manifestation of *Klebsiella* virulence decreases in the following order: *aer*, *rmpA*, *allR*, *kfu, uge*. This result concerning

Table 1. Minimal inhibitory concentrations of beta-lactams for *K. pneumoniae* strains carrying different sets of beta-lactamase genes.

		MICs of beta-lactams (number of strains)						
Beta-lactamase genes	Number of strains	AMS	CXM	CRO	CAZ	CFP	FEP	IPM
bla_{SHV} bla_{TEM} bla_{CTX-M} bla_{OXA} bla_{NDM}	1	>32(1)	>64(1)	>64(1)	>64(1)	>64(1)	16(1)	>16(1)
bla_{SHV} bla_{TEM} bla_{CTX-M} bla_{OXA}	31	>32(31)	>64(31)	>64(31)	>8(31)	>16(31)	>8(31)	>16(9) 8(5) ≤2(17)
bla_{SHV} bla_{TEM} bla_{CTX-M}	38	>32(38)	>64(38)	>64(38)	>16(38)	>8(38)	>4(37) 2(1)	>16(4) 8(2) ≤2(32)
bla_{SHV} bla_{TEM} bla_{OXA}	2	>32(2)	>16(2)	32(1) 2(1)	≤1(2)	>32(2)	≤2(2)	8(1) ≤2(1)
bla_{SHV} bla_{CTX-M} bla_{OXA}	15	>32(15)	>64(15)	>64(15)	>64(15)	>16(15)	>8(15)	>16(4) 8(7) ≤2(4)
bla_{SHV} bla_{TEM}	3	>32(3)	>16(2) ≤16(1)	>16(2) ≤1(1)	>64(2) ≤1(1)	≤1	128(1) 2(1) <1(1)	>256(1) ≤1(2)
bla_{SHV} bla_{CTX-M}	19	>32(19)	>64(19)	>64(19)	>64(19)	>16(19)	>16(19)	>16(2) 4(1) ≤1(16)
bla_{SHV} bla_{OXA}	7	>32(7)	>16(5) ≤16(2)	2(4) ≤1(3)	>64(1) ≤1(6)	>32(6) ≤1(1)	<1(1)	>16(6) ≤1(1)
bla_{SHV}	18	>32(18)	>16(2) ≤16(16)	>2(3) ≤1(15)	>64(4) ≤1(14)	>1(1) ≤1(17)	>4(4) ≤1(14)	>16(4) ≤1(14)
bla_{CTX-M}	6	>32(6)	>64(6)	>64(6)	>64(6)	>16(6)	>16(6)	≤1(6)
–	8	>32(4) ≤32(4)	>16(4) ≤16(4)	>2(4) ≤1(4)	>4(1) ≤1(7)	>1(4) ≤1(4)	>4(4) 4(4)	>16(2) 4(2) ≤1(4)
Total	148	>32(144) ≤32(4)	>16(125) ≤16(23)	>2(120) 2(5) ≤1(23)	>4(118) ≤1(30)	>1(123) ≤1(25)	>4(124) 4(6) ≤1(18)	>16(33) 4-8(18) ≤1(97)

Notes: AMS, amoxicillin-sulbactam; CXM, cefuroxime; CRO, ceftriaxone; CAZ, ceftazidime; CFP, cefoperazone-sulbactam; FEP, cefepime; IPM, imipenem.

Table 2. Virulence gene combinations of *K. pneumoniae* strains.

Virulence genes combination	Set of virulence genes	Number of strains
7	*rmpA*, *aer*, *uge2*, *wabG*, *kfu*, *fimH*, *allR*	10
6	*rmpA*, *aer*, *uge2*, *wabG*, *kfu*, *fimH*	2
5a	*rmpA*, *aer*, *uge2*, *wabG*, *fimH*	7
5b	*rmpA*, *aer*, *wabG*, *kfu*, *fimH*	3
5c	*rmpA*, *uge2*, *wabG*, *kfu*, *fimH*	1
5d	*aer*, *uge2*, *wabG*, *kfu*, *fimH*	1
4a	*uge2*, *wabG*, *kfu*, *fimH*	26
4b	*rmpA*, *aer*, *wabG*, *fimH*	8
3a	*uge2*, *wabG*, *fimH*	68
3b	*rmpA*, *wabG*, *fimH*	4
2a	*wabG*, *fimH*	4
2b	*uge2*, *wabG*	5
1	*fimH*	1
0	–	8
Total		148

Note: '-' gene was not detected.

virulence in mice does not conflict with the conclusion concerning *Klebsiella* virulence for human [31].

Two strains, *K. pneumoniae* KPB550 and KPB584, have the identical virulence gene profile (*rmpA, aer, wabG, kfu,* and *fimH*), and capsular type K57, but differ in the HV phenotype, resistance phenotype and the degree of virulence in mice. Sequencing of *rmpA* gene (in three replicates) revealed a deletion of one G nucleotide at the position 286 leading to a shift in the reading frame and generation of a stop codon in non-virulent *K. pneumoniae* strain KPB584 (Figure 4). This mutation apparently explains the absence of hypermucoviscosity and virulence in mice of this strain and confirms the importance of the *rmpA* gene for *K. pneumoniae* virulence. Another intrapersonal mutation of *rmpA* gene (insertion of one nucleotide at the polyG tract) was reported as a reason for negative hypermucoviscosity phenotype and low virulence of *rmpA*-positive *K. pneumoniae* isolates in 2015 [32].

Sequence types and clonal complexes

Nine previously described sequence types (STs) of *K. pneumoniae* strains ST23 (n = 12), ST218 (n = 7), ST86 (n = 4), ST147 (n = 4), ST48 (n = 2), ST395 (n = 2), ST20 (n = 1), ST65 (n = 1), and ST833 (n = 1) were identified in this study. Moreover, novel ST2280 (n = 1) presenting a new allele profile of 7 housekeeping genes; and novel ST2174 (n = 1) carrying a novel allele of the glyceraldehyde-3-phosphate dehydrogenase gene, *gapA125* (GB KU510247) were first identified in this study (Table 3). The obtained sequence types were assigned to ten

Table 3. Features of virulent and avirulent in mice *Klebsiella pneumoniae* strains ($n = 38$).

Strain	Year	Source	String test	K-type	ST	Resistance to antibacterials: number groups (functional classes)	Beta-lactamase genes	Virulence genes array[a]	LD_{50}, CFU
KPM9[b]	2011	Water	+	K20	1544	1 (BLA)	SHV-1	6	10
KPS73	2016	Lung	+	K1	23	1 (BLA)	SHV-1	7	44
KPB1802K	2013	Trachea	+	K1	23	1 (BLA)	SHV-190	7	86
KPI1683	2014	Trachea	+	K1	23	2 (BLA NIT)	SHV-1	7	26
KPI261	2014	Trachea	+	K1	23	3 (BLA AMI NIT)	SHV-1, CTX-M-15	7	16
KPB1493-1	2013	Trachea	+	K1	23	6 (BLA TET QNL AMI SUL NIT)	SHV-11, TEM-1, CTX-M-15	7	4×10^2
KPB463K-13	2013	Trachea	+	K1	23	6 (BLA QNL AMI SUL NIT PHO)	SHV-11, TEM-1, CTX-M-15, OXA-48	7	1×10^4
KPB475-14	2014	Urine	+	K1	23	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-11, TEM-1, CTX-M-15, OXA-48	7	1×10^3
KPB594-14	2014	Wound	+	K1	23	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-11, TEM-1, CTX-M-15, OXA-48	7	3×10^3
KPB1103-14	2014	Urine	+	K1	23	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-190, TEM-1, CTX-M-15, OXA-48	7	3×10^2
KPB2580-14	2014	Urine	+	K1	23	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-11, TEM-1, CTX-M-15, OXA-48	7	17
KPI1748	2014	Trachea	+	K2	65	5 (BLA QNL CHL AMI SUL)	SHV-11	6	83
KPB4010	2013	Cerebrospinal fluid	+	K2	2280	3 (BLA TET NIT)	SHV-11	5a	25
KPI1627	2014	Trachea	+	K2	86	2 (BLA NIT)	SHV-11	5a	14
KPI6208	2014	Trachea	+	K2	86	3 (BLA TET NIT)	SHV-11	5a	40
KPB492-16	2016	Wound	+	K2	86	1 (BLA)	SHV-11	5a	1×10^3
KPB463-16	2016	Trachea	+	K2	86	1 (BLA)	SHV-11	5a	1×10^3
KPI3014	2014	Trachea	+	K2	2174	6 (BLA TET QNL CHL AMI NIT)	SHV-11, TEM-1	5d	1×10^4
KPB550	2013	Urine	+	K57	218	5 (BLA QNL PHO NIT SUL)	SHV-11, TEM-1, CTX-M-15, OXA-48	5b	2×10^3
KPB584	2013	Urine	–	K57	218	4 (BLA SUL NIT PHO)	SHV-11, TEM-1, CTX-M-15, OXA-48	5b	$>10^6$
KPB500	2013	Trachea	+	K57	218	5 (BLA QNL SUL NIT PHO)	SHV-11, TEM-1, CTX-M-15, OXA-244	4b	$>10^6$
KPB811K	2013	Cerebrospinal fluid	+	K57	218	5 (BLA QNL SUL NIT PHO)	SHV-11, TEM-1, CTX-M-15, OXA-244	4b	$>10^6$
KPB757K	2013	Urine	+	K57	218	8 (BLA TET QNL CHL AMK SUL NIT PHO)	SHV-11, TEM-1, CTX-M-15, OXA-244	4b	$>10^6$
KPB612-1	2013	Trachea	+	K57	218	7 (BLA QNL AMI SUL PHO NIT CST)	SHV-11, TEM-1, CTX-M-15, OXA-48	4b	$>10^6$
KPB690-14K	2014	Urine	–	K57	218	5 (BLA QNL CHL AMI NIT)	SHV-11, CTX-M-15, OXA-48	2b	$>10^6$
KPB542-15	2015	Urine	–	K57	23	7 (BLA QNL CHL AMI SUL NIT PHO)	SHV-11, CTX-M-15	3b	$>10^6$
KPI112	2014	Trachea	–	K57	23	4 (BLA QNL AMI SUL)	SHV-11, TEM-1, CTX-M-55	5c	$>10^6$
KPB1493-2	2013	Trachea	–	K62	48	4 (BLA CHL AMI NIT)	SHV-11, CTX-M-15	3a	$>10^6$
KPB420-14	2014	Urine	–	K62	48	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-11, CTX-M-15	2a	$>10^6$
KPB1224	2012	Trachea	–	K47	395	6 (BLA TET QNL CHL AMI SUL)	SHV-11, TEM-1, CTX-M-15, OXA-48	4a	$>10^6$
KPB1667	2013	Urine	–	K47	395	6 (BLA QNL CHL AMI SUL NIT)	SHV-11, TEM-1, CTX-M-15, OXA-48	4a	$>10^6$
KPB958-14	2014	Urine	–	K14	147	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-11, CTX-M-15, OXA-48	4a	$>10^6$
KPB417-16	2016	Trachea	–	K14	147	4 (BLA QNL AMI NIT)	SHV-11, TEM-1, CTX-M-15, OXA-48, NDM-1	3a	$>10^6$
KPB941-14	2014	Trachea	–	K27	833	7 (BLA TET QNL CHL AMI SUL NIT)	SHV-11, TEM-1, OXA-48	2b	$>10^6$

(*Continued*)

Table 3. (*Continued*).

Strain	Year	Source	String test	K-type	ST	Resistance to antibacterials: number groups (functional classes)	Beta-lactamase genes	Virulence genes array[a]	LD_{50}, CFU
KPB591-15	2015	Cerebrospinal fluid	–	K28	20	2 (BLA NIT)	SHV-11, OXA-48	3a	$>10^6$
KPB54-14	2014	Urine	–	K60	NI	7 (BLA TET QNL CHL AMK SUL NIT)	SHV-11, TEM-1, CTX-M-15	4a	$>10^6$
KPB944-14	2014	Urine	–	*wzi420*[c]	147	6 (BLA TET QNL CHL AMI NIT)	SHV-11, CTX-M-15, OXA-48	3a	$>10^6$
KPB711-14	2014	Wound	–	*wzi420*[c]	147	6 (BLA TET QNL CHL AMI NIT)	SHV-11, CTX-M-15, OXA-48	0	$>10^6$

[a]The designations correspond to those presented in Table 2.
[b]Reference strain for virulence in mice; ST – sequence type; NI – non identified.
[c]Gene allele *wzi420* is not associated with any known K-serotype in the Pasteur Institute Database; BLA – beta-lactams; TET – tetracyclines; QNL – quinolones; CHL – chloramphenicol; AMK – aminoglycosides; SUL – sulfonamides.

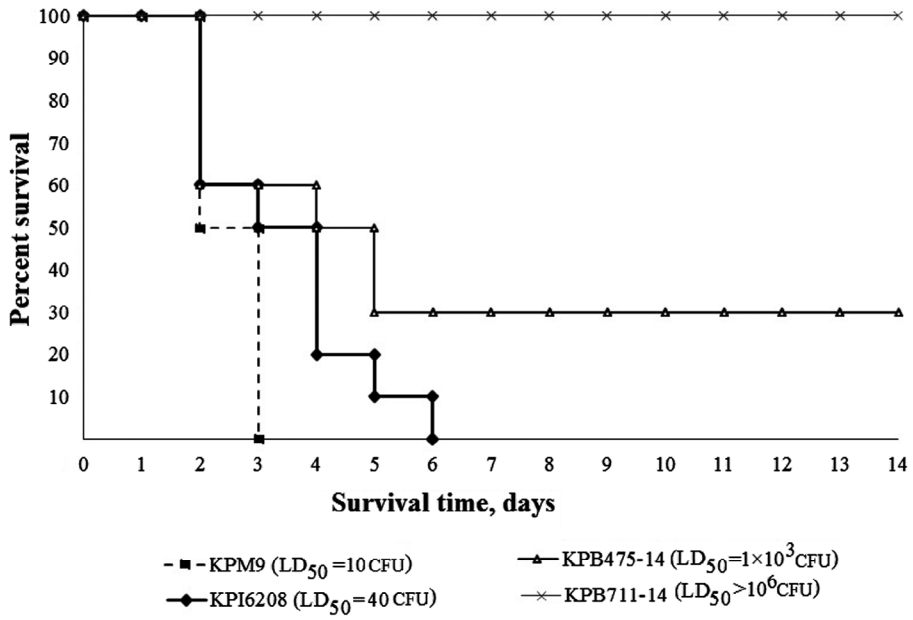

Figure 2. Kinetics of survival in intraperitoneally inoculated mice in infection dose of 1×10^4 CFU by *K. pneumoniae* strains: KPM9 – reference virulent for mice strain isolated from fresh water; KPI6208 – hypervirulent clinical strain; KPB475-14 – virulent clinical strain; KPB711-14 – non-virulent clinical strain.

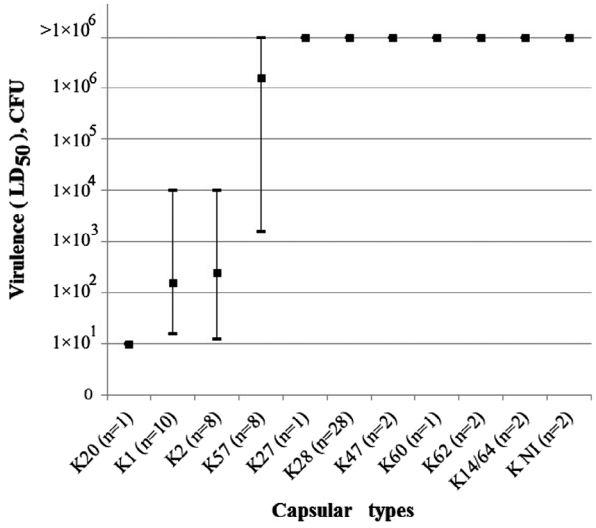

Figure 3. Virulence in mice for *K. pneumoniae* strains attributed to capsular types K1, K2, K20, K27, K28, K47, K57, K60, K62, K14/62 and non-identified capsular types (KNI).

clonal complexes (CCs) based on the allelic identity of 6 housekeeping genes using eBURST software and the MLST PASTEUR database: CC23 (ST23 and ST218), CC65 (ST65 and ST2280), CC11 (ST833), CC14 (ST2174), CC15 (STNI), CC20 (ST20), CC48 (ST48), CC86 (ST86), CC147 (ST147), and CC395 (ST395) (Supplementary Figure 1). Sequence type ST1544 of reference virulent for mice strain *K. pneumoniae* KPM9 was not related to any CC presented in the database. CCs identified in this study were presented by 1030 *K. pneumoniae* strains in the MLST PASTEUR database on the date 1 September 2017. Among them, CC11 (40% of strains) contains the most globally distributed cKP strains; CC65 (16%) and CC23 (15%) include the most globally distributed hvKP strains (Supplementary Figure 2).

In our study, 'classical' CC11 was presented by one strain of $ST833^{K27}$ (non-hypermucoviscous, non-virulent in mice, MDR). Moreover, strains of $ST48^{K62}$, $ST395^{K47}$,

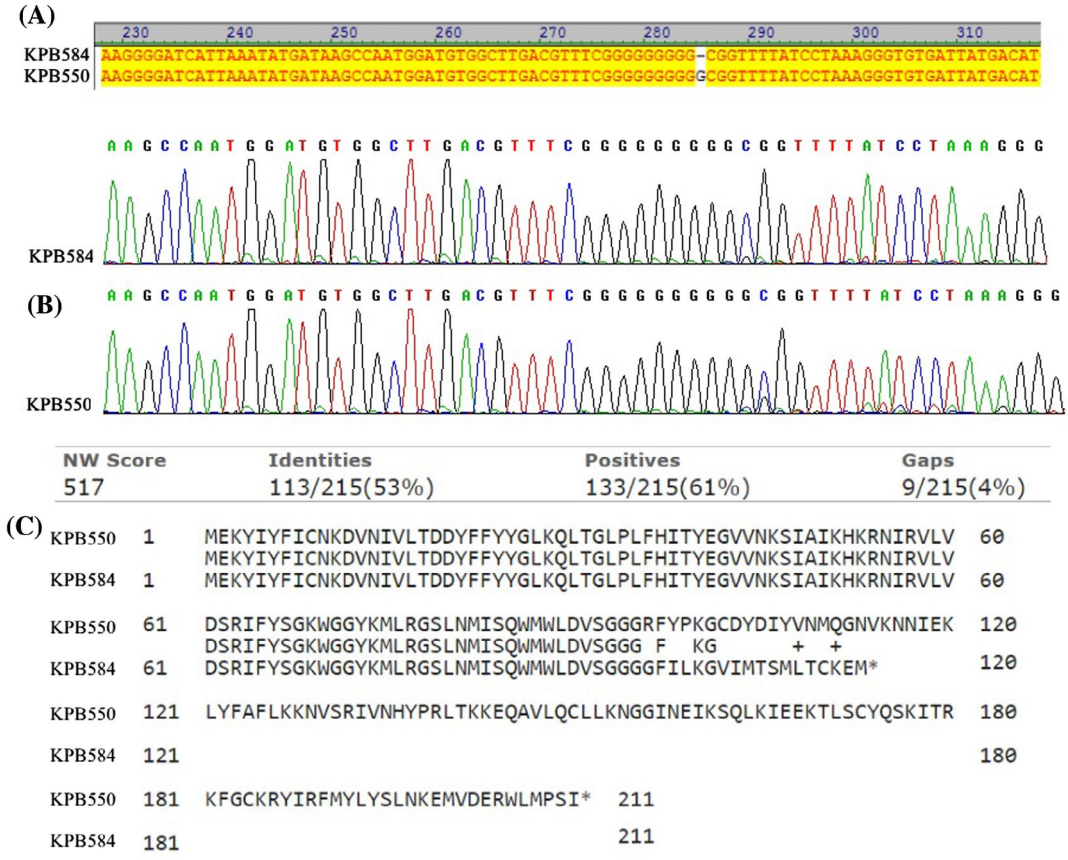

Figure 4. Comparison of *rmpA* gene sequences in *K. pneumoniae* strains KPB-584 and KPB-550. (A) Nucleotide alignment of the partial *rmpA* gene sequences; (B) Chromatogram of *rmpA* gene selected regions (C) Amino acid alignment of the gene translation products.

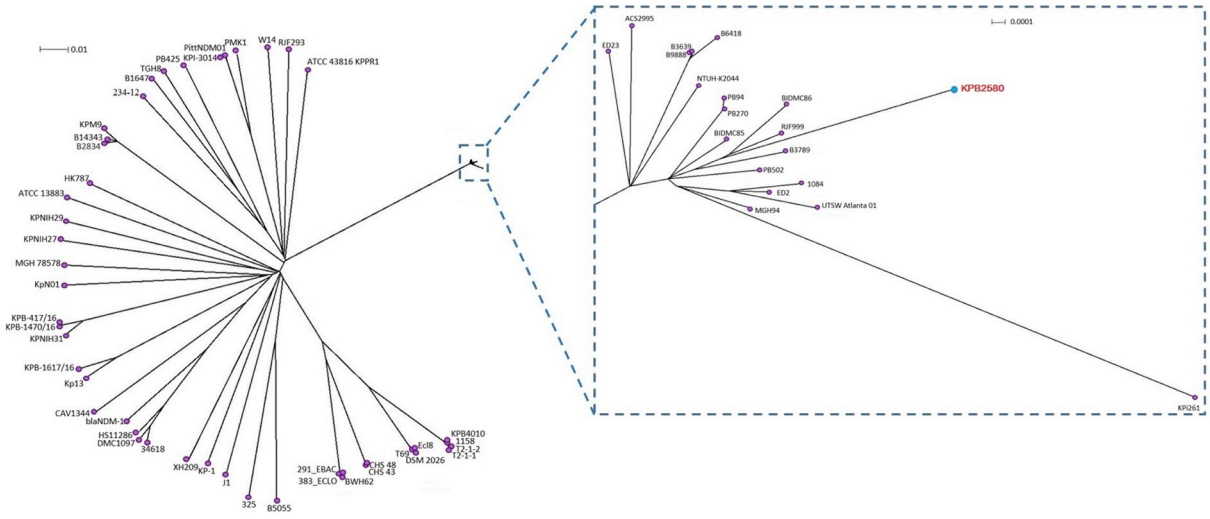

Figure 5. Phylogenetic tree of 65 *K. pneumoniae* genomes based on the core single nucleotide polymorphism (SNP) generated by the program Wombac 2.0. Phylogenetic trees were constructed using the neighbor-joiningmethod with 1000 bootstrap resamplings. The genome of KPB2580 strain is highlighted in red.

ST147^{K14}, ST20^{K28}, STNI^{K60} and ST147^{KNI} (non-hypermucoviscous, non-virulent in mice) were attributed to this group of *K. pneumoniae*. 'Hypervirulent' CC65 was presented by two strains of ST65^{K2} and ST2280^{K2} (hypermucoviscous, hypervirulent in mice). The CC23 was more heterogenic – presented by 10 strains of ST23^{K1} (hypermucoviscous, virulent in mice); two strains of ST23^{K57} (non-hypermucoviscous, non-virulent in mice); one strain of ST218^{K57} (hypermucoid, virulent in mice); four strains of ST218^{K57} (hypermucoviscous, non-virulent in mice), two strains of ST218^{K57} (non-hypermucoviscous, non-virulent in mice). Moreover, four strains

of ST86^{K2} (hypermucoviscous, virulent in mice) and one strain of ST2174^{K2} (hypermucoviscous, virulent in mice) were attributed to highly virulent *K. pneumoniae* (Table 3). Of particular interest are hypervirulent, ST23^{K1}, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}48}$-positive *K. pneumoniae* strain KPB2580-14 and four closely related, virulent in mice strains (KPB463K-13, KPB475-14, KPB594-14, and KPB1103-14). *K. pneumoniae* strain KPB2580-14 which is distinguished from others by its hypervirulence for humans and in mice, was selected for whole genome sequencing analysis.

Genome characteristic of hypervirulent K. pneumoniae of ST23 acquired pCTX-M-15 and pOXA-48 plasmids

As a result of *K. pneumoniae* strain KPB2580-14 whole genome sequencing 360 contigs were obtained, the total length of the genome was 5773846bp. The GenBank accession number is PUXF00000000.1. Raw data accession number is SRR6785071. Chromosomal fragment with putative iron transport and phosphotransferase function genes (20430bp) and the chromosomal region associated with allantoin metabolism (63291bp) were identified, as well as sequence type ST23 was confirmed.

Phylogenetic analysis showed that the strain KPB2580 was clustered into the same clade with K1 type strains of clonal complex CC23 causing liver abscess such as 1084 [33], NTUH_K2044 [34], ED2 and ED23 [35] isolated in Taiwan. In addition, this group includes our previously isolated hypermucoviscous, multy-drug resistant strain KPi261 [13] and four hvKp strains B6418, B3639, B3789, and B9888 (isolate No. Kp2, Kp3, Kp4, and Kp5, respectively) that were isolated from community and hospital acquired bloodstream infection in India [36]. All these strains also belonged to the clonal complex CC23 and capsule type K1 (Figure 5).

Three plasmids were detected among KPB2580 contigs. The first was virulence plasmid homologous to pLVPK (GenBank: AY378100) carrying *rmpA, iroN, iroD, iroC, iroB, fecI, fecA, rmpA2, iutA, iucD, iucC*, and *iucB* genes (Supplementary Figure 3). The second was the plasmid homologous to pCTXM15 (GenBank: CP016925) carrying $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ genes (Supplementary Figure 4). The third was the plasmid homologous to pOXA48 (GenBank: JN626286) carrying $bla_{OXA\text{-}48}$ gene (Supplementary Figure 5).

Conclusions

Three groups of *K. pneumoniae* strains isolated in 2012–2016 were identified in this study: (i) cKP characterized by a high level of antibacterial resistance and avirulent in mice; (ii) hypermucoviscous, virulent in mice *K. pneumoniae* that are not multidrug resistant; (iii) hypermucoviscous, virulent in mice *K. pneumoniae* that are also multidrug resistant. This study is the first report about hvKP ST23^{K1} strain harboring extended-spectrum beta-lactamase CTX-M-15 and carbapenemase OXA-48 genes.

Disclosure statement

No potential conflict of interest was reported by the authors.

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