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Synergistic Antimicrobial Activity of Bacteriophages and Antibiotics against *Staphylococcus aureus*

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Abstract This study was designed to assess the synergistic antimicrobial effect of phages combined with antibiotics against *Staphylococcus aureus*. The phage-antibiotic synergy (PAS) effect was evaluated using the fractional inhibitory concentration (FIC) and flow cytometric analysis. The determined minimum inhibitory concentration (MIC) values varied from 0.125 to 128 μg/mL for *S. aureus* KACC 13236 (SA^S) and from 0.25 to >256 μg/mL for *S. aureus* CCARM 3080 (SA^R). The PAS effect was more pronounced in SA^S treated with phage SA11 in the presence of cefoxitin (FIC=0.62), chloramphenicol (FIC=0.54), and polymyxin B (FIC=0.38). SA^S and SA^R cells were injured when exposed to asublethal concentration of ciprofloxacin, whereas these cells were highly susceptible to the phage-antibiotic combined treatment, showing 96% of relative percentages of injured and dead cells. The results suggest that the combined treatment of phages and antibiotics can be used to improve antimicrobial efficacy against antibiotic-resistant bacteria.

Keywords: phage-antibiotic synergy, fractional inhibitory concentration, *Staphylococcus aureus*, antibiotic resistance, bacteriophage

Introduction

Over the last two decades, *Staphylococcus aureus* has become a leading cause of nosocomial and community-acquired infections, including staphylococcal scalded skin syndrome, toxic shock syndrome, endocarditis, osteomyelitis, abscesses, meningitis, and wound infections (1,2). Recently, there has been a significant increase in the prevalence of antibiotic-resistant *S. aureus* infections due to the overuse and misuse of antibiotics (3,4). The rapid emergence and spread of antibiotic-resistant *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA), has become a great concern for public health worldwide. Although much effort has been focused on the discovery of new antibiotics, the acquisition of antibiotic resistance has preceded the development of novel antibiotics (5,6). Furthermore, unfortunately, investment in the development of novel antibiotics has been continuously declined due to the low potential for profitability in the pharmaceutical industry (5). Therefore, new approaches are needed to lessen the threat of growing antibiotic resistance.

Interest in bacteriophages (phages) has been renewed for controlling pathogenic bacteria because of their host-specific characteristics with no detrimental effects on human cells (7-10). Recently, phages have received much attention as a possible alternative to antibiotics in the treatment of antibiotic-resistant bacterial infections (11,12). The practical problem in confronting antibiotic resistance is that current antibiotics are quickly becoming useless against emerging antibiotic-resistant bacteria. To overcome this drawback, phages can be used in combination with current antibiotics. Many researchers have demonstrated that combining phages with antibiotics can be a promising way to improve antimicrobial activity, known as phage-antibiotic synergy (PAS) (13-17). The lytic activity of phageshas been shown to be synergistically enhanced in the presence of antibiotics (14). However, this phenomenon is still not clearly understood, and relatively little knowledge exists on antibiotic-resistant *S. aureus*. Therefore, this study aimed to evaluate the PAS effect of phage SA11 and antibiotics (cefoxitin, chloramphenicol, ciprofloxacin, polymyxin B, and tetracycline) against antibiotic-resistant *S. aureus*.

Materials and Methods

Bacterial strains and culture conditions Strains of *S. aureus* KACC 13236 and *S. aureus* CCARM 3080 were purchased from the Korean Agricultural Culture Collection (KACC, Suwon, Korea) and Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea), respectively. The strains were cultured in trypticase soy broth (TSB;

BD, Becton, Dickinson and Co., Sparks, MD, USA) at 37°C for 20 h. The cultures were collected by centrifugation at 3,000×*g* for 20 min at 4°C. The harvested cells were then washed twice with phosphate-buffered saline (PBS, pH 7.2) and were diluted to approximately 2×10^5 CFU/mL for inoculation.

Bacteriophage propagation Bacteriophage SA11 (PB Number: BP 6002) was purchased from the Bacteriophage Bank at Hankuk University of Foreign Studies (Yongin, Korea) and propagated onthe host strain (*S. aureus* KACC 13236) in TSB at 37°C for 24 h. After propagation, the culture was centrifuged at 5,000×*g* for 10 min and filtered through a 0.2 μm sterilized filter. The bacteriophage titer was determined using a soft-agar overlay method. Briefly, the harvested phage culture was serially (1:10) diluted with PBS, and was gently suspended in 10 mL 0.5% TSB soft-agar containing the host cells (10^8 CFU/mL) and poured onto a prewarmed base. The plates were incubated at 37°C for 24 h to enumerate phage SA11, expressed as plaque-forming units (PFU/mL).

Preparation of antibiotic stock solutions The antibiotics used in this study (Table 1) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The stock solutions of ampicillin (water), piperacillin (water), cephalothin (water), cefoxitin (water), cefotaxime (water), chloramphenicol (ethanol), ciprofloxacin (glacial acetic acid), erythromycin (ethanol), novobiocin (water), polymyxin B (water), and tetracycline (ethanol) were prepared by dissolving in appropriate solvents at a concentration of 1,024 mg/mL.

Antibiotic susceptibility assay The antibiotic susceptibilities of *S. aureus* KACC 13236 and *S. aureus* CCARM 3080 were determined using a broth dilution method (18). The antibiotic stock solutions were serially diluted (1:2) from 256 mg/mL with TSB. The strains (10^5 CFU/mL each) were cultured in the diluted antibiotic solutions at 37°C for 20 h. Minimum inhibitory concentrations (MICs) were determined at concentrations of antibiotics that inhibited the visible growth of bacterial strains.

Antibiotic activity assay The antimicrobial activity of selected antibiotics (cefoxitin, chloramphenicol, ciprofloxacin, polymyxin B, and tetracycline) combined with phage SA11 was evaluated based on the growth properties of *S. aureus* KACC 13236 and *S. aureus* CCARM 3080. The strains (10^5 CFU/mL each) were cultured in 96-well microtiter plates (BD Falcon, San Jose, CA, USA) with TSB containing 1/4 MIC of each antibiotic and different levels (10 to 10^7 PFU/mL) of phage SA11, at 37°C for 20 h. The culture growth was measured at 600 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA)

Fractional inhibitory concentration assay The antimicrobial interaction of the antibiotics combined with phages on the growth of *S. aureus* KACC 13236 and *S. aureus* CCARM 3080 was evaluated according to the fractional inhibitory concentration (FIC) indices. Each antibiotic (cefoxitin, chloramphenicol, ciprofloxacin, polymyxin B, and tetracycline) was serially diluted (1:2) from the MIC, and the phage SA11 was serially diluted (1:10) from 10^6 PFU/mL. The strains of *S. aureus* KACC 13236 and *S. aureus* CCARM 3080 (10^5 CFU/mL each) were inoculated in TSB prepared by the combination of an antibiotic (0 to MIC) and phage SA11 (0 to 10^6 PFU/mL). The inoculated plates were incubated for 18 h at 37°C. The dose-response curves for bacterial growth were analyzed to estimate the antimicrobial parameters at which the bacterial growth was inhibited by 90% compared with the growth of the control using the Nonlinear Curve Fitting Function of Microcal Origin version 7.5 (Microcal Software Inc., Northampton, MA, USA). The FIC indices were estimated by the following equation: $FIC=FIC_{antibiotic}+FIC_{phage}=C_{antibiotic}/MIC_{antibiotic}+C_{phage}/MIC_{phage}$, where $MIC_{antibiotic}$ and MIC_{phage} are the respective MICs of the antibiotic and the phage alone, and $C_{antibiotic}$ and C_{phage} are the respective concentrations of the antibiotic and phage in combination (19).

Flow cytometric analysis To assess the membrane permeability, the cells treated with ciprofloxacin (1/4 MIC) and phage SA11 (10^5 PFU/mL) were analyzed using a flow cytometry system (FACS Calibur;

Table 1. MIC (mg/mL) of antibiotics against *S. aureus* KACC 13236 and *S. aureus* CCARM 3080

Antibiotic	Classification	Target site	MIC[1)]	
			S. aureus KACC 13236	*S. aureus* CCARM 3080
Ampicillin	β-lactam	Cell wall	2 (S)	128 (R)
Piperacillin	β-lactam	Cell wall	2 (S)	128 (R)
Cephalothin	β-lactam	Cell wall	0.5 (S)	64 (R)
Cefoxitin	β-lactam	Cell wall	8 (S)	128 (R)
Cefotaxime	β-lactam	Cell wall	2 (S)	>256 (R)
Chloramphenicol	Amphenicol	Peptide bond	16 (I)	16 (I)
Ciprofloxacin	Fluoroquinolones	DNA gyrase	1 (S)	32 (R)
Erythromycin	Macrolides	tRNA	0.5 (S)	>256 (R)
Novobiocin	Aminocoumarin	DNA gyrase	0.125 (S)	0.25 (I)
Polymyxin B	Peptides	LPS	128 (I)	256 (R)
Tetracycline	Tetracyclines	rRNA	1 (S)	64 (R)

[1)]S, I, and R represent sensitive, intermediate, and resistant bacteria to each antibiotic, respectively.

Becton Dickinson, Mountain View, CA, USA) at the Central Laboratory of Kangwon National University. The treated cells were centrifuged at 5,000×*g* for 10 min and then washed twice in PBS. LIVE/DEAD BacLight kits (Invitrogen, Eugene, OR, USA) were used to assess the cell viability by discriminating between live cells with intact cytoplasmic membranes and dead cells with injured membranes. The collected cells were stained according to the manufacturer's instructions. The prepared cells (1 mL) were stained with green fluorescence from membrane-permeable SYTO 9 (1.5 mL) and redfluorescence from membrane-impermeable propidium iodide (PI; 1.5 mL), having maximum excitation and emission at 480 and 500 nm, respectively, for SYTO 9 (FL1 channel), and 490 and 635 nm, respectively, for PI (FL3 channel).

Statistical analysis All the experiments were independently performed three times with duplicates. Data were analyzed using Statistical Analysis System software (SAS Institute, Cary, NC, USA). Significant differences in the means were determined with Fisher's least significant difference test when a *p* value of <0.05 was found.

Results and Discussion

This study demonstrates the synergistic effect of phages and antibiotics on the inhibition of *S. aureus*. Recently, the synergistic phage-antibiotic combination has been emerging as a highly promising approach for enhancing antimicrobial activity against antibiotic-resistant bacteria and reducing the dosage of antibiotics used to treat bacterial infection. In this study, the synergistic activity of sublethal concentrations of antibiotics and phage SA11 was evaluated against antibiotic-sensitive and -resistant *S. aureus*.

Antibiotic susceptibility profile of *S. aureus* strains used in this study To determine the antibiotic susceptibility, the MIC values of different classes of antibiotics (ampicillin, piperacillin, cephalothin, cefoxitin, cefotaxime, chloramphenicol, ciprofloxacin, erythromycin, novobiocin, polymyxin B, and tetracycline) were estimated against *S. aureus* KACC 13236 and *S. aureus* CCARM 3080 (Table 1). The strains tested were defined as sensitive (S), intermediate (I), and resistant (R) bacteria based on the sensitive and resistant MIC breakpoints (20). *S. aureus* KACC 13236 was susceptible to most antibiotics, except for chloramphenicol andpolymyxin B, whereas *S. aureus* CCARM 3080 showed high levels of resistance to mostof the antibiotics tested. The MIC values for cefotaxime and erythromycin were >256 mg/mL against *S. aureus* CCARM 3080. The MIC values of ampicillin, piperacillin, cephalothin, cefoxitin, cefotaxime, ciprofloxacin, erythromycin, novobiocin, polymyxin B, and tetracycline against *S. aureus* CCARM 3080 were 64-, 64-, 128-, 16-, >128-, 32-, >512-, 2-, 2-, and 64-fold higher than those against *S. aureus* KACC 13236, respectively. According to the MIC results, *S. aureus* KACC13236 and *S. aureus* CCARM 3080 were assigned to antibiotic-sensitive *S. aureus* (SA^S) and multiple antibiotic-resistant *S. aureus* (SA^R), respectively.

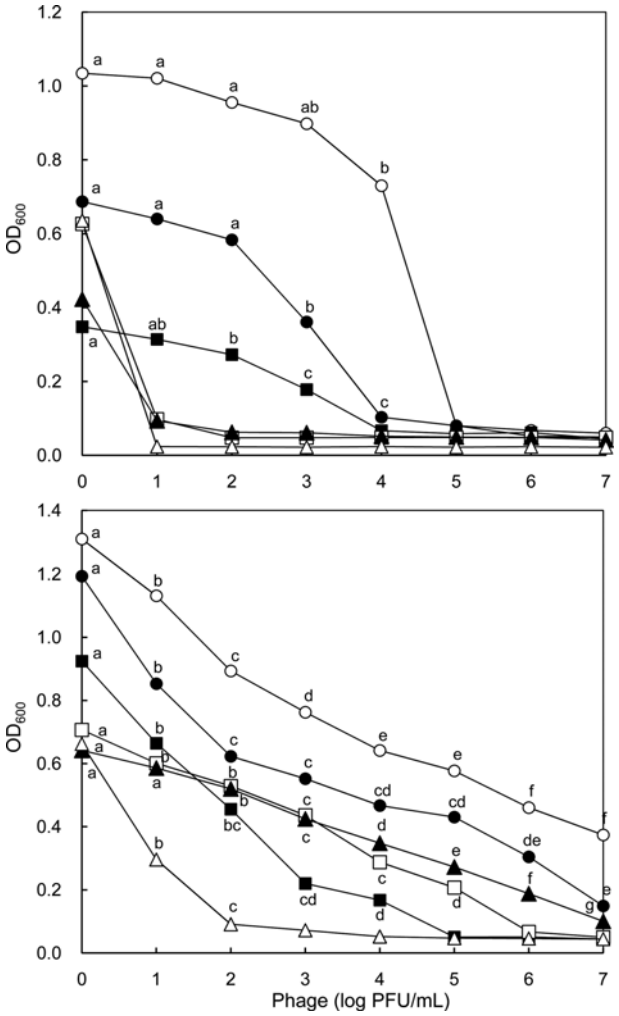

Fig. 1. Synergistic inhibitory effects of antibiotics (1/4 MIC; control, ○; ciprofloxacin, ●; tetracycline, ■; chloramphenicol, □; cefoxitin, ▲; polymyxin B, △) combined with phage SA11 on the growth of *S.aureus* KACC 13236 (SA^S; A) and *S. aureus* CCARM 3080 (SA^R; B). Means with different letters (a-g) within treatment are significantly different (p<0.05).

Evaluation of the combined antimicrobial activity of antibiotics and phages The PAS effects of phage SA11 and antibiotics (cefoxitin, chloramphenicol, ciprofloxacin, polymyxin B, and tetracycline) against SA^S and SA^R were evaluated at a sublethal concentration (1/4 MIC) of each antibiotic (Fig. 1). Phage SA11 improved the antimicrobial effect of antibiotics in a concentration-dependent manner. Relative to the growth of control, the growth of phage-antibiotic (PA)-treated SA^S and SA^R was significantly inhibited after 24-h incubation at 37°C (p<0.05). SA^S was most susceptible to cefoxitin, chloramphenicol, and polymyxin B, showing a significant decrease in the SA^S growth at 1 log PFU/mL (Fig. 1A). This is in good agreement with previous studies showing that sublethal concentration of antibiotics stimulated phage production and thus enhanced phage

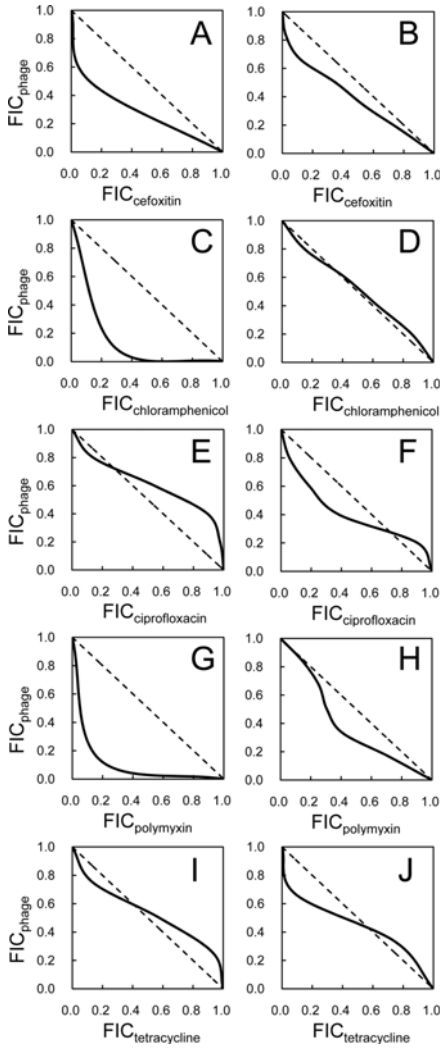

Fig. 2. Isobolograms of antimicrobial interactions between antibiotics [cefoxitin (A, B); chloramphenicol (C, D); ciprofloxacin (E, F), polymyxin B (G, H), and tetracycline (I, J)] and phage SA11 against *S. aureus* KACC 13236 (A, C, E, G, I) and *S. aureus* CCARM 3080 (B, D, F, H, J). Dashed line indicates additive interactions.

lytic activity (14,15). The lytic activity was positively influenced by the changes in the state of the host cells exposed to antibiotics, resulting in enhanced free phages (15). The growth of PA-treated SA^R was gradually inhibited with increasing phage concentration (Fig. 1B). SA^R was most susceptible to polymyxin B (1/4 MIC) treated with phage SA11 (<2 log PFU/mL). A previous study reported that the PAS effects were dependent of the combination composition of antibiotic, phage, and host strain (13).

The antimicrobial interactions between phage SA11 and antibiotics (cefoxitin, chloramphenicol, ciprofloxacin, polymyxin B, and tetracycline) were evaluated against SA^S and SA^R (Fig. 2). PAS effects were observed in SA^S treated with cefoxitin (Fig. 1A), chloramphenicol (Fig. 1C), and polymyxin B (Fig. 1G) and phage SA11, showing a concave shape (FIC indices <1). For chloramphenicol (Fig. 2E) and tetracycline (Fig. 2I), the synergistic inhibition of SA^S occurred at the lower concentrations of each antibiotic. The growth of SA^R was synergistically inhibited by the PA-combined treatment, with the exception of chloramphenicol, which showed an additive effect (FIC index=1.01). Antibiotics maybe majorly responsible for the increase in phage burst size, leading to the enhanced lytic activity in the presence of both antibiotics and phages (15). PAS was relatively more effective against SA^S than SA^R, suggesting that the antibiotic-induced alteration in bacterial membrane proteins may result in a modification in the phage receptors (21,22). The synergistic activity against SA^S and SA^R may be attributed to the different mechanisms of phages and antibiotics, preventing the development of phage and antibiotic resistance. The results suggest that the combination of phages and antibiotics can be used to control antibiotic-resistant bacteria (13).

Flow cytometric evaluation of *S. aureus* treated with phages and antibiotics The heterogeneous subpopulations of SA^S and SA^R treated with ciprofloxacin, phage SA11, and ciprofloxacin combined with phage SA11 were evaluated using the flow cytometric assay (Fig. 3). From those values in the control, the numbers of SA^S and SA^R were reduced by 0.75 and 0.42 log units for ciprofloxacin alone, 0.99 and 0.86 log units for phage SA11 alone, and 3.49 and 1.86 log units for PA-combined treatments, respectively. As shown in Fig. 3A and 3B, the SA^S and SA^R cells were more likely to be injured than killed when exposed to the sublethal concentration of ciprofloxacin. SA^S and SA^R were highly susceptible to the PA-combined treatment, showing 96 and 95% of injured SA^S and SA^R cells, respectively (Fig. 3E and 3F). This maybe due to the increased phage lytic activity stimulated by antibiotics (14). The results suggest that the PA-combined treatment can reduce the amount of antibiotics used therapeutically and eventually decrease the emergence of antibiotic resistance in bacteria exposed to sublethal concentrations of antibiotics (23).

In conclusion, to the best of our knowledge, no comparable observations have been reported for antibiotic-sensitive and -resistant *S. aureus* treated with the combination of phages and various antibiotics. The results of the study showed that the combined treatment of phage SA11 with antibiotics was found to be synergistic in inhibiting the growth of SA^S and SA^R. Therefore, the most significant finding in this study was that the PAS effect can be used as a potential alternative to control antibiotic-resistant bacteria at decreased antibiotic concentrations and to reduce the emergence of antibiotic and phageresistance. However, further study is needed to understand the mechanism of the PAS effect; this would help to develop new, promising antimicrobial strategies for the control of antibiotic-resistant bacteria.

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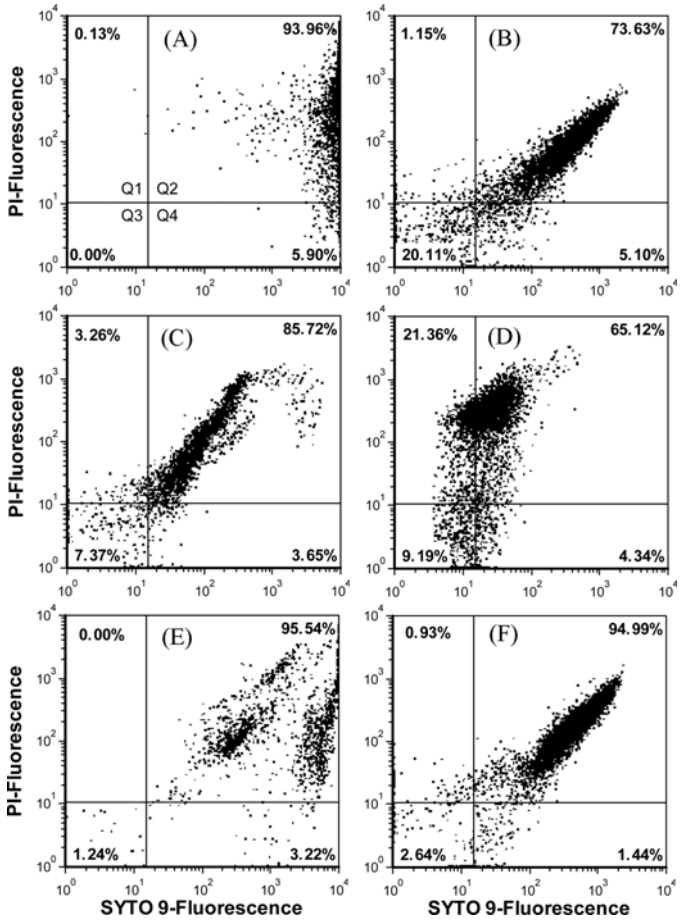

Fig. 3. Flow cytometric dot plots of *S. aureus* KACC 13236 (SA^S; A, C, E) and *S. aureus* CCARM 3080 (SA^R; B, D, F) treated with ciprofloxacin (A, B), phage SA11 (C, D), or ciprofloxacin combined with phage SA11 (E, F). The treated cells were stained with PI and SYTO 9. The quadrants represent the relative percentages of cells for positive red fluorescence only (Quadrant 1, dead cells; PI^+, SYTO 9), positive red and green fluorescence (Quadrant 2, injured cells; PI^+, SYTO 9^+), negative red and green fluorescence (Quadrant 3; PI, SYTO 9), and for positive green fluorescence only (Quadrant 4, live cells; PI, SYTO 9^+).

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Disclosure The authors declare no conflict of interest.

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