

Clinical Efficacy, Antibiotic Resistance Genes, Virulence Factors and Outcome of Hospital-Acquired Pneumonia Induced by *Klebsiella pneumoniae* Carbapenemase 2-Producing with Tigecycline Treatment in the ICU

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Purpose: Tigecycline is an agent for carbapenemase-producing *Klebsiella pneumoniae* (KPC-KP), given its penetration into lung tissues. Our study focused on the molecular and clinical efficacy of tigecycline for hospital-acquired pneumonia (HAP) in the ICU.

Patients and Methods: A retrospective cohort study of 52 adult KPC-KP HAP patients by searching hospital medical records from January 2018 to December 2020 was established to investigate the epidemiology of KPC-KP infections for tigecycline treatment and the associated clinical efficacy of tigecycline. The KPC-KP isolates underwent multilocus sequence typing. Molecular typing, antimicrobial resistance, and virulence profiling were also analyzed by whole-genome sequencing of KPC-KP.

Results: Among 52 patients with KPC-KP, the ICU mortality rate was 14/52 (27%), and there was no significant statistical difference in mortality between the effective group and failure group ($p = 0.754$). However, the duration of tigecycline was statistically different between the two groups of patients (14.4 vs 10 days, $p=0.046$). The total bacterial clearance rate was 6/52 (11.5%). There was no significant statistical difference in both groups ($p=0.416$). Antibiotic resistance genes (*aac3iia*) and virulence gene (*AREO-iutA*, *Capsule-wzc*) were negatively correlated with clinical efficacy ($p = 0.011$, OR = 1.237).

Conclusions: *Bla_{kpc}* was the main carbapenemase in all *K. pneumoniae* strains. ST11-KL64 KPC-KP was the most common virulence factors in KPC-KP isolates. This study suggested that antibiotic resistance genes (*aac3iia*) and virulence gene (*AREO-iutA*, *Capsule-wzc*) were independent mortality risk factors for patients with *Klebsiella pneumoniae* carbapenemase-2 producing *K. pneumoniae* infections, when during the tigecycline treatment. Molecular analysis of *K. pneumoniae* may provide an option when choosing the antimicrobial treatment.

Keywords: *Klebsiella pneumoniae*, carbapenemase, virulence factors, resistance genes, hospital-acquired pneumonia

Introduction

Klebsiella pneumoniae is the most clinically relevant *Klebsiella* species, often exhibiting multidrug resistance and high virulence. Globally, *K. pneumoniae* infections are leading healthcare-associated infection.¹ The most common are

pneumonia, urinary tract and abdominal infections, any of which can progress to bacteremia. In the last 10 years, carbapenem-resistant *K. pneumoniae* (CR-Kp) had increased from 3% in 2005 to 25.3% in 2019 in China.²

The management of nosocomial infections due to CR-Kp infections has become challenging. The mortality rates of CR-Kp were between 23% and 75%, which are attributed to inappropriate empirical therapy and underlying comorbidities of patients.³ A cohort study in ICU suggested that empirical antibiotic therapy, the use of piperacillin-tazobactam, was associated with mortality, and CR-Kp increased 20% of adjusted mortality.⁴

The most mechanism of carbapenem resistance was carbapenem production in *Klebsiella pneumoniae*, particularly KPC in China.⁵ Based on understanding of carbapenem resistance mechanisms can guide the selection of treatment option for CR-Kp.^{6,7}

The clinical evidence seems to indicate tigecycline has promising for KPC- *Klebsiella pneumoniae* (KPC-Kp) infections, but its clinical efficacy is varied.³ The current study believes that the insufficient dose recommended by the manual is one of the reasons for the poor efficacy of tigecycline. A large number of studies show that high-dose (100mg bid) tigecycline is an effective treatment option.⁸ A meta analysis study indicated that the monotherapy tigecycline had a higher mortality compared to the combination therapy group.⁹ When new treatment options were not available, high dose tigecycline combination therapy could be an option.¹⁰ In vitro and vivo data indicated that tigecycline combination with carbapenem can decrease bacterial loads.¹¹ A multicenter retrospective cohort study suggested that combination of tigecycline and meropenem was associated with lower mortality.¹² Therefore, the current lack of alternative treatment option suggests that tigecycline benefit–risk continues to be positive.

In addition, genomic studies provide a clue for the interpretation of studies aimed at the pathogenicity and/or virulence of *K. pneumoniae*.¹³ Yersiniabactin (ybt, irp, fyuA), which form the Yersinia high-pathogenicity island (HPI), was the most prevalent virulence-associated locus and strongly predictive of infection. Similarly, all *K. pneumoniae* encoding aerobactin (iucABCD, iutA), salmochelin (iroN, iroBCD), and rmpA/rmpA2 were isolated from human infections. The combination of salmochelin, aerobactin, and rmpA was frequently linked to the presence of genes from the known *K. pneumoniae* virulence plasmids pLVPK and pK2044.¹⁴ In China, sequence type (ST) 11 carbapenem-resistant hypervirulent *K. pneumoniae* is still the dominant clone.¹⁵ ST23 has also been identified in China.¹⁶ A study indicated that an ST16 clone is associated with high mortality for adult KPC-KP bloodstream infection.¹⁷ However, it is unknown for HAP. Thus, we conducted a study to understand the molecular of KPC-KP infections for tigecycline treatment, and associated with the clinical efficacy of tigecycline with HAP.

Materials and Methods

Study Population

We conducted a retrospective cohort study of adult KPC-KP HAP cases in Xuanwu hospital from January 2018 to December 2020. We retrieved the case by searching the microbiology database adults aged >18 years old whose respiratory tract specimens (Bronchoalveolar Lavage or Endotracheal Aspirates) were KPC-KP who had taken more than three days of tigecycline. Respiratory tract secretion test was performed daily. Data were extracted from the medical records in our hospital computer health-care system. The variables collected included patient demographic characteristics (gender, age, BMI, comorbidities), and Charlson's comorbidity index, medical history, surgery during hospitalization. We also collected medication, including tigecycline dosage: High Dose (HD) (loading dose 200mg, maintenance dose 100mg q12h), Standard Dose (SD) (loading dose 100mg, maintenance dose 50mg q12h) and course of treatment, and the combined use of antibacterial drugs. Vital signs, such as body temperature, respiration rate, pulse, and laboratory test, drug sensitivity results (tigecycline, amikacin and compound trimethoprim) were also collected within 48 hours before and after tigecycline treatment. The endpoint was 72 hours after tigecycline withdrawal or transfer from the ICU ward. Patients were excluded if they had combined other sites of infection, bacteremia or adjusted the dose of tigecycline during the study, the main outcome indicators cannot be evaluated, liver or kidney insufficiency. Hospital-Acquired Pneumonia was diagnosis based on IDSA guideline published in 2016.¹⁸

Patient group: All patients were divided into effective (body temperature, symptoms and signs improved, and lung imaging improved or not progressed) group and failure group (body temperature, vital signs have not improved or

worsened, lung imaging progressed or the patient died due to infection).¹⁹ The outcomes were evaluated by two authors separately. When the results were inconsistent, another two authors negotiated together.

Outcomes

All-cause death in the ICU: The patient died during the whole follow-up.

Clearance of pathogens: The microbiology efficacy was divided into clearance (bacterial culture results are negative), hypothetical clearance (clinically effective, not checked due to the inability to obtain respiratory specimens), and replacement (carbapenemase 2–Producing K was clear, other bacteria are detected) and re-infection (carbapenemase 2–Producing K was still detected in the culture). The microbiology clearance rate = (number of cleared cases + assumed number of cleared cases + number of replacement cases)/total number of cases × 100%. The duplicated cases (multiple isolates in the same patient) were not counted.²⁰

Microbiological Analysis

The isolates of *K. pneumoniae* were cultivated overnight on MacConkey agar media at 37°C for further sequence typing. The *K. pneumoniae* strains were subjected to antimicrobial susceptibility tests comprising 13 different antibiotics by VITEK-2 compact system (bioMérieux, France). The minimum inhibitory concentration (MIC) was routinely determined in accordance with the general guidance of the Clinical and Laboratory Standards Institute (CLSI).²¹ Tigecycline susceptibility breakpoints were determined according to the FDA standard (MIC <2mg/L, sensitive; MIC = 4 intermediate, MIC ≥8mg/L, resistant) by using the E-test method (BIO-KONT, Wenzhou, China).²²

Carbapenemase Test

The VITEK2 Compact system (bioMérieux, France) was used for antimicrobial susceptibility test and further confirmed by using E-test. The MIC results of carbapenems were interpreted strictly followed the guidelines of the Clinical and Laboratory Standards Institute (CLSI). *Escherichia coli* ATCC25922 was used as quality control strain. Confirmation of suspected carbapenemase production in *K. pneumoniae* isolates was performed using a modified carbapenem inactivation method (mCIM). Phenotypic screening for the presence of carbapenemases was performed using a double-disk synergy test (DDST) with phenylboronic acid or ethylenediaminetetraacetic acid with meropenem as previously described. The mCIM analysis and DDST are biochemical assay for carbapenemase activity, and the results were positive. The blaKPC gene was detected in all isolates with PCR and CARBA NP test.

Genome sequencing

Extraction of Genome DNA

Bacterial genomic DNA was extracted and quantified by Qubit[®] 2.0 Fluorometer (Thermo Scientific).

Library Construction and Sequencing

Sequencing libraries were generated using a NEBNext Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences to each DNA sample fragmented by sonication to a size of 350 bp. Libraries were analyzed for size distribution by an Agilent2100 Bioanalyzer and quantified using real-time PCR. The GeneMarkS program was used to retrieve the related coding gene. The whole genome of *K. pneumoniae* was sequenced using Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd. Genomes were assembled with SOAP denovo, SPAdes, Abyss and gapclose software. Antibiotic Resistance Genes Database (ARDB), Pathogen Host Interactions (PHI) and Virulence Factor Database (VFDB) were used to perform the pathogenicity and drug resistance analyses. Then, multilocus sequence typing (MLST) database (<https://www.pasteur.fr/fr/mlst>) with various allelic profiles was performed to determine the genetic diversity of *K. pneumoniae*. In addition, we also used Kaptive to search for the K-loci of *K. pneumoniae*, which allowed for defining the capsule serotype (K and KL), thus narrowing the isolate classification. Finally, the results obtained by the programs mentioned above are summarized and presented in a concise tabular form.

Statistical Analysis

Categorical variables was used χ^2 or Fisher's exact test and *t*-test or Mann–Whitney *U*-test for continuous variables. Multivariate analysis to determine the impact of covariates on clinical efficacy was performed by binary logistic regression, adjusting for confounding factors, and using IBM SPSS Statistics 25.0 (Armonk, New York). The critical value of $p < 0.2$ in univariate analysis is used to select the covariates of the multivariate model, and the Horner-Lemeshow goodness-of-fit test is applied. The entire study (two-tailed) showed precise *p*-values. The statistical significance was established at $p < 0.05$.

Institutional Review Board Statement: The study was carried out in accordance with the Declaration of Helsinki criteria and was approved by the Ethics Committee of Xuanwu Hospital Capital Medical University (IRB, protocol number: 2020038 6/20/2020). All patients gave written informed consent. The clinical trial registration number is ChiCTR2000041036

Results

In total, 3477 patients were observed from January 2018 to December 2020 in ICU. Among them, 3.5% (120/3477) of the cases were *Klebsiella pneumoniae* with Tigecycline treatment. A total of 87 patients were diagnosis of Hospital-Acquired Pneumonia. Twenty-four patients were carbapenem-sensitive *Klebsiella pneumoniae*. Tigecycline dose adjustment was performed in 11 patients. At last, 52 patients were included in the final analysis. The distribution of these patients is shown in Figure 1.

The mean \pm standard deviation age of all patients was 62.3 ± 15.2 years, with 44.2% (23/52) was female. Mechanical ventilation patients were 65.4% (34/52). Combining therapy with tigecycline was a major treatment option, with 46.2% (24/52) of patients being on carbapenems in our study. 38.5% (20/52) patients were treated with high-dose tigecycline. The treatment days of tigecycline were 10 in the treatment failure group and 14.5 days for the patients in the effective treatment group, $p = 0.046$. The clinical characteristics are listed in Table 1.

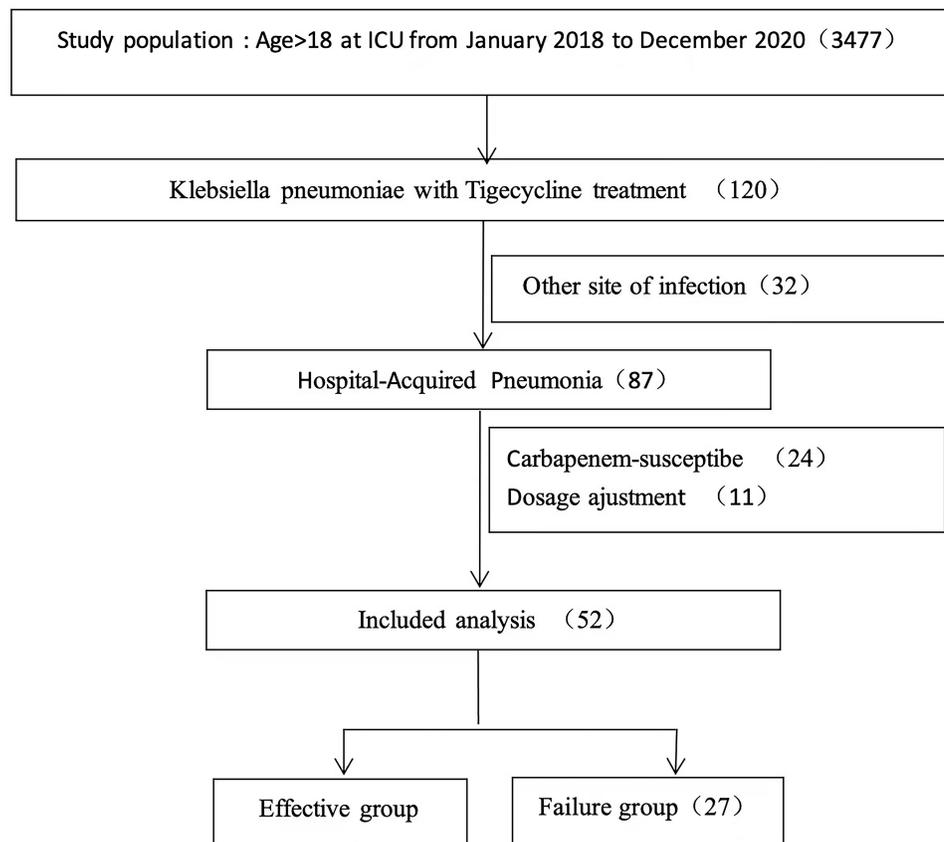


Figure 1 Flowchart of patient inclusion.

Table 1 Characteristics of Patients with HAP Caused by KPC with TG in Both Groups

	Effective Group N = 25	Failure Group N = 27	p-value
Characteristic			
Age (years) median (IQR)	59.1 (44–74.5)	65.5 (56.2–78)	0.356
BMI	25.36 (20.1–26.9)	23.6 (21.6–27.8)	0.763
Female sex	9	14	0.578
CCI score median (IQR)	2.25	2 (0–3)	0.788
Tumor	5	4	0.621
DM	4	3	0.606
COPD	5	3	0.375
Surgery	7	11	0.335
Mechanical ventilation	16	18	0.840
Length of ICU days (IQR)	41 (31–54)	30 (22–51)	0.077
Days of TG treatment (IQR)	14.5 (9–28.75)	10 (5–16)	0.046
Dose of TG (100mg q12h)	8	12	0.357
Combine therapy			
Carbapenems	11	13	0.236
β-Lactamase inhibitors	6	8	0.647
Ceftazidime	1	3	0.317
SMZ	2	5	0.267
Amikacin	5	4	0.621

Abbreviations: BMI, Body Mass Index; CCI, Charlson Comorbidity Index; IQR, Inter-Quartile Range; DM, diabetes mellitus; COPD, chronic obstructive pulmonary disease; TG, tigecycline; Carbapenems (Imipenem/cilastatin, Meropenem, Biapenem); SMZ, sulfamethoxazole.

AST and Isolated Antibiotic Resistance Genes

All isolates were all resistant to IPM: (Imipenem), MEM: (meropenem), AMP (ampicillin), CAZ: (ceftazidime), CTX (cefotaxime), CRO (ceftriaxone), PIP (piperacillin), TZP (piperacillin and tazobactam), MNO (minocycline), and ATM (aztreonam) except CT (Clostin) and AMK (amikacin). Sixteen isolates (30.8%) showed MIC=1mg/L, 15 (28.4%) MICs = 2 mg/L, 6 (11.5%) MIC = 0.5 mg/L, 8 (15.4%) MIC = 0.25 mg/L, 5 (9.6%) MIC ≤0.125 mg/L; among them, 50 (96.0%) was susceptible, and 2 (4%) was intermediate to tigecycline (MIC = 4 mg/L).

The phenotype studies showed that 52 isolates were ampicillin-resistant, which corresponds well with their possession of SHV-type beta-lactamase genes known to be chromosome-encoded. All isolates were also resistant to other beta-lactam antibiotics and carbapenems. The prevalent *blaKPC-2* gene was detected in all 52 isolates, whereas the *blaNDM* genes were not detected. The prevalent β-lactamase genes *blaSHV*, *blaCTX-M*, and *blaTEM* were mostly expressed by the CRKP isolates at ratios of 100%, 100%, and 90.4% (47/52), respectively. Each CRKP isolate in this study expressed at least three resistance genes, whereas 63.5% (33/52) of the CRKP strains co-expressed the *blaKPC-2*, *blaCTX-M-15*, *blaSHV*, and *blaTEM* genes. The β-lactamase resistance genes *blaOXA-1* and *blaTEM-1B*, the phenicol resistance gene *catB4*, the sulphonamide resistance gene *sul2*, and the trimethoprim resistance gene *dfiA14* were also expressed in the CRKP isolates. Three isolates expressed *blaTET* (5.8% each). The data obtained are summarized in Figure 2.

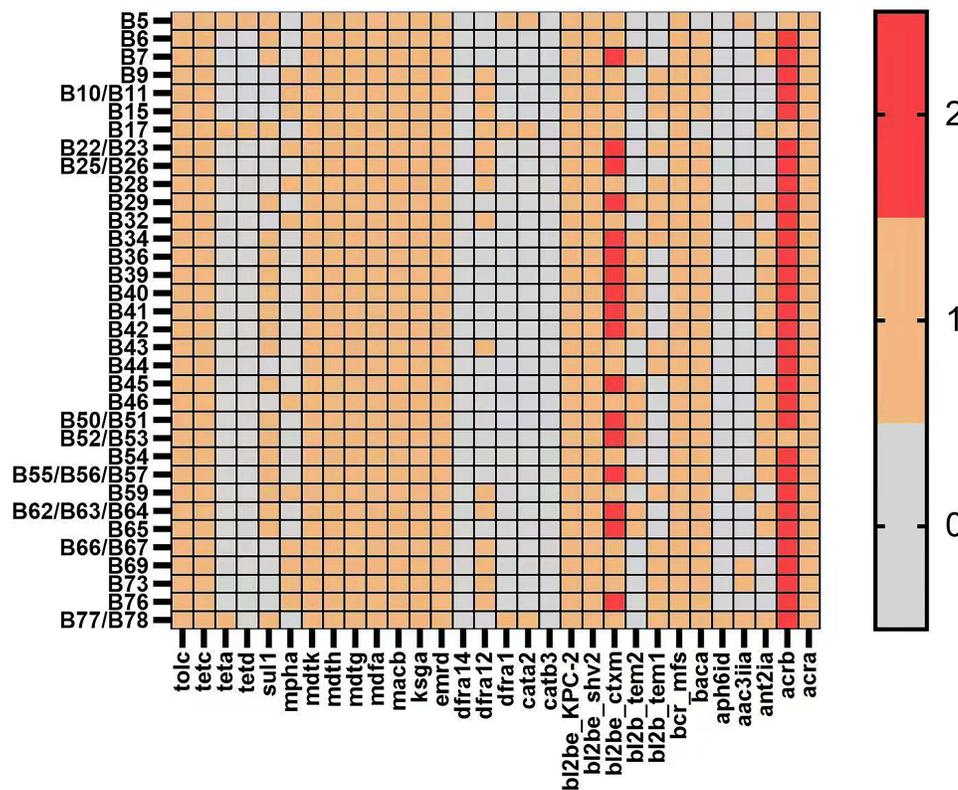


Figure 2 Distribution map of drug resistance genes. (1) Multi-drug resistance genes were detected from studied strains, including multidrug resistance efflux pump genes—major facilitator superfamily transporter (MFS), resistance-nodulation-cell division transporter (RND), and β -lactamase genes. Among them, KPC-2, SHV, CTX-M, MFS (mdth, mdtg, mdtk) and RND (acrb, tolc) genes existed in all strains. (2) The number of tetracycline resistance genes contained in the studied strains was different, in which the detection rates of teta, tetd and tetc were 6%, 4% and 100%, respectively.

Virulence Factors

All strains had *aero*, *salmonellin*- and *yersiniabactin*-related genes, and 69% of the isolates contained the *rmpA* (regulator of mucoid phenotype A) gene. In addition, almost all of the isolates contained genes of the *mrk* and *fim* clusters, which are involved in types 3 and 1 fimbriae formation, respectively. Most isolates contained four or more virulence factors, suggesting that they could be dangerous in clinical settings, requiring further investigation. The main virulence factors given above are shown in Figure 3.

Typing and Classification

Two STs were identified among the 52 KPC-KP isolates, which included 37 isolates for ST11 (71.1%), the most prevalent ST, belonged to CC258, while the others (40%) were novel sequence types with *phoE* gene allelic profiles 419.

There were five different KL types among the isolates, with two isolates belonging to the KL47 type. The KL types identified were KL47, KL64, k20, k50, and k25, respectively. The most frequent K type was KL64 (88%), which is usually considered to be associated with high virulence in China.²³ However, there was no significant difference between the two groups in the mortality in ICU for ST11-KL64 KPC-KP ($P > 0.05$).

Among the 52 patients with KPC-KP, the ICU mortality rate was 22/52 (27%), and there was no significant difference in mortality between the two groups ($p = 0.754$). The total bacterial clearance rate was 6/52 (11.5%), but there was no statistical difference between the two groups of patients ($p = 0.578$). See Table 2 for more information.

Clinical Efficacy Predictor Analysis

We performed an analysis for factors expected to influence clinical efficacy. See Table 3. The results showed that the dose, course of treatment and combination of tigecycline had no significant correlation with clinical efficacy. As for the

Table 2 The Mortality, Clearance of Pathogens and Distribution of Antibiotic Resistance Genes, Virulence Factors in Both Groups

	Effective Group N = 25	Failure Group N = 27	p-value
Mortality			
All-cause death in the ICU	6	16	0.010
Clearance of pathogens			
At 3 days after stopping TG	12	16	0.416
Virulence factors			
rmpA	16	15	0.535
rmpA2	15	18	0.618
AREO-iutA	10	19	0.028
Capsule-wzc	16	20	0.432
Capsule-wza	18	20	0.866
LPS-wbbO	4	14	0.009
Antibiotic Resistance Genes			
sulI	17	12	0.087
mpha	7	13	0.136
dfra12	11	10	0.609
aac3iia	6	1	0.046
ant2ia	15	11	0.165

Abbreviations: ICU, Intensive Care Unit; TG, TG, tigecycline.

Shelenkovis et al's study analyzed 36 cases of KPC-Kp for virulence factors and resistance genes. The results showed that 32 cases were multi-drug resistant strains and 5 cases were polymyxin-resistant strains. The rare type was ST 377.³⁰ Our study showed that all strains are resistant to carbapenem, β -lactamase inhibitor combinations, third-generation cepheims, aminoglycosides, and levofloxacin. The detection rate of the key enzyme of the carbapenem resistance gene *blaKPC-2* and the extended-spectrum β -lactamases *blaSHV*, *blaCTX-M*, and *blaTEM* were $\geq 90\%$, which support the contention of the expression of multiple resistance genes of the CRKP strains. In the study, ST11 was the predominant clone (60%) in CRKP strains, but emerging novel ST with *phoE* gene allelic profiles 419 worthy of continued attention.³¹ This was consistent with numbers of study in China and other countries.^{32,33} ST11 type CRKP strains, which belong to one of clonal group 258 (CG258) type of CRKP, is a high risk resistant clone with transmissible characteristic of carrying co-resistance determinants and virulence factors.³⁴ It poses enormous challenges to infection control. In our study, the ST11- KL64 *K. pneumoniae* strains (88%) were the dominant K types of the most important clinical pathogens, which is consistent with previous studies.³⁵ The KL64 strains contained the most virulence genes, including *iucA* and *rmpA*. ST11-KL64 *K. pneumoniae* strains seem to have no higher mortality and poor clinical efficacy. This may be due to the small sample size in our study.

Hypervirulent *Klebsiella pneumoniae* (HvKP) strains are characterized by the presence of capsular polysaccharides (K antigen), fimbriae, lipopolysaccharides (O antigen), and siderophores (aerobactin and yersiniabactin).³⁶ Herein, we investigated 42 virulence-associated genes in 52 KPC-KP isolates. According to the aforementioned criteria, these isolates were identified as carbapenem-resistant hypervirulent *K. pneumoniae* strains with a ST11 KL64/KL47 serotype. The *mrkD* gene encodes type 3

Table 3 Univariate and Multivariate Analyses of the Risk Factors Associated with Clinical Efficacy in Patients with *Klebsiella Pneumoniae* Carbapenemase 2-Producing *K. Pneumoniae* Infections

Univariate Analysis				Multivariate Analysis		
Covariate	OR	95% CI	p-value	OR	95% CI	p-value
Age	0.975	0.943–1.008	0.130			
Male	0.745	0.248–2.232	0.599			
BMI	0.969	0.872–1.076	0.551			
CCI	0.921	0.766–1.108	0.382			
Mechanical ventilation	0.818	0.267–2.510	0.726			
Combined with carbapenems	0.361	0.114–1.145	0.084	0.377	0.211–1.179	0.248
High-dose TG	0.989	0.967–1.012	0.358			
Days of TG	1.002	0.963–1.035	0.912			
sulI	2.222	0.729–6.777	0.160			
mpha		0.132–1.328	0.139			
dfraI2	1.336	0.44–4.05	0.610			
aac3iia	8.221	0.91–73.95	0.060	0.541	0.106–2.758	0.088
ant2ia	2.182	0.72–6.61	0.068	0.740	0.218–2.897	0.024
rmpA	1.422	0.466–4.337	0.536			
rmpA2	0.750	0.242–2.325	0.618			
AREO-iutA	0.281	0.089–0.887	0.03	0.055	0.004–0.731	0.028
Capsule-wzc	0.142	0.027–0.745	0.021	0.003	0.000–0.262	0.010
Capsule-wza	0.206	0.038–1.108	0.066	3.194	0.196–6.12	0.178
LPS-wbbO	0.177	0.048–0.655	0.009	1.109	0.170–7.217	0.914

Abbreviations: BMI, Body Mass Index; CCI, Charlson Comorbidity Index; TG, tigecycline; Carbapenems (Imipenem/cilastatin, Meropenem, Biapenem).

fimbriae, which play critical roles in adhesion to the respiratory tract, often detected in cases of ventilator-associated pneumonia caused by *K. pneumoniae*.³⁷ A previous study reported that biofilm formation requires the type 3 fimbriae and adhesion factor *mrkD*.³⁸ Biofilm formation inhibits the penetration of drugs, thus increasing antibiotic resistance, which further complicates the clinical treatment of *K. pneumoniae* infections.³⁹ Our study found that *ant2ia* gene was correlated with clinical efficacy. *Nucleotidyltransferase (ant2ia)* gene regions suggested it was resistance to aminoglycosides. However, all *Klebsiella pneumoniae* were sensitive to amikacin, and 9 patients were treated with amikacin, accounting for 17.3% (9/52), and the detection rate of *ant2ia* gene was 36.5% (19/52). It is suggested that the selection of drugs based solely on the drug susceptibility results has certain limitations, and the monitoring of antibiotic resistance genes has certain significance for the selection of antibiotics. However, this still needs to be verified by large-scale clinical studies. Our results suggested that virulence gene (*AREO-iutA*, *Capsule-wzc*) was related to the clinical efficacy. The *AREO-iutA* gene encodes a membrane protein receptor involved in biofilm formation in *Klebsiella pneumoniae*.⁴⁰ *Capsule-wzc* is produced through a Wzy-dependent process for which the conserved machinery is encoded by genes that are present in most K-antigen biosynthesis loci.⁴¹

The outbreak of KPC-KP strains and the emergence of hypervirulence forces us to promote awareness and to strengthen epidemiological surveillance and infection control measures in our hospital, especially regarding ST11 KPC-producing hypervirulent strains.

Conclusion

In conclusion, we found that there was no significant difference in mortality for effective group and failure group. Antibiotic resistance genes (aac3iia) and virulence gene (AREO-iutA, Capsule-wzc) were independent mortality risk factors for patients with KPC-KP. However, there was no significant difference between the two groups in the mortality in ICU for ST11-KL64 KPC-KP. ST11-KL64 KPC-Kp had a significantly higher mortality rate than other CRKP-infected patients in the ICU. There is a need for further study to confirm the association between ST11-KL64 KPC-KP and the dose of tigecycline.

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; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This study was funded by Beijing Municipal Science and Technology Commission Special Funding Project (D181100000218002); National Clinical Research Center for Geriatric Disorders, Beijing, China. Beijing Municipal Commission of Health and Family Planning, No. PXM2018_026283_000002; Beijing Municipal Administration of Hospitals Incubating Program (PX2020038); Beijing High-level Public Health Technical Talent Development Plan (2022-01-023).

Disclosure

The authors declare no competing interests.

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