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RESEARCH ARTICLE

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Carbapenem antibiotic stress increases *bla*$_{KPC-2}$ gene relative copy number and bacterial resistance levels of *Klebsiella pneumoniae*

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Abstract

Background: The clinical isolation rates of carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) continue to increase. In China, clinical CR-KP isolates are mainly attributed to the *bla*$_{KPC-2}$ gene carried on plasmids, and the *bla*$_{KPC-2}$ copy number correlates with the expression of KPC enzymes, which can cause elevated carbapenem MICs.

Methods: Thirty-seven CR-KP isolates were collected at the Second People's Hospital of Lianyungang City between January 2020 and March 2021, with no duplicate isolates, and were screened for the *bla*$_{KPC-2}$ gene with PCR. Analysis of current CRKP resistance to clinically relevant antimicrobials using the bioMérieux VITEK® 2 bacterial identification card. The multilocus sequence types of the strains were confirmed with PCR and DNA sequencing. Recombinant plasmids pET20b-*bla*$_{KPC-2}$ and pET20b-CpsG were constructed, and the copy numbers of the recombinant plasmids per unit volume was calculated based on the molecular weight of the plasmids. After the genomes DNA of clinical isolates of *K. pneumoniae* carrying the *bla*$_{KPC-2}$ gene were purified, the *bla*$_{KPC-2}$ gene relative copy number in individual *K. pneumoniae* strains was indicated by the double standard curve method. Detection of MIC values changes of *K. pneumoniae* under imipenem selection pressure by broth microdilution method.

Results: Among the 37 CR-KP strains isolated, only the *bla*$_{KPC-2}$ gene was detected in 30 strains, three strains were positive for the *bla*$_{NDM-1}$ gene, two strains carried both the *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ genes, and two strains without detectable carbapenem resistance genes. The ST11 clone was predominant among the 37 carbapenem-resistant *K. pneumoniae* isolates. Drug sensitivity testing showed that except for polymyxins (100% susceptible) and tigecycline (75.7% intermediate), the 37 CR-KP strains were resistant to almost all antimicrobial drugs. The *bla*$_{KPC-2}$ relative copy number in nine ST11 clinical isolates of *K. pneumoniae* was 7.64 ± 2.51 when grown on LB plates but 27.67 ± 13.04 when grown on LB plates containing imipenem. Among these nine isolates, five CRKP strains exhibited elevated MICs to imipenem, while the remaining four strains showed unchanged MIC values to imipenem.

Conclusion: Carbapenem-resistant *Klebsiella pneumoniae* isolates may have multiple pathways to achieve high levels of carbapenem resistance, and moderate carbapenem pressure can increase the copy number of KPC enzyme genes in CRKP strains and enhance the degree of carbapenem resistance in the strains.

KEYWORDS
carbapenemase, drug resistance, *Klebsiella pneumoniae*, relative gene copy number

1 | INTRODUCTION

Klebsiella pneumoniae is one of the commonest pathogens causing hospital-acquired infections, especially in critically ill patients in intensive care units.[1] In recent decades, the widespread use of antimicrobial drugs, immunosuppressive agents, and invasive procedures has led to a significant increase in the clinical isolation of carbapenem-resistant *K. pneumoniae* (CR-KP). The emergence of drug-resistant strains of *K. pneumoniae* has made clinical treatment extremely difficult, leading to a significant increase in mortality among infected patients.[2–5] Carbapenemase production is a common mechanism of resistance to carbapenem killing in these strains, with *K. pneumonia* carbapenemase (KPC) predominating.[6] The presence of the carbapenemase gene is usually predictive of carbapenem resistance phenotypes; however, recent studies have shown that strains with similar carbapenemase genes may differ significantly in the degree of phenotypic resistance (MIC) to carbapenem antibiotics.[7] Therefore, the timely monitoring of drug resistance in *K. pneumonia* and understanding the dynamic changes in its drug resistance and the clinical distribution of this resistance are extremely important for the rational use of antimicrobial drugs and the prevention and control of nosocomial infections, to reduce patient mortality.

In this study, we examined the current drug resistance profile of CR-KP, molecular prevalence characteristics, and the relative number of copies of the bla_{KPC-2} gene contained in individual bacteria as indicated by the *bla*KPC-2/CpsG ratio to assess whether copy number is influenced by carbapenem pressure and the role on carbapenem resistance MIC levels, providing data to support the development of clinical anti-infective strategies.

2 | MATERIALS AND METHODS

2.1 | Clinical strain collection and definitions

CR-KP strains were defined as isolates with resistance or moderately susceptible to at least one type of carbapenem (imipenem, ertapenem, or meropene). Thirty-seven CR-KP strains isolated from the Second People's Hospital of Lianyungang City between January 2020 and March 2021 were selected for this study. The strains mainly originated from the intensive care unit, cerebrovascular surgery department, or respiratory medicine department. The specimen types were sputum and bronchial secretions (53.3%), urine (23.3%), blood (13.3%), and incisional secretions (10%). There were no duplicate isolates from the same patient among the 37 strains.

2.2 | Strain antimicrobial susceptibility testing

The Vitek® 2 system (bioMérieux, Marcy l'Étoile, France) bacterial analyser was used to detect the resistance of 37 carbapenem-resistant *K. pneumoniae* strains to 16 antimicrobial drugs: polymyxin, tigecycline, imipenem, meropenem, amikacin, levofloxacin, ciprofloxacin, minocycline, cotrimoxazole, tobramycin, doxycycline, aminotrans, ceftazidime, cefoperazone/sulbactam, cefepime, and ticarcillin/clavulanic acid. Minimum inhibitory concentrations were interpreted according to the CLSI supplement M100 31th edition. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

2.3 | Preparation of genomic templates

The genomic DNA of the 37 strains was extracted with the boiling method. Two or three single colonies were picked and mixed in an Eppendorf tube containing 200 μl of sterile water, heated at 100°C for 10 min, and centrifuged at 12,000 × *g* for 10 min. The supernatant was used as the template for the subsequent PCR (Table 1). The qPCR template was purified from the bacterial genome kit (Tiangen,).

2.4 | Detection of genotypes

The presence of genes encoding carbapenemases (KPC, NDM, IMP, VIM, or OXA-48) was detected with PCR, using the standard

TABLE 1 Configure 5 resistance genes (KPC-2, IMP, NDM-1, VIM, OXA-48) and 7 MLST housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) according to the reaction system

Reagent	Volume (for 1 sample)
2 × PCR Master Mix	12.5 μl
Forward primer (10 μM)	1 μl
Reverse primer (10 μM)	1 μl
Supernatant (template)	2 μl
ddH2O	8.5 μl
Total	25 μl

TABLE 2 Carbapenem resistance gene primers

PCR name	Sequence (5′-3′)	Tm (°C)	Amplicon size (bp)
KPC-2	TGCTTGTCATCCTTGTTA	50	796
	TAGTTCTGCTGTCTTGTC		
IMP	CATGGTTTGGTGGTTCTTGT	53	488
	ATAATTTGGCGGACTTTGGC		
NDM-1	ATTAGCCGCTGCATTGAT	53	241
	ATTAGCCGCTGCATTGAT		
VIM	GATGGTGTTTGGTCGCATA	55	865
	CGAATGCGCAGCACCAG		
OXA	TTTTCTGTTGTTTGGGTTTT	50	519
	TTTCTTGGCTTTTATGCTTG		

thermal cycling program recommended by the manufacturer (ABI, Veriti96,): 95°C for 10 min, followed by 30 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 1 min. The primers were designed with the Primer Premier 5 program and are shown in Table 2. The 37 PCR products were sequenced (Youkang,) and confirmed with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5 | Multilocus sequence typing (MLST) analysis

Seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) were PCR amplified (Table 3) from all CR-KP isolates, as previously described.[8] The allele numbers and sequence types (STs) were assigned at the MLST website (http://bigsdb.pasteur.fr/klebsiella/).

2.6 | Primer design and synthesis

The single-copy *K. pneumoniae* podoconiosis-associated gene *CpsG* was selected as the chromosomal internal reference gene, according to the published National Center for Biotechnology Information genome sequence ID: CP040993.1 complement (4757121.0.4758491), and the bla_{KPC-2} gene on plasmid pHS10842 ID: KP125892.1 complement (8451.0.9332) was the target sequence examined. Seven pairs of primers were designed and synthesized (Table 4), among which primers 1 and 2 were used for the construction of the recombinant plasmids, and primers 3,4, 5, 6, and 7 were used for amplification with fluorescent qPCR.

2.7 | Construction of recombinant plasmids

TIANprep Mini Plasmid Kit II (Tiangen,) was used to extract the laboratory-stored pHS10842 from DH5α as the template for PCR, and the bla_{KPC-2} gene (between nucleotides 9 and 440) in the plasmid was PCR amplified with primers 1-F and 1-R. The whole genome of *K. pneumoniae* was extracted with the boiling method from a strain of *K. pneumoniae* type ST11(1/30) and used as the template to PCR amplify the *CpsG* gene (between nucleotides 113 and 517) with primers 2-F and 2-R. The PCR system and procedure refer to Detection of Genotypes above. The PCR products were recovered with 1% agarose gel electrophoresis, and excision from the gel. The recovered target fragments were doubly digested with *Xho*I and *Nde*I (NEB, USA). The fragments were then ligated overnight with T4 ligase (NEB, USA) to the pET20b plasmid also doubly digested with *Xho*I and *Nde*I. *E. coli* DH5α (biomed, Beijing, China) was transformed with the individual plasmids. The universal primers T7 and T7T were used to amplify the target sequence from each plasmid to confirm that it was inserted correctly. The successfully constructed plasmids carrying bla_{KPC-2} and *CpsG* were designated pET20b-KPC and pET20b-CpsG, respectively.

2.8 | Real-time PCR conditions

The StepOnePlus™ Real-Time PCR System (Applied Biosystems,) was used in this study. The PCR reaction system components (Tiangen,) are shown in Table 5. The optimal qPCR cycling conditions were 30 s at 95°C and 40 cycles of 10 s at 95°C and 30 s at 60°C. A melting curve analysis was then performed to determine the specificity of the primers. The thermal cycling parameters were 95°C for 15 s and 60°C for 1 min, followed by ramping to 95°C at a rate of 0.3°C/s, resulting in a melting curve in which the fluorescent signal decreased with increasing temperature. Each sample was quantified in triplicate.

2.9 | Construction of standard curves

To generate standard curves for the bla_{KPC-2} and *CpsG* genes, plasmid pET20b-KPC and pET20b-CpsG concentrations (ng/μl) were determined using a nucleic acid detector Quawell Q9000(USA), their corresponding copy numbers were calculated according to the following formula[9], and then diluted 10-fold to 10^8, 10^7, 10^6, 10^5, 10^4 copies/μl (2 μl per reaction) and used as reaction templates for qPCR when establishing the standard curve in triplicate with three independent replicates.

$$(6.02\times10\,(23))\times(\text{ng}/\mu\text{l}\times10[-9])/(\text{DNA length}\times660) = \text{copies}/\mu\text{l}.$$

TABLE 3 Seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*)

Gene	Direction	Primer Sequence	Amplicon size (bp)
gapA	F	TGAAATATGACTCCACTCACGG	662
	R	CTTCAGAAGCGGCTTTGATGGCTT	
infB	F	CTCGCTGCTGGACTATATTCG	468
	R	CGCTTTCAGCTCAAGAACTTC	
mdh	F	CCCAACTCGCTTCAGGTTCAG	756
	R	CCGTTTTTCCCCAGCAGCAG	
pgi	F	GAGAAAAACCTGCCTGTACTGCTGGC	566
	R	CGCGCCACGCTTTATAGCGGTTAAT	
phoE	F	ACCTACCGCAACACCGACTTCTTCGG	602
	R	TGATCAGAACTGGTAGGTGAT	
rpoB	F	GGCGAAATGGCWGAGAACCA	1075
	R	GAGTCTTCGAAGTTGTAACC	
tonB	F	CTTTATACCTCGGTACATCAGGTT	539
	R	ATTCGCCGGCTGRGCRGAGAG	

TABLE 4 The underlined portion of the primer is the introduced restriction endonuclease recognition site. Primers 1 and 2 were used for the construction of recombinant plasmids, and primers 4, 5, 6, and 7 were used for the amplification of fluorescent quantitative PCR

Primer name	Sequence (5′ - 3′)	Target gene	Restriction sites	Amplicon size (bp)
1-F	GGAATTCCATATGCGTTGACGCCCAATCCCT	KPC	Nde I	432
1-R	CCGCTCGAGCGGCCTTCATGCGCTCTAT		Xho I	
2-F	GGAATTCCATATGTCAGCGGACGGGTGAAGTT	CpsG	Nde I	405
2-R	CCGCTCGAGCCGGGAAGATAGTGGTGGGT		Xho I	
3-F	CGTTGACGCCCAATCCCT	KPC-2		432
3-R	CGGCCTTCATGCGCTCTAT			
4-F	CGTTGACGCCCAATCCCT	KPC-2		239
4-R	AAACACGACCGGCAACCAC			
5-F	GCCTCGCTGTGCTTGTCA	KPC-2		123
5-R	CGGAACCTGCGGAGTGTAT			
6-F	TCAGCGGACGGGTGAAGTT	CpsG		405
6-R	CCGGGAAGATAGTGGTGGGT			
7-F	AGGTGGAAAGTGGCGAAGTAA	CpsG		139
7-R	TAGTGGTGGGTGGCGATGT			

TABLE 5 Primer final concentration of 0.3 μl gives good amplification; all RT-PCR reaction solution configurations were performed on ice

Reagent	Volume (for 1 sample)
2×SuperReal PreMix Plus	10 μl
Forward primer (10 μM)	0.6 μl
Reverse primer (10 μM)	0.6 μl
Supernatant (template)	2 μl
ROX Reference Dye	2 μl
RNase-free ddH_2O	4.8 μl
Total	20 μl

2.10 | Calculation of bla_{KPC-2} gene relative copy number

We used the random number method in the excel function "RANDBETWEEN (1, 30)"to evaluate the copy number of blaKPC-2 gene in 9 ST11 isolates randomly selected from 30 strains of CR-KP with positive KPC-2 gene amplification.

Colonies of nine CR-KP strains were cultured overnight on normal Luria–Bertani (LB) plates or LB plates supplemented with 2.7 μg/ml imipenem. Three colonies were picked with sterile tips, suspended in 400 μl of sterile water, heated at 100°C for 10 min, and centrifuged at 12,000×*g* for 10 min. An aliquot (2 μl) of each supernatant

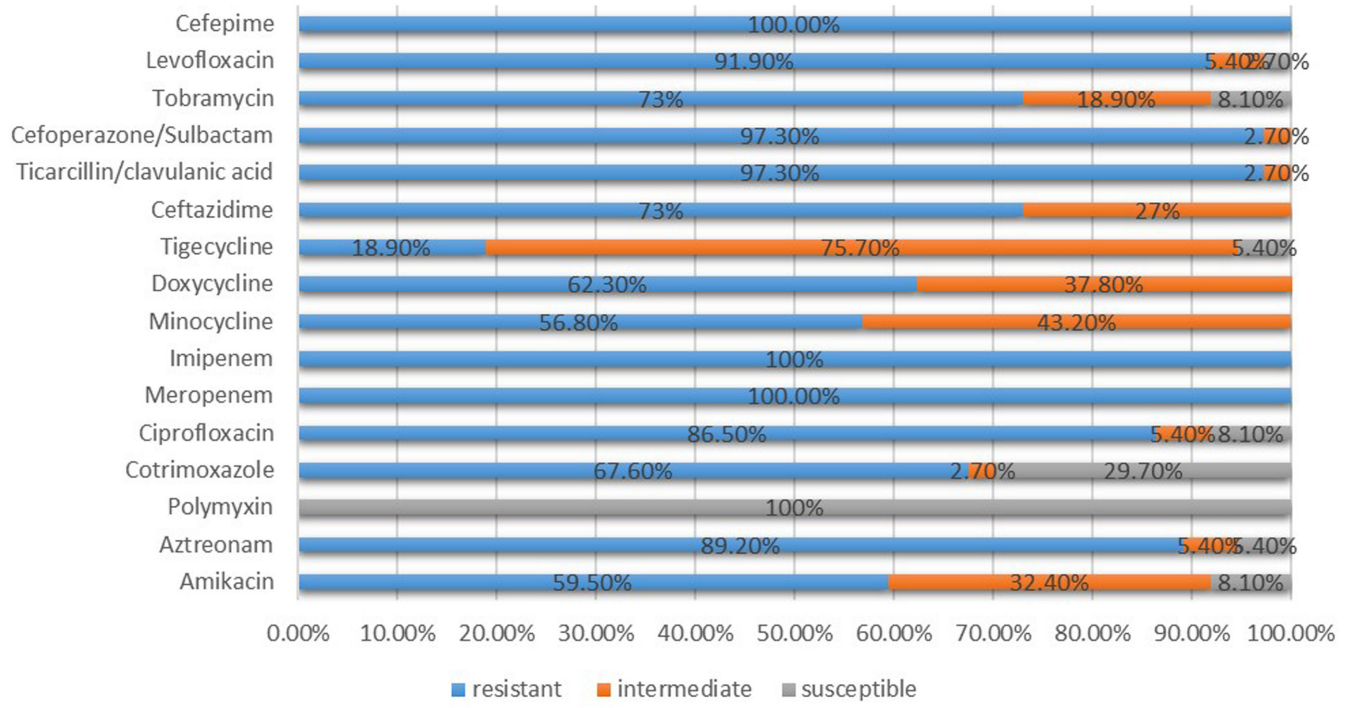

FIGURE 1 Drug susceptibility results of 37 carbapenase-resistant *Klebsiella pneumoniae* strains

was taken as the PCR template so that the cycle threshold (C_T) value fell exactly in the centre of the standard curves for the recombinant plasmids pET0b-CpsG and pET20b-KPC-2. The plasmid copy number was calculated with the absolute quantitative method[10], and the relative copy number of the target gene was calculated by dividing the absolute amount of the target gene by the absolute amount of the endogenous gene in the same sample. Samples with C_T values of <26 and that differed by less than 0.3 between different wells were used to calculate the relative copy numbers. The initial amount of target gene in each bacterium was calculated with the equation: copies of bla_{KPC-2} = amount bla_{KPC-2}/amount *CpsG*.

2.11 | Minimum inhibitory concentration measurements

Minimum inhibitory concentrations (MICs) were [11,12]determined using the previously described broth microdilution method. After grown overnight on Mueller–Hinton (MH) agar plates at 37°C, the CR-KP bacteria were harvested in mid-exponential growth phase. All these antimicrobial agents were serially diluted with MHB from 512 to 1 μg/ml. The bacterial suspension was diluted with phosphate-buffered saline (PBS, pH 7.4) to adjust the turbidity approximately to the standard McFarland 0.5, which nearly corresponds to the concentration of 1×10^8 colony-forming unit (CFU)/ml. Then, this suspension was further diluted by 100-fold with MHB. Subsequently, 100 μl of the diluted bacterial suspension (1×10^6 CFU/ml) was mixed with 100 μl of antimicrobial agent dilution and incubated for 18 h at 37°C. Finally, the MIC was determined as the lowest concentration of these antimicrobial agents, at which no turbidity was observed with unaided eyes. MH broth was utilized as the negative control, and each experiment was performed in triplicates for three times.

3 | RESULTS AND ANALYSIS

3.1 | Antimicrobial susceptibility testing of CR-KP isolates

The 37 strains of CR-KP isolated at our hospital were resistant to most antimicrobial drugs, although some strains showed intermediate sensitivity to antibiotics such as levofloxacin, ciprofloxacin, tobramycin, aminoglutethimide, ceftazidime, and tigecycline. Thirty-seven drug-resistant strains showed zero resistance to polymyxin, which is the last line of defence against clinical infection (Figure 1).

3.2 | Detection of antimicrobial resistance genes

Among the 37 CR-KP strains, carbapenemase gene amplification was positive for the bla_{KPC-2} gene in 30 strains, for the bla_{NDM-1}gene in three strains, and for the bla_{KPC-2} and bla_{NDM-1} genes in two strains. No carbapenemase gene was detected in two strains (Supplementary material).

3.3 | MLST results

The 37 strains were classified into eight sequence types (STs) (Figure 2): ST11 (78.4%, 29/37), ST641 (5.5%, 2/37), ST35, ST76,

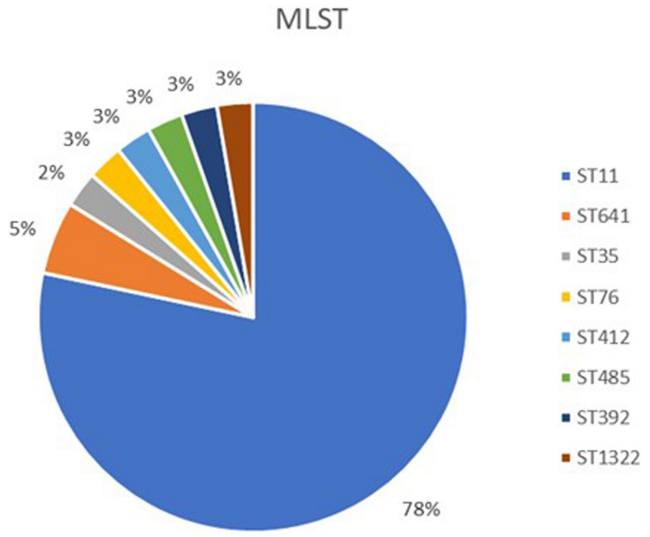

FIGURE 2 MLST analysis results of 37 carbapenem-resistant *Klebsiella pneumoniae* strains

ST412, ST485, ST392, and ST1322 (2.7%, 1/37). Among the 37 strains, ST11 was overwhelmingly dominant, with the most concentrated distribution and relatively homogeneous clonal strains.

3.4 | Construction of standard curves for plasmid copy number determination

The recombinant plasmids pET20b-KPC and pET20b-CpsG were diluted within the concentration range of 10^3–10^7 copies/μl, and the measured C_T values were plotted against the template copy number. The results are shown in Figure 3.

3.5 | Relative copy number of *bla*$_{KPC-2}$ gene

The ratio *bla*$_{KPC-2}$ copies/*CpsG* copies was determined as the copy number of the *bla*$_{KPC-2}$ gene in a single cell of *K. pneumonia*. We tested nine clinical CR-KP strains carrying the *bla*$_{KPC-2}$ gene grown on LB agar in the presence or absence of 2.7 μg/ml imipenem to determine the copy number of the *bla*$_{KPC-2}$ gene. The *bla*$_{KPC-2}$ copy number on normal LB plates was 7.64 ± 2.51, but 27.67 ± 13.04 on the LB plates containing 2.7 μg/ml imipenem (Figure 4).

3.6 | Detection of minimum inhibitory concentration value

We examined the above nine carbapenem-resistant *K. pneumoniae* strains using the broth microdilution method (micro-double dilution method), and the same isolates on the antibiotic plates exhibited elevated relative *bla*$_{KPC-2}$ copy numbers compared to isolates grown on LB plates; however, not all of these nine isolates exhibited elevated carbapenem resistance, and only five CR-KP strains exhibited elevated MICs to imipenem, while the other four strains showed no change in MIC values to imipenem (Figure 5).

4 | DISCUSSION

In recent years, ST11 CR-KP has become the dominant clone in China[13]. We confirmed this finding and demonstrated that 78.4% of the CR-KP clinical isolates collected in this study belonged to the ST11 clone. ST641, ST35, ST76, and other STs were also detected among these isolates.

Carbapenem resistance can result from multiple mechanisms, including the production of carbapenemases, such as KPC (*K. pneumoniae* carbapenemase), NDM (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-β-lactamase), IMP(Imipenem), and OXA-48 (oxacillin-hydrolysing), a combination of pore protein defects, Enterobacteriaceae-produced extended spectrum β-lactamases (ESBLs), or AmpC enzyme production.[14] However, the production of KPC is the commonest mechanism and the genes encoding these carbapenemases can be transferred horizontally,[15] allowing them to spread widely. Notably, *bla*$_{KPC-2}$ is commonly isolated in adult patients in China, followed less frequently by *bla*$_{NDM-1}$, whereas the *bla*$_{NDM-1}$ gene is commonly present in paediatric patients, followed less frequently by *bla*$_{KPC-2}$ and *bla*$_{OXA-48}$[13]. We also detected two strains in which both *bla*$_{KPC-2}$ and *bla*$_{NDM-1}$ were present, suggesting that horizontal gene transfer drives the evolution of incurable "superbugs" by concentrating antibiotic resistance genes in the same cell. This has resulted in a yearly increase in the carbapenem-resistant bacteria detected in clinical settings.

In this study, 37 strains of *K. pneumoniae* were fully resistant to carbapenem antibiotics, whereas aminoglycosides, tetracyclines, and cephalosporin antibiotics remained somewhat effective (albeit <50% effective). Treatments for CR-KP infections are limited to antibiotics with high potential toxicity, such as polymyxins, or those with poor pharmacokinetic or pharmacodynamic profiles, such as tigecycline. The use of these antibiotics is associated with the emergence of resistance to them.[16] Only seven tigecycline-resistant strains were identified among our collection, and the increased use of this drug in recent years, especially as a monotherapy, may have contributed to this situation. In systematic studies[17], the success rates of combination therapies with aminoglycosides (75%), polymyxins (73%), and tigecycline (71%) were higher than those of monotherapies with carbapenems (40%) or polymyxins (14%). For these reasons, most authors recommend the use of combinations of different classes of antibiotics to improve treatment efficacy and prevent the development of further resistance.[18]

Although resistance studies of CR-KP have been extensively reported, the plasmid copy numbers of these clinical strains have been unclear until now. In this study, we developed a sensitive qPCR technique for determining copy numbers of resistance gene in CR-KP. Quantitative PCR[19] is an important and widely used analytical tool. Moreover, more papers have reported qPCR methods to detect the relative copy number of bacteria, plants, and even human cells.[20–23]

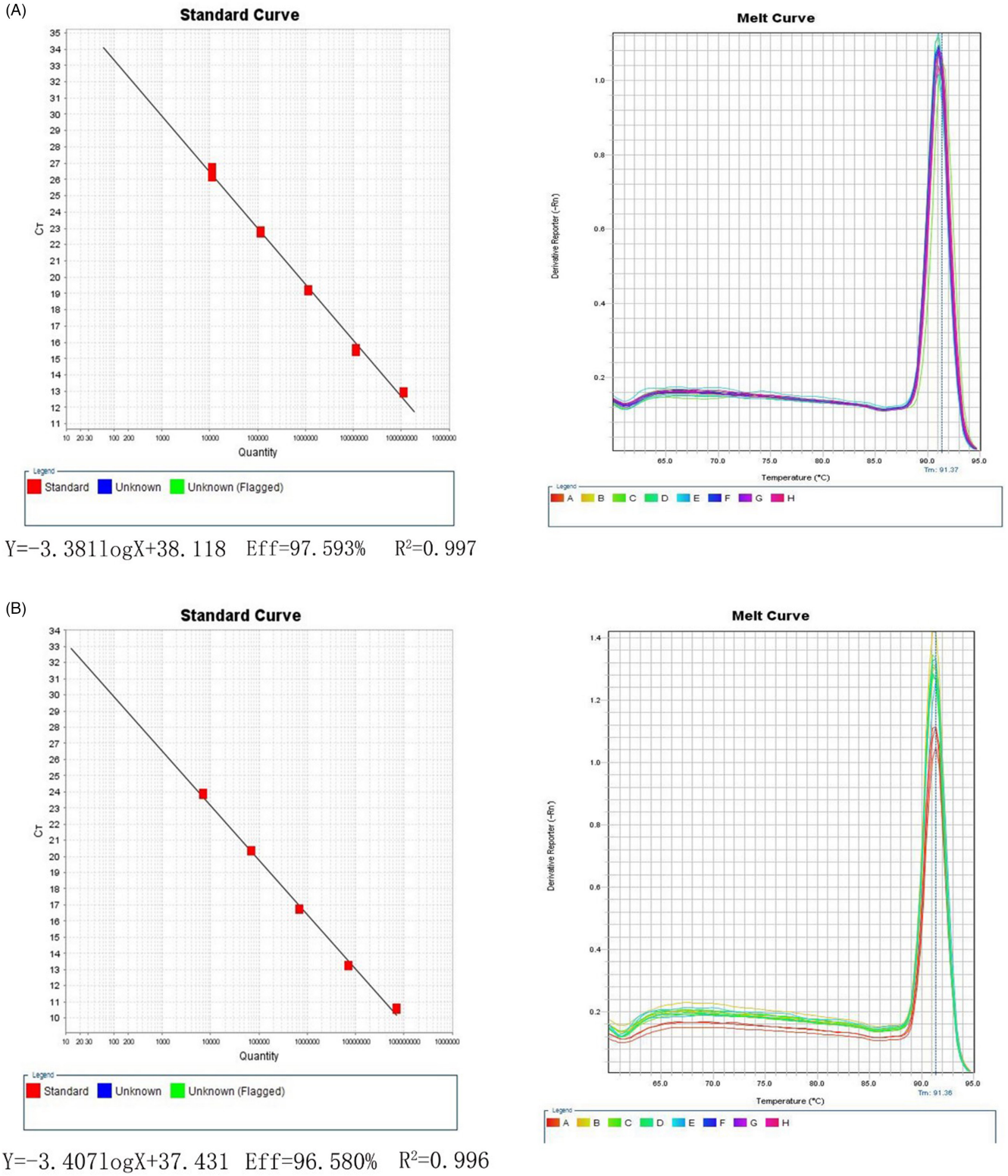

FIGURE 3 (A) and (B) are the standard and melting curves of bla_{KPC-2} and *CpsG*, respectively. The amplification efficiency was between 90% and 110% for fluorescence PCR with $R^2 > 0.99$. In this study, the optimal primer pairs 3-F/3-R and 6-F/6-R were obtained based on three primer pairs designed for the bla_{KPC-2} gene and two primer pairs designed for the *CpsG* gene for fluorescence quantitative PCR, which were judged based on the amplification efficiency of both primers, primer specificity, and comparable amplification efficiency of both

In general, the appropriate assay design, template preparation, and analytical method are essential for accurate quantitative gene amplification.[24] In this experiment, we used the "three identical" principle—same vector plasmid, same batch of samples, and the same fluorescence threshold to obtain the C_T values—and standard curves for the target gene bla_{KPC-2} and the single-copy gene *CpsG*.

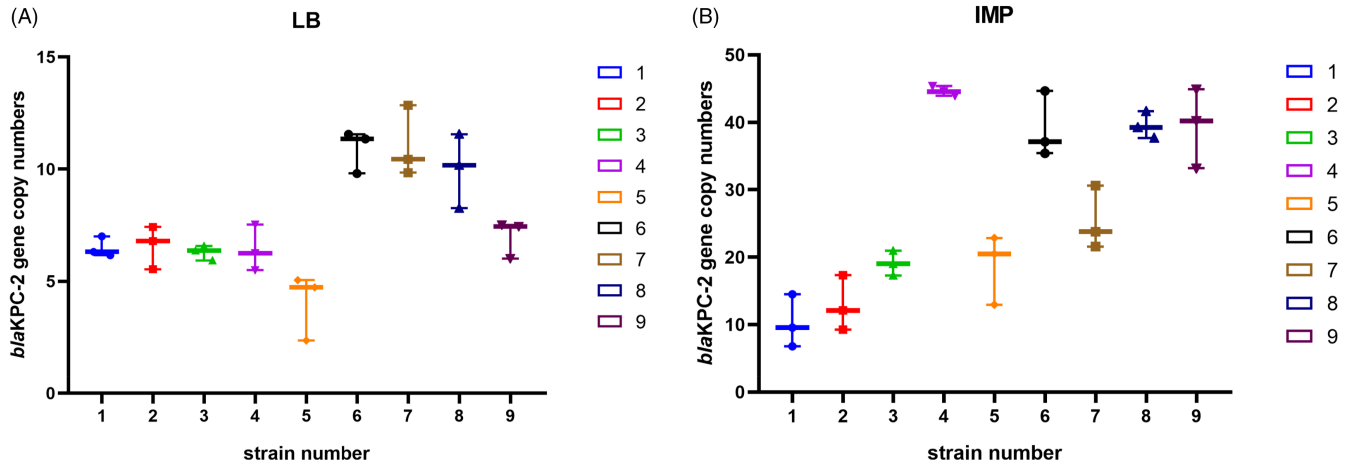

FIGURE 4 A and B are the bla_{KPC-2} gene copy numbers in CR-KP grown on LB plate and LB plate with imipenem (2.7 μg/mL), respectively. Nine ST11-type KPC-2 positive strains were subjected to absolute quantification of bla_{KPC-2} and *cpsG* genes simultaneously, with three replicates per sample, and their average Ct values were used to calculate the starting copy number of each gene. Copy numbers of individual bacterial bla_{KPC-2} genes were obtained based on copies of bla_{KPC-2} = amount bla_{KPC-2}/amount *CpsG*

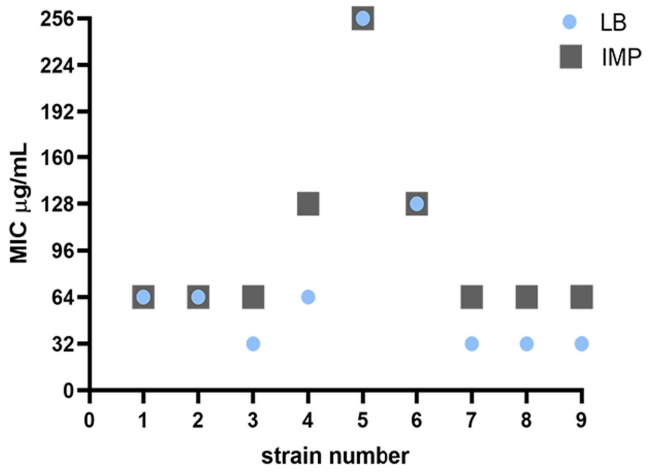

FIGURE 5 The broth microdilution method was used to detect the minimum inhibitory concentration of nine CRKP isolates grown on LB agar plates and LB agar plates containing antibiotic imipenem, and the wells without bacteria were used as negative controls, three replicate wells were set up for each experiment, and the experiment was repeated three times for each strain. As a result, the lowest drug concentration that appeared clear and bright was used as the MIC of the drug

The *CpsG* gene is a capsular polysaccharide[25,26] gene of *K. pneumoniae*, located on its chromosome. In selecting the internal reference gene, we considered 16S rRNA gene sequences, but each genome of *E. coli* contains seven copies of 16S rDNA,[27] resulting in a much greater amplification efficiency than for the target gene, and even an inverse ratio. PCR amplification efficiency is an important factor when using relative quantification methods, and the slope of the ideal standard curve should be $X = 3.32$ for 100% PCR efficiency. In addition, the PCR efficiency needs to be similar for all genes when using the relative copy method, preferably greater than 90%.[28]

The absolute copy number of the endogenous gene was determined using the target gene in the same recombinant plasmid subsequently used to calculate the relative copy number of the target gene. The PCR amplification efficiency for both genes was >95%, which were high enough for copy number calculations.[29] For the bla_{KPC-2} and *cpsG* standard curves, the mean correlation coefficients (R^2) were 0.997 and 0.996, respectively, suggesting a good fit between the experimental data and the fitted function, and the absolute copy numbers of bla_{KPC-2} and *cpsG* in each KPC-2-positive clinical specimen were calculated from the C_T values based on the corresponding standard curves. When performing quantitative PCR assays, especially when testing bacteria,[22,23,30,31] since bacterial genomic DNA is single copy, as long as the copy number of a conserved gene in bacterial genomic DNA can be determined, the number of other genes in bacteria can eventually be quantified relative to each other. Because qPCR has the advantages of simplicity, rapidity and accuracy, this experiment strictly follows the quantitative PCR-related copy number detection method, and the shortcoming is the lack of validation of WGS, digital PCR, classical qPCR and other detection methods, which can be used as a reference for the relative copy number detection of bla_{KPC-2} gene.

Nine bla_{KPC-2}-positive strains were selected for quantitative analysis in this study, and the bla_{KPC-2} copy number in the strains grown on LB plates was 7.64 ± 2.51. We found 3.62-fold increase in the carbapenemase gene bla_{KPC-2} when the bacterial cells were exposed to selective pressure exerted by the antibiotic imipenem. We speculate that under antibiotic selective pressure, the copy number of plasmids carrying the bla_{KPC-2} gene in bacteria increases, leading to increased levels of bacterial resistance. There is strong evidence[6,32,33] that plasmid copy number regulation contributes to the regulation of plasmid-bearing bacteria during antibiotic treatment and promotes host survival. For the same host bacterium, the strength of drug resistance mainly depends on the resistance gene present, with high copy number expression strains producing optimal expression rates. A survey of plasmids[34] in *E. coli* confirmed that antibiotic resistance genes are rarely found on small plasmids. Although some (often

large) plasmids exist only as single copies within bacterial cells, many plasmids exist in multiple copies per cell, sometimes in hundreds of copies.[35] The importance of the plasmid copy number on the evolutionary dynamics of plasmid-encoded genes and the implications for bacterial adaptation have recently received increased attention.[36] One study hypothesized that the adaptation of bacteria depends on the intracellular concentrations of antibiotics and plot the corresponding dose–response curves. The rate of antibiotic degradation increased as the number of antibiotic plasmids in the bacterial cell increased.[37]

The copy number of the bla_{KPC-2} gene has been described to have an effect on the level of carbapenem resistance,[6] and we examined those isolates that showed a significant increase in gene copy number after exposure to IMP for their increased imipenem MIC values using the broth microdilution method (micro-double dilution method). Compared to CR-KP isolates grown on LB plates, not all nine isolates exhibited elevated carbapenem resistance, only five CR-KP isolates exhibited elevated MICs to imipenem, from 32 μg/ml to 64 μg/ml or 64 μg/ml to 128 μg/ml, while the other four exhibited unchanged MICs to imipenem. Due to the methodological shortcomings of the broth microdilution method itself, with a large inter-well span, we were unable to obtain exact MIC values, but it is not difficult for us to find that moderate antibiotic pressure can increase the MIC levels of CR-KP to imipenem, and this also suggests that KPC enzymes alone are not always sufficient to confer carbapenem resistance, but that multiple resistance mechanisms coexist (production of carbapenem hydrolases,[38–40] reduction of carbapenem membrane permeability,[41] loss of bacterial channel proteins,[42] overexpression of bacterial active efflux systems,[43] etc.). For example, the copy number of strain 5 in this study was relatively low, but its drug resistance level reached 256 μg/ml. These factors causing carbapenem resistance are usually studied individually, and few data are available to explore their combined effect on carbapenem MICs in clinical CR-KP strains.

In summary, our results suggest that moderate carbapenem stress can increase the copy number of the KPC enzyme gene in CR-KP strains and increase the degree of carbapenem resistance of the strains. These results also suggest that KPC-positive isolates that cause elevated carbapenem MICs may have multiple pathways to achieve high levels of carbapenem resistance. Furthermore, as plasmids carrying the resistance gene bla_{KPC-2} continue to spread in *K. pneumoniae*, KPC-producing isolates with high levels of carbapenem resistance may become more common. Our analysis confirms the complexity of the carbapenem resistance phenotype, enhances our understanding of the many factors that contribute to carbapenem resistance in *K. pneumoniae*, and highlights the threat will continue to pose to our patient health.

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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SUPPORTING INFORMATION

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