Generation of Newly Discovered Resistance Gene mcr-1 Knockout in Escherichia coli Using the CRISPR/Cas9 System

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Introduction

The mcr-1 gene is a new “superbug” gene discovered in China in 2016 that makes bacteria highly resistant to the last-resort class of antibiotics. The mcr-1 gene raised serious concern about its possible global dissemination and spread. Here, we report a potential anti-resistant strategy using the CRISPR/Cas9-mediated approach that can efficiently induce mcr-1 gene knockout in Escherichia coli. Our findings suggested that using the CRISPR/Cas9 system to knock out the resistance gene mcr-1 might be a potential anti-resistant strategy. Bovine myeloid antimicrobial peptide-27 could help deliver plasmid pCas::mcr targeting specific DNA sequences of the mcr-1 gene into microbial populations.

Keywords: mcr-1, knockout, E. coli, CRISPR/Cas9 system, BMAP-27

Materials and Methods

The clinical isolate bacterial strain E. coli NJ-15-3 used in this work was identified by 16S rDNA sequencing and biochemistry analysis and grown in LB medium at 37°C. The colistin resistance phenotype was detected by minimum inhibitory concentration (MIC) according to the recommendations of the Clinical and Laboratory Standards Institute document M100-S25 [14]. Then, the NJ-15-3 plasmids were isolated by employing manual extraction with the alkaline lysis method, and the mcr-1 gene was detected by PCR (primers located upstream and downstream of the mcr-1 gene target MCRF-ATGATGCAGCATACTTCTGTG, MCRR-TCGGTCTGTAGGGCATTTTGGAG; primers located upstream and downstream of the mcr-1 gene MCRUF-GTATAATTG
CCGTAATTATC, MCRUR-ATAATACGAATGGAGTGTGC) with Taq DNA Polymerase (M0495L; New England Biolabs, USA)

Plasmid pCas::mcr containing the mcr-1 gene target was transferred into 10⁶ CFU/ml cells containing the mcr-1 gene. After electroporation, cultures were screened for surviving recipient cells on LB plates and LB + chloramphenicol (Cm), LB + colistin (Cl) for selection of transconjugants (Cm resistance is encoded by the pCas::mcr plasmid).

E. coli NJ-15-3 cells were co-incubated with phosphate buffered saline (PBS, pH 7.2) or Bovine myeloid antimicrobial peptide-27 (BMAP-27) labeled with WGA 610-X conjugate (Invitrogen, USA). After washing three times with PBS, the E. coli NJ-15-3 cells were observed by laser confocal microscopy.

We co-incubated the E. coli NJ-15-3 (OD₆₀₀ = 0.3–0.5) with the BMAP-27 (0.1–1 µM) and pCas::mcr (0.1–1 ng/µl) complex in PBS. After 5 h, we screened 10 E. coli strains on LB and detected the mcr-1 gene by PCR and the colistin resistance phenotype by MIC.

Results and Discussion

In clinical isolate bacterial strain E. coli NJ-15-3 as wild type, the colistin resistance phenotype was detected by MIC, and the mcr-1 gene was identified by PCR (data not shown). Based on the fact that Liu et al. [1] successfully acquired a large plasmid (pHNSHP45) from a pig E. coli isolate, we attempted to subject the NJ-15-3 strain to plasmid isolation by employing manual extraction with the alkaline lysis method. It is the same as plasmid pHNSHP45 by sequencing the whole plasmid (data not shown).

Here, we first designed chimeric sgRNA that encodes sequences complementary to a target protospacer of mcr-1 (shown in Fig. 1A). For sgRNA design, the mcr-1 gene sequence edited was screened for the presence of a 20-bp guide and PAM sequence. A guide sequence for sgRNA had to meet the rules governing efficient and precise initiation of the T7 promoter. To establish functionality for CRISPR in mediating sequence-specific gene editing, we designed sgRNA to induce a DSB in the mcr-1 gene, which encodes broad-spectrum and pan-resistance to the colistin antibiotic. Bacterial Cas9-sgRNA expression plasmid pCas was constructed with two T7 promoters to ensure independent expression of Cas9 and sgRNA (Fig. 1A), the same as plasmid BPK764 [15].

Plasmid pCas::mcr containing the mcr-1 gene target was transferred into 10⁶ CFU/ml cells containing the mcr-1 gene, and then cultures were screened for surviving recipient cells on LB plates and LB+ chloramphenicol (Cm) (Fig. 1B). Transformation of E. coli NJ-15-3 with pCas::mcr containing a copy of mcr-1 resulted in the same viable recipients in transformation efficiency compared with pCas cells (Fig. 1B). We have repeated the transformation 10 times, and found that the viable recipients of Cm resistance were sensitive to colistin (Cl) for those 10 times (Fig. 1C). These

![Fig. 1. Design of sgRNA targeting the mcr-1 gene, and double strand break analysis of the mcr-1 gene fragment.](image-url)

(A) Design of sgRNA targeting the mcr-1 gene. (B) Electric transfer of pCas::mcr into 10⁶ CFU/ml cells containing the mcr-1 gene. After transformation, cultures were plated on LB for surviving recipient cells, and LB + chloramphenicol (Cm) and LB + Colistin (Cl) to select for transconjugants (C).
results indicated that bacterial plasmid pCas::mcr enabled knockout of the mcr-1 gene in the E. coli isolate with the help of electroporation.

Owing to the application limitation of electroporation, we attempted to explore a plasmid-delivery system. Cathelicidins are small, cationic, antimicrobial peptides found in mammalian species. BMAP-27 contains 27 amino acid residues and an α-helical C-terminus with structural attributes of antimicrobial activity. It was demonstrated that BMAP-27 induces mitochondrial permeability by forming transition pores [16]. Here, we investigated whether BMAP-27 could enhance the efficiency of plasmid transfer. As shown in Fig. 2, with the help of BMAP-27, pCas::mcr exhibited better efficient and specific antimicrobial effects against strains harboring plasmid or chromosomal target sequences. To determine how BMAP-27 work it out for the gene transfer, E. coli NJ-15-3 cells were co-incubated with PBS or BMAP-27 labeled with WGA 610-X conjugate. The labeled BMAP-27 was observed in E. coli NJ-15-3 cells but not in control cells by laser confocal microscopy (Fig. 2A).

**Fig. 2.** Cas9-sgRNA delivered by Bovine myeloid antimicrobial peptide-27 (BMAP-27) exhibits efficient and specific antimicrobial effects against strains harboring plasmid target sequences. (A) BMAP-27 delivered pCas::mcr into cells. E. coli NJ-15-3 cells were co-incubated with PBS or BMAP-27 labeled with WGA 610-X conjugate (Invitrogen, USA). The labeled BMAP-27 was observed in E. coli NJ-15-3 cells but was not found in control cells by laser confocal microscopy. (B–D) mcr-1 gene analysis of E. coli NJ-15-3 mcr-1 knockout monoclonal. E. coli NJ-15-3 (OD_{600} = 0.3–0.5) was co-incubated with the BMAP-27 (0.1–1 µM) and pCas::mcr (0.1–1 ng/µl) complex in PBS. After 5 h, we screened 10 E. coli strains on LB and found that 3 E. coli strains were colistin-sensitive, and no mcr-1 gene was detected by PCR using primers located upstream and downstream of the mcr-1 gene target and mcr-1 gene in screened E. coli NJ-15-3. Consequently, we determined the sensitivity of the screened E. coli strains to a range of concentration of colistin. The results indicated the screened E. coli strains recovered to be sensitive to colistin, and the minimum inhibitory concentration (MIC) of the wild-type E. coli NJ-15-3 was 8 µg/ml of colistin.
We co-incubated the *E. coli* NJ-15-3 (OD₆₀₀ = 0.3–0.5) with the BMAP-27 (0.1–1 μM) and pCas::mcr (0.1–1 ng/μl) complex in PBS. After 5 h, we screened 10 *E. coli* strains on LB and found that 3 *E. coli* strains were colistin-sensitive and no *mcr-1* gene was detected by PCR using primers located upstream and downstream of the *mcr-1* gene target and the *mcr-1* gene in screened *E. coli* NJ-15-3 (Figs. 2B–2C). Consequently, we determined the sensitivity of the screened *E. coli* strains to a range of concentration of colistin according to the recommendations of the Clinical and Laboratory Standards Institute document M100-S25 [14]. The *E. coli* strain NJ-15-3 (wild type) was used for quality control. The results indicated that the screened *E. coli* strains recovered to be sensitive to colistin, and the MIC of the wild-type *E. coli* NJ-15-3 was 8 μg/ml of colistin (Fig. 2D). These results demonstrated that the *mcr-1* gene in *E. coli* NJ-15-3 was successfully knocked out using the CRISPR/Cas9 system, and with BMAP-27, the CRISPR/Cas9 system exhibited better efficiency of intracellular gene editing. BMAP-27, as a small, cationic, antimicrobial peptide, can combine with the plasmid and deliver it to the bacteria.

The CRISPR/Cas9 system can be developed into a new microbial gene therapy technology; however, these highly anionic nucleic acids and proteins could not penetrate the cell membrane into the cell by themselves. Because of the lack of an efficient delivery system for CRISPR/Cas9 systems, this technique is limited to the in vitro treatment [17]. Citorik *et al.* [18] delivered CRISPR/Cas9 systems into cells by phage or conjugation plasmid. However, the phage host spectrum is narrow, there is the risk of gene recombination, and the efficiency of the conjugation plasmid has the risk of the existence and recombination of drug resistance genes. CRISPR/Cas9-mediated knockout of the *mcr-1* gene in *E. coli* NJ-15-3 in our study provides a potential solution to resistance genes. Because CRISPR/Cas9 systems are widely conserved in bacteria, the development and optimization of delivery vehicles will be required to improve the efficiency of Cas9-sgRNA targeting in other strains.

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**References**


