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CRISPR interference for bla_{NDM-1} and bla_{OXA-48} genes suppression in carbapenemase-resistant *Klebsiella pneumoniae* strains

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ABSTRACT

One treatment option for multidrug-resistant strains, such as carbapenemase-producing *Klebsiella pneumoniae*, is the CRISPR interference (CRISPRi) technique, developed to address issues caused by these pathogens. This system includes a gRNA and a dCas9. This study investigates the effectiveness of the CRISPRi system in reducing meropenem resistance in *K. pneumoniae* harboring β-lactamase strains, specifically the bla_{NDM-1} and bla_{OXA-48} genes, both separately and simultaneously, by optimizing sgRNA designs. We designed and incorporated two optimal sgRNAs, which were then cloned into a pJMP1363 plasmid with the CRISPRi system to target the expression of the bla_{NDM-1} and bla_{OXA-48} genes separately and simultaneously. Antimicrobial resistance was evaluated using the MIC test, and gene expression was measured with qRT-PCR. The MIC test results showed a decrease in meropenem resistance in *K. pneumoniae* strains carrying the pJMP1363 construct (sgRNA+). The qRT-PCR analysis indicated a reduction in mRNA expression for strains carrying the construct (sgRNA+), with a 67-fold and 31-fold decrease for bla_{OXA-48} and bla_{NDM-1}, respectively, and a 100-fold decrease for strains with both bla_{OXA-48} and bla_{NDM-1}. The decrease in meropenem resistance was more noticeable in strains harboring both resistance genes compared to strains with either gene alone. The study also demonstrated that both $sgRNA_{OXA-48}$ and $sgRNA_{NDM-1}$, targeting the non-template and template strands of the respective genes, effectively lowered transcription levels of the target genes. Notably, $sgRNA_{OXA-48}$ significantly reduced the expression of the bla_{OXA-48} gene.

1. Introduction

Antibiotic resistance is a major challenge in modern medicine, often referred to as a silent tsunami (Exner et al., 2017). The rising prevalence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is one of the most pressing global public health concerns (Jin et al., 2021). Infections caused by *K. pneumoniae* are primarily hospital-associated, and the situation is worsened by antibiotic resistance. CRKP strains, in particular, are resistant to all clinically relevant antibiotics, complicating treatment options. Mortality rates linked to infections caused by carbapenem-resistant *K. pneumoniae* are relatively high (Kobayashi et al., 2018). CRKP isolates are frequently categorized as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pan-drug-resistant (PDR), each presenting distinct treatment challenges. According to the European Centre for Disease Prevention and Control (ECDC), MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories; XDR refers to non-susceptibility to at least one agent in all but two antimicrobial categories; and PDR is non-susceptibility to all agents in all antimicrobial categories (Jin et al., 2021; Karampatakis et al., 2023). Understanding the molecular epidemiology of CRKP isolates is crucial, as it can inform treatment strategies (Petrosillo et al., 2013). Beta-lactam resistance in bacteria arises through various mechanisms. Beta-lactamase production, altered permeability, and efflux pumps are common mechanisms contributing to resistance in Gram-negative bacteria. Efflux systems play a particularly important role in the development of antibiotic resistance (Beceiro et al., 2013). Carbapenemases responsible for carbapenem resistance are classified into three main classes: Class A (KPCs), Class B (IMP, VIM, and NDM), and Class D (OXA). The bla_{NDM-1} and bla_{OXA-48} are among the most clinically significant carbapenem resistance genes found in

Abbreviations: K. pneumoniae, *Klebsiella pneumoniae*; CRISPRi, clustered regularly interspaced short palindromic repeats interference; sgRNA, single guide RNA; dCas9, dead Cas9; OXA-48, Oxacillinases β-lactamases 48; NDM-1, New Delhi metallo-β-lactamases 1.

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K. pneumoniae. These genes confer resistance to the last-resort antibiotics for treating multidrug-resistant Gram-negative infections, which presents a major global health threat (Al-Agamy et al., 2018; Solgi et al., 2020).

Given the complexity of combating antibiotic resistance, novel approaches are essential for tackling these multidrug-resistant pathogens. One promising strategy involves the use of the CRISPR-Cas system, which has gained considerable attention for its potential in genetic manipulation and targeted genome editing. The CRISPR-Cas system is a powerful immune mechanism found in most bacteria and archaea (Deveau et al., 2010). This system consists of CRISPR repeat spacers transcribed into crRNA and tracrRNA, along with Cas genes that encode Cas proteins with endonuclease activity (Xu and Li, 2020). The CRISPR-Cas system is widely used as a fast and cost-effective biotechnology tool (Kundar and Gokarn, 2022). The Class II CRISPR-Cas9 system, which includes the sgRNA and the Cas9 protein, was employed in this study (Brouns et al., 2008). The sgRNA is a fusion of crRNA and tracrRNA, forming a Cas9 ribonucleoprotein (RNP) that binds to DNA targets for cleavage (Jinek et al., 2012). Additionally, the Cas9 protein requires a protospacer adjacent motif (PAM) sequence for binding to the target DNA. The CRISPR-Cas9 system (Type II) was initially derived from Streptococcus (Peters et al., 2016). The CRISPR-Cas9 system has the capability to edit multiple genes simultaneously by using multiple sgRNAs, allowing for targeted editing at multiple genomic locations (Barrangou, 2014).

In addition to its gene-editing capabilities, the CRISPR-Cas system can be adapted for gene knockdown through CRISPR interference (CRISPRi), a method that targets specific pathogenic genes (Banta et al., 2020a). CRISPRi employs a modified Cas9 protein known as dead Cas9 (dCas9), which carries two amino acid substitutions (D10A and H841A) that eliminate its endonucleolytic activity (Qi et al., 2013). Although dCas9 can target DNA in combination with sgRNA, it does not alter the DNA sequence. This RNA-directed transcriptional repression system has been shown to achieve up to 99 % suppression of gene expression, resulting in consistent phenotypic outcomes (Schultenkämper et al., 2020). CRISPRi presents several advantages over other approaches, as it allows for precise, reversible gene silencing without introducing permanent mutations. Unlike conventional antibiotics, which apply selective pressure that drives resistance, CRISPRi directly reduces the expression of resistance genes, potentially restoring antibiotic susceptibility. Furthermore, CRISPRi can be customized to target various resistance determinants across different bacterial species, making it a versatile tool in antimicrobial research.

This study aims to investigate the effectiveness of the CRISPRi system in reducing meropenem resistance in *K. pneumoniae* strains with β-lactamase production by optimizing the design of sgRNAs. We separately and simultaneously examined the expression of the $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ genes. Two optimal sgRNAs were designed and incorporated into a pJMP1363 plasmid containing the CRISPRi system. Final assessments were conducted using in vitro assays to determine the level of meropenem resistance in *K. pneumoniae* (OXA-48+), *K. pneumoniae* (NDM-1+), and *K. pneumoniae* (OXA-48 + NDM-1) strains.

2. Material and methods

2.1. Strains and vectors

Clinical isolates of *Klebsiella pneumoniae* (OXA-48+), *K. pneumoniae* (NDM-1+), and *K. pneumoniae* (OXA-48 + NDM-1) were isolated from urinary tract infections at Loghman Hakim Hospital (Iran) between September 2021 and November 2022 and were confirmed as carbapenem-resistant based on their resistance gene profiles ($bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$). Standard biochemical laboratory methods were used to identify the clinical isolates. The plasmid pJMP1363 (#119279) was used as the vector for this study. Plasmid pJMP1363 is a CRISPR interference (CRISPRi) vector designed for targeted gene repression in Gram-negative bacteria, featuring a catalytically inactive dCas9, a chloramphenicol resistance marker for selection, an R6Kgamma origin of replication for stability, and multiple cloning sites for sgRNA insertion (Peters et al., 2019).

2.2. Design of sgRNA

The sgRNAs were designed using the CHOPCHOP web tool (https://chopchop.cbu.uib.no/) (Hajizadeh et al., 2024). The nucleotide sequences of the $bla_{\text{OXA-48}}$ (accession #KY213890.1) and $bla_{\text{NDM-1}}$ (accession #KU985245.1) genes were inputted to design sgRNAs capable of efficiently inhibiting the expression of each gene. High GC content and lack of self-complementarity were key considerations for the sgRNA design. Two sgRNAs, $\text{sgRNA}_{\text{OXA-48}}$ and $\text{sgRNA}_{\text{NDM-1}}$, were developed to target specific regions of the $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ genes, respectively.

- $\text{sgRNA}_{\text{OXA-48}}$: Targets the non-template strand near the 5′ end of the $bla_{\text{OXA-48}}$ gene.
- $\text{sgRNA}_{\text{NDM-1}}$: Targets the template strand near the 5′ end of the $bla_{\text{NDM-1}}$ gene.

The designed sequences of both sgRNAs are shown in Fig. 1.

The designed sgRNA sequences were 298 bp in length and included restriction enzyme sites for *Xho*I (5′_CTCGAG_3′), *Eco*RI (5′_GAATTC_3′), and *Xba*I (5′_TCTAGA_3′), along with two sgRNA components (sgRNA_1 and sgRNA_2).

First, sgRNA was inserted between *Xho*I and *Xba*I restriction enzymes in pJMP1363. The designed sgRNA sequence (298 bp) includes the *Xho*I enzyme site, sgRNA_1 (lac promoter, $\text{sgRNA}_{\text{OXA-48}}$, and scaffold), the *Eco*RI enzyme site, sgRNA_2 (lac promoter, $\text{sgRNA}_{\text{NDM-1}}$, and scaffold) and the *Xba*I enzyme site, respectively (Fig. 2). The lac promoter sequence is intended to enhance the function and efficiency of sgRNA in inhibiting gene expression. A 20 bp sgRNA region is designed for specific DNA binding, and the scaffold sequence (dCas9 handle hairpin) for the dCas9 protein binding (Hajizadeh et al., 2024; Banta et al., 2020b; Larson et al., 2013). sgRNAs transcription can begin at its 5′ end. Finally, the designed sgRNA cloned from 5′ to 3′ direction of *Xho*I to *Xba*I restriction enzymes in the pJMP1363 plasmid. Also, we included the empty vector pJMP1363 as a negative control.

To evaluate the potential for off-target interactions, we implemented in silico prediction tools prior to sgRNA design. Specifically, we used the CHOPCHOP web tool to select sgRNAs with high specificity and minimal predicted off-target binding. Additionally, algorithms such as CRISPRscan were used to screen for potential off-target sequences by comparing the sgRNA sequences against the entire genome.

2.3. Design of primers

The primers for sgRNA amplification were designed as follows: The forward primer (21 nt) was selected from the 5′ end of the 298 bp sgRNA sequence, while the reverse primer (22 nt) was a reverse complementary sequence from the 3′ end of the 298 bp sgRNA sequence (Table 2). The primer sequences were verified for specificity using the BLAST web tool (http://www.ncbi.nlm.nih.gov/blast/).

2.4. Transformation

The pJMP1363 construct (5 μL, 0.1 μg/μL) was used to transform competent bacterial strains. Transformed strains were streaked onto LB agar plates supplemented with 40 μL of ampicillin (100 mg/mL) and incubated overnight at 37 °C (Hajizadeh et al., 2024; Hanahan et al., 1991).

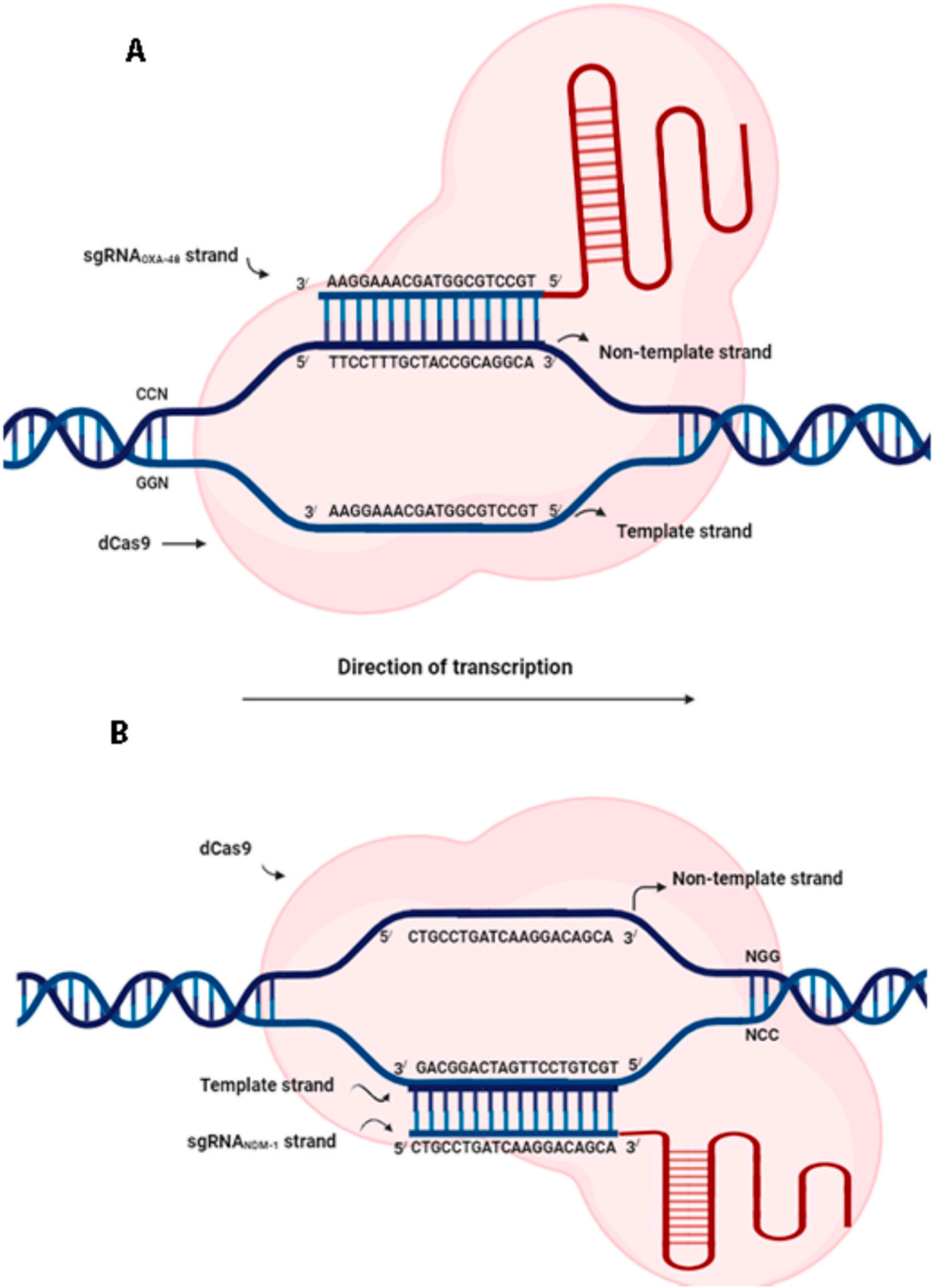


Fig. 1. Specific targeting of non-template and template DNA strands by sgRNAs. A: sgRNA targets the non-template strand of the *bla*$_{OXA-48}$ gene, being reverse complementary to the transcribed sequence. B: sgRNA targets the template strand of the *bla*$_{NDM-1}$ gene, having the same sequence as the transcribed region.

2.5. Antibiotic susceptibility tests

Antimicrobial susceptibility testing was performed using the minimum inhibitory concentration (MIC) method (Weinstein and Lewis 2nd, 2020). Meropenem was used as the test antibiotic, and its MIC was determined using the Müller-Hinton broth (MHB) dilution method. *Escherichia coli* ATCC 25922 served as the quality control strain. All experiments were conducted in triplicate.

2.6. Molecular detection of bla genes

Plasmid DNA extraction was performed to identify carbapenemase genes, including *bla*$_{NDM-1}$ and *bla*$_{OXA-48}$. PCR assays confirmed the transformation of the pJMP1363 construct (sgRNA+) in *K. pneumoniae* (OXA-48+), *K. pneumoniae* (NDM-1+), and *K. pneumoniae* (OXA-48 + NDM-1). Each PCR assay was prepared with a total reaction volume of 20 µl (primer sequences in Table 1). PCR products were verified using the BLAST web tool available at http://www.ncbi.nlm.nih.gov/blast/.

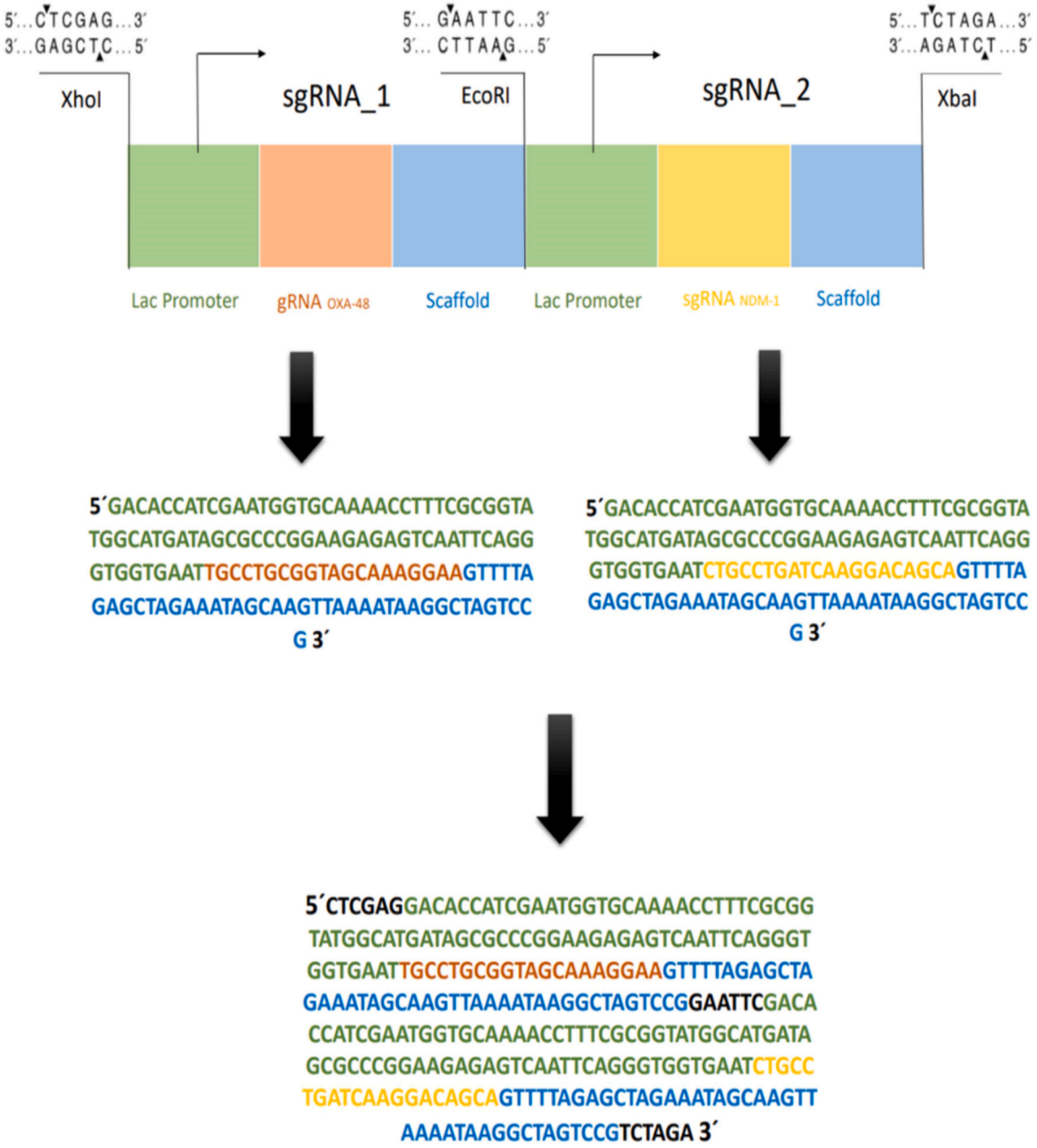


Fig. 2. Structure of the 298-bp sgRNA sequence. The 298 bp sequence contains the following components in order: *Xho*I enzyme site (6 bp, 5′_CTCGAG_3′), sgRNA_1 (140 bp, consisting of lac promoter (78 bp), $sgRNA_{OXA-48}$ (20 bp), and scaffold (42 bp)), *Eco*RI enzyme site (6 bp, 5_GAATTC_3′), sgRNA_2 (140 bp, consisting of lac promoter (78 bp), $sgRNA_{NDM-1}$ (20 bp), and scaffold (42 bp)), and *Xba*I enzyme site (6 bp, 5′_TCTAGA_3′).

Table 1
PCR forward and reverse primers.

Genes	sequences	Product sizes (bp)	References
bla_{OXA-48}_F bla_{OXA-48}_R	5′ TTGGTGGCATCGATTATCGG 3′ 5′ TGAGCACTTCTTTTGTGATGGC 3′	745	(Hajizadeh et al., 2024)
bla_{NDM-1}_F bla_{NDM-1}_R	5′ GGTTTGGCGATCTGGTTTTC 3′ 5′ CGGAATGGCTCATCACGATC 3′	621	(Poirel et al., 2011)
sgRNA_F sgRNA_R	5′ CTCGAGGACACCATCGAATGG 3′ 5′ TCTAGACGGACTAGCCTTATTT 3′	298	

2.7. Digestion

The digestion process was carried out using a 20 μl master mix consisting of 9 μl of water, 2 μl of Tango buffer, 2 μl of the *Xba*I restriction enzyme, 2 μl of the *Mlu*I restriction enzyme, and 5 μl of plasmid DNA. Initially, the plasmid DNA was mixed with Tango buffer, *Xba*I enzyme, and water in a tube and incubated at 37 °C for 1 h. Following this, the *Mlu*I restriction enzyme was added to the same tube and incubated again at 37 °C for another hour.

2.8. qRT-PCR (quantitative real-time PCR)

RNA was extracted using a commercial RNA extraction kit as per the manufacturer's instructions. cDNA synthesis was performed using a 2× RT-PCR Pre-Mix, and qRT-PCR was conducted with SYBR Green Master

Mix. Gene expression was analyzed using a relative quantification method. All qRT-PCR assays (primer sequences in Table 2) were prepared in a total volume of 20 µl. Relative abundance was calculated using the standard curve $2^{-\Delta\Delta ct}$ method, with 16S rRNA as the reference gene. Experiments were performed in triplicate (Livak and Schmittgen, 2001; Schefe et al., 2006).

2.9. Statistical analysis

Data were analyzed using Microsoft Excel 2013, and graphs were created using GraphPad Prism. To compare differences between experimental groups, we performed a one-way analysis of variance (ANOVA). A p-value of <0.05 was considered statistically significant, indicating a meaningful difference between the groups.

3. Results

3.1. Digestion of the pJMP1363 construct (sgRNA+)

The pJMP1363 construct (sgRNA+) was digested using *Xba*I and *Mlu*I restriction enzymes, followed by gel electrophoresis. Two distinct bands at 3933 bp and 6113 bp were observed, confirming the presence of the 298 bp sgRNA fragment (Fig. 3). The successful digestion with specific restriction enzymes of the plasmid construct (10,094 bp) resulted in two distinct fragments, whose combined size exceeded that of the original plasmid (9796 bp), confirming the integration of the 298 bp sgRNA fragment.

3.2. Transformation of the pJMP1363 construct (sgRNA+)

To further validate, we conducted a PCR assay targeting the 298 bp sgRNA fragment, which successfully verified its presence in the plasmid. PCR assays revealed three distinct bands at 298 bp in *K. pneumoniae* (OXA-48+), *K. pneumoniae* (NDM-1+), and *K. pneumoniae* (OXA-48 + NDM-1) strains (Fig. 4). The successful transformation of the pJMP1363 construct (sgRNA+) was confirmed.

3.3. Inhibition of meropenem resistance via CRISPRi plasmid

In this study, we included the empty vector pJMP1363 (lacking sgRNA) as a negative control to confirm that the observed inhibition of antibiotic resistance was specifically due to sgRNA expression and not the presence of the plasmid itself or other unintended effects. Moreover, to assess toxicity, we monitored bacterial growth before and after introducing the CRISPRi plasmid. Specifically, we compared two groups: The selected *K. pneumoniae* strains and *K. pneumoniae* strains harboring empty vector pJMP1363 as a negative control. Our results demonstrated that both groups of bacteria exhibited normal growth patterns, with no significant differences observed in growth rates between two groups.

Quantitative real-time PCR (qRT-PCR) was used to assess mRNA expression levels. Results demonstrated that the expression levels of OXA-48 mRNA, NDM-1 mRNA, and OXA-48 + NDM-1 mRNA carrying the pJMP1363 construct (sgRNA+) were reduced by 67-fold, 31-fold, and 100-fold, respectively, upon the induction of the meropenem antibiotic. Statistical analysis using a one-way ANOVA confirmed these significant reductions, as shown in Fig. 5A.

According to the CLSI 2021 guidelines, meropenem resistance is defined by a minimum inhibitory concentration (MIC) of ≥4 µg/mL. Prior to transformation, *Klebsiella pneumoniae* strains producing OXA-48, NDM-1, or both (OXA-48 + NDM-1) exhibited high resistance to meropenem. After introducing the pJMP1363 construct (sgRNA+), the MIC values for these strains significantly decreased. Specifically, in *K. pneumoniae* (OXA-48+), the MIC decreased from 64 mg/L to 4 mg/L; in *K. pneumoniae* (NDM-1+), the MIC dropped from 256 mg/L to 128 mg/L; and in *K. pneumoniae* (OXA-48 + NDM-1), the MIC reduced dramatically from 32 mg/L to 0.5 mg/L. In comparison, strains transformed with the empty pJMP1363 vector exhibited no significant changes in resistance, with MIC values remaining as follows: 64 mg/L for OXA-48-producing strains, 256 mg/L for NDM-1-producing strains, and 32 mg/L for OXA-48 + NDM-1-producing strains.

These findings highlight the significant impact of the pJMP1363 construct (sgRNA+) in reducing meropenem resistance. In particular: OXA-48-producing strains showed a 16-fold reduction in MIC (from 64 mg/L to 4 mg/L), NDM-1-producing strains showed a 2-fold reduction (from 256 mg/L to 128 mg/L), and OXA-48 + NDM-1-producing strains showed a remarkable 32-fold reduction (from 32 mg/L to 1 mg/L) (Fig. 5B).

Table 2
qRT-PCR forward and reverse primers.

Gene	sequences	Reference
16S rRNA_F	5′ TTACCTGGGCTTGACATGGAC 3′	(Cleto et al., 2016)
16S rRNA_R	5′ GCTGGCAACATAAGACAAGGG 3′	
bla_{OXA-48}_F	5′ GTAGCAAAGGAATGGCAA 3′	(Naas et al., 2013)
bla_{OXA-48}_R	5′ CCTTGCTGCTTATTGTCA 3′	
bla_{NDM-1}_F	5′ TTTGGCGATCTGGTTTTCCG 3′	(Smiljanic et al., 2017)
bla_{NDM-1}_R	5′ ATCAAACCGTTGGAAGCGAC 3′	

4. Discussion

Infections caused by carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates pose a significant threat to public health, especially among critically ill patients, where they are associated with elevated mortality rates (Karampatakis et al., 2023). The resistance of CRKP is primarily attributed to plasmids carrying multiple resistance genes, including those encoding extended-spectrum β-lactamases (ESBLs) (Yao et al., 2022). Alarmingly, nearly 200,000 hospitalized patients in the United States are affected annually by *Enterobacteriaceae* infections caused by ESBL-producing strains, representing a 50 % increase in the past five years—a concerning trend (Kadri, 2020). Therefore, reducing antibiotic resistance associated with beta-lactamase genes is essential for improving global public health outcomes.

The CRISPR interference (CRISPRi) presents several advantages over other approaches, as it allows for precise, reversible gene silencing without introducing permanent mutations. Unlike conventional antibiotics, which apply selective pressure that drives resistance, CRISPRi directly reduces the expression of resistance genes, potentially restoring antibiotic susceptibility. Furthermore, CRISPRi can be customized to target various resistance determinants across different bacterial species, making it a versatile tool in antimicrobial research (Angeles Flores et al., 2025).

The CRISPRi system has shown promise in overcoming drug resistance mechanisms. Studies have demonstrated its ability to reduce *Escherichia coli*'s sensitivity to rifampicin and tetracycline. These findings highlight the CRISPRi system's efficacy and ease of use in mitigating the development of multidrug resistance (MDR) in *E. coli* (Wan et al., 2022). Similarly, in this study, we utilized the CRISPRi technique to effectively reduce meropenem resistance in *K. pneumoniae* strains.

We designed two single-guide RNAs (sgRNAs): $sgRNA_{OXA-48}$, targeting the bla_{OXA-48} gene on the non-template strand, and $sgRNA_{NDM-1}$, targeting the bla_{NDM-1} gene on the template strand. Experimental data revealed that meropenem resistance was reduced in strains carrying the pJMP1363 construct (sgRNA+). However, the reduction was more pronounced in strains with the bla_{OXA-48} gene. The efficiency of targeting template strands remains a subject of debate; some studies suggest inefficiency, while others report effective transcriptional repression (Cleto et al., 2016). Additionally, previous research indicates that sgRNAs targeting the non-template strand may be more effective (Yao et al., 2022). In our study, inducing the pJMP1363 construct (sgRNA+) in strains resulted in decreased meropenem resistance, with $sgRNA_{OXA-48}$ significantly reducing the expression of the bla_{OXA-48} gene.

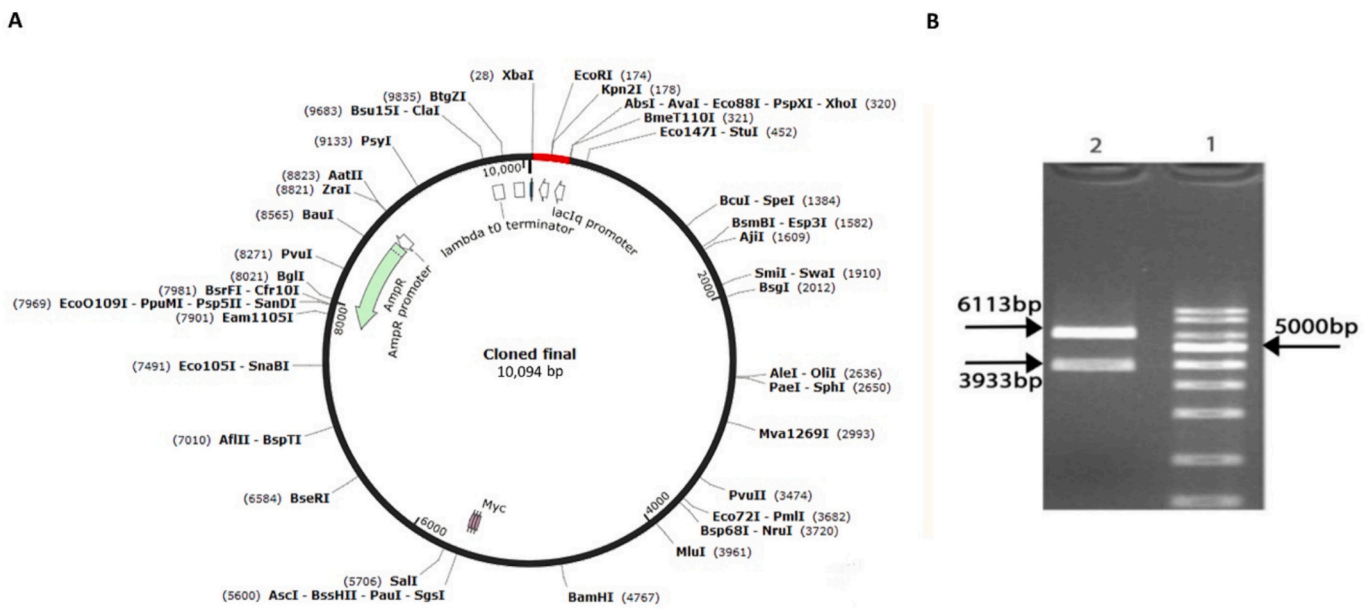


Fig. 3. pJMP1363 Construct (sgRNA+) Digestion. A: The 298 bp sgRNA fragment was cloned between *Xho*I and *Xba*I enzyme sites in the pJMP1363 plasmid. B: Gel electrophoresis revealed two sharp bands at 3933 bp and 6113 bp, indicating the addition of the 298 bp sgRNA fragment. **Lane 1**: 1 kb marker (Fermentas); **Lane 2**: Digested pJMP1363 plasmid with XbaI and *Mlu*I.

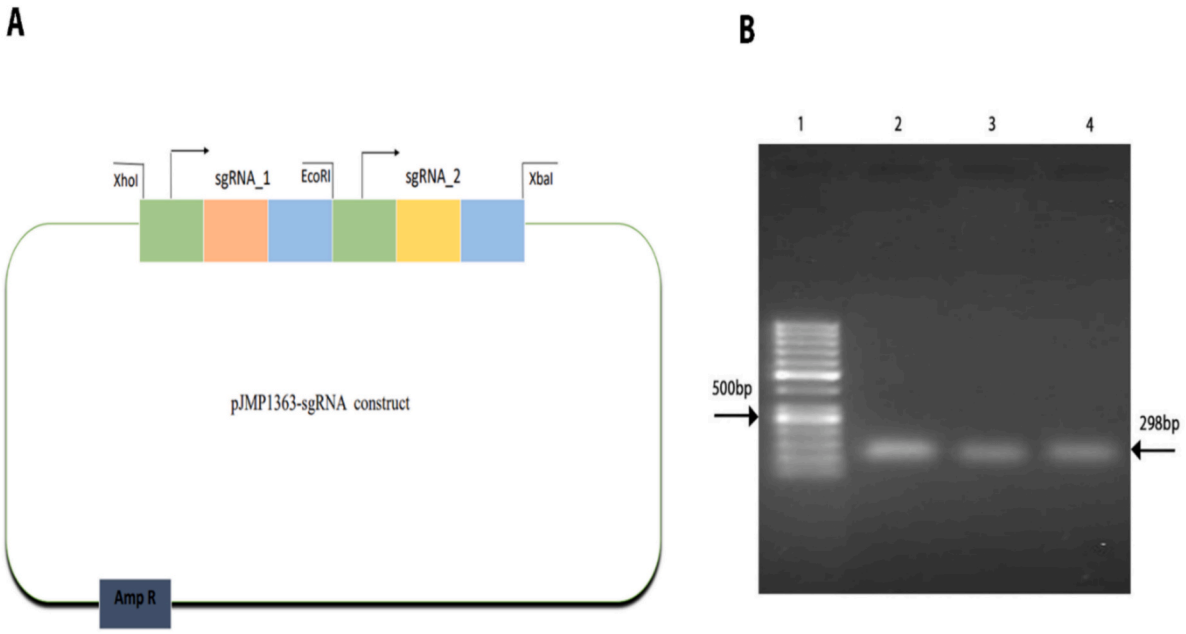


Fig. 4. Confirmation of sgRNA Presence in Transformed Strains. A: The designed sgRNA (298 bp fragment) contained two sgRNA sequences (sgRNA_1 and sgRNA_2) and restriction enzyme sites. B: PCR assays showed the presence of the sgRNA fragment in *K. pneumoniae* strains. **Lane 1**: 100 bp marker (Fermentas); **Lane 2**: Transformed sgRNA fragment (298 bp) in OXA-48; **Lane 3**: Transformed sgRNA fragment (298 bp) in NDM-1, and **Lane 4**: Transformed sgRNA fragment (298 bp) in OXA-48 + NDM-1.

Furthermore, we designed the sgRNAs to target sites at the 5′ end of the genes, as successful target binding often requires a well-defined 5′ end (Larson et al., 2013). Targeting sites at the 5′ end in bacterial genes has been consistently shown to yield optimal results, aligning with prior findings (Banta et al., 2020a; Larson et al., 2013; Cui et al., 2018).

We tested meropenem resistance levels in the target strains. Compared to the control or empty vector, the resistance levels varied significantly upon meropenem induction. Among the strains tested, *K. pneumoniae* (OXA-48 + NDM-1) exhibited the most dramatic reduction in resistance (100-fold), consistent with corresponding decreases in mRNA expression levels.

We also explored whether CRISPRi could simultaneously suppress the expression of multiple resistance genes. The CRISPRi system can accommodate multiple sgRNA cassettes and targets, enabling simultaneous regulation of different genes. For this purpose, we designed and cloned $sgRNA_{OXA-48}$ and $sgRNA_{NDM-1}$ into the pJMP1363 plasmid. Testing this combination revealed a significant reduction in mRNA expression—by up to 100-fold—and a corresponding 32-fold decrease in meropenem resistance, from 32 μg/mL to 1 μg/mL. These findings demonstrate the potential of using multiple sgRNAs to control resistance

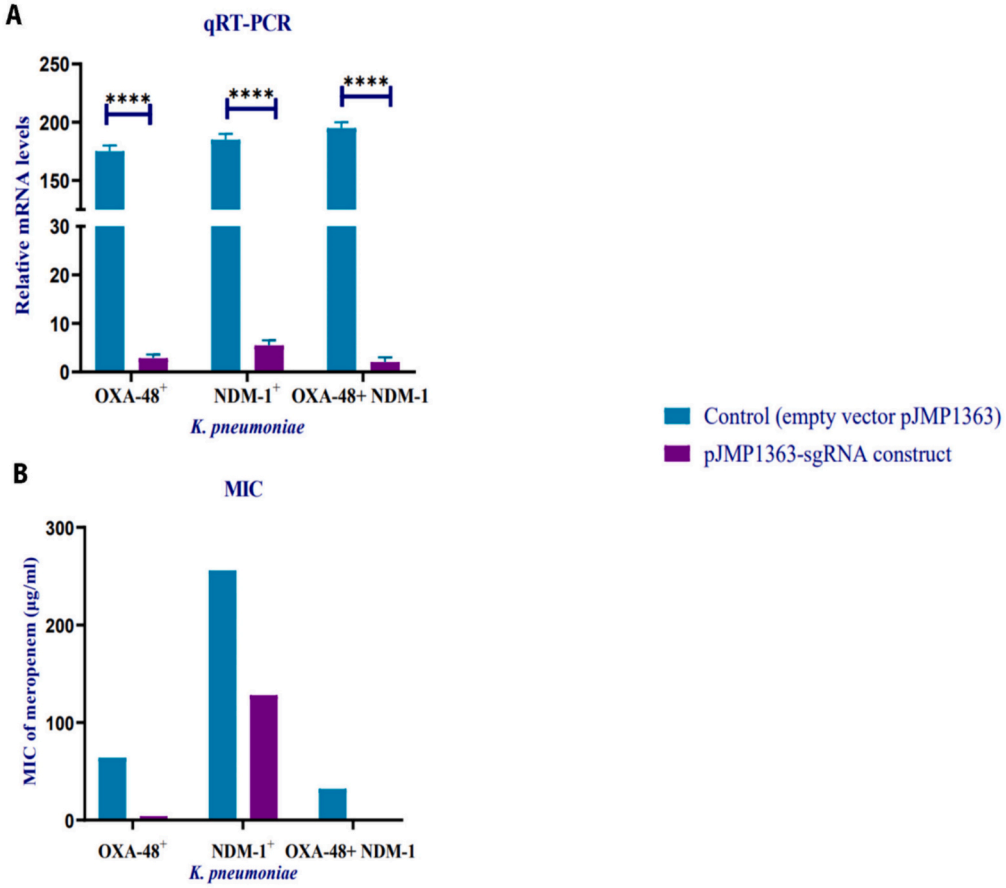


Fig. 5. Inhibition of $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ Resistance Genes. A: qRT-PCR results demonstrate reductions in mRNA expression: 67-fold for OXA-48, 31-fold for NDM-1, and 100-fold for OXA-48 + NDM-1, all carrying the pJMP1363 construct (sgRNA+). B: MIC analysis shows significant decreases in meropenem resistance: 16-fold for OXA-48 (64 to 4 mg/L), 2-fold for NDM-1 (256 to 128 mg/L), and 32-fold for OXA-48 + NDM-1 (32 to 1 mg/L) when carrying the pJMP1363 construct (sgRNA+). Statistical analysis was performed using one-way ANOVA with GraphPad (****, $P < 0.0001$).

genes concurrently (Larson et al., 2013). The 100-fold reduction in *K. pneumoniae* OXA-48 + NDM-1 (the double-positive strain) is likely due to synergistic suppression when both $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$ are simultaneously targeted by CRISPRi. In contrast, targeting each gene individually may not achieve the same level of suppression due to residual expression from secondary regulatory pathways.

This study highlights the effectiveness of CRISPRi and the designed sgRNAs in reducing the expression of carbapenem resistance genes in *K. pneumoniae* strains carrying $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$, or both. Our findings underscore the importance of thoughtful sgRNA design in enhancing the efficacy of CRISPRi for combating antibiotic resistance.

5. Conclusion

Antibiotic-resistant bacteria pose a significant challenge in the clinical management of infections, contributing to increased healthcare costs, mortality, and morbidity. The rising prevalence of antibiotic resistance necessitates the development of innovative approaches to combat this threat. Genetic manipulation, particularly through CRISPRi, has emerged as a promising tool to reduce bacterial antibiotic resistance. In this study, the CRISPRi system demonstrated its potential in reducing meropenem resistance in *Klebsiella pneumoniae* strains. The use of an effective single-guide RNA (sgRNA) significantly lowered meropenem resistance, with the *K. pneumoniae* (OXA-48 + NDM-1) strain showing the most pronounced reduction (100-fold), corresponding to the observed decrease in mRNA expression levels. Furthermore, the sgRNAs targeting the non-template ($\text{sgRNA}_{\text{OXA-48}}$) and template strands ($\text{sgRNA}_{\text{NDM-1}}$) of the genes successfully downregulated the expression of the OXA-48 and NDM-1 resistance genes. Notably, the design of the sgRNA, particularly its location at the 5′ end of the gene, proved critical for effective inhibition. This strategy for designing sgRNAs could be a highly efficient method for targeting multi-drug-resistant bacteria, offering a potential approach to prevent the development and spread of resistance in clinical settings.

CRediT authorship contribution statement

Yeganeh Hajizadeh: Writing – original draft, Data curation. **Mana Oloomi:** Writing – review & editing, Supervision, Conceptualization.

Consent for publication

All authors have consented to publish this manuscript.

Ethics approval

Approval was granted by the Pasteur Institute of Iran (Code number: IR.PII.REC.1400.050).

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Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

All data and materials are available in the article. Other data will be provided upon request.

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