

A comparative analysis of CRISPR systems, virulence factors, and antibiotic resistance genes in carbapenem-sensitive and carbapenem-resistant *Klebsiella pneumoniae*

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae* is a major cause of healthcare-associated infections, particularly in immunocompromised patients. This study compares the CRISPR systems, virulence factors, and antibiotic resistance genes in carbapenem-sensitive (CSKP) and carbapenem-resistant (CRKP) clinical isolates.

Materials and Methods: Carbapenemase-producing isolates were identified by mCIM/eCIM. PCR and RT-qPCR detected key genes, including cas3, involved in CRISPR-Cas function. In silico analyses included STRING for protein interactions, CRISPRCasdb for CRISPR subtype distribution, and Phyre2/AlphaFold for cas3 structure prediction.

Results: Among the isolates, 35.2% were resistant to carbapenems. Among CRKP strains, high prevalence of *bla*-NDM-1 (82%) and *bla*-OXA-48 (64%) was observed. The cas3 expression was significantly upregulated in resistant isolates ($P = 0.002$). CRISPR subtype I-E was identified in 16% of CRKP and 36% of CSKP isolates. Structural-functional analysis supported the integrity of Cas3 and revealed interactions with regulatory and iron acquisition proteins. Statistically significant differences in virulence and resistance gene profiles were found between CRKP and CSKP groups ($P < 0.05$).

Conclusion: This study highlights key differences between CRKP and CSKP isolates, particularly in CRISPR-Cas systems, resistance, and virulence. The findings suggest that cas3 plays a critical role in genomic adaptation and resistance mechanisms in *K. pneumoniae*, offering insights for future therapeutic strategies.

Keywords: *Klebsiella pneumoniae*; CRISPR-cas systems; Drug resistance; Virulence factors; Gene expression regulation

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INTRODUCTION

Klebsiella pneumoniae is a pathogenic bacteria that serves as a principal etiological agent of nosocomial infections in immunocompromised individuals (1). *K. pneumoniae* primarily induces conditions such as pneumonia, bloodstream infections, and urinary tract infections (UTIs), and its development of antibiotic resistance mechanisms poses a significant concern to global public health (2-4). Carbapenems are one of the few antibiotics that are effective against infections caused by *K. pneumoniae*, which is why they are considered the last resort due to their potent antibacterial activity. However, the appearance of carbapenem-resistant *K. pneumoniae* (CRKP) strains has made treatment fail and raised the risks of morbidity and mortality (5, 6). Carbapenem resistance has many causes, such as the creation of carbapenemases, extended-spectrum beta-lactamases (ESBLs), and changes in proteins that make up the outer membrane (7-9). CRKP has become a growing concern due to its ability to spread through hospital environments and its association with poor clinical outcomes (10). While much research has focused on carbapenem-resistance mechanisms, there is an increasing interest in understanding the differences in virulence factors between carbapenem-resistant and carbapenem-sensitive *K. pneumoniae* (CSKP) strains (11).

While considerable research has focused on the molecular mechanisms underlying carbapenem resistance, less is known about how resistance correlates with virulence factors. Virulence factors, such as capsular polysaccharides, siderophores, fimbriae, and lipopolysaccharides, play a critical role in bacterial pathogenicity and host immune evasion (12). Understanding differences in virulence between CRKP and CSKP strains can offer insights into their clinical behavior and inform therapeutic strategies. Recent attention has turned to the role of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas systems in bacterial evolution and resistance (13). CRISPR-Cas systems, identified initially as a bacterial immune defense mechanism against phages, have been implicated in influencing the horizontal transfer of resistance genes (14, 15). *Klebsiella* spp. exhibit CRISPR-Cas systems, specifically type I (including types I-F, I-E, and I-E*) and type IV (predominantly type IV-A) systems. The type I CRISPR system is predominantly found on chromo-

somes, while the type IV system is solely prevalent in plasmids (16-19).

The Type I-E CRISPR-Cas system is located in the *cysH-iap* region and consists of a *cas* operon, 29 bp direct repeats, and a CRISPR array (CRISPR1) downstream of the *cas* genes. In contrast, the Type I-E* system, which is more variable, is found within the ABC transport system-glyoxalase region and often contains a transposase-encoding gene within the *cas* operon. Additionally, Type I-E* includes two CRISPR arrays, CRISPR2 and CRISPR3, flanking the *cas* genes (20-22). In the type I CRISPR-Cas system, the *cas3* gene functions as the marker gene. The *cas1* gene is a ubiquitous *cas* gene present in all types of CRISPR-Cas systems (23-25). Although CRISPR systems in *K. pneumoniae* have been associated with limiting plasmid uptake, their dual role in regulating resistance and virulence remains inadequately explored (26). Despite these advances, there is a clear gap in understanding how CRISPR systems might simultaneously influence antibiotic resistance and virulence in CRKP and CSKP strains. Addressing this gap is essential because the interplay between resistance mechanisms and virulence factors may affect infection severity, transmission dynamics, and treatment outcomes. This study sought to conduct a comparative examination of CRISPR systems, virulence factors, and antibiotic resistance genes in CRKP and CSKP strains. By examining these genomic and phenotypic traits concurrently, we aim to clarify whether CRISPR-Cas systems influence the acquisition of resistance and virulence factors. We hypothesize that variations in CRISPR-Cas system profiles between CRKP and CSKP strains are associated with differences in both resistance gene content and virulence factor expression. This study represents the first comparative analysis of CRISPR-Cas systems and the distribution of virulence genes in *K. pneumoniae* isolates from Iran. Given the growing significance of *K. pneumoniae* as a hospital-associated pathogen and its increasing resistance to various antibiotics, understanding the role of CRISPR-Cas systems, particularly in relation to virulence and resistance genes, plays a crucial role in advancing our knowledge of molecular mechanisms and identifying potential therapeutic targets. Furthermore, elucidating the relationships between CRISPR-Cas systems and antibiotic resistance or virulence factors may contribute to a deeper understanding of bacterial pathogenicity and resistance mechanisms.

MATERIALS AND METHODS

Patients and specimens. In this cross-sectional study that was conducted for one year from April 2023 to June 2024, 142 samples (urine, sputum, blood, and wound) were obtained from patients with various infections from Imam Reza Hospital (a large teaching hospital in Tabriz, Iran).

Microbiological procedure. The specimens were transferred to the microbiology laboratory. The obtained samples were cultured on sheep blood agar (SBA) and incubated at 37°C for 24 to 48 hours. The identification of *K. pneumoniae* involved the utilization of Gram staining and biochemical tests, such as Esculin hydrolysis, oxidase, methyl red, acid generation from glucose, motility, maltose, and lactose, as well as urease assays.

Antibiotic resistance profiles and demographic data of the patients. As previously mentioned, in this study, 142 samples were collected from patients with various infections at Imam Reza Hospital. The isolates were evaluated for their antibiotic resistance profiles using the modified carbapenem inactivation method (mCIM) and ethylenediaminetetraacetic acid (EDTA)-modified carbapenem inactivation method (eCIM) methods. Demographic data of the patients (including age, gender, and clinical condition) were collected in detail (Table 1). Furthermore, the majority of the isolates were obtained from nosocomial infections, which significantly influenced the severity of antibiotic resistance.

Phenotypic assays: the mCIM and eCIM. Phenotypic verification of carbapenemase synthesis was conducted via the modified carbapenem inactivation method (mCIM). The EDTA-modified carbapenem inactivation method (eCIM) was employed to distinguish metallo-beta-lactamases (MBLs) from serine carbapenemases. Both experiments utilized a meropenem disk (10 µg, Liofilchem, Italy), with *Escherichia coli* ATCC 25922 serving as the indicator

strain. The methodology for mCIM and eCIM was as follows: A 1-µL aliquot of the *K. pneumoniae* isolate was suspended in two microtubes, each containing 2 mL of tryptic soy broth (TSB). The suspension was vortexed for 15 seconds. One microtube was made without EDTA (mCIM), whereas the other contained 0.5 M EDTA (eCIM). A meropenem disk (10 µg) was inserted into each microtube with sterile forceps, and the tubes were incubated at 37°C in ambient air for 4 hours. The disks were extracted from the mixture with a 10 µL inoculating loop and positioned onto Muller Hinton agar (MHA) plates that had been previously infected with a suspension of *E. coli* ATCC 25922, a carbapenem-susceptible strain employed as the mCIM indicator organism. The plates were subsequently incubated at 37°C for 18 to 24 hours. After incubation, the inhibition zones were evaluated with the conventional disc diffusion technique. The proliferation of *E. coli* adjacent to the meropenem disc signified carbapenem breakdown (positive mCIM), whereas a distinct inhibitory zone surrounding the disk indicated preserved antibiotic efficacy (negative mCIM). An elevation of 5 mm or greater in the zone diameter for eCIM relative to mCIM indicated the presence of metallo-β-lactamases.

Polymerase chain reaction. The DNA was isolated from bacterial colonies that had grown on the MHA by using the boiling method. The PCR procedure was conducted using a final volume of 20 µL, which consisted of 10 µL of a 2X master mix including Taq DNA polymerase, MgCl₂, and dNTPs (Ampliqon Co, Denmark), and 1 µL of each primer. Furthermore, 2 µL of DNA template was utilized in each PCR reaction. The components were amalgamated and adjusted to a final volume of 20 µL using sterile distilled water. Amplification reactions were conducted using a BIO-RAD T100™ thermocycler.

In this study, the frequency of genes associated with carbapenem resistance, including *bla-OXA-48*, *blaK-PC*, *bla-NDM-1*, *bla-IMP*, *bla-VIM*, *bla-AIM*, *bla-GIM*, *bla-BIC*, *bla-SIM*, *bla-DIM*, and *bla-SPM*, was investigated. Additionally, the frequency of CRISPR

Table 1. Demographic data of the patients

Gender	Age range (Years)	Clinical condition	Infection source
Male = (65%)	12-60	Underlying Disease (e.g., dia-betes, hypertension) = 68%	Nosocomial (Hospital-acquired) = 83%
Female = (35%)		No Underlying Disease = 32%	Community-acquired = 17%

genes such as CRISPR1, CRISPR2, CRISPR3, *cas1*, and *cas3* was examined. Isolates that exhibited at least one CRISPR locus or any of the *Cas* genes were classified as CRISPR-Cas positive, while those lacking these genes were considered CRISPR-Cas negative. Furthermore, the study investigated the presence of virulence genes such as *kfu*, aerobactin, *silS*, *ironN*, *iucB*, *lutA*, *ybtS*, *terW*, *entB*, *repA*, and *rmpA2*, as well as resistance genes like *blaTEM*, *blaSHV*, and *blaC-TX-M* in the collected samples (the primer sequences for each gene are listed in Table 2). The PCR results were analyzed using electrophoresis on a 1% agarose gel, stained with a safe stain, and viewed with a gel documentation system (UVP, USA).

Quantitative reverse transcription PCR (RT-qPCR) assay. The RT-qPCR test was performed by using Applied Biosystems StepOnePlus™ real-time PCR equipment. The experiment employed the SYBR Premix EX Taq II, Tli RNaseH Plus (Takara Bio Inc.). The RNA was isolated using a Favorgen kit from Pingtung, Taiwan. The DNA-free RNA was then utilized to produce cDNA following the directions provided by the manufacturer, Yekta Tajhiz Azma from Tehran, Iran (Cat. No.: YT4500). In the present study, to investigate the variations in *cas3* gene expression between CRKP and CSKP groups, a specific primer was designed (Table 2). The qRT-PCR experiment was conducted in 96-well strips, with each reaction

Table 2. The sequence of primers used for PCR

Target gene	Primer sequence (5'-3')	Product size (bp)
<i>bla-OXA-48</i>	F: GCGTGGTTAAGGATGAACAC R: CATCAAGTTCAACCCAACCG	438
<i>bla-KPC</i>	F: CGTCTAGTTCTGCTGTCTTG R: CTTGTCATCCTTGTTAGGCG	798
<i>bla-NDM-1</i>	F: GGTTTGGCGATCTGGTTTTTC R: CGGAATTGGCTCATCACGATC	621
<i>bla-IMP</i>	F: GGAATAGAGTGGCTTAAAYTCTC R: GGTTTAAAYAAAACAACCACC	232
<i>bla-VIM</i>	F: GATGGTGTTTGGTCGCATA R: CGAATGCGCAGCACCAG	390
<i>bla-AIM</i>	F: CGAATGCGCAGCACCAG R: GTTCGGCCACCTCGAATTG	322
<i>bla-GIM</i>	F: TCGACACACCTTGGTCTGAA R: AACTTCCAACCTTGCCATGC	477
<i>bla-BIC</i>	F: TATGCAGCTCCTTTAAGGGC R: TCATTGGCGGTGCCGAACAC	537
<i>bla-SIM</i>	F: TACAAGGGATTCGGCATCG R: TAATGGCCTGTTCCCATGTG	570
<i>bla-DIM</i>	F: GCTTGTCTTCGCTTGCTAACG R: CGTTCGGCTGGATTGATTTG	699
<i>bla-SPM</i>	F: AAAATCTGGGTACGCAAACG R: ACATTATCCGCTGGAACAGG	271
<i>CRISPR1</i>	F: CGGTTCTTCGGGCTTAAACG R: CTGCTGCAATGACGCCAG	391
<i>CRISPR2</i>	F: TGTTCCGCGCTGAGTTTATG R: TACCACGCCAGTTACTACGC	459
<i>CRISPR3</i>	F: GACGCTGGTGGCATTCTTGAG R: CGCAGTATTCCTCAACCGCCT	1598
<i>cas1</i>	F: CTTTTGGCACGACGGAATCA R: TGGCGCTGGATGATGATTTG	381
<i>cas3</i>	F: GTCCCGACTAAAATGCGTCC R: CGTTGATGGCGGTGATGAAT	598

Table 2. Continuing...

<i>kfu</i>	F: GGCCTTTGTCCAGAGCTACG R: GGGTCTGGCGCAGAGTATGC	638
<i>lutA</i>	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	300
<i>ybtS</i>	F: GACGAAACAGCACGGTAAA R: GAGCATAATAAGGCGAAAGA	242
<i>terW</i>	F: ATGCAATTAACACCAGACAG R: CTCATTCTCTTGAGTGTTTTC	239
<i>aerobactin</i>	F: GCATAGGCGGATACGAACAT R: CACAGGGCAATTGCTTACCT	556
<i>entB</i>	F: GTCAACTGGGCCTTTGAGCCGTC R: TATGGGCGTAAACGCCGGTGAT	400
<i>repA</i>	F: GGCCAATGATAACAATCAG R: GAATGACCAGTACATAATCC	857
<i>silS</i>	F: CATAGCAAACCTTCCAGGC R: ATCGGCAGAGAAATTGGC	803
<i>iroN</i>	F: AAGTCAAAGCAGGGGTTGCCCG R: TGACGCCGACATTAAGACGCAG	669
<i>iucB</i>	F: ATGTCTAAGGCAAACATCGT R: TTACAGACCGACCTCCGTGA	948
<i>rmpA2</i>	F: CTTTATGTGCAATAAGGATGTT R: CCTCCTGGAGAGTAAGCATT	451
<i>blaTEM</i>	F: TGC GTATTATCCCGTGTTG R: TCGTCGTTTGGTATGGCTTC	296
<i>blaSHV</i>	F: AGCCGCTTGAGCAAATTAAC R: ATCCCGCAGATAAATCACCAC	713
<i>blaCTX-M</i>	F: TTTGCGATGTGCAGTACCAGTA R: CGATATCGTTGGTGGTGCCATA	544
<i>cas3</i>	F: GTCCCGACTAAAATGCGTCC R: TTGAGAACACCATTGCCGAA	199
Universal (16s rRNA)	F: TGGAGCATGTGGTTTAATTCGA R: TGCGGGACTTAACCCAACA	159

including 10 μ L of Master Mix, 1 μ L of forward primer, 1 μ L of reverse primer, 2 μ L of template cDNA, and 6 μ L of DEPC water, resulting in a final volume of 20 μ L.

The RT-qPCR parameters for the *cas3* gene were as follows: The hot spot Taq polymerase reaction includes a hold phase at 95°C for 15 minutes. An initial denaturation occurs at 95°C for 30 seconds, succeeded by 40 cycles of annealing at 60°C for 45 seconds, and concluding with an extension at 72°C for 45 seconds. The amplification parameters for the universal (16s rRNA) were as follows: The Taq polymerase reaction includes a hold phase at 95°C for 15 minutes. The procedure commences with an initial denatur-

ation at 95°C for 20 seconds, succeeded by 40 cycles of annealing at 60°C for 20 seconds, and concludes with an extension at 72°C for 30 seconds.

Bioinformatic analysis. Publicly available databases and computational tools were used to perform in silico analyses that supported the experimental findings. Protein-protein interaction (PPI) networks were constructed using the STRING v11.5 platform, based on the annotated *K. pneumoniae* MGH78578 reference genome, to explore potential functional relationships among *cas3*, *repA*, *kfu*, *ybtS*, and *rmpA2*. The accession number for the *K. pneumoniae* MGH78578 genome is NC_009648, and it was select-

ed from the NCBI database (National Center for Biotechnology Information). CRISPR-Cas subtype I-E distribution was evaluated using CRISPRCasdb and literature data to compare global trends with the studied isolates. Additionally, homology-based 3D structure prediction for *cas3* was conducted via Phyre2 and AlphaFold to assess its domain organization and functional conservation. These analyses provided supportive evidence for the roles of CRISPR systems in modulating resistance and virulence-related pathways in *K. pneumoniae*.

Statistical analysis. The statistical analyses were conducted using SPSS v.27.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism (v.9.4.1). Before applying parametric tests, normality of the data was assessed using the Shapiro-Wilk test. For comparisons between groups, a t-test was used for normally distributed variables, while non-parametric tests (Mann-Whitney U test) were applied for skewed data. To control for multiple comparisons, the Bonferroni correction was used, and exact P-values are reported. Effect sizes (Cohen's d) and 95% confidence intervals (CIs) are provided for all relevant analyses. Furthermore, the relative expression analysis was performed using the Pfaffl approach, which combines elements of the standard curve and $\Delta\Delta CT$ method. A P-value below 0.05 was deemed statistically significant.

Ethical consideration. This study was conducted in accordance with the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of Tabriz University of Medical Sciences, Iran (Approval Number: Tabriz, Iran, No. IR.TB-ZMED.VCR.REC.1400.096).

RESULTS

The mCIM and eCIM. The results of the mCIM test showed that 142 samples collected from patients with various infections, 50 (35.2%) samples were positive in the mCIM test, indicating resistance to carbapenems. Fifty CRKP isolates were tested using the eCIM because the eCIM test is relevant for carbapenem-resistant isolates. The eCIM test specifically identifies carbapenemase production, which is a critical factor in determining carbapenem resistance. To ensure a valid comparison, we selected 50 CSKP isolates

from the 92 available CSKP strains. These 50 CSKP isolates were chosen to match the CRKP isolates in terms of clinical and demographic characteristics. This allowed for a meaningful comparison between the two groups, with the primary aim of evaluating differences in resistance and virulence profiles. For 50 samples that were mCIM positive, the eCIM test was used to check Serine carbapenemase and Metallo- β -lactamase. Out of 50 mCIM positive samples, 32 (64%) were Metallo- β -lactamase and 18 (36%) were Serine carbapenemase. In contrast, the remaining 92 (64.8%) samples did not demonstrate this resistance and were sensitive to carbapenem antibiotics (Fig. 1).

PCR. The PCR results indicated the frequency of genes associated with carbapenem resistance as follows: 32 (64%) samples included the *bla-OXA-48* gene, 41 (82%) samples contained the *bla-NDM-1* gene, and 23 (46%) samples contained both *bla-OXA-48* and *bla-NDM-1* genes. Other genes involved in resistance to carbapenem antibiotics (*blaKPC*, *bla-IMP*, *bla-VIM*, *bla-AIM*, *bla-GIM*, *bla-BIC*, *bla-SIM*, *bla-DIM*, and *bla-SPM*) were not detected. Furthermore, the frequency of other genes in *K. pneumoniae* strains producing carbapenemase was as follows: subtype I-E CRISPR-cas system; 8 (16%), *kfu*; 21 (42%), *aerobactin*; 9 (18%), *Sils*; 37 (74%), *IroN*; 10 (20%), *IutA*; 9 (18%), *iucB*; 10 (20%), *repA*; 19 (38%), *rmpA2*; 12 (24%), *entB*; 49 (98%), *ybtS*; 29 (58%), *terW*; 35 (70%), *blaTEM*; 23 (46%), *blaCTX-M*; 27 (54%), and *blaSHV*; 44 (88%). In comparison, the gene frequencies among *K. pneumoniae* strains that did not produce carbapenemase were: subtype I-E CRISPR-cas system; 18 (36%), *kfu*; 11 (22%), *aerobactin*; 2 (4%), *Sils*; 17 (34%), *iroN*; 3 (6%), *iucB*; 3 (6%), *IutA*; 2 (4%), *repA*; 6 (12%), *rmpA2*; 3 (6%), *entB*; 46 (92%), *terW*; 19 (38%), *ybtS*; 18 (36%), *blaTEM*; 9 (18%), *blaCTX-M*; 18 (36%), and *blaSHV*; 27 (54%) (Fig. 2).

Based on the percentages mentioned above, it was determined that the prevalence of *kfu*, *aerobactin*, *iroN*, *IutA*, *iucB*, *repA*, *rmpA2*; *ybtS*, *terW*, and *blaTEM* genes in CRKP strains was more than in CSKP strains ($P < 0.05$). In contrast, the prevalence of the subtype I-E CRISPR-cas system in CSKP was more than in CRKP strains ($P < 0.05$), and the prevalence of *entB*, *blaSHV*, and *blaCTX-M* genes between the groups did not show statistically significant differences ($P > 0.05$).

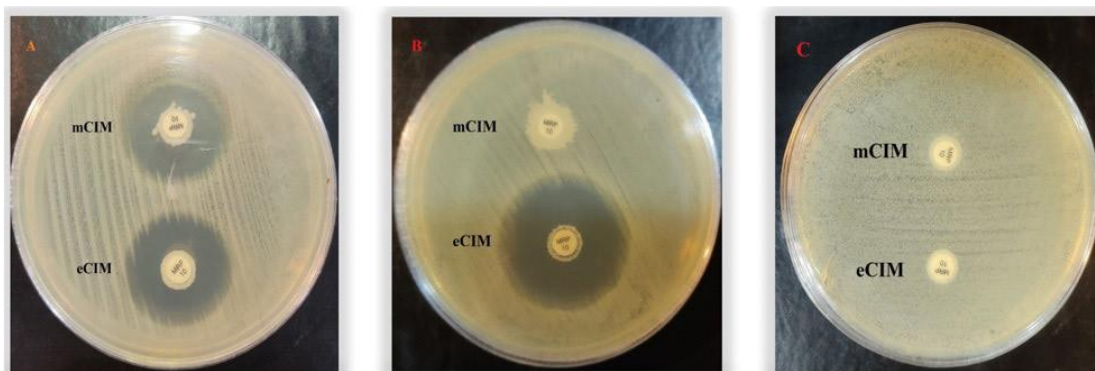


Fig. 1. A: The negative findings of the mCIM and eCIM tests indicate that the bacteria are sensitive to carbapenems. B: *K. pneumoniae* strains resistant to carbapenems are identified when there is an increase of 5 mm or more in the zone diameter for eCIM compared to mCIM, signifying the presence of metallo-β-lactamase. C: *K. pneumoniae* strains resistant to carbapenemase are indicated by the absence of a 5 mm or greater increase in the zone diameter for eCIM relative to mCIM, which signifies the presence of serine carbapenemase.

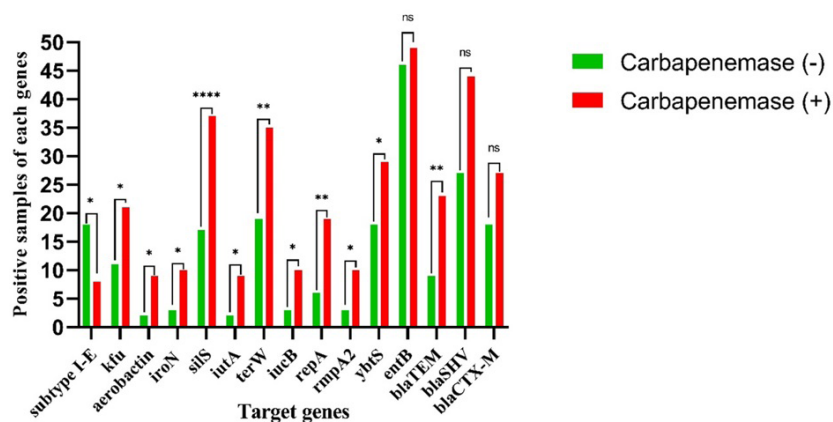


Fig. 2. Distribution of CRISPR-associated, virulence, and antimicrobial-resistance genes among carbapenem-sensitive and carbapenem-resistant *K. pneumoniae* isolates. Bars represent the number of gene-positive samples for each target gene. Significance levels: ns = not-significant, *P = 0.01 – 0.04, **P = 0.001 – 0.003, ****P < 0.0001.

RT-qPCR. RT-qPCR analysis revealed a significant increase in the relative expression of the *cas3* gene in carbapenem-resistant CRKP strains compared to CSKP, with a p-value of 0.002 (Fig. 3). This suggests that *cas3* expression is upregulated in response to factors associated with carbapenem resistance. Given the role of *cas3* as a key component of the CRISPR-Cas system, its higher expression in CRKP strains may indicate an active defense mechanism against foreign genetic elements such as plasmids, which often carry resistance genes. These findings point to a possible association between *cas3* expression and the maintenance or regulation of resistance determinants, warranting further investigation into its potential role. It is worth noting that in the present study, relative gene

expression analysis was performed using the Pfaffl approach, which combines elements of the standard curve and the $\Delta\Delta CT$ method. This method allows for accurate calculation of relative gene expression levels in RT-qPCR experiments. For data normalization, 16S rRNA was used as the housekeeping gene. The validation of 16S rRNA as a stable housekeeping gene was performed by analyzing its expression across all samples, and no significant variation was observed, confirming its stability for normalization purposes. To evaluate the efficiency of the PCR, efficiency values for the target gene (*cas3*) and the reference gene (16S rRNA) were determined using standard curves. The PCR efficiencies for the target and reference genes were found to be 95% and 97%, respectively, indicat-

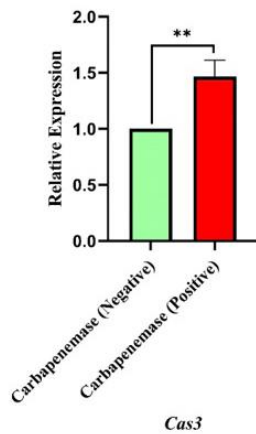


Fig. 3. The relative expression levels of the *cas3* gene were compared in carbapenem-sensitive and carbapenem-resistant *K. pneumoniae* isolates. The bar graph displays the mean relative expression of *cas3* in the two bacterial groups. A significantly higher expression level of *cas3* was detected in carbapenem-resistant isolates compared with carbapenem-sensitive isolates (** $P = 0.002$), indicating a potential association between *cas3* transcriptional activity and carbapenem resistance.

ing optimal performance in the qPCR reactions. For the RT-qPCR analysis, three biological replicates and three technical replicates were conducted for each sample to ensure the correctness and reliability of the data. Each sample underwent triplicate analysis, and the average CT values were utilized for evaluation.

Bas. A PPI analysis was conducted using STRING v11.5 to explore the functional connectivity among five key proteins in *K. pneumoniae* (strain MGH78578): *cas3*, *repA*, *kfu*, *ybtS*, and *rmpA2*. The PPI network revealed significant functional clustering, with an average node degree of 6.2 and a clustering coefficient of 0.88. *cas3* was centrally connected to transcriptional regulators and hypothetical proteins (e.g., KPN_01787 and KPN_01789), suggesting potential roles beyond adaptive immunity in cellular processes. Enrichment analysis demonstrated that these proteins are significantly involved in iron acquisition, biofilm formation, oxidative stress response, and polysaccharide metabolism. The PPI network's enrichment p-value (3.89×10^{-8}) indicated that these interactions are highly non-random, highlighting the biological interdependence between CRISPR-Cas systems and virulence and resistance determinants. The computational analysis, including PPI networks and structural pre-

dictions, provided valuable insights into the potential functional relationships between *cas3*, *repA*, *ybtS*, and *rmpA2*. The PPI network constructed using STRING v11.5 revealed significant interactions between *cas3* and other key proteins involved in virulence and resistance mechanisms. Experimental data from our study demonstrated a significant upregulation of *cas3* expression in carbapenem-resistant CRKP strains compared to CSKP. This suggests that the increased expression of *cas3* could be linked to the bacterial response to antibiotic pressure, which may contribute to the regulation of virulence factors. Furthermore, the interaction between *cas3* and *repA*, a protein involved in plasmid replication, supports the hypothesis that *cas3* may play a role in limiting the horizontal gene transfer of resistance genes, particularly plasmids carrying virulence factors such as *rmpA2*. This interaction could potentially explain the increased virulence observed in CRKP strains, which often carry *rmpA2* and other virulence-associated genes. Our experimental results showing higher virulence in CRKP strains with elevated *cas3* expression are consistent with the computational prediction that *cas3* could regulate the expression of virulence factors, such as *rmpA2*, through its interactions with *repA* and other regulatory proteins. These findings suggest that *cas3* may not only be involved in bacterial immunity through CRISPR-Cas systems but may also play a critical role in modulating virulence in *K. pneumoniae* under conditions of antibiotic resistance. Furthermore, to validate the distribution of subtype I-E among *K. pneumoniae* strains, data from CRISPRCasdb and recent literature were compared with our findings. In this study, 36% of CSKP isolates and 16% of CRKP isolates carried the I-E CRISPR-Cas system. These results align with global trends, where CRISPR-Cas systems, particularly subtype I-E, are more prevalent in non-resistant or plasmid-limited strains. Previous studies have shown a negative correlation between CRISPR presence and the acquisition of resistance plasmids, particularly those carrying the *bla-KPC* gene. This trend reinforces the hypothesis that CRISPR-Cas systems may function to limit horizontal gene transfer, thereby affecting plasmid-mediated resistance gene acquisition. The reduced presence of subtype I-E in CRKP isolates may facilitate the accumulation of resistance determinants, especially in high-risk clones such as ST258.

The amino acid sequence of *cas3* was submitted to Phyre2 and AlphaFold for 3D structure prediction. *cas3* comprises two main functional domains: an

N-terminal HD nuclease domain and a C-terminal SF2 helicase domain, which together mediate processive degradation of foreign DNA in a CRISPR-targeted manner. The predicted structure showed high conservation (>95% alignment confidence) with *cas3* from *E. coli* and *Pseudomonas* spp., with intact ATP-binding motifs and metal-ion coordination residues, indicating full enzymatic activity. These findings support the observed upregulation of *cas3* expression in CRKP strains and suggest a link between active CRISPR systems and antibiotic pressure. Bioinformatics analyses confirm that the *cas3* component is structurally intact and functionally active, with differential expression and network connectivity indicating its role in genomic immunity and the evolution of resistance and virulence in *K. pneumoniae*. Further functional validation is needed, with potential applications in CRISPR-based antimicrobial strategies.

DISCUSSION

The emergence of CRKP has become a global public health concern, especially in health care, where the spread of multidrug-resistant organisms may lead to outbreaks and treatment failures. The importance of CRKP does not relate only to its resistance to one of the most potent classes of antibiotics, the carbapenems, but also to the capability of retaining or enhancing its virulence, thereby making infections more serious and challenging to treat (6, 27). This study aimed to comprehensively analyze the genetic and phenotypic traits of CRKP and CSKP, emphasizing antibiotic-resistance genes, CRISPR-Cas systems, and virulence determinants. This study aims to compare the different characteristics of these two groups (CRKP and CSKP) to contribute to the growing knowledge needed to develop new strategies to treat CRKP-induced infections. Our study identified a high prevalence of the *bla-NDM-1* (82%) and *bla-OXA-48* (64%) genes in CRKP strains, both of which are key mediators of carbapenem resistance.

The findings of this study are consistent with previous research, highlighting the global spread of resistance genes such as *bla-NDM-1* and *bla-OXA-48* in *K. pneumoniae* (28). *bla-NDM-1* is particularly concerning due to its association with metallo-beta-lactamase (MBL) production, conferring resistance to almost all beta-lactam antibiotics, including carbapenems (29). Similarly, *bla-OXA-48* encodes an ox-

acillinase that specifically degrades carbapenems without affecting expanded-spectrum cephalosporins, further complicating treatment options (30). In our study, 46% of the CRKP strains co-carried *bla-NDM-1* and *bla-OXA-48*, suggesting a synergistic effect in enhancing resistance mechanisms. This aligns with concerns about the rapid emergence of MBL-producing *K. pneumoniae*, which significantly limits the efficacy of current antibiotics.

Our phenotypic testing confirmed the presence of MBLs in 64% of the CRKP strains, reinforcing the findings by Tu et al., who identified MBLs as a major contributor to carbapenem resistance (31). Furthermore, serine carbapenemases were detected in 36% of the resistant strains, revealing the multifactorial nature of resistance in CRKP. The coexistence of different carbapenemases within the same bacterial population adds a layer of complexity to treatment strategies, highlighting the urgent need for precise and rapid diagnostic methods. While the focus of our study was on carbapenem resistance, we also explored the potential involvement of CRISPR-Cas systems in influencing resistance and virulence. Traditionally, CRISPR-Cas systems are known as adaptive immune mechanisms in bacteria, targeting and cleaving foreign genetic material (32). Recent findings indicate that CRISPR-Cas systems may also promote the horizontal transfer of resistance genes and other mobile genomic elements (33, 34). The increased expression of *cas3* in CRKP strains likely reflects a dual role in both genomic defense and stress adaptation. On one hand, *cas3* is involved in bacterial immunity by limiting the integration of foreign genetic material, such as resistance genes. On the other hand, its upregulation may also be a response to environmental stress, such as antibiotic pressure, suggesting a role in adapting to adverse conditions. Therefore, it may contribute to both defense mechanisms and stress responses in CRKP strains. Our work revealed that *cas3*, a crucial element of the Type I CRISPR-Cas system, was markedly over-expressed in CRKP strains ($P = 0.002$), indicating a possible function in restricting the acquisition of resistance genes via horizontal gene transfer. However, the exact mechanisms by which CRISPR-Cas systems interact with resistance determinants are not fully understood and warrant further investigation. The presence of CRISPR-Cas systems, particularly subtype I-E, plays a key role in limiting horizontal gene transfer in *K. pneumoniae*. In CSKP strains,

these systems prevent the uptake of plasmids carrying resistance genes. However, in CRKP strains, the reduced frequency of CRISPR-Cas may facilitate the acquisition of resistance genes, particularly via plasmids, contributing to the spread of resistance. This decrease in CRISPR-Cas presence in CRKP isolates likely enhances horizontal gene transfer, promoting the dissemination of resistance determinants. In our study, the I-E CRISPR-Cas subtype was present in 16% of resistant strains and 36% of sensitive strains, which is consistent with the findings of Pursey et al., who proposed that this system could play a role in horizontal gene transfer (34). While these results suggest a link between CRISPR-Cas systems and antibiotic resistance, more studies are needed to clarify whether this system acts as a direct modulator of resistance or primarily limits gene acquisition. Regarding virulence, our study revealed several significant differences between CRKP and CSKP strains. *kfu*, involved in iron acquisition, was present in 42% of resistant strains, compared to 22% in sensitive strains, a finding consistent with Dogan et al. (35). Similarly, the aerobactin gene, detected in 18% of resistant strains and 4% of sensitive strains, suggests its important role in pathogenicity, as it aids in iron acquisition. This is supported by the work of et Lam al., who also identified a correlation between aerobactin and virulence (36). Additionally, *silS* and *repA*, genes involved in resistance to silver and plasmid replication, respectively, were more prevalent in resistant strains, further supporting their roles in resistance and virulence (37). The *terW* and *ybtS* genes, linked to oxidative stress resistance and iron acquisition, were more frequent in CRKP strains, with *terW* conferring survival under oxidative stress conditions (38, 39), and *ybtS* contributing to enhanced bacterial growth and virulence (40). We also identified higher prevalence of iron-acquisition genes like *iucB*, *iutA*, and *iroN* in CRKP strains, which facilitate survival in conditions where iron is sequestered by the host immune system (41, 42). These findings reinforce the association between iron acquisition and increased virulence in resistant strains. Moreover, *rmpA2*, a gene associated with hypervirulence, was more prevalent in CRKP strains, which is consistent with studies suggesting its role in the production of polysaccharide capsules that help bacteria evade the host immune system (43). Interestingly, we did not find significant differences in the prevalence of *entB*, *blaSHV*, and *blaCTX-M* genes between CRKP and

CSKP strains, although they were present in both groups. These genes may not be directly linked to carbapenem resistance but rather reflect broader resistance mechanisms or general survival strategies in *K. pneumoniae*. The *blaCTX-M* gene, for instance, is more commonly associated with cephalosporin resistance and may not play a significant role in carbapenem resistance (44). This work, for the first time, evaluates the involvement of CRISPR-Cas systems in carbapenem-resistant and carbapenem-sensitive *K. pneumoniae* isolates from Iran, elucidating their correlation with resistance and virulence genes. This comparative analysis provides novel insights into the role of CRISPR-Cas in the genetic evolution and horizontal gene transfer of resistance determinants in *K. pneumoniae*, offering valuable perspectives for future research and therapeutic strategies. Notwithstanding the thoroughness of this investigation, certain limitations must be recognized. The clinical isolates utilized may not comprehensively reflect the genetic variety of *K. pneumoniae* strains globally, hence constraining the generalizability of our results. Second, while our study investigates associations between CRISPR-Cas systems, resistance, and virulence factors, it does not provide direct evidence of causality. Functional assays or gene knock-out experiments are needed to establish whether CRISPR-Cas systems directly influence resistance and virulence. Additionally, our study was based on genomic data, so phenotypic variations influenced by environmental factors were not assessed, which could impact the interpretation of resistance and virulence profiles. Finally, the study's focus on CRKP and CSKP strains from a specific geographic region limits its applicability to other settings with different antibiotic usage patterns or local epidemiologies. Future research should address these limitations by incorporating a broader range of strains, functional analyses, and longitudinal studies to validate and expand upon our findings.

CONCLUSION

Our findings reveal significant differences between CSKP and CRKP strains in terms of CRISPR system distribution, virulence factors, and antibiotic resistance genes. Notably, CRKP strains showed a higher prevalence of resistance genes and more potent virulence factors compared to their sensitive coun-

terparts. The reduced frequency of CRISPR-Cas systems in CRKP strains may facilitate plasmid-mediated gene transfer, contributing to the spread of both resistance and virulence traits. Additionally, the upregulation of *cas3* in CRKP strains suggests a compensatory role in genomic regulation, potentially enhancing the bacteria's adaptability to antibiotic pressure and promoting stress adaptation. These results highlight the complex interplay between resistance mechanisms, virulence factors, and CRISPR-Cas systems, which are crucial for understanding bacterial evolution under antibiotic selection pressure. Future studies should focus on exploring the mechanisms by which CRISPR systems influence virulence and resistance, particularly in different environmental and therapeutic conditions. A deeper understanding of these interactions could pave the way for developing targeted therapeutic strategies to combat antibiotic resistance and improve clinical outcomes.

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