R0322

Impact of *Staphylococcus aureus* on colistin susceptibility of *Acinetobacter baumannii*

Goeun Lee, Hee Won Han, Kwan Soo Ko

Department of Microbiology, Sungkyunkwan University School of Medicine, Suwon, Republic of Korea

Abstract

BACKGROUND : The rise of multidrug-resistant bacterial pathogens poses a severe global health threat. These pathogens are often co-isolated at the same infection site, but it remains unclear whether such co-existence affects antibiotic susceptibility. In this study, we investigated the impact of co-culture with *Staphylococcus aureus* on colistin activity against *Acinetobacter baumannii*.

METHODS : *A. baumannii* was cultured alone or co-cultured with *S. aureus* (5×10^5 CFU/mL each) for 12 h. Time-kill assays were performed under no antibiotic, 0.5× MIC, and 1× MIC colistin, with CFU determined over time. Colistin MIC and expression of lipid A biosynthesis and modification-related genes (*lpxA, lpxC,* and *pmrAB*) were analyzed in *A. baumannii* recovered at 12 h. In addition, *S. aureus* was fractionated into cell pellet and cell-free culture medium (CFCM) to assess their effects on *A. baumannii* survival. Outer membrane permeability was evaluated using an NPN uptake assay under colistin with or without CFCM.

RESULTS : In the absence of antibiotics, *A. baumannii* growth was similar in monoculture and co-culture. However, under 0.5× and 1× MIC colistin, co-culture significantly reduced *A. baumannii* survival compared to monoculture, while the MIC of surviving cells at 12 h remained unchanged. Expression levels of *pmrAB, lpxA,* and *lpxC* in *A. baumannii* recovered at 12 h showed no significant differences between monoculture and co-culture under antibiotic-treated conditions. When *A. baumannii* was exposed to 0.5× MIC (0.25 mg /L) colistin in the presence of *S. aureus* pellet or CFCM, monoculture grew to ~3×10^9 CFU/mL at 12 h, whereas co-culture with either fraction maintained CFU at inoculum levels, indicating that secreted *S. aureus* factors enhance colistin-mediated killing. Outer membrane permeability analysis using the NPN uptake assay showed no difference between monoculture and co-culture in the absence of colistin. However, in the presence of both colistin and CFCM, membrane permeability was significantly increased compared to colistin

CONCLUSION : *S. aureus* secretes substances that enhance colistin-mediated killing of *A. baumannii*, highlighting polymicrobial interactions as important modulators of antibiotic efficacy.

Methods

Bacterial strains culture condition: Clinical isolates of *S. aureus* and *A. baumannii* were obtained from patients and stored at −80 °C in glycerol stocks. For experiments, strains were subcultured on sheep blood agar and adjusted to the required inoculum using phosphate-buffered saline. All in vitro assays, including antimicrobial susceptibility testing and time-kill experiments, were performed in cation-adjusted Mueller–Hinton broth. For co-culture experiments, *A. baumannii* was selectively quantified on Mueller–Hinton agar supplemented with ciprofloxacin (10 mg/L).

Antibiotic susceptibility testing: The minimum inhibitory concentrations (MICs) of colistin and ciprofloxacin were determined by broth microdilution according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were included as quality control strains. All assays were performed in duplicate.

Time-kill assay: Time-kill assays were performed according to CLSI guidelines. *A. baumannii* monocultures and co-cultures with *S. aureus* were prepared at a final inoculum density of 5×10^5 CFU/mL for each species. Cultures were exposed to colistin at 0.5× and 1× MIC of *A. baumannii* (0.25 mg/L and 0.5 mg/L, respectively), with a no-antibiotic control included. Incubation was carried out at 35 ± 2 °C with shaking at 220 rpm. Viable *A. baumannii* counts were determined at 0, 2, 4, 6, 8, and 12 h using the spot plating method.

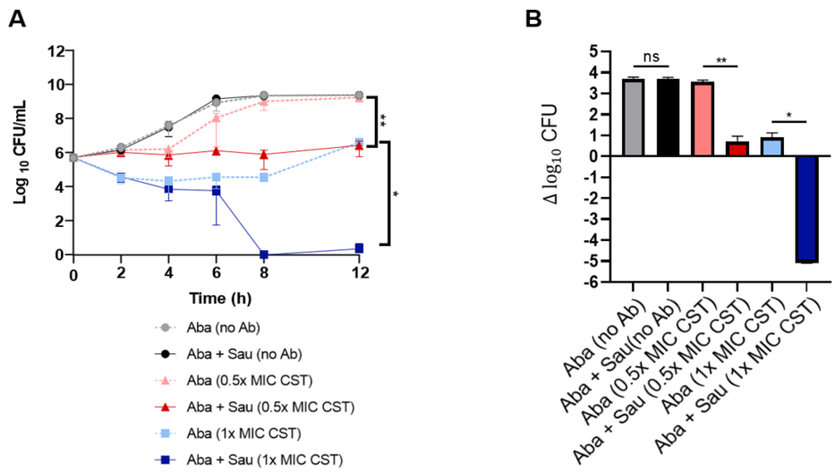
RNA extraction and qRT-PCR: Relative expression of *pmrAB, lpxA,* and *lpxC* in *A. baumannii* was analyzed after 12 h of time-kill assays under monoculture and co-culture conditions with *S. aureus*. Samples were collected from both no-antibiotic controls and colistin-treated groups (0.5× MIC). Total RNA was extracted using the S-16 Nucleic Acid Extraction System and reverse-transcribed into cDNA according to the manufacturer's protocol. qRT-PCR was performed using TB Green® Premix Ex Taq™ on a QuantStudio™ 6 Flex Real-Time PCR System. Each reaction included gene-specific primers, with *rpoB* used as the internal reference gene. Relative expression levels were calculated using the $2^{-}\Delta\Delta Ct$ method. All experiments were performed with three independent biological replicates.

Evaluation of *S. aureus* cell pellet and CFCM: *S. aureus* was cultured for 8 h and then separated into cell pellet and cell-free conditioned medium (CFCM) fractions. Briefly, cultures were centrifuged (13,000 × g, 2 min), and the supernatant was filtered through a 0.22 µm syringe filter to obtain CFCM. The bacterial pellet was resuspended in fresh cation-adjusted Mueller–Hinton broth and adjusted to 0.5 McFarland (~5×10^5 CFU/mL). For the CFCM condition, filtered supernatant was mixed with fresh broth to a final concentration of 20% (v/v).*A. baumannii* (5×10^5 CFU/mL) was then inoculated into each condition and subjected to time-kill assays with colistin at 0.5× MIC (0.25 mg/L). Viable counts were determined at 6, 8, and 12 h post-exposure.

NPN uptake assay: Outer membrane permeabilization was assessed using the N-phenyl-1-naphthylamine (NPN) uptake assay, adapted from previously described methods. *A. baumannii* (5×10^5 CFU/mL) was incubated for 4 h under no antibiotic or 0.5× MIC colistin, with or without 20% (v/v) *S. aureus* CFCM, under conditions identical to the time-kill assay. Following incubation, cells were washed and resuspended in HEPES buffer, and fluorescence was measured after incubation with NPN (10 µM). CFU-normalized fluorescence was used to quantify outer membrane permeability. All experiments were performed in triplicate.

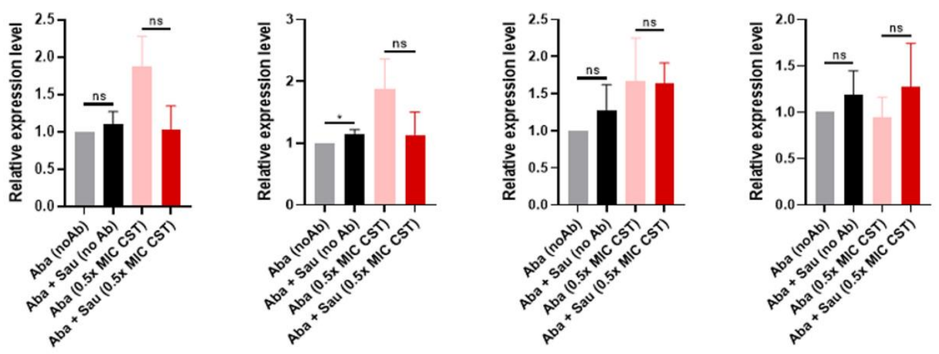
Results

Figure 1. Time-kill analysis and survival of *A. baumannii* in mono- and co-culture with *S. aureus* under colistin treatment.



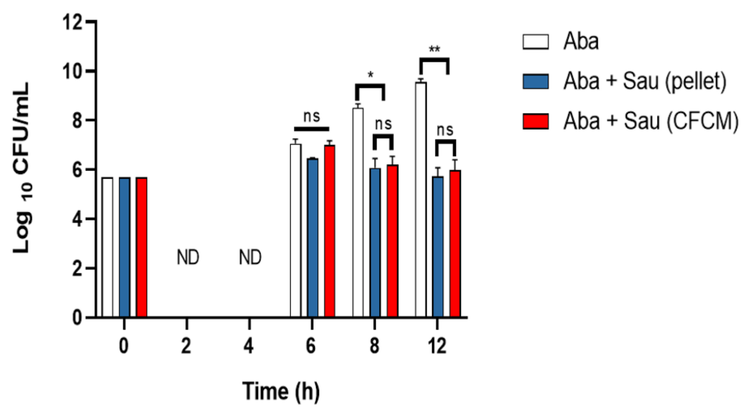
▶ In the presence of colistin, *A. baumannii* showed lower survival in co-culture with *S. aureus* than in monoculture. no Ab, no antibiotic.

Figure 2. Expression of *pmrAB*, *lpxA*, and *lpxC* in *Acinetobacter baumannii* under mono- and co-culture conditions at 12 h.



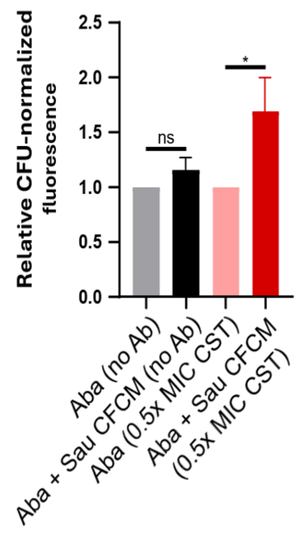
▶ Expression levels of *pmrAB, lpxA,* and *lpxC* in *A. baumannii* recovered at 12 h did not differ significantly between monoculture and co-culture under colistin-treated conditions. no Ab, no antibiotic.

Figure 3. Effects of *S. aureus* cell pellet and cell-free culture medium (CFCM) on the survival of *A. baumannii* under colistin treatment.



▶ Both live *S. aureus* and CFCM inhibited *A. baumannii* growth, with no significant difference between the two conditions, suggesting that secreted factor(s) from *S. aureus* are responsible for the observed effect. ND, not determined.

Figure 4. Outer membrane permeability of *A. baumannii* in response to *S. aureus* CFCM under colistin treatment.



▶ Under 0.5× MIC colistin treatment, *A. baumannii* exposed to *S. aureus* CFCM showed significantly increased NPN fluorescence compared to monoculture.

Conclusion

S. aureus–derived secreted factors enhance colistin-mediated killing of *A. baumannii* without inducing changes in MIC or resistance-associated gene expression, likely through increased outer membrane permeability. These findings highlight polymicrobial interactions as important modulators of antibiotic susceptibility.