Infection and Drug Resistance

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ORIGINAL RESEARCH

Molecular Profiling of a Multi-Strain Hypervirulent *Klebsiella pneumoniae* Infection Within a Single Patient

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Background: The rising prevalence of infections caused by carbapenem-resistant and hypervirulent *Klebsiella pneumoniae* (CR-hvKP) has outpaced our understanding of their evolutionary diversity. By straining the antimicrobial options and constant horizontal gene transfer of various pathogenic elements, CR-hvKP poses a global health threat.

Methods: Six *KP* isolates (KP1~KP6) from urine, sputum and groin infection secretion of a single patient were characterized phenotypically and genotypically. The antimicrobial susceptibility, carbapenemase production, hypermucoviscosity, serum resistance, virulence factors, MLST and serotypes were profiled. Genomic variations were identified by whole-genome sequencing and the phylogenetic differentiation was analyzed by Enterobacterial repetitive intergenic consensus (ERIC)-PCR.

Results: All *KP* strains were multi-drug resistant. Four of them (KP1, KP3, KP5 and KP6) belonged to ST11-K64, with high genetic closeness (relatedness coefficient above 0.96), sharing most resistance and virulence genes. Compared with KP1, the later isolates KP3, KP5 and KP6 acquired $bla_{\text{KPC-1}}$ and lost $bla_{\text{SHV-182}}$ genes. KP2 and KP4 had the same clonal origin of ST35-K16 (relatedness coefficient 0.98), containing almost identical genes for resistance and virulence. They were non-mucoid and carried $bla_{\text{NDM-5}}$ gene.

Conclusion: A co-infection with two types of CR-hvKP affiliated with different clades within a single patient amplified the treatment difficulties. In addition to source control and epidemiological surveillance, investigation of the in-host interactions between CR-hvKP variants may provide valuable treatment solutions.

Keywords: *Klebsiella pneumoniae*, carbapenem-resistance, virulence, ST11-K64, ST35-K16, MLST, whole-genome sequencing

Introduction

Klebsiella pneumoniae (*KP*) is a common gram-negative bacillus ubiquitously present in natural environment and different hosts. *KP* also constitutes the normal flora of human mucosal surfaces such as intestine, skin and nasopharynx.[1] Community-acquired or nosocomial *KP* has emerged as one of the principal pathogens of infectious diseases including pneumonia, urinary tract infections, sepsis, liver abscesses, purulent pericarditis, and wound site infections.[2–4]

Decades of extensive use of antimicrobials has facilitated the rapid evolvement and dissemination of *KP* that produces extended-spectrum ß-lactamases (ESBLs) and carbapenemases (KPCs). These are enzymes that break down the entire last line of weapons against infectious diseases, by hydrolysing ß-lactams, fluoroquinolones and aminoglycosides, as well as penicillins, cephalosporins and monobactams, etc.[5,6] Primary mechanisms adopted by carbapenem-resistant *KP* (CR-KP) to deactivate carbapenems are classified into the Ambler classes A (KPC), B (the metalloenzymes NDM, VIM and IMP) and D (oxacillin enzyme, OXA).[4]

The morbidity and mortality of *KP* infection are also dictated by its virulence. An arsenal of virulence factors such as capsule, lipopolysaccharide (LPS), siderophore, urease and fimbriae, manifest as phenotypic traits of adherence, immune

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evasion, hypermucoviscosity and nutrients scavenge. High virulence equates to greater ability of bacterial invasion, survival and proliferation.[7] The distinct antigenic capsular polysaccharide of different strains has been utilized for serotype classification. From the normally opportunistic classical *KP* strains (cKP), the versatile evolution of *KP* has prompted the differentiation of hypervirulent *KP* (hvKP). Although the distinction between them is less definitive and tends to become blurred, they often differ in demographic and clinical distribution propensity. While the elderly people and hosts with comorbidities such as immunosuppression, diabetes mellitus and chronic renal insufficiency are at a greater risk for severe, sometimes even fatal infection of cKP, hvKP infection significantly affects younger and otherwise healthy population.[3] Infections with hvKP are more often emanated from community exposure, bearing a high geographical variation (higher in Taiwan and China than in western countries and Europe).[8] Besides, hvKP more likely possesses elevated iron acquisition systems and causes more metastatic phenotypes including multiple-site infection and debilitating syndromes.[9–11] Although the terms hypermucoviscous *KP* (hmKP) and hvKP are often interchangeably used, researchers have found that mucoviscosity cannot be sufficiently used to infer hypervirulence.[9] Instead, genotypic delineation of virulent factors such as *iucA, iroB, peg-344*, $_{p}$*rmpA* and $_{p}$*rmpA2*, and their combination patterns, are more accurate.[12,13]

In recent years, carbapenem-resistant hypervirulent *KP* (CR-hvKP) emerges and thrives on the convergence of both the drug resistance- and hypervirulence-encoding elements through plasmid fusion or conjugation.[14] CR-hvKP has been relentlessly impoverishing our last resort of antimicrobials and is showing an alarming trend to disseminate from Asian pacific rim to the whole globe to cause intractable infections in individuals of all ages.[3,10] While an accurate prevalence of CR-hvKP as an infectious agent is hard to measure, due to the lack of standardised diagnostic markers, devastating regional outbreaks of CR-hvKP in healthcare facilities have been reported around the world.[15–20] In particular, CR-hvKP is a major cause for ventilator-associated pneumonia, with a very high mortality rate. ICU, where tracheostomy and other indwelling medical devices are regularly applied, represents a unique niche in favour of biomaterial-associated infections. Opportunistic bacteria in this environment, predominantly *KP* strains, can aggregate and embed themselves in a thick layer of self-produced extracellular biofilm matrix. The adherence of the biofilms to biotic or abiotic surfaces protects the bacteria from host defence and bactericidal reagents.[2,21] In addition, the mobility and diversity of large plasmid/transposon burden of *KP* enable it to be dangerously active in inter-species transfer of the resistance elements via bacterial conjugations between different Gram-negative bacteria such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.[22]

The pathogenic colonization of *KP* in some cases and some period of infection can be occult and asymptomatic, which heavily compounds the tracking of its inter- or intra-patient evolution and spread.[3,19] Using whole-genome sequencing, researchers were able to chart a putative transmission map of multidrug-resistant *KPs* within a hospital environment and pin down the index patient who was discharged three weeks prior to the stealthily developed outbreak.[19] However, how various *KP* strains interact and adapt to survive along the course of the infection within a single host is not much understood.

In this study, we identified two distinct colonies of CR-hvKPs with sequence types ST11-K64 and ST35-K16, from six *KP* isolates obtained successively from the urine, sputum and secretion samples of a single patient with acute respiratory failure. Molecular characterization of the drug resistance, virulence and genome sequencing were performed to discern the mechanisms conferring bacterial survival, at the cost of the host's survival.

Materials and Methods

Bacterial Isolates and the Case History

The patient was admitted to the ICU at the local hospital in July 2021 due to recessive severe cough and long-term ventilator-dependency. A total of six strains of *Klebsiella pneumoniae* (*KP*) were recovered from isolates successively collected from the urine, sputum and groin infection secretion (Figure 1), and were labelled as KP1, KP2, KP3, KP4, KP5 and KP6 in chronological order. Considering the high risk of lower respiratory tract and multi-site infection due to tracheotomy the patient underwent, anti-infection treatment of piperacillin/tazobactam was delivered at the initial stage of hospitalization, but without significant improvement. KP1 was isolated from urine samples when urinary tract infection

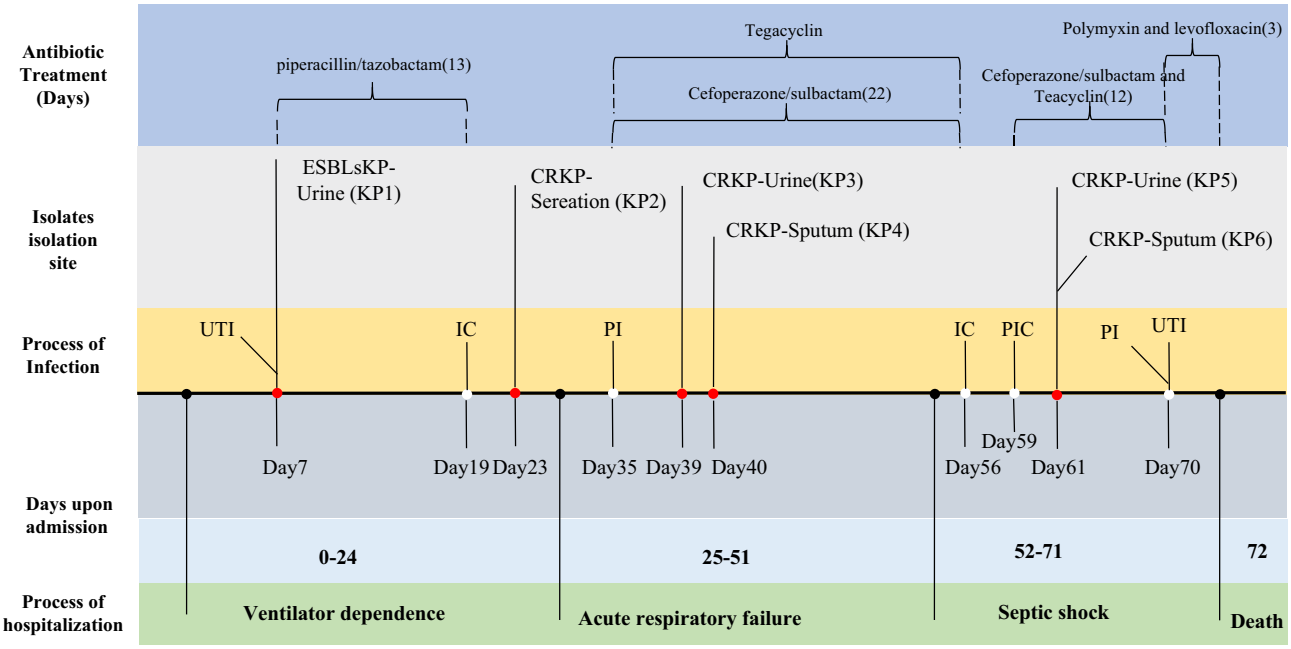

Figure 1 Timeline to summarize the case history of the patient admission, treatment, and isolate collection.
Abbreviations: UTI, Urinary tract infection; IC, Infection control; PI, Pulmonary infection; PIC, Pulmonary infection control.

was diagnosed at day 7. By immediate implementation of piperacillin/tazobactam treatment, the infection was controlled by day 19. On day 23, patches of bilateral groin erythema occurred, where KP2 was collected from the purulent secretion, which was considered to be a colonization and no specific treatment was applied. On day 35, the pulmonary infection deteriorated into acute respiratory failure. Despite prompt anti-infection treatment with cefoperazone/sulbactam + tigecycline, the infection was not controlled effectively. KP3 (urine) and KP4 (sputum) were detected during this period. The patient's condition continued to advance into septic shock on day 52. The infection was largely controlled after an additional 22 days of treatment with cefoperazone/sulbactam + tigecycline but then relapsed 3 days afterwards. Meanwhile, KP5 and KP6 were identified from urine and sputum, respectively. Extended treatment with cefoperazone/sulbactam + tigecycline regimen for another 12 days did not stop the progress of the patient's pulmonary and urinary tract infections, nor did the alternative treatment with colistin + levofloxacin. The patient ultimately succumbed to severe infections and multiple organ failure.

KP Identification and Antimicrobial Susceptibility Test

KP identification and antimicrobial susceptibility were performed using Micro MIC method on the VITEK 2 automated microbial identification and antibiotic susceptibility system (bioMérieux, France), according to the manufacturer's instructions. *Escherichia coli* ATCC25922 and *Klebsiella pneumoniae* ATCC700603 (Microbiologics, America) served as quality control strains. Antimicrobials included in the panel were as follows: amikacin, ampicillin/sulbactam, ampicillin, cefazolin, cefepime, cefoperazone/sulbactam, cefotaxime, cefoxitin, ceftazidime, ceftazidime/clavulanate sodium, ceftriaxone, cefuroxime, ciprofloxacin, ertapenem, gentamicin, imipenem, meropenem, levofloxacin, nitrofurantoin, piperacillin/tazobactam, polymyxin E (colistin), tigecycline, tobramycin, and trimethoprim/tazobactam. The data were interpreted in accordance with the guidelines recommended by CLSI (Clinical and laboratory Standards Institute, 2020 version).

Detection of Carbapenemase and Its Encoding Genes

The NG-Test CARBA-5 immunochromatographic assay (Changsha Zhongsheng Zhongjie Biotechnology Co., Ltd) was performed to detect the five most prevalent carbapenemase families, ie Klebsiella pneumoniae carbapenemase (KPC), oxacillin-hydrolyzing β-lactamase (OXA-48), verona integron-encodes metallo-β-lactamase (VIM), imipenem-resistant phenotype (IMP), and New Delhi metallo-β-lactamase (NDM), as described previously.[23] Colony samples from bacteria

grown on blood agar culture were taken with a 1-μL loop and seeded into 150 μL extraction buffer and mixed by vortexing. Next, 100 μL of the mixture suspension was transferred into NG-Test Carba 5 sample well marked with 'S' in the test cassette. After 15 min of incubation at room temperature, the presence or absence of the control and test lines was visualized within 20 min. Positive controls for each target were provided in the test kit.

The carbapenem resistance genes (bla_{kpc}, bla_{OXA-48}, bla_{vim}, bla_{IMP}, bla_{NDM}) encoding these five carbapenemases were examined by PCR, as described in the literature.[24] The bacterial DNA template was extracted by boiling procedure. PCRs were carried out in 20 μL volumes containing 1μL extracted DNA, 9.5 μL Premix Taq DNA polymerase (Dalian Bao Biological Engineering Co., Ltd) and 1μL of each primer. Reaction conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55–58°C for 60 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. The PCR products were separated through 2% agarose gel, stained in GoldViewTM nucleic acid dye and photographed under UV by GenoSensl500 transilluminator (Shanghai Qinxiang Scientific Instrument Co., Ltd). The detail of the PCR primers and the corresponding annealing temperature are listed in Table S1 (Supplemental Material).

Detection of Virulence Factors

The presence of six hypervirulence genes including rmpA (regulator of mucoid phenotype A), mrkD (mannose-specific adhesin subunit of type 3 fimbriae), entB (enterobactin biosynthesis), ybtS (yersiniabactin biosynthesis), wcaG (endotoxin-related gene) and KfuBC (iron acquisition system-related gene) was determined by PCR. The primer information is listed in Table S1. The PCR was conducted as described in Detection of Carbapenemase and Its Encoding Genes.

String Test

String test was conducted to assess the mucoviscosity of the KP strains, as established elsewhere.[13] The formation of a >5 mm viscous filament of the KP colonies on a blood agar plate, stretched upwards by an inoculation loop, was classified as hypermucoviscous phenotype.

Multi-Locus Sequence Typing (MLST)

The nucleotide sequence-based serotyping MLST was performed to determine the sequence types (ST) of the KP strains. The primers used for PCR amplification of seven housekeeping gene fragments (gapA, rpoB, mdh, pgi, phoE, infB and tonB) were designed according to the Institute Pasteur Klebsiella MLST scheme (https://bigsdb.pasteur.fr/klebsiella/primers-used/). The PCR products were sequenced and aligned with online MLST database (https://bigsdb.pasteur.fr/).

Capsular Serotyping

The capsular serotypes of the KP isolates were determined using the PCR-based capsular antigen as previously described.[25] The primers used are shown in Table S1. These assays differentiate KP isolates into seven major serotypes: K1, K2, K5, K20, K54, K57 and K64. The serotype of KP2 and KP4 was not determined by this panel and was identified by genome sequencing (Whole-Genome Sequencing (WGS)).

Molecular Genotyping by ERIC-PCR

To evaluate the genetic relatedness among the KP strains, ERIC (enterobacterial repetitive intergenic consensus) analysis was performed as described previously.[26,27] Genomic DNA extracted from the bacteria was amplified using the primers ERIC1: 5'-ATGTAAGCTCCTGGGGATTCA-3'and ERIC2: 5'-AAGTAAGTGACTGGGGTGAGCG-3'. PCR was performed in total volumes of 25μL, containing 1μL DNA template, 12.5 μL Premix Taq DNA polymerase (Dalian Bao Biological Engineering Co., Ltd) and 1μL of each primer. Reaction conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 45 seconds, 52°C for 50 seconds, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. The PCR products were subject to agarose gel electrophoresis and analysis system. The bacterial community fingerprint bands of the strains were inspected using Quantity One software. The unweighted pair-group method with arithmetic mean (UPGMA) was adopted to determine the similarity clustering among the obtained *KP* types. An inter-strain relatedness with genetic similarity coefficient >0.90 was considered highly homogeneous.

Serum Survival Assay

The survival of the bacteria in pooled serum from healthy individuals was investigated using a standard assay.[28,29] Each experiment was performed with duplicates. Briefly, bacterial growing in exponential-phase were suspended at 1×10^6 CFU/mL into healthy human serum (1:3 volume ratio). The response of bacteria to serum killing was determined by viable colony counts obtained at 0, 1, 2, and 3 h upon the co-incubation at 37°C with shaking. The sensitivity levels were scored into six grades and classified into three levels, ie serum-sensitive (grade 1 or 2), intermediately sensitive (grade 3 or 4), or serum-resistant (grade 5 or 6). The grade criteria were as follows. Grade 1: viable counts <10% of the inoculum at 1, 2 h and <0.1% at 3 h; grade 2: viable counts 10-100% of the inoculum at 1, 2 h and <10% at 3 h; grade 3: viable counts >100% of inoculum at 1 h and <100% at 2, 3 h; grade 4: viable counts >100% of inoculum at 1, 2 h and <100% at 3 h; grade 5: viable counts >100% of the inoculum at 1, 2, and 3 h, but decrease afterwards; and grade 6: viable counts >100% of the inoculum at 1, 2, and 3 h, and keep increasing. A graph was drawn using Graphpad prism software, where the original data of experiment duplicates (number of colonies on the plate x dilution factor) were calculated, logarithmically transformed, and expressed in the form of mean ± error.

Whole-Genome Sequencing (WGS)

Genomic DNA of all strains was extracted with a TIANamp Bacteria DNA Kit (Beijing TIANGEN Biotech Co., Ltd) following the manufacturer's instructions. The extracted gDNA was interrupted using Covaris LE220-PLUS (Covaris, Woburn, MA, United States) and the 300–500 bp fragments were selected as templates for library construction by VAHTS DNA Clean Beads. The double-stranded DNA was repaired and dA was added to the 3 'end. VAHTS DNA Clean Beads were used for purification. After purification, the WGS library was prepared using the VAHTS® Universal DNA Library Prep Kit for Illumina V3 (Vazyme Biotech Co., Ltd, United States). The sequencing library was sequenced using the MGI DNBSEQ-T7 (Shenzhen MGI Co., Ltd) with PE150 model.

Results

All KP Strains, Except for KP1, are Resistant to Carbapenems

As revealed by the antimicrobial susceptibility test, all the *KP* strains were ESBLs-producing and resistant to ampicillin, ampicillin/sulbactam, ceftriaxone, ceftazidime, cefotaxime, cefepime, cefazolin, cefoperazone/sulbactam, cefuroxime, ciprofloxacin, levofloxacin, nitrofurantoin, gentamicin, piperacillin/tazobactam, and trimethoprim/sulfa. Two antibiotics, tigecycline and colistin, were inhibitory to all six strains. KP1 was sensitive to cefoxitin and carbapenems including ertapenem, imipenem and meropenem, whereas all the other five strains were resistant. KP2 and KP4 had intermediate sensitivity to tobramycin. In opposition to KP2 and KP4, which were sensitive to aztreonam and resistant ceftazidime/avibactam, other strains had inverted responses to these antibiotics. In addition, the strains displayed various responses to amikacin, with only KP2, KP4 and KP5 showing sensitivity. Details of MIC (minimum inhibitory concentration) and sensitivity interpretation are listed in Table 1.

Table 1 Antimicrobial Susceptibility Test of Six *Klebsiella pneumoniae* Isolates

Antibiotic Class	Antibiotic	MIC (μg/mL) of Each Isolate					
		KP1	KP2	KP3	KP4	KP5	KP6
Monobactams	ATM	≥64 (R)	2 (S)	≥64 (R)	4 (S)	≥64 (R)	≥64 (R)
Penicillins	AM	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)
Cephalosporins	CRO	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)
	CAZ	32 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)
	CTX	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)	>32 (R)
	CZ	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)	>16 (R)
	FEP	≥32 (R)	≥32 (R)	≥32 (R)	≥32 (R)	≥32 (R)	≥32 (R)
	FOX	≤8 (S)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)
	CXM	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)

(*Continued*)

Table 1 (Continued).

Antibiotic Class	Antibiotic	MIC (μg/mL) of Each Isolate					
		KP1	KP2	KP3	KP4	KP5	KP6
Carbapenems	ETP	≤1 (S)	≥8 (R)	≥8 (R)	≥8 (R)	≥8 (R)	≥8 (R)
	IPM	≤0.25 (S)	≥16 (R)	4 (R)	≥16 (R)	4 (R)	≥16 (R)
	MEM	0.5 (S)	≥16 (R)	≥16 (R)	≥16 (R)	≥16 (R)	≥16 (R)
Fluoroquinolones	CIP	≥4 (R)	≥4 (R)	≥4 (R)	≥4 (R)	≥4 (R)	≥4 (R)
	LVX	≥8 (R)	4 (R)	≥8 (R)	4 (R)	≥8 (R)	≥8 (R)
Aminoglycosides	AN	≥64 (R)	≤2 (S)	≥64 (R)	≤2 (S)	≤2 (S)	≥64 (R)
	GM	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)	>8 (R)
	TM	≥16 (R)	8 (I)	≥16 (R)	8 (I)	≥16 (R)	≥16 (R)
Others	TZP	≥128 (R)	≥128 (R)	≥128 (R)	≥128 (R)	≥128 (R)	≥128 (R)
	SXT	>2/38 (R)	>2/38 (R)	>2/38 (R)	>2/38 (R)	>2/38 (R)	>2/38 (R)
	TGC	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)	1 (S)
	FM	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)	>64 (R)
	SCF	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)	≥64 (R)
	CZA	24 (S)	17 (R)	25 (S)	16 (R)	25 (S)	27 (S)
	PE	≤0.5 (S)	≤0.5 (S)	≤0.5 (S)	≤0.5 (S)	≤0.5 (S)	≤0.5 (S)

Notes: Micro MIC method was used for antimicrobial susceptibility test.

Abbreviations: MIC, Minimum inhibitory concentration. S, susceptible; I, intermediate; R, resistant. ATM, Aztreonam; AM, Ampicillin; CRO, Ceftriaxone; CAZ, Ceftazidime; CTX, Cefotaxime; CZ, Cefazolin; FEP, Cefepime; FOX, Cefoxitin; CXM, Cefuroxime; ETP, Otapenem; IPM, Imipenem; MEM, Meropenem; CIP, Ciprofloxacin; LVX, Levofloxacin; AN, Amikacin; GM, Gentamicin; TM, Tobramycin; TZP, Piperacillin/tazobactam; SXT, Trimethoprim/sulfamethoxazole; TGC, Tigecycline; FM, Furantoin; SCF, Cefoperazone/sulbactam; CZA, Ceftazidime/avibactam; PE, Polymyxin E (Colistin).

In agreement, KP1 did not contain any of the five carbapenemase enzymes examined by the NG- CARBA 5 assay. KP3, KP5 and KP6 were positive for KPC (Figure 2A), while NDM was detected in KP2 and KP4. The broad substrate spectrum of KPC enzyme covers all beta-lactam classes, including nitrocefin, cefotaxime, cephalothin, cephaloridine, benzylpenicillin, ampicillin and piperacillin.[5] NDM is known to inactivate all β-lactams except aztreonam,[30] which explained why KP2 and KP4 were sensitive to aztreonam in the antimicrobial test.

PCR data further confirmed the carriage of bla_{NDM} gene in KP2 and KP4, and bla_{KPC} gene in KP3, KP5 and KP6 (Figure 2B).

All Six *KP* Strains Harbour Virulence Factors, but KP2 and KP4 are Non-Mucoid

The presence of six virulence genes that are among the most recognised factors contributing to hypervirulence of *KP*[31–33] were characterized by PCR amplification. We found that all strains carried *mrkD, entB, kfuBC* and *ybtS* genes, which are

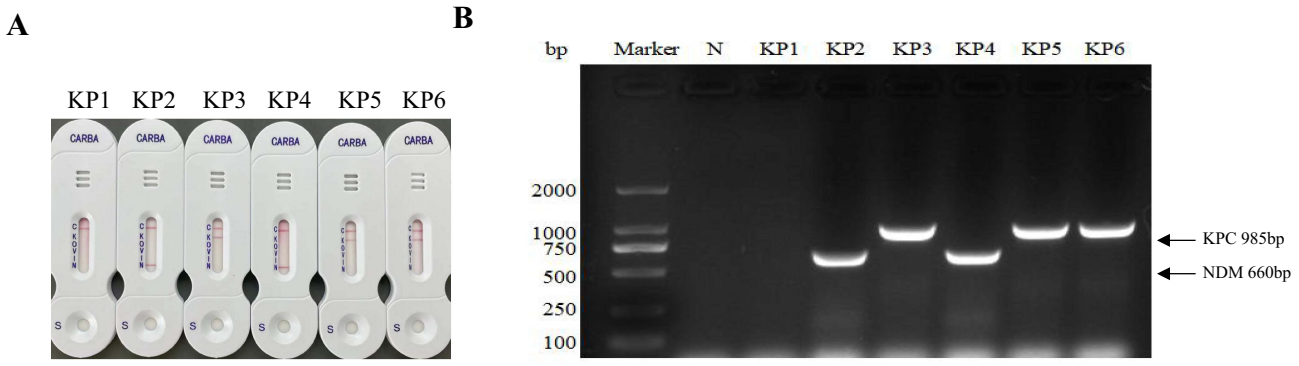

Figure 2 Determination of carbapenem resistance of the *KP* strains by measuring carbapenemase activity using NG-test CARBA 5 kit (**A**), and carbapenem resistance genes using PCR (**B**). C: positive control, K: KPC, O: OXA-48-like, V: VIM, I: IMP, N: NDM.

Table 2 Summary of the Genetic and Phenotypic Profiles of the *Klebsiella pneumoniae* Isolates

Isolate	KP1	KP2	KP3	KP4	KP5	KP6
Isolation origin	Urine	Wound	Urine	sputum	Urine	sputum
ESBLs	+	+	+	+	+	+
CR-genes	-	bla_{NDM-5}	bla_{KPC-1}	bla_{NDM-5}	bla_{KPC-1}	bla_{KPC-1}
Virulence genes	*rmpA*	*mrkD*	*rmpA*	*mrkD*	*rmpA*	*rmpA*
	mrkD	*entB*	*mrkD*	*entB*	*mrkD*	*mrkD*
	entB	*ybtS*	*entB*	*ybtS*	*entB*	*entB*
	ybtS	*kfuBC*	*ybtS*	*kfuBC*	*ybtS*	*ybtS*
	kfuBC	*wcaG*	*kfuBC*	*wcaG*	*kfuBC*	*kfuBC*
Hypermucoviscosity	+	-	+	-	+	+
MLST	ST11	ST35	ST11	ST35	ST11	ST11
Serotype	K64	K16	K64	K16	K64	K64

Notes: +, positive; -, negative.

responsible for production of pathogenic molecules of adhesin subunit, enterobactin, iron transporter and siderophore-dependent yersiniabacin, respectively. However, *rmpA* was present only in KP1, KP3, KP5 and KP6, while the fucose-synthesizing gene *wcaG* was detected only in KP2 and KP4 (Table 2).

Hypermucoviscosity is a phenotypic manifestation of virulence that is significantly associated with *rmpA* plasmid and can be measured by string test. Among all strains, visible strings longer than 5mm were stretched from the colonies of KP1, KP3, KP5 and KP6, but not KP2 and KP4 (Figure 3), which matched the status of *rmpA* gene.

The *KP* Strains Develop Resistance to Serum Killing Along the Course of Infection

The resistance of the bacteria to the healthy defence mechanism of human serum, typically the serum compliment killing, is an important aspect to ensure their survival in the host. As demonstrated by the logarithmic number of viable colonies counted at different timepoints (Figure 4), KP1, KP2 and KP4 were sensitive (both at grade 2) to serum killing and were almost completely eradicated after three hours of co-incubation. KP6 had an intermediate grade 4 sensitivity. KP3 and KP5 displayed strong resistance to serum killing, scored grade 5 and 6, respectively. According to the percentage of

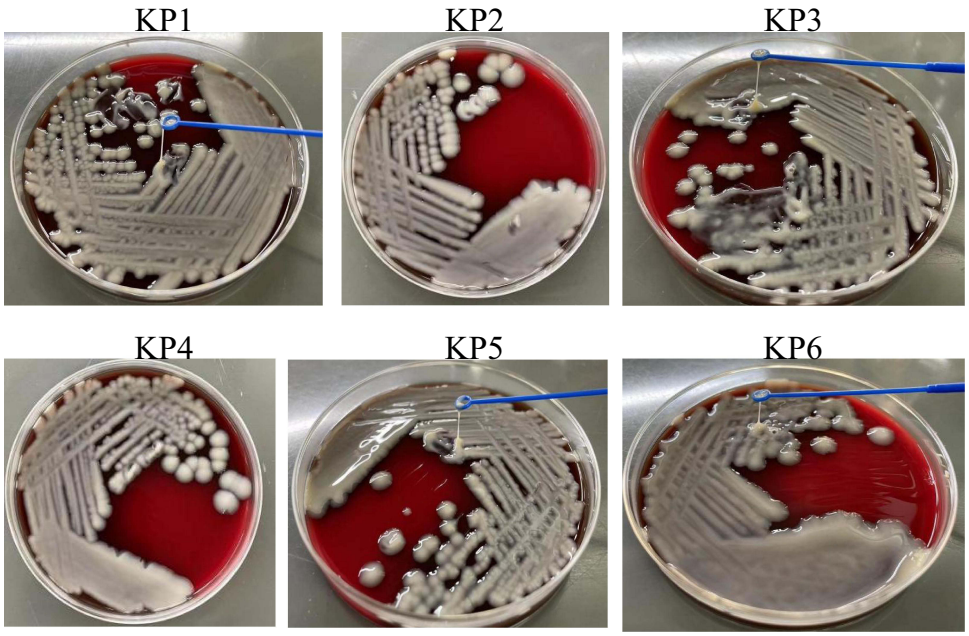

Figure 3 Images of hypermucoviscous phenotype assessed by string test.

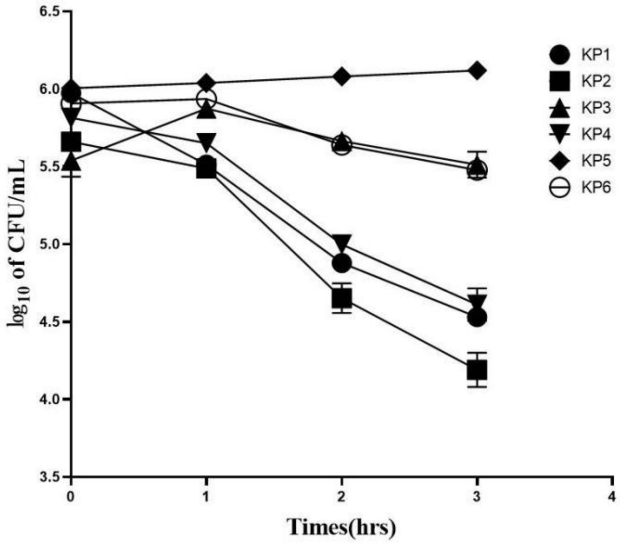

Figure 4 Logarithmic calculation of viable colony counts (CFU/mL) of the *KP* strains subjected to co-incubation with pooled healthy serum for three hours. The values were expressed in the form of mean ± error, and log 10 was used for logarithmic conversion to simplify the data.

surviving *KP* colonies at the end of three hours in comparison to the initial time point, the order of serum susceptibility was KP2>KP1>KP4>KP6>KP3>KP5 (Table 3). KP1 and KP2 were killed by two thirds and one third, respectively, after 1 hour and mostly wiped out after 3 hours of co-incubation (3.82% and 3.48% left, respectively), while other strains demonstrated various resilience to the serum killing machinery. The survival of KP5 colonies was not inhibited but even encouraged throughout the co-incubation (viable counts > 100%). Comparing KP2 and KP4, which shared all phenotypic and genotypic attributions, KP4 had slightly higher survivability than KP2 at each time point. The results suggested a gradual accretion of serum resistance from KP1 to KP5, and from KP2 to KP4.

Genomic and WGS Analyses Reveal Two Distinct Types of the *KP* Clones with Various Pathogenic Mechanisms

By MLST classification, KP1, KP3, KP5 and KP6 isolates were identified as ST11, whereas KP2 and KP4 were ST35. Subsequent capsular serotyping by PCR indicated that KP1, KP3, KP5 and KP6 belonged to K64. The serotype of KP2 and KP4 was not detected by the panel of common capsular serotypes covered in the PCR test but was determined separately by whole-genome sequencing (WGS). By aligning the sequence data with the MLST online database, both KP2 and KP4 were identified as K16 (Figure 5). WGS further revealed the subtypes of carbapenemase-coding genes in KP3, KP5 and KP6 as $bla_{\text{KPC-1}}$, while that in KP2 and KP4 was $bla_{\text{NDM-5}}$. Like common *KP* strains that contain $bla_{\text{KPC-1}}$, KP6 was heavily resistant to imipenem, with MIC≥16 μg/mL. Noticing that the MIC of KP3 and KP5 to imipenem was 4 μg/mL, potential point mutations in the genes were suspected. But no base difference was found, the discordance of their imipenem susceptibility remained unexplained.

Table 3 Viable Counts (CFU/mL) and Percentage of Living Colonies in Serum Killing Assay

Isolate	0h	1h (%)	2h (%)	3h (%)
KP1	236	82 (34.74)	19 (8.05)	9 (3.81)
KP2	115	77 (66.96)	12 (10.43)	4 (3.48)
KP3	89	187 (210.11)	116 (130.33)	83 (93.26)
KP4	164	113 (68.90)	25 (15.24)	11 (6.71)
KP5	254	274 (107.87)	301 (118.50)	330 (129.92)
KP6	202	216 (106.93)	110 (54.46)	76 (37.62)

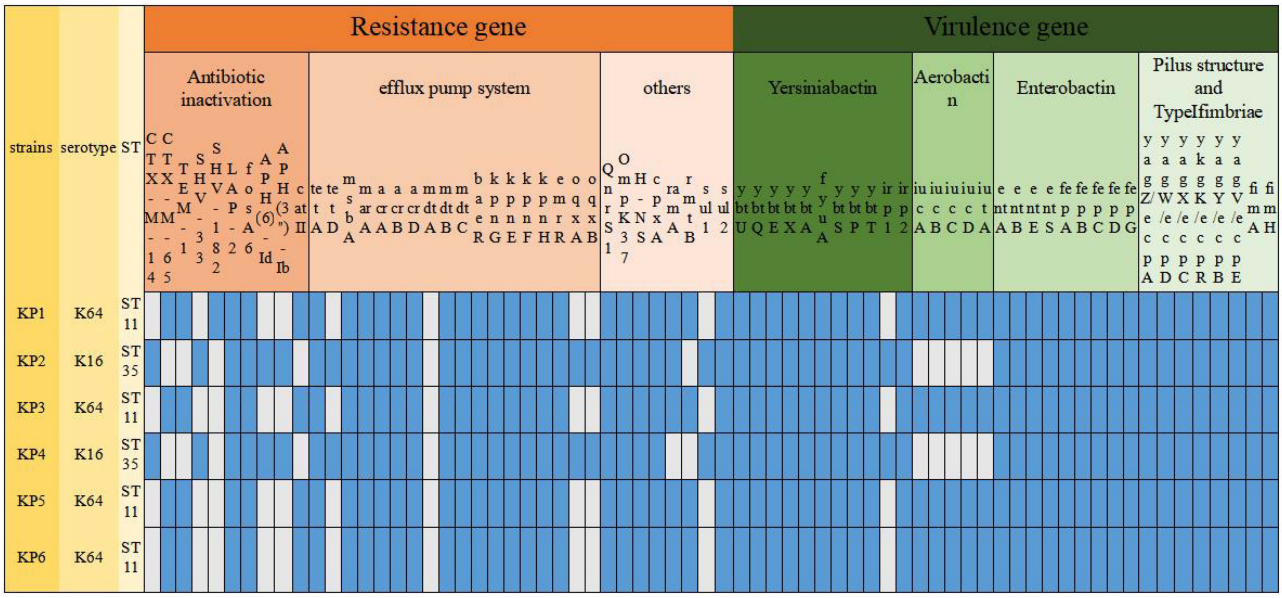

Figure 5 Capuslar serotypes, ST types, and the distribution of genetic features of the strains revealed by Whole genome sequencing (WGS). The presence and absence of the gene were represented by blue and gray squares, respectively.

To identify the mechanisms responsible for the drug resistance and virulence of the *KP* strains, the WGS data were annotated against the Comprehensive Antibiotic Resistance Database (CARD) and the Virulence Factor Database (VFDB). Two distribution patterns of genetic features regarding resistance and virulence were distinguished between the ST11-K64 type (KP1, KP3, KP5 and KP6) and ST35-K16 type (KP2 and KP4) (Table 2).

Overall, three main resistance mechanisms were revealed as follows. 1) Antibiotic inactivation (enzyme inactivation). Firstly, all strains (KP1~KP6) carried various genes encoding broad-spectrum β-lactamase (CTX- M, TEM, and SHV). The NDM-5-producing strains KP2 and KP4 carried $bla_{\text{CTX-M-14}}$ and $bla_{\text{SHV-33}}$. KP1 carried $bla_{\text{CTX-M-65}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-182,}}$ while the KPC-1-producing strains (KP3, KP5 and KP6) carried $bla_{\text{CTX-M-65}}$ and $bla_{\text{TEM-1}}$. Secondly, KP2 and KP4 carried aminoglycoside resistance factors AAC(3)-IId and APH(3")-Ib, which encode aminoglycoside acetyltransferase and phosphotransferase, respectively, to act on the specific amino groups of aminoglycoside antibiotics or hydroxyl groups. The KPC-1-producing strains did not have these genes. However, KP1, KP3, KP5 and KP6 harboured *cat II* gene, encoding chloramphenicol acetyltransferase to act against chloramphenicol. 2) Antibiotic export systems, especially genes belonging to the Resistance-nodulation-division (RND) pumps (AcrA/B) and the Major Facilitator Superfamily (MFS) efflux pumps (tetracycline resistance proteins TetA and emrR) were present in all strains. Moreover, KP2 and KP4 carried Tet(D) gene and oqxA/B genes encoding RND-family transporters to facilitate resistance to fluoroquinolones. 3) Mutations via target site modification or reduced permeability, with two sequence type-specific genetic polymorphisms. All isolates contained several genes that enable the annihilation of antimicrobial inhibition by means of mutation. For instance, *rmtB* gene that encodes 16S rRNA methylase to disable the binding of aminoglycoside on 16S rRNA, *sul1/2* gene that encodes a mutated dihydrofolate synthase (DHPS) to confer the resistance to sulfa drugs, and point mutations in chloramphenicol acetyltransferase gene cat II. In addition, all strains had *OmpK37* gene, encoding a product of an outer membrane porin that reduces the permeability to limit antibiotics uptake.

The strains carried numerous virulence factors, including bacteriocins involved in iron uptake systems (yersinicins, ybtU/Q/E/X/A/S, irp1/2 etc.), enterobactins (entA/B, fepA/B/C, etc.), as well as genes encoding type 1 pilus (fimA/H) and structural subunits of pilus (yagZ/ecpA, etc.). Aerobactins (iucA/B/C/D and iutA) were present in all strains except for the NDM-5-producing KP2 and KP4.

The genetic and phenotypic profiling of carbapenem resistance, virulence, MLST and serotyping are summarised in Table 2.

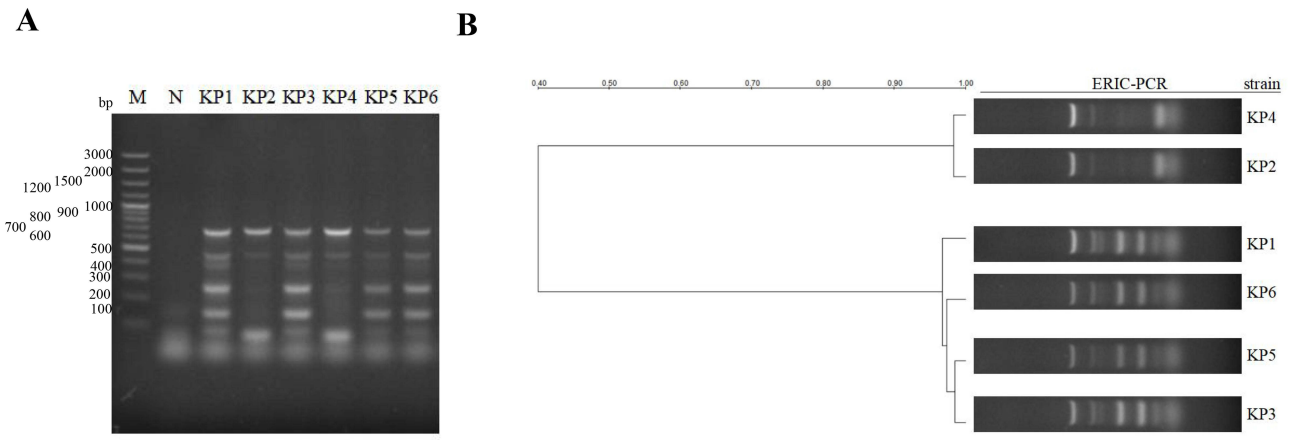

Figure 6 ERIC-PCR banding fingerprints of the *KP* strains (**A**). Lane M is the DNA molecular weight marker. The homology among the strains was calculated with the UPGMA clustering algorithm and illustrated as a dendrogram (**B**).

Differentiated ERIC-PCR Fingerprint Patterns Infer a Heterogenous *KP* Infection in the Patient

Two distinct ERIC-PCR banding patterns of the six *KP* strains were shown (Figure 6A), one was shared among KP1, KP3, KP5 and KP6, and the other between KP2 and KP4. As indicated by the phylogenetic tree, the rates of genetic relatedness between KP1, KP3, KP5 and KP6 was over 0.96, while that of KP2 and KP4 was 0.98 (Figure 6B). In conjunction with the conspicuous pattern discerned above from the phenotypic and genomic profiles, it was evident that the resultant antimicrobial responses and disease development of the single patient were a collected manifestation of an infection of multi-strain *KPs* with intraspecific genetic diversity.

Discussion

Infections caused by simultaneously carbapenemase-producing and hypervirulent *Klebsiella pneumoniae* (CR-hvKP) are often lethal, as evidenced by our previous clinical experience[3] and the present study. The mortality rate is as high as 50% around the world.[6,8,34] A massive challenge arises from the genetic plasticity of the bacteria and the extremely limited choice of antibiotics. In the absence of systematic or evaluated treatment guidelines, an empiric treatment regimen needs to be tailored rapidly.

Based on the phenotypic and genotypic characteristics of the six *KP* isolates from the patient, we were able to reconstruct the infection route in perspective of the bacterial evolvement. KP1 was first detected from urine and belonged to ST11-K64. Although it was ESBL-producing, mucoid and contained several other virulence genes, it was highly sensitive to carbapenem and serum killing. Two more ST11-K64 strains from urine, KP3 and KP5, were respectively detected 32 days later during the episode of respiratory failure and 54 days later following the onset of septic shock. Compared with KP1, both had gained a CR factor $bla_{\text{KPC-1}}$, retained ESBL-coding gene $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-65}}$, but lost the bla_{SHV182} gene. A strain from the same clade, KP6, was identified from sputum at the same time of the detection of KP5. Notwithstanding the same genomic fingerprints shared with KP3, the resistance to serum killing and imipenem had advanced and peaked at KP5 and KP6, respectively. Parallelly, a genotypically distinct strain KP2, an NDM5-producing ST35-K16 CR-hvKP strain, was isolated from the groin purulent secretion 16 days after KP1 identification. This isolate was non-mucoid and susceptible to serum killing. KP4, bearing the same genotypic identity with KP2, was detected from sputum 17 days later, with enhanced serum resistance. As KP2 and KP4 had different isolation sites, there should be bacterial dissemination from the groin to the respiratory tract or *vice versa*. However, this clone of *KP* disappeared in the later samples of sputum, which might suggest a growth inhibition by the antibiotics or out-competition by the other *KP* type.

Although all isolates were sensitive to tigecycline, which has been suggested to be effective to treat various strains of CR-KP,[6] a combination of tigecycline with cefoperazone-sulbactam did not achieve adequate efficacy to control the infection. The antibiogram indicated that the NDM-type KP2 and KP4 strains were sensitive to aztreonam and levofloxacin. Studies using

in vitro model have shown that combining avibactam can reduce the susceptible breakpoint concentration of aztreonam, thereby enhancing its killing activity against *KP*s producing metallo- β-lactamases (MBLs) such as NDM and IMP.[35] Successful treatment of infection with NDM-positive *KP* with a combination of aztreonam-ceftazidime-avibactam was also reported in a clinical case.[36] However, due to the concurrent infection of the KPC-bearing CR-hvKP strain, they could not be used successfully to control the infection progress of the present patient. Although NDM-carrying *KP* may be susceptible to colistin and tigecycline, the former has noxious nephrotoxicity and the latter displays inconsistent efficacy among various subgroups of pneumonia patients.[37] The last attempt with a reserve regime of colistin + levofloxacin did not curb the infection, although the synergistic killing of CR-KPs by colistin-based combination therapy with high efficacy was documented.[38,39] The pan-drug resistance and the sum of diverse virulence of the two different clones of *KP*, compounded by the adverse side effects of antibiotics, contributed to the treatment failure.

ST11 is the predominant CR-KP strain in Asia and the serotype K64 constitutes its major sub-lineage.[40–42] ST11-K64 infection is identified from different sources, such as sputum and liver abscess samples. Persistent colonization of ST11-K64 KP in gastrointestinal tract can aggravate gut dysbiosis, as shown in a mouse model.[43] ST11-K64 is also the most common CR-hvKP clonal group causing nosocomial infections in China.[44] With acquisition of $bla_{\text{KPC-1}}$, *rmpA*/*rmpA2*, and siderophore gene clusters, ST11-K64 CR-hvKP possesses strengthened virulence and multi-drug resistance, which has earned its qualification for highly pathogenic "superbug" to challenge clinicians.[45,46] In contrast, ST35 KP represents the major KP type in poultry species[47] and is among the most prevalent KP types composing the gastrointestinal microbiome in humans.[48] However, increased gut colonization of CR-KP can provide a major reservoir for healthcare-associated infectious diseases including pneumonia and bloodstream infection.[49] Infections with multi-drug resistant ST35 KP were reported in different countries such as France,[50] Yemen[51] and China,[52] but the pathogenicity of this strain was less understood. A ST35-K108 strain RJY9645, containing *rmpA* and $bla_{\text{NDM-5}}$, was identified from the sample of a patient with bloodstream infection who was successfully treated with imipenem and levofloxacin.[52] Meanwhile, K16 hvKP is less associated with MLST types[53] and its implication in invasive infections is reported sporadically.[54–56] Despite the initial assumption, we could not determine whether or not the ST35-K16 strains (KP2 and KP4) were derived from the patient's gut colonization.

Infections involving multiple microbial species or different clones of the same species can exhibit magnified drug resistance and exacerbated disease progression, compared to single-strain infections. Both *KP* clones detected from the patient were equipped with a full range of antimicrobial resistance mechanisms, ie decrease of drug intake, modification of drug target, drug inactivation, drug extrusion, and shift in metabolic pathways.[57] However, they might have unequal survivability. All the main biosynthetic genes for siderophores including yersiniabactin, enterobactin and aerobactin, which enable them to extract iron from the host, were present in the ST11-K64 strains. Notably, as aerobactin was proposed to be the most important determinator for the fitness and virulence of KP,[58] its presence in ST11-K64 strains and absence in ST35-K16 strains, may partially explain why KP2 and KP4 had relatively weaker resistance to serum killing and were eliminated in sputum at later stage of the infection.

Only a few multi-strain infections of *KP* have been documented,[19,59,60] and our understanding of the dynamic in-host interaction between the clones is meagre. The co-existing bacterial strains could compete to survive and proliferate. The combined modulation in the overall virulence and microbial resistance may favour the prosperity, dissemination, and pathogenesis of certain strains. Although exchange of genetic elements between the two types of *KP* strains was not suggested by the WGS results, crosstalk between them cannot be excluded. A mixed infection was reported in a case of necrotizing fasciitis (NF) caused by four *Aeromonas Hydrophila* strains originated from two distinct paraphyletic lineages NF1 and NF2.[61] Further in vitro and mouse model investigations demonstrated that the functional type 6 secretion system (T6SS), possessed by NF1, not only was associated with multi-drug resistance but also mediated the elimination of NF2 by NF1. Conversely, NF2 assisted the rapid metastatic dissemination of NF1 by destruction of the local tissue barrier, through the exotoxin A (*ExoA*) virulence factor carried by NF2.[62,63] In another study, mutations via insertion of IS (Insertion Sequences) elements at *cps* (capsular polysaccharide synthesis) locus were detected within a single host, which was suggested to be responsible for the survival competition between the mucoid form of *KP* and the non-mucoid *KP* strains.[60]

The unresolved but interesting questions are: 1) What are the infection sources of the *KP* strains? 2) Where did KP3 acquire $bla_{\text{KPC-1}}$ gene from and how? 3) What are the mechanisms and clinical consequences of the loss bla_{SHV182}? 4)

Can the survival competition between co-existing *KP* strains turn to our advantage in treatment solutions? They can only be answered by pursuing tight clinical surveillance and quantitative assessment of the pathogenic traits.

Conclusion

Two distinct *Klebsiella pneumoniae* clones, KPC-1-producing CR-hvKP ST11-K64 and NDM-5-producing ST35-K16, were identified in a single patient. Both clones had rich possession of resistance and virulence factors, which aggregated to the treatment failure. Along the course of infection development, the strains demonstrated a propensity for expanded drug resistance spectrum and strengthened serum survival. The large repertoire of pathogenic gene factors detected in the strains raises an alarm for the lurking circulation of dangerous *KP*s.

Abbreviations

KP, *Klebsiella pneumoniae*; ESBLs, extended-spectrum ß-lactamases; hvKP, hypervirulent *KP*; hmKP, hypermucoviscous *KP*; cKP, classical *KP*; CR-hvKP, carbapenem resistant hyper-virulent *KP*; KPC, *Klebsiella pneumoniae* carbapenemase; OXA-48, oxacillin-hydrolyzing β-lactamase; VIM, verona integron-encodes metallo-β-lactamase; IMP, imipenem-resistant phenotype; NDM, New Delhi metallo-β-lactamase; MLST, multi-locus sequence typing; ST, sequence type; ERIC (enterobacterial repetitive intergenic consensus); PCR, polymerase chain reaction; CFU, colony-forming units; WGS, whole-genome sequencing.

Ethics Approval and Patient Consent

The study was approved by the Ethics Committee of the Affiliated Hospital of Guizhou Medical University. Since the patient is deceased, written informed consent for publication of his clinical details and clinical images was obtained from the next of kin. We confirm that informed consent obtained from all study participants prior to study commencement, and Guidelines outlined in the Declaration of Helsinki were followed.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas. HC and SL have contributed equally to the work, and are co-first authors. YF and HC conceived and designed the study. HC, SL and CZ collected the clinical specimen and performed the laboratory analyses. SL took part in drafting, and critically reviewing the article. HC and BL substantially reviewed the data analyses and revised the manuscript. All authors gave final approval of the version to be published. All authors have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no potential conflict of interest.

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