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ORIGINAL RESEARCH

The Evolution of Virulence of Carbapenem-Resistant *Klebsiella pneumoniae* from the Same Source Under the Pressure of Omadacycline Treatment

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Introduction: *Klebsiella pneumoniae* (KP) is a common Gram-negative bacterium in clinical practice and can cause various infectious diseases, including pneumonia, liver abscess and bloodstream infection. Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become a major threat to global health due to its high incidence and mortality rates, especially the ST11-CRKP strain prevalent in China.

Methods: The age, main clinical diagnosis, previous health and immune status of the two patients with ST11-CRKP-related infections during the same period reported in this study were similar.

Results: The antibiotic treatment regimens for the two patients were the same in terms of the type and dosage of antibiotics, except for omadacycline. Meanwhile, PFGE, multilocus sequence typing (MLST), and K typing confirmed that the two strains had the same genetic background. Through experiments on serum resistance, biofilm formation, the *Galleria mellonella* infection model and the hypermucus phenotype, it was found that CRKP1 showed a hypervirulent phenotype, while CRKP15 showed a hopervirulent phenotype. Whole-genome sequencing further revealed the differences in virulence genes between the two strains and further confirmed the virulence phenotypes of the two strains. Single nucleotide polymorphism (SNP) analysis showed that the *terw* gene was one of the key genes for the virulence difference of the two strains with the same genetic background under the therapeutic difference of omadacycline. In addition, the effect of omadacycline on the expression of the *terw* gene was evaluated by qRT-PCR technology. The interaction between the *terw* gene and omadacycline was confirmed through molecular docking.

Conclusion: To sum up, these findings suggest that under the therapeutic stress of omadacycline, CRKP may adjust virulence through adaptive evolution, and the *terw* gene may be the key factor for the differences in virulence within this bacterial population.

Keywords: *Klebsiella pneumoniae*, CRKP, omadacycline, *terw*, virulence

Introduction

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has become an important challenge for global medical institutions due to its high infectivity, high incidence and high mortality rate. Recent research data show that the ST11 clone is the

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main dominant strain of CRKP in China and poses a serious threat to clinical anti-infection treatment. The incidence and mortality rate of ST11-CRKP infection in hospitalized patients are as high as 33.5%. Although various treatment strategies have been applied at present, there are still many problems in the treatment of CRKP infection with traditional antibiotics, such as high drug prices and poor safety, which bring great uncertainty to clinical treatment.

Omadacycline, as a new type of third-generation tetracycline antibiotic, is not only effective against both Gram-positive and Gram-negative bacteria, but also, due to its long half-life, broad antibacterial spectrum and outstanding efficacy against atypical pathogens, is regarded as a potential option for the treatment of *Klebsiella pneumoniae* infection. However, due to the high adaptation cost of the ST11-CRKP strain, drug resistance and the evolution of virulence plasmids are prone to occur during the clinical anti-infective treatment process, further increasing the complexity of the treatment.

The two cases of ST11-CRKP infection reported in this study during the same period were similar in age and main clinical diagnosis, and neither of the patients had an obvious history of underlying diseases. The antibiotic regimens (except for omadacycline) received by the two patients were exactly the same in type and dosage. The study confirmed through PFGE, MLST, K typing and SNP evolutionary analysis that the two strains had the same clonal relationship. However, further virulence phenotypic experiments and whole-genome sequencing revealed the differences in virulence genes between the two strains and further confirmed the virulence phenotypes of the two strains. Single nucleotide polymorphism (SNP) analysis showed that the *terw* gene was one of the key genes for the virulence difference of the two strains with the same genetic background under the therapeutic difference of omadacycline. In addition, the effect of omadacycline on the expression of the *terw* gene was evaluated by qRT-PCR technology. The interaction between the *terw* gene and omadacycline was confirmed through molecular docking. To sum up, these findings suggest that under the therapeutic stress of omadacycline, CRKP may adjust virulence through adaptive evolution, and the *terw* gene may be the key factor for the differences in virulence within this bacterial population.

In conclusion, this study has revealed the possible mechanism of changes in the virulence of CRKP strains under the therapeutic stress of omacycline, providing an important scientific basis for optimizing the treatment strategy of CRKP infection and controlling the evolution of drug resistance. With the continuous evolution of drug-resistant strains, the existing antibiotic treatment strategies are facing increasingly greater challenges. Therefore, exploring the impact of new antibiotics (such as omacycline) on the virulence evolution of *Klebsiella pneumoniae* infection is of great significance for optimizing clinical treatment plans, preventing the further spread of drug-resistant strains, and reducing the mortality rate of patients.

Materials and Methods

Strain Identification and Clinical Data Collection

This study collected two cases of ST11-CRKP infection of similar age during the same period. The initial diagnosis of both cases was pulmonary infection (*Klebsiella pneumoniae* infection), accompanied by fever symptoms. The patient received combined treatment with multiple antibiotics during hospitalization. Among them, the antibiotics used for patients with CRKP1 infection were meropenem 1g q8h, coperazone sodium and sulbactam sodium 3g bid, meropenem 1.0g q8h, piperacillin and tazobactam 4.5g q8h, vancomycin 500mg q8h, and polymyxin 150mg q12h. The antibiotics used for patients infected with CRKP15 are meropenem 1g q8h, cefoperazone sodium and sulbactam sodium 3g bid, polymyxin 75wu q12h, piperacillin and tazobactam 4.5g q8h, vancomycin 1000mg q8h, and omadacycline 1000mg qd. And the sample was obtained 5 days after omadacycline treatment (the specific clinical data of the two patients in this study are shown in Table 1). Two clinical strains were isolated from the sputum specimens of the patients and used the French Vitek 2 system (Biomerieux) for strain identification and antibacterial drug sensitivity tests. According to relevant literature, the minimum inhibitory concentrations (MIC) of imipenem (IMP), meropenem (MEM), colistin (COL), amikacin (AMK), ciprofloxacin (CIP), and piperacillin-tazobactam (PTZ) were determined by the AGAR dilution method. The minimum inhibitory concentrations (MIC) of ceftazidime/avibactam (CAZ), ceftriaxone (CRO), and tigecycline (TGC) were determined by the E-test method, but TGC was determined in accordance with the standards of the United States Food and Drug Administration (USFDA). Except for

Table 1 General Clinical Characteristics of the Two Patients

Patient ID	Age	Gender	Diagnosis	Antibiotic Used	Outcome
CRKP1-patient	77	Male	Pulmonary infection	MEM, CAZ, PB, VAN, PTZ	Improvement
CRKP15-patient	60	Male	Pulmonary infection	MEM, CAZ, PB, VAN, PTZ, OMC	Improvement

Abbreviations: MEM, Meropenem; CAZ, ceftazidime-avibactam; PB, PolymyxinB; VAN, Vancomycin; CTX, Ceftriaxone; PTZ, Piperacillin-Tazobactam; OMC, Omadacyclin.

TGC, the rest all refer to the standards of the standardization protocol guidelines of the American Society for Clinical and Laboratory Standards (M100-S32). These isolates were obtained from patients as part of the hospital's routine procedures and informed consent and ethical approval from patients for the use of these samples have been obtained [Approving Ethics Committee: The First Affiliated Hospital of Nanchang University, Ethics Number: No. (2023) CDYFYYLK(01-033)].

Virulence Assessment

Serum Resistance Test

The experimental method was adjusted with reference to the relevant literature,[1] and KP1, KP15, ATCC700603 and NUHL30457 were inoculated into autoclaved LB broth, and placed in 37°C to oscillate overnight, and then the concentration was adjusted to 10^6 cfu/mL on the next day. Healthy serum were obtained from a tertiary hospital in Jiangxi Province. Healthy serum is inactivated in a water bath at 56°C for 30 min. Two kinds of sera (healthy and inactivated serum) were added into the diluted bacterial solution to oscillate and incubate for two hours, and then the bacterial suspension was inoculated onto the agar plates. The bacterial survival rate was calculated by counting the number of colonies on the two plates, and the virulence of the strains was further hypothesized.

Biofilm Formation Assay

The biofilm formation assay was modified with reference to the method reported in the literature (Liao, 2021).[2] The concentration of the bacterial suspension cultured overnight was adjusted to 0.5 McFarland turbidity, and it was diluted to a certain proportion and added to 96-well plate, and 200uL/well (3 replicate wells). The 96-well plate was incubated at 37°Cfor 24h, then stained with crystal violet, and the biofilm was lysed with ethanol, and finally the product of the previous step was transferred to the 96-well plate, and the optical density (OD) value of each well was determined, and the negative control value (Nc) was taken as the mean ± 3 times the standard deviation ($\bar{x} \pm 3s$). The results were assessed as follows: strong positive ($4 \times Nc < OD$); positive ($2 \times NC < OD \leq 4 \times Nc$); weak positive ($Nc < OD \leq 2 \times Nc$); negative ($OD \leq Nc$).

Galleria mellonella Infection Model

The virulence of the experimental strains was assessed using the infection model of the *Galleria mellonella* with reference to the literature.[3] A total of 10 *Galleria mellonella* weighing about 250 mg (purchased from Tianjin Hui You De Biotechnology Co., Ltd., Tianjin, China) were used to test the virulence of each strain. Each strain was injected with 10 μL of injection solution at a concentration of 1×10^6 cfu/mL, and the survival rate of the *Galleria mellonella* was recorded every 12 hours for three consecutive days. All experiments were performed three *times*. Hypervirulent *Klebsiella pneumoniae* (hvKP) *strains* NUHL30457 and ATCC700603 were used as controls for high and low virulence strains, respectively.

Pulsed-Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) was used to assess the clonal relationships of CRKP isolates. The two isolates were digested with restriction endonuclease XbaI for 19 hours and electrophoresis using the CHEF Mapper XA system (PFGE electrophoretic band similarity was greater than 80%).

Multilocus Sequence Typing

Multilocus sequence typing (MLST) of the two CRKP strains were performed according to the related literature.[1,2] Seven housekeeping genes (*gapA, infB, mdh, pgi, rpoB, phoE, tonB*) of KP were located from the website (https://bigsdb.pasteur.fr/klebsiella/) for amplification and sequencing. The results were also entered into the website (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) to search for strain ST.

SNP Evolutionary Analysis

To further determine whether two strains of the same clone, by BLAST (https://blast.ncbi.nlm.nih.gov/), analyzes the 17 strains klebsiella pneumonia bacteria genome sequence, SNP evolution analysis of its genome. 15 strains of whole genome sequences from NCBI web site (https://www.ncbi.nlm.nih.gov/genome/). MEGE software is used to construct maximum likelihood phylogenetic trees.

Whole Genome Sequencing

The genomic DNA of the experimental strains was extracted using Tiangen DNA (DP302-02) extraction kit, and the PacBio Sequel library was firstly constructed, and then the target DNA fragments were broken, purified, ligated and repaired by Covarisg-TUBE and magnetic beads. The constructed library was then measured with Agilent 2100 to quantify the DNA fragment size. Sequencing, fragment assembly and proofreading were then performed on the PacBio platform using HiSeq data.

Real-Time Fluorescence Quantitative PCR

The mRNA expression levels of the *terw* gene in CRKP1 and CRKP15 bacteria were analyzed by RT-qPCR. The bacteria were cultured overnight at 37°C until the logarithmic growth phase. Extract total RNA from cells and reverse transcribe it into cDNA. The RNA concentration was detected by Nanodrop 2000C (Thermo, USA). The internal reference gene (*16s rRNA*) was selected as the internal reference, and the experiment was repeated three times.

Molecular Docking Analysis

The crystal structure corresponding to the terw protein was predicted using AlphaFold3. The obtained terw protein crystals were processed using Schrodinger software. First, the SiteMap module was used to predict the best binding sites, then the most suitable Enclosing box was set to perfectly wrap the predicted binding sites, and on this basis, the active sites of the terw protein were obtained. The processed ligand compound omadacycline was subjected to molecular docking with the active site of the terw protein.

Statistical Analysis

Statistical analysis and image visualization was performed using IBM SPSS Statistics Software (ver 24.0) and GraphPad Prism Software (ver 9.0). All tests were two-tailed, and a p-value <0.05 was considered to indicate a statistically significant difference.

Results

Clinical Features

From Table 1, we can see that there is little difference in age, gender, diagnosis, and prognosis. The significant difference was in the use of antibiotics, which were the same in both patients except omadacycline.

Resistance Phenotype and Virulence of CRKP Isolates

The two CRKP strains exhibited similar antimicrobial profiles and were resistant to most clinical antibiotics, but were susceptible to TGC, CZ, CXM, CL (Tables 2 and 3). Experiments with biofilms, serum resistance, and the *Galleria mellonella* infection model revealed that CRKP15 exhibited low virulence, whereas CRKP1 exhibited hypervirulence, with reference to the hypervirulence standard strain NUHL30457 (Figure 1).

Table 2 The Drug Resistance Phenotype of CRKP Isolates was Determined by MIC Method

Antibacterial Drug	CRKP1-MIC (μg/mL)	Phenotypic Change	CRKP15-MIC (μg/mL)	Phenotypic Change
TIM	≥128	R	≥128	R
CAZ	≥64	R	≥64	R
FEP	≥32	R	≥32	R
LEV	≥8	R	≥8	R
AMK	≥64	R	≥64	R
CIP	≥4	R	≥4	R
COL	≥16	R	≥16	R
MIN	≥16	R	≥16	R
TZP	≥128	R	≥128	R
CFP	≥64	R	≥64	R
ATM	≥64	R	≥64	R
CXM	6	S	6	S
TMP	≥16	R	≥16	R
GEN	≥320	R	-	-
IMP	≥16	R	≥16	R
MEM	≥16	R	≥16	R
CL	≤0.5	S	2	S

Abbreviations: TIM, Ticarcillin-Clavulanate; CAZ, Ceftazidime; FEP, Cefepime; LEV, Levofloxacin; AMK, Amikacin; CIP, Ciprofloxacin; COL, Colistin; MIN, Minocycline; TZP, Ceftriaxone; CFP, Cefuroxime; ATM, Amoxicillin; CXM, Cefoxitin; TMP, Trimethoprim; GEN, Gentamicin; IMP, Imipenem; MEM, Meropenem; CL, Chloramphenicol; R, Resistance; S, Susceptible; MIC, Minimum Inhibitory Concentration.

Table 3 The Drug Resistance Phenotype of CRKP Isolates was Determined by the KB Method

Antibacterial Drug	CRKP1-KB (mm)	Phenotypic	CRKP15-KB (mm)	Phenotypic
CZ	6	R	-	-
CRO	6	R	6	R
CXM	6	R	6	R
TGC	14	S	18	S

Abbreviations: CZ, Cefazolin; CRO, Ceftriaxone; TGC, Tigecycline.

PFGE Analysis

According to the literature,[2,3] identical PFGE typing was defined as electrophoretic band similarity greater than 80%. From Figure 2, it can be seen that the two strains belong to the same PFGE typing (identical clonal typing).

Genomic Analysis

Whole genome sequencing results showed that the 2 CRKP isolates belonged to the same clone type ST11-KL2, and a total of multiple antibiotic resistance genes carrying multiple resistance efflux proteins such as AcrB, MdtK, MdtG, and multiple antibiotic resistance genes, such as *KPC, qnrS, Sul2* (Figure 3 and Supplementary Table 1); as well as virulence genes, such as *fimA, fimD, fimC* and *CitB* were identified; no conventional virulence factors such as iron acquisition, adhesion, anti-phagocytosis, serum resistance, secretion system, etc. were found, and they were not highly virulent strains. Among them, the *KPC* gene (ISKPn27 upstream and IS1182 downstream, see Figure 4) was located on a plasmid of 86841 bp size, and the plasmid replicon typed as IncFII. Notably, the differential genes of the two strains after SNP comparison with the hypervirulent NUHL30457 was *terw* gene (Figure 5 and Supplementary Table 2). The results of evolutionary tree analysis showed that the 15 strains of *Klebsiella pneumoniae* were mainly distributed in 5 different clades. Among them, the two strains CRKP1 and CRKP2 in this study are located in the adjacent evolutionary clades, and the separation time is in 2023. The evolutionary tree is clustered in the third clade, and there are differences in the evolutionary clades, and the similarity is 99.8%. Combined with the same PFGE, ST and K typing of the two strains, it is

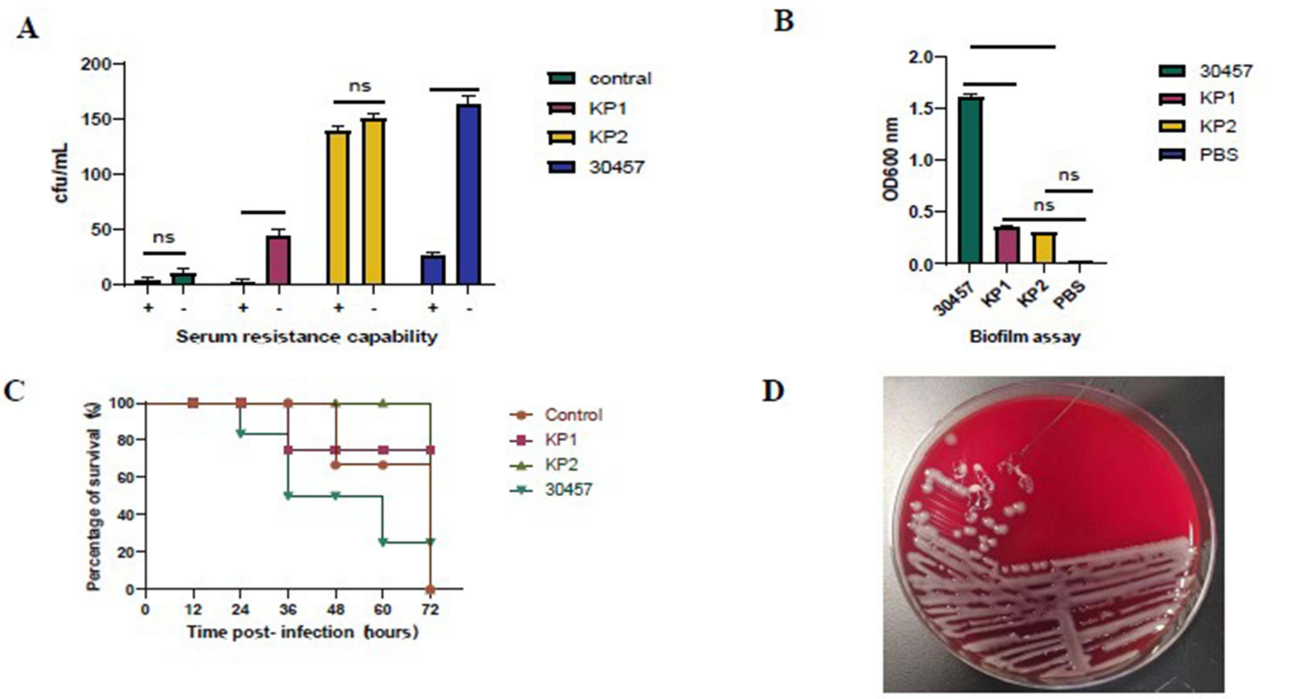

Figure 1 CRKP virulence characterization. (**A**) Serum resistance capability of KP1 and KP15. The survival ability of strains KP1 and KP15 in positive (+) and negative (-) serum was little different from that of control, but significantly different from that of NUHL30457. (**B**) Biofilm formation ability of KP1 and KP15. (**C**) Virulence analysis of the strain KP1 and KP15 in the *Galleria mellonella* infection model. (**D**) The string test of KP1 shows that the mucus filaments<5 mm.***p<0.001.

inferred that these two strains of *Klebsiella pneumoniae* are more closely related and come from the same clone, as shown in Figure 6.

Real-Time Fluorescence Quantitative PCR

qRT-PCR was performed to evaluate the effects of CRKP1 and CRKP15 strains on the expression level of the *terw* gene under differences in omacycline treatment. The results indicated that the expression of CRKP1 strains under stress treated with omacycline was significantly increased compared with CRKP15 strains that were not treated with omacycline (Figure 7).

Molecular Docking Analysis

Molecular docking of the processed ligand compound omadacycline with the active site of the terw protein revealed that Omadacycline penetrated deep into the active pocket of the terw protein. The terw protein residues such as LEU110, ILE112, LEU117, ILE120, and VAL135 form hydrophobic forces on omadacycline. This ligand forms two hydrogen bonds with the residue LEU110 and a salt bridge with the residue GLU136 (Figure 8). The lower the scores of both, the lower the binding free energy of the compound with the protein and the higher the binding stability. Combining the results of XP docking and MM-GBSA analysis, the docking score of omadacycline with terw was −4.790, while the result of MMGBSA analysis was −37.33 kcal/mol. The binding free energy was low and the docking score was relatively low, indicating that the binding of omadacycline with terw was relatively stable.

Terw Gene is the Possible Loci Causing the Effect of Omadacycline on CRKP Virulence

The *terw* gene, the first known functional ter gene product, is primarily associated with tellurium resistance and is often considered an important virulence factor in highly pathogenic bacteria.[4] Research has shown that *terw* proteins control tellurite resistance levels in *Escherichia coli* by inducing overexpression of the gene. Additionally, Xiufeng Wu et al

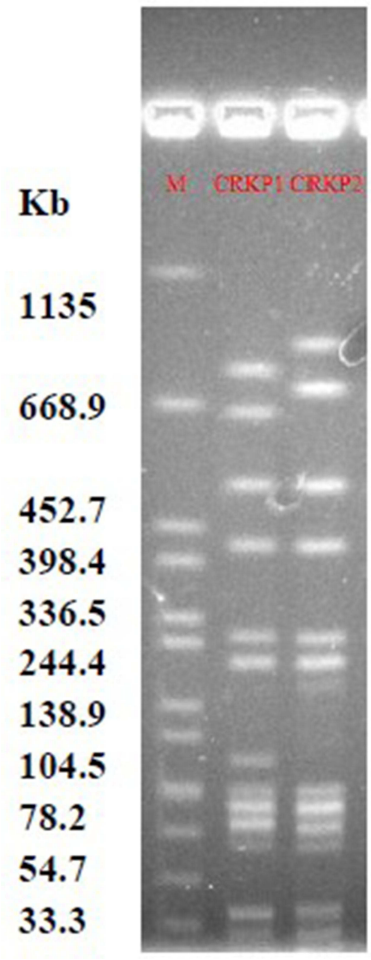

Figure 2 PFGE analyze. The PFGE of CRKP1 and CRKP15 appears as linearized fragments on the gel (the similarity of the PFGE electrophoresis bands is greater than 80%, which is the same source). Lane M, reference standard strain brenderup H9812 serotype, Xbal restricted.

found that the detection rate of the *terw* gene in the majority of hypervirulent *Klebsiella pneumoniae* (hvKP) strains was significantly higher than that in classical *Klebsiella pneumoniae* (cKP), and it was proposed that *terw* exerts its virulence by producing intracellular reactive oxygen species (ROS), suggesting that *terw* is a virulence-associated gene in hvKP.[4,5]

Discussion

In China, ST11 has gradually evolved into a major clone of hospital-acquired CRKP infection, and according to relevant reports, the infection rate of ST11-CRKP accounts for 60–90% of clinical CRE infections, with the mortality rate increasing year by year.[6] With the widespread use of antibiotics, ST11-CRKP has gradually evolved into a more virulent and drug-resistant phenotype, especially to a variety of antibiotics, such as aminoglycosides and β-lactams, which show different degrees of resistance.[7] Along with the emergence of hypervirulent and drug-resistant ST11-CRKP, there are many uncertainties in the treatment of CRKP infection in China. Omadacycline, as a third-generation new tetracycline, has a long half-life and a broad antimicrobial spectrum, which can cover a variety of pathogens at the same time, and is effective in the treatment of KP infections. The safety and effectiveness of omadacycline have been gradually demonstrated in the course of long-term anti-infective treatment compared with the high resistance of conventional clinical antibiotics.

In this study, the two patients were similar in age and major clinical diagnoses, and had no previous underlying diseases. The chest CT scan before admission showed multifocal patch ground glass opacity and consolidation in the lower lobes of both lungs, suggesting a high possibility of community-acquired pneumonia (CAP). Therefore, in this

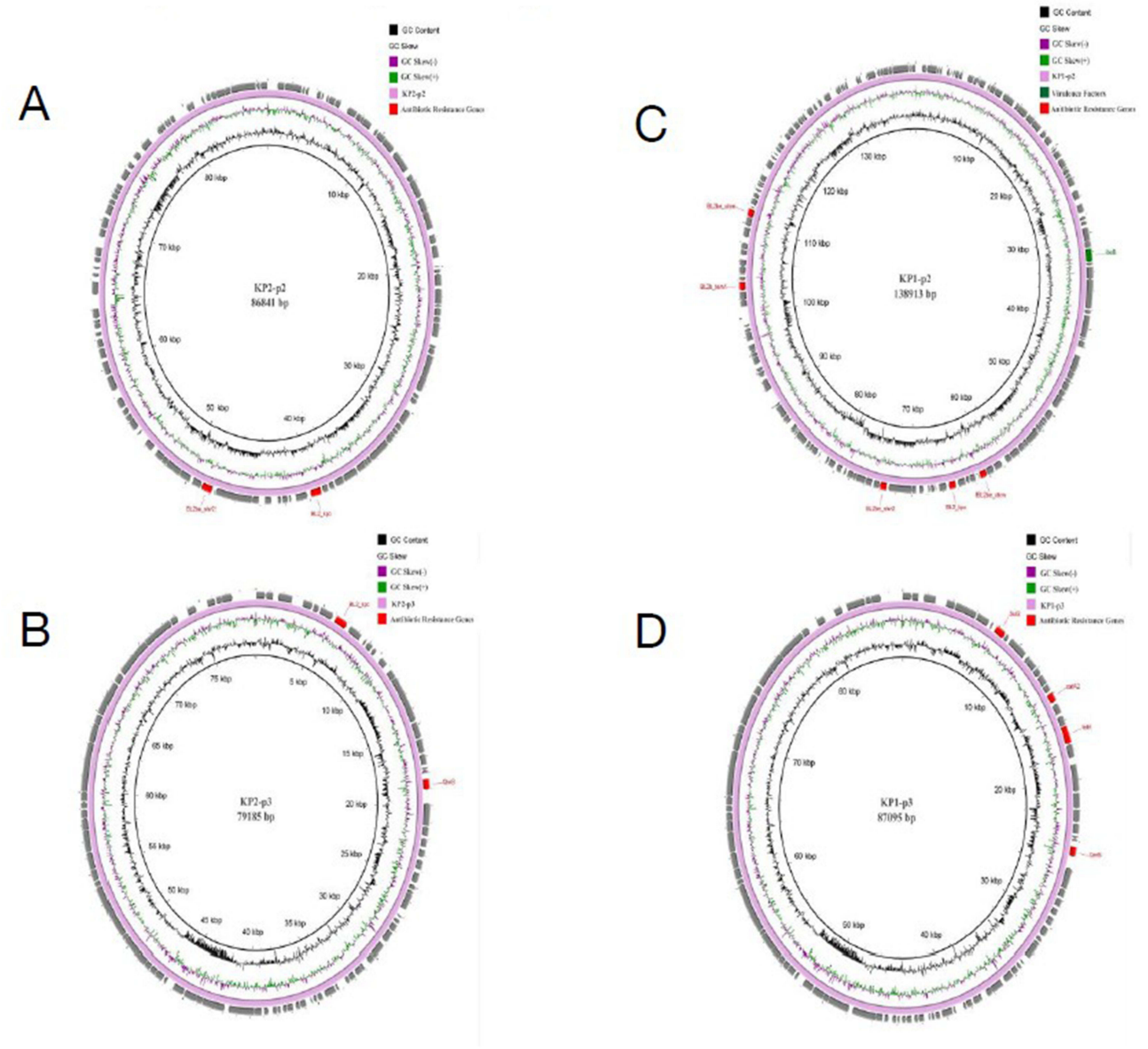

Figure 3 Circular genome diagram of CRKP1 and CRKP15 strains. Each ring contains multiple virulence and resistance genes. The outermost circle is the position coordinates of the genome sequence. (**A**) Plasmid map of strain CRKP15-P1 (**B**) Plasmid map of strain CRKP15-P2 (**C**) Plasmid map of strain CRKP1-P2 (**D**) plasmid map of strain CRKP1-P3.

study, omadacycline was empirically administered to the patients upon admission. During their hospital stay, bacterial culture and susceptibility testing revealed that both strains were carbapenem-resistant *Klebsiella pneumoniae* (CRKP). Despite treatment with omadacycline in combination with various antibiotics, including aminoglycosides, for a period of time, the patients showed poor outcomes. This unfavorable prognosis may be related to the patients' advanced age, multiple comorbidities, compromised immune function, and the high uncertainty of external factors that triggered the infection.

The virulence and resistance of bacteria are influenced by different genetic mechanisms. Virulence is typically regulated by the expression of virulence genes and the genomic composition of the strain itself, while resistance is primarily influenced by the presence and expression levels of resistance genes.[8] According to the whole-genome sequencing (WGS) results, the strains in this study only carried the carbapenem resistance gene bla_{KPC}. Considering that omadacycline belongs to the tetracycline class of antibiotics, the primary resistance genes associated with it are tet

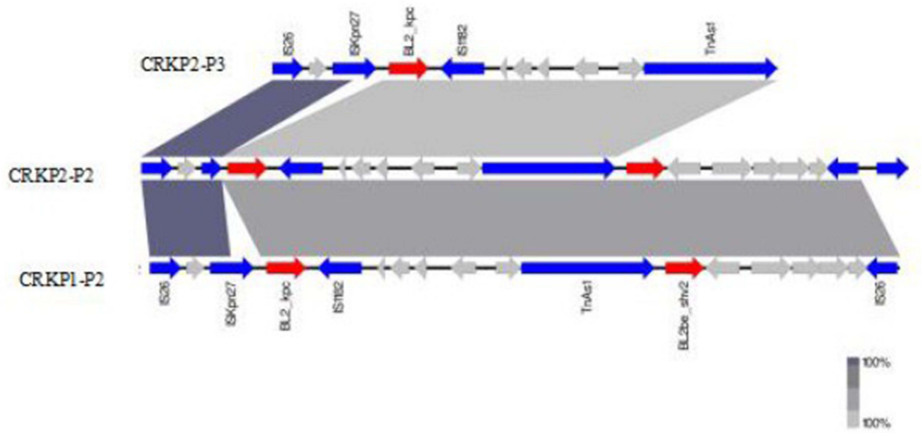

Figure 4 Genetic environment surrounding the *KPC* gene. Comparison of pKPC sequences carried by strains CRKP15 and CRKP1. Red arrows indicate antibiotic resistance genes; blue arrows indicate transposons; arrow direction refers to orientation on the genome.

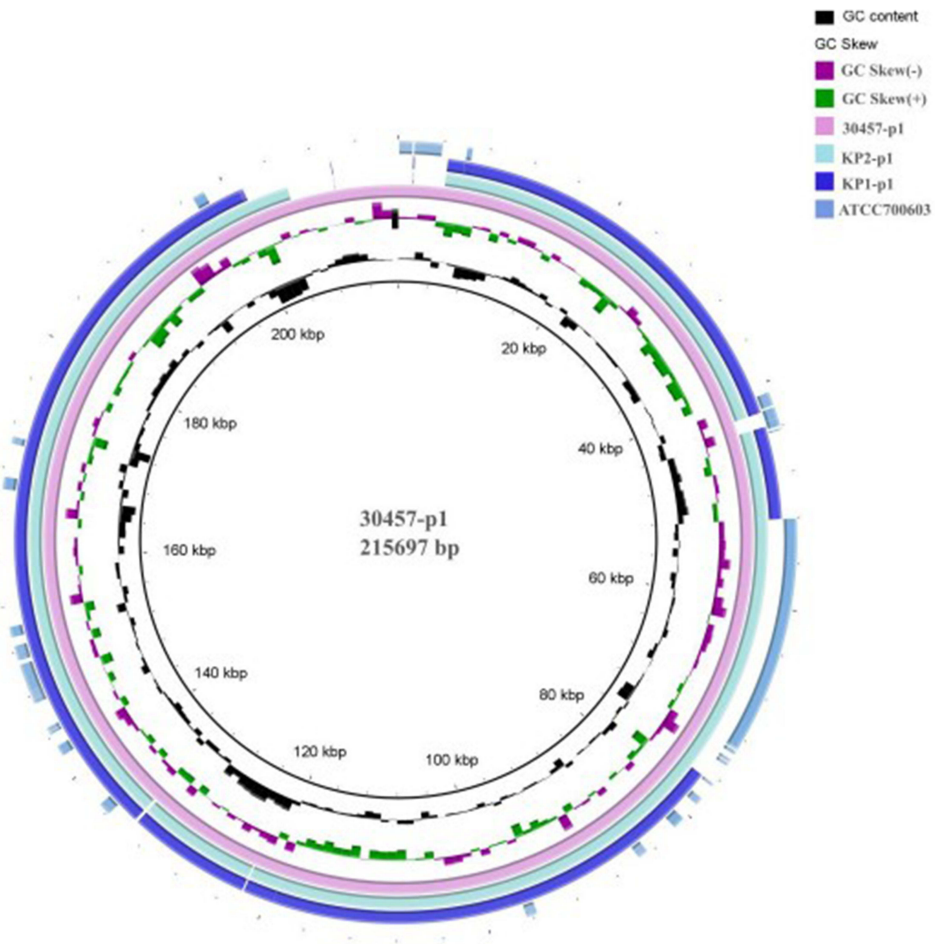

Figure 5 The whole-genome Circos maps of CRKP1 and CRKP15. Comparative genomic analysis of strains CRKP1, CRKP15 and NUHL30457. Each circle contains multiple virulence and resistance genes. The innermost circle is the positional coordinates of the genomic sequence. The gene circle diagrams for the 30457-p1 plasmid, CRKP1-p1 plasmid, and CRKP15-p1 plasmid are shown in order from inner to outer.

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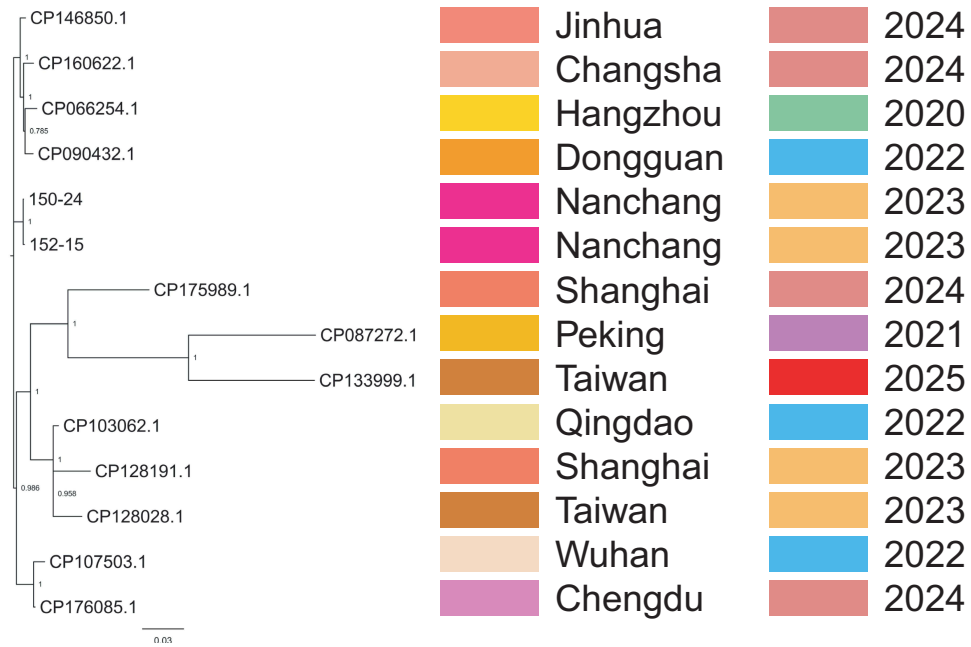

Figure 6 SNP evolutionary analysis.

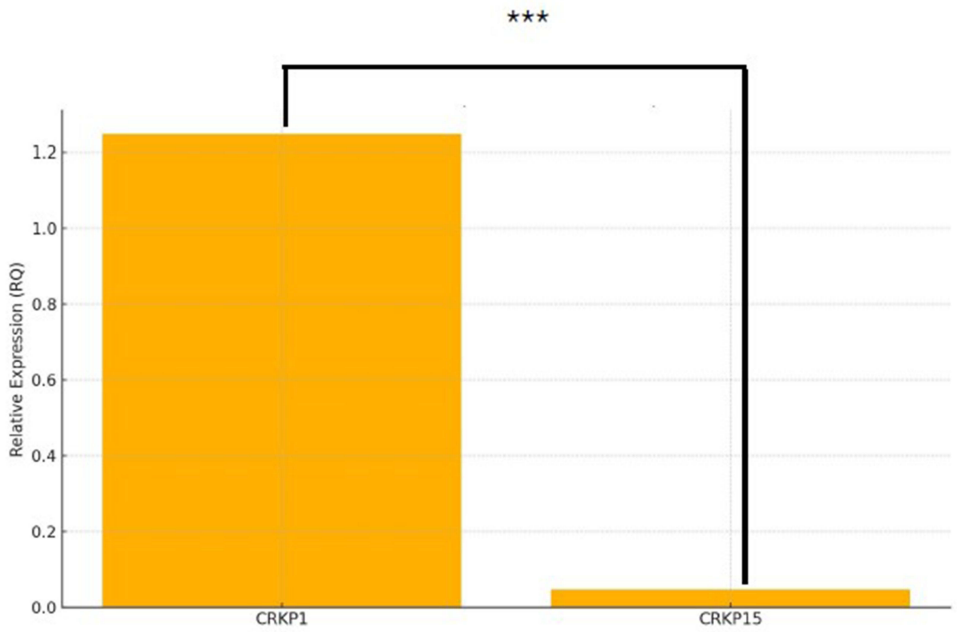

Figure 7 The expression levels of terw gene mRNA in CRKP1 and CRKP15. qPCR of the terw genes of the two strains revealed that the expression of the terw gene in the CRKP1 strain treated with omacycline under stress was significantly increased compared with the CRKP15 strain that was not treated with omacycline.

genes. Therefore, the impact of omadacycline on the evolution of carbapenem resistance plasmids is likely to be minimal. It is also possible that the short time since omadacycline's introduction, as well as the duration and dosage of treatment, have not yet significantly affected the bacterial resistance mechanisms in the short term. Consequently, we will continue to track the clinical data of patients treated with omadacycline and collect corresponding CRKP strains. By increasing the sample size and extending the duration of treatment, we aim to focus on studying the induced bacterial resistance before and after omadacycline use. Through the virulence characterization of the two CRKP strains, significant differences were

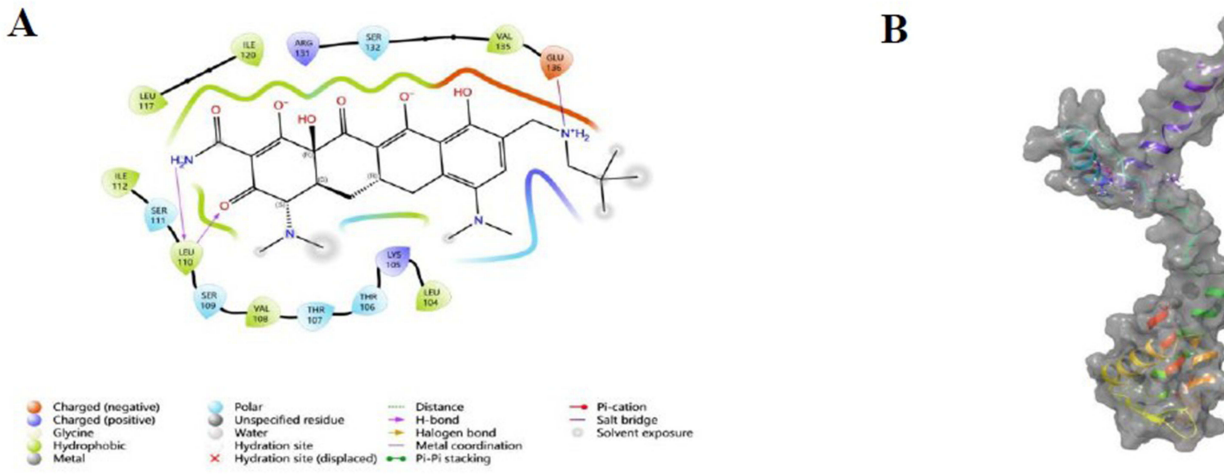

Figure 8 (**A**) 2D diagram of the docking of omadacycline with terw protein; (**B**) 3D diagram of the docking of omadacycline with terw protein. Omadacycline penetrates deep into the living mouth pocket of the terw egg white. terw egg white residues such as LEU110, ILE112, LEU117, ILE120, and VAL135 exert hydrophobic forces on omadacycline. This ligand forms two hydrogen bonds with the residue LEU110 and a salt bridge with the residue GLU136.

observed in biofilm formation, serum resistance, and lethality in the Galleria mellonella infection model. Specifically, CRKP1 was identified as a high-virulence strain, while CRKP15 was a low-virulence strain.

Combining two strains of *Klebsiella pneumoniae* with the same PFGE, ST and K types, it is speculated that the two strains have a close genetic relationship and come from the same clone. On this basis, the clinical data of the two infected patients were analyzed. It was found that the two patients were hospitalized at the same time and had the vast majority of similarities in terms of age, main clinical diagnosis, previous health, and immune status. The antibiotic medication regimens of the two patients were analyzed. Except for omacycline, the types and dosages of other antibiotics were the same. Therefore, the main difference between the two strains in clinical practice lies in the therapeutic stress of omacycline. Meanwhile, the genomic analysis of the two strains revealed that there was not much difference in the gene functions of the two strains, mainly involving cell metabolism, environmental information processing and genetic information processing. Among them, the differences in carbohydrate metabolism and amino acid metabolism were the most significant, which had little impact on the virulence difference of the strains. Furthermore, the qRT-PCR experiment found that compared with the CRKP15 strain not treated with omacycline, the expression of the CRKP1 strain treated with omacycline under stress increased significantly. Molecular docking confirmed the interaction between the *terw* gene and omalamecycline. On this basis, the SNPs of the two strains further confirmed that the *terw* gene is a differential gene between the virulence plasmids of the same clone of the two strains. Therefore, we believe that these two strains of *Klebsiella pneumoniae* have the same genetic and environmental background. These factors excluded the influences of the environment, host immune status and comorbidities on the virulence differences between the two strains. To sum up, these findings suggest that under the therapeutic pressure of omacycline, CRKP may regulate virulence through adaptive evolution, and the *terw* gene may be the key factor for the differences in virulence within this microbiota. In future studies, we need to further explore the genomic composition of the ST11-CRKP strain after omalamecycline treatment and the potential mechanisms of its drug resistance and virulence.

The results of whole-genome sequencing showed that both strains contained four plasmids, including blaKPC. The genes on the IncFII plasmid replicator. Analyze the surrounding environment. The upstream insertion component is ISKPn27, and the downstream insertion component is IS1182. Similar to previous studies, ISKPn27 is a conserved genetic factor. However, the downstream IS1182 is different from the core structure of the popular domestic ISKpn27-blaKPC-ISKpn6.[9] This difference in transposons indicates the variability and diversity of the genetic environment of blaKPC. This might help blaKPC spread rapidly. Another hypothesis holds that genetic variations in the environment may enhance the persistence of antibiotic resistance, making the host more adaptable to changes in the external environment. Therefore, the horizontal transmission of transposons poses a significant threat to clinical practice. In

this study, the genetic annotations of the two strains showed that the types and quantities of resistance genes were similar, but the virulence phenotypes were significantly different. The only clinically significant difference in virulence between the two strains with the same genetic background was the use of omacycline in the anti-infective treatment regimen. The SNP results indicated that the *terw* gene was a differential gene between the virulence plasmids of the two homologous strains. In 2022, Wu Xiufeng et al reported that the *terw* gene is a virulence related gene of highly pathogenic *Klebsiella pneumoniae* (hvKP).[4,5,10] The results of qPCR and molecular docking further proved the correlation between omacycline and the *terw* gene. Based on these findings, we further hypothesize that the virulence evolution of CRKP from the same source under the therapeutic stress of omacycline might be mediated by the *terw* gene.

In conclusion, omacycline treatment may affect the expression of virulence genes or genomic composition of *Klebsiella pneumoniae* for a certain period of time, but drug resistance usually gradually forms during the long-term use of antibiotics. In this study, the clinical differences between the two strains with the same genetic background mainly lie in the treatment with omacycline. Based on the SNP results and the fact that *terw* is a virulence related gene of highly pathogenic *Klebsiella pneumoniae* (hvKP), combined with qPCR and molecular docking analysis, we further speculate that the virulence evolution of CRKP from the same source under the therapeutic stress of omacycline may be caused by the *terw* gene. The specific mechanism of action awaits further verification and analysis. In conclusion, these findings suggest that under the therapeutic stress of omacycline, the evolution of CRKP virulence may be mediated by the *terw* gene. However, our sample size is relatively small, which may limit the universality of our conclusion. This kind of restriction mainly stems from strict inclusion criteria. Nevertheless, our research provides new insights as there is still a scarcity of previous literature on this topic. To solve this problem, we will subsequently collect a large amount of Klebsiella pneumoniae from the same patient before and after the use of omacycline to better support the current research results.

Data Sharing Statement

Article-related data are available from the corresponding author.

Ethics Approval

This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University (Approval Number: No. (2023) CDYFYYLK (01-033)). All procedures adhere to the ethical principles of the Helsinki Declaration (revised in 2013). The written informed consent of all participants was obtained.

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This study did not involve clinical research.

Author Contributions

Jianhua Fang conceptualized and drafted the manuscript; Qiong Liu and Huade Chen collected the strains, performed strain characterization and collected clinical data; Jingyi Huang, Hongyi Lai and Jiayue Li helped with strain characterization and clinical data analysis; Yilin Xu helped with study design; Na Cheng and Tianxin Xiang conceptualization, writing – review and editing. 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.

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Disclosure

The authors report no potential conflicts of interest in this work.

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