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CRISPR-Cas based plasmid design for multidrug-resistant *Klebsiella pneumoniae* isolates

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Abstract

Antimicrobial resistance is a major global health concern that requires innovative therapeutic strategies. This study aimed to address this challenge by designing Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated protein (CRISPR-Cas)-based plasmid systems for potential genome editing applications in multidrug-resistant (MDR) *Klebsiella pneumoniae* clinical isolates. Minimum inhibitory concentrations (MICs) of imipenem, meropenem, and ertapenem were determined according to European Committee on Antimicrobial Susceptibility Testing guidelines. All isolates ($n = 5$) were resistant, with MIC ranges of 4–128 μg/ml for imipenem, 8–64 μg/ml for meropenem, and 8–256 μg/ml for ertapenem. Resistance gene analysis revealed $bla_{\text{OXA-48}}$-like and $bla_{\text{CTX-M-15}}$ in all isolates, while $bla_{\text{NDM-1}}$ was detected in one isolate. Two CRISPR-based plasmid systems, CRISPR-Cas9 and CRISPR-assisted cytidine deaminase, were designed. Target genes were amplified by polymerase chain reaction, and guide RNA (gRNA) sequences were designed from selected regions. Apramycin (50 μg/ml) was identified as a suitable selection marker. The pSGKP–AmpR(Pro)–ApmR plasmid was successfully constructed, whereas Cas9 and APOBEC constructs could not be cloned. Overall, this study highlights technical challenges in developing CRISPR-based tools for MDR *K. pneumoniae* and emphasizes the need for isolate-specific plasmid design and gRNA optimization.

Keywords carbapenemase, CRISPR-Cas systems, *Klebsiella pneumoniae*, plasmids

Introduction

Antimicrobial resistance (AMR) constitutes a major global public health threat, contributing to millions of deaths annually and posing an increasing burden on healthcare systems worldwide (O'Neill 2016, GBD 2021 Antimicrobial Resistance Collaborators 2024). Often referred to as a "silent pandemic," AMR increases morbidity and mortality and places a substantial burden on public health systems and the global economy (Shallcross et al. 2015).

In response to this threat, the World Health Organization (WHO) has published a priority pathogen list based on the urgency of new antibiotic development. Carbapenem-resistant *Enterobacteriaceae* (CRE), including *Klebsiella pneumoniae*, are classified among the most critical due to their resistance to last-resort antibiotics, which severely limits therapeutic options and necessitates urgent alternative strategies against multidrug-resistant (MDR) *K. pneumoniae* (Antimicrobial Resistance Collaborators, 2022, WHO 2017, Bengoechea and Sa Pessoa 2019).

Although new antibiotics continue to be developed, antimicrobial discovery alone is insufficient to address resistance, highlighting the importance of rational antibiotic use and complementary strategies such as phage therapy and genome-editing technologies (Bikard et al. 2014, Bengoechea and Sa Pessoa 2019, Antimicrobial Resistance Collaborators 2017, 2022).

The Clustered Regularly Interspaced Short Palindromic Repeats—CRISPR-associated protein (CRISPR-Cas) system, originally identified as a bacterial adaptive immune mechanism, has emerged as a powerful gene-editing platform (Ishino et al. 1987, Jinek et al. 2012). It consists of a CRISPR array, Cas genes organized in operons, and spacer sequences interspersed with repeats (Bhaya et al. 2011, Wiedenheft et al. 2012). In the context of AMR, this system underscores the importance of resistance profile-specific plasmid design, which is the primary focus of the present study.

In our study, we focused on plasmid design tailored to the resistance profile of clinical *K. pneumoniae* isolates. These strains were selected based on their carbapenem resistance and production of extended-spectrum beta-lactamases (ESBLs), features commonly linked to MDR phenotypes (WHO 2017, Salgado-Caxito et al. 2021, Ramatla et al. 2023). The $bla_{\text{CTX-M}}$ gene, one of the most

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widespread ESBL genes globally, was of particular interest due to its dominant presence in resistant *Enterobacteriaceae* (Hawkey and Jones 2009, Lascols et al. 2012). Additionally, we investigated $bla_{\text{NDM-1}}$, a metallo-β-lactamase identified in 2009, and $bla_{\text{OXA-48}}$, an oxacillinase endemic in our country since its first report in 2001 (Poirel et al. 2004, Carrer et al. 2008, Yong et al. 2009, Nordmann et al. 2011).

The aim of this study was to detect $bla_{\text{CTX-M-15}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{OXA-48}}$-like genes in MDR *K. pneumoniae* isolates and to establish a CRISPR-Cas-based plasmid design approach for potential genome engineering applications.

Materials and methods

Study design and ethics

Approval was received from the Clinical Research Ethics Committee of Istanbul Faculty of Medicine on 21 February 2020. This retrospective study included *K. pneumoniae* isolates ($n = 5$) found to be resistant to carbapenems by the disk diffusion method from various clinical samples sent to the Istanbul Faculty of Medicine, Medical Microbiology Department, Bacteriology Laboratory, between June and August 2020.

Antibiotic susceptibility tests

The minimum inhibitory concentration (MIC) values of the isolates for imipenem, meropenem, and ertapenem were investigated using the broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing criteria (EUCAST valid from 1 January 2021). We determined which strains were resistant to imipenem, ertapenem, and meropenem. *Escherichia coli* ATCC 25922 and *E. coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. Istanbul University Microorganism and Culture Collections Center (KUKENS) provided control strains.To make a selection in cloning, their susceptibilities to kanamycin, spectinomycin, streptomycin, hygromycin, chloramphenicol, and apramycin at concentrations of 1–0.0625 μg/ml were investigated by the broth microdilution method.

Colony polymerase chain reaction

Colony polymerase chain reaction (PCR) was performed to detect $bla_{\text{OXA-48}}$-like, $bla_{\text{NDM-1}}$, and $bla_{\text{CTX-M-15}}$ genes in MDR *K. pneumoniae* isolates using a modified protocol on a BioRad T100 Thermal Cycler. PCR conditions were initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 60 s, primer annealing at 56°C ($bla_{\text{OXA-48}}$-like), 60°C ($bla_{\text{NDM-1}}$), and 62°C ($bla_{\text{CTX-M-15}}$) for 45 s; extension at 72°C for 1 min; and a final extension at 72°C for 7 min (Poirel et al. 2004, Aktas et al. 2008). Primers for OXA-48 (Aktas et al. 2008), NDM-1 (Hidalgo et al. 2013), and CTX-M-15 (Karim et al. 2001) are listed in Table 1. Amplicons were confirmed by Sanger sequencing.

Plasmid construction and design

Plasmids used in this study, pCasKPapr (ID: 117231), pSGKP-spe (ID: 117234), and pBECKP-km (ID: 117235), were commercially obtained from Addgene (Wang et al. 2018).

Following sequence verification of resistance genes (OXA-48-like, NDM-1, and CTX-M-15), alignments were performed using SnapGene and ClustalX2, and specific guide RNA (gRNA) sequences were designed for each gene (Jinek et al. 2012). The presence of PAM (protospacer adjacent motif) sites, specifically NGG for Cas9, was assessed in conserved regions. Twenty-nucleotide conserved sequences following PAM were selected as target sites.

Target site scoring was performed via the Benchling web server (https://www.benchling.com/). Forward (5′-TAGT-N20) and reverse (5′-AAAC-N20) primers were synthesized from high-scoring targets (Cong et al. 2013, Hu et al. 2018).

A two-plasmid CRISPR-Cas system was designed: CRISPR-Cas9 for chromosomal targeting and a CRISPR-assisted cytidine deaminase system for plasmid-based targeting (Wang et al. 2018). The plasmids utilized and newly constructed in this study, along with their corresponding target regions, are summarized in Table 2.

Plasmid construction and PCR amplification

The AmpR(Pro) (ampicillin resistance promoter) from pUC19 (Addgene ID: 50005) (Fig. 1), the araBAD region from the pCasKP-apr plasmid (Fig. 2a), and the ApmR region (Fig. 2b) were amplified (Table 3). All three PCR reactions shared the same amplification mixture and cycling program. The mixture contained 5 μl of 5X Phusion Buffer, 1 μl DNA (20 ng), 0.5 μl of 10 mM dNTPs, 2.5 μl primers (forward/reverse), 0.5 μl Phusion High-Fidelity DNA Polymerase (2 U/μl), and 15.5 μl distilled water, totaling 25 μl. The PCR program was initial denaturation at 98°C for 2 min, 30 cycles of 98°C for 15 s, 56°C for 10 s, 72°C for 20 s, and a final extension at 72°C for 2 min.

Cas9 protein from pCasKP-apr plasmid (Fig. 2c), and Cas9 (D10A) nickase–cytidine deaminase (APOBEC1) from pBECKP-km (Fig. 3) were also amplified (Table 3) using a shared PCR mixture and program. The mixture included 5 μl 5X Q5 Reaction Buffer, 5 μl 5X Q5 High GC Enhancer, 2.5 μl primers, 0.5 μl 10 mM dNTPs, 1 μl DNA (20 ng), 0.25 μl Q5 Hot Start High-Fidelity DNA Polymerase, and 10.5 μl distilled water (25 μl total). PCR cycling: 98°C for 2 min; 10 cycles of 98°C for 15 s, 66°C for 30 s, and 72°C for 5 min; followed by 15 cycles of 98°C for 15 s, 56°C for 30 s, and 72°C for 5 min; final extension at 72°C for 5 min. PCR products were purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey Nagel, Germany).

For cloning, PCR products were inserted into the pJET1.2 vector. To linearize the vector, *Eco32I* restriction enzyme—cutting at a single site—was used. The digestion mix contained 7 μl pJET1.2 (50 ng), 2 μl FastDigest Green Buffer (10X), 0.5 μl *Eco32I*, and 20 μl distilled water. Incubation was at 37°C for 1 h. The digested vector was run on a 1% agarose gel at 120 V for 30 min, visualized under UV, and the desired band was gel-extracted and purified. DNA concentrations (∼150–300 ng/μl) were measured using a Nanodrop spectrophotometer (DeNovix DS-11, USA). Purified DNA was stored at −20°C.

Preparation of competent cells, ligation, and transformation

Competent *E.coli* DH10B cells were prepared using the calcium chloride ($CaCl_2$) chemical method (Chang et al. 2017). The ligation reaction mixtures were prepared using PCR products with a vector-

Table 1 Primers used to detect OXA-48-like, NDM-1, and CTXM-15 genes.

Primer	Nucleotide sequence (5′→3′)	Amplicon size (bp)
OXA-48-like-F	TTGGTGGCATCGATTATCGG	743
OXA-48-like-R	GAGCACTTCTTTTGTGATGGC	
NDM-1-F	TCTCGACATGCCGGGTTTCGG	475
NDM-1-R	ACCGAGATTGCCGAGCGACTT	
CTX-M-15-F	CGCTTTGCGATGTGCAG	550
CTX-M-15-R	ACCGCGATATCGTTGGT	

Table 2 Plasmids used and constructed in this study and their target regions.

Plasmid	Purpose	Target region	Figure
pUC19	Cloning vector	AmpR(Pro) (resistance promoter)	1
pCasKP-apr	Bacterial expression of Cas9 nuclease and apramycin resistance	AraBAD (*araC* gene promoter)	2(a)
		ApmR (apramycin resistance)	2(b)
		Cas9 (Cas9 nuclease)	2(c)
pBECKP-km	A cytidine deaminase-mediated base-editing plasmid in *K. pneumoniae*	Cas9(D10A)-APOBEC1 (cytidine deaminase)	3
pSGKP-spe	A single-guide RNA expression plasmid for genome editing in *K. pneumoniae*	Spectinomycin-free pSGKP backbone	4

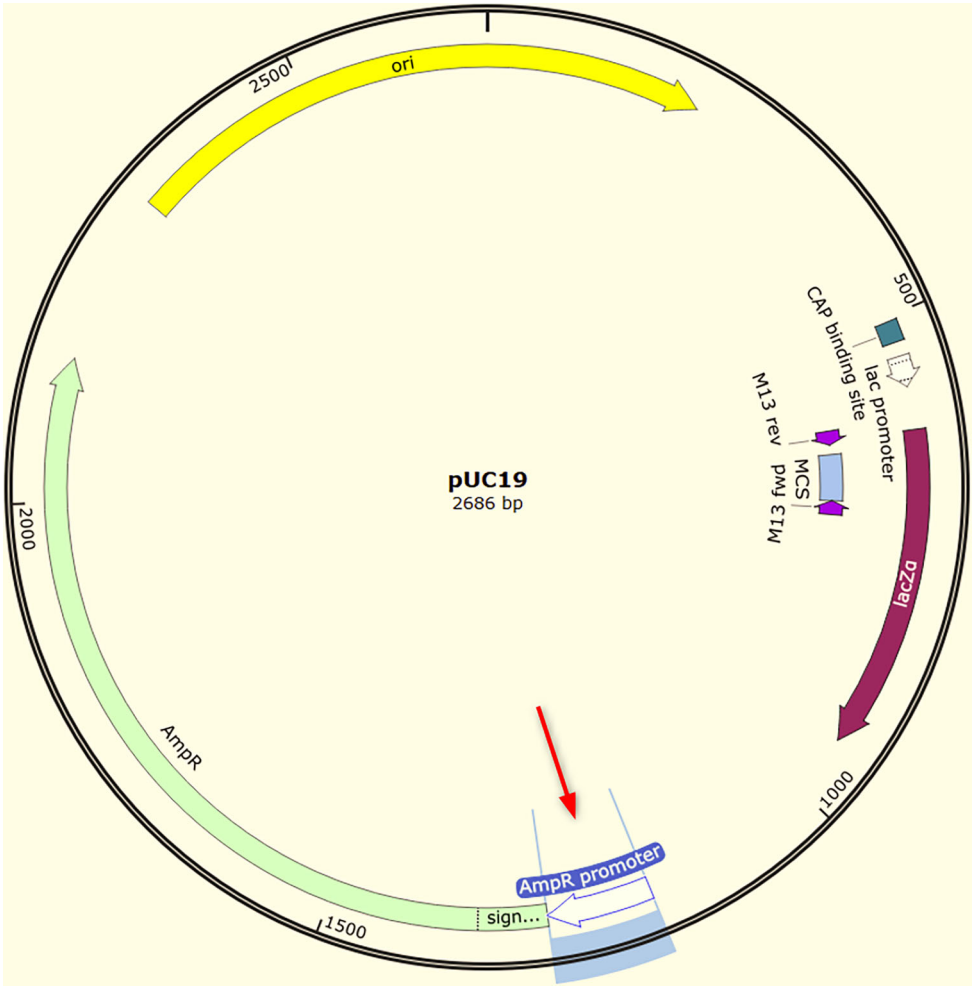

Figure 1 AmpR promoter in pUC19.

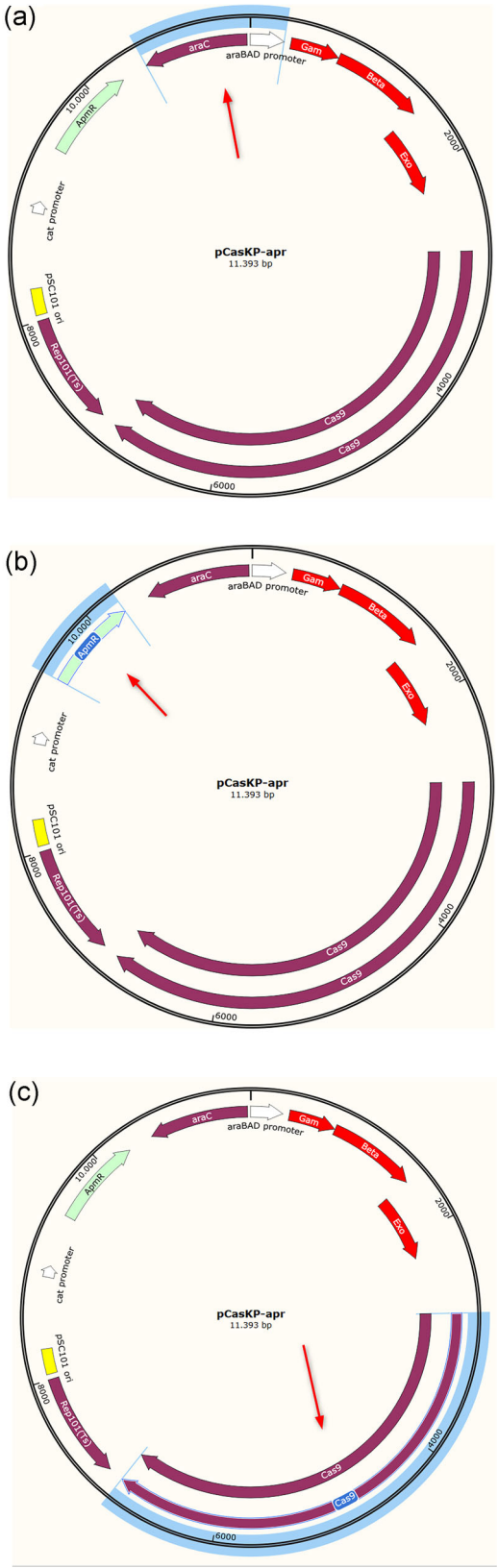

Figure 2 (a) AraBAD in the pCasKP-apr plasmid. (b) Apramycin resistance in the pCasKP-apr plasmid. (c) Cas9-containing pCasKP-apr plasmid.

to-insert molar ratio of 1:3, as shown in Table 4. A negative control ligation mixture, containing sterile distilled water instead of a PCR product, was also prepared. Ligation was incubated at room temperature for at least 1 h or overnight at 16°C (Wang et al. 2018).

For transformation, 50 μl of competent *E. coli* DH10B cells were aliquoted into 1.5 ml microcentrifuge tubes on ice. Five microliters of the ligation mixture or control mixtures were added. For positive control, 0.5 μl of pJET1.2 plasmid was added to competent cells. After incubation on ice for 10 min, tubes were heat-shocked at 42°C for 45 s, then placed on ice for 2 min. Next, 450 μl of Luria–Bertani (LB) medium was added, and tubes were incubated at 37°C for 1 h.

Transformed cells (250 μl) were spread onto LB agar plates containing ampicillin (100 μg/ml) and incubated for 18 h at 37°C. Single colonies were picked and cultured in 4 ml LB broth with ampicillin (100 μg/ml) at 37°C, 180 rpm for 18 h (Tu et al. 2005, Chang et al. 2017). Plasmid DNA was extracted using the Plasmid Plus DNA Purification Mini Spin Column Kit (Genaxxon, Germany), and DNA concentration was measured by a Nanodrop spectrophotometer. Purified plasmids were stored at −20°C.

For verification, plasmids were digested with selected restriction enzymes based on plasmid maps and incubated at 37°C for 2 h (Table 5). The resulting DNA fragments were analyzed by gel electrophoresis and compared with expected sizes.

Plasmid construction (pSGKP–AmpR(Pro)–ApmR–Cas9/APOBEC1)

The ApmR gene (154 bp), cloned into the pJET1.2 plasmid, was excised and subcloned into the pUC19 vector. The digestion reaction was prepared using 10 μl (500 ng) of pJET1.2 + ApmR vector, 2 μl of 10X Green Buffer (Thermo Fisher Scientific, USA), 0.2 μl of BpiI, and distilled water to a final volume of 20 μl. Similarly, the pUC19 + AmpR(Pro) vector (15 μl, 1.5 μg) was digested with 2 μl of 10X Green Buffer, 0.2 μl of BpiI, 1 μl of FastAP Thermosensitive Alkaline Phosphatase (1 U/μl), and water to a final volume of 20 μl. Both reactions were incubated at 37°C for 2 h. Digestion products were separated on a 1% agarose gel stained with ethidium bromide, run at 120 V for 30 min, and visualized under UV light. The desired bands were excised and purified according to the manufacturer's protocol using a purification kit. DNA concentrations were measured using a microvolume spectrophotometer, and samples were stored at −20°C until use. Ligation reactions (Table 4) were prepared and incubated at room temperature for 1 h, followed by transformation into chemically competent *E. coli* DH10B cells. Plasmid DNA was subsequently isolated and stored at −20°C.

The pSGKP-spe vector was amplified by PCR (Fig. 4), excluding the spectinomycin resistance region, to generate the backbone vector for further cloning. The PCR mix contained 5 μl of 5X Q5 Reaction Buffer, 5 μl of 5X Q5 High GC Enhancer, 2.5 μl of forward and reverse primers, 0.5 μl of 10 mM dNTPs, 1 μl of template DNA (20 ng), 0.25 μl of Q5 Hot Start High-Fidelity DNA Polymerase, and distilled water to 25 μl. The cycling conditions were initial denaturation at 98°C for 2 min; 35 cycles at 98°C for 10 s, 60°C for 10 s, and 72°C for 135 s; final extension at 72°C for 5 min, then held at 10°C. AmpR(Pro) and ApmR inserts cloned into pUC19 were digested with *BglII* and *MssI* restriction enzymes prior to cloning.

Table 3 Primers of the target regions to be used in the vector (pSGKP–AmpR(Pro)–ApmR-Cas9/AraBAD).

Target region	Nucleotide sequence (5′→3′)	Amplicon size (bp)
AmpR(Pro)	F-CGCGGAACCCCTATTTGTTTATTTTTC R-TCACTCTTCCTTTTTCAATATTATTGAAGC	154
AraBAD	F-TTATGACAACTTGACGGCTACATC R-ATGGAGAAACAGTAGAGAGTTGC	1190
ApmR	F-GAGAGTATGTCATCAGCGGTGGAGTG R-AGTAAACTCAGCCAATCGACTGGCGAG	829
Cas9	F-ATGGATAAGAAATACTCAATAGGC R-TCAGTCACCTCCTAGCTGAC	4108
Cas9(D10A)–APOBEC1	F-ATGAGTAGCGAAACCGGTC R-TCAGTCACCTCCTAGCTGAC	4841

Table 4 Ligation mix.

Component	Quantity
Vector DNA	50 ng
Insert DNA	X ng
10X T4 DNA Ligase Buffer (NEB)	1 μl
T4 DNA Ligase (NEB)	0.5 μl
Nuclease-free water	Up to 10 μl

Cloning of AmpR(Pro) and ApmR into vector by T5 exonuclease DNA assembly method

One milliliter of 5X T5 Exonuclease DNA Assembly (TEDA) solution was prepared by adding 0.5 M Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 50 mM DTT (dithiothreitol), 0.25 g PEG (polyethylene glycol) 8000, and 1 μl of 10 U/μl T5 exonuclease. Aliquots of 20 μl were dispensed into 200 μl PCR tubes and stored at −80°C until use (Xia et al. 2019). The vector backbone (pSGKP-spe, lacking spectinomycin resistance) was amplified by PCR and assembled with AmpR(Pro) and ApmR inserts using the TEDA cloning method.

Ligation of gRNAs and cloning into vector

For the preparation of the CRISPR vector pBECKP, 2 μl of FastDigest Green Buffer, 2 μg of plasmid DNA, 0.5 μl of Eco31I, 0.5 μl of FastAP, and distilled water were combined to a final volume of 20 μl and incubated at 37°C for 5 h. The reaction products were run on a 1% agarose gel at 100 V for 30 min and visualized under UV light. Plasmid DNA was extracted from the gel and quantified.

Annealing of gRNA oligonucleotides was performed by mixing 1 μl of each 100 μM gRNA, 1 μl of 10X T4 ligation buffer, 1 μl of 10 mM ATP, 0.25 μl of T4 Polynucleotide Kinase (10 U/μl), and 5.75 μl of sterile distilled water. The mixture was incubated at 37°C for 30 min, heated at 95°C for 5 min, and gradually cooled to 25°C over 5 min. The annealed gRNA pairs were diluted 1:200 in sterile distilled water.

Ligation reactions were assembled by combining 50 ng of linearized vector DNA, 1 μl of diluted gRNA duplex, 1 μl of 10X T4 DNA ligase buffer, 0.2 μl of T4 DNA ligase, and sterile distilled water to a final volume of 10 μl, and incubated at room temperature for 1 h. Following ligation, 5 μl of the reaction mixture was added to 50 μl of competent *E. coli* DH10B cells on ice, and the cells were transformed as previously described (Sanjana et al. 2014).

Results

The MICs of the isolates ($n = 5$) ranged from 4 to 128 μg/ml for imipenem, 8 to 64 μg/ml for meropenem, and 8 to 256 μg/ml for ertapenem. All isolates were resistant to imipenem, meropenem, and ertapenem. PCR analysis revealed the presence of the $bla_{\text{OXA-48}}$-like, and $bla_{\text{CTX-M-15}}$ genes in all isolates (100%). The $bla_{\text{NDM-1}}$ gene was detected in only one isolate (20%) (Table 6).

All isolates exhibited resistance to kanamycin (1 μg/ml), spectinomycin (>1 μg/ml), streptomycin (>1 μg/ml), hygromycin (1 μg/ml), and chloramphenicol (>1 μg/ml) while remaining susceptible only to apramycin at concentrations ranging from 1 to 0.0625 μg/ml.

Based on sequence alignments of $bla_{\text{OXA-48}}$-like, $bla_{\text{NDM-1}}$, and $bla_{\text{CTX-M-15}}$, gRNA target sequences were designed and are presented in Table 7.

The araBAD and ApmR regions were successfully amplified from the pCasKP-apr plasmid by PCR and gel purified. Each PCR product was cloned individually into the pJET1.2 vector, followed by transformation and plasmid DNA isolation. Restriction digestion of the pJET1.2-AmpR plasmid was performed using SacI and HindII, whereas the pJET1.2-AraBAD plasmid was digested with Eco32I and HindIII (Fig. 5a and b). Restriction enzyme digestion confirmed the correct insert sizes via agarose gel electrophoresis.

The AmpR(Pro) region was excised from the pJET1.2 vector using HindIII and PstI, and the pUC19 plasmid was digested with the same enzymes. Four colonies were selected (Fig. 6a), cultured in LB broth, and incubated at 37°C with shaking at 180 rpm for 18 h. Plasmid DNA was extracted from each culture and digested with appropriate enzymes, yielding expected band patterns (Fig. 6b).

Subsequently, the AmpR(Pro) and ApmR regions were cloned into the pSGKP vector, which contains the CRISPR-Cas9 system, using the TEDA method. To confirm successful cloning, two restriction digestions were performed (Fig. 7a). The first digestion using KpnI and BoxI produced bands of 3910 and 805 bp, respectively, and the second digestion with HindIII and SalI yielded bands of 2846 and 1869 bp, respectively, consistent with the expected sizes (Fig. 7b).

Table 5 Reactions prepared for verification.

	AmpR(Pro)	AraBAD	ApmR	Cas9	Cas9(D10A)–APOBEC1
FastDigest Green Buffer (10X)	2 μl	2 μl	2 μl	2 μl	2 μl
Plazmid (1.5–2 μg)	x	x	x	x	x
Restriction enzyme (0.5 μl)	MssI	Eco32I HindIII	SacI HindIII	BglII Eco32I BamHI	BglII Eco32I BamHI
ddH_2O	Up to 20 μl				

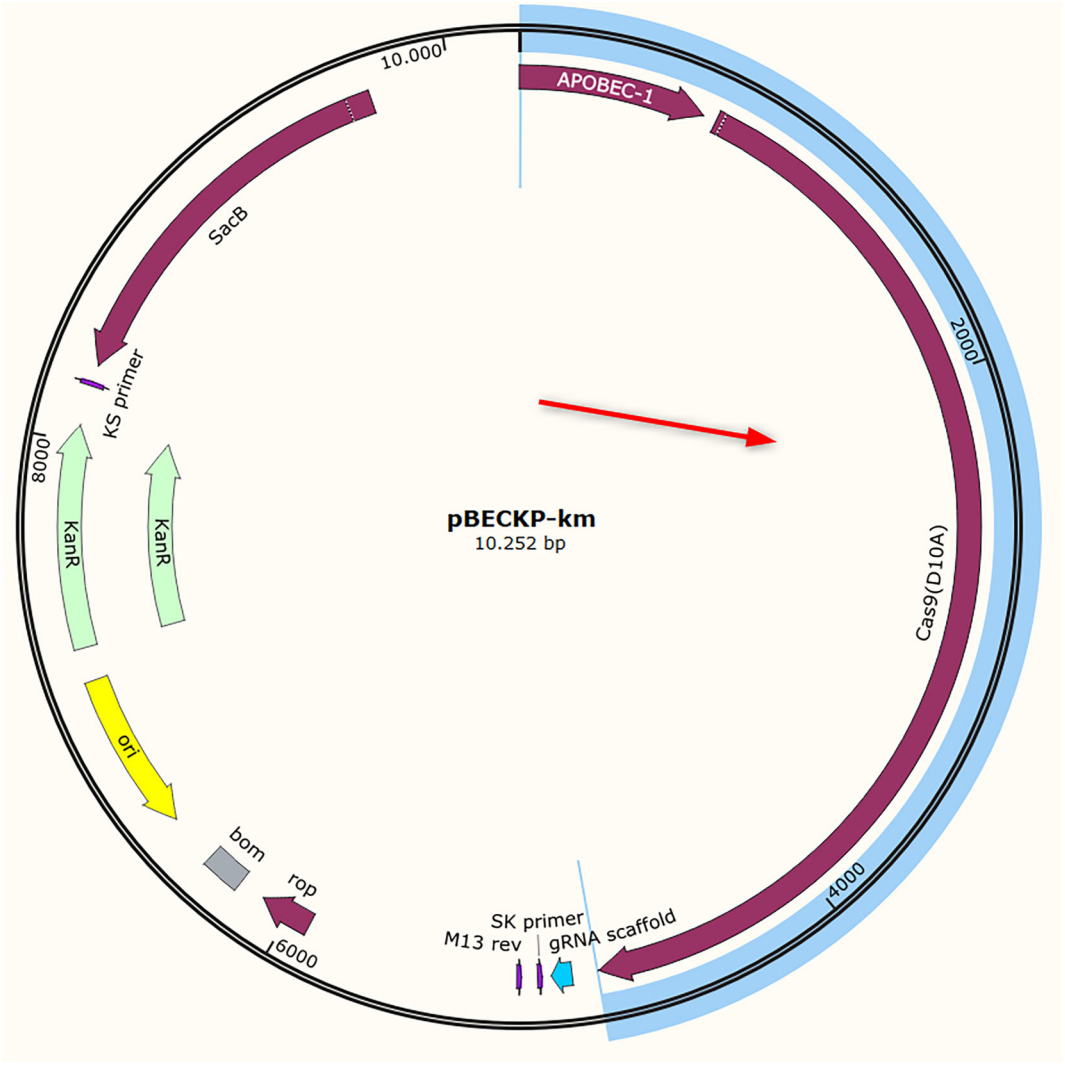

Figure 3 APOBEC1–Cas9(D10A) in the pBECKP-km plasmid.

Discussion

AMR is one of the major public health threats of the 21st century. CRE, including *K. pneumoniae*, are listed as "urgent threats" by the WHO, and accurate mortality rate estimates for CRKP remain insufficient (Antimicrobial Resistance Collaborators 2022). Despite increasing antibiotic resistance, treatment options are limited, highlighting the urgent need for novel therapeutic strategies to address this crisis. CRISPR-Cas technology represents a revolutionary genome-editing tool, emerging as a promising approach to combat antibiotic resistance and demonstrating significant impact in molecular biology.

Globally, OXA-48 and NDM-1 remain the most prevalent carbapenemases in *K. pneumoniae*. Therefore, treatment strategies—including CRISPR-Cas-mediated gene editing—are actively being explored (Isler et al. 2022, Kurt et al. 2024, Qala Nou et al. 2025). Gene editing using the CRISPR-Cas system offers an innovative approach to counter antimicrobial resistance.

Targeting resistance genes in MDR bacteria requires the design of CRISPR-Cas systems that incorporate a CRISPR array and mul-

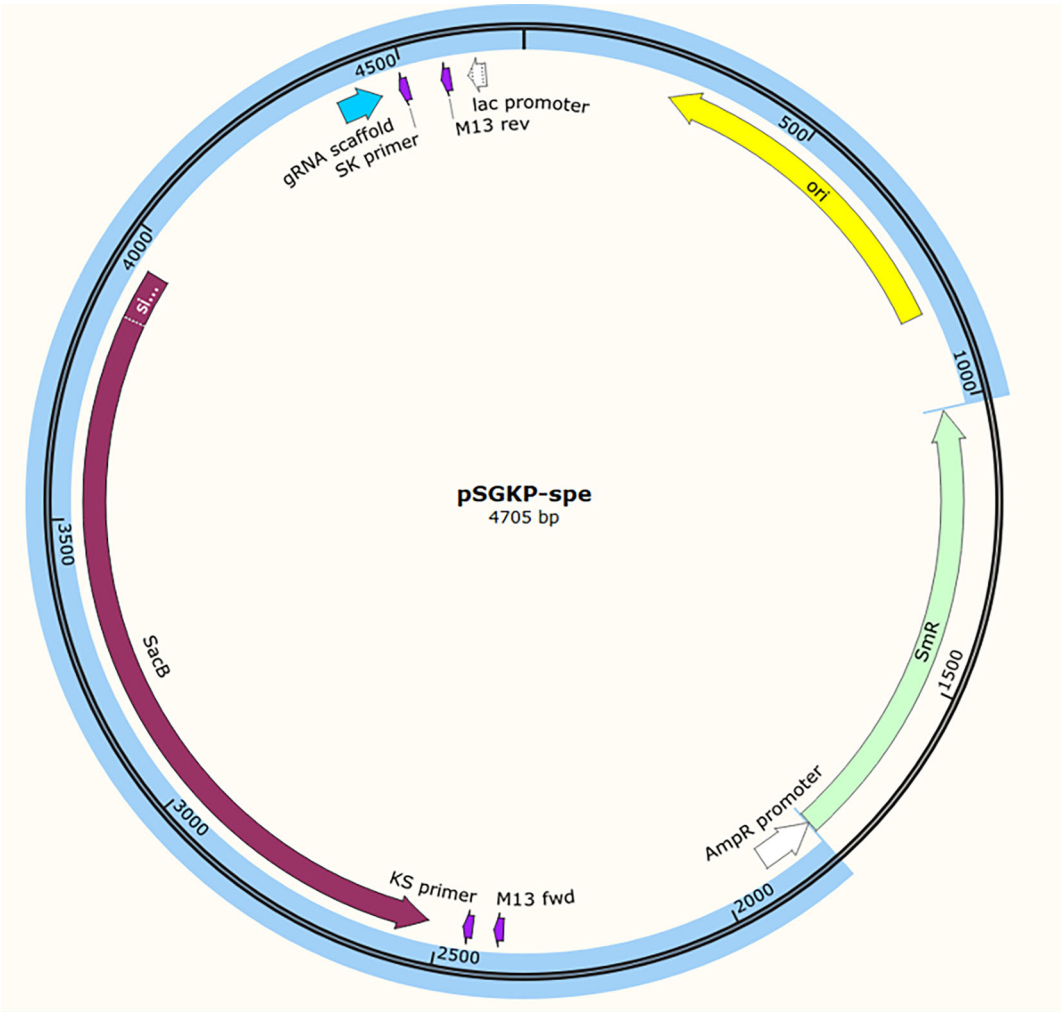

Figure 4 Part of the vector obtained from the pSGKP-spe plasmid.

Table 6 MIC values, antimicrobial susceptibility, resistance genes, and clinical sample types of the isolates.

	MIC (μg/ml) values and susceptibilities of the isolates			Resistance genes detected in isolates			Type of sample
Strain	İmipenem (μg/ml)[*]	Meropenem (μg/ml)[*]	Ertapenem (μg/ml)[*]	NDM-1	OXA-48 like	CTX-M-15	
1	128 [R]	64 [R]	256 [R]	+	+	+	Catheter tip
2	4 [R]	16 [R]	128 [R]	–	+	+	Blood
3	32 [R]	8 [R]	64 [R]	–	+	+	Blood
4	8 [R]	8 [R]	8 [R]	–	+	+	Blood
5	4 [R]	8 [R]	64 [R]	–	+	+	Catheter tip

*Susceptibility R: resistant.

tiple gRNAs (Wu et al. 2021). In this study, gRNAs were designed based on sequence analyses of $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{CTX-M-15}}$ genes from MDR *K. pneumoniae* isolates, highlighting the importance of customized plasmid construction for CRISPR-Cas applications in MDR bacteria.

Our results indicate that commercially available CRISPR plasmids are not universally applicable to clinical isolates, as their selectable antibiotic resistance markers often conflict with intrinsic resistance profiles. Such incompatibility compromises clone selection and limits the feasibility of genome engineering approaches. Given the diversity of resistance mechanisms among clinical *K. pneumoniae* isolates, plasmid constructs should therefore be designed on an isolate-specific basis rather than assuming species-wide compatibility.

Within this context, recent studies provide important proof-of-concept evidence for the potential of CRISPR-based approaches

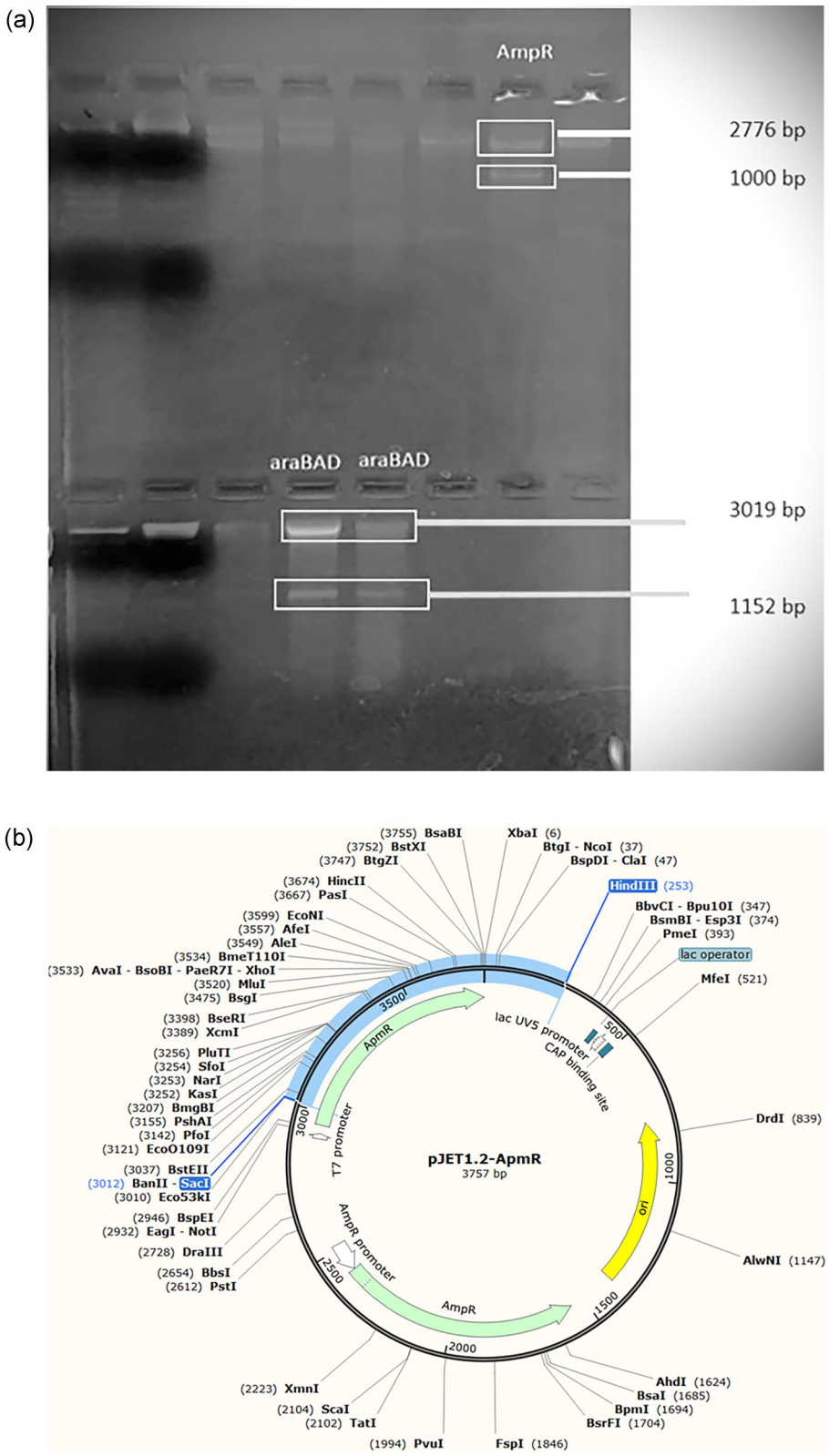

Figure 5 (a) Agarose gel images showing verification of ApmR and AraBAD cloned into pJET1.2. (b) SacI and HindIII restriction enzyme cleavage sites in the pJET1.2-ApmR vector.

Table 7 gRNA sequences of bla_{OXA-48}-like, bla_{NDM-1}, and $bla_{CTX-M-15}$ genes.

CRISPR/base editing	Primer sequences (5′→3′)
Kp_OXA-48_gRNA_CRISPR_F	TAGTTTTACTGAACATAAATCACA
Kp_OXA-48_gRNA_CRISPR_R	AAACTGTGATTTATGTTCAGTAAA
Kp_OXA-48_gRNA_Edit_F	TAGTAATGGCAAGAAAACAAAAGT
Kp_OXA-48_gRNA_Edit_R	AAACACTTTTGTTTTCTTGCCATT
Kp_NDM-1_gRNA_CRISPR_F	TAGTATTGCCCAATATTATGCACC
Kp_NDM-1_gRNA_CRISPR_R	AAACGGTGCATAATATTGGGCAAT
Kp_NDM-1_gRNA_Edit_F	TAGTATTGGCCAGCAAATGGAAAC
Kp_NDM-1_gRNA_Edit_R	AAACGTTTCCATTTGCTGGCCAAT
Kp_CTX-M-15_gRNA_CRISPR_F	TAGTACTTCCTAACAACAGCGTGA
Kp_CTX-M-15_gRNA_CRISPR_R	AAACTCACGCTGTTGTTAGGAAGT
Kp_CTX-M-15_gRNA_Edit_F	TAGTCCAGTTCACGCTGATGGCGA
Kp_CTX-M-15_gRNA_Edit_R	AAACTCGCCATCAGCGTGAACTGG

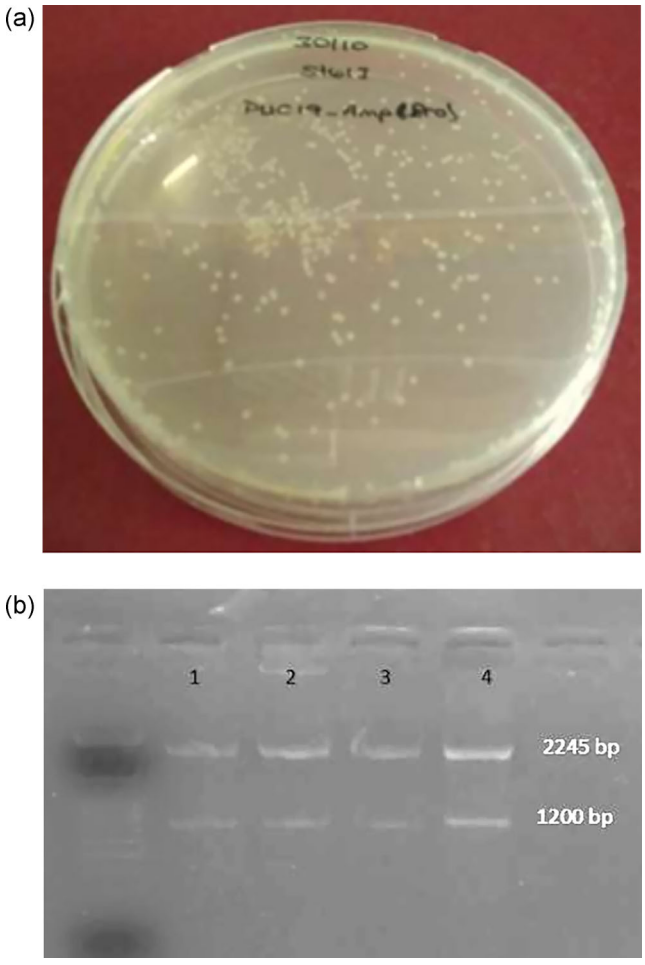

Figure 6 (a) Colonies of pUC19-AmpR(Pro) after transformation. (b) Agarose gel image of pUC19-AmpR(Pro)plasmid (3445 bp) digested with BglII and MssI.

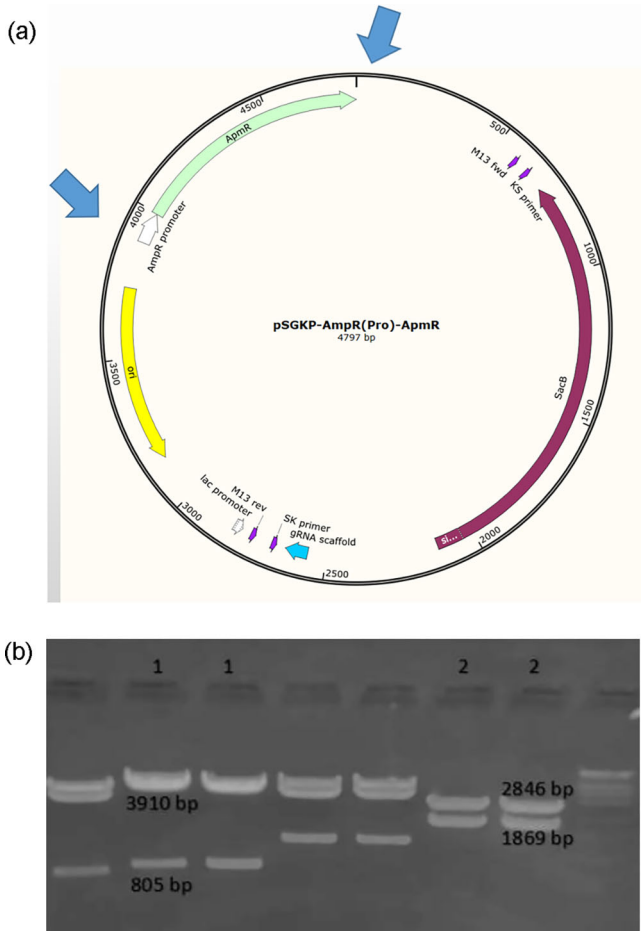

Figure 7 (a) pSGKP–AmpR(Pro)–ApmR plasmid. (b) Restriction digestion of pSGKP–AmpR(Pro)–ApmR plasmid with KpnI/BoxI and HindIII/SalI.

in *K. pneumoniae*. For example, a recent study by Hajizadeh and Oloomi (2025) demonstrated that genetic manipulation approaches, particularly CRISPR interference (CRISPRi), can effectively modulate antibiotic resistance in *K. pneumoniae*. In that study, CRISPRi-mediated targeting of carbapenem resistance genes resulted in a marked reduction in meropenem resistance, which correlated with decreased mRNA expression levels of bla_{OXA-48} and bla_{NDM-1}. Notably, careful design of single-guide RNAs (sgRNAs) was shown to be critical for effective transcriptional repression. These findings highlight the potential of CRISPRi-based strategies as functional tools for investigating and

modulating resistance mechanisms in MDR clinical isolates. Importantly, such studies underscore the necessity of precise sgRNA design and robust genetic tools, reinforcing the relevance of optimized plasmid construction as a foundational step for implementing CRISPR-based methodologies in MDR *K. pneumoniae* (Hajizadeh and Oloomi 2025).

The number of studies addressing this topic remains limited, and those that do explore it involve differing resistance genes and bacterial profiles. Wang et al. (2018) used the two-plasmid pCasKP–pSGKP system to individually delete carbapenemase and ESBL genes in a hypermucoviscous, carbapenem-resistant *K. pneumoniae* strain. In our study, we designed a two-plasmid CRISPR system for gene editing based on these plasmids. The first critical step was to identify suitable antibiotics that could serve as effective selection markers during cloning. However, the presence of resistance genes such as kanamycin, spectinomycin, and hygromycin in the commercial plasmids used by Wang et al. and in other studies posed a significant challenge, as all *K. pneumoniae* isolates were resistant to these antibiotics, rendering them ineffective for selection (Sun et al. 2019, Wang et al. 2019, Tagliaferri et al. 2020). While CRISPR-based genome editing has been explored in bacterial systems, establishing such approaches in MDR clinical isolates remains technically challenging. Accordingly, the present study primarily focused on plasmid design and methodological optimization as essential preliminary steps toward establishing functional CRISPR-Cas tools in MDR *K. pneumoniae*. Within this framework, the identification of apramycin as a compatible selection marker represents a key methodological outcome, underscoring the necessity of isolate-specific optimization rather than the direct application of existing plasmid constructs. These challenges further highlight the technical limitations of current DNA assembly methods when applied to complex plasmid constructs in MDR clinical isolates. If these components had been successfully assembled, the resulting construct would have been introduced into the isolates by electroporation, followed by MIC testing for ertapenem, meropenem, and imipenem.

Another study by Hao et al. (2020) used the pSGKP-km and pCasKP plasmids to delete *bla*KPC, *bla*NDM, and $bla_{\text{OXA-48}}$ genes in CRE, thereby restoring their susceptibility to carbapenems. However, the ligation methods used for plasmid construction were not described in the study (Hao et al. 2020).

Moreover, a study conducted in Canada in 2022 reported a similar technical challenge, in which a 125 bp fragment containing a lac promoter, lac operator, and catabolite activator protein could not be successfully cloned into the 3520 bp pSPPH21 vector. The researchers systematically evaluated various parameters such as the competent bacterial strain, incubation duration, ligation mixture, and vector-to-insert ratios, ultimately proposing an optimized protocol to support future efforts (MacPherson et al. 2022). Similarly, in our study, Cas9 and APOBEC1 fragments initially cloned into the pJET1.2 intermediate vector were PCR-amplified and subsequently subjected to ligation into the final construct (pSGKP–AmpR(Pro)–ApmR). Ligation reactions were carried out at 22°C for 1 h, 16°C overnight, and using thermal cycling between 10°C and 30°C for 10 h. For each ligation condition, vector-to-insert ratios of 1:1 to 1:5 were tested. Although colonies were obtained following transformation and plasmid DNA was isolated, restriction enzyme digestion failed to produce bands of the expected size, indicating unsuccessful integration of the target fragments.

In the present study, antibiotic-based selection was employed to ensure experimental simplicity and reproducibility. Nevertheless, alternative neutral or nonantibiotic selection strategies are recognized tools in CRISPR-based genome engineering (Jiang et al. 2013, Stukenberg et al. 2022). Approaches such as metabolic complementation or fluorescent reporter-based selection can reduce selective pressure and eliminate the need for antibiotic resistance markers, which is particularly important in studies involving MDR clinical isolates (Li et al. 2015). Accordingly, the integration of marker-free or neutral selection systems into CRISPR-Cas platforms may reduce selective pressure and improve compatibility with MDR clinical isolates. Future studies building on the plasmid design presented here may benefit from incorporating such selection strategies to further CRISPR-mediated applications in *K. pneumoniae* and other MDR pathogens.

CRISPR-Cas adaptation in bacteria still lags behind its use in eukaryotes. Effective application in prokaryotes demands isolate-specific system optimization. Cas9 cytotoxicity and off-target effects are documented concerns; further research into cytidine deaminases such as APOBEC is also needed (Lee et al. 2016, Zhang and Voigt 2018, Alberti and Corre 2019, Arroyo-Olarte et al. 2021). In our study, the inability to clone Cas9 and APOBEC into the backbone vector may similarly be attributed to these adverse effects. The designed CRISPR system was tailored for MDR *K. pneumoniae* isolates using specific gRNAs targeting resistance genes. If cloning had been successful, each gRNA would have been introduced separately with the corresponding construct for individual gene editing.

Conclusions

Limitations of our study include technical challenges in constructing CRISPR plasmids for MDR isolates, the limited body of literature in this niche, and restricted funding. Nonetheless, to our knowledge, this is the first reported CRISPR-Cas-based gene editing study on MDR *K. pneumoniae* clinical isolates in our country. Our results underscore the necessity of isolate-specific vector design and alternative selection markers for successful editing.

These findings not only contribute to the molecular understanding of resistance mechanisms but also lay a foundation for advanced applications such as phage engineering, functional genomics, and targeted antimicrobial strategies. As CRISPR-Cas systems advance, the development of adaptable, strain-specific editing tools will be essential for integrating genome editing into microbiological research and clinical practice.

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Conflicts of interest

None declared.

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