

Targeted Elimination of *bla*_{NDM-5} Gene in *Escherichia coli* by Conjugative CRISPR-Cas9 System

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Purpose: Plasmid-borne carbapenem resistance gene *bla*_{NDM-5} accelerates the dissemination of carbapenem-resistant Enterobacteriaceae. To efficiently eliminate the *bla*_{NDM-5}-harboring plasmid and sensitize the antibiotic-resistant bacteria to meropenem, we used the CRISPR-Cas9 system for combating the carbapenem-resistant *Escherichia coli* (*E. coli*).

Methods: A series of CRISPR-Cas9 plasmids was constructed, and specific guide RNAs (sgRNA) were designed to target the *bla*_{NDM-5} gene. We used chemically transformation or conjugation delivery methods, and the elimination efficiency in each recipient strains was evaluated by plate counting, PCR and quantitative real-time PCR (qPCR). Antimicrobial susceptibility test was carried out by using the broth microdilution method. In addition, we assessed the effect of the CRISPR-Cas9 system of adaptive immunity on the prevention of the exogenous resistant plasmids pNDM-5 by introducing the system into *E. coli* J53.

Results: The results showed that pCas9, pCas9-oriT and pBAD-Cas9-oriT can effectively eliminate *bla*_{NDM-5} in *E. coli* with >94.00% elimination efficiency. The *bla*_{NDM-5}-harboring *E. coli* successfully restored their susceptibility to meropenem, with eight-fold reduction of minimum inhibitory concentration (MIC) values (from 16 µg/mL to 0.06 µg/mL). The *E. coli* J53 strain containing plasmid pCas9-N reduced the number of transconjugants by 26-fold.

Conclusion: The CRISPR-Cas9 system achieved plasmid clearance and simultaneous re-sensitization to meropenem in *E. coli*. The CRISPR-Cas9 system could block the horizontal transfer of plasmid pNDM-5. The conjugative delivery of CRISPR-Cas9 provides a new tool for the removal of resistance plasmids and sensitize the recipient to carbapenem. It provides a therapeutic approach to counteract the propagation of *bla*_{NDM-5} gene among clinical pathogens.

Keywords: antimicrobial resistance, eliminating efficiency, plasmid conjugation, re-sensitization

Introduction

Antimicrobial resistant microorganisms have become a public health concern in recent decades.¹ Horizontal gene transfer (HGT) is the main contributor to the rapid dissemination of antibiotic resistance genes (ARGs).² The emergence and dissemination of carbapenem resistance in Enterobacteriaceae mediated by plasmid-borne carbapenemase genes such as *bla*_{NDM-5} have been a concern.³ The *bla*_{NDM-5} gene is carried by conjugative plasmids that can horizontally transfer, resulting in multidrug or extensively drug-resistant phenotypes.⁴ Therefore, novel strategies to combat plasmid-mediated antimicrobial resistance should be developed.

The CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats – CRISPR-associated protein-9 nuclease) system provides an effective tool to eradicate ARGs and plasmids carrying ARGs by cleaving double-stranded DNA.⁵ After the sgRNA targets specific sequences and recruits Cas9 protein to form functional complex, the Cas9 protein acts as a nuclease and generates a blunt end double-strand break (DSB). Considering the lack of the error-prone non-homologous end-joining

pathway in prokaryotes, Cas9-mediated DSB cannot be repaired spontaneously, resulting in DSB in plasmid sequence and thus triggering plasmid elimination.⁶

Existing bacterial CRISPR-Cas systems can target ARGs and observe a CRISPR-Cas mediated cytotoxicity in different bacteria.^{7–9} Subsequent studies have developed several CRISPR-Cas9 systems for specific elimination of plasmids in bacterial hosts that carry multiple resistant plasmids.^{10–13} The mobile genetic elements (MGE)-associated CRISPR-Cas9 may serve as a therapeutic approach to control the dissemination of antibiotic resistance among clinical pathogens.^{14,15} These works support that CRISPR-Cas9 system can solve the problem of multi-drug resistance in clinical and environmental settings. However, concerning the CRISPR-Cas9 system in clinical application, the delivery method must be developed and optimized.¹⁶ Bacterial conjugation may be a feasible way to deliver the CRISPR-Cas9 into bacteria in their natural environment.^{17–19} In the present study, we investigated the effect of CRISPR-Cas9 system on the elimination *bla*_{NDM-5} gene and explored the potential of CRISPR-Cas9 system deliver methods via bacterial conjugative transfer to control the dissemination of *bla*_{NDM-5} resistance.

Materials and Methods

Bacterial Strains and Plasmids

The strains and plasmids used in this study are listed in Table 1, while the primers used in this study are listed in Table 2. *E. coli* 01 containing *bla*_{NDM-5} and *E. coli* J53 were prepared for conjugation experiments.²⁰ Donor strain *E. coli* 01 and recipient strain *E. coli* J53 grown separately in LB medium were cultured at 37°C. Equal volumes of the donor culture and the recipient culture were mixed, and then incubated at 37 °C. After 12 h, dilutions of the mixtures were plated on Luria-Bertani (LB) agar plates. Transconjugants, named as *E. coli* 02, were incubated at 37 °C in Luria-LB agar plates containing with chloramphenicol (50 µg/mL) and sodium azide (200 µg/mL). *E. coli* 02 was verified by PCR via using the primers NDM-5-F/R. *E. coli* strains were grown in LB broth and agar plates at 37 °C. When needed, antibiotics such as chloramphenicol (50 µg/mL), meropenem (1 µg/mL) and sodium azide (200 µg/mL) were added.

Plasmid Construction

A 20 nt sgRNA (N-F/R) targeting the *bla*_{NDM-5} gene was designed using the CHOPCHOP tool while adding *Bsa*I digestion sites. The primers were annealed and ligated with *Bsa*I-digested pCas9 by using T4 DNA ligase (NEB), and the

Table 1 Bacterial Strains and Plasmids Used in This Study

Bacterial Strains or Plasmids	Relevant Characteristics	Source or Reference
Bacterial Strains		
<i>E. coli</i> DH5α	F-,φ80dlacZΔM15,Δ(lacZYA-argF)UI69,deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, λ-, thi-1, gyrA96, relA1	Laboratory stock
<i>E. coli</i> S17-1	with an integrated RP4-2-Tc::Mu-Km::Tn7	Laboratory stock
<i>E. coli</i> ATCC 25922	Quality control strains for antimicrobial susceptibility	Laboratory stock
<i>E. coli</i> J53	F-ara Δ(lac-proAB) rpsL(Str){φ80 dlacΔ(lacZ)M15	Laboratory stock
<i>E. coli</i> 01	Isolated with plasmid pNDM-5	Laboratory stock
<i>E. coli</i> 02	J53+pNDM-5 with IncFII-type	In this study
<i>E. coli</i> 03	J53+pUC19-NDM-5	Laboratory stock
<i>E. coli</i> 04	J53+pNDM-5 with IncX3-type	Laboratory stock
Plasmids		
pCas9(#42876)	pSC101 ori, Cas9 expression plasmid, Cm ^R	Laboratory stock
pBBR1MCS-2	Broad-host-range mobilizable plasmid, ori _{TRP4} , Kan ^R	Laboratory stock
pCas9-N	pCas9 with sgRNA targeting <i>bla</i> _{NDM-5} , Cm ^R	In this study
pCas9-oriT-N	pCas9 with ori _{TRP4} and sgRNA targeting <i>bla</i> _{NDM-5} , Cm ^R	In this study
pBAD-Cas9-oriT-N	pCas9 with ori _{TRP4} and sgRNA targeting <i>bla</i> _{NDM-5} , arabinose pBAD Promoter, Cm ^R	Laboratory stock

Abbreviations: Cm^R, chloramphenicol-resistant; Kan^R, kanamycin-resistant.

Table 2 Primers Used in This Study

Primer Name	Primer Sequence (5'-3')
N-F	<u>AAACGATCCAGTTGAGGATCTGGGG</u>
N-R	<u>AAAACCCCGATCCTCAACTGGATC</u>
DR-F	CACGCATTGATTTGAGTCAG
DR-R	GGTGATGTCGGCGATATAGG
oriT-XbaI-F	<u>CGGTCTAGACAGCTGGCAATTCGGTTTCG</u>
oriT-XbaI-R	<u>CGGTCTAGATGGGTATGTGGTCGAAGGCTG</u>
NDM-5-F	GCTCTAGAATGGCTCCAGATGACAAACAT
NDM-5-R	GCTCTAGATGGGTCGAGGTCAGGATAGG
I6S-F	CGGTGAATACGTTTCYCGG
I6S-R	GGWTACCTTGTACGACTT
qPCR-NDM-F	TTTGGCGATCTGGTTTTCCG
qPCR-NDM-R	ATCAAACCGTTGGAAGCGAC
pCas9-F	AACACGCATTGATTTGAG
pCas9- R	ATAGGAAGGTATCCGACT

Note: The underline indicates the enzyme cutting site.

recombinant plasmids named pCas9-N.²¹ The recombinant plasmids were transformed into *E. coli* DH5 α by using the heat-shock method,²² and the inserted sgRNA sequences were verified using primers DR-F/R. The plasmid pCas9 without the sgRNA cassette was used as a control plasmid.

Bacterial conjugation via plasmid RP4 requires the origin of transfer (oriT_{RP4}), while the other proteins necessary for conjugation were obtained from *E. coli* S17-1, which contains chromosomally integrated genes from the natural conjugative plasmid RP4. The OriT_{RP4} fragment was amplified from pBBR1MCS-2 by using primers oriT-*XbaI*-F/R and inserted into *XbaI*-digested pCas9 and pBAD-Cas9, resulting in conjugative plasmids pCas9-oriT and pBAD-Cas9-oriT.²³ Subsequently, 20 nt sgRNA ligated with *BsaI*-digested pCas9-oriT and pBAD-Cas9-oriT were prepared and named as pCas9-oriT-N and pBAD-Cas9-oriT-N by using T4 DNA ligase (NEB).²¹ The recombinant plasmids were transformed into *E. coli* S17-1 by using the heat-shock method.^{17,22} The plasmid pCas9-oriT and pBAD-Cas9-oriT, which lack the sgRNA cassette, were used as a control plasmid.

Plasmid Transformation and pNDM-5 Eliminative Efficiency

Chemically competent *E. coli* 02 samples were prepared using 0.1 M MgCl₂-CaCl₂ and competent cells were transformed with plasmids pCas9-N by using the heat-shock method at 42 °C for 90s.²² In addition, different plasmid types have different replicons and backbone structures, and the effects of different plasmid types (IncFII and IncX3) were analyzed.^{4,24} In brief, chemically competent *E. coli* 03 was transformed with plasmids pCas9-N. The culture was diluted and plated on LB agar plates with chloramphenicol (50 μ g/mL) only or the combination of chloramphenicol (50 μ g/mL) and meropenem (1 μ g/mL) to determine the number of colony-forming units (CFU). Elimination efficiency was calculated using the equation (1 – colonies grown on chloramphenicol and meropenem plates/ colonies grown on chloramphenicol plates) \times 100%. The empty plasmid pCas9 was used as the negative control. Each experiment was performed in triplicate.

To further analyze the efficiency of pCas9 on eliminating *bla*_{NDM-5} gene, we detected the change of pNDM-5 copy number at each time point via qPCR. Genomic DNA was extracted using TIANamp bacteria DNA kit (Tiangen, Beijing, China). qPCR was performed using SYBRP remix ExTaqII (Takara, Dalian, China) with primers I6S-F/-R and qPCR-NDM-F/R.²⁵ The 2^{- $\Delta\Delta$ CT} method was used to calculate the change of pNDM-5 copy number in the experimental group compared with the control group. All reactions were run in triplicate.

Conjugation Assays Plasmids and Eliminating Experiment

Donor strain *E. coli* S17-1 containing plasmid pCas9-oriT-N and recipient strain *E. coli* 02 grown separately in LB medium were cultured overnight and grew at an OD₆₀₀ value of 0.4 at 37 °C.^{17,26} Equal volumes of the donor culture and the recipient culture

were mixed, and then incubated at 37 °C. After 12 h, dilutions of the mixtures were plated on LB agar plates with chloramphenicol (50 µg/mL) only or the combination of chloramphenicol (50 µg/mL) and sodium azide (200 µg/mL) to select donor and transconjugant clones. The plates were incubated overnight at 37 °C. Colonies grown on the plates were enumerated, and the CFU were calculated. Conjugation efficiency was calculated by dividing the number of transconjugant strains by the number of donor strains. A total of 24 transconjugant strains were randomly picked for evaluating the eliminating efficiency via PCR. The plasmids pCas9-oriT and pNDM-5 were detected with primers pCas9-F/R and NDM-5-F/R, respectively. The empty plasmid pCas9-oriT was used as negative control. Each experiment was performed in triplicate.

Eliminating Condition – Incubation Time and Arabinose Concentrations

We verified the feasibility of CRISPR-Cas9 system via conjunctive delivery method. Furthermore, we determined whether efficient clearance can be achieved in other conjunctive plasmids. Notably, we then evaluated the target eliminating efficiency under different arabinose induction concentrations and incubation time.²⁷ After introducing pBAD-Cas9-oriT-N into *E. coli* 02 via conjugation, transconjugant strains were selected using LB agar plates supplemented with chloramphenicol (50 µg/mL), and PCR confirmation was carried out using primers pCas9-F/R and NDM-5-F/R. Overnight culture of pBAD-Cas9-oriT-N positive *E. coli* 02 was diluted 100-fold in 2 mL of LB broth containing 0.01%, 0.10%, 1.00% or 2.00% arabinose in combination with chloramphenicol (50 µg/mL). The cultures were incubated for 6 h at 37 °C with shaking at 180–200 rpm. After 6 h, the cultures were diluted and simultaneously plated on LB agar plates containing chloramphenicol (50 µg/mL) only or combination of chloramphenicol (50 µg/mL) and meropenem (1 µg/mL) to determine the number of CFU. Elimination efficiency was calculated using the equation (1–colonies grown on chloramphenicol and meropenem plates/ colonies grown on chloramphenicol plates) × 100%. The experiments were conducted using three randomly picked pBAD-Cas9-oriT-N positive *E. coli* 02 colonies.

Thereafter, the target eliminating efficiency under different arabinose incubation times was evaluated. Similarly, the overnight cultures of pBAD-Cas9-oriT-N positive *E. coli* 02 colonies were diluted 100-fold in 2 mL of LB broth containing 1.00% arabinose and chloramphenicol (50 µg/mL), followed by incubation at 37 °C with shaking for 16 h. At 0, 2, 4, 6, 8 and 16 h, 100 µL of culture were removed, diluted, and plated out for colony counting. The curing efficiency was calculated as above.

Antimicrobial Susceptibility Tests

The MIC of meropenem was determined using the broth microdilution method in accordance with the guide lines of Clinical and Laboratory Standards Institute.²⁸ The strains contained original *E. coli* 02 and the corresponding plasmid-eliminated strains eliminating *bla*_{NDM-5} gene. *E. coli* ATCC 25922 was routinely used as quality control strain. Each experiment was performed in triplicate.

The CRISPR-Cas9 System Preventing Plasmid Acquisition

We used *E. coli* J53 containing plasmid pCas9 as the recipient to assess whether the integration of CRISPR-Cas9 can impede the acquisition of pNDM-5 via conjugation, and the donor was *E. coli* J53 contained plasmid pNDM-5. The conjugation assay was performed as described above. The blocking of *bla*_{NDM-5} transfer was evaluated based on the number of transconjugants. Each experiment was performed in triplicate.

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism software (version 6, GraphPad Software, CA, United States).

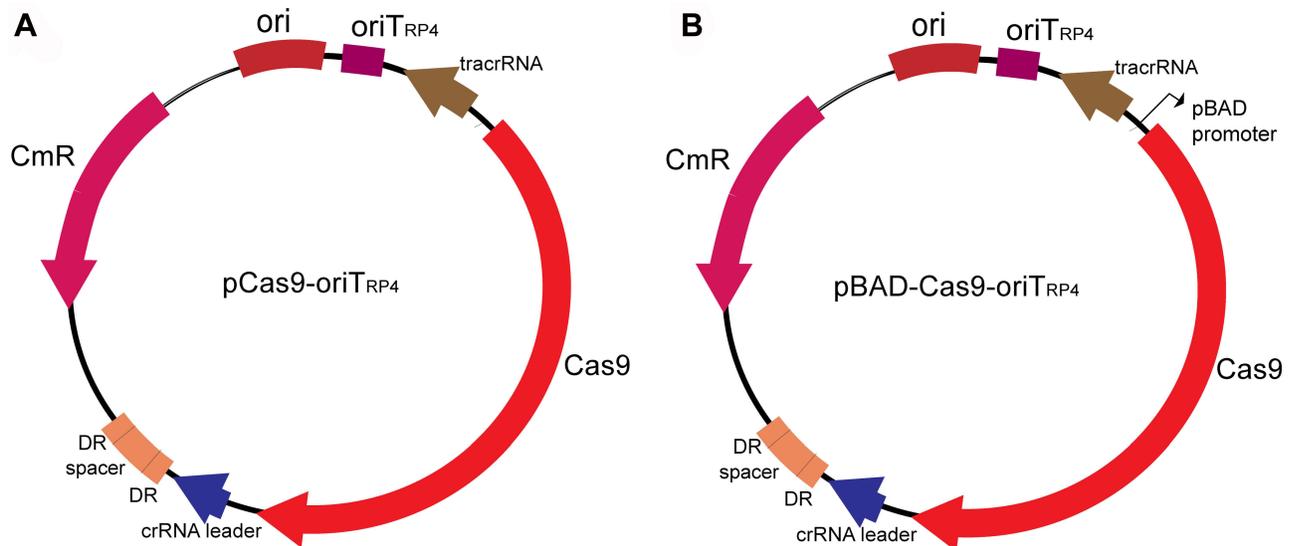


Figure 1 Plasmid map of pCas9-oriT and pBAD-Cas9-oriT. (A) Plasmid pCas9-oriT containing Cas9, tracrRNA, crRNA, specific guide RNAs (sgRNA), origin of transfer (ori_{TRP4}) and chloramphenicol resistance gene (CmR). DR: direct repeats (B) The pBAD-Cas9-oriT containing the Cas9, tracrRNA, crRNA, sgRNA, ori_{TRP4} , the arabinose inducing promoter pBAD and CmR.

Results

Construction of CRISPR-Cas9 Plasmids Targeting bla_{NDM-5}

Plasmid pCas9-N harbors essential elements, including Cas9, tracrRNA and crRNA, in combination with the 20 nt sgRNA. To test the utility of the CRISPR-Cas9 system via conjugation, we used pCas9 and pBAD-Cas9 as the backbone to construct the pCas9-oriT and pBAD-Cas9-oriT, which ligate the ori_{TRP4} , and plasmid mapping as shown in Figure 1.

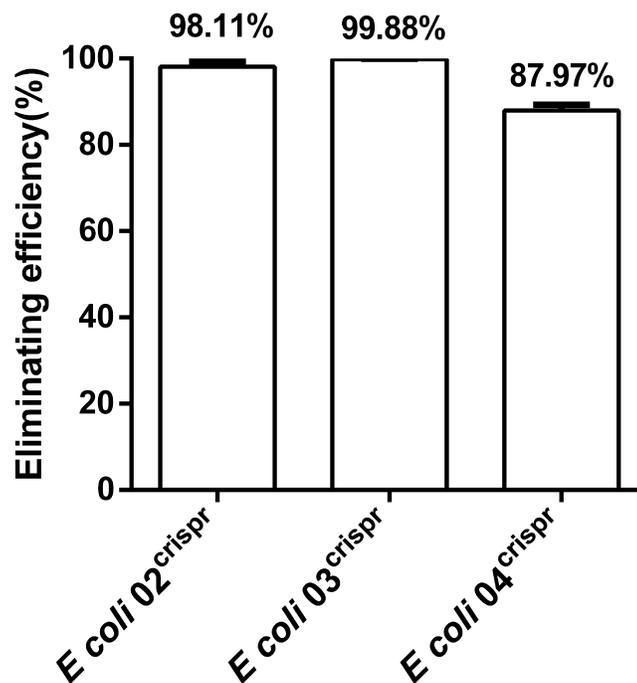


Figure 2 Effect of the pCas9 transformation into *E. coli* harboring different pNDM-5. *E. coli* 02 bears the plasmid pNDM-5 with IncFII-type. *E. coli* 03 contains the high copy numbers plasmid pUC19- bla_{NDM-5} . *E. coli* 04 bears the plasmid pNDM-5 with IncX3-type. Data points represent the mean value of three biological replicates, in which error bars showing the standard deviation.

Elimination of Plasmid by Using the pCas9-sgRNA

We firstly examined whether plasmid elimination can be achieved by targeting *bla*_{NDM-5}. The pCas9-N were transformed into *E. coli* 02 bearing pNDM-5 plasmid with IncFII-type. The result showed that 98.11% bacterial population lost their corresponding target genes (Figure 2). Then, the elimination efficiency under high copy number pressure was verified by transforming the pCas9-N into *E. coli* 03 containing the high copy numbers plasmid pUC19-*bla*_{NDM-5}. The result showed that 99.88% bacterial lost their corresponding resistant genes. In addition, pCas9-N targeted the different plasmid type, and the pCas9-N was transformed into *E. coli* 04 with IncX3-type. pCas9-N targeted IncFII and IncX3 could also remove pNDM-5 plasmids, and the elimination efficiency reached 87.00%. Plasmid removal could be achieved through CRISPR-Cas9 mediated cleavage with different pNDM-5 plasmid.

To accurately evaluate the elimination efficiency, qPCR-based detection of the *bla*_{NDM-5} gene was conducted at each time point in pCas9-N and pCas9 control groups, and the results showed that the elimination efficiency of the experimental group was greater than 80.00% at the 16th hour and continued to increase until the 24th hour, and a substantial plasmid reduction had occurred in *E. coli* 02 (Figure 3).

Elimination of Plasmid Using the pCas9-oriT-sgRNA via Conjugation

E. coli S17-1 samples containing pCas9-oriT-N and pCas9-oriT were used as donor cells. *E. coli* 02 samples were used as recipient cells. The conjugation efficiency was estimated at $(2.29 \pm 0.77) \times 10^{-2}$, while the elimination efficiency was $(1 - 1/24) \times 100\% = 95.83\%$ (Figure 4). Therefore, the CRISPR-Cas9 system via conjunctive delivery method could be achieved with high elimination efficiency.

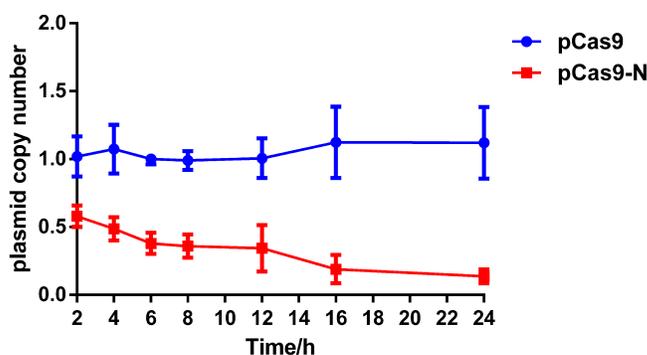


Figure 3 Relative copy number of plasmid pNDM-5 at each time point. pCas9-N transformed into competent *E. coli* 02 served as the experimental groups. Plasmid pCas9 transformed into competent *E. coli* 02 served as the control group. Data points represent the mean value of three biological replicates, in which the error bars showing the standard deviation.

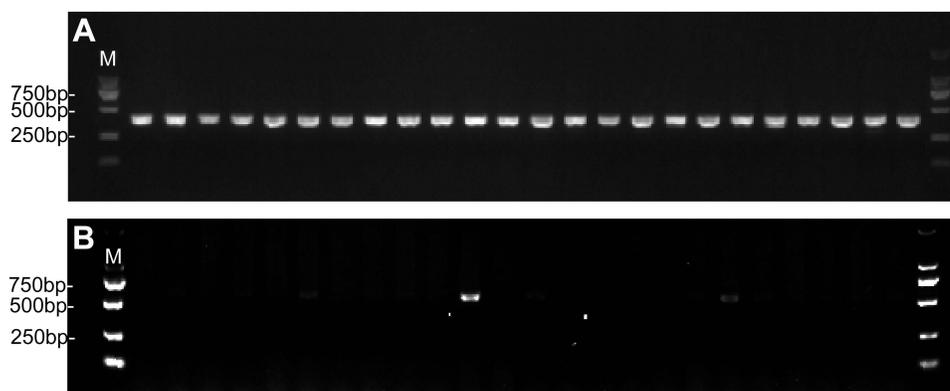


Figure 4 Confirmation of Cas9 and *bla*_{NDM-5} gene presence in *E. coli* 02 by PCR amplification with primer pCas9-F/R and NDM-5-F/R. M indicates the 2000 bp marker. (A) Cas9 was detected in the pCas9-oriT-N; (B) *bla*_{NDM-5} was detected in the pCas9-oriT-N.

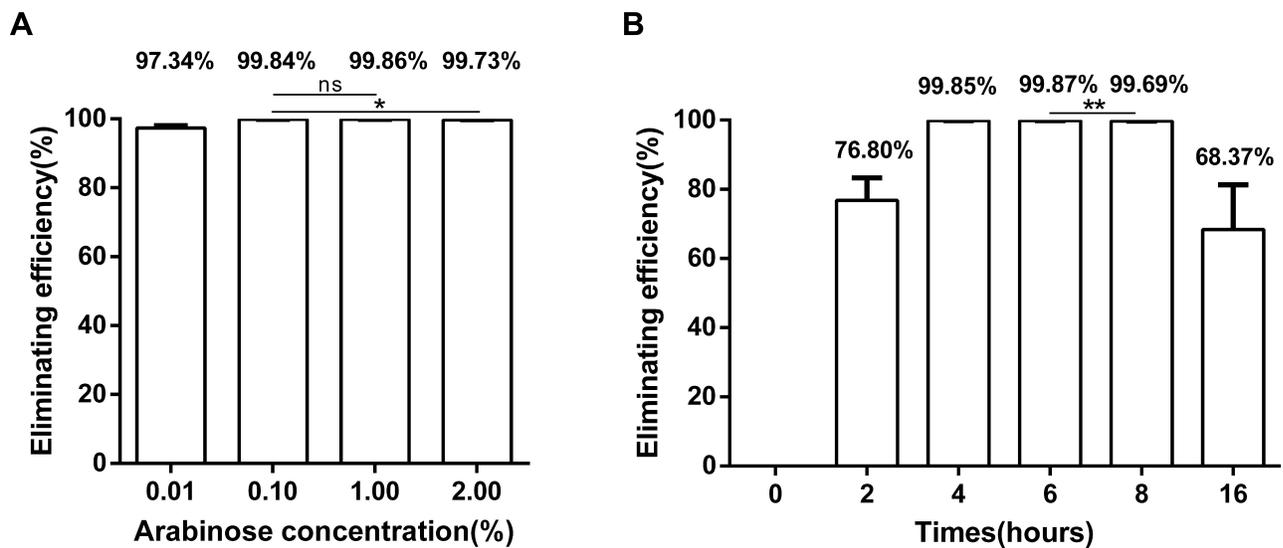


Figure 5 *bla*_{NDM-5} elimination efficiency with different arabinose concentrations and incubation time. **(A)** Elimination efficiency of *bla*_{NDM-5} with four different concentrations of arabinose (0.01%, 0.10%, 1.00% and 2.00%) at 6 h of incubation. **(B)** Elimination efficiencies at induction times of 0, 2, 4, 6, 8 and 16 h with 1.00% arabinose induction. Each experiment was performed in triplicate. Data points represent the mean value of three biological replicates, in which the error bars indicate the standard deviation. **p*<0.05, ***p*<0.01.

Elimination of Plasmid by Using the pBAD-Cas9-oriT-sgRNA via Conjugation

We initially investigated pBAD-Cas9-oriT-N elimination efficiency in *E. coli* 02 under different arabinose induction concentrations (0.01%, 0.10%, 1.00% and 2.00%). The results showed that after 6 h of incubation, the elimination efficiencies reached 97.34%, 99.84%, 99.86% and 99.74%. (Figure 5A). In addition, we examined elimination efficiencies at different incubation times. The results showed that under 1.00% arabinose treatment, the elimination efficiencies reached 76.80%, 99.85%, 99.87%, 99.69% and 68.37% at 2, 4, 6, 8 and 16 h, respectively (Figure 5B). Based on both arabinose concentration and incubation time, 1.00% arabinose and induction time of 6 h were the best eliminating conditions with 99.00% pNDM-5 removal, which achieved efficient clearance with conjunctive plasmids by CRISPR-Cas9.

Antimicrobial Susceptibility Tests

We examined the MIC of meropenem for *E. coli* 02 and corresponding plasmid-eliminated strain. The results showed that the MIC of meropenem in the eliminated isolates were reduced by eight-fold from 16 µg/mL to 0.06 µg/mL. The elimination of *bla*_{NDM-5} gene effectively restored the carbapenem susceptibility.

Plasmid Conjugation Blocking

E. coli 02, which carries the conjugation plasmid pNDM-5, was used as a donor. The *E. coli* J53, which was transformed with a control plasmid pCas9 or a recombinant plasmid pCas9-N, was used as recipient. As shown in Figure 6, the *E. coli* J53 containing plasmid pCas9-N reduced the number of transconjugants by 26 times compared with the control plasmid pCas9 (1.31E+07 vs 5.00E+05 CFU/mL; *p*<0.0001). The results demonstrated that the CRISPR-Cas9 system in the recipient cell could block the horizontal transfer of the conjugative plasmid pNDM-5.

Discussion

The HGT of ARGs plays an essential role in disseminating antimicrobial resistance.²⁹ The occurrence and dissemination of carbapenem resistance gene *bla*_{NDM-5} in bacteria makes the treatment of clinical infections challenging.³⁰ The re-sensitization of the sensitivity to traditional antibiotics might be more effective than searching for new antimicrobials.³¹ Therefore, new strategies to prevent the dissemination of *bla*_{NDM-5} should be developed.

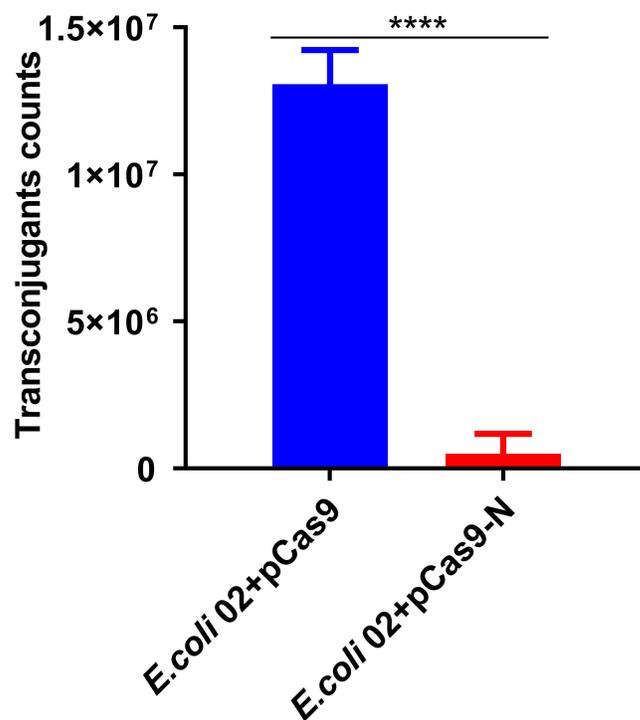


Figure 6 *E. coli* J53 harboring pCas9-N precluded the conjunctive plasmid pNDM-5. *E. coli* 02 as donor and *E. coli* J53+pCas9-N or *E. coli* J53+pCas9 as the recipient strain. Data points represent the mean value of three biological replicates, in which the error bars showing indicate the standard deviation. **** $p < 0.0001$.

In the present study, we investigated the potential of the CRISPR-Cas9 system to counteract plasmid-borne *bla*_{NDM-5} gene in wild-type plasmids (pNDM-5) and the high-copy numbers of plasmids (pUC19-NDM-5). The results showed that our pCas9 system could efficiently remove several carbapenem resistance plasmids by targeting *bla*_{NDM-5} genes and re-sensitize them to meropenem after the removal of plasmids pNDM-5. Comparing engineered high copy number plasmid (pUC19-NDM-5) to wild-type plasmid (pNDM-5) in elimination efficiency, The elimination efficiency of the high-copy plasmid is slightly higher than that of the wild-type plasmid. We guess that the high-copy plasmid is an engineered plasmid with a clear plasmid background, while the wild-type plasmid is clinically isolated and the background is more complicated. The pCas9 system can also eliminate other plasmid types such as IncX3. Hence, the content of plasmid backbone may have an influence on elimination efficiency. Our results were consistent with the previous findings that showing CRISPR-Cas9 can eliminate the resistance plasmid.^{15,32–34} Designing multiple sgRNAs that target different ARG subtypes and even different ARG types can prevent antimicrobial resistance.^{35,36} Multiple sgRNA for potentially targeting different gene, such as replication, partition, conjugation and plasmid stability, is a new strategy.¹²

However, an optimal deliver strategy to introduce the CRISPR-Cas9 system into the target bacterial populations remains a great challenge for plasmid or resistance gene elimination in the clinical application.³⁷ Most of the previous studies used physical or chemical methods, such as electroporation and chemical transformation, but these approaches are restricted to the Laboratory research and are not be applied in the clinical application. Considering that bacterial conjugation is critical for the dissemination of antimicrobial resistance, we aimed to explore conjugative plasmids to eliminate antibiotic resistance plasmids in bacteria.¹⁷ Our results demonstrated that the *bla*_{NDM-5}-harbouring plasmids in the recipient can be eliminated and sensitized to antibiotics via conjugative delivery of CRISPR-Cas9. For two recombinant conjugative plasmids, the elimination efficiencies reached 95.00%. Moreover, the conjugative delivery of CRISPR-Cas9 antimicrobials may be adaptable for the exact targeting of defined multidrug-resistant bacteria.^{15,17} Although our conjugative system has achieved compelling results, some limitations, such as the low conjugation efficiency (usually $< 10^{-1}$), limits the utility of this conjugative system to neutralize resistance at a population level. Conjugation efficiency may be influenced by time and biofilm structure.³⁸ Research has shown that transfer efficiency can be artificial increased using glass beads in vitro.³⁹ Similarly, F plasmid as a helper plasmid mediates efficient

transfer.¹⁸ Further research should focus on screening broad-host-range conjugative plasmids and enhance conjugation efficiency.¹⁷ Considering that bacterial conjugation is a naturally occurring process, this strategy could be optimized for clinical application. Furthermore, phages and nanoparticles can be effectively used for the delivery of the CRISPR-Cas9 system into the bacterial pathogens.^{13,40} In addition to plasmid elimination, the CRISPR-Cas9 could further provide immunity in *E. coli* against the acquisition of plasmid-mediated ARGs.^{14,34}

Conclusion

We provide a proof of principle that a novel CRISPR-Cas9 system eliminate carbapenem genes and plasmids to interrupt the HGT of ARGs. This method aims to achieve proactive prevention of the dissemination of carbapenem resistance in clinical pathogens.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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