

Exploration of Antimicrobial Peptides in the Treatment of Gentamicin-Resistant *Klebsiella pneumoniae* Infection

Xiaochun Chen^{1,*}, Benhong Zhang^{2,*}, Jin He^{3,*}, Xiaohong Rui⁴, Tian He⁴, Lizhu Zhang⁵, Junfeng Bao⁶, Yanfei Jing⁷, Futao Cao⁸

¹Department of Laboratory Medicine, Taizhou Second People's Hospital, Taizhou, People's Republic of China; ²Department of Laboratory Medicine, Hangzhou Gongshu District Integrated Traditional Chinese and Western Medicine Hospital, Hangzhou, People's Republic of China; ³Department of Laboratory Medicine, Hangzhou Yuhang Jiamu Nursing Home, Hangzhou, People's Republic of China; ⁴Department of Laboratory Medicine, Affiliated Wuxi Fifth Hospital of Jiangnan University, Wuxi, People's Republic of China; ⁵Department of Research, Nanxin Pharm, Nanjing, People's Republic of China; ⁶Department of Laboratory Medicine, Wuxi Maternal and Child Health Care Hospital, Women's Hospital of Jiangnan University, Wuxi, People's Republic of China; ⁷Department of Function, Affiliated Wuxi Fifth Hospital of Jiangnan University, Wuxi, People's Republic of China; ⁸Department of Emergency, Jiangnan University Medical Center, Wuxi, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yanfei Jing, Department of Function, Affiliated Wuxi Fifth Hospital of Jiangnan University, Wuxi, Jiangsu, 214005, People's Republic of China, Email tengfeiba0002@163.com; Futao Cao, Department of Emergency, Jiangnan University Medical Center, Wuxi, Jiangsu, 214000, People's Republic of China, Email caofutao999@163.com

Introduction: The emergence of multidrug-resistant *Klebsiella pneumoniae* (*K. pneumoniae*) and the decline of effective antibiotics lead to the urgent need for new antibacterial agents. The aim of this study is to investigate the therapeutic effect of antimicrobial peptides against gentamicin-resistant (RT) *K. pneumoniae* and to screen effective antimicrobial peptides.

Methods: In this study, the RT strains were induced by gradient gentamicin, and the RT strains were selected by detecting the expression levels of efflux pump genes, porin genes, and biofilm formation genes of the strains combined with their effects on the cells. Then the effects of four antimicrobial peptides on the efflux pump activity, biofilm formation level and cell condition after infection were detected to explore the effects of antimicrobial peptides on RT strains. Finally, the RT strain was used to induce a mouse model of pneumonia, and the four antimicrobial peptides were used to treat pneumonia mice for in vivo experiments. The pathological changes in lung tissues in each group were detected to explore the antimicrobial peptide with the most significant effect on the RT strain in vivo.

Results: The results showed that the minimal inhibitory concentrations of the RT strains (strain C and strain I) were significantly higher than those of the wild-type strain, and the expression of efflux pump, porin and biofilm formation genes was significantly increased. The antimicrobial peptides could effectively inhibit the biofilm formation and efflux pump protein function of the RT strains. In addition, the antimicrobial peptides showed promising antibacterial effects both in vitro and in vivo.

Discussion: Our study provided a theoretical basis for the treatment of gentamicin resistant *K. pneumoniae* infection with antimicrobial peptides, and found that KLA was significantly superior to LL37, Magainin I, KLA and Dermaseptin (10 µg/mL in cells, 50 µg in mice).

Keywords: *Klebsiella pneumoniae*, *K. pneumoniae*, antibiotics, antimicrobial peptide, gentamicin-resistant, RT, microenvironment

Introduction

As a gram-negative bacterium with opportunistic pathogenicity, *Klebsiella pneumoniae* (*K. pneumoniae*) is the leading cause of hospital-acquired infections (HAIS),^{1,2} such as urinary tract infection, pneumonia and bloodstream infection in hospitalized or other immunocompromised patients.³⁻⁶ In 2019, the global death toll caused by and related to antimicrobial drug resistance of *K. pneumoniae* was approximately 200,000 to 650,000 cases, ranking third among the death tolls caused by drug resistance of various antibacterial drugs.⁷ The mortality rate of hypervirulent *K. pneumoniae* in

endemic areas is 12% to 45%.⁸ Moreover, the isolation rate of *K. pneumoniae* in China is also increasing year by year.⁹ At present, carbapenems, β -lactams, quinolones, aminoglycosides and other drugs are commonly used for the treatment of *K. pneumoniae* infection.¹⁰ However, due to the abuse of antimicrobial drugs, multidrug-resistant strains have appeared in *K. pneumoniae*, which has aroused great clinical and social attention.^{1,11} The resistance mechanism of *K. pneumoniae* mainly includes (1) producing enzymes to inactivate antibiotics;¹² (2) change in target sites of antimicrobial agents;¹³ (3) decrease in drug penetration;¹⁴ (4) overexpression of efflux pump gene.¹⁵ Among them, increasing efflux by efflux pumps (EPs) plays an important role in the multidrug resistance of bacteria.^{16,17}

Gentamicin, one of the highly effective broad-spectrum aminoglycosides antibiotics active against various Bacilli, *Pseudomonas aeruginosa* and Gram-positive cocci, has been used in the clinical treatment of bacterial infections.^{18,19} Like other aminoglycosides, gentamicin exerts antimicrobial effects by interfering with bacterial protein synthesis. Gentamicin can bind 16S rRNA on the 30S subunit of bacterial ribosomes, interfering with the attachment of formyl-methionyl-tRNA to 30S rRNA and blocking bacterial protein synthesis.²⁰ Due to its wide application, the increasing resistance of bacteria to gentamicin poses a challenge to human health.³ Moreover, the clinical application of gentamicin is limited by its significant nephrotoxic and neurotoxic effects.²¹

Antimicrobial peptides (AMP), a type of small-molecule weight short peptides in organisms, are generated by specific gene transcription.²² They are active molecules generated and retained during biological evolution. With broad antibacterial and antiviral activities, antimicrobial peptides regulate innate immunity, clear mutant cells, and are essential to the biological innate immune system.^{23–26} Increasing attention has been attracted to the research of antimicrobial peptides for clinical application due to their diverse bactericidal mechanisms, biological activity and excellent properties, and inhibition of bacteria resistance to various antibiotics.²⁷

LL37, the only known human antimicrobial peptide (CAMP), as an endogenous host defense peptide, has attracted a lot of research attention due to its broad-spectrum antibacterial activity and immunomodulatory properties,²⁸ and is considered as a promising choice for the treatment of biomembrane-associated infectious diseases, which can prevent *K. pneumoniae* from forming biofilms by down-regulating biofilm-related genes and degrading biofilm matrix²⁹ and has great potential as an antibiotic against carbapenem-resistant *K. pneumoniae*.³⁰

Magainin I is an antimicrobial peptide that specifically interacts with bacteria.³¹ The results of Mayram Hacıoglu et al showed that magainin (100 μ g/mL) was ineffective against *K. pneumoniae*,³² while Laura Bedin Denardi et al showed that magainin-2 (80–160 mg/L) had strong bactericidal activity against carbapenem-resistant *K. pneumoniae*.³⁰

KLA is an antimicrobial peptide that is modified to form a cyclic structure to limit helicity and thus reduce cytotoxicity,³³ and KLA mediated by neutrophil membrane-coated nanoparticles (KLA-Nnps) was found to be effective in inhibiting the development of pneumonia caused by drug-resistant *K. pneumoniae*.³⁴

Dermaseptins, a type of α -helical polycationic peptides isolated from Hylid frogs, have antibacterial effects against bacteria, parasites, protozoa and viruses in vitro. In addition, a 10-peptide derivative of the N-terminal domain of Dermaseptin-PC showed significant antimicrobial activity against *K. pneumoniae* and showed synergistic antimicrobial activity with gentamicin against carbapenem-resistant *K. pneumoniae*.³⁵

All the above four antimicrobial peptides have a certain effect on *K. pneumoniae* resistance, but the effect on gentamicin-resistant *K. pneumoniae* has not been studied. In this study, ten *K. pneumoniae* strains isolated from patients were screened with gentamicin at gradient concentrations to obtain drug-resistant strains. Therefore, in this study, 10 strains of *K. pneumoniae* isolated from patients were screened by gradient concentration of gentamicin to obtain drug-resistant strains, and their efflux pump activity and biofilm formation ability were detected to analyze the reasons for drug resistance of *K. pneumoniae*, and the effects and differences of antimicrobial peptides LL37, Magainin I, KLA and Dermaseptin against *K. pneumoniae* infection in vitro and in vivo were studied.

Materials and Methods

Bacterial Strains and Reagents

A total of ten *K. pneumoniae* strains (A-J) were obtained from the biological samples (including blood, throat, rectum, and catheter) of the patients in Wuxi Fifth People's Hospital, from January 2014 to June 2020. This study was approved by the

Table 1 The Antibiotic Resistance Profile of *Klebsiella pneumoniae*

| Strains | Antibiotic resistance |
|-----------|-----------------------|
| ATCC13883 | None |
| A | Piperacilina |
| B | Imipenem |
| C | None |
| D | Cefazolin, |
| E | Piperacilina |
| F | None |
| G | Tazobactam |
| H | Piperacilina |
| I | None |
| J | Ciprofloxacin |

Ethics Committee of our hospital (No. 2021–021-1). All strains were identified using the VITEK 2 system (bioMérieux, France). The resistance profile of the strains used in this study is presented in Table 1. The standard strain *K. pneumoniae* ATCC13883 was purchased from the Clinical Laboratory Center of the Ministry of Health (Beijing, China). Mueller–Hinton broth, MH agar, Luria-Bertani (LB) broth, carbonyl cyanide m-chlorophenylhydrazine (CCCP), ethidium bromide (EtBr), sodium acetate, crystal violet and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Darmstadt, Germany). Antibacterial AgNPs were obtained from Yamei nanotech (Zhejiang, China). Antimicrobial peptides (LL37, Dermaseptin, Magainin 1 and KLA) were designed and synthesized by China Peptides Co., Ltd (Shanghai, China). Mouse TNF- α , IL-1 β and IL-6 ELISA kit, Hematoxylin and Eosin (HE) Staining Kit and DAB (Diaminobenzidine) Horseradish Peroxidase Color Development Kit were purchased from Beyotime (Shanghai, China). Anti-Ly6G antibody (ab261916) and Goat Anti-Rabbit IgG H&L (HRP) (ab6721) were purchased from Abcam (Cambridge, England).

Cell Culture

Human lung cell line (HLF-a) was purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Yeasen, Shanghai, China) containing 10% fetal bovine serum (FBS, Sigma-Aldrich, Darmstadt, Germany) with 1% Penicillin/Streptomycin in a humidified incubator containing 5% CO₂ at 37°C.³⁶

Drug-Resistant Strain Induction

The *K. pneumoniae* strains (A–J) were inoculated on fresh agar plates for 18–24 h at 37°C. A single colony was selected and inoculated in 10 mL LB liquid medium for oscillating culture for 18 h. The supernatant was removed by centrifugation. The bacteria solution was resuspended with sterile normal saline and adjusted to 1.5×10^8 CFU/mL. Then, 150 μ L of bacteria solution was evenly spread on the surface of the Mueller–Hinton (MH) agar plate containing gentamicin (concentration of 0.5–256 mg/L). After being incubated in an incubator at 37°C for 3 days, the colonies on the plate were picked up and adjusted to 1.5×10^8 CFU/mL.

Similarly, 150 μ L of bacteria solution was evenly spread on the next gradient concentration plate for further induction, gradient by the gradient. After incubation for four weeks, the colony number in each gradient was calculated, and the monoclonal was screened out for further incubation in LB medium with corresponding concentrations of gentamicin. The drug-resistant strains were obtained in the 10th generation of induction, respectively. Drug susceptibility test and PCR analysis were performed for the expression of efflux pump genes, porin genes and biofilm formation genes to verify the resistant strains.³⁷

Cytotoxicity Assay

The toxicity of the antimicrobial peptides to HLF-a cells was detected by the release of lactate dehydrogenase (LDH).³⁸ HLF-a cells were grown in 96-well plates and cultured overnight. Subsequently, 100 μ L of the peptides (LL37, Magainin

I, KLA and Dermaseptin; concentration range: 1000–0.98 µg/mL) diluted in DMEM was added into the plates and incubated at 37°C for 24 h.^{39,40} The control group was cultured in DMEM without peptides or in medium with 0.5% Triton X-100 (Thermo Scientific, Shanghai, China). The plates were added with 50 µL of LDH reaction mixture (Pierce, Rockford, IL, USA) and cultured for 30 min at room temperature. Next, each well was added with 50 µL stop solution. Finally, the OD values at 490 nm were measured for LDH activity using a microplate reader (BioTek, Biotek Winooski, VT, USA).

Detection of the Drug Resistance and Biofilm Forming Genes

The mRNA levels of gentamicin-resistance related genes⁴¹ (*acrA*, *acrB*, *tolC*, *acrR*, *ompK35* and *ompK36*) and biofilm forming genes⁴² (*mrkA*, *luxS*, *pga*, *wbbM*, and *wzm*) of *K. pneumoniae* were detected by real-time PCR (RT-PCR). In brief, the genomic DNA of *K. pneumoniae*, as the template for PCR reaction, was extracted according to the instructions of the Bacterial Genomic DNA Extraction Kit (Solarbio, Beijing, China). The concentration and quality of RNA were measured using a spectrophotometer. Then 2 µg of total RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). RT-qPCR was performed using a SYBR Green PCR Kit (Vazyme, Nanjing, China) on ABI 7500 Rapid real-time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal cycle consisted of reverse transcription at 42°C for 5 min, initial denaturation at 95°C for 10s, 45 cycles of denaturation at 95°C for 5 s, and annealing/extension at 55°C for 10s. The mRNA expression of *acrA*, *acrB*, *tolC*, *acrR*, *ompK35* and *ompK36* gene was normalized by the expression of 16S rRNA. The quantitative analysis was obtained using the $2^{-\Delta\Delta CT}$ method.⁴³ The primers used in the study are listed in Table 2.

Minimal Inhibitory Concentration (MIC) Assay

The minimal inhibitory concentration (MIC) of the 10 strains for gentamicin was detected by the two-fold broth microdilution method in 96-well microtiter plates according to the method recommended by the Clinical and

Table 2 Primer Sequences Used for RT-PCR

| Genes | | Primer sequences (5'-3') |
|---------------|---------|----------------------------|
| <i>acrA</i> | Forward | TCTGATCGACGGTGACATCC |
| | Reverse | TCGAGCAATGATTTCTGCG |
| <i>acrB</i> | Forward | CGATAACCTGATGTACATGTCC |
| | Reverse | CCGACAACCATCAGGAAGCT |
| <i>tolC</i> | Forward | TACCAGCAGGCACGCATCA |
| | Reverse | GCTGTGCGGATAGCCATTGT |
| <i>acrR</i> | Forward | AGTCGTCAAGACGATTTTCAATTT |
| | Reverse | AGTGTTTCCGGCGTCATTAG |
| <i>ompK35</i> | Forward | GCAGTTAACGACTGCAGCTTAAGAA |
| | Reverse | TCAGACGTAACAGTATTTCCGT |
| <i>ompK36</i> | Forward | AAGGTATTAAGCCAGTACAGTAAG |
| | Reverse | TAACAGCTGACATTGAGCTTAAC |
| <i>mrkA</i> | Forward | CGACTTAAGATAACGATTGACG |
| | Reverse | TAACGATTAGACTGATATCCGA |
| <i>luxS</i> | Forward | GCAAGTTAACGTTCACTAAGCAAT |
| | Reverse | GGACCTTTCAGACCTAACCGTTCA |
| <i>pga</i> | Forward | ATTGATAACGATGCAATTGCCGAAT |
| | Reverse | GACAACTAGCTTTCGAGCAATAACG |
| <i>wbbM</i> | Forward | TTGCGACTGAATACACTTGAACGTTA |
| | Reverse | AAGCAGTTACAAGATTCTTGACA |
| <i>wzm</i> | Forward | GAACCTAGACTTACTAGAATCACG |
| | Reverse | TTACAGAATTCGCGATCAATGACAC |
| 16sRNA | Forward | GGCCGCAAGGTTAAACTCAAATG |
| | Reverse | AACCGCTGGCAACAAAGGATAAGG |

Laboratory Standards Institute (CLSI, 2021). Briefly, the bacterial colonies were suspended and grown in Muller Hinton broth. The suspension was adjusted to McFarland standard 0.5 to obtain a concentration of 10^5 CFU/mL. An equal amount of 0.05 mL was transferred to each well of a 96-well plate containing 0.05 mL gentamicin at a concentration twice as diluted as that continuously prepared in a nutrient solution medium. After the incubation at 37°C for 16–18 h, MIC results were recorded. The experiments were performed three times.²²

Biofilm Assay

K. pneumoniae were cultured in LB broth overnight and diluted to a density of 1×10^7 CFU/mL. 100 μ L of the diluted bacteria were added to the 96-well microtiter plate containing gentamicin and 10 μ g/mL antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) and inoculated for 24 h at 37°C.^{44,45} The solution was removed, and the wells were washed with PBS thrice to remove the bacteria. The biofilm was fixed with sodium acetate and then stained with crystalline violet (0.1%) for 10 min. The plates were rinsed to remove excess dye and dried. The combined crystalline violet was then dissolved by 200 μ L of 95% ethanol. Furthermore, the $A_{570\text{nm}}$ was measured on a microplate spectrophotometer (Biotek, Biotek Winooski, VT, USA). Moreover, the %age biofilm inhibition of gentamicin treated experiment was calculated as follows: %age biofilm inhibition = $1 - \text{OD}_{570}$ of cells treated with Gentamicin/ OD_{570} of nontreated cells $\times 100$.^{2,46}

Ethidium Bromide (EtBr) Accumulation Assay

As previously described, ethidium bromide (EtBr) was used to evaluate the efflux capacity of strains.² Briefly, *K. pneumoniae* strains were cultured in nutrient broth until the logarithmic growth phase. The bacteria were collected and washed with $1 \times$ PBS 3 times. The bacteria were then resuspended and OD_{600} was adjusted to 0.3. Next, 200 μ L of bacterial solution was added to a 96-well black plate. EtBr (50 μ M) and the CCCP (100 μ M) or antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin; 10 μ g/mL) were added to each well and incubated at 37°C for 30 min with 5% glucose. Each sample was set to three repeats. The light was avoided during the experiment. After adding EtBr, fluorescence detection was performed immediately using a Multiskan MK3 multifunctional enzyme plate analyzer (Thermo Fisher Scientific, Waltham, MA, USA), measured every 2 min for a total duration of 30 min. The excitation wavelength was set at 530 nm, and the emission spectrum was set at 585 nm.

Fluorescence Detection

Logarithmic phase cells (1×10^5 cells/well) were inoculated in 6-well Chamber Slide (Thermo fisher Scientific, Waltham, MA, USA), incubated with buffer mixed with *Klebsiella pneumoniae* for 24 h. After washing twice with PBS, lung cells were fixed on ice with 4% paraformaldehyde for 15 min, then infiltrated with 0.5% TritonX-100 at room temperature for 30 min. Gentamicin (32 mg/L) and 10 μ g/mL antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) were treated in different groups for 1 h. Next, lung cells infected with *Klebsiella pneumoniae* were incubated with hybridization buffer mixed with fluorescence in situ hybridization (FISH) probe (Ribobio, red, Guangzhou, China). The nuclei were stained (blue) with 6-diamino-2-phenylindole (DAPI). Lung cells infected with *Klebsiella pneumoniae* were observed under a confocal fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA).^{47,48}

Flow Cytometry

The cell apoptosis was detected by flow cytometry analysis. In brief, the *K. pneumoniae*-infected HLF-a cells at the logarithmic growth stage (4×10^6 /mL) were inoculated into a 6-well plate, and 80 μ M of RES containing 10% fetal bovine serum was added and cultured at 37°C and 5% CO_2 . After 48 h, the cells were collected and then suspended in 195 μ L of binding buffer (1×10^6 cells/mL). The cells were then incubated with 5 μ L of AnnexinV-Alexa Fluor 647 (Alexa Fluor® 647, 1:200, Abcam, Cambridge, England) for 15 min and then co-incubated with 5 μ L of PI at room temperature. FITC+ and PI- cells were defined as apoptotic cells. Finally, the apoptosis rate was measured using a flow cytometer (Beckman Coulter, CA, USA).⁴⁹

Animals

Specific-pathogen-free (SPF) grade 6–8 weeks old C57BL/6 mice were purchased from Shanghai Laboratory Animal Centre (Shanghai, China). All mice were routinely raised in the Animal Experiment Center of Jiangnan University. The experimental scheme was approved by the Animal Ethics Committee of the Jiangnan University. After one week of adaptive feeding, we anesthetized mice using 1–1.5% isoflurane inhalation method (IsoFlo, Abbott Laboratories) for 2–3 min and intubated them endotracheally using a blunt tip feeding needle. The 50 μ L of *K. pneumoniae* solution (concentration: 1×10^6 CFU/mL) was administered by blunt needle inoculation. After 6 h of infection, the mice were divided into 6 groups randomly ($n = 6$ for each group). The mice were then injected via the tail vein with gentamicin (1.5 mg/kg), 50 μ g of antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) or an equivalent volume of saline for infected mice, respectively. The mice were euthanized with 1–1.5% isoflurane at 16 h or 48 h after treatment, and the lung tissues were collected^{50,51} after confirmation of death. Under aseptic conditions, the lungs of each group of mice were ground into homogenate and diluted 100 times with sterile PBS. Then, 100 μ L of the homogenate was spread onto MH agar plates and incubated at 37°C. The colonies were counted after 24h.

Detection of the Cytokines in Lung

The lung tissue homogenate of the mice sacrificed 48h after the induction of pneumonia was repeatedly frozen and thawed twice, and then centrifuged at 5000 g for 5 min at 4°C to collect the supernatant.⁴⁶ The corresponding ELISA kit (MultiSciences, Beijing, China) was used to detect the cytokines TNF- α , IL-1 β and IL-6 in the supernatant. The samples and standards were added to the antibody-coated plates, and the biotinylated antibody was added after incubation. The HRP-labeled streptavidin was added after binding, and the color development was conducted with tetramethylbenzidine (TMB). The A_{450} was immediately detected after adding the stop solution. The concentration of cytokines in the sample was calculated according to the standard curve.

Pathological Analysis of Lung Tissue

For hematoxylin-eosin (HE) staining, lung tissues collected from the six mouse groups were fixed with 10% formalin and embedded in paraffin. Then, paraffin sections (4 μ m) were stained with hematoxylin and eosin to observe lung tissue lesions. Ly-6G is a neutrophil antigen, and the neutrophils are bactericidal. The immunohistochemical staining was performed to observe the expression of Ly-6G. In short, after dewaxing and hydration, the endogenous peroxidase activity was inactivated. The antigen was retrieved, and then the slices were incubated with Ly-6G monoclonal antibody (1/2000, Abcam, Cambridge, England). Next, HRP-labeled secondary antibody (1/2000, Abcam, Cambridge, England), and finally, color development was conducted with DAB-H₂O₂.

Statistical Analysis

Data are expressed as the means \pm standard deviation of the mean (SD). All experiments were carried out three times independently. Comparisons between the untreated control and treated groups were analyzed with the one-way ANOVA followed by the Bonferroni post-hoc test. A *P*-value of <0.05 was considered statistically significant.

Results

This study aimed to investigate the effect of the antimicrobial peptide on the resistance of *K. pneumoniae* to gentamicin. The gentamicin-resistant strains were screened in this study. We hypothesized that the drug resistance of *K. pneumoniae* was caused by the enhancement of efflux pump activity and biofilm forming ability of *K. pneumoniae*. We also investigated the effects of antimicrobial peptides on *Klebsiella pneumoniae* induced infection *in vitro* and *in vivo*.

Screening and Identification of the Drug-Resistant *K. Pneumoniae* Strains

The antimicrobial susceptibility of the *K. pneumoniae* collection against gentamicin based on MIC values for each strain before and after induction was depicted in Figure 1A and B. Before induction, all wild-type isolates (A-J) were phenotypically susceptible to gentamicin (MIC <2 mg/L) (Figure 1A). However, after gentamicin induction, among the

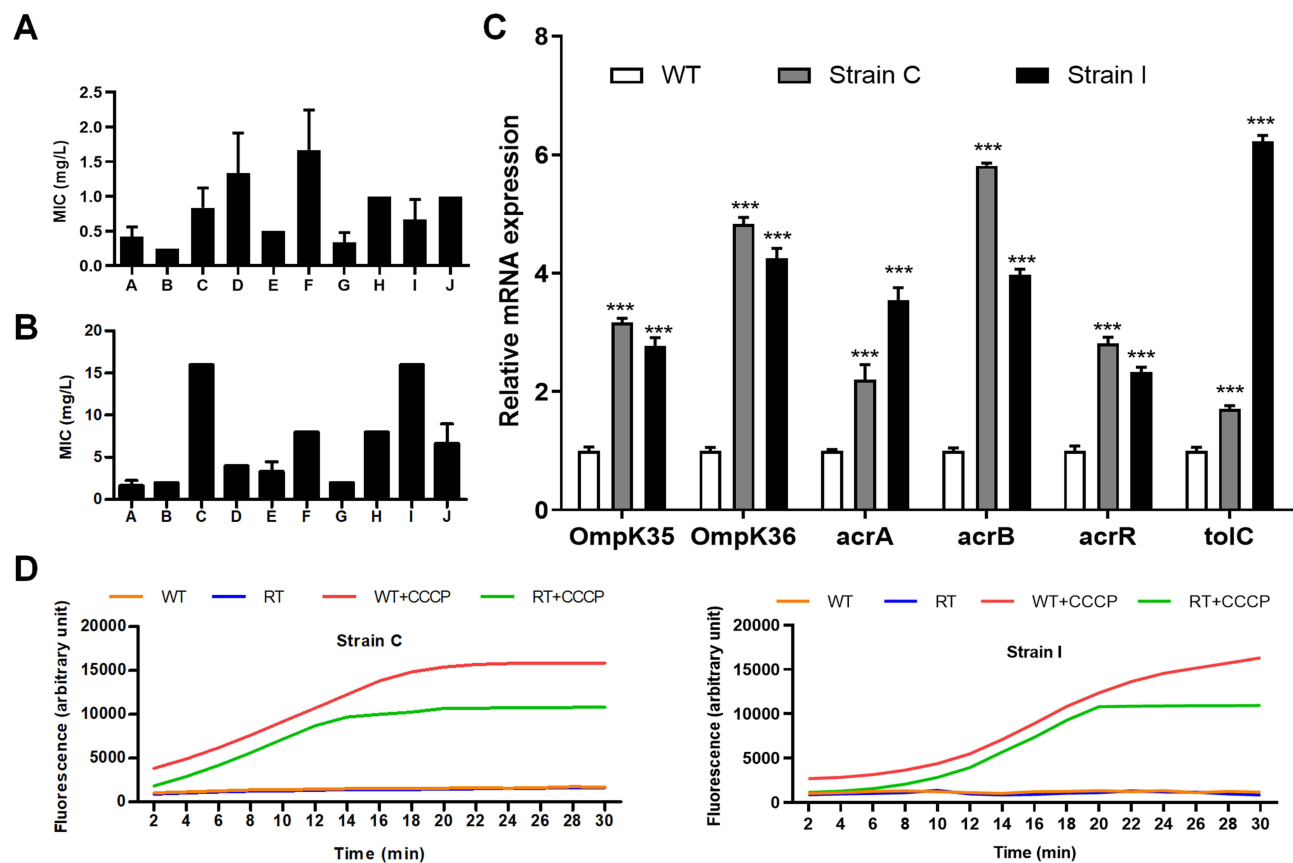


Figure 1 Screening and identification of the drug-resistant *K. pneumoniae* strains. MIC values of *K. pneumoniae* strain before (A) and after (B) with gentamicin overuse. (C) Gene expression of *OmpK35*, *OmpK36*, *acrA*, *acrB*, *tolC*, and *acrR* in WT and RT strains. (D) Fluorescence of EtBr accumulation in WT strain and RT strains C and I in absence or presence of CCCP. *** $P < 0.001$ vs WT group.

ten *K. pneumoniae* isolates, five induced strains were found to be gentamicin-resistant (MIC>5 mg/L), especially in C and I isolate (MIC>10 mg/L) (Figure 1B). Therefore, gentamicin-resistant strains C and I were chosen for subsequent experiments. To testify the resistance of *K. pneumoniae*, we detected the mRNA levels of efflux pump genes (*acrA*, *acrB*, and *tolC*), the porin genes (*OmpK35* and *OmpK36*) and pump regulators (*acrR*) by RT-PCR. Compared with the wild-type isolates (WT), the expression of *OmpK35*, *OmpK36*, *acrA*, *acrB*, *acrR* and *tolC* genes was significantly increased in the gentamicin-resistance (RT) strains C and I (Figure 1C, $P < 0.001$). Next, fluorimetry was performed to evaluate the efflux activity of these *K. pneumoniae* strains by ethidium bromide (EtBr). In the absence of efflux pump inhibitor CCCP, all strains had roughly the same fluorescence value, which suggested that the efflux capacity of all strains was similar. However, the fluorescence value of strains with CCCP was significantly higher than that of strains without CCCP, which revealed that EtBr accumulation was increased in the presence of CCCP. Moreover, the fluorescence value of RT strains was lower than that of WT strains in the presence of CCCP (Figure 1D).

Evaluation of Biofilm-Forming Ability of Drug-Resistant *K. Pneumoniae* Strains

In order to explore whether the drug resistance of RT strains C and I was mediated by the formation of biofilms, we used RT-PCR to detect the mRNA levels of biofilm-forming genes (*mrkA*, *luxS*, *pga*, *wbbM*, and *wzm*). Compared with the WT isolates, the expressions of *mrkA*, *luxS*, *pga*, *wbbM*, and *wzm* genes were all up-regulated in RT strains C and I (Figure 2A, $P < 0.001$). Furthermore, the inhibition rate of gentamicin on the biofilm formation of RT strains C and I was about 20%, while that of WT was more than 40% (Figure 2B, $P < 0.01$).

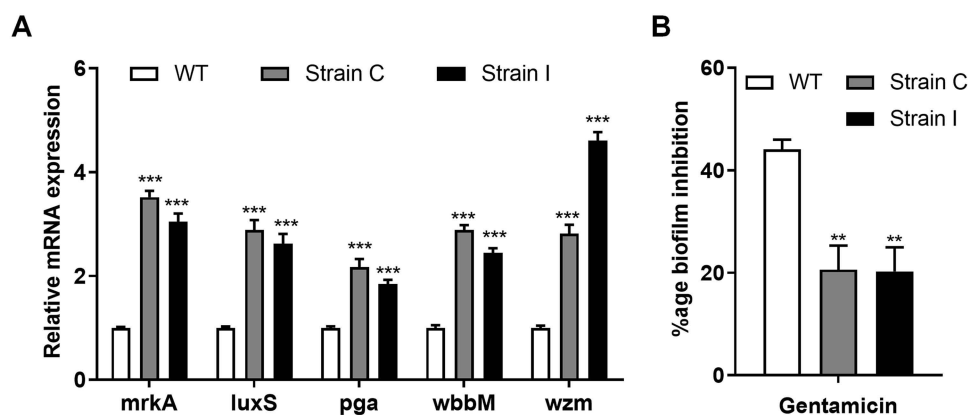


Figure 2 Biofilm-forming ability of drug-resistant *K. pneumoniae* strains. (A) Expression of biofilm-forming genes *mrkA*, *luxS*, *pga*, *wbbM* and *wzm* in WT and RT strains. (B) The inhibition rate of gentamicin on the biofilm formation of WT and RT strains. ** $P < 0.01$, *** $P < 0.001$ vs WT group.

Antibacterial Effect of Antibacterial Peptides in vitro

The toxicity of antibacterial peptides to lung cells was first investigated. As shown in Figure 3A, the LL37, KLA and Dermaseptin reached 50% release of LDH at nearly 0.5 mg/mL, while the LD50 for Magainin I was above 1 mg/mL. We then investigated the inhibitory effects of the antimicrobial peptides LL37, Magainin I, KLA and Dermaseptin on the biofilm formation of RT strains C and I. Compared with the negative control group, the treatment of antimicrobial peptides had a significant inhibitory effect on the biofilm formation of the RT strains C and I. Among the four antimicrobial peptides, KLA had the strongest inhibition ability for biofilm formation, which was superior to gentamicin (Figure 3B and C, $P < 0.001$). In addition, EtBr fluorescence accumulation in the RT strains C and I treated with multiple antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) was detected. Gentamicin was used as a control. As shown in Figure 3D and E, the fluorescence value was similar in the PBS and gentamicin groups of strains C and I. However, in strain C, LL37, Magainin I, KLA and Dermaseptin groups showed significantly increased fluorescence values, while in strain I, only Magainin I, KLA and Dermaseptin groups showed significantly increased fluorescence value. For strain C and strain I, the fluorescence accumulation in the KLA treatment group was the highest. This result demonstrated that antimicrobial peptides inhibited the efflux activity of the RT strains.

Antimicrobial Peptides Inhibit the Infection of *K. pneumoniae* on Lung Cells

To further investigate the effects of antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) on RT *K. pneumoniae*, fluorimetry was performed in lung cells infected with RT strain C then treated with antimicrobial peptides including LL37, KLA, Magainin and Dermaseptin. As shown in Figure 4A, no significant difference was observed between PBS and Gentamicin group, which indicated that *K. pneumoniae* strain C developed resistance to gentamicin. Compared with PBS and Gentamicin, four antimicrobial peptides significantly reduced the red fluorescence in *K. pneumoniae*-infected lung cells, especially in KLA ($P < 0.05$, $P < 0.01$ and $P < 0.001$), which demonstrated that these antimicrobial peptides protected lung cells by clearing *K. pneumoniae*. Furthermore, flow cytometry results showed that annexin V positive cell density in LL37, KLA and Dermaseptin groups was lower than that in PBS or Gentamicin groups. At the same time, Magainin I had no significant effect on *K. pneumoniae*-infected lung cells (Figure 4B, $P < 0.01$ and $P < 0.001$). It is indicated that RT *K. pneumoniae* strain C developed resistance to gentamicin, and the antimicrobial peptides including LL37, KLA and Dermaseptin can effectively inhibit the increased annexin V positive cells caused by *K. pneumoniae* infection and resistance to gentamicin.

Antibacterial Effect of Antibacterial Peptides in vivo

In order to investigate the inhibition of antimicrobial peptides on RT *K. pneumoniae* in vivo, we used RT strain C to induce pneumonia in mice. The four kinds of antimicrobial peptides were used to treat pneumonia mice, and the

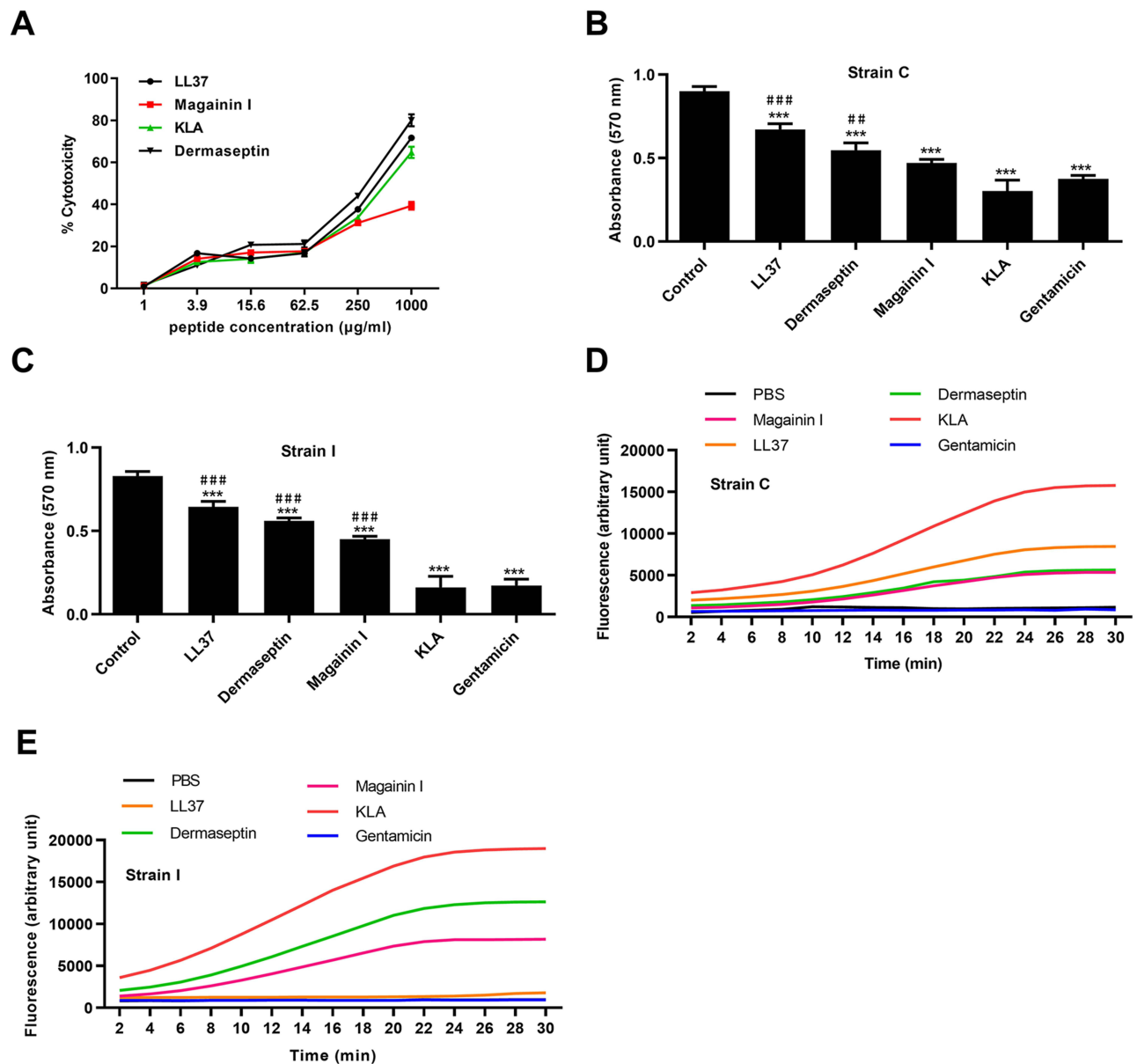


Figure 3 Antibacterial effect of the antimicrobial peptides in vitro. (A) Toxicity of antimicrobial peptides to lung cells. Antimicrobial peptides inhibit the biofilm formation of strain C (B) and I (C). Fluorescence of EtBr accumulation in RT strains C (D) and I (E) treated with antimicrobial peptides. *** $P < 0.001$ vs Control group, ** $P < 0.01$, **** $P < 0.001$ vs Gentamicin group.

therapeutic effect was then evaluated. As shown in Figure 5A, after antimicrobial peptide treatment for 16 h or 48 h, the bacterial burden in the lungs of mice was significantly decreased in the LL37, KLA, Magainin I and gentamicin groups compared with that in the control group ($P < 0.01$ and $P < 0.001$). In addition, after 48 h of treatment, the levels of the inflammatory cytokine TNF- α , IL-1 β and IL-6 also decreased (Figure 5B, $P < 0.05$, $P < 0.01$ and $P < 0.001$). At the same time, we performed pathological analysis on the lung tissues. The results of HE staining showed that the lesions of the lung tissue of mice treated with several antimicrobial peptides were improved compared with a large amount of inflammatory cell infiltration in the control group (Figure 5C). Immunohistochemical staining of the neutrophil antigen LY6G in lung tissue indicated that the antimicrobial peptides reduced the accumulation of neutrophils in lung tissue (Figure 5D).

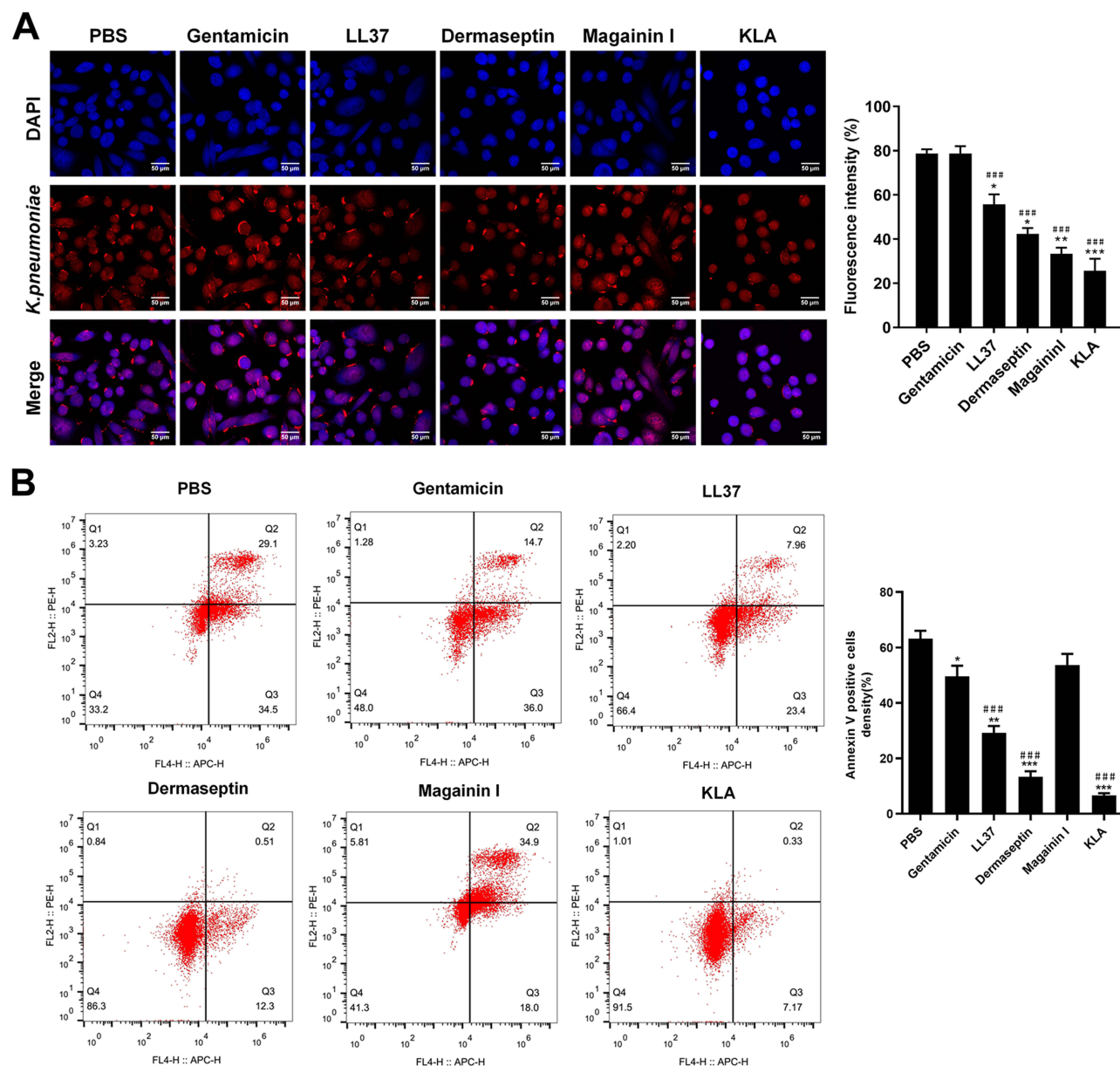


Figure 4 Antimicrobial peptides inhibit the infection of *K. pneumoniae* on lung cells. **(A)** The fluorescence of lung cells infected by *K. pneumoniae* was treated with PBS, Gentamicin, and the four antimicrobial peptides. Scale bar = 100 μ m. **(B)** The apoptosis of lung cells and the Annexin V positive cells density. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs PBS group, #### $P < 0.001$ vs Gentamicin group.

Discussion

In the present study, we aimed to study the effects of multiple antimicrobial peptides on gentamicin-resistant *K. pneumoniae* and *K. pneumoniae*-infected lung cells so as to discover the dominant antimicrobial peptides for the treatment of gentamicin-resistant *K. pneumoniae*. Our results demonstrated that antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) potentially exerted antimicrobial effects by inhibiting efflux activity and the biofilm formation of gentamicin-resistant strains. Among them, KLA (10 μ g/mL) showed better antibacterial activity than the other three antimicrobial peptides against gentamicin-resistant *K. pneumoniae*.

As one of the important pathogens of community and nosocomial acquired infections, *K. pneumoniae* was ubiquitous in the natural environment. Due to the large and irregular use of antimicrobial agents, multiple drug-resistant strains appeared rapidly. The overall 14-day mortality rate of 249 patients infected with *Klebsiella pneumoniae* pneumonia in the intensive care unit was 28.9%.⁵² To determine drug-resistant *K. pneumoniae* and the resistance mechanisms, we compared the MIC value

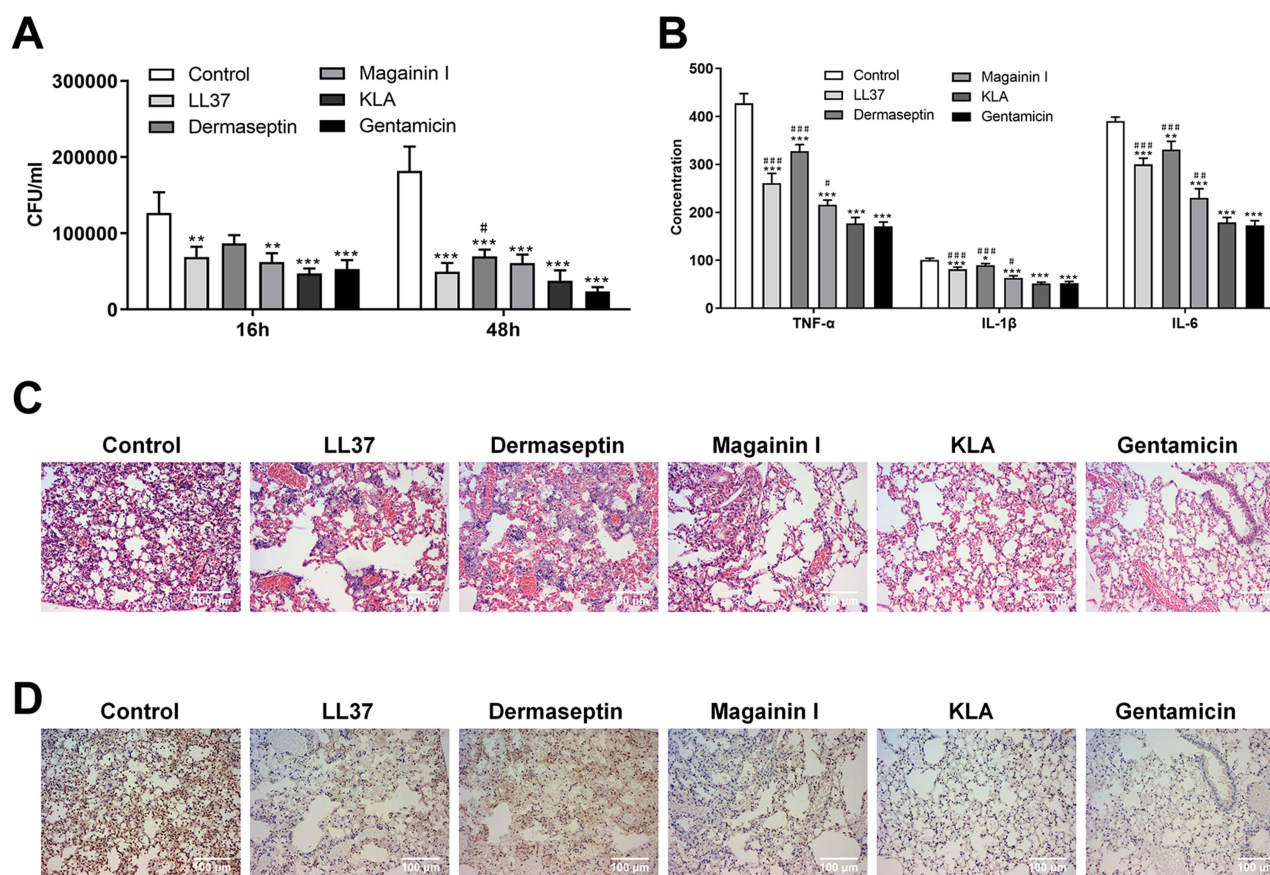


Figure 5 Antibacterial effect of antimicrobial peptides in vivo. **(A)** Bacterial loads in the lungs of mice 16h and 48h after treatment with antimicrobial peptides. The levels of inflammatory cytokines **(B)**, HE staining **(C)** and Immunohistochemical staining of Ly-6G **(D)** in lung tissue 48h after treatment with antimicrobial peptides. Scale bar = 100 μm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs Control group, # $P < 0.05$, ### $P < 0.01$, #### $P < 0.001$ vs Gentamicin group.

between strains screened by stepwise increasing concentrations of gentamicin and untreated strains. In our study, we found that five strains showed gentamicin resistance, especially the strain C and I.

The existence of the active efflux pump accounted for the multiple drug resistance of *K. pneumoniae*. Since the substrates of the active efflux system are not specific and very broad, some strains may constitute multiple types of different efflux systems simultaneously. When the efflux of these systems is enhanced, various antibacterial drugs are excreted through this system, resulting in *K. pneumoniae* resistance to multiple antibiotics, that is, multiple-drug resistance.⁵³ The active efflux system consists of efflux proteins, fusion proteins and outer membrane channel proteins. Studies have shown that as the most important efflux system, AcrAB-TolC is composed of AcrA, AcrB and TolC,⁵⁴ and widely exists in *K. pneumoniae*, accounting for the multiple drug resistance of *K. pneumoniae*.⁵⁵ In addition, the porin genes (*OmpK35* and *OmpK36*) and pump regulators (*acrR*) are closely related to drug resistance of *K. pneumoniae*.⁵⁶ Our data showed that the mRNA levels of efflux pump genes (*acrA*, *acrB*, and *tolC*), the porin genes (*OmpK35* and *OmpK36*) and pump regulators (*acrR*) were significantly increased in RT strains compared with WT strains. In addition, to further demonstrate whether the resistance of *K. pneumoniae* was attributable to the enhancement of efflux pump activity, CCCP was used to stimulate the RT strains and WT strains. As an efflux pump inhibitor, CCCP destroyed the proton gradient across the proteoliposome membrane of bacteria,⁵⁷ resulting in the loss of energy of transporters and the increase in drug accumulation concentration in bacteria, thereby inhibiting drug efflux. In our study, EtBr accumulation assay demonstrated that the fluorescence value of EtBr in RT strains and WT strains was remarkably increased in the presence of CCCP, and the fluorescence value of RT strains was lower than that of WT strains in the presence of CCCP, suggesting that the drug resistance of *K. pneumoniae* was mediated by efflux pump activity, and the RT strains had stronger tolerance to drugs compared with the WT strains. The formation of biofilm is also one of the mechanisms of drug resistance.⁵⁸

Biofilms block the infiltration of antimicrobial agents, making the antibacterial drugs ineffective. It has been reported that biofilm formation genes (*mrkA*, *luxS*, *pga*, *wbbM*, and *wzm*) are involved in *K. pneumoniae* biofilm.⁴² In this study, we found that the expression of biofilm formation genes (*mrkA*, *luxS*, *pga*, *wbbM*, and *wzm*) in strain C and I was significantly increased. The inhibition of gentamicin on the biofilm formation of RT strain C and I was weaker than that of WT strain.

In this study, we did not sequence the RT strains to detect DNA mutations. However, we found significant differences in the expression of genes related to efflux activity and biofilm formation between the RT strains and the WT strains. Future studies need to further analyze the genetic changes of RT strains and whether there are mutations in genes related to efflux activity and biofilm formation. To improve the conclusion of this paper.

Antibacterial peptides are an essential part of the natural immune system, characterized by high antibacterial efficiency and broad-spectrum. Unlike antibiotics, antimicrobial peptides rarely cause drug resistance to pathogens. Moreover, antimicrobial peptides have a certain killing effect on fungi, viruses, protozoa and tumor cells. Thus, the great potential of antimicrobial peptides is indicated for new drug development.^{59–63} According to statistics, 3099 natural antimicrobial peptides have been identified and isolated in the antimicrobial peptide database (as of May 2020, <http://aps.unmc.edu/ap/main.php>). In our study, four antimicrobial peptides (LL37, Magainin I, KLA and Dermaseptin) used to treat gentamicin-resistant *K. pneumoniae* infection significantly inhibited both biofilm formation and efflux activity of the RT strain (vs Gentamicin, $P < 0.001$). In addition, compared with the other three antimicrobial peptides, KLA was more effective in treating *K. pneumoniae*-infected lung cells and *K. pneumoniae*-induced pneumonia in mice. KLA significantly reduced the fluorescence intensity and annexin V-positive cell density of *K. pneumoniae*-infected lung cells ($P < 0.001$). These results indicate that KLA is superior among the four antimicrobial peptides for the treatment of gentamicin-resistant *K. pneumoniae* infection. However, this study only compared the effects of gentamicin and four antimicrobial peptides on the treatment of gentamicin-resistant *K. pneumoniae* infection, and the lack of comparison with other antibiotics in the same class makes it difficult to determine the potential of KLA in the treatment of gentamicin-resistant *K. pneumoniae* infection. Therefore, other antibiotics and antimicrobial peptides will be added in the future to determine the advantages of antimicrobial peptides in the treatment of gentamicin-resistant *K. pneumoniae* infection. In addition, there is a lack of comparison of the differences in the effects of antimicrobial peptides on RT strains and WT strains in this study, which needs to be further studied in combination with the genetic changes of RT strains.

For all we know, *K. pneumoniae* is a pathogen of clinical relevance and can induce the inflammatory response of lung,⁶⁴ while in vivo experiments revealed that KLA significantly reduced the bacterial load and levels of inflammatory cytokine in the lungs of mice with pneumonia and reduced the degree of lesions and the accumulation of neutrophils in the lung tissues, indicating that KLA could reduce inflammatory response to protect lung cells by destroying *K. pneumoniae*. It has been pointed out that the in vitro activity of antimicrobial peptides usually cannot be translated into in vivo efficacy due to the interference of the host microenvironment on peptide stability/efficacy.⁶⁵ However, a recent studies have shown that neutrophil membrane coated nanoparticles mediated KLA peptides can evade immune system clearance and achieve efficient targeted aggregation and drug delivery at bacterial infection sites, effectively inhibiting the development of drug-resistant *K. pneumoniae* pneumonia.³⁴ Therefore, this study needs to be in for a particular infection in parts of the body model system to assess the effectiveness of antibacterial peptides, with more realistic to predict their successful treatment in clinical trials, and can be combined with the new body delivery method to maximize the effectiveness of antimicrobial peptides.

In addition, it has been shown that the activation of Toll and IMD pathways can induce the gene expression of various antimicrobial peptides,⁶⁶ and NF- κ B is also involved in the regulation,⁶⁷ and Toll, IMD and NF- κ B pathways are involved in oxidative stress⁶⁸ and immune inflammation.^{69–71} In conclusion, antimicrobial peptides can significantly reduce the levels of inflammatory cytokines, which may be achieved through the Toll, IMD and NF- κ B signaling pathway, that is, antimicrobial peptides may regulate inflammatory cytokines through the Toll, IMD and NF- κ B signaling pathway to protect the lung tissue infected with gentamicin-resistant *K. pneumoniae*. In the future, we will further explore the potential mechanisms of the four antimicrobial peptides destroying *K. pneumoniae*, and supplement the downstream targets or signaling pathways of the four antimicrobial peptides regulating gentamicin-resistant *K. pneumoniae*, so as to provide new ideas for the treatment of drug-resistant bacteria.

Conclusion

In Conclusion, the study revealed the effects of several antimicrobial peptides on gentamicin-resistant *K. pneumoniae*. Our data showed that four antimicrobial peptides exhibited antimicrobial activity against gentamicin-resistant *K. pneumoniae* strains, especially KLA, indicating that KLA was a promising antimicrobial peptide candidate for treating infections caused by *K. pneumoniae*.

Data Sharing Statement

Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All experiments were conducted with the informed consent of the subjects, and the experimental protocol complied with the Declaration of Helsinki was approved by the Medical Ethics Committee of Wuxi Fifth People's Hospital (2021-021-1). All animal experiments complied with the welfare of laboratory Animals and the ethical requirements of animal testing (Laboratory animal—Guideline for ethical review of animal welfare, GB/T 35892-2018). The animal experiment protocol was approved by the Animal Ethics Committee of Jiangnan University (20220302-WY001). All experiments were conducted with the informed consent of the subjects.

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Disclosure

The authors report no conflicts of interest in this work.

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