BMC Microbiology

RESEARCH

Open Access

Activity of polymyxin B combined with cefepime-avibactam against the biofilms of polymyxin B-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* in in vitro and in vivo models

Miaomei Tian[1,3], Bingqian Yan[1,3], Rong Jiang[1,2,3], Candi Liu[4], You Li[1], Bing Xu[1,2,3], Siwei Guo[1,2,3*] and Xin Li[1,2,3*]

Abstract

Bacterial biofilms, often forming on medical devices, can lead to treatment failure due to their increased antimicrobial resistance. Cefepime-avibactam (CFP-AVI) exhibits potent activities against *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Klebsiella pneumoniae (K. pneumoniae)* when used with polymyxin B (PMB). However, its efficacy in biofilm-related infections is unknown. The present study aimed to evaluate the activity of PMB combined with CFP-AVI against the biofilms of PMB-resistant Gram-negative bacteria. Five *K. pneumoniae* strains and three *P. aeruginosa* strains known to be PMB-resistant and prone to biofilm formation were selected and evaluated. Antimicrobial susceptibility assays demonstrated that the minimal biofilm inhibitory and eradication concentrations of PMB and CFP-AVI for biofilms formed by the eight strains were significantly higher than the minimal inhibitory concentrations of the antibiotics for planktonic cells. The biofilm formation inhibition and eradication assays showed that PMB combined with CFP-AVI cannot only suppress the formation of biofilm but also effectively eradicate the preformed mature biofilms. In a modified in vitro pharmacokinetic/pharmacodynamic biofilm model, CFP-AVI monotherapy exhibited a bacteriostatic or effective activity against the biofilms of seven strains, whereas PMB monotherapy did not have any activity at 72 h. However, PMB combined with CFP-AVI demonstrated bactericidal activity against the biofilms of all strains at 72 h. In an *in vivo Galleria mellonella* infection model, the 7-day survival rates of larvae infected with biofilm implants of *K. pneumoniae* or *P. aeruginosa* were 0-6.7%, 40.0-63.3%, and 46.7–90.0%, respectively, for PMB alone, CFP-AVI alone, and PMB combined with CFP-AVI; the combination therapy increased the rate by 6.7–33.3% ($P < 0.05$, $n = 6$), compared to CFP-AVI monotherapy. It is concluded that PMB combined with CFP-AVI exhibits effective anti-biofilm activities against PMB-resistant *K. pneumoniae* and *P. aeruginosa* both in vitro and in vivo, and thus may be a promising therapeutic strategy to treat biofilm-related infections.

*Correspondence:
Siwei Guo
siwei-guo@cssdsyy.com
Xin Li
xin-li@cssdsyy.com

Full list of author information is available at the end of the article

Keywords Biofilm, Cefepime-avibactam, Polymyxin B-resistance, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*

Introduction

Severe infections caused by multidrug-resistant Gram-negative bacteria (MDR-GNB) are on the rise globally and pose a significant threat to public health. 62% of the causative agents of septic shock include MDR-GNB bacterial strains [1–3]. Patients with severe infections often require the insertion of biocompatible materials, such as central venous catheters, closed thoracic drainage tubes, and tracheal intubation. Certain MDR-GNBs, including *P. aeruginosa* and *K. pneumoniae*, readily adhere to the surfaces of these biomaterials, forming complex biofilms and leading to biomaterial-related infections [4–7]. Compared to planktonic cells, bacteria within biofilms demonstrate stronger adhesion and fertility, with antibiotic resistance that is 10-1000 times higher, and thus can evade clearance by both the host immune system and antibiotics. Therefore, biofilm-related infections are characterized by low cure rates and high mortality, especially when caused by multidrug-resistant bacteria [8–12].

Polymyxin B (PMB), a polypeptide antibiotic known as the "last line of defense" against MDR-GNB, is widely used in clinical practice for severe infections of MDR-GNB [13]. PMB has been shown to inhibit the biofilm of PMB-sensitive MDR *Acinetobacter baumannii* [14]. However, with the widespread use of PMB, bacteria resistant to PMB have proliferated globally. For example, a retrospective hospital-based study indicated that the resistance rate to PMB in *P. aeruginosa* was 25.1% in Nepal [15], and the rate of polymyxin E-resistant *K. pneumoniae* increased from 36 to 50% from 2011 to 2015 in Italy [16]. In addition, it has been reported that 5-40% of carbapenem-resistant *Enterobacteriaceae* are also resistant to PMB [17]. Treating biofilm infections caused by PMB-resistant bacteria presents more significant challenges than those caused by PMB-sensitive bacteria. Some studies have demonstrated the effectiveness of colistin (a PMB congener) in combination with other antimicrobial agents, such as fosfomycin, gentamycin, mefloquine, and several new compounds, including PFK-158, ethylenediaminetetraacetic acid, or furanone C-30, against biofilms formed by colistin-resistant Gram-negative bacteria [18–23]. However, studies evaluating on PMB-based combinations with other antimicrobial agents for combatting PMB-resistant biofilms remains limited. An in vitro study reported that PMB, when used in combination with meropenem, was effective against the biofilm of PMB-resistant *P. aeruginosa* [24]. Our previous study demonstrated that PMB plus a new β-lactam/β-lactamase inhibitor (BL/BLI) combination restored PMB activity against PMB-resistant *K. pneumoniae* and *P. aeruginosa* [25]. Moreover, we further observed that among various BL/BLI combinations, CFP-AVI exhibited a highest synergistic rate (52.8% in the checkerboard assay), and obvious synergistic effects for all eight tested PMB-resistant clinical isolates [25]. These preliminary findings suggest that PMB plus CFP-AVI is potentially effective against the biofilm of PMB-resistant *K. pneumoniae* and *P. aeruginosa*. However, the impact of this combination on the biofilm has not yet been elucidated.

Therefore, the present study was performed to evaluate the activities of PMB in combination with CFP-AVI against the biofilms formed by PMB-resistant *K. pneumoniae* and *P. aeruginosa*.

Materials and methods

Bacterial strains

PMB-resistant clinical strains, comprising five strains of *K. pneumoniae* (K001-K005) and three strains of *P. aeruginosa* (P001-003), were obtained from patients at the Third Hospital of Changsha, Changsha, Chain and Sir Run Run Shaw Hospital, Zhejiang, China. These strains were identified using the automated Vitek 2 system (bioMérieux, Lyon, France). Reference strains, *K. pneumoniae* ATCC700603 and *P. aeruginosa* ATCC27853 were used for routine quality control.

Antimicrobial agents

Reference substances of PMB (lot number: 130313–201310) and cefepime (lot number: 130524–202005) were acquired from the China National Institutes of Food and Drug Control. Avibactam, which was used as another reference substance, was generously provided by Nanjing Yoko Bio-Pharma Co., Ltd. (lot number: 1192491-61-4). These substances were used in antimicrobial susceptibility assays. For PK/PD experiments, the active pharmaceutical ingredients of PMB (no. A1411171, Xellia Pharmaceuticals), CFP (no. 0074MJ86D8, Nanjing Yoko Bio-Pharma Co., Ltd.), and AVI (no. 1192491-61-4, Nanjing Yoko Bio-Pharma Co., Ltd.) were used. All antimicrobial stock solutions were freshly prepared according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and filtered using a 0.22 μm sterile filter membrane (Millex-GP 33 mm PES 0.22 μm Sterile; Millipore, USA) prior to use.

Biofilm formation assays

Overnight cultures of the tested strains were adjusted to a 0.5 McFarland using sterile saline and then diluted 1:100 in fresh tryptic soy broth (TSB). A standardized inoculum of 200 μL, equivalent to 10^6 cfu/mL, was added to each well of a 96-well plate (flat bottom, Corning, USA) and incubated at 37 °C for various time points

(6, 12, 24, 48, 56, and 72 h). After incubation, the wells were rinsed three times with sterilized water to remove planktonic bacteria, and then 200 μL of 1% crystal violet (AnYan, China) was added into each well for 15 min, followed by two washes with sterilized water. After air drying at room temperature, 200 μL of 95% ethanol (KESHI, China) was added into each well to solubilize the bound crystal violet. The formation of biofilm biomass was detected and quantified with a microplate reader at an optical density of 595 nm (OD595). Negative controls consisted of wells without any inoculum. The mean OD595 values of the biofilms at different time points were used to assess changes in biofilm biomass over time, while the mean OD595 values of the biofilms at 24 h were used to evaluate the biofilm-formation capacity of the strains. The optical density cut-off (ODc) was defined as three standard deviations above the mean OD of the negative controls. Accordingly, the strains were categorized according to their biofilm formation capacity as non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2ODc), moderate biofilm producers (2 ODc < OD ≤ 4ODc), and strong biofilm producer (OD > 4 ODc).

Antimicrobial susceptibility assays for P. Aeruginosa and K. pneumoniae bacteria and biofilms

The minimal inhibitory concentrations (MICs) of PMB and CFP-AVI were determined by using the broth microdilution method, following the CLSI guidelines [26]. Antimicrobial susceptibility tests were conducted in 96-well microplates, with each well containing a final bacterial concentration of approximately 10^5 cfu/mL in Mueller-Hinton broth medium (Oxoid, Hampshire, England). All antibiotics were prepared using the serial two-fold dilution method, with the concentrations tested ranging from 0.25 to 128 μg/mL for PMB and 0.0625–256 μg/mL for CFP-AVI. The results were recorded after an incubation period of 18–22 h at 37 °C.

Biofilm antimicrobial susceptibility tests were performed by using the Calgary Biofilm Device (Innovotech Inc. Canada), which consists of a standard 96-well plate and a plastic lid with 96 pegs [27]. Biofilms were formed on the pegs by culturing bacterial suspension with a final concentration of 10^6 cfu/mL at 37℃ for 24 h. After incubation, the lid was washed three times with sterilized water. The lid pegs were then transferred to a new sterile 96-well plate with TSB containing different concentrations of antibiotics (PMB or CFP-AVI alone) and incubated at 37 °C for 18–22 h. The minimal biofilm inhibitory concentration (MBIC) was identified as the lowest concentration of antibiotics that did not show visible growth or turbidity [28]. Subsequently, the lid was rinsed three times with sterilized water and placed in another sterile 96-well plate containing 200 μL of fresh TSB without any antibiotics, and then incubated at 37 °C for 24 h. The minimal biofilm eradication concentration (MBEC) was determined by visually identifying the lowest concentration of antibiotics with no observable bacterial growth [28].

Biofilm formation inhibition and eradication assays

Briefly, bacterial suspensions were prepared at a final concentration of approximately 10^6 cfu/mL in a 96-well plate with 1/4 MICs of PMB and CFP-AVI, alone or in combination. Negative controls consisted of wells containing the inoculum without any antibiotics. After incubation at 37 °C for 24 h, 1% crystal violet was added into each well for 15 min, and the formation of biofilm biomass was detected and quantified as described above.

For biofilm eradication experiment, the inoculum was prepared in a 96-well plate as described above. Following a 24-h incubation at 37 °C, the unattached bacteria were removed by washing twice with sterilized water. The mature biofilms preformed were then exposed to 1/2 MIC of all antibiotics alone or in combination at 37 °C for another 24 h. Negative controls did not contain any antibiotics. The quantity of biofilm biomass was assessed as described above.

Confocal laser scanning microscopy

Two representative strains, *K. pneumoniae* K002 and *P. aeruginosa* P002, were selected for confocal laser scanning microscopy (CLSM) to further observe the biofilm formation inhibition and eradication effects of PMB and CFP-AVI, alone or in combination. Briefly, biofilms of the two strains treated with the antibiotics in the above experiments were cultured in a 24-well plate (Jet Bio-Filtration Co., Ltd, China) with each well containing one cell climbing slice. Subsequently, each cell climbing slice was thoroughly rinsed with sterilized water and stained with the fluorescent dye 4′, 6′-diamidino-2-phenylindole for 15 min in the dark. Biofilms without the treatment (as negative controls). CLSM was used to observe the biofilms on the cell climbing slices.

In vitro biofilm PK/PD model

An in vitro biofilm PK/PD model was used to determine the activities of PMB in combination with CFP-AVI against the biofilms formed by PMB-resistant *K. pneumoniae* and *P. aeruginosa*.

Modification of the in vitro biofilm PK/PD model

Briefly, during the biofilm culture phase, 1 mL of bacterial suspension (10^8 cfu/mL) was inoculated into a CDC biofilm reactor (BioSurface Technologies, Bozeman, MT), which contained eight rods with each rod housing three removable coupons where bacterial biofilms would form, followed by a 24-h static batch culture at 37°C [29].

Subsequently, fresh sterile TSB was continuously pumped into the reactor at a constant flow rate, calculated by dividing the reactor volume (315 mL) by the residence time [30]. Upon the completion of the biofilm culture phase, the therapeutic phase began. For monotherapy, an antibiotic solution was introduced into the reactor using an infusion pump (B. Braun Medical (Shanghai) International Trading Co., Ltd.), while a peristaltic pump (Baoding Lead Fluid Technology Co., Ltd.) was used to pump fresh medium, simulating the antibiotic half-life. For combination therapy, we innovatively incorporated a compensation chamber to compensate for drugs with longer half-lives, deviating from conventional methods. This chamber facilitated the simulation of the simultaneous half-lives of both drugs, thereby aligning the concentration changes in the reactor more closely with the real concentration changes following combination therapy in the human body.

Simulated PK profiles in the in vitro biofilm PK/PD model

A total of four regimens were evaluated on each of the eight clinical strains over a 72-h treatment period. These included: (i) PMB alone (2.5 mg/kg load, 1.5 mg/kg q12h, 2-h infusion) with an assumed f_{Cmax} of 4.1 mg/L, 50% protein binding, and targeting an adult half-life of 11.9 h; (ii) CFP-AVI alone (2 g–0.5 g q8h, 2-h infusion), with CFP having an f_{Cmax} of 68.73 mg/L, 16% protein binding, and targeting an average adult half-life of 1.98 h, and AVI has an f_{Cmax} of 22.19 mg/L, 6.95% protein binding, and the same half-life; (iii) a combination of PMB (2.5 mg/kg load, 1.5 mg/kg q12h, 2-h infusion) and CFP-AVI (2 g–0.5 g q8h, 2-h infusion); and (iv) a drug-free group. The doses and PK parameters of PMB and CFP-AVI were determined based on the findings of our previous research, particularly in the context of severe infections in critically ill individuals [31–35].

PK samples (1 mL) of PMB and CFP-AVI were collected from the reactor exhaust port at various time points (0, 1, 2, 3, 4, 6, 8, 10, 12 h after the first dose, and before and after each subsequent dose). All samples were filtered through a 0.22 μm sterile filter membrane and stored at -80 °C until analysis.

The concentrations of PMB and AVI were measured by liquid chromatography-tandem mass spectrometry as our previously described [36, 37]. The linearity ranges for PMB1 and PMB2 were 0.1–10.0 mg/L (y = 1.74123x-0.390872, R^2=0.9992) and 0.01-1.00 mg/L (y = 4.45587x + 0.00447435, R^2=0.9991), respectively, with lower quantification limits of 0.10 and 0.01 mg/L. Intra-day and inter-day assay precisions for PMB1 and PMB2 ranged from 1.23 to 4.82% and 2.57–7.00%, respectively. For AVI, the linearity range was 0.1–25 mg/L (y = 0.322873x + 0.0435643, R^2=0.9991), with a lower quantification limit of 0.08 mg/L and intra-day and inter-day assay precision ranging from 1.50 to 3.71%. CFP concentration was measured using high performance liquid chromatography with a linearity range of 0.5–120.0 mg/L (y = 0.114973x + 0.054603, R^2=0.9992), a lower quantification limit of 0.50 mg/mL, and assay precision ranging from 0.21 to 5.04%.

PD analysis in the in vitro biofilm PK/PD model

One rod was aseptically removed from the CDC biofilm reactor at 0, 8, 24, 32, 48, 56, and 72 h. The coupons were then rinsed twice with sterile saline to remove unbound bacteria. Each coupon was transferred into 10 mL of normal saline, and biofilm bacteria were recovered by sonication, followed by serial dilution in saline. A 100 μL aliquot of the bacterial suspensions was seeded in Mueller-Hinton agar plates using an automatic spiral plater (easySpiral; Interscience, Cantal, France). The bacterial colonies were counted after incubation at 37 °C for 18–22 h. Each experiment was performed three times, with a reliable detection limit of 50 cfu/mL.

Time-kill curves were constructed by plotting log10 cfu/mL against time (h) to determine the anti-biofilm activity of the different dose regimens. Bactericidal and effective activities were, respectively, defined as a ≥ 3-log, and ≥ 1-log and < 3-log reduction in bacterial density at 72 h compared to the initial inoculum. Synergy and additivity in the treatment were defined as a ≥ 2-log reduction and a ≥ 1-log to < 2-log reduction, respectively, in bacterial density caused by combination therapy compared to the most active antimicrobial alone at 72 h [30].

Galleria mellonella larvae biofilm implant infection model

A *Galleria mellonella* larvae infection model was also used to determine the activities of PMB in combination with CFP-AVI against the biofilms formed by PMB-resistant *K. pneumoniae* and *P. aeruginosa*.

Galleria mellonella larvae were obtained from Huiyude Biological Technology (Tianjin, China), specifically selected for their weight, approximately 300 mg per larva. The lethal dose for 90% mortality (LD_{90}) was first determined for each strain in the larvae, and subsequently used as the initial inoculum for the *Galleria mellonella* larvae infection model. Then, the *Galleria mellonella* larvae implant biofilm infection model was established as previously described [38]. Briefly, commercially available hard nylon toothbrush bristles were cut into 1 cm segments and sterilized in an autoclave before use. A single bristle segment was carefully inserted into the last left proleg of each larva, while a 10 μL LD90 bacterial suspension was injected into the last right proleg using a Hamilton syringe.

To evaluate the effect of PMB combined with CFP-AVI on the survival of the larvae with biofilm infection, three groups of larvae with biofilm infection were

administered with the following three dose regimens 1 h after bristle insertion and infection: (i) a single dose of PMB (2.5 mg/kg), according to the loading dose in clinical settings, producing in vivo levels of 3.53 mg/L; (ii) a single dose of CFP-AVI (21 mg/g), based on a previous in vivo study [39], producing in vivo levels of 42.3 mg/L; and (iii) a combination of PMB and CFP-AVI (2.5 mg/kg+21 mg/g). In addition, larvae with and those without biofilm infection received normal saline without any antibiotic treatment served as positive and negative controls, respectively. Each group included 30 larvae. The larvae were incubated at 37 °C, and their survival rates were observed every 24 h for 7 days. Thirty larvae were used in each experimental group.

Statistical analysis

Phoenix WinNonlin 6.0 software (Pharsight Co. Ltd., Missouri, USA) was utilized for the PK analysis. Differences in the OD595 values between single-drug and combination groups in biofilm inhibition and eradication experiments were assessed using a one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was considered statistically significant.

Results

Biofilm formation of K. pneumoniae and P. Aeruginosa

The ODc was 0.141, and all strains were identified as strong biofilm producers with the OD595 values ranging from 0.647 to 1.230, and strain P002 demonstrating the strongest biofilm formation ability (Fig. 1A). Figure 1B illustrates the OD595 values for all strains at various time points. A gradual increase in OD595 was observed in all strains up to 48 h. The peak OD595 values was reached at 48 h for all strains, followed by a subsequent decline. However, the OD595 values of the experimental strains remained higher than those of the control at 72 h.

Effects of PMB and CFP-AVI on planktonic cells and biofilms of K. pneumoniae and P. Aeruginosa

Table 1 presents the MICs, MBICs, and MBECs of antibiotics. All strains exhibited resistance to PMB. The MICs of PMB ranged from 4 to 128 μg/mL. The MBIC and MBEC values of PMB and CFP-AVI for biofilms formed by the eight strains were significantly higher than the MIC values of the antibiotics for planktonic cells of all strain, with the MBECs tested exceeded 1,024 μg/mL for all antibiotics.

Inhibitory and eradication effects of PMB combined with CFP-AVI on bacterial biofilms

As shown in Fig. 2A, the OD595 values of all strains were significantly reduced in the group treated with PMB-based combinations compared to those of the control and monotherapy groups ($P<0.05$). Figure 2B shows that the combination of PMB and CFP-AVI effectively eradicated preformed biofilms in all strains ($P<0.05$).

In addition, CLSM revealed significant biofilm formation and eradication effects (Fig. 3). The combination of PMB and CFP-AVI exhibited a notable inhibitory effect on biofilm formation, as indicated by the density of the reduced cell arrangement within the biofilm (Fig. 3A&B). Furthermore, co-administration of both drugs effectively eliminated mature biofilms, as indicated by the significant reduction in biofilm thickness (Fig. 3C&D).

PK/PD of PMB and CFP-AVI detected in the modified in vitro *biofilm PK/PD model* The targeted and measured concentrations of PMB and CFP-AVI in the in vitro biofilm PK/PD model are shown in Fig. 4. The measured and targeted drug time curves were nearly identical (within ±20% of the targeted value).

Figure 5 shows the bacterial counts in the biofilms with or without antibiotics for all strains. After 48 h of pre-culturing the biofilm, the initial bacterial load of all strains on the sample coupons ranged from 8.17±0.13

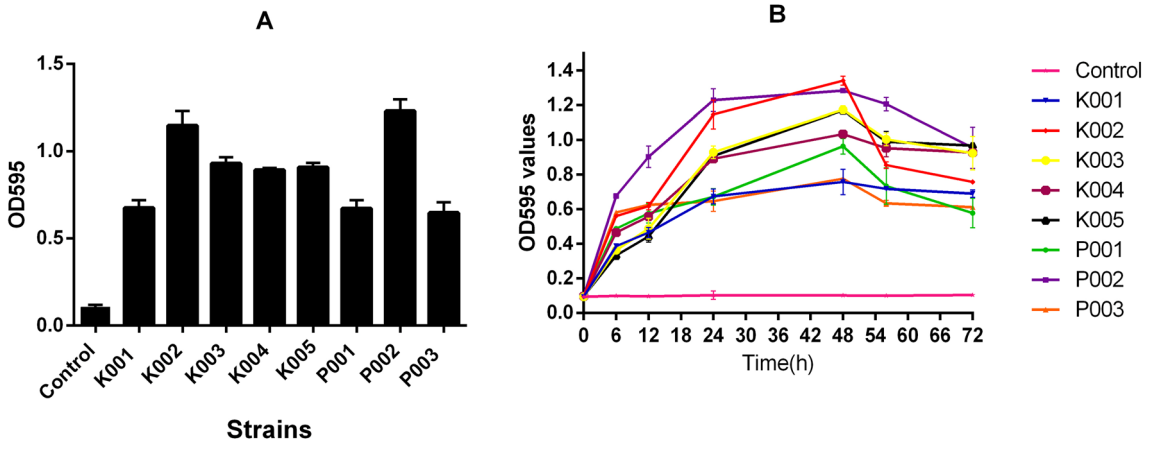

Fig. 1 **A**: The mean OD595 values of eight experimental strains. The bars represent standard deviations. **B**: Biofilm formation at different time points of eight experimental strains. The results are expressed as means± standard deviations

Table 1 The minimal inhibitory concentrations (MIC_S), minimal biofilm inhibitory concentration ($MBIC_S$), and minimal biofilm eradication concentration ($MBEC_S$) of polymyxin B (PMB) and cefepime-avibactam (CFP-AVI)

Strains	PMB			CFP-AVI (4:1)**		
	MIC*	MBIC	MBEC	MIC	MBIC	MBEC
Klebsiella pneumoniae						
K001	128	512	> 1024	8/2	512/128	> 1024/256
K002	32	256	> 1024	16/4	512/128	> 1024/256
K003	64	1024	> 1024	8/2	512/128	> 1024/256
K004	16	256	> 1024	8/2	128/32	> 1024/256
K005	16	64	> 1024	4/1	128/32	> 1024/256
Pseudomonas aeruginosa						
P001	4	128	> 1024	8/2	512/128	> 1024/256
P002	4	> 1024	> 1024	8/2	1024/256	> 1024/256
P003	4	32	> 1024	2/0.5	512/128	> 1024/256

*, The breakpoint values defining the bacterial resistance to PMB is ≥ 4 (mg/L)

**, The ratio of CFP to AVI is 4:1 in this study; and an MIC of 8/2 indicates the MIC of 8 μg/ml for CFP and 2 μg/ml for AVI for the combination formulation

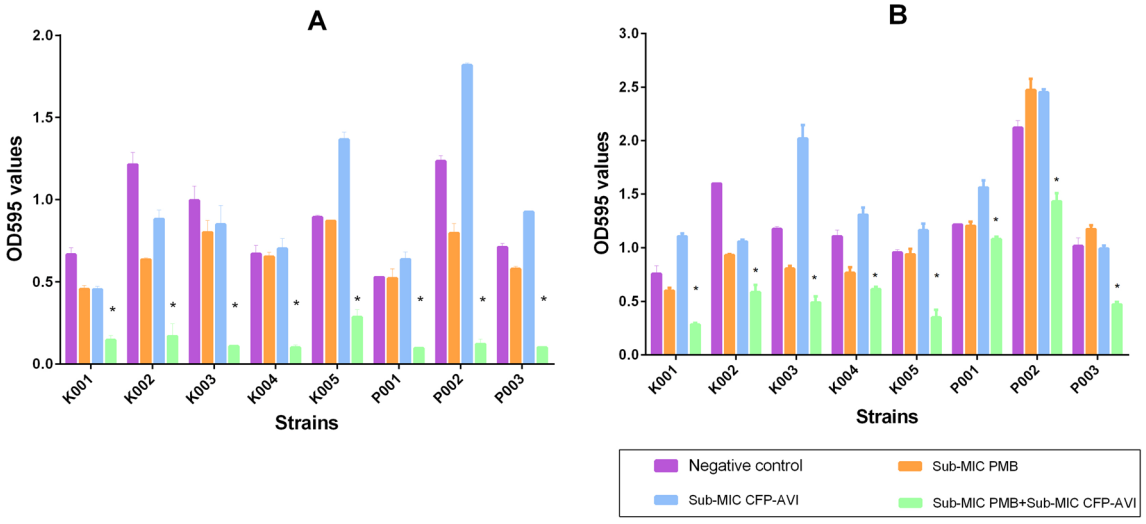

Fig. 2 Biofilm inhibitory (**A**) and eradication (**B**) effects of polymyxin B (PMB) and cefepime-avibactam (CFP-AVI). The results are expressed as the mean ± standard deviation. *, $P < 0.05$, compared with all other groups

to 9.56±0.12 log10 cfu/mL. PMB monotherapy did not effectively kill all strains after 72 h, as bacterial counts in biofilms remained similar to those in the control. In contrast, CFP-AVI monotherapy exhibited effective activity against most strains (Δlog10 cfu/mL = -2.560 to -1.245), except for P002. The combination of PMB and CFP-AVI demonstrated significant bactericidal activity against all biofilm-producing strains at 72 h (Δlog10 cfu/mL = -5.345 to -3.243). Compared to the most active single agent, the combination of PMB and CFP-AVI showed additivity (K003, Δlog10 cfu/mL = -1.621; K005, Δlog10 cfu/mL = -1.676) or synergy (in all strains except K003 and K005, with Δlog10 cfu/mL ranging from −5.089 to -2.019).

The therapeutic effect of antibiotic in Galleria mellonella larvae biofilm implants infection model

The LD_{90} values for the strains tested in the larval infection model ranged from 10^7 to 10^9 cfu/mL for *K. pneumoniae* and from 10^3 to 10^4 cfu/mL for *P. aeruginosa* (Table 2).

As shown in Fig. 6, there was no signigfcant difference in the 7-day survival rates between larvae injected with PMB or CFP-AVI only and those in the control group ($P > 0.05$). The survival curves of the infected larvae with biofilms are shown in Fig. 7. PMB monotherapy was not effective in rescuing biofilm-infected larvae for all strains, with a 7-day survival rate ranging from 0 to 6.7%. In the groups treated with CFP-AVI monotherapy, the 7-day survival rates for larvae infected with biofilm-forming strains were between 40.0% and 63.3%. However, in the groups receiving combined PMB and CFP-AVI treatment, the larvae survival rate increased between 46.7%

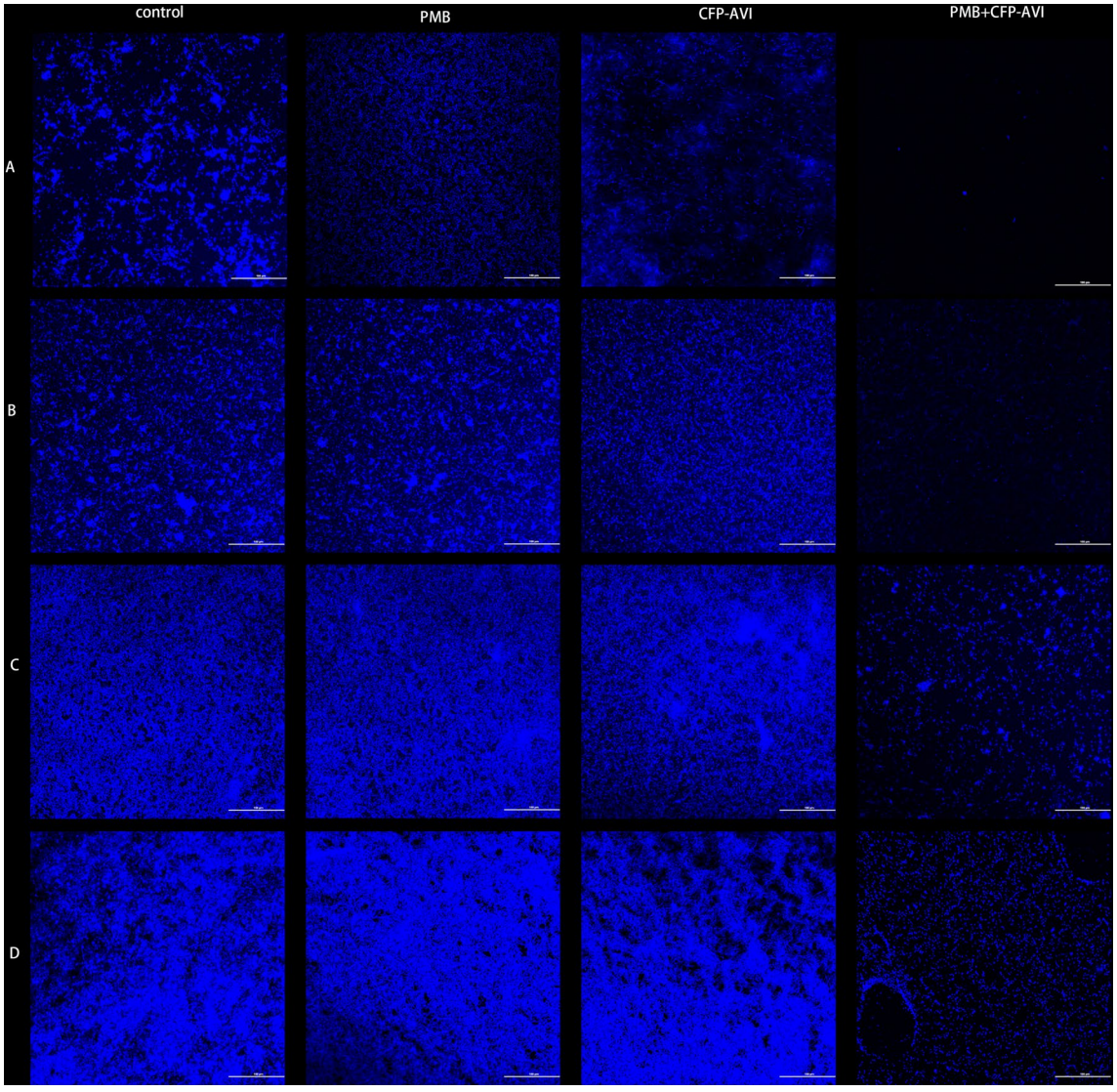

Fig. 3 CLSM images reveal the inhibitory (**A**&**B**) and eradication (**C**&**D**) effects of polymyxin B (PMB), cefepime- avibactam (CFP-AVI) on biofilm formation by *Klebsiella pneumoniae* K002 (A&C) and *Pseudomonas aeruginosa* P002 (**B**&**D**). Scale bar, 100 μm

and 90.0%. Compared to the CFP-AVI monotherapy groups, the combination treatment groups showed significantly increased survival rates ($P<0.05$, $n=6$).

Discussion

The present study investigated the anti-biofilm effects of PMB combined with CFP-AVI on PMB-resistant biofilms using biofilm formation inhibition and eradication assays, modified in vitro biofilm PK/PD model and *in vivo Galleria mellonella* larvae biofilm implant infection model. Our results revealed, for the first time, that the combination of PMB and CFP-AVI exhibited a significant synergistic effect against PMB-resistant *K. pneumoniae* and *P. aeruginosa* biofilms, both in vitro and in vivo. In the antimicrobial susceptibility assays, PMB-resistant *K. pneumoniae* and *P. aeruginosa* biofilms demonstrated considerably higher resistance to PMB and CFP-AVI compared to planktonic bacterial cells. The MBICs and MBECs of all antibiotics were 4 to over 256 and 16 to over 1,024 times higher than their MICs, respectively, suggesting that conventional antibiotic concentrations are insufficient to eradicate the biofilms. The effective steady-state concentration of PMB in clinical practice is 2–4 mg/L [13]. In our biofilm formation inhibition and eradication assays, the combination of PMB with CFP-AVI demonstrated a synergistic anti-biofilm effect, with PMB concentrations (1/4 MIC or 1/2 MIC) in the combination ≤ 4 mg/L for three strains of *P. aeruginos*a. These findings further substantiate the potential clinical applicability of the combination.

The biofilm development mainly includes four stages: initial adhesion of bacteria, formation of microcolonies,

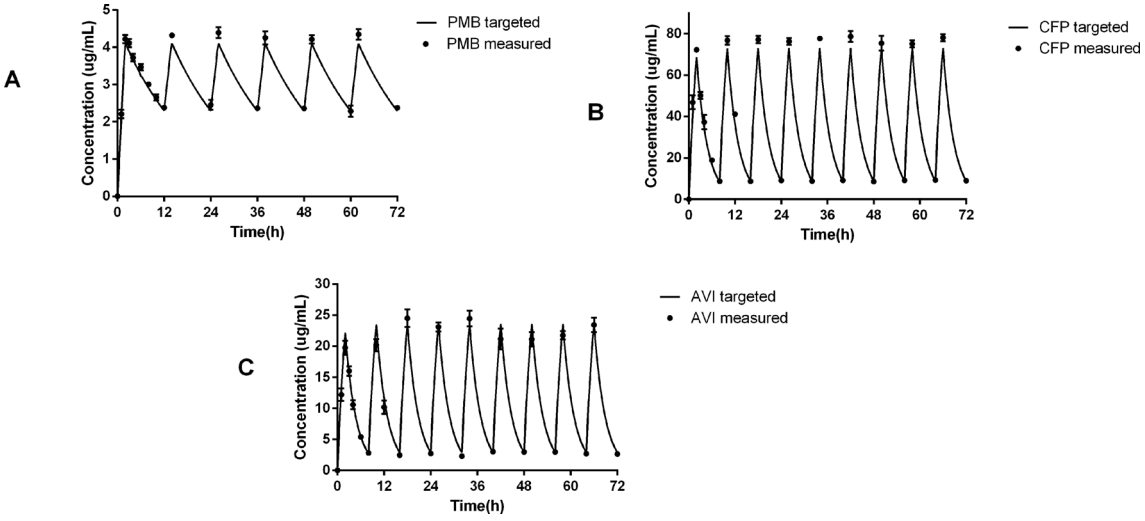

Fig. 4 The targeted and measured concentrations of polymyxin B (PMB) (A), cefepime (CFP) (B) andavibactam (AVI) (C) in the in vitro biofilm PK/PD model.

maturation of biofilm, and dispersion of biofilm. There are mainly two anti-biofilm strategies: inhibiting the formation of biofilm and eradicating mature biofilm. Sub-MIC PMB has an inhibitory effect on the formation of biofilm but could not eradicate the already formed biofilm. This may be due to the fact that in the early stages of biofilm formation, the biofilm structure is not yet mature, and bacteria have relatively weak resistance to antibiotics. Mature biofilms can enhance bacterial tolerance due to their structural integrity and the permeability barrier effect of extracellular polysaccharides. Previous studies have shown that sub-MIC doses of cefepime can upregulate the expression of biofilm-related genes and enhance biofilm by *P. aeruginosa* clinical isolates [40]. The present study also showed similar results, that is, sub-MIC CFP-AVI increased biofilm formation in monotherapy in the case of several *P. aeruginosa.* However, whether it also upregulates the expression of related genes needs to be further investigated.

CFP-AVI is a new BL-BLI antibiotic with high susceptibility rates against Gram-negative bacteria, specifically targeting extended-spectrum β-lactamase-, AmpC-, KPC-, and OXA-48-producing *Enterobacteriaceae*, and multidrug-resistant *P. aeruginosa* [41, 42]. Our previous static in vitro time-kill study demonstrated that CFP-AVI alone exhibited bacteriostatic activities against PMB-resistant *P. aeruginosa* and *K. pneumoniae* [25]. However, its efficacy against biofilm-related infections has not been well-established. In the present study, the modified in vitro PK/PD biofilm model showed that CFP-AVI had bacteriostatic or effective activities against the majority of biofilms formed by PMB-resistant *P. aeruginosa* and *K. pneumoniae* despite no obvious bactericidal effect. Additionally, in the *Galleria mellonella* larvae biofilm implant infection model, the survival rate of larvae treated with CFP-AVI ranged from 40.0 to 63.3%, which is a significant improvement compared to the positive control, where no larvae survived after 7 days. Our results suggest that CFP-AVI has potential value in treating biofilm-related infections. In contrast, one other study [43] found that the new BL-BLI ceftazidime-avibactam did not exhibit reliable anti-biofilm activity. Thus, further investigation is required to assess the anti-biofilm activities of BL-BLI antibiotics. It is acknowledged that a nylon toothbrush bristle segment was inserted in the last left proleg of each larva to allow biofilm formation but the effect of the antibiotic treatments on the in vivo biofilms was assessed without consideration of the bristle segment, which is a limitation of this study design. We will keep this issue in mind and test the biofilm on the implanted bristles in the future study, which will provide a more intuitive conclusion.

In the present study, the LD_{90} values were significantly different between *K. pneumoniae* (10^7-10^9 cfu/mL) and *P. aeruginosa* (10^3-10^4 cfu/mL), a finding consistent with other studies [44]. The high virulence of *P. aeruginosa* may be attributed to its secretion of various virulence factors, such as outer membrane proteins, alginate, flagellum, type IV pili, and protein secretion systems [45].

In our modified in vitro biofilm PK/PD model, PMB and CFP-AVI exhibited synergistic or additive effects when administered at clinical doses (PMB at a 2.5 mg/kg loading dose, followed by 1.5 mg/kg every 12 h; CFP-AVI at 2 g every 8 h through a 2-h infusion). However, the in vitro biofilm PK/PD model cannot fully simulate the in vivo environment to reflect the human immune response. Therefore, we applied the established *Galleria mellonella* larvae biofilm implant infection model as *Galleria mellonella* has both cellular and humoral defenses that are analogous to the human immune system, making

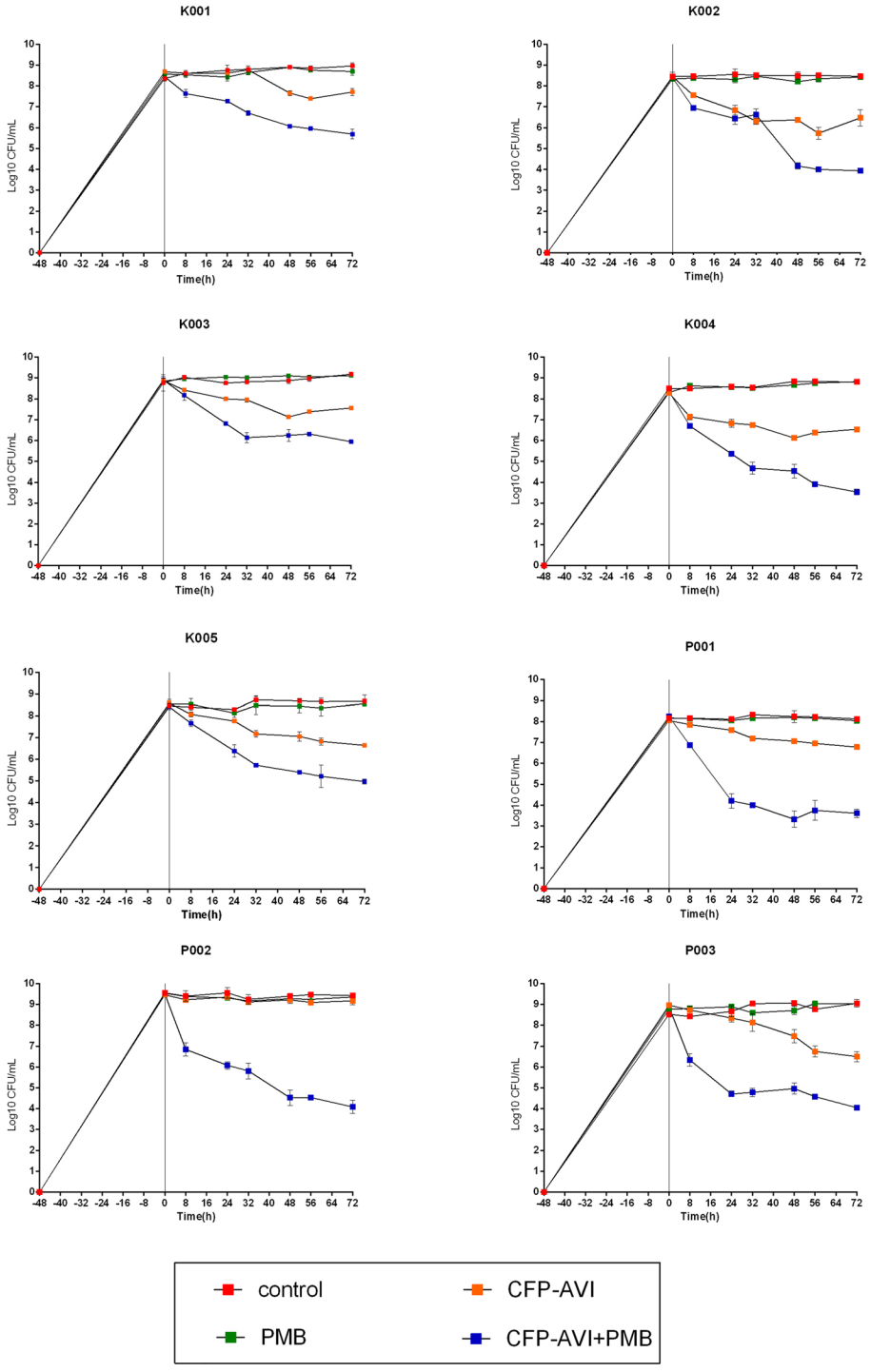

Fig. 5 Bacterial killing by different treatments against biofilm-embedded bacteria of *K. pneumoniae* and *P. aeruginosa* in the in vitro biofilm PK/PD model after 72 h treatment

it widely used in models of biofilm-related infections. These characteristics would compensate for the limitations of the in vitro dynamic PK/PD model. Previous studies found consistent results between larvae and mammals [46, 47]. We observed that the PMB and CFP-AVI combination treatment significantly increased the survival rate in group, which confirmed the findings in the in vitro biofilm PK/PD model. It should be mentioned that although the humoral immunity of larvae is similar to that of humans, the adaptive immune system of larvae is different from that of mammals, and thus the results

Table 2 The lethal doses for 90% mortality (LD_{90}) of the eight strains of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* for *Galleria mellonella* larvae

Strains	LD_{90}
K. pneumoniae	
K001	~ 10^7cfu/mL
K002	~ 10^9cfu/mL
K003	~ 10^9cfu/mL
K004	~ 10^9cfu/mL
K005	~ 10^7cfu/mL
P. aeruginosa	
P001	~ 10^3cfu/mL
P002	~ 10^3cfu/mL
P003	~ 10^4cfu/mL

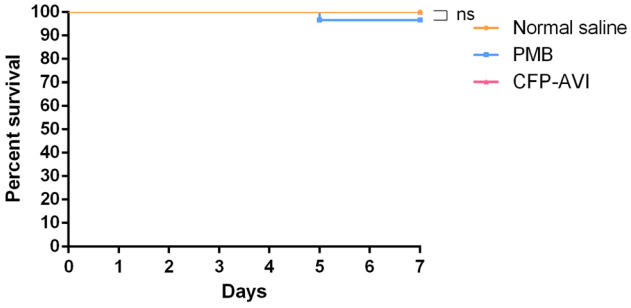

Fig. 6 Survival of *G. mellonella* larvae treated only with polymyxin B (PMB) and cefepime-avibactam (CFP-AVI) without infection. ns, no statistical differences, compared between normal saline and antibiotics

obtained in the present study need to be further verified in clinical practice.

The synergistic effects produced by the combination may be based on the following two possible mechanisms. First, subpopulations in biofilms have different metabolic activities. PMB could kill the subpopulation exhibiting low metabolic activity in the inner layers of biofilm, whereas the β-lactam antibiotics could specifically kill the metabolically active subpopulation in biofilm because the activity of these antibiotics is highly dependent on the rate of bacterial growth [48]. Therefore, the rationale for the combination of these two types of drugs is based on the potential for subpopulation synergy, with each drug in combination targeting a different subpopulation. Second, it is well known that poor antibiotic penetration into biofilms plays an important role in biofilm resistance. PMB can bind to the lipopolysaccharides (LPS) of Gram-negative bacteria, which may disrupt the outer membrane and facilitate the penetration of other antibiotics in the biofilm [49, 50].

In conclusion, the combination of PMB and CFP-AVI exhibits effective anti-biofilm activities against PMB-resistant *K. pneumoniae* and *P. aeruginosa* both in vitro and in vivo. This study provides valuable preclinical evidence supporting the use of PMB and CFP-AVI in combination as a promising strategic approach to overcome the challenges associated with treating biofilm-related infections.

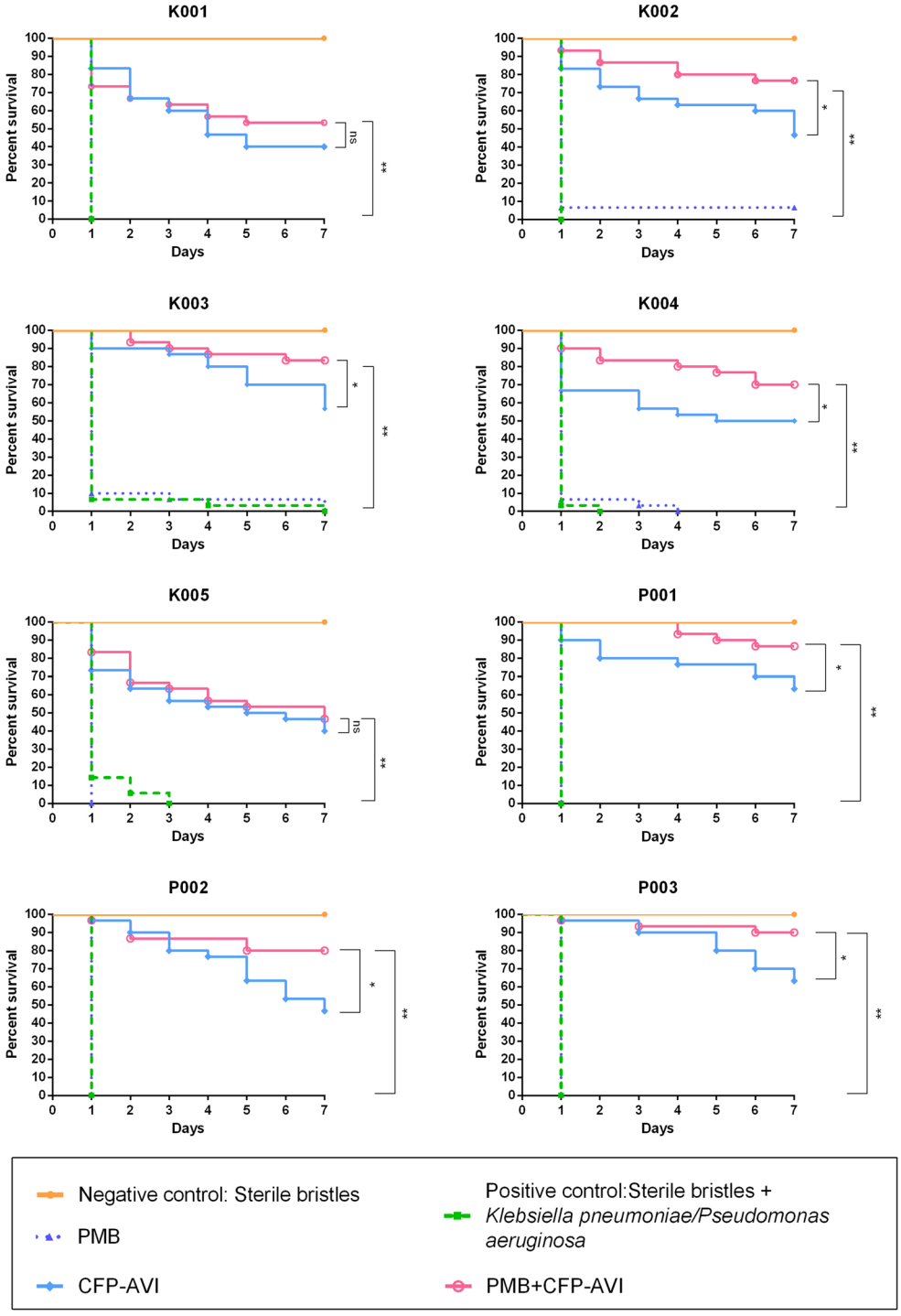

Fig. 7 Survival of *G. mellonella* larvae infected with biofilm implants, with or without polymyxin B (PMB) and cefepime-avibactam (CFP-AVI) treatment. Experiment was conducted with 30 larvae per group. *, $P < 0.05$, compared between CFP-AVI monotherapy and PMB combined with CFP-AVI; **, $P < 0.001$, compared between positive controls and PMB combined with CFP-AVI; ns, no statistical differences, compared between CFP-AVI monotherapy and PMB combined with CFP-AVI

Abbreviations

BL/BLI	β-lactam/β-lactamase inhibitor
CFP-AVI	Cefepime-avibactam
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
LD_{90}	Lethal dose for 90% mortality
MBEC	Minimal biofilm eradication concentration
MBIC	Minimal biofilm inhibitory concentration
MDR-GNB	Multidrug-resistant Gram-negative bacteria
MIC	Minimal inhibitory concentrations
PMB	Polymyxin B

Acknowledgements
Not applicable.

Author contributions
All authors contributed to the study conception and design. Miaomei Tian, Xin Li and Siwei Guo designed the experiments; Miaomei Tian, Bingqian Yan, You Li and Rong Jiang performed experiments and collected data; Candi Liu and Bing Xu analysed data. All authors read and approved the final manuscript.

Funding
This study was supported by the Hunan Provincial Key Laboratory of Ant-Resistance Microbial Drugs, the third hospital of Changsha (No:2023TP1013), the Hunan Provincial Science and Technology Department Foundation, China [No. 2016SK4008, No. 2020SK52901].

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Pharmacy, The Third Hospital of Changsha, 176 Western Laodong Road, Tianxin District, Changsha, Hunan Province 410015, People's Republic of China
[2]Institute of Clinical Application of Antibiotics, Changsha, Hunan Province, People's Republic of China
[3]Hunan Provincial Key Laboratory of Anti-Resistance Microbial Drugs, Changsha, Hunan Province, People's Republic of China
[4]Hunan Drug Inspection Center, Changsha, Hunan Province, People's Republic of China

Received: 1 August 2024 / Accepted: 7 October 2024
Published online: 16 October 2024

References

1. Walsh TR. Toleman MA.The emergence of pan-resistant gram-negative pathogens merits a rapid global political response[J]. J Antimicrob Chemother. 2012;67(1):1–3.
2. Laxminarayan R, Duse A, Wattal C et al. Antibiotic resistance-the need for global solutions[J]. Lancet Infect Dis. 2013;13(12):1057–98.
3. Vincent JL. International study of the prevalence and outcomes of infection in intensive care units. JAMA. 2009;302(21):2323.
4. Harron Katie,Mok Quen,Hughes Dyfrig. Generalisability and cost-impact of antibiotic-impregnated central venous catheters for reducing risk of bloodstream infection in paediatric intensive care units in England[J]. PLoS One. 2016;11(3):e0151348.
5. Rateb Hina Hedaya,Mcdowell Joan R. S.Minimising central line-associated bloodstream infection rate in inserting central venous catheters in the adult intensive care units[J]. J Clin Nurs. 2017;23-24:26.
6. Li YLXH, Yu et al. Biological and Physiochemical methods of Biofilm Adhesion Resistance Control of Medical-Context Surface[J]. Int J Biol Sci. 2021;15(7):1769–81.
7. Percival SL, Kite P. Intravascular catheters and biofilm control[J]. J Vasc Access. 2007;8(2):69.
8. Thi MTT. Wibowo D,Rehm BHA.Pseudomonas Aeruginosa Biofilms[J]. Int J Mol Sci. 2020;21(22):8671.
9. Hall-Stoodley L. Costerton JW,Stoodley P.Bacterial biofilms: from the natural environment to infectious diseases[J]. Nat Rev Microbiol. 2004;2(2):95–108.
10. Hall CW. Mah TF.Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria[J]. FEMS Microbiol Rev. 2017;41(3):276–301.
11. Uruén C, Chopo-Escuin G, Tommassen J et al. Biofilms as promoters of Bacterial Antibiotic Resistance and Tolerance[J]. Antibiotics (Basel).2020;10(1):3.
12. Høiby N, Bjarnsholt T, Moser C et al. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014[J]. Clin Microbiol Infect. 2015;21 Suppl 1.
13. Tsuji BT, Pogue JM, Zavascki AP et al. International Consensus Guidelines for the Optimal Use of the Polymyxins: Endorsed by the American College of Clinical Pharmacy (ACCP), European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Infectious Diseases Society of America (IDSA), International Society for Anti-infective Pharmacology (ISAP), Society of Critical Care Medicine (SCCM), and Society of Infectious Diseases Pharmacists (SIDP)[J]. Pharmacotherapy.2019;39(1):10–39.
14. Ruan SW, Wang H-X, Zhang M-Y, Jiang W-J. Effect of polymyxin B on the biofilm formation of Acinetobacter baumannii. Pract Pharm Clin Remedies. 2020;23(05):54-57.
15. Baral S, Pokharel A, Subramanya SH, et al. Clinico-epidemiological profile of Acinetobacter and Pseudomonas infections, and their antibiotic-resistant pattern in a tertiary care center, Western Nepal[J]. Nepal J Epidemiol. 2019;9(4):804–11.
16. Giani T, Arena F, Vaggelli G, et al. Large nosocomial outbreak of Colistin-Resistant, carbapenemase-producing Klebsiella pneumoniae traced to clonal expansion of an mgrB. Deletion Mutant[J] J Clin Microbiol. 2015;53(10):3341–4.
17. Poirel L, Jayol A,Nordmann PP. Antibacterial activity, susceptibility testing, and Resistance mechanisms encoded by plasmids or Chromosomes[J]. Clin Microbiol Rev. 2017;30(2):557–96.
18. Chen L, Yu K, Chen L, et al. Synergistic activity and biofilm formation effect of Colistin combined with PFK-158 against colistin-resistant gram-negative Bacteria[J]. Infect Drug Resist. 2021;14:2143–54.
19. Zhang Y, Lin Y, Zhang X, et al. Combining colistin with Furanone C-30 rescues Colistin Resistance of Gram-negative Bacteria in Vitro and in Vivo[J]. Microbiol Spectr. 2021;9(3):e0123121.
20. Zhang X, Zhao Y, Feng L et al. Combined with mefloquine, resurrect colistin active in colistin-resistant Pseudomonas aeruginosa in vitro and in vivo[J]. Front Microbiol. 2021;12:790220.
21. Shein AMS, Wannigama DL, Higgins PG, et al. Novel colistin-EDTA combination for successful eradication of colistin-resistant Klebsiella pneumoniae catheter-related biofilm infections[J]. Sci Rep. 2021;11(1):21676.
22. Memar MY, Adibkia K, Farajnia S et al. In-vitro effect of Imipenem, Fosfomycin, Colistin, and Gentamicin Combination against Carbapenem-resistant and Biofilm-forming Pseudomonas aeruginosa isolated from burn Patients[J]. Iran J Pharm Res. 2021;20(2):286–96.
23. Boncompagni SR, Micieli M, Di Maggio T, et al. Activity of fosfomycin/colistin combinations against planktonic and biofilm gram-negative pathogens[J]. J Antimicrob Chemother. 2022;77(8):2199–208.
24. Wickremasinghe H, Yu HH, Azad MAK et al. Clinically relevant concentrations of Polymyxin B and Meropenem synergistically kill Multidrug-Resistant Pseudomonas aeruginosa and minimize Biofilm Formation[J]. Antibiotics (Basel). 2021;10(4):405.
25. Li Y, Guo S, Li X et al. Evaluation of the in vitro synergy of polymyxin B-based combinations against polymyxin B -resistant gram-negative bacilli[J]. Microb Pathog. 2022;166:105517.
26. Wayne PA. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing[J]. 2022.
27. Ceri H, Olson M, Morck D, et al. The MBEC Assay System: multiple equivalent biofilms for antibiotic and biocide susceptibility testing[J]. Methods Enzymol. 2001;337:377–85.

28. Macias-Valcayo A, Aguilera-Correa JJ, Broncano A, et al. Comparative in Vitro Study of Biofilm formation and Antimicrobial susceptibility in Gram-negative Bacilli isolated from Prosthetic Joint. Infections[J] Microbiol Spectr. 2022;10(4):e0085122.
29. Gambino E, Maione A, Guida M et al. Evaluation of the pathogenic-mixed biofilm formation of Pseudomonas aeruginosa/Staphylococcus aureus and treatment with limonene on three different materials by a dynamic Model[J]. Int J Environ Res Public Health. 2022;19(6):3741.
30. Gómez-Junyent J, Benavent E, Sierra Y, et al. Efficacy of ceftolozane/tazobactam, alone and in combination with colistin, against multidrug-resistant Pseudomonas aeruginosa in an in vitro biofilm pharmacodynamic model[J]. Int J Antimicrob Agents. 2019;53(5):612–9.
31. Li Y, Yan B, Guo S, et al. Pharmacokinetics of YK-1169 in healthy subjects and pharmacokinetic/pharmacodynamic analysis by Monte Carlo simulation[J]. Br J Clin Pharmacol. 2023;89(10):3067–78.
32. Zavascki AP, Goldani LZ, Cao G et al. Pharmacokinetics of intravenous polymyxin B in critically ill patients[J]. Clin Infect Dis. 2008;47(10):1298–304.
33. Sandri AM, Landersdorfer CB, Jacob J, et al. Population pharmacokinetics of intravenous polymyxin B in critically ill patients: implications for selection of dosage regimens[J]. Clin Infect Dis. 2013;57(4):524–31.
34. Barbhaiya RH, Forgue ST, Gleason CR, et al. Pharmacokinetics of cefepime after single and multiple intravenous administrations in healthy subjects[J]. Antimicrob Agents Chemother. 1992;36(3):552–7.
35. Merdjan H, Rangaraju M, Tarral A. Safety and pharmacokinetics of single and multiple ascending doses of avibactam alone and in combination with ceftazidime in healthy male volunteers: results of two randomized, placebo-controlled studies[J]. Clin Drug Investig. 2015;35(5):307–17.
36. Deng Y, Gu JY, Li X, et al. Does Monitoring total and free polymyxin B1 plasma concentrations predict Polymyxin B-Induced Nephrotoxicity? A retrospective study in critically ill Patients[J]. Infect Dis Ther. 2022;11(4):1591–608.
37. Yan BQ, Guo SW, Li Y, Tian MM. Development and validation of a method for quantitation of cefepime /avibactam in MH Broth: application to antibacterial activity using in vitro PK/PD Model[J]. Chin J Clin Pharmacol. 2024;29(1):52–60.
38. Campos-Silva R, Brust FR, Trentin DS et al. Alternative method in Galleria mellonella larvae to study biofilm infection and treatment[J]. Microb Pathog. 2019;137:103756.
39. Nath S, Moussavi F, Abraham D, et al. In vitro and in vivo activity of single and dual antimicrobial agents against KPC-producing Klebsiella pneumoniae[J]. J Antimicrob Chemother. 2018;73(2):431–6.
40. Hagras SAA, et al. Effect of sub-inhibitory concentrations of cefepime on biofilm formation by Pseudomonas aeruginosa. Can J Microbiol[J]. 2021;67(12):894–901.
41. Karaiskos I, Galani I,Souli M et al. Novel β-lactam-β-lactamase inhibitor combinations: expectations for the treatment of carbapenem-resistant Gram-negative pathogens[J]. Expert Opin Drug Metab Toxicol. 2019;15(2):133–49.
42. Aktaş Z. Kayacan C,Oncul O.In vitro activity of avibactam (NXL104) in combination with β-lactams against Gram-negative bacteria, including OXA-48 β-lactamase-producing Klebsiella pneumoniae[J]. Int J Antimicrob Agents. 2012;39(1):86–9.
43. Papalini C, Sabbatini S, Monari C, et al. In vitro antibacterial activity of ceftazidime/avibactam in combination against planktonic and biofilm carbapenemase-producing Klebsiella pneumoniae isolated from blood[J]. J Glob Antimicrob Resist. 2020;23:4–8.
44. Benthall G, Touzel RE, Hind CK et al. Evaluation of antibiotic efficacy against infections caused by planktonic or biofilm cultures of Pseudomonas aeruginosa and Klebsiella pneumoniae in Galleria mellonella[J]. Int J Antimicrob Agents. 2015;46(5):538–45.
45. Jurado-Martín I, Sainz-Mejías. M,McClean S.Pseudomonas Aeruginosa: An Audacious Pathogen with an adaptable Arsenal of Virulence Factors[J]. Int J Mol Sci. 2021;22(6):3128.
46. Cruz LIB, Lopes LFF, de Camargo Ribeiro F et al. Anti-candida albicans activity of thiazolylhydrazone derivatives in invertebrate and murine Models[J]. J Fungi (Basel). 2018;4(4):134.
47. Jeon J. Park JH,Yong D.Efficacy of bacteriophage treatment against carbapenem-resistant Acinetobacter baumannii in Galleria mellonella larvae and a mouse model of acute pneumonia[J]. BMC Microbiol. 2019;19(1):70.
48. Pamp SJ, Gjermansen M, Johansen HK, Tolker-Nielsen T. Tolerance to the antimicrobial peptide colistin in Pseudomonas aeruginosa Biofilms is linked to metabolically active cells, and depends on the pmr and mexAB-oprM genes[J]. Mol Microbiol. 2008;68(1):223–40.
49. Lora-Tamayo J, Murillo O, Ariza J. Clinical use of Colistin in Biofilm-Associated Infections[J]. Adv Exp Med Biol. 2019;1145:181–95.
50. Slingerland CJ, Kotsogianni I, Wesseling CMJ, Martin NI. Polymyxin Stereochemistry and its role in antibacterial activity and outer membrane Disruption[J]. ACS Infect Dis. 2022;8(12):2396–404.

Publisher's note