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Role of phage-antibiotic combination in reducing antibiotic resistance in *Staphylococcus aureus*

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Abstract This study was designed to evaluate the effect of phage-antibiotic synergy in reducing antibiotic resistance. The initial numbers of *Staphylococcus aureus* treated with ciprofloxacin, phages, and combination were significantly reduced by 3.47, 4.62, and 5.75 log CFU/mL, respectively, at the early 12 h of incubation. The combination treatment most effectively inhibited the growth of *S. aureus*, showing more than 4 log reduction in 18 h of incubation at 37°C. The significant reduction in biofilm formation by *S. aureus* was observed at the combination treatment (3.91 log). Ciprofloxacin-treated *S. aureus* cells became resistant to both ciprofloxacin and phage, showing the mutant frequencies of 27% and 25%, respectively, whereas no antibiotic- and phage-resistant *S. aureus* cells were observed at the combined treatment of ciprofloxacin and phages. These results provide useful information for reducing the risk of antibiotic resistance in human and food animals.

Keywords: phage-antibiotic combination, ciprofloxacin, *Staphylococcus aureus*, antibiotic resistance, mutant frequency

Introduction

For a long time, the emergence of antibiotic-resistant *Staphylococcus aureus* has been a great concern in hospitals because the nosocomial infections with methicillin-resistant *S. aureus* (MRSA) are easily transmitted between patients and furthermore become difficult to treat with common antibiotics (1). MRSA is a main cause of growing public health problems with a high mortality worldwide (2). In addition, *S. aureus* is able to form surface-associated bacterial communities on medical devices and implants known as biofilms (3). Since biofilm cells are highly resistant to antibiotics, the biofilm-associated infections with antibiotic-resistant *S. aureus* can lead to several serious clinical problems (4). Over the past few decades, the development of new antibiotics has lagged behind the acquisition of antibiotic resistance in bacteria (5). Therefore, novel antimicrobial strategies are essential to effectively control antibiotic resistance. Recently, bacteriophages (phages) have received much attention as an alternative over antibiotics because of their host specificity and safety (6).

The virulent phages can effectively destruct bacterial biofilms and inhibit antibiotic-resistant bacteria (7). Despite the efficacy of phages, the major drawback still remains on the emergence of biofilm-associated and phage-resistant bacteria (8). The bacterial resistance of phages is caused by the alteration of production of glycocalyx, phage receptors, and the restriction-modification systems (9,10). Recently, novel approaches have been proposed to overcome the major problem of using phages, including phage cocktails and phage-antibiotic combinations (11). Several studies have confirmed the synergistic effect of phages and antibiotics against bacteria. However, relatively few studies have directly evaluated the fates of phage-induced resistant bacteria in the treatment of phage-antibiotic combination. The study that characterizes the bacterial resistance to phage is essential for the practical application of phage-based control in animal foods. Therefore, the objective of this study was to evaluate the ability of *S. aureus* to form biofilms and the induction of phage-resistant *S. aureus* under the combination treatment of phages and ciprofloxacin.

Materials and Methods

Bacterial strain and culture condition Strain of *S. aureus* KACC 13236 was provided by the Korean Agricultural Culture Collection (KACC; Suwon, Korea). The strain was cultured in trypticase soy broth (TSB; Becton, Dickinson and Company [BD], Franklin Lakes, NJ, USA) at 37°C for 20 h. The culture was centrifuged at 3,500 × *g* for 15 min at 4°C. The harvested cells were washed with phosphate buffered

saline (PBS, pH 7.2) and diluted to approximately 2×10^6 CFU/mL for further assay.

Phage propagation Phage SA11 (PB Number: BP 6002) was purchased from the Bacteriophage Bank at Hankuk University of Foreign Studies (Yongin, Korea) and propagated on the *S. aureus* KACC 13236 as the host bacteria in TSB. After incubation for 24 h at 37°C, the culture was centrifuged at 5,000 × *g* for 10 min and then filtered through a 0.2 μm sterilized filter to remove host cells and lysates. The harvested phages were serially (1:10) diluted with PBS, gently suspended in 10 mL TSB (0.5% agar) containing the host cells (10^8 CFU/mL), and poured onto a pre-warmed TSA plates. After incubation for 24 h at 37°C, the phages were enumerated. Phage titer was expressed as plaque-forming unit (PFU/mL).

Antibiotic susceptibility assay The stock solution of ciprofloxacin (Sigma Chemical Co., St. Louis, MO, USA) was prepared by dissolving in water to a final concentration of 1,024 mg/mL. The antibiotic susceptibility of *S. aureus* KACC 13236 was evaluated using a broth dilution method (12). The ciprofloxacin stock solution was serially diluted in one-half (1:2) from 256 mg/mL with TSB. The bacterial strain (10^5 CFU/mL) was cultured in the diluted ciprofloxacin solutions. After incubation for 20 h at 37°C, minimum inhibitory concentration (MIC) was determined at the ciprofloxacin concentration at which no visible growth of *S. aureus* was observed.

Disk diffusion susceptibility test The synergistic effect of phage and antibiotic against *S. aureus* was determined using a disk diffusion susceptibility test. The ciprofloxacin disks (5 mg) were placed on the Muller-Hinton agar plates containing *S. aureus* (10^6 CFU/mL) alone and with phage (10^6 PFU/mL). After 18-h incubation at 37°C, the diameter of the clear inhibition zone was measured using an electronic caliper (The L.S. Starrett Co., Athol, MA, USA).

Time-kill curve analysis The antimicrobial activity of ciprofloxacin alone, phage alone, and combination was evaluated by the time-kill assay. Ciprofloxacin (1/2 MIC; 0.5 mg/mL), phage (10^6 CFU/mL), and combination (0.5 mg/mL and 10^6 CFU/mL) prepared in TSB were inoculated with *S. aureus* (7×10^6 CFU/mL). The inoculated samples were cultured in a shaking incubator for 60 h at 37°C. Bacterial survivors were measured every 4 h until 60 h using an Autoplate® Spiral Plating System and QCount Colony Counter (Spiral Biotech, Norwood, MA, USA). The cultured samples were used for further analyses, including bacterial motility, biofilm-forming ability, and Congo red agar at 24 h, and mutant frequency assays at 60 h.

Biofilm enumeration The biofilm-forming ability was evaluated after 24-h cultivation of *S. aureus* in the control, ciprofloxacin, phage, and combination (ciprofloxacin and phage). The cultured cells in the microtiter plates were rinsed twice with PBS to remove non-attached cells. The attached cells were scrapped with a cell scrapper. The collected biofilm cells were serially (1:10) diluted with PBS, plated on TSA using an Autoplate Spiral Plating System (Spiral Biotech), and incubated at 37°C for 24 h to enumerate viable biofilm cells using a QCount® Colony Counter (Spiral Biotech).

Bacterial spreading ability assay The colony formation of *S. aureus* treated with ciprofloxacin, phages, and combination was evaluated on the soft agar plates. The bacterial cells cultured for 24 h in the control, ciprofloxacin, phage, and combination (ciprofloxacin and phage) were suspended in PBS and diluted to 5×10^3 CFU/mL. The diluted cells (2 mL each) were stabbed onto the soft agar plates (0.45% agar). The plates were incubated for 20 h at 37°C and then the colony morphology was observed.

Congo red agar assay The slime-producing property of *S. aureus* cells was evaluated using a Congo red agar (CRA) method (13). In brief, the CRA was mixed with 36 g sucrose and 0.8 g Congo red stain in 1 L of brain heart infusion agar (BHI). Approximately 10^5 CFU/mL of *S. aureus* (50 μL each) containing ciprofloxacin, pre-adsorbed phages (10 min), or combination was spread on the surface of pre-prepared CRA. The colony morphology was observed after 24-h incubation at 37°C.

Mutant frequency assay The mutation rates of *S. aureus* cultured in the control, ciprofloxacin (1/2 MIC), phages, and combination (ciprofloxacin and phages) for 60 h were estimated on the TSA containing ciprofloxacin (1 MIC) or phages (10^6 PFU/mL). The cultured cells were plated on TSA with and without ciprofloxacin (or phages) and incubated for 24 h at 37°C. The mutant frequencies were estimated as the proportion of the numbers of surviving colonies on the TSA with and without ciprofloxacin (or phages).

Statistical analysis All analyses were performed in duplicate on three replicates. Data were analyzed using the Statistical Analysis System software (SAS, Cary, NC, USA). The general linear model and least significant difference (LSD) procedures were used to compare means of treatments, including the control, ciprofloxacin, phage, and combination (ciprofloxacin and phage). Significant mean differences among treatments or incubation times were calculated by Fisher's LSD at $p<0.05$.

Results and Discussion

Since the increased incidence of multidrug-resistant bacteria has become a global issue because of the lack of newly developed antibiotics, new therapeutic strategies are needed to solve clinical and public health problems associated with infections caused by multidrug-resistant bacteria. The application of phages has been known to be one potential alternative. However, the emergence of phage resistance in the host bacteria still remains in negative

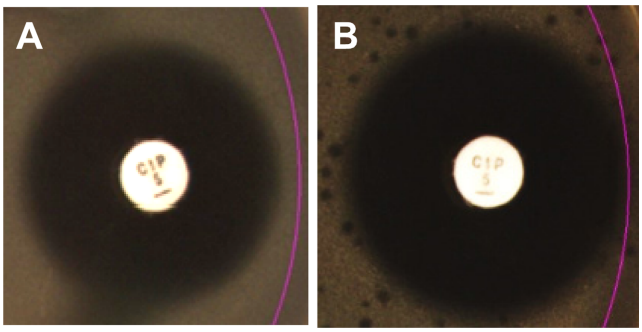

Fig. 1. Ciprofloxacin (5 mg) disk susceptibility of *S. aureus* in the absence (A) and presence (B) of phages.

towards phage application. Thus, this study describes the possibility of using phage-antibiotic synergy (PAS) as an alternative method to reduce the level of antibiotic and phage resistance.

Combined antimicrobial effect of phage and ciprofloxacin against *S. aureus* The PAS effect against *S. aureus* was evaluated using ciprofloxacin disk diffusion assay in the absence and presence of phages (Fig. 1). The size of clear zone was increased to 10% in the presence of phages when compared to the clear zone in the absence of phages. The increased clear zone might be attributed to the plaques produced by phage burst release. The sublethal levels of antibiotics can synergistically stimulate the phage growth (14). In this study, the PAS effect might be highly considered if the sublethal concentration of ciprofloxacin was used for the disk diffusion assay. Thus, the relatively large plaques were observed in the zones close to the ciprofloxacin disk. The MIC value for ciprofloxacin was 1 mg/mL against *S. aureus* used in this study. One-half of MIC was used in the time-kill curve experiment. The antimicrobial effect of ciprofloxacin alone, phages alone, and combination (ciprofloxacin and phages) was evaluated against *S. aureus* for 60 h at 37°C as displayed in Fig. 2. Compared to the control, all treatments (ciprofloxacin, phages, and combination) significantly inhibited the growth of *S. aureus* up to the early 12 h of incubation, resulting in 3.47-, 4.62-, and 5.75-log reductions, respectively. The number of *S. aureus* treated with ciprofloxacin alone rapidly increased after 12 h of incubation, whereas those treated with phages alone and combination decreased continuously up to 24 h, followed by a steady growth. Although the *S. aureus* cells were recovered at all treatments, the recovery rate was significantly delayed in combination. The growth of *S. aureus* was most effectively inhibited by the combination treatment throughout the incubation period. The results confirm that the PAS effect can effectively decrease the occurrence of phage- and antibiotic-resistant *S. aureus* (11). Similar to PAS, phage cocktail has also been proposed as an effective way to retard the phage resistance (15).

Physiological properties of *S. aureus* treated with a combination of phage and ciprofloxacin The adhesion ability of *S. aureus* cells

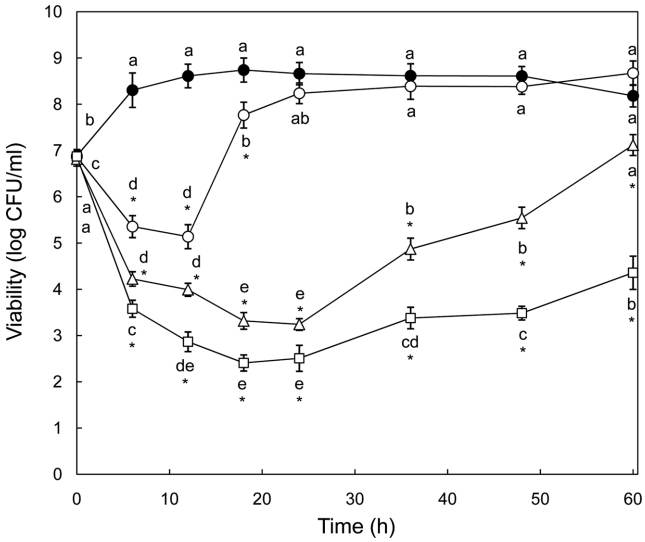

Fig. 2. Survival of *S. aureus* cultured in TSB (control; ●) containing one-half of MIC of ciprofloxacin (○), phages (△), and combination (ciprofloxacin + phages; □) for 60 h at 37°C. Means with different letters (a-e) within each treatment are significantly different at $p<0.05$. Asterisk (*) indicates that means within each incubation time are significantly at $p<0.05$ when compared to the control.

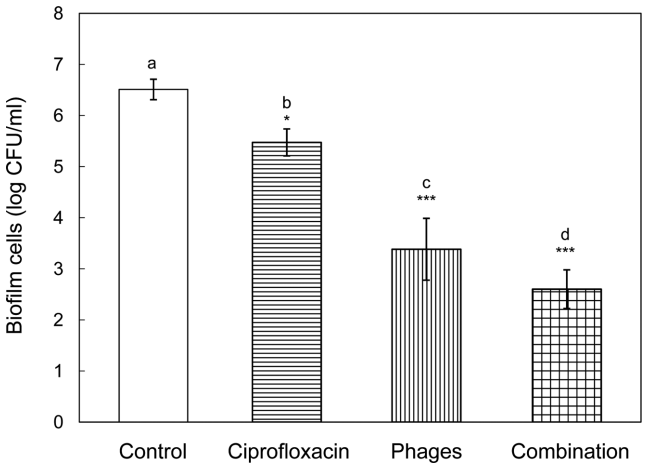

Fig. 3. Biofilm formation abilities of *S. aureus* cultured in TSB (control) containing one-half of MIC of ciprofloxacin, phages, and combination (ciprofloxacin + phages) for 24 h at 37°C. Means with different letters (a-d) on the bars are significantly different at $p<0.05$. Asterisks indicate that means are significantly at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***) when compared to the control.

treated with on-half of MIC of ciprofloxacin, phages, and combination (ciprofloxacin and phages) was evaluated after 24 h of incubation at 37°C (Fig. 3). Compared to the control (6.51 log CFU/mL), the number of adhered *S. aureus* cells significantly reduced by 1.04, 3.13, and 3.91 log, respectively, for ciprofloxacin alone, phages alone, and combination. Combination was most effective to inhibit the biofilm formation, followed by phages and ciprofloxacin, corresponding to the inhibition of planktonic cells (Fig. 2). Bacteria biofilms are highly resistant to antibiotics because of structural rigidity and reduced

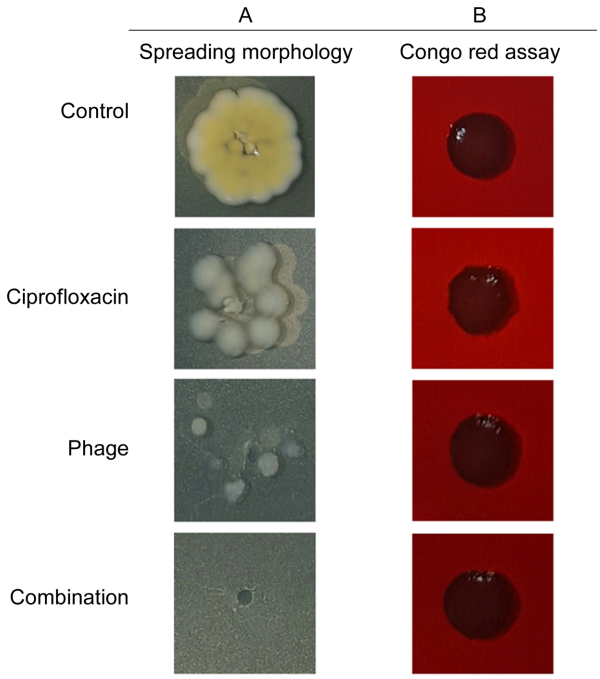

Fig. 4. Phenotypic variation in spreading morphology (A) and slime production (B) of *S. aureus* cultured in TSB (control) containing one-half of MIC of ciprofloxacin, phages, and combination (ciprofloxacin + phages) for 24 h at 37°C.

metabolic activity, which can cause serious persistent and chronic infections (16). Previous study reported that the bacterial cell components, including extracellular DNA, can promote biofilm formation (17). In this study, the phage-induced bacterial lysates, however, did not have a great influence on the induction of biofilm formation by *S. aureus* in combination. This observation indicates that ciprofloxacin can synergistically act with phages rather than mutually exclusive in increasing susceptibility of *S. aureus* biofilms (14,18).

The colony morphology of *S. aureus* treated with ciprofloxacin, phages, and combination was evaluated on the soft agar base (0.45% agar) (Fig. 4A). *S. aureus* treated with the phages produced less dense colonies, followed by the ciprofloxacin alone. No colonies were observed in the combination treatment. This result confirms that *S. aureus* planktonic and biofilm cells were effectively inhibited by the combination treatment as indicated in Fig. 2 and 3. The slime production of *S. aureus* treated with ciprofloxacin, phages, and combination was qualitatively evaluated as indicated in Fig. 4B. On CRA, the slime-positive *S. aureus* cells developed black colonies, whereas the slime-negative *S. aureus* cells developed red colonies. No considerable difference in phenotype was observed among all treatments. *S. aureus* treated with ciprofloxacin, phages, and combination developed black colonies on CRA, indicating slime producing phenotype. Mostly circular colonies were observed for the control, whereas *S. aureus* cells treated with ciprofloxacin, phages, and combination produced slightly irregular colonies (Fig. 4B). The slime layer plays an important role in antibiotic resistance because it mediates the adhesion to abiotic or biotic surface, leading to multi-layered biofilms (19). Therefore, the slime-positive bacteria are responsible for increased virulence and antibiotic resistance (20). However, the decreased biofilm-forming ability in this study was mainly because of the considerable inhibitory activity of phages and combination against *S. aureus*.

Table 1. Mutant frequencies of *S. aureus* cultured in the control, ciprofloxacin, phages, and combination (ciprofloxacin + phages) for 60 h followed by exposure to ciprofloxacin and phages

Treatment	Mutant frequency (%)	
	After exposure to ciprofloxacin	After exposure to phages
Control	ND[1)]	ND
Ciprofloxacin	26.85±2.62	24.80±5.63
Phage	ND	81.61±8.33
Combination	ND	ND

[1)]ND indicates that colonies were not detected on the TSA plates containing ciprofloxacin (or phages).

Change in the frequency of antibiotic resistance in *S. aureus* treated with a combination of phage and ciprofloxacin The mutant frequency was estimated to evaluate the induced resistance of *S. aureus* to ciprofloxacin and phage during incubation (Table 1). *S. aureus* cells treated with ciprofloxacin for 60 h at 37°C acquired resistant to ciprofloxacin (27%) and phage (25%). The increased ciprofloxacin resistance observed in this study is in good agreement with the previous report that ciprofloxacin could induce resistance at high frequency (21). Antibiotic alone and phage alone are more likely to induce phage- and antibiotic-resistant bacterial cells (8). The mechanisms of ciprofloxacin resistance in *S. aureus* include 1) point mutations in essential bacterial enzymes (topoisomerase IV and DNA gyrase) and 2) alteration in bacterial efflux system (NorA) (22). The phage resistance is directly associated with the change in the receptors for phages (9). The alteration of phage receptors in ciprofloxacin-resistant *S. aureus* might result in the interruption of phage-host interaction and decrease in phage adsorption (10). The high mutant frequency against phages was observed in the phage-treated *S. aureus* cells (82%). This result suggests that the application of phage alone for controlling pathogenic bacteria is most frequently confronted with the phage resistance under selective pressure (23). Ciprofloxacin- and phage-resistant *S. aureus* cells were not induced in the control and combination treatment. This suggests that combination can be better at treating pathogenic bacteria than antibiotic alone and phage alone. The combination treatment effectively inhibited the growth of *S. aureus* and significantly decreased the mutant frequency of mutant production, resulting from the mutual inhibitory activity of ciprofloxacin and phage against *S. aureus* (8).

In conclusion, the most significant finding in this study was that PAS can be a potential alternative to effectively control antibiotic-

resistant *S. aureus* with the least frequency of phage or antibiotic resistance. The emergence of phage- and ciprofloxacin-resistant *S. aureus* cells was not observed at the combination treatment. The decreased biofilm-forming ability and low mutant frequency were attributed to the mutual inhibitory activity of ciprofloxacin and phage against *S. aureus*. However, these results are not enough to understand the inhibitory activity of PAS. Therefore, further study is needed to elucidate the mechanisms of antibiotic and phage resistance in planktonic and biofilm cells under food system.

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