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ORIGINAL RESEARCH

Molecular Characterization and Risk Factors of Carbapenem-Resistant Hypervirulent *Klebsiella pneumoniae* Isolated from Chinese Tertiary Hospital

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Background: Therefore, the objectives of this study were to investigate the prevalence of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* (CR-hvKp) in Fujian Medical University Union Hospital, identify their genetic characters, characterize their resistance profiles, and identify risk factors for their infection to improve prevention and treatment strategies for CR-hvKp in the area.
Methods: Between January 2021 and January 2022, clinically identified carbapenem-resistant *Klebsiella pneumoniae* (CRKp) isolates were collected. A PCR assay was used to detect the K capsule type, virulence genes, carbapenemase genes, and membrane pore protein. ERIC-PCR was carried out for homology analysis. Antimicrobial susceptibility test was used to determine drug resistance. Logistic multivariate regression analysis was conducted to confirm the risk factors for CR-hvKp infection.
Results: In total, 239 CRKp isolates were obtained. The virulence genes with the highest detection rates were *mrkD, iucA*, and *rmpA2*. Of these isolates, 54 (22.59%) carried both *iucA* and *rmpA2*, thus classifying them as CR-hvKp. All CR-hvKp isolates carried bla_{KPC}. Furthermore, capsular serotypes K64 (94.44%) and K47 (3.70%) were detected. Resistance was observed against most common antibiotics, with the exception of complete sensitivity to ceftazidime-avibactam. ERIC-PCR indicated a potential clonal spread among CR-hvKp. Multivariate analysis found that changing beds was a risk factor for CR-hvKp infection.
Conclusion: Currently, the hospital predominantly carries K64 CR-hvKp that harbors the bla_{KPC}. Our study found that changing patient beds was an independent risk factor for CR-hvKp infection.
Keywords: carbapenem-resistant, hypervirulent, *Klebsiella pneumoniae*, virulence, resistance, risk factor

Introduction

Klebsiella pneumoniae (Kp) is a major pathogen that causes infections acquired in the community and in hospitals,[1] such as urinary tract infections, pneumonia, and meningitis.[2] Carbapenem-resistant *Klebsiella pneumoniae* (CRKp) strains have emerged due to the extensive use of antimicrobial agents, with the acquisition of mobile genetic elements carrying different antimicrobial resistance genes being a significant factor in the evolution of Kp into CRKp.[3] According to the China Antimicrobial Surveillance Network (CHINET), the resistance rate of carbapenems in Kp has significantly increased from 2.9% to 25.3%.[1] In addition to the issue of antimicrobial resistance, the emergence and progression of hypervirulent *Klebsiella pneumoniae* (hvKp) is also a matter of significant concern.

Since its initial discovery in Taiwan in 1986,[4] hvKp has garnered increasing attention, with studies indicating that pLVPK/pLVPK-like virulent plasmids play a significant role in its pathogenicity.[5] The connection between the

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hypervirulent phenotype and the virulence plasmid has been established in previous research.[6] The emergence of drug-resistant plasmids and virulence plasmids has led to the development of a highly resistant and difficult-to-treat "superbug", whether it acquires a low-virulence CRKp with a virulence plasmid or a highly sensitive hvKp with a drug-resistant plasmid.[7]

Since its initial report in China in 2018, the occurrence of CR-hvKp bacteria harboring pLVPK-like virulence plasmids has been documented in multiple countries in recent years.[8–11] Danxia Gu et al[12] found that CRKp can evolve into CR-hvKp by acquiring pLVPK/pLVPK-like virulence plasmids. These plasmids enable the rapid dissemination of hypervirulence and enhance environmental survival.[11,13] Currently, the main virulence factors on these plasmids are *iucA, peg-344, iroN, rmpA* and *rmpA2*.[14] This study primarily identified hvKp strains using the virulence genes *iucA* and *rmpA2* on pLVPK-like virulence plasmids.

CRKp poses a significant challenge in terms of treatment, while hvKp is associated with a high mortality rate. As we all know, the isolates of *Klebsiella pneumoniae* that have both high virulence and carbapenem resistance are called "superbugs", and CR-hvKp has the characteristics of multi-drug resistance, high virulence and high transmission, which will pose a serious threat to human health.[15] CR-hvKp is a newly reported nosocomial pathogen, which was first reported and mainly reported in China.[12] Currently, the scarcity of research on CR-hvKp in China can be attributed to its low detection rate, with the infection rate ranging from 0% to 25.8%.[13] Relevant studies have shown that there are significant regional differences in CR-hvKp prevalence. This study analyzed the distribution of CR-hvKp in six regions of Zhejiang, Jiangsu, Beijing, Henan, Shandong and Hebei, among which Zhejiang and Jiangsu accounted for the largest proportion, accounting for half of the six regions.[15] Regional differences might also exist in the risk factors and molecular epidemiological characteristics of CR-hvKp. Earlier studies indicated that cardiovascular and cerebrovascular diseases were independent predictors of CR-hvKp infections.[7,16] Nonetheless, research on the risk factors and molecular epidemiology of CR-hvKp in this region of Fujian is insufficient. Therefore, this study aimed to analyze CR-hvKp strains isolated from Fujian Medical University Union Hospital to reveal their clinical characteristics, assess their resistance, and identify risk factors for their infection, it is crucial to investigate these factors to improve prevention and treatment strategies for CR-hvKp in the area.

Methods

Research Setting and Ethics Statement

The study was conducted from January 2021 to January 2022 at Fujian Medical University Union Hospital. The cultured isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS; Bruker Daltonics Inc., Billerica, Massachusetts) to confirm strains of Kp. The disk diffusion method was used to perform a carbapenem antimicrobial susceptibility test and classify Kp as CRKp if it was resistant to meropenem or imipenem.[17] A total of 239 unique strains were collected. The collected CRKp strains were divided into experimental group (CR-hvKp group) and control group (CRKp group) for study. The medical details such as age, gender, admission date, number of beds, surgeries and invasive procedures, and antibiotic treatments were collected during the patient's stay. Each case or control was included only once. Informed consent was obtained from all participants.

String Test

The strains were left to incubate overnight on blood agar plates at 37 °C. Subsequently, fresh colonies that had been cultured overnight were carefully pulled outward with the inoculation ring. If mucus filaments were detected and traction length was greater than 5 mm, the phenotype was recognized as mucosal hyperplasia, with a focus on the hypermucous phenotype.[7]

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test employed microbroth dilution to ascertain the minimum inhibitory concentration (MIC) of various antibiotics, including ceftazidime-avibactam, amoxicillin/clavulanate, piperacillin/tazobactam, cefazolin, ceftriaxone, cefepime, cefoxitin, aztreonam, imipenem, amikacin, gentamicin, tobramycin, ciprofloxacin,

levofloxacin and trimethoprim-sulfamethoxazole. The interpretation of the results was based on the 2022 Clinical and Laboratory Standards Institute (CLSI 2022) guidelines. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were utilized as quality control standards.

Molecular Detection of Resistance and Virulence Genes

The polymerase chain reaction (PCR) was employed to identify specific genes associated with the type K capsule (K1, K2, K47, K57, and K64) and virulence (*mrkD, iucA, rmpA2, rmpA, allS, iroN, wcaG, magA*, and *aerobactin*). Additionally, resistance genes were detected, including carbapenemase genes (bla_{KPC}, bla_{IMP}, bla_{VIM}, bla_{NDM}, and bla_{OXA-48}) as well as resistance-related genes include membrane pore protein genes (*OmpK35, OmpK36*, and *OmpK37*). The amplification conditions included a 2-minute initial denaturation at 95°C, followed by 35 cycles of 95°C for 20 seconds, 52°C for 40 seconds and 72°C for 1 minute with a single final extension cycle at 72°C for 5 minutes. The primers used for this analysis can be found in Supplementary Data: Table S1.

DNA Fingerprint Technology

DNA templates for the ERIC-PCR assay were extracted by the boiling centrifugation method.[18] Universal primers ERIC-F (5′-ATGTAAGCTCCTGGGGATTAC-3′) and ERIC-R (5′-AAGTAAGTGACTGGGGTGAGCG-3′) were used to amplify DNA. The ERIC- PCR conditions were modified based on the findings reported by Smith et al.[19] The DNA fingerprint was examined using Image Lab software, while clustering was conducted using NTSYSpc 2.10 software, with clusters being defined using a cutoff value of 92% similarity. The band-matched Dice coefficient was employed to assess the similarity between species, and the tree map for each species was generated using the unweighted arithmetic mean pairing group method (UPGMA).

Statistical Analysis

Data were analyzed using IBM SPSS ver. 21.0 statistical software (IBM Co., Armonk, NY, USA). Frequency tables (n, %) for categorical variables and descriptive statistics (mean, median, standard deviation) for numerical variables were used. Comparisons of categorical variables were analyzed by the Chi square test. Logistic regression (Backward LR) methods (univariate, multivariate) were used to determine the risk factors for CR-hvKp infection. Statistical significance was assigned to a P value of less than 0.05.

Results

Molecular Characteristics of CRKp and CR-hvKp

Among the 239 CRKp strains, 91.18% (218/239) carried the bla_{KPC} gene, 5.88% (14/239) had the bla_{NDM} gene, 0.42% (10/239) contained the bla_{IMP} gene, and neither bla_{OXA-48} nor bla_{VIM} was detected (Figure 1A). The presence of porin genes was as follows: *OmpK35* in 96.65% (231/239), *OmpK36* in 98.32% (235/239), and *OmpK37* in 99.16% (237/239) (Figure 1B). Among the virulence genes, *mrkD* was the most commonly carried (96.65%, 231/239), with *iucA* (27.20%, 65/239), *rmpA2* (23.01%, 55/239), *allS* (2.09%, 5/239), *rmpA* (1.67%, 4/239), and *iroN* (0.42%, 1/239) being less frequent (Figure 1C). Out of these, 54 strains that contained both *iucA* and *rmpA2* were classified as CR-hvKp. The predominant serotype was K64 (94.44%, 51/54), followed by K47 (3.70%, 2/54). None of the strains showed porin deficiency (Figure 1D). All CR-hvKps contained the carbapenemase bla_{KPC} (100%) (Figure 1D). Three strains tested positive in the string test.

Distribution of the CR-hvKp and the Ratio of Specimen Types

With a separation rate of 22.59%, CR-hvKp cases were primarily found in the ICU (44.44%, 24/54), with other cases in hematology (11.11%, 6/54), emergency surgery (11.11%, 6/54), burns (9.26%, 5/54), gastric surgery (9.26%, 5/54), neurosurgery (5.56%, 3/54), basic surgery (5.56%, 3/54), and other departments (3.70%, 2/54) (Figure 2A). In the 54 CR-hvKp cases, 46.30% (25/54) were retrieved from sputum, 11.11% (6/54) from drainage fluid, 11.11% (6/54) from blood, 9.26% (5/54) from secretion, 7.41% (4/54) from urine, 3.70% (2/54) from pus, 3.70% (2/54) from perianal swabs, and 7.41% (4/54) from other sources (Figure 2B).

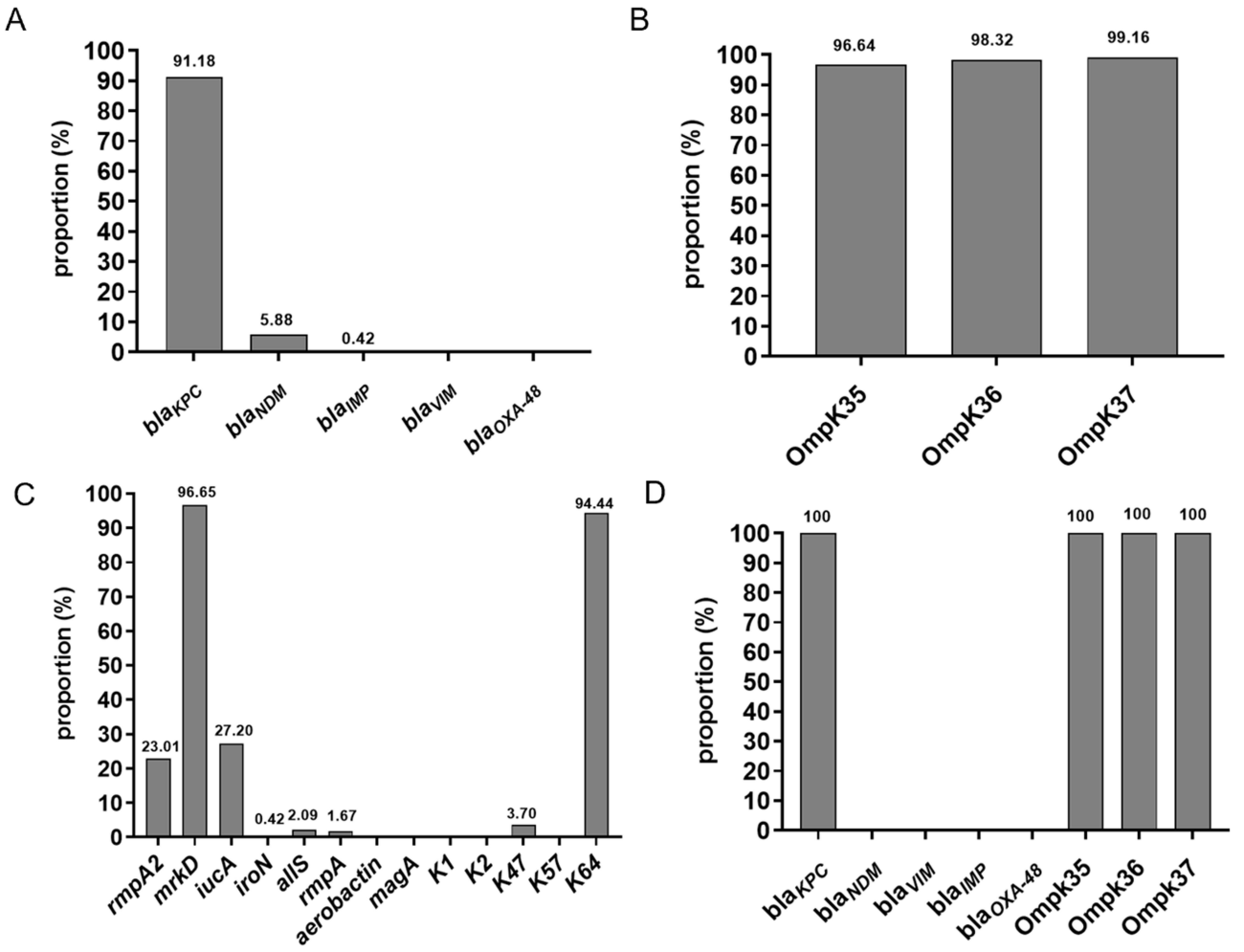

Figure 1 Molecular characteristics of clinical isolates were detected by PCR. (**A**) Carbapenemase genes in carbapenem-resistant *Klebsiella pneumoniae* isolates. (**B**) Porin genes in carbapenem-resistant *Klebsiella pneumoniae* isolates. (**C**) Virulence factors in carbapenem-resistant *Klebsiella pneumoniae* isolates. (**D**) Carbapenemase genes and porin genes in carbapenem-resistant hypervirulent *Klebsiella pneumoniae* isolates.

Antimicrobial Resistance Characteristics

All isolates were resistant to piperacillin/tazobactam, cefazolin, ceftriaxone, cefepime, cefoxitin, aztreonam, imipenem, ciprofloxacin, and levofloxacin. Ceftazidime-avibactam was effective against all 54 CR-hvKp isolates, and 51.90% (28/54) were susceptible to trimethoprim/sulfamethoxazole. The resistance rates for amikacin, gentamicin, and tobramycin were 90.90% (49/54), 90.90% (49/54), and 92.50% (50/54), respectively (Figure 3).

ERIC-PCR DNA Fingerprint Analysis of CR-hvKp

Using NTSYSpc software, a dendrogram was developed to organize all CR-hvKp isolates based on their ERIC-PCR patterns. The isolates generated multiple amplicons with similarity levels from 68% to 100%, forming 14 major clusters (I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV). Most of the isolates were located in clusters VIII (27.78%, 15/54) and XII (24.07%, 13/54) (Figure 4). For specific electrophoretic images, see Supplementary Data: Figure S1.

Analysis of Risk Factors Associated with CR-hvKp Infection

Among those infected with CR-hvKp, adults represented a significant majority (98.15%, 53/54), with men being the predominant gender (77.78%, 42/54). Gastrointestinal diseases (29.63%, 16/54) and lung diseases (22.22%, 12/54) were the most frequently observed conditions (Table 1). Univariate analysis identified antibiotic use history and bed changing

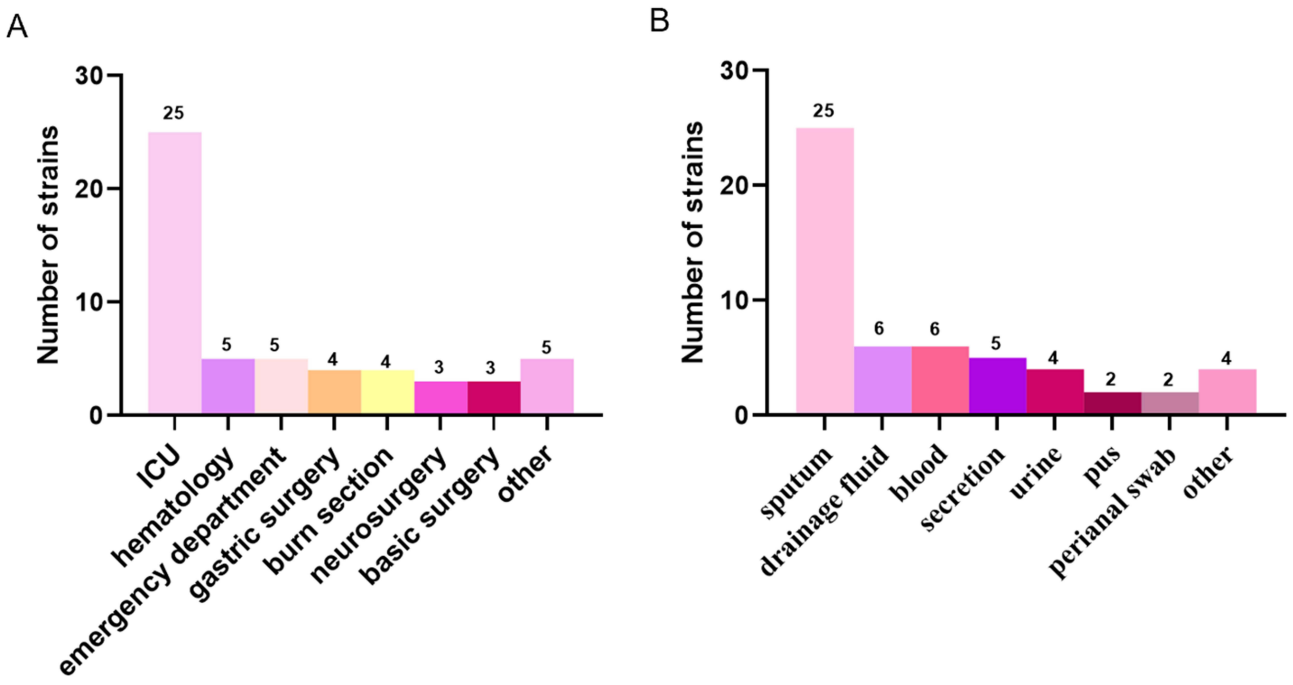

Figure 2 Clinical distribution (**A**) and specimen types (**B**) of carbapenem-resistant hypervirulent *Klebsiella pneumoniae*.

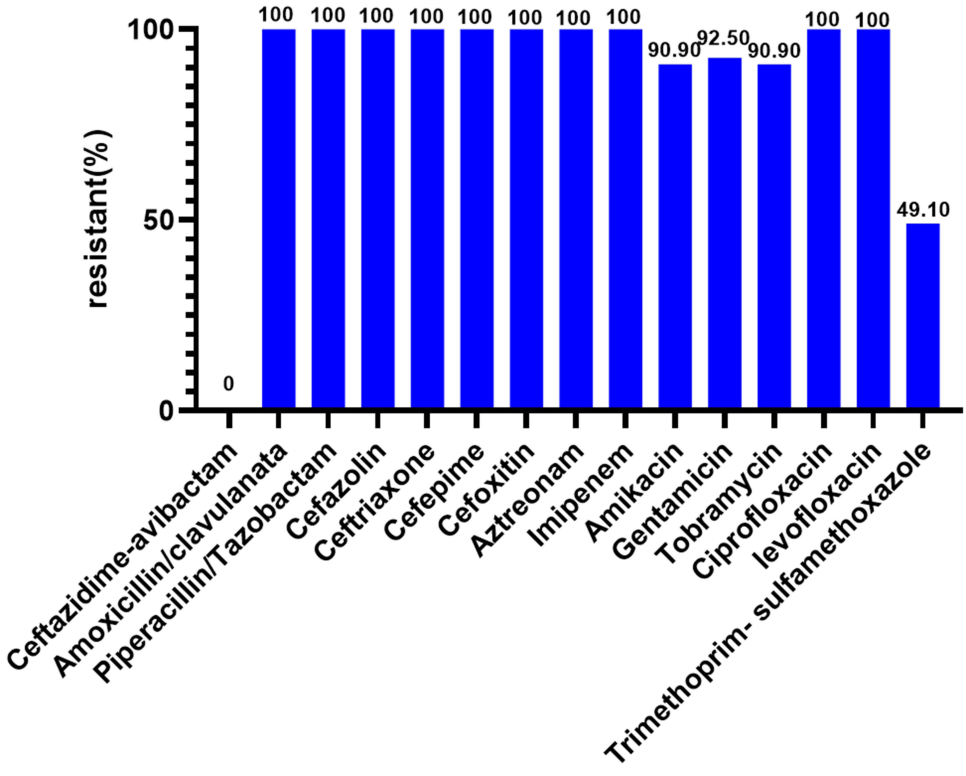

Figure 3 Resistant rate of carbapenem-resistant hypervirulent *Klebsiella pneumoniae*.

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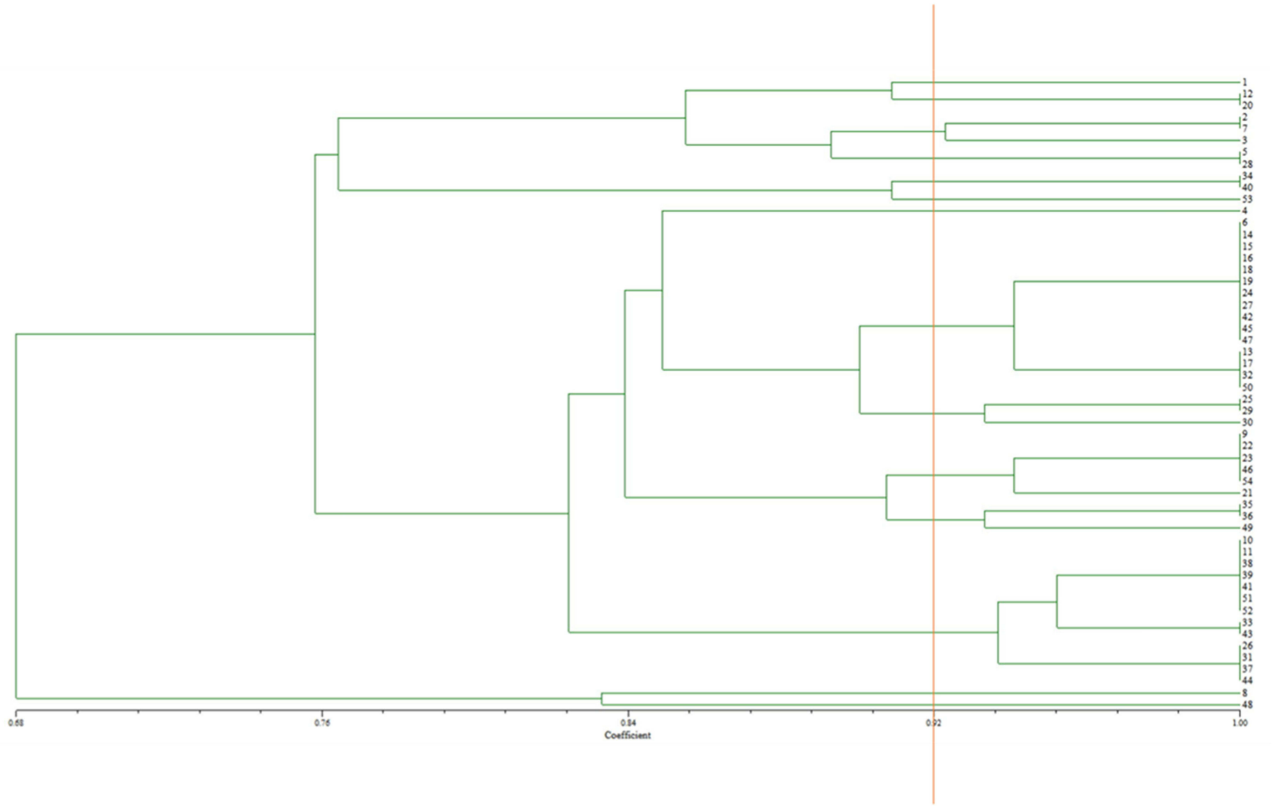

Figure 4 ERIC-PCR dendrogram of all carbapenem-resistant hypervirulent *Klebsiella pneumoniae* strains.

experience as risk factors for CR-hvKp infection. Multivariate analysis confirmed bed changing as a significant risk factor (odds ratio [OR] 4.538; 95% confidence interval [CI]: 1.664–12.377; $P<0.05$) (Table 2).

Discussion

The increased occurrence of CRKp and hvKp might contribute to the development of organisms that are both resistant to carbapenem antibiotics and highly virulent.[9,20] This study investigated the molecular epidemiology of CR-hvKp and its associated risk factors among clinically isolated strains obtained from a tertiary hospital in Fujian.

Out of 239 CRKp strains, those testing positive for both *iucA* and *rmpA2* via PCR were identified as hypervirulent *Klebsiella pneumoniae*.[12] In this study, 54 strains of CR-hvKp were identified. Previous research indicated that the severity of hvKp was associated with its virulence-related genes and hypermucous phenotype.[21] *RmpA* and *rmpA2* were identified as regulators necessary for the hypermucous phenotype in hvKp strains.[22] Only 3 out of 54 CR-hvKp isolates were hypermucous, implying that the *rmpA*/*rmpA2* genes do not always correlate with capsule expression. Previous studies had shown that K1 and K2 capsule serotypes were frequently linked to enhanced virulence, and correspondingly, the most prevalent serotypes in hvKp were K1 and K2.[23] In this research, K64 was the predominant serotype of CR-hvKp, followed by K47, with no K1/K2 serotypes detected, suggesting a difference in the main serotypes between CR-hvKp and hvKp. Consequently, it was assumed that their virulence could also fluctuate. Importantly, K64 Kp has been associated with suppurative liver abscesses and endogenous endophthalmitis,[24] and ST11-K64 clones were found to independently predict CR-hvKp infection,[16] aligning with our experimental findings. The majority of virulence-related genes were found on pLVPK or pK2044-like virulence plasmids.[25] Some investigations, however, revealed that K47 CR-hvKp contained a plasmid similar to the rare pLVPK-like plasmid in CR-hvKp, highlighting the importance of sending the discovered K47 CR-hvKp for whole genome sequencing.[26]

Carbapenem resistance in Kp was primarily caused by carbapenemase production and reduced expression or loss of outer membrane proteins, with carbapenemase being the key mechanism.[27] Carbapenemase were categorized into three

Table 1 Demographic Characteristics of CR-hvKp

No. of Cases (n=54)	CR-hvKp (n=54)
Age	
Pediatric	1 (1.85%)
Adult	53 (98.15%)
Sex	
Male	42 (77.78%)
Female	12 (22.22%)
Diagnosis	
Leukemia	7 (12.96%)
Gastrointestinal disease	16 (29.63%)
Pulmonary disease	12 (22.22%)
Bone and joint diseases	1 (1.85%)
Heart disease	1 (1.85%)
Endocrine disease	2 (3.70%)
Cerebral disease	9 (16.67%)
Sepsis	3 (5.56%)
Burn	3 (5.56%)
Discharge	
Yes	30 (55.56%)
No	24 (44.44%)
Operation	
Yes	46 (85.19%)
No	8 (14.81%)

Table 2 Univariate Analysis and Multivariate Logistic Regression Analysis of Risk Factors for CRKp Vs CR-hvKp Infection

Variable	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Sex	2.140 (0.864–5.302)	*P*>0.05		
Age	0.425 (0.168–1.073)	*P*>0.05		
Invasive procedures	3.146 (0.314–31.484)	*P*>0.05		
Antibiotic use	0.079 (0.010–0.648)	*P*<0.05	0.242 (0.040–1.444)	*P*>0.05
Hypertension	1.000 (0.414–2.415)	*P*>0.05		
Diabetes	1.629 (0.526–5.044)	*P*>0.05		
Hospital stays	1.441 (0.622–3.338)	*P*>0.05		
Bed change	4.459 (1.661–10.921)	*P*<0.05	4.538 (1.664–12.377)	*P*<0.05
Transfer	1.739 (0.745–4.059)	*P*>0.05		
Prognosis	1.369 (0.595–3.152)	*P*>0.05		

Abbreviations: *P*, test significance; OR, odds ratio; 95% CI, confidence interval.

groups: A, B and D according to the Ambler classification. The most prevalent carbapenemase, bla_{KPC}, bla_{NDM} and bla_{OXA-48}, were classified under groups A, B and D respectively.[28] In CRKp, bla_{KPC} was the most prevalent class A enzyme.[29] This study found that all 54 CR-hvKp strains had bla_{KPC} and retained their membrane proteins. Consequently, bla_{KPC} was identified as the primary reason for carbapenem resistance in this area, consistent with earlier studies.[30]

Sputum was the main specimen type for CR-hvKp strains, consistent with Kp's role as a respiratory pathogen.[31] Due to their critical condition, ICU patients often require lengthy hospitalizations and invasive surgeries, raising the risk of antibiotic resistance and increasing the likelihood of CR-hvKp detection.[32] During February, March, and April, 32 strains

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of CR-hvKp were identified (59.26%), suggesting that CR-hvKp might be more prevalent in the spring. Thus, it is crucial to improve management and screening for CR-hvKp in this season.

Research on 54 CR-hvKp isolates uncovered complete resistance to carbapenems, fluoroquinolones, and cephalosporin antibiotics, with only a low level of sensitivity to sulfonamides and aminoglycosides. The resistance rate of the CR-hvKp strain closely matches that of CRKp.[33] The treatment of the CR-hvKp strains may follow the treatment approach for CRKp strains infection. The treatment with ceftazidime-avibactam has been highly effective in China, the United States, and Europe, achieving high rates of clinical cures and survival.[34,35] This study showed that all 54 CR-hvKp strains were fully sensitive to ceftazidime-avibactam, suggesting that antibiotic usage is well managed.

The fingerprint results revealed that 54 strains of CR-hvKp had mostly similar strains, with the majority belonging to clusters VIII and XII. Cluster VIII isolates were spread out, but most inspections occurred in February and March, with 73.33% (11/15) of isolates having 100% similarity, indicating a possible epidemic of this clonal strain during that time. Twelve strains of cluster XII were found in the ICU, with inspections mainly conducted in April and May, indicating potential clonal transmission of CR-hvKps in this department.

According to the clinical data of CR-hvKp infected patients, 77.78% of CR-hvKp infected patients were male. In accordance with the colonization environment of *Klebsiella pneumoniae*, 51.85% of CR-hvKp infected patients primarily suffered from gastrointestinal and lung-related diseases. Some studies have reported that potential complications such as diabetes and chronic diseases were the main risk factors for CR-hvKp infection,[36] while in this study, only 9 patients (16.67%) in the CR-hvKp group were found to have diabetes, which is not a risk factor for CR-hvKp infection. Surprisingly, multivariate analysis revealed that bed change was a risk factor for CR-hvKp infection. There are three possible reasons for this phenomenon: First of all, this may be due to the fact that severely ill patients with weakened immunity often need to be transferred from the general ward to the ICU. If they are slightly better, they can be transferred from the ICU to the general ward, a process that increases the number of beds changes and makes them more susceptible to pathogens. Moreover, patients who often change beds may meet more patients, which could result in CR-hvKp cross-infection. Ultimately, the inadequate disinfection practices of medical personnel and a poor understanding of aseptic techniques could lead to a potential risk of hospital-acquired CR-hvKp infection. Therefore, there are two recommendations: one is that for severe patients who are more likely to require frequent bed changes, we recommend providing them with a separate room, as their immunity is weaker than that of ordinary patients, making them more prone to cross-infection. For ordinary patients who often change beds, we recommend minimizing unnecessary bed changes. The other is to enhance the awareness of infection surveillance among medical staff to minimize the CR-hvKp infection caused by changing beds.

The study has several limitations. Firstly, due to the lack of a definitive hvKp identification method, it is best to detect the virulence plasmid of the strains by whole genome sequencing as a means of determination, and to detect the virulence of the strains by in *vitro* and in *vivo* experiments to further determine the strain is hvKp. Secondly, the study is limited to a single center and is retrospective in nature, spanning a duration of one year. Finally, the patient sample size is fairly limited. In future studies, we plan to continue to collect samples to expand the sample size and further validate and expand the current findings.

Conclusion

In conclusion, the hospital primarily harbors K64 CR-hvKp, a strain that generates bla_{KPC}, with CR-hvKp being predominantly found in the ICU. Multivariate analysis has indicated that bed changing poses a risk factor. Consequently, it is advisable for hospitalized patients to minimize bed changes, enhance surveillance of bacterial infections in individuals without underlying health conditions and prevent the transition of bacterial colonization to infection.

Ethical Approval

The Medical Ethics Committee of Fujian Medical University Union Hospital (2023KY104) thoroughly reviewed and granted approval for all procedures pertaining to human subjects, including individuals, medical records, human samples,

and clinical isolates, in this study. We affirm that the execution of this study adhered to the principles outlined in the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest to declare.

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