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Synergistic Effect of Bacteriophage and Antibiotic against Antibiotic-Resistant *Salmonella* Typhimurium

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ABSTRACT - In this study, we investigated the efficacy of *Salmonella* phage P22 combined with antibiotics to inhibit antibiotic-resistant *S*. Typhimurium CCARM 8009. The synergistic effect of phage P22 and antibiotics was evaluated by using disk diffusion and broth dilution assays. The development of Antimicrobial resistance was determined after time-kill assay. The antibiotic susceptibility assay showed the inhibition zone sizes around the antibiotic disks were increased up to 78.8% in the presence of phage (cefotaxime; 13.6%, chloramphenicol; 19.3%, ciprofloxacin; 12.7% and erythromycin; 78.8%). The minimum inhibitory concentration values of the combination treatment significantly decreased from 256 to 64 mg/mL for tetracycline, 8 to 4 mg/mL for chloramphenicol, 0.0156 to 0.0078 mg/mL for ciprofloxacin, 128 to 64 mg/mL for erythromycin and 512 to 256 mg/mL for streptomycin. The number of *S*. Typhimurium CCARM 8009 was approximately 4-log lower than that of the control throughout the combination treatment with phage P22 and ciprofloxacin delete at 37°C for 20 h. The results indicate that the development of antimicrobial resistance in *S*. Typhimurium could be reduced in the presence of phage treatment. This study provides promising evidence for the phage-antibiotic combination as an effective treatment to control antibiotic-resistant bacteria.

Key words: Bacteriophage, Antimicrobial resistance, *Salmonella*, Phage-antibiotic combination, Ciprofloxacin

Salmonella is one of the major foodborne pathogens that cause public health concern worldwide. The estimated 1.35 million cases associated with *Salmonella* infections has been reported annually in the U.S.[1)]. Among >2500 serovars, *Salmonella enterica* which presents the majority of *Salmonella* infections in humans accounted for more than half of the number[2)]. In the past several decades, using of antibiotic treatments to control foodborne pathogens in agricultural industry is mis- and over-used, leading to critical problem related antibiotic-resistant development in bacteria[3)]. The infections associated with antimicrobial resistance have resulted in the healthcare costs of $20 billion and additional society costs for productivity loss of $35 billion each year in the U.S.[4)]. More than 100,000 illnesses associated antibiotic-resistant *Salmonella* has been reported annually. The study reported that *Salmonella* isolates conferring resistance to ≥5 antibiotics (i.e. ceftriaxone, ciprofloxacin, streptomycin, tetracycline and erythromycin) accounted for more than 66,000 illnesses from 2009 to 2011 in the U.S[5)]. The emergence of multiple antibiotic-resistant *Salmonella* serovars is responsible for the interruption of antibiotic treatment. The antibiotic-resistant *Salmonella* infections might increase in mortality rates[2)]. The development of effective control method is essential step to solve or decrease the spread of antimicrobial resistance in food production chain.

Bacteriophage (phage) is a type of viruses that have the outstanding properties over antibiotic (e.g. specificity to target hosts)[6,7)] or chemical agents which have some negative effects on human health[8)]. Several phages have been approved as GRAS (Generally Recognized AS Safe)[9)]. Phage application is recognized as the potential tool as either an alternative or supplemental to antibiotic treatments[10)]. The desirable efficacy of phage-antibiotic combination has been reported and continuously investigated. Synergistic effect of *Salmonella* phage P22 and ciprofloxacin has been investigated on the inhibition of antibiotic-sensitive *S*. Typhimurium. The remarkable antimicrobial efficacy of more than 5 log reductions was reported. Moreover, the expression level of genes related to antimicrobial resistance was decreased following the combination treatment[11)]. In

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order to solve the problem related antibiotic-resistant development in foodborne pathogens, the synergistic effect of phage and antibiotic is an interesting approach to reduce the expression of antimicrobial resistance in antibiotic-resistant bacteria. This study investigated the antimicrobial efficacy of *Salmonella* phage P22 in combination with several antibiotics (ceftriaxone, chloramphenicol, ciprofloxacin, streptomycin, tetracycline and erythromycin) to inhibit the growth of antibiotic-resistant *Salmonella* Typhimurium. The antimicrobial efficacy was assessed by the antimicrobial susceptibility assays, time-kill curve, and the development of resistance upon exposure to antimicrobial agents.

Materials and Methods

Bacterial strains and culture conditions

In this study, *Salmonella enterica* subsp. enterica serovar Typhimurium CCRAM8009 from the Culture Collection of Antibiotic Resistant Microbes (CCARM, Seoul, Korea) was used as the targeted bacteria. For each experiment, *Salmonella* strain was cultured in trypticase soy broth (TSB; BD, Becton, Dickinson and Co., Sparks, MD, USA) and incubated at 37°C for 20 h. Bacterial cells in the stationary phase were centrifuged at 3000×g for 20 min at 4°C, which was washed and diluted with phosphate buffered saline (PBS, pH 7.2) to 10^8 CFU/mL.

Bacteriophage propagation and titer determination

Salmonella bacteriophage P22 (ATCC 97541) was propagated in TSB containing the suggested host *S.* Typhimurium LT2 (ATCC 19585), purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) at the multiplicity of infection (MOI) of 0.1. The mixture was incubated at 37°C for 24 h. For harvesting, the incubated mixture was centrifuged at 5000×g for 10 min and filtered through a 0.2-μm syringe filter. The phage titers were determined by the double-layer agar technique according to the protocol previously described[12]. Briefly, the serial dilution of phages were performed in PBS buffer. Each phage dilution was mixed with soft agar (0.45% TSA) containing host cells approximately 10^7 CFU/mL. The mixture was then poured onto the bottom layer (1.5% TSA). Titers of phage particles as plaque forming unit (PFU) were enumerated after incubated at 37°C for 24 h.

Disk diffusion assay

To determine the synergistic effect of phage P22 and antibiotics by the disk diffusion method, the mixture of *S.* Typhimurium CCARM (10^5 cfu/mL) and phage P22 (10^6 pfu/mL) was streaked on Muller–Hinton agar plates. The antibiotic disks, including cefotaxime (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), streptomycin (10 μg) and tetracycline (30 μg), were placed on the bacterium lawn and then incubated at 37°C for 18 h. The inhibition zones (mm) were measured using a digital vernier caliper (The L.S. Starrett Co., Athol, MA, USA).

Broth dilution assay

All antibiotics tested were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and prepared as the stock solutions at the concentration of 1024 mg/mL. Cefotaxime and streptomycin solutions were prepared in water. Chloramphenicol, erythromycin and tetracycline were prepared in absolute ethanol. Ciprofloxacin was prepared in glacial acetic acid. The susceptibility of *S.* Typhimurium CCARM8009 to each antibiotic was evaluated in the presence and absence of phage P22. Each antibiotic with and without phage P22 was diluted in TSB as two-fold serial dilution in 96-well microtiter plates. The number of *S.* Typhimurium (10^5 cfu/mL) was then inoculated in the prepared plates and incubated for 18 h at 37°C. The density of *S.* Typhimurium was observed at 600 nm[13] and fitted to exponential function using Microcal Origin® 8.0 (Microcal Software Inc., Northampton, MA, USA).

Time-kill assay

Antimicrobial efficacy of phage P22 alone, ciprofloxacin alone and combination of phage P22 and ciprofloxacin to reduce the number of antibiotic-resistant *S.* Typhimurium CCARM8009 was evaluated at 37°C for 20 h. Phage P22 (10^6 pfu/mL), ciprofloxacin (1×MIC; 0.016 μg/mL), and the combination was inoculated with *S.* Typhimurium (10^5 cfu/mL) in TSB. The number of *S.* Typhimurium was enumerated at every 4 h by a standard spread plate on TSA. After 20 h culture, sample from each treatment was collected for antimicrobial resistance analysis.

Antimicrobial resistance development

In this study, agar disk diffusion assay was used to determine the changes of antimicrobial resistance in *S.* Typhimurium CCARM 8009 after the time-kill assay. *S.* Typhimurium cells cultured without antibiotic and phage treatments as the control, with phage P22 alone or ciprofloxacin alone, and the combination was streaked onto Muller–Hinton agar plate. Ciprofloxacin disks were placed on bacterium lawn and incubated at 37°C. The inhibition zone sizes were measured after 18 h of incubation.

Statistical analysis

All experiments were conducted in duplicate for three replicates. The Analysis of variance (ANOVA) was used to

determine significant mean differences at $P<0.05$ using the Statistical Package for Social Science (SPSS 10.0 for windows, SPSS Inc., Chicago, IL, USA). Comparison of means was carried out by Duncan's multiple range tests.

Results and Discussion

Antibiotic susceptibility profiles of antibiotic-resistant *S.* Typhimurium in the presence of phages

The susceptibility of *S.* Typhimurium CCARM8009 to several classes of antibiotics was evaluated using two standard methods, disk diffusion and broth dilution assays (Table 1 and Fig. 1). In the presence of phage P22, the inhibition zone sizes of cefotaxime, chloramphenicol, ciprofloxacin and erythromycin disks were increased by 13.6%, 19.3%, 12.7% and 78.8%, respectively as compared to the treatment without phage. However, streptomycin and tetracycline disks showed no changes in the inhibition zone sizes against the targeted bacteria. Phage P22 combined with antibiotics against *S.* Typhimurium CCARM8009 showed the decrease in MIC values of antibiotics up to three folds for tetracycline (from 256 to 64 mg/mL). The decrease in two folds was observed for other antibiotics, chloramphenicol (from 8 to 4 mg/mL), ciprofloxacin (from 0.0156 to 0.0078 mg/mL), erythromycin (from 128 to 64 mg/mL) and streptomycin (from 512 to 256 mg/mL). By the broth dilution technique, both conditions (presence and absence of phage P22) showed similar MIC value of cefotaxime (0.25 mg/mL).

Several previous studies reported the remarkable

Table 1. Inhibition zone diameter (mm) of *S.* Typhimurium CCARM 8009 with antibiotic alone and combination of phage-antibiotic

Classification	Antibiotic	*S.* Typhimurium CCARM 8009	
		Antibiotic alone	Combination
β-lactam	Cefotaxime	24.3±0.2	27.6±0.2
Phenicol	Chloramphenicol	18.1±0.3	21.6±0.4
Quinolone	Ciprofloxacin	26.0±0.2	29.3±0.1
Macrolide	Erythromycin	3.3±0.2	5.9±0.2
Aminoglycoside	Streptomycin	nc*	0±0.0
Tetracycline	Tetracycline	nc	0±0.0

* nc describes no clear zone.

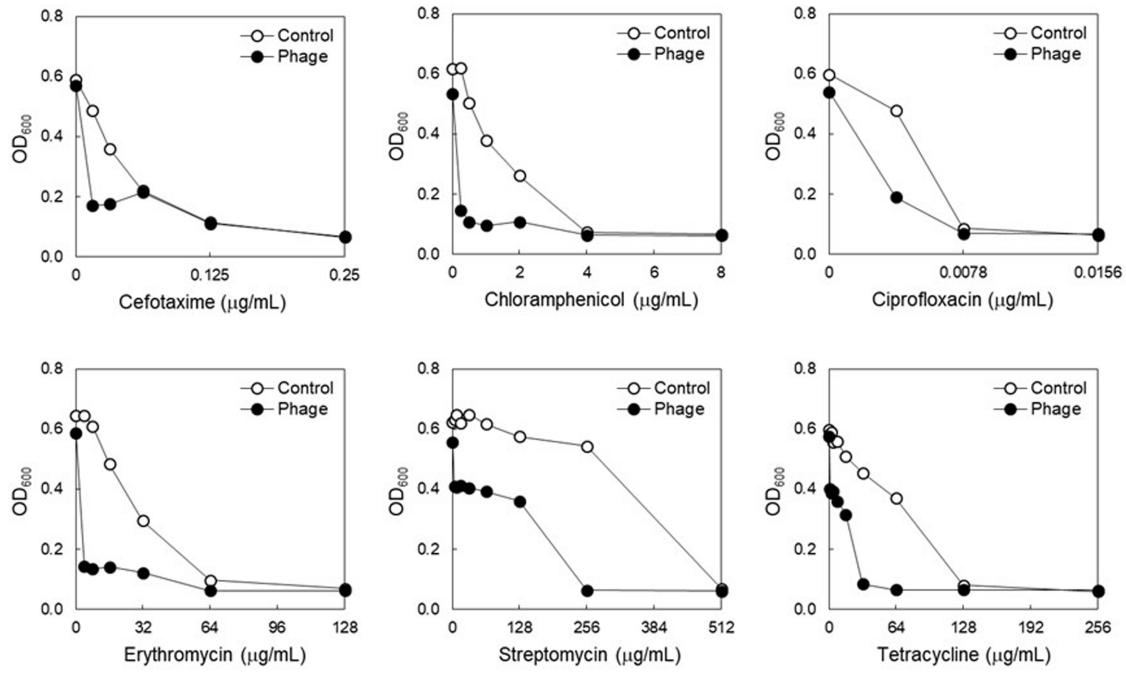

Fig. 1. Minimum inhibitory concentration (MIC, mg/mL) of *Salmonella* Typhimurium CCARM 8009 treated with antibiotic alone (control) or the antibiotic and phage combination (phage).

achievement to increase the susceptibility of bacterial cells on the combination effects of phages and antibiotics (Phage-Antibiotic Synergistic; PAS). The potential of synergistic effect of PAS to reduce the development of antibiotic-resistance in antibiotic-sensitive *S.* Typhimurium has been previously reported[11]. The increase of inhibition zone sizes of several antibiotic disks and the decrease of MIC values of those several antibiotic classes indicate the potential of phage P22 to promote antimicrobial efficacy of the combination. More than 10% in size of the inhibition zone of antibiotic disks and two-fold decrease in MIC values were observed in the combination treatment[11]. Dickey and Perrot investigated the effect of PAS compared to using of antibiotics alone applied to *in-vitro* biofilms of *Staphylococcus aureus*. The results showed that at 2×MIC, most antibiotics tested were ineffective, whereas the combination also improved the antimicrobial efficacy[14]. Auturk et al. reported higher antimicrobial efficacy of phage-antibiotic combination compared to the treatment of phage alone or antibiotic alone. Time of treatment and antibiotic concentration have been suggested as the major keys on PAS[15]. The results indicated that the susceptibility of bacterial cells could be increased by the presence of phages particles combined with antibiotic substance.

Synergistic effect of phage and antibiotic on the inhibition of antibiotic-resistant *S.* Typhimurium

The synergistic effect of phage P22 and ciprofloxacin on the inhibition of *S.* Typhimurium CCARM8009 was evaluated at 37°C for 20 h (Fig. 2). Treatments of the phage alone, antibiotic alone and no treatment (control) were evaluated in parallel for comparison. The cell numbers of *S.* Typhimurium CCARM8009 were significantly decreased by all treatments (phage alone, antibiotic alone and combination) during the 20-h incubation period. Phage alone exhibited the highest antimicrobial efficacy to reduce *S.* Typhimurium CCARM8009 at the first 4 h of incubation by 3.9 log cfu/mL. The antimicrobial efficacy of phage alone against *S.* Typhimurium CCARM8009 was rapidly decreased after 4 h, leading to approximately 1 log lower than the control during the incubation period. Treatment of antibiotic alone showed the steady decrease of *S.* Typhimurium CCARM8009 throughout the 20-h incubation period (1.4 to 3 log cfu/mL). Overall, the remarkable decrease was obtained from the combination treatment as indicated by the decrease of the number of *S.* Typhimurium CCARM8009 by approximately 4 log cfu/mL, when compared to the control, throughout the 20-h incubation period.

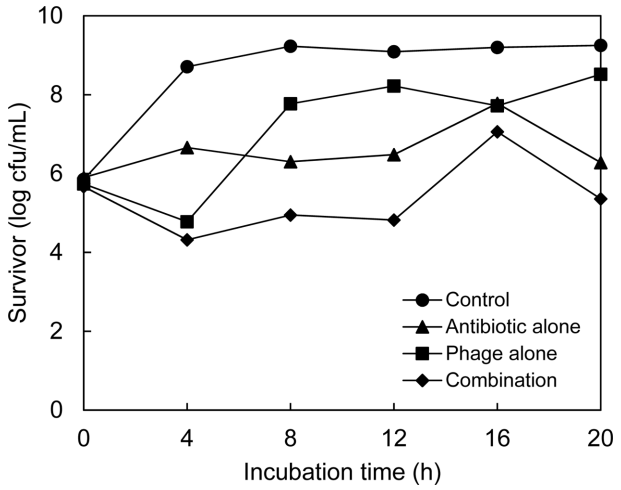

Fig. 2. Survival of *Salmonella* Typhimurium CCARM 8009 cultured in the control (no treatment), ciprofloxacin alone, phage alone, and combination (ciprofloxacin and phage) for 20 h at 37°C.

The results in this study generally provide evidence similar to the previous studies. More than 5 logs of antibiotic-sensitive *S.* Typhimurium were reduced by the combination of bacteriophage P22 and 1×MIC ciprofloxacin[11]. A previous study inactivated *E. coli* by the synergistic effect of phages and antibiotics. The results showed that after 8 h of treatment, combined phage and ciprofloxacin could decrease the bacterial cells by 8 log cfu/mL, whereas phages or the antibiotic alone could decrease the bacterial cells by 4 log cfu/mL and 1.2 log cfu/mL, respectively[16]. The study related PAS indicated that antibiotic could be a functional key on the consequence of distribution and lysis process of phage particles. The effect of antibiotic (cefotaxime) was investigated on the appearance of plaque sizes of phage φMFP on *E. coli* MFP, showing that plaque sizes in the presence of antibiotic were significantly larger than when there was no phage in the treatment[17]. Overall, the results of this study provide the evidence which in agreement to the previous study. Suggesting that the combination of phage and antibiotic provides the potential to improve the antimicrobial efficacy against bacterial pathogens.

Development of antimicrobial resistance of *S.* Typhimurium treated with phage and antibiotic

The development of antimicrobial resistance in *S.* Typhimurium was evaluated after antibiotic, phage, and combination treatments (Fig 3). Compare to the control, phage treatment did not show the development of antimicrobial resistance. However, the development of antimicrobial resistance was increased by 10.5% and 7.3%, respectively, antibiotic and combination treatments. The superior strategy for pathogen control is the expected advantage of the combined method that leads to the reduced bacterial capacity to the development of phage and/or

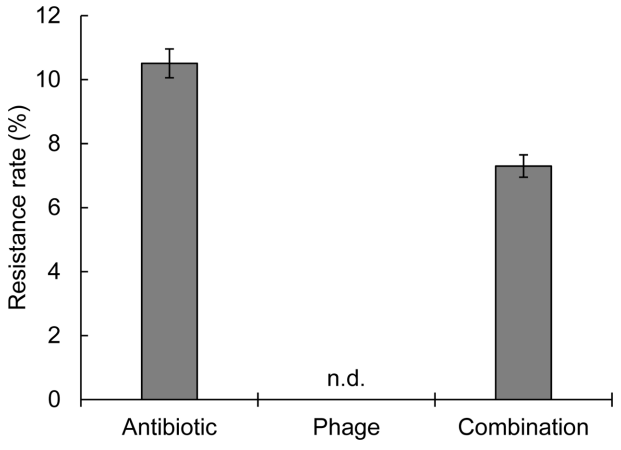

Fig. 3. Development of antimicrobial resistance (%) of *S.* Typhimurium CCARM 8009 after treatment with ciprofloxacin alone, phage alone, or combination (ciprofloxacin and phages) for 20 h at 37°C as compared to the control. n.d. indicates no significant difference when compared to the control.

antimicrobial resistance[18].

In this study, the treatment of phage alone showed the remarkable potential to decrease antimicrobial resistance development in bacterial cells. Whereas, the treatment of antibiotic alone showed the highest percentage of antimicrobial resistance development among all treatments. Interestingly, antimicrobial resistance development in the combination treatment showed the lower percentage compared to the treatment of antibiotic alone. The mode of function to kill bacteria of both ciprofloxacin and phage have major influence on bacterial DNA. Ciprofloxacin functions on the inhibition of DNA replication targeting DNA gyrase[19]. Whereas phage particles synthesize the new progenies by using bacterial DNA machinery[20]. The phenomenon observed in this study could be hypothesized that the mechanism of phage to lyse bacterial cells might be affected to genes related antimicrobial resistance in bacteria, resulting the less expression of antimicrobial resistance development. The observed results indicating that the presence of phage particles in the system could decrease the virulence of antimicrobial resistance development in bacterial cells. Data in this study suggests that the effect of phage and antibiotic combination provided the desirable antimicrobial to control antibiotic-resistant *Salmonella*. Moreover, this strategy could decrease the development of antimicrobial resistance in *Salmonella* compared to treatment of antibiotic alone.

In conclusion, this study demonstrates the effective antimicrobial efficacy of phage and antibiotic combination to reduce the number of antibiotic-resistant *Salmonella* and the occurrence of antimicrobial resistance development after treatment. The results showed that presence of phage particles in the system of antibiotic treatment could provide the positive effects to solve the concerned issue. Phages in the treatment could affect the improvement of antibiotic susceptibility of *S.* Typhimurium CCARM8009 by two to three folds. The effective inactivation of *Salmonella* growth was significantly higher in the treatment of phage-antibiotic combination than those treatments of phage alone or antibiotic alone. This study provides evidence that phages could link to the key functional properties which could decrease the expression of antimicrobial resistance after treatments. Since the function of antibiotics still provides the benefit on microbial control and the advantages of phage application are of interest, this study suggests that PAS should be applied as the effective alternative tool for reducing and controlling antibiotic-resistant bacteria.

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국문요약

본 연구는 항생제 내성 *Salmonella* Typhimurium CCARM 8009을 저해하기 위한 phage와 항생제 조합처리의 효과를 평가하였다. 디스크 확산법과 액체배지 희석법에 의해 phage와 항생제의 상승 저해효과를 측정하였고 배양을 통한 항생제 내성 유도를 평가하였다. Phage를 처리한 cefotaxime, chloramphenicol, ciprofloxacin, erythromycin의 디스크의 저해 구역은 각각 13.6%, 19.3%, 12.7%, 78.8%로 증가되었다. Phage와 항생제 조합 처리에 의해 tetracycline, chloramphenicol, ciprofloxacin, erythromycin, streptomycin의 최소생육억제농도는 각각 64, 4, 0.0078, 64, 256 mg/mL으로 감소되었다. Phage와 항생제의 조합 처리는 항생제 내성 *S.* Typhimurium CCARM 8009을 효과적으로 저해하였다 (4 log reduction). 본 결과는 phage와 항생제의 조합처리는 항생제 내성균을 제어하기 위한 방법으로 충분히 응용가치가 높음을 보여주고 있다.

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