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An abnormal multidrug-resistant and hypervirulent *Klebsiella pneumoniae* clinical isolate without *rmpA* or *rmpA2*

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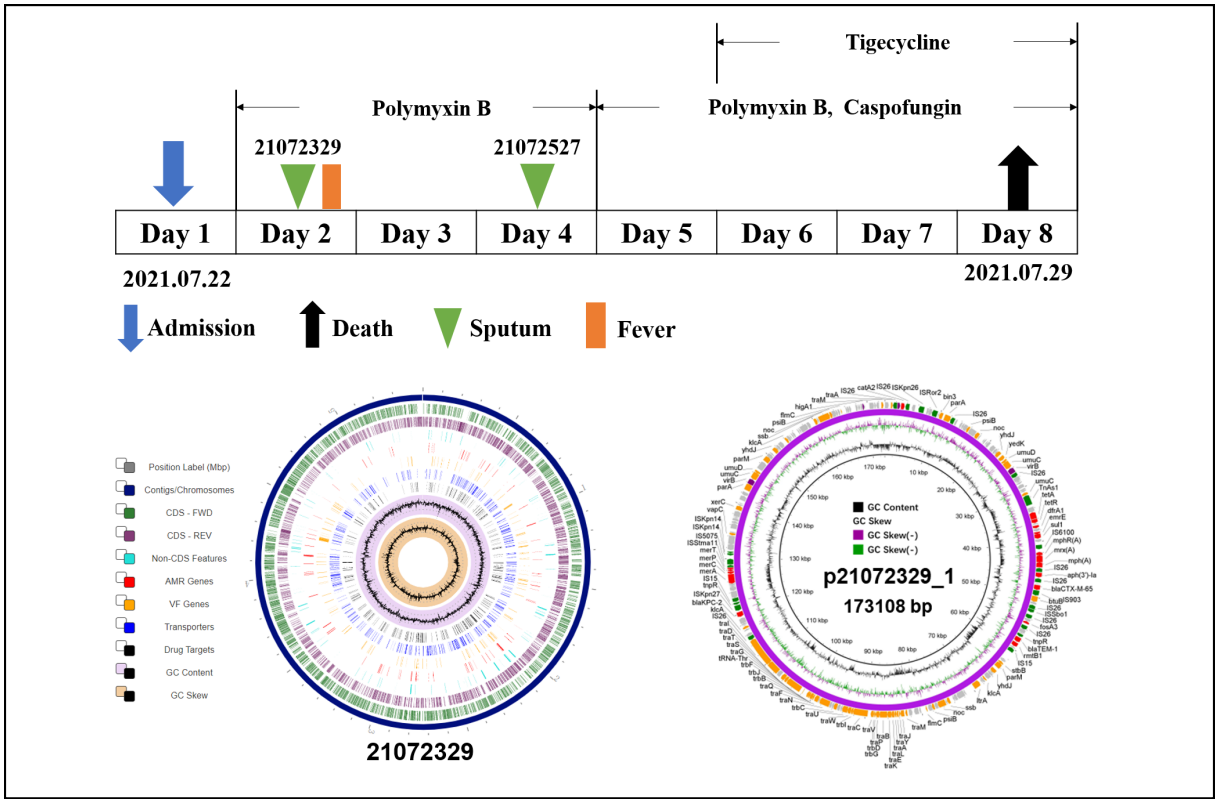
Graphical abstract



Multidrug-resistant hypervirulence Klebsiella pneumoniae 21072329 without rmpA and rmpA2 isolated from the sputum of patients with chronic obstructive pulmonary disease.

Public summary

■ A fatal multidrug-resistant hypervirulent *K. pneumoniae*, 21072329, was reported.

■ Multidrug-resistant hypervirulent *K. pneumoniae* 21072329 without *rmpA* and *rmpA2*.

■ Multidrug-resistant hypervirulent *K. pneumoniae* 21072329 carried only one siderophore of yersiniabactin.

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An abnormal multidrug-resistant and hypervirulent *Klebsiella pneumoniae* clinical isolate without *rmpA* or *rmpA2*

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Abstract: *Klebsiella pneumoniae* is a notorious opportunistic pathogen, especially hypervirulent *K. pneumoniae* (hvKp). Fortunately, most classical hvKp strains are antibiotic-susceptible. However, in recent years, reports of multidrug-resistant hvKp (MDR-hvKp) have increased dramatically, threatening the health and safety of people worldwide. Here, we report the discovery of MDR-hvKp without *rmpA* and *rmpA2* in a 92-year-old patient with chronic obstructive pulmonary disease. The patient died on the eighth day of hospitalization. Phenotyping experiments and whole-genome sequencing of *K. pneumoniae* isolate 21072329 isolated from the patient's sputum were performed. Moreover, 21072329 belongs to ST11-KL47 MDR-hvKp, which was highly lethal to *Galleria mellonella*. Meanwhile, 21072329 had a strong viscosity, and it was difficult to completely centrifuge it; 21072329 carried ESBL genes ($bla_{\text{CTX-M-65}}$, $bla_{\text{SHV-158}}$, and $bla_{\text{TEM-1}}$) and a carbapenemase gene ($bla_{\text{KPC-2}}$), and it was resistant to carbapenem antibiotics and third- and fourth-generation cephalosporins. Although 21072329 had the characteristics of hvKp, *rmpA* and *rmpA2* could not be found in its genome; it also only carried a siderophore of yersiniabactin. This may indicate that other hypervirulence factors promote the formation of hvKp. MDR-hvKp has already brought an enormous burden to global medical care, and those carrying unknown hypervirulence factors are new threats, so urgent prevention and control with research are urgently needed.

Keywords: multidrug-resistant and hypervirulent *Klebsiella pneumoniae* (MDR-hvKp); *Klebsiella pneumonia*; ST11; whole-genome sequencing

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1 Introduction

Hypervirulent *Klebsiella pneumoniae* (hvKp) was first reported in 1986[1]. It can infect healthy individuals, causing community-acquired liver abscesses[2, 3]. HvKp is generally hypermucoviscous, and colonies can be stretched by at least 5 mm[4], but this is not the only basis for judgment[5]. The identification of hvKp using the biomarkers *peg-344*, *rmpA*, and *rmpA2* appears to have a higher accuracy than using the hyperviscous phenotype[6]. The virulence determinants of hvKp are mainly the overproduction of capsules, extra siderophores, and a hypervirulence plasmid. Generally, the MLSTs of classical hvKp are ST23, ST65, and ST86, and the capsule types are KL1 and KL2[7].

Most hvKp are ST23-KL1, which is mostly reported in Asia and is sensitive to most antibiotics[8]. However, recently, there have been increasing reports of multidrug-resistant hvKp (MDR-hvKp), which are distributed worldwide[9, 10]. Classical hvKp can be treated with carbapenems or third-generation cephalosporins. However, most MDR-hvKps are resistant to these drugs and can only be treated with polymyxins or through combination antimicrobial susceptibility testing to select treatment methods. In addition, compared with classical hvKp, MDR-hvKp caused higher mortality[11, 12]. MDR-hvKp has become a major threat to global medical and health security, threatening the health of people worldwide.

2 Materials and methods

2.1 Ethics statement

Ethical approvals were obtained from the Ethics Committee of the University of Science and Technology of China (USTCACUC182301015). All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of the University of Science and Technology of China.

2.2 Bacterial strains, growth conditions and minimum inhibitory concentration

The isolates were plated on blood agar plates and incubated at 37 °C to isolate bacterial clones. The VITEK 2 Compact System (bioMérieux, France) was used to identify positive culture strains and determine the minimum inhibitory concentration (MIC) of isolates. NTUH-K2044 (classical hypervirulent *K. pneumoniae*) was used as a hypervirulent positive control,

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and classical *K. pneumoniae* (cKp) 19PDR22 was used as a negative control[13, 14].

2.3 Genomic analysis

Whole genome sequencing of *K. pneumoniae* was performed using a PacBio RS II and an Illumina HiSeq 4000 platform (Anhui, China). Four SMRT cell zero-mode waveguide arrays for sequencing were used with the PacBio platform to generate the subread set. PacBio subreads (length < 1 kb) were removed. The Pbdagcon program (https://github.com/PacificBiosciences/pbdagcon) was used for self-correction. Draft genomic unitigs, which are uncontested groups of fragments, were assembled using the Celera Assembler against a high-quality corrected circular consensus sequence subread set. To improve the accuracy of the genomic sequences, GATK (https://www.broadinstitute.org/gatk/) and SOAP tool packages (SOAP2, SOAPsnp, SOAPindel) were used to make single-base corrections. De novo hybrid assembly of short Illumina reads and long PacBio reads was performed using Unicycler v0.4.8 and SPAdes 3.0.0[15, 16]. Annotation was performed using Prokka[17]. Plasmid maps were drawn using BRIG[18]. Acquired antimicrobial resistance genes (ARGs) were identified using ABRicate 1.0.1 (https://github.com/tseemann/abricate) by aligning the genomic sequences to the ResFinder database and NCBI database[19]. The virulence factors were identified using Kleborate[20].

2.4 Nucleotide sequence accession number

The whole-genome sequence of isolates in this study has been submitted to the GenBank nucleotide sequence database under BioProject PRJNA823907.

3 Results

3.1 Case report

In July 2021, a 92-year-old male with cognitive decline and cough with sputum production for six years was admitted to a local tertiary hospital in China for treatment (Fig. 1a). The patient had a five-year history of chronic obstructive pulmonary disease and had been bedridden for the past two years. On admission, the patient had cough, expectoration, coarse breath sounds in both lungs, dry and wet rales, no fever, no abdominal distension, normal bowel movements, no obvious skin petechiae, and a heart rate of 74 beats/min. The initial diagnosis was vascular dementia, a chronic obstructive pulmonary disease with acute lower respiratory tract infection, multiple cerebral infarcts, and type 2 diabetes. A day after

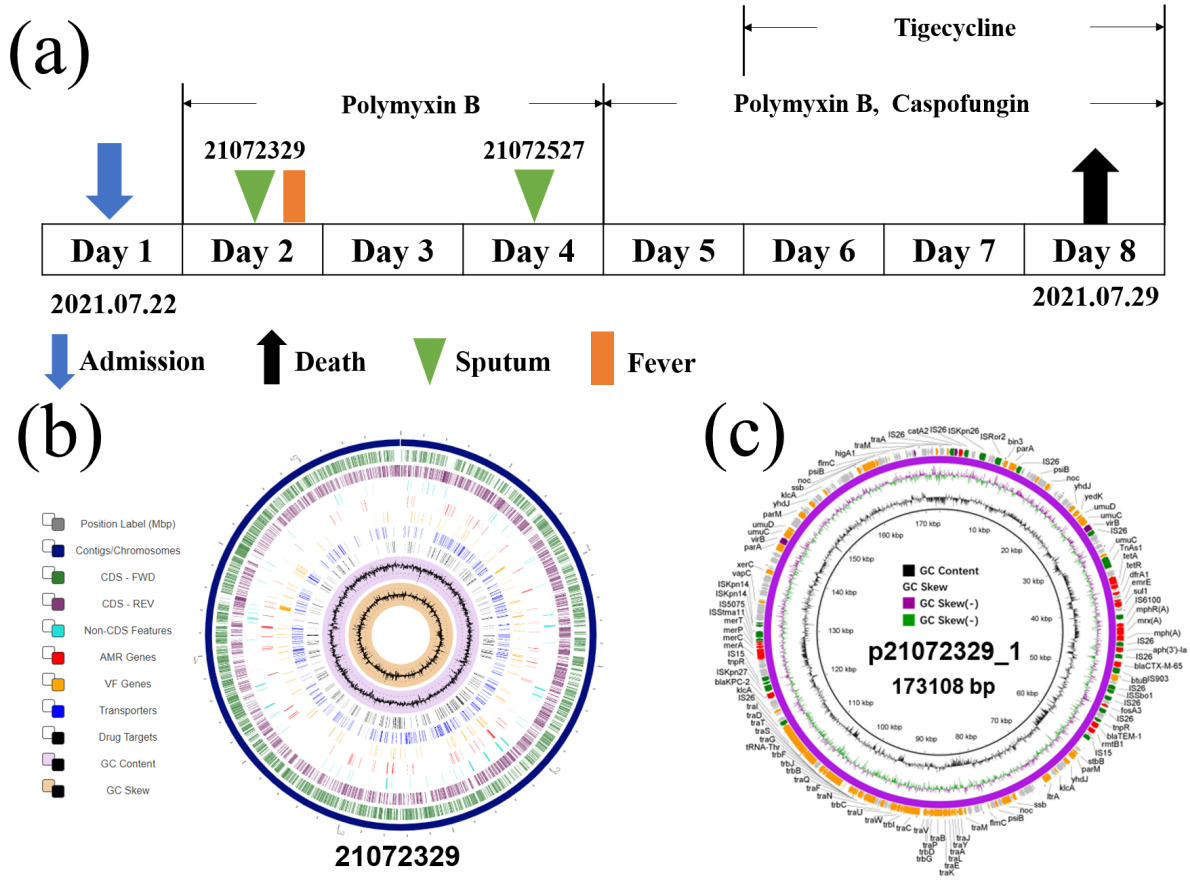

Fig. 1. Clinical data of the patient infected with 21072329 and genetic features of the genome and ESBL plasmid (p21072329_1) of *K. pneumoniae* isolate 21072329. (a) Time and site of *K. pneumoniae* isolation from patients infected with 21072329 and antibiotic treatment. (b) Chromosome map of 21072329. Different types of functional genes are indicated by different colors. (c) Plasmid map of p21072329_2 using a BLAST Ring Image Generator. Drug resistance genes are indicated by red, transposons are indicated by green, virulence factors are indicated by purple, genes of unknown function are indicated by gray, and other genes are indicated by orange.

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admission, the patient developed fever (body temperature, 38 °C), and his sputum culture was positive for *K. pneumoniae*. Drug susceptibility testing showed that it was resistant to carbapenems and third-generation and fourth-generation cephalosporins, but it was sensitive to polymyxin B and tigecycline. Polymyxin B (25 mg/dL) was administered for treatment. During hospitalization, the patient had repeated infections and frequent fever, and the anti-infection effect of polymyxin B was poor. It was considered that severe pneumonia may be accompanied by a fungal infection. On the fifth day after admission, polymyxin B was administered in combination with caspofungin for anti-infection treatment. However, no fungi were cultured from the patients' urine and sputum for five days. On the sixth day after admission, tigecycline was administered; however, due to the patient's blood pressure, pulse oxygen instability, respiratory failure, and critical condition, he was considered to be transferred to the ICU for emergency treatment, but his family refused. Despite the best efforts of the medical staff, the patient died in the early morning of the eighth day of admission.

3.2 Phenotype of *K. pneumoniae* isolates

Two *K. pneumoniae* isolates were isolated from the patient's sputum: 21072329[21], ST11-KL47, and 21072527, ST1883-KL47. Both isolates were resistant to 14 antibiotics (ceftriaxone, amoxicillin/clavulanic acid, amikacin, ceftazidime, cefoperazone/sulbactam, cefuroxime axetil, cefuroxime, ertapenem, cefepime, cefoxitin, imipenem, levofloxacin, trimethoprim/sulfamethoxazole, and piperacillin/tazobactam) based on susceptibility tests and were only susceptible to ceftazidime/avibactam, polymyxin B, and tigecycline (Table 1). 21072329 was isolated on the second day after the patient was admitted to the hospital, and 21072527 was isolated on the fourth day after the patient was admitted to the hospital. Although the two isolates had different MLST types, they shared the same capsular type. Meanwhile, among the seven genes used for MLST in 21072329 and 21072527, only *rpoB* had a mutation (the adenine (A) at position 1657 was mutated to cytosine (C)). This suggested that the genetic backgrounds of the two isolates were highly similar. Of these, we specifically focused on 21072329, which exhibited a hyperviscous phenotype. We performed India ink staining, a *Galleria mellonella* infection model, a microviscosity assay, and a growth curve for 21072329 using NTUH-K2044 and 19PDR22 (ST11-KL47) as controls. The staining results showed that NTUH-K2044 had the thickest capsule, followed by 21072329. The capsule of 19PDR22 was the thinnest (Fig. S1). The survival rate of *G. mellonella* at three different bacterial concentrations was tested (Fig. 2a–c). At all three concentrations, the lethality of 21072329 to *G. mellonella* was much higher than that of the other two strains. The OD_{600} of the overnight bacterial solution was adjusted to 1 OD_{600}, 1 mL, 2 OD_{600}, 1 mL, and 3 OD_{600}, 1 mL, and it was centrifuged at 2350 g for 5 min. NTUH-K2044 and 19PDR22 were completely centrifuged after 3 min, and the supernatant was completely transparent (Fig. 2d–f). Moreover, 21072329 was difficult to centrifuge down (Fig. S2). After the OD_{600} of 21072329 was adjusted to 3, the supernatant of 21072329 remained opaque even after 5 min of centrifugation (Fig. 2d–f, and Fig. S1). In LB medium, NTUH-K2044 had the fastest growth rate, while 19PDR22 and 21072329 had similar growth rates (Fig. S3). All indications showed that 21072329 had the hypervirulence characteristics of hvKp.

Table 1. Minimum inhibitory concentration of different antibiotics on isolates 21072329 and 21072527.

Antibiotics (μg/mL)	21072329	21072527
Ceftriaxone	64 (R)	64 (R)
Amoxicillin/Clavulanic acid	32 (R)	32 (R)
Amikacin	64 (R)	64 (R)
Ceftazidime	64 (R)	64 (R)
Cefoperazone/Sulbactam	64 (R)	64 (R)
Cefuroxime axetil	64 (R)	64 (R)
Cefuroxime	64 (R)	64 (R)
Ertapenem	8 (R)	8 (R)
Cefepime	32 (R)	32 (R)
Cefoxitin	64 (R)	64 (R)
Imipenem	16 (R)	16 (R)
Levofloxacin	8 (R)	8 (R)
Trimethoprim/Sulfamethoxazole	320 (R)	320 (R)
Piperacillin/Tazobactam	128 (R)	128 (R)
Polymyxin B	0.5 (S)	0.5 (S)
Tigecycline	0.5 (S)	0.5 (S)

Hospital categorization: R, resistant; S, susceptible.

3.3 Genomic analysis and phylogeny

Whole-genome sequencing results showed that the chromosome size of 21072329 was 5,450,821 bp, the GC content was 57.43%, and there were 5,544 coding sequences, 87 tRNAs, and 25 rRNAs (Fig. 1b). Five plasmids, 17 drug-resistance genes, and 21 virulence genes were detected in 21072329. IncR plasmid p21072329_1 had 173,108 bp and carried ESBL genes ($bla_{CTX\text{-}M\text{-}65}$, $bla_{SHV\text{-}158}$, and $bla_{TEM\text{-}1}$), a carbapenemase gene ($bla_{KPC\text{-}2}$), other drug-resistance genes (*aph(3')-Ia*, *catA2*, *dfrA1*, *fosA3*, *mph(A)*, *rmtB1*, *sul1*, and *tet(A)*), and heavy metal tolerance genes (mercury tolerance gene *merACPT*) (Fig. 1c). The IncFII plasmid p21072329_2 (58,972 bp) carried the aminoglycoside antibiotic resistance gene aac(3)-IId and arsenic resistance gene *arsABCD* (Fig. S4a). The plasmid p21072329_3 (40,479 bp) carried *lexA* and phage protein-encoding genes; it had 100% homology and coverage with the plasmid pMTY12128 from Japan (Fig. S4b). The ColRNAI plasmid p21072329_4 (10,060 bp) carried genes encoding toxin proteins and lysis proteins (Fig. S4c). The ColRNAI plasmid (5,596 bp) p21072329_5 carried the *mbeBD*, *mobC*, and toxin protein-encoding genes (Fig. S4d). The drug-resistance genes *aadA2*, $bla_{SHV\text{-}158}$, *fosA6*, and *oqxAB* were encoded on chromosomes. hvKp

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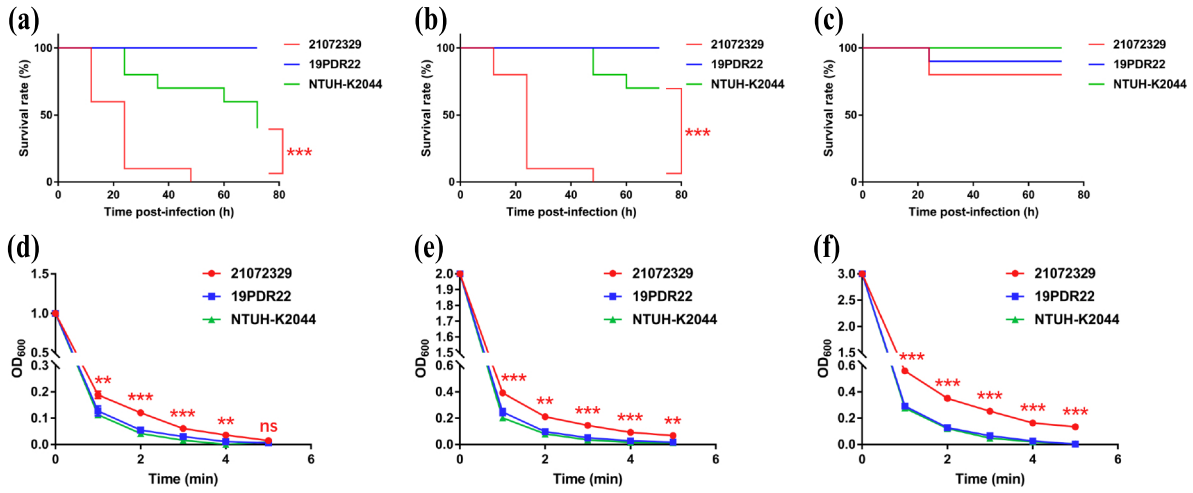

Fig. 2. *K. pneumoniae* isolate 21072329 showed high virulence and high visibility. The *G. mellonella* infection model was used to evaluate the virulence of strains at three different concentrations (1×10^6 CFU: (a); 1×10^5 CFU: (b); 1×10^4 CFU: (c)). Their viscosity was evaluated by measuring the OD_{600} of the supernatant after centrifugation (initial OD_{600} adjusted to 1: (d); initial OD_{600} adjusted to 2: (e); initial OD_{600} adjusted to 3: (f)). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ns: no significant difference.

generally carried at least three kinds of siderophores, *rmpA* and *rmpA2*, which are important factors for the hypervirulence production of hvKp. Surprisingly, genes encoding yersiniabactin were found on chromosome 21072329 (*ybtS*, *ybtX*, *ybtQ*, *ybtP*, *ybtA*, *irp2*, *irp1*, *ybtU*, *ybtT*, *ybtE*, *fyuA*), but other siderophore-encoding genes were not found. Meanwhile, *rmpA*, *rmpC*, *rmpD*, and *rmpA2* were also not detected. These phenomena did not explain at the genomic level how 21072329 exhibited hypervirulence characteristics similar to other hvKp. This may suggest the existence of other hypervirulence factors that lead to the occurrence of hvKp.

4 Discussion

Compared with cKp, hvKp had stronger virulence, stronger viscosity, and higher lethality. Fortunately, the MLST of most hvKp was ST23, which was sensitive to antibiotics[2]. However, with the continuous spread of hvKp strains and hypervirulence plasmids worldwide, the MLST of hvKp also showed a trend of diversification. The common features of these hvKp strains were *rmpA*, *rmpA2*, and various siderophores. *rmpA* and *rmpA2* can enhance the expression of capsules, while siderophores confer the ability of strains to colonize various parts of the host. However, recently, a hospital in Sudan reported an MDR-hvKp isolate that did not carry *rmpA*, *rmpA2*, or siderophores[22]. There may also be other factors that contribute to hvKp formation.

In this study, we describe an ST11-KL47 MDR-hvKp isolate, 21072329, with a hypervirulent phenotype of hvKp and lacking the siderophores *rmpA* and *rmpA2*. Despite the older age of infected patients, this isolate is quite threatening because 21072329 was resistant to 14 antibiotics. It carried five plasmids and included the ESBL plasmid p21072329_1 (carried $bla_{CTX\text{-}M\text{-}65}$, $bla_{SHV\text{-}158}$, $bla_{TEM\text{-}1}$, and $bla_{KPC\text{-}2}$). Although it was sensitive to polymyxin B and tigecycline, their clinical therapeutic effects were not ideal. 21072329 carried only one siderophore and did not carry the hvKp markers *rmpA* and *rmpA2* but still exhibited stronger virulence than NTUH-K2044. This suggests that in addition to *rmpA*, *rmpA2* and siderophores, there may be virulence factors that can determine the hypervirulent phenotype of *K. pneumoniae*.

Another *K. pneumoniae* isolate, 21072527, was also isolated the day after 21072329 was isolated. It was identical to the capsular type of 21072329, and it differed from the MLST of 21072329 by only one allele. Meanwhile, it had the same drug resistance spectrum as 21072329. This may suggest that 21072329 and 21072527 have high homology and similar genetic backgrounds. Since 21072527 was isolated after 21072329, we speculated that the capsular polysaccharide synthesis gene cluster of 21072329 may have mutations that lead to reduced viscosity, thus deriving 21072527. However, the whole genome of 21072527 was not sequenced in this study, and the evolutionary developmental relationship between the two isiolates cannot be determined.

5 Conclusions

In conclusion, we identified an hvKp isolate that did not carry biomarkers of hypervirulence and characterized the isolate epidemiologically and genomically. Although we have not identified the cause of the hypervirulent phenotype of this isolate, our results laid the foundation for further in-depth analysis of this isolate and proved that *rmpA* and *rmpA2* are not the only hvKp markers.

Availability of data and materials

The whole-genome sequences generated in the current study are available in the NCBI database (BioProject: PRJNA823907). https://www.ncbi.nlm.nih.gov/bioproject/PRJNA823907.

Supporting information

The supporting information for this article can be found online at https:// doi.org/10.52396/JUSTC-2023-0085.

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Conflict of interest

The authors declare that they have no conflict of interest.

Biographies

Zhien He is currently a doctoral candidate in the Division of Life Sciences and Medicine, University of Science and Technology of China, under the supervision of Prof. Baolin Sun. His research mainly focuses on the epidemiology of hypervirulent *Klebsiella pneumoniae* and *Acinetobacter baumannii*.

Baolin Sun is a Professor at the University of Science and Technology of China. He received his Ph.D. degree from Michigan State University in 1999. In 2004, he was selected into the Hundred Talents Program of the Chinese Academy of Sciences. His research interests include the expression regulation of virulence genes of pathogens, the regulatory mechanisms of the occurrence and transfer of bacterial resistance, and the interaction mechanisms between pathogens and hosts.

Yujie Li is currently an Associate Research Fellow at the University of Science and Technology of China. He received his Ph.D. degree in Microbiology from Southwest University in 2020 under the supervision of Prof. Yan Pei. He then joined Prof. Baolin Sun's group as a postdoctoral fellow in July 2020. His scientific interests include the bacterial drug resistance and pathogenicity and bacteria-host interaction.

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